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Nature of Proton Cycling during Gramicidin Uncoupling of Oxidative Phosphorylation†

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ABSTRACT: Addition of gramicidin D to liver mitochondria, incubated in low- or high-salt media, results in stimulation of respiration in the absence or presence of depression of $\Delta\tilde{\mu}_H$, respectively. Gramicidin D concentrations 2 orders of magnitude higher are required in the low-salt media with full uncoupling at 1 nmol of gramicidin·mg^{-1}. The stimulation of respiration is not accompanied by increased passive proton influx in low-salt media. In high-salt media, the extent of respiratory stimulation and the extent of $\Delta\tilde{\mu}_H$ depression differ according to the nature and concentration of cation. The flow–force relationship is very steep when gramicidin D induced uncoupling occurs in low-salt media and much less steep in high-salt media. A multiplicity of flow–force relationship, respiratory rate vs $\Delta\tilde{\mu}_H$, is obtained, the slope of which depends on the nature and concentration of cation, and which can be reproduced by computer simulation by introducing a variable extent of proton cycling either in the membrane or in the pump. The *apparent* proton conductance, as analyzed in the relationship of $J_e/\Delta\tilde{\mu}_H$ vs $\Delta\tilde{\mu}_H$, increases in the so-called ohmic and nonohmic regions according to whether gramicidin D is added in high-salt or low-salt media, respectively. Titration with antimycin of the respiratory control ratio (RCR) in gramicidin D treated mitochondria leads to a depression of the RCR in high-salt but not in low-salt media. The view is discussed that in low-salt media the gramicidin D induced uncoupling is due to a cycling of protons within a proton domain operationally located at or near the proton pump. This leads to a $\Delta\tilde{\mu}_H$-independent uncoupling different from the $\Delta\tilde{\mu}$-dependent uncoupling due to proton cycling through the lipid bilayer.

The mechanism of uncoupling is one of the most fundamental problems of oxidative phosphorylation and as such has always received utmost attention in studies of energy coupling. In fact, if any acceptable mechanism of oxidative phosphorylation must explain how ATP is made, it must provide an equally satisfactory explanation as to why ATP is not made. Both questions have been answered by the chemiosmotic hypothesis, and this has been the main reason for its success.

That uncoupling agents increase the conductance for protons of the inner mitochondrial membrane and of black lipid membranes may be considered an established fact (Mitchell & Moyle, 1967; Hopfer et al., 1968). Whether the mem-

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brane–proton cycling mechanism explains completely the uncoupling phenomenon is, on the other hand, less secure. Mitchell and Moyle (1967) have made a rough calculation of the correspondence between rates of respiration and of proton translocation. Such a calculation (Luvisetto et al., 1987) was based on measurements of $\Delta\tilde{\mu}_H$[1] and of H^+/O stoichiometry which were far from correct. If one introduces in the experimental data of Mitchell and Moyle (1967) the more recent measurements of the H^+/O stoichiometry and of the values of $\Delta\tilde{\mu}_H$, a discrepancy is found between the rates of respiration and of proton conductance. Furthermore, there is a large discrepancy (100–1000×) between the rates of proton transport in black lipid membranes and in mitochondria [McLaughlin & Dilger, 1980; cf. also Kell and Westerhoff (1985)].

The interpretation of uncoupling in mitochondria has undergone a marked change in view of three significant developments, two theoretical and one experimental. The first development has been the proposal that uncoupling may be due not only to proton cycling across the membrane but also to electron or proton cycling within the pump (Pietrobon et al., 1981). It has been noted that there is no physical justification for assuming that the pumps must always be tightly coupled, and the capacity to undergo cycles of electron transfer without vectorial proton translocation has been denoted *intrinsic uncoupling* or *slip*. With this proposal, uncoupling is not only a membrane but also a pump property. The second development has been the application of a nonequilibrium thermodynamic approach and of Hill kinetic methods to the analysis of the proton pump and of the overall process of oxidative phosphorylation. The availability of mathematical models has allowed a quantitative comparison between predictions and observations (Pietrobon et al., 1986; Zoratti et al., 1986). The third development has been the systematic comparison of the relationship between the rates of the various reactions of oxidative phosphorylation and the level of $\Delta\tilde{\mu}_H$. This comparison has led to the discovery, although not without dispute, that the extent of *uncoupling* is not related in a unique manner to the depression of $\Delta\tilde{\mu}_H$ [Zoratti et al., 1982; Rottenberg, 1983; Luvisetto et al., 1987; Pietrobon et al., 1987; cf., however, Woelders et al. (1986)].

In the earlier times, the basis for the proposal that uncoupling does not depend only on the lipid bilayer was very indirect, for example, the specific interaction of uncoupling agents with some proteins of the inner membrane (Hanstein & Hatefi, 1974; Hanstein, 1976; Katre & Wilson, 1978). Two groups of findings then provided direct support to this concept. On one side, it is observed that the FCCP-induced increase of membrane conductance (as calculated indirectly from the rate of electron transfer and H^+/O) was titrated down upon inhibition of the redox pumps (Pietrobon et al., 1981; Walz, 1983). On the other, Rottenberg and collaborators (Rottenberg, 1983; Rottenberg & Hashimoto, 1986) pointed out that some agents, such as anesthetics and fatty acids, were able to uncouple oxidative phosphorylation with litle depression of $\Delta\tilde{\mu}_H$. Luvisetto et al. (1987) and Pietrobon et al. (1987) then conducted a systematic analysis of the mechanism of uncoupling by anesthetic and fatty acids and showed that the *uncoupling* effect of anesthetics was not accompanied by an increase of membrane proton conductance. Furthermore, the entire set of uncoupling effects, obtained with FCCP, fatty acids, and anesthetics, was compared with the predictions of a kinetic model of oxidative phosphorylation (Pietrobon & Caplan, 1985) and could not be accounted for exclusively by a membrane proton cycling mechanism (Luvisetto et al., 1987; Pietrobon et al., 1987).

The interpretation of these findings was not unequivocal. On one side, Rottenberg (1983) favored the view that this anomalous behavior was the reflection of an uncoupling effect acting at the level of intramembrane processes of energy coupling between redox and ATPase proton pumps, whereby these agents were denoted as *decouplers*. On the other side, Pietrobon et al. [1987; cf. also Pietrobon et al. (1981) and Walz (1983)] favored the view that the anomalous behavior was due to an *intrinsic uncoupling*, i.e., that these agents were increasing the extent of uncoupling in the proton pump domain. However, Pietrobon et al. (1987) noticed that in fact neither the experiments nor the computer simulations could satisfactorily distinguish between the two interpretations.

The development of the nonequilibrium thermodynamic approach to oxidative phosphorylation has raised considerable interest on the flow–force relationships. In brief, if the rates of oxidation and of phosphorylation are not coupled directly but only through $\Delta\tilde{\mu}_H$, it might be expected that the rates of oxidation and phosphorylation should be independent of the way in which $\Delta\tilde{\mu}_H$ is varied; i.e., a unique relation between rates of oxidation or of phosphorylation should be found. However, this has not been the case (Azzone et al., 1977; Baccarini-Melandri et al., 1977; Zoratti & Petronilli, 1985). This finding has been very controversial. In some cases, the flow–force relationships measured with other experimental systems did yield a unique relationship whatever means was used to vary (Sorgato et al., 1985). Finally, it was reported that the nonunique relationships might be due to differences in the methods used to assay $\Delta\tilde{\mu}_H$, i.e., that they were essentially experimental artifacts (Woelders et al., 1986). It has been suggested that the different patterns of the flow–force relationship were due to the fact that there exists a local $\Delta\tilde{\mu}_H$ which differs in magnitude from, but communicates with, the bulk-phase $\Delta\tilde{\mu}_H$. The pattern of the relationship would thus depend on the extent to which the local or the bulk-phase $\Delta\tilde{\mu}_H$ is affected.

The present investigation provides new experiments useful to distinguish between the membrane– and pump–proton cycling mechanism of uncoupling. Furthermore, it also aims to clarify some experimental discrepancies. For example, gramicidin was reported to act by Rottenberg and Hashimoto (1986) as a membrane–proton cycling uncoupler in mitochondria and by Pick et al. (1987) as a decoupler in chloroplasts. Gramicidin has been used in other studies as a membrane–proton cycling uncoupler in submitochondrial particles (Herweijer et al., 1987). We find that gramicidin D acts as a membrane–proton cycling uncoupler in high-salt media and as a pump–proton cycling uncoupler in low-salt media. In this first paper, we shall essentially concentrate on the analysis of the effects of gramicidin D on the proton flows and conductances and on the flow–force relationships, while in the following paper (Luvisetto & Azzone, 1989) we shall concentrate on the synthesis of ATP.

Materials and Methods

Materials. Rat liver mitochondria were prepared according

[1] Abbreviations: J_o^{sh}, rate of respiration in static head; J_o^{max}, maximal rate of respiration; J_e, rate of electron transfer; J_K^{eff}, rate of K^+ efflux; J_H^l, proton flux through the leaks; L_H^l, membrane proton conductance; $L_e(c)$, redox phenomenological conductance; Z, redox phenomenological stoichiometry; q, decree of coupling; n_e, H^+/e^- stoichiometry; n, H^+/O stoichiometry; $\Delta\psi$, transmembrane electrical potential gradient; ΔpH, transmembrane pH gradient; $\Delta\tilde{\mu}_H$, transmembrane proton electrochemical potential gradient; P_i, inorganic phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

to standard procedures (Massari et al., 1972), and all the 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 the reagents were of maximal purity commercial grade. Inhibitors, valinomycin and gramicidin D, were obtained from Sigma.

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

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. For measurements of J_K^{eff}, mitochondria were suspended in 3 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) 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 a stationary state (with succinate), and after incubation with gramicidin D, 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. For each set of determinations, suitable calibrations of the rates of K^+ efflux were determined.

Determination of $\Delta\tilde{\mu}_H$. The transmembrane electrical potential, $\Delta\psi$, was evaluated from the distribution of the lipophilic ion triphenylmethylphosphonium ($TPMP^+$) as described in 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. 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 were measured also by the distribution of K^+ as continuously monitored by using a K^+ electrode. The $\Delta\psi$ was calculated from Nernst's law by assuming an internal matrix concentration of K^+ equal to 50% of medium osmolarity. All the experiments of the present work were conducted in the presence of 5 mM P_i/Tris, a condition which even during cation uptake has been found to result, by direct measurement of ΔpH with the DMO technique (Azzone et al., 1984), in negligible ΔpH changes. In the figures, the term $\Delta\psi$ is used instead of $\Delta\tilde{\mu}_H$ solely to indicate that this was the parameter directly measured. It is, however, implicit that under the prevailing experimental conditions the two terms $\Delta\psi$ and $\Delta\tilde{\mu}_H$ are interchangeable.

Results

Uncoupling Effect of Gramicidin D. Figure 1 shows a summary of the uncoupling effects of gramicidin D on the rates

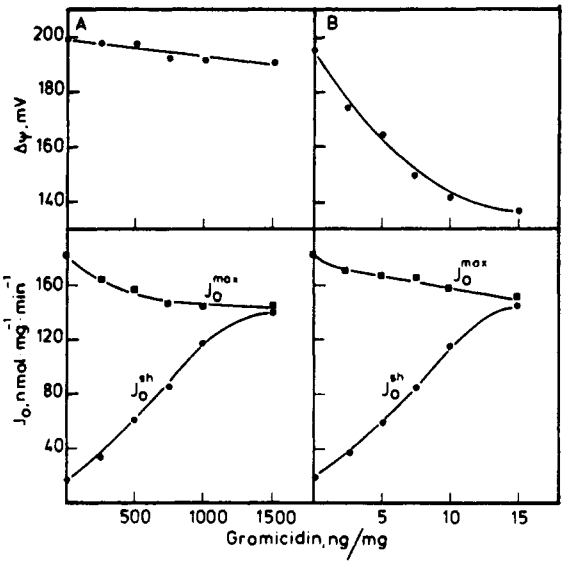

FIGURE 1: (Lower panels) Mitochondrial rate of respiration in static head, J_o^{sh} (●), and maximal rate of respiration, J_o^{max} (■), as a function of increasing concentrations of gramicidin in low-salt media (panel A) and in high-salt media (panel B). (Upper panels) Difference of electrical potential across the inner mitochondrial membrane in static head as a function of the same gramicidin concentrations. Standard (low salt) medium composition was 0.2 M sucrose, 10 mM succinate (Tris salt), 30 mM Tris/MOPS, 5 mM P_i/Tris, 1 mM EDTA, and 5 μM rotenone, pH 7.4, T = 25 °C. In high-salt media, 5 mM KCl was added to the standard medium. After 2 min of incubation of rat liver mitochondria (1 mg of protein/mL), gramicidin was added. After 2 min of incubation, FCCP was added, and J_o and $\Delta\tilde{\mu}_H$ were measured.

of respiration as measured under two basic conditions (e.g., in the resting state or in the presence of an excess of uncoupler) and on the level of $\Delta\tilde{\mu}_H$. All the effects of gramicidin D were tested either in low-salt or in high-salt media. It is seen in Figure 1A that addition of gramicidin D in low-salt media resulted in a stimulation of the resting respiration which reached a maximum at around 1.5 μg of gramicidin/mg of protein (equivalent to about 1 nmol of gramicidin·mg^{-1}). The stimulation of the resting respiration was accompanied by a negligible depression of $\Delta\tilde{\mu}_H$. On the other hand, addition of gramicidin D to mitochondria incubated in the presence of 5 mM K^+ resulted in a maximal stimulation of the resting respiration at gramicidin D concentrations which were 2 orders of magnitude lower, i.e., 15 ng/mg of protein. In the presence of 5 mM K^+, the stimulation of the resting respiration by gramicidin D was accompanied by a large depression of $\Delta\tilde{\mu}_H$. Results similar to that shown in Figure 1B could be obtained with other univalent cations such as Na^+ and Li^+, although at higher concentrations. The experiments of Figure 1 characterize two distinct effects of gramicidin D: one occurring in the absence of univalent cations, requiring high gramicidin D concentrations and not accompanied by $\Delta\tilde{\mu}_H$ depression; another occurring only in the presence of univalent cations, requiring much lower gramicidin D concentrations (equivalent to about 10–20 pmol of gramicidin·mg^{-1}) and accompanied by $\Delta\tilde{\mu}_H$ depression.

Effect of Gramicidin D on the Flow of Protons across the Membrane. Consider the mitochondria in the resting state (static head condition). These mitochondria respire at a low rate without net movement of protons. However, respiration means, in a system where electron transfer in the proton pumps is obligatorily coupled to vectorial proton translocation, ejecton of protons in the cytosolic space. According to the definition of a chemiosmotic system, the lack of net proton movement then means, in the stationary state and under the assumption

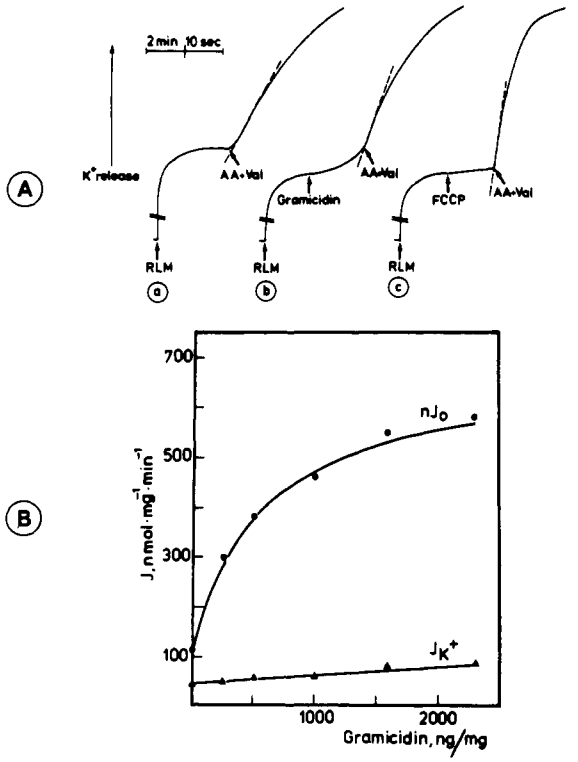

FIGURE 2: (A) Comparison between typical K^+ electrode recording as obtained in the presence of FCCP (130 pmol/mg) and gramicidin D (1500 ng/mg). Experimental conditions as in Figure 1. Where indicated, antimycin A and valinomycin were added. The dashed line represents the rate of initial K^+ efflux. (B) Comparison between the stimulation of respiration multiplied by the H^+/O stoichiometry and of proton permeability as induced by increasing gramicidin concentrations. After incubation of mitochondria for 2 min with the medium, gramicidin was added, and after 30 s, the rate of respiration and in a parallel sample the initial rate of K^+ efflux immediately after the addition of antimycin A (0.05 μg/mg) and valinomycin (0.15 μg/mg) were measured. n was taken equal to 7.

of completely coupled proton pumps, that so many protons are extruded from the mitochondria as they reenter the mitochondria:

$$n_e J_e = J_H^i \quad (1)$$

where n_e is the H^+/e^- stoichiometry, J_e is the respiratory rate, and J_H^i is the passive proton influx driven by the proton electrochemical gradient. Equation 1 states that the respiratory rate must be accounted for by an equivalent influx of protons through the membrane and, furthermore, that any increase of the respiratory rate must be accompanied by an equivalent increase of the passive proton influx.

Luvisetto et al. (1987) have recently reported the parallel titrations of the rates of respiration and of passive proton influx against increasing concentrations of various types of uncoupling agents (chloroform, oleic acid, and FCCP). With all three uncouplers, the rate of passive proton influx did not account for the rate of respiration, and the discrepancy increased with the increase of the uncoupler concentration. However, the extent of discrepancy was not identical with the three uncouplers. In the case of chloroform, there was an increase of the respiration with practically no increase of the rate of passive proton influx.

Figure 2A shows the effect of gramicidin D on the rate of K^+ efflux as obtained from antimycin- plus valinomycin-treated mitochondria. As discussed elsewhere (Zoratti et al., 1986), the rate of K^+ efflux from antimycin- plus valinomycin-treated mitochondria is a reliable measure of the inner membrane permeability for protons under conditions where no other major permeant ion species is present in the system. The question arises as to whether this is also true with mitochondria incubated in low-salt media but in the presence of increasing concentrations of gramicidin D which can act as an ionophore for protons and univalent cations. Figure 2A shows three traces of K^+ efflux as obtained always with antimycin- plus valinomycin-treated mitochondria but under three different experimental conditions, namely, in the absence of gramicidin, in the presence of gramicidin, and in the presence of a concentration of FCCP ensuring a respiratory stimulation identical with that given by gramicidin. Figure 2A, trace a, shows that in the control mitochondria there was no K^+ movement before the addition of antimycin plus valinomycin while after antimycin plus valinomycin there was a rate of K^+ efflux of about 40 nmol of $K^+ \cdot mg^{-1} \cdot min^{-1}$. This is the rate of K^+ efflux corresponding to the physiological proton leak in native mitochondria. Figure 2A, trace b, then shows that addition of gramicidin in high concentration, before antimycin plus valinomycin, caused only a slight increase of the rate of K^+ efflux which tended, however, to increase with time. The independence of the gramicidin-induced efflux from the addition of valinomycin indicates that it is due to an increased permeability for both protons and cations. Quantitatively, the gramicidin effect is very small as can be judged by comparing the rate of K^+ efflux before and after the addition of antimycin plus valinomycin. However, what is more important is that the rate of K^+ efflux in antimycin- plus valinomycin-treated mitochondria is only slightly modified by the presence of gramicidin D. This can be seen more clearly by comparison with the increase of the rate of K^+ efflux caused by FCCP. In Figure 2A, trace c, is also reported the K^+ efflux occurring when mitochondria were supplemented with FCCP added at a concentration causing a respiratory stimulation equivalent to that caused by gramicidin D. Note that as discussed previously (Luvisetto et al., 1987) even FCCP enhances the rate of K^+ efflux much less markedly than might be expected from the stimulation of the respiratory rate. The experiment of Figure 2A thus shows that under comparable conditions, i.e., at FCCP and gramicidin concentrations inducing similar respiratory stimulations, the increase of the rate of K^+ efflux is considerably lower in the presence of gramicidin than of FCCP.

Figure 2B shows a complete titration carried out with gramicidin D in a low-salt medium. It is seen that an increase of the gramicidin D concentration in the range between zero and 2000 ng/mg of protein resulted in a stimulation of the rate of respiration of almost 5-fold while under the same conditions the increase of the passive proton influx was not higher than 30%. It may be argued that the comparison made in Figure 2 is erroneous because rates of respiration and of passive proton influx should be compared at equal values of $\Delta\tilde{\mu}_H$. However, this objection is invalid for two reasons. First, Luvisetto et al. (1987) have shown that a parallelism between the rate of proton pumping and of passive proton influx does exist if, for example, the redox pump is replaced with the ATPase proton pump. Thus, the lack of parallelism is the consequence of a specific effect of the uncoupler on the redox proton pump. Second, the experiment is carried out in such a way to measure the rate of proton influx immediately after addition of antimycin to respiring mitochondria, i.e., under conditions where K^+ efflux and proton influx initiate immediately after the membrane potential falls below the level of the resting state. That the value of the membrane potential at the initiation of K^+ efflux is equal to that of the resting state can be controlled by parallel measurements with the TPMP

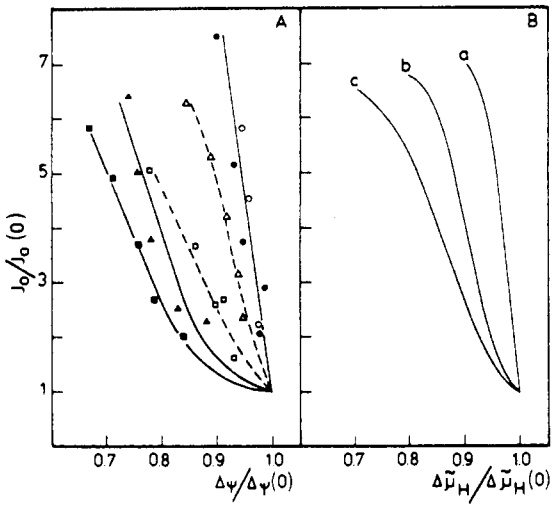

FIGURE 3: Comparison between normalized experimental flow–force relationships as obtained in low- and high-salt media and simulated flow–force relationships. (Panel A) Flow–force relationships as obtained in presence of gramicidin (0–2 μg/mg) in low-salt media (●, ○) and gramicidin (0–25 ng/mg) in 1 mM Li (△), 10 mM Li^+ (□), 0.5 mM K^+ (▲), and 5 mM K^+ (■) supplemented media, respectively. The simulations in panel B are performed by using the six-state proton pump model of Pietrobon and Caplan (1985) and an ohmic parallel pathway for passive diffusion of protons (leak). Kinetic parameters are as in Pietrobon et al. (1986). Curve a was obtained by increasing the rate constant of slip transition on the redox proton pump, curve b by increasing in parallel the rate constant of slip transition and the membrane proton conductance, and curve c by increasing only the membrane proton conductance.

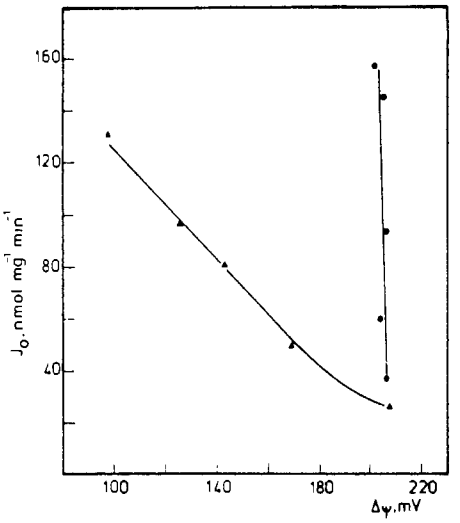

FIGURE 4: Flow–force relationship in low-salt media (●) and increasing concentrations of gramicidin (0–2 μg/mg) or at increasing concentrations of K^+ but in the presence of 1.5 ng/mg of gramicidin (▲). The values of $\Delta\psi$ were measured on the K^+ distribution with a K^+ electrode. Experimental conditions as in Figure 1 except that 200 pmol·mg^{-1} valinomycin was also present in all samples.

electrode. The extent of discrepancy between rates of respiration and of proton influx can be further evaluated by confronting the data of Figure 2B with the similar experiment carried out with FCCP [cf. Figure 3 in Luvisetto et al. (1987)] where an increase of the rate of respiration from 20 to 100 natoms·mg^{-1}·min^{-1} is accompanied by an increase of the passive proton influx from 40 to 240 natoms·mg^{-1}·min^{-1}.

Effect of Gramicidin D on the Flow–Force Relationship. The relationship between the rate of respiration and $\Delta\tilde{\mu}_H$ has been measured at increasing concentrations of gramicidin D either in the presence or in the absence of univalent cations. The comparison was carried out by adding to the medium 0.5 or 5 mM K^+, or 1 and 10 mM Li^+, respectively. Addition of K^+ not only resulted in stimulation of the respiration at much lower concentrations of gramicidin D, in accord with what was already shown in Figure 1, but also rendered the slope of the flow–force relationship much less steep as compared to the case in the absence of cations. Also, the higher the cation concentration, the less steep was the slope of the flow–force relationship. Similar results were obtained when K^+ was replaced by Li^+ as cation in the medium. In practice, a multiplicity of flow–force relationships could be obtained depending on the nature of the cation and its concentration.

In Figure 3A are reported the data of the flow–force relationships after normalization in order to allow a better comparison of the effects of the cation nature and concentration. Furthermore, in Figure 3B is also reported a computer simulation of the flow–force relationship carried out as described in Luvisetto et al. (1987) as based on the proton pump model of Pietrobon and Caplan (1985). It is seen that the slope of the flow–force relationship was very steep when the stimulation of respiration was achieved by increasing the rate constant of slip transition while on the other hand it was much less steep when obtained by increasing the extent of leak in the membrane. Intermediate slopes were obtained by obtaining the stimulation of the respiration partly by increasing the slip and partly the leak. The data of Figure 3B indicate that the pattern of the flow–force is not a reflection of the dependence of a redox proton pump on its output force but rather of the mechanism of uncoupling.

Woelders et al. (1986) have suggested that the multiplicity of flow–force relationships is a consequence of the use of TPMP for the assay of the membrane potential since a unique relation is observed when the membrane potential is assayed on the basis of the K^+ distribution. Also in the experiments of Figure 3, one may object that addition of cations changes the extent of TPMP binding, thus producing an artifact in the $\Delta\psi$ assay. This interpretation is however unlikely for two reasons. First, all calculations of $\Delta\psi$ where done by assuming as zero potential the value of the TPMP electrode in the presence of FCCP. This automatically corrects for the passive TPMP binding. Second, it is true that at lower potentials there is a lower inorganic cation uptake which could lead to higher matrix binding of TPMP. However, due to the osmotic behavior of mitochondria, large changes in inorganic cation distribution do not result in changes in the matrix inorganic cation concentrations. A more direct approach to the question whether the difference in flow–force relationship can be due to changes in TPMP binding is provided in experiments where the data for the flow–force relationship are obtained by measuring on the K^+ and not on the TPMP distribution. In the experiments of Figure 4, respiration was stimulated either with gramicidin D in low-salt medium or by increasing concentrations of K^+, in the presence of a constant gramicidin D concentration, and the membrane potential was always assayed with the K^+ electrode in the presence of valinomycin as suggested by Woelders et al. (1986). It is seen that the slope of the plot was dramatically different in the two cases: very steep in the former and much less steep in the latter case. The experiment further supports the concept that the shape of the plot depends on the mechanism of uncoupling and not on the assay procedure for $\Delta\psi$.

The comparison between the data of Figures 3 and 4 on the flow–force relationships obtained at increasing uncoupler concentrations with the flow–force relationships obtained at increasing respiratory inhibitor concentrations, in the absence and presence of uncouplers (Azzone et al., 1984), suggests a certain correspondence between the slopes of the former and

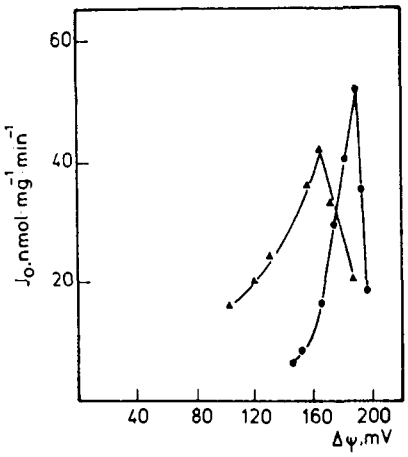

FIGURE 5: Relationship between rate of respiration and output force as obtained first in the presence of increasing gramicidin concentrations either in the absence of cation (●) or in the presence of 5 mM K^+ (▲) and then in the presence of increasing concentrations of antimycin (0–12.5 ng/mg) and a constant amount of gramicidin (250 ng/mg in low-salt media; 5 ng/mg in K^+-supplemented media).

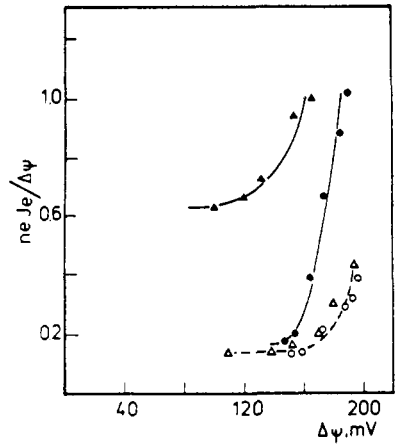

FIGURE 6: Ratio between the rate of electron transfer from succinate to oxygen (multiplied by n_e, the H^+/e^{-1} stoichiometry) and $\Delta\psi$ in static head as a function of $\Delta\psi$ in titrations with antimycin in the presence of a constant amount of gramicidin [(●) 250 ng/mg in low-salt media; (▲) 5 ng/mg in 5 mM K^+-supplemented media] and in the absence of gramicidin (open symbols). The units of $n_e J_e/\Delta\psi$ are nanomoles per milligram per minute per millivolt. n_e is taken as equal to 4. Mitochondria (1 mg/mL) were incubated for 5 min in the presence of increasing concentrations of antimycin (0–12.5 ng/mg) and for 2 min with gramicidin. Then succinate (10 mM) was added, and the rate of respiration and $\Delta\psi$ were measured.

of the latter. This is shown in Figure 5 which reports the shape of the relationship between rate of respiration and output force first in the presence of increasing gramicidin D concentrations and then in the presence of increasing respiratory inhibitor concentrations. It is seen that the values of the membrane potential at all extents of respiratory stimulation, as well as of respiratory inhibition, depended on whether the gramicidin D addition had taken place in low- or high-salt media. Figure 5 thus shows that in all types of flow–force relationships there is always a multiplicity of slopes depending on the mechanism by which energy is drained from the system. However, the slopes of the flow–force relationships are always steeper when uncoupling does not occur by membrane–proton cycling.

Effect of Gramicidin on the Pump and Membrane Conductances. The *conductance of the inner mitochondrial membrane for protons* may be defined as the ratio between the rate of passive proton influx and $\Delta\tilde{\mu}_H$:

$$J^{\perp}_H/\Delta\tilde{\mu}_H \qquad (2)$$

By a similar reasoning, the *apparent conductances of the redox and of the ATPase proton pump for protons* may be defined by the ratio between the rates of respiration and of ATPase activity and $\Delta\tilde{\mu}_H$:

$$n_e J_e/\Delta\tilde{\mu}_H \qquad (3)$$

$$n_p J_p/\Delta\tilde{\mu}_H \qquad (4)$$

In the stationary state and with completely coupled pumps, the rates of respiration and of passive proton influx must be equal (eq 1), and remembering that the rate of passive proton influx is given by $L^{\perp}_H\Delta\tilde{\mu}_H$ ($L^{\perp}_H$ = the leak conductance of the membrane for protons), one obtains

$$L^{\perp}_H\Delta\tilde{\mu}_H = n_e J_e \qquad (5)$$

Equation 5 predicts that titration of the respiratory rate with a respiratory inhibitor should yield a proportional depression of the respiratory rate, $n_e J_e$, and of $\Delta\tilde{\mu}_H$. This, however, was not found, and the discrepancy was explained by Nicholls (1974) by introducing the concept of nonohmic membrane conductance, i.e., that the value of $L^{\perp}_H$ tends to increase in the region of high $\Delta\tilde{\mu}_H$ values. The dependence of the values of $L^{\perp}_H$ on $\Delta\tilde{\mu}_H$ leads to a nonlinear relationship between $J_e/\Delta\tilde{\mu}_H$ vs $\Delta\tilde{\mu}_H$, denoted as nonohmicity of the membrane.

The concept that the proton pumps may not be completely coupled (slipping pumps) leads, on the other hand, to another formulation of eq 5:

$$J_e = L_e(c)Z(1/q - q) + L^{\perp}_H/qZ \qquad (6)$$

where $L_e(c)$ is the phenomenological conductance of the respiratory chain as a function of the respiratory inhibitor concentration, Z is the phenomenological stoichiometry of the pump, and q is the degree of coupling. Equation 6 reduces to eq 5 for $q = 1$, in the case of completely coupled pumps. When $q < 1$, the function $n_e J_e/\Delta\tilde{\mu}_H$ is given by the sum of two terms: one, $L^{\perp}_H/qZ$, is a measure of the leak conductance, and the other, $L_e(c)Z(1/q - q)$, is a measure of the intrinsic uncoupling of the pump.

The comparison between eq 5 and 6 indicates that while in eq 5 the nonproportionality is a property of $L^{\perp}_H$, and thus of the lipid bilayer, in eq 6 the nonproportionality is a property also of the proton pump. Furthermore, while in eq 5 the rates of respiration and of passive proton influx should always be equivalent, this is not the case in eq 6 where the rate of respiration exceeds that of passive proton influx by a factor given by the extent of slip within the proton pump.

In the experiment shown in Figure 6, we have measured the rate of respiration and $\Delta\tilde{\mu}_H$ at increasing concentrations of antimycin with rat liver mitochondria incubated either in the absence of gramicidin D or in the presence of gramicidin but in low-salt or in high-salt media. The concentration of gramicidin D was chosen so to obtain an equivalent rate of respiration in the two media. The data are reported as the ratios of $n_e J_e/\Delta\psi$ as a function of $\Delta\psi$ in view of the fact that the changes of $\Delta\tilde{\mu}_H$ reflect strictly those of $\Delta\psi$. It is seen that in both incubation media the presence of gramicidin D resulted in a marked modification of the plot. This was essentially due to the fact that, in the high-salt medium, both the respiratory stimulatin caused by gramicidin D and the respiratory inhibition caused by antimycin were accompanied by a larger depression of $\Delta\tilde{\mu}_H$, as compared to the low-salt medium. As a consequence, the slope of the plot of $n_e J_e/\Delta\tilde{\mu}_H$ was very steep in the low-salt medium and much more smooth in the high-salt

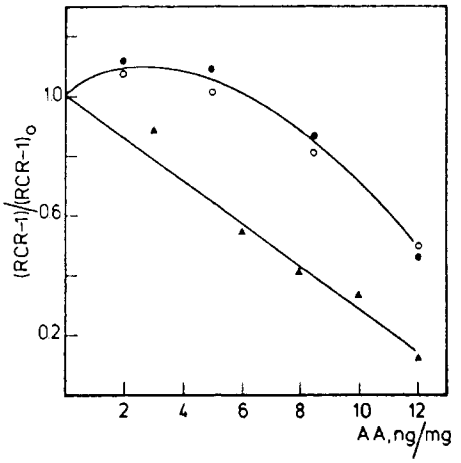

FIGURE 7: Normalized respiratory control ratio (RCR) as a function of antimycin in the presence of a constant amount of gramicidin [(●) 250 ng/mg in low-salt media; (▲) 5 ng/mg in 5 mM K^+-supplemented media] and in the absence of gramicidin (open symbols). Mitochondria (1 mg/mL) were incubated for 5 min in the presence of increasing amounts of antimycin and for 2 min in the presence of gramicidin. Succinate (10 mM) was then added, and after 1 min, FCCP (0.2 μM) was added. The RCR is calculated as the ratio between the maximal rate of respiration in the presence of FCCP and the rate of respiration in its absence. $(RCR - 1)_0$ refers to the value in the absence of antimycin. The RCR in the absence of antimycin was the following: control (open symbols), $(RCR)_0 = 8.78$; in low-salt media with 250 ng/mg of gramicidin, $(RCR)_0 = 3.64$; in K^+-supplemented media with 5 ng/mg of gramicidin, $(RCR)_0 = 3.60$.

medium. Figure 6 indicates that in the low-salt media addition of gramicidin D resulted in an increase of the apparent nonohmicity of the membrane, which becomes an expression of the extent of intrinsic uncoupling in terms of eq 6. On the other hand, in cation-supplemented media, addition of gramicidin D results in an increase of what is defined both in eq 5 and in eq 6 as ohmic conductance or leak. Thus, whatever the interpretation, the experiment of Figure 6 shows that gramicidin D, in low-salt media, does not increase the proton conductance of the membrane. This is what should have been expected from Figure 2.

Respiratory Control Ratio. In a recent paper, Pietrobon et al. (1987) have analyzed the effect of increasing antimycin concentrations on the respiratory control ratio (RCR) in the absence and in the presence of various uncoupling agents. In the range of the low antimycin concentrations, the RCR was found to slightly increase in the controls or in the presence of chloroform, and to markedly decrease in the presence of classical membrane–proton cycling agents. The decline of the RCR in the presence of proton cycling agents was interpreted as being due to the fact that the uncoupled rate of respiration, controlled only by the activity of the redox enzymes, is more sensitive to the addition of respiratory inhibitors than the resting respiration, controlled by the thermodynamic potential. That in the presence of chloroform the pattern of the RCR as a function of antimycin was more similar to that of the control mitochondria than to the FCCP-treated mitochondria was then considered to favor the view that chloroform acts by increasing the redox or proton cycling at the level of the pumps rather than the membrane–proton cycling. Figure 7 shows an experiment where the RCR of mitochondria treated with gramicidin D in low-salt and high-salt media was measured at increasing antimycin concentrations. It is seen that the RCR showed a marked decline, similarly to the FCCP–treated mitochondria (Luvisetto et al., 1987), when gramicidin D was added in high-salt media, and first an increase and then a smaller decline, only at high antimycin concentrations, when gramicidin D was added in low-salt media.

Discussion

Gramicidin D as Uncoupler. Gramicidin has been one of the first ionophores used in biological systems (Chappell & Crofts, 1965) and one of the most studied with respect to its mechanism of action (Lauger, 1980). It is generally accepted that gramicidin D is a channel-forming ionophore capable of transporting univalent cations as well as protons although not with the same affinity. As a prototype of delocalized uncoupling agents, gramicidin was used also by Herweijer et al. (1986) in submitochondrial particles and by Rottenberg and Hashimoto (1986) in rat liver mitochondria. However, it was later reported by Pick et al. (1987) that gramicidin D caused uncoupling in chloroplasts without depression of $\Delta\tilde{\mu}_H$, a behavior suggested to be due to interference of gramicidin D with H^+ transfer between primary and secondary proton pumps. In none of these studies was the dependence of the nature of the gramicidin effect on the presence or absence of cations observed. The present results are in agreement with Rottenberg and Hashimoto (1986) as to the delocalized uncoupling by gramicidin in the presence of cations and with Pick et al. (1987) as to the localized uncoupling by gramicidin D in chloroplasts. On the other hand, they are in disagreement with Pick et al. (1987) since in this work the localized uncoupling by gramicidin D was observed in high-salt media. In the present study, we distinguish two mechanisms of gramicidin D induced uncoupling in mitochondria, one membrane dependent and another pump dependent, according to whether the proton cycling occurs via the membrane or at or near the redox proton pump.

The question arises as to whether the so-called membrane-independent gramicidin D induced uncoupling is not a consequence of a heterogeneous distribution of gramicidin D among mitochondria. This would leave part of the mitochondrial population fully, or almost fully, coupled with consequent large uptake of the $\Delta\psi$ probe TPMP. However, fully uncoupling concentrations of gramicidin D are obtained at about 1 nmol·mg^{-1}, and 1 nmol·mg^{-1} is also the sum of the redox proton pumps in muscle mitochondria (Capaldi et al., 1988). The sum of the redox pumps in liver mitochondria is probably slightly lower. If we consider that liver mitochondria contain about 20 000 respiratory chains per mitochondrion (Estabrook & Holowinski, 1961), this leads to an amount of 20 000 molecules of gramicidin per mitochondrion for full uncoupling. An uneven distribution of gramicidin D molecules leaving part of the mitochondrial population free of gramicidin is therefore unlikely.

Gramicidin D as Proton Pump Uncoupler. In the present study, we report four groups of observations with respect to the mechanism of uncoupling by gramicidin D: (1) as shown in Figure 1, the uncoupling of respiration is accompanied by a depression of $\Delta\tilde{\mu}_H$ in high-salt media and by the absence of $\Delta\tilde{\mu}_H$ depression in low-salt media; (2) as shown in Figure 2, the stimulation of the respiration in low-salt media is not accompanied by an increased rate of passive proton influx; (3) as shown in Figures 3–5, the slope of the plot of respiratory rate vs output force depends on whether respiration is stimulated in the presence or absence of cations, very steep in the latter case and less steep in the former case; and (4) as shown in Figure 6, the uncoupling effect is accompanied by an increase of the *apparent* proton conductance in its ohmic part in high-salt media, and in its nonohmic part in low-salt media.

The above observations indicate that the mechanism of uncoupling of gramicidin D differs in low- and high-salt media. In high-salt media, the effects of gramicidin D are those

predicted for an agent causing uncoupling by means of a membrane–proton cycling mechanism. In this case, one would expect a marked depression of $\Delta\tilde{\mu}_H$, a proportional increase of passive proton influx, and an increase of proton conductance in the linear, ohmic, part of the plot of $n_e J_e/\Delta\tilde{\mu}_H$. On the other hand, the same lines of evidence indicate that the uncoupling effect of gramicidin D in low-salt media requires a different explanation.

Flow–Force Relationships. As mentioned in the introduction, the relationship between respiratory rate and $\Delta\tilde{\mu}_H$ has been taken as a reflection of the intrinsic dependence of the redox proton pump on its output force, and therefore the nonunique dependence of this relationship on $\Delta\tilde{\mu}_H$ taken as a reflection of two types of energy drains one more and another less delocalized (Azzone et al., 1977). In contrast, the multiplicity of the flow–force relationship has been considered as being simply a consequence of the assay procedure (Woelders et al., 1986). It appears now that this latter interpretation is not correct. In fact, the present data favor the view that the multiplicity of flow–force relationship is a consequence of the extent of localization of the energy drain and hence of the mechanism of uncoupling. This may be understood quantitatively in terms of intrinsic uncoupling by noting that the slope of the flow–force relationship reflects the value of the cross-coupling coefficient L_{eH} according to the simplified formulation of the phenomenological equations. However, in the analysis of Pietrobon and Caplan (1985), this cross-coupling coefficient may be shown to be affected and varied by the presence of a variable extent of intrinsic uncoupling in the proton pumps, and in particular by the reaction cycle; i.e., an increase of the intrinsic uncoupling causes an increase of the cross-coupling coefficient, and this results in an increased slope of the plot of J_e vs $\Delta\tilde{\mu}_H$.

Pump and Membrane Conductance. In contrast with the interpretation suggested by Nicholls (1974), we have proposed that the lack of proportionality between the depressions of respiration and of titrations with respiratory inhibitors, as predicted by a straightforward application of eq 5, is due to the presence of intrinsic uncoupling in the proton pumps (Pietrobon et al., 1981, 1983, 1986; Walz, 1983; Zoratti et al., 1986). The suggestion was based on two findings. First, one would expect that if the nonproportionality is a membrane property its range should not vary depending on which proton pump is under operation, and vice versa if the nonproportionality is a proton pump property. However, it was found that the range of nonproportionality was shifted to a lower range of about 30 mV when the redox pump was replaced by the ATPase proton pump (Pietrobon et al., 1983). Second, one would expect that if eq 5 holds in the static head, the rate of electron transfer multiplied by the H^+/e^- stoichiometry should be accounted for completely by the rate of passive proton influx at equal $\Delta\tilde{\mu}_H$. Again, this was found not to be the case (Zoratti et al., 1986; Pietrobon et al., 1986). In a recent paper (Pietrobon et al., 1987), we have shown that the changes caused by the uncouplers in the plots of $n_e J_e/\Delta\tilde{\mu}_H$ vs $\Delta\tilde{\mu}_H$ can be reproduced by computer simulations in two ways: the increase in the linear part of the plot, in the low range of $\Delta\tilde{\mu}_H$, by increasing the extent of leak; the increase in the nonlinear part, in the high $\Delta\tilde{\mu}_H$ range, by increasing the rate constant for the intrinsic uncoupling.

The comparison between the results of Figures 2 and 3 is strong evidence against the view that, in low-salt media, gramicidin D acts by increasing the *apparent* nonohmic membrane conductance for protons. If this nonohmic conductance had been a reflection of the membrane conductance for protons, there should have been a strict correlation between stimulation of the respiration and increase of the rate of passive proton influx. Instead, this correlation was not found, and the effect of gramicidin D on the passive proton influx was negligible. This result therefore becomes the third line of evidence against the concept that the nonlinear part of the plot of $n_e J_e/\Delta\tilde{\mu}_H$ be due to nonohmic conductance of the lipid bilayer.

Intrinsic Uncoupling, Decoupling, and Proton Domains. The concept of local coupling, i.e., by means of local protonic circuits or domains, is a long-standing view and considered as an alternative to overcome the inconsistencies arising from the strictly delocalized proton circuits (Azzone et al., 1977; Westerhoff et al., 1981, 1984; Hitchens & Kell, 1983; Rottenberg, 1983; Slater et al., 1985; Slater, 1987; Kamp et al., 1988). The concept of intrinsic uncoupling has been introduced by Pietrobon et al. (1981) as indicative of transfer of electrons within the pump without proton translocation or of protons without electrons. The concept of decoupling has been introduced by Rottenberg (1983) as uncoupling occurring at the level of an intramembranal pathway of protein nature, transferring protons between redox and ATPase proton pumps. The question arises as to whether these three terms, intrinsic uncoupling, local coupling or proton domains, and decoupling, can be sufficiently distinguished both at the theoretical and at the experimental level. It would seem that the concepts of *decoupling* and of *intrinsic uncoupling* have more stringent implications with respect to that of *local coupling or proton domain*; in *decoupling*, the emphasis is at the level of the protein pathway conducting the protons between the primary and secondary proton pumps; in *intrinsic uncoupling*, there is a kinetic step which allows the process of uncoupling to take place within the proton pump itself. In contrast, the concept of *local coupling or of proton domain* implies that somewhere and somehow in the membrane proteins there are areas where the protons assume a particular thermodynamic role with respect to the coupling mechanism. Clearly, the proton domain concept is much more flexible and may cope with almost any mechanism of coupling or of uncoupling.

While the evidence for a mechanism of uncoupling not depending on depression of bulk $\Delta\tilde{\mu}_H$ and on increase of proton conductance, and then on proton cycling through the membrane, is overwhelming, the distinction between the various concepts discussed above is not clear. Independently of the fact that no experimental evidence exists in favor of the proton conducting networks postulated in the decoupling concept, we note that all the effects reported in the present and in previous papers, such as stimulation of respiraton without depression of $\Delta\tilde{\mu}_H$, lack of increase of passive proton flux and of membrane proton conductance during the respiratory stimulation, and lack of decline of RCR at increasing antimycin concentrations, are compatible with all three concepts. In addition, it may also be conceived, as discussed in a recent paper (Pietrobon et al., 1987), that the same transitions within the pumps, which give rise to pump uncoupling when the two pumps operate independently, are responsible for local uncoupling or decoupling when the two pumps operate in conjunction to catalyze ATP synthesis. As we will show in the following paper (Luvisetto & Azzone, 1989), the effects of gramicidin D on ATP synthesis provide new significant information for understanding the primary event of energy coupling and hence the mechanism of coupling between primary and secondary proton pumps.

Operational Description of Gramicidin D Uncoupling. None of the present experiments is relevant to establish the location of gramicidin D in the inner mitochondrial membrane.

All of them, however, suggest that, in low-salt media, gramicidin D causes proton cycling at or near the redox proton pump. Such an effect of gramicidin can be simulated in the kinetic model of Pietrobon and Caplan (1985) by increasing the rate constants of the slip transition proportionally to the gramicidin D concentration. In fact, a slip transition operating at high rate allows electrons to be transported through the pump without concomitant vectorial proton translocation. This is equivalent to what is observed in the presence of gramicidin D where the redox proton pump operates without net transfer of protons between the bulk phases.

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