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Mechanism of Uncoupling of Oxidative Phosphorylation by Gramicidin[†]

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ABSTRACT: The mechanism of the uncoupling of oxidative phosphorylation in rat liver mitochondria by gramicidin and truncated gramicidin derivatives was investigated. The derivatives desformylgramicidin and des(formylvalyl)gramicidin are not expected to form head to head, dimeric, ion-conducting channels, and thus allow an evaluation of the relevance of the stimulation of transmembrane cation conductance (and the resulting collapse of the proton electrochemical gradient) to the uncoupling of oxidative phosphorylation. When assayed for the enhancement of the passive diffusion of KSCN, gramicidin was 100-fold more potent than desformylgramicidin and 50-fold more potent than des(formylvalyl)gramicidin. Yet, in a medium devoid of alkalai cations, all three compounds were nearly equally potent uncouplers at low concentrations. Moreover, this uncoupling was not associated with stimulation of cation transport or a reduction of the magnitude of the proton electrochemical potential. In the same medium, gramicidin stimulated ^{86}Rb uptake 50-fold more than desformylgramicidin and 10 times more than des(formylvalyl)gramicidin. At higher concentrations, gramicidin induced further uncoupling, which was associated with reduction of membrane potential (and presumably with transport of alkali cations), while the truncated derivatives were considerably less effective than gramicidin in this range. Thus, with the truncated derivatives, a better separation between decoupling (i.e., uncoupling not associated with reduction of $\Delta\tilde{\mu}_H$) and uncoupling is observed. In the same medium, gramicidin, but not the truncated derivatives, strongly inhibits the formation of both the membrane potential and ΔpH by the H^+-ATPase. This finding suggests direct interaction of gramicidin with the H^+-ATPase. The truncated derivatives stimulated the ATPase without collapsing the membrane potential. We conclude that decoupling of oxidative phosphorylation by gramicidin does not depend on transmembrane cation transport but results from specific interference with direct proton transfer to the H^+-ATPase.

The linear gramicidins are short hydrophobic peptide antibiotics produced by *Bacillus brevis*. Gramicidin A, the major component of the commercially produced mixture, is a peptide chain of 15 amino acids with alternating amino acids of opposite chirality. The amino head-group (valine) is blocked by a formyl, and the carboxy tail-group is blocked by ethanolamine. When incorporated into lipid bilayer membranes, the peptide may assume several helical conformations. A "channel" conformation in which two monomers form head to head dimers with left-handed single-stranded $\beta^{6.3}$-structure, which traverses the membrane from surface to surface, is believed to be the major ion conducting channel (Weinstein et al., 1980; Urry et al., 1983). The channel conducts small monovalent cations with relatively low selectivity ($H^+ > K^+ > Na^+$). Another structure which may form a transmembrane channel, which is called "Pore", is a left-handed antiparallel double-helix dimer in which the intertwined monomers traverse the membrane in opposite directions of each other. This form is believed to be of minor importance in ion conducting. The structures and conductances of the gramicidin channel, the simplest and best characterized membrane channel, have been studied extensively [reviewed in Andersen (1984), Cornell (1987), and Urry (1985)].

It is generally believed that the biological activities of the gramicidins (e.g., inhibition of bacterial growth, uncoupling of oxidative phosphorylation, and photophosphorylation) are due to the activity of the dimer channel (Bakker, 1979). It is assumed that the conductance of monovalent cations, or protons, leads to the collapse of either $\Delta\psi$ or ΔpH which results in reduction of the proton electrochemical potential and hence the uncoupling. This belief is based on the demonstration, many years ago, that in mitochondria gramicidin at high concentrations, and in the presence of monovalent cations, induces massive cation uptake and swelling (Pressman, 1965; Chappell & Crofts, 1965). In this, the gramicidin effect is similar to valinomycin and likewise is associated with reduction of $\Delta\psi$, the major component of $\Delta\tilde{\mu}_H$ in mitochondria (Rottenberg, 1973). Similarly, in chloroplasts, in which ΔpH is the major component of $\Delta\tilde{\mu}_H$, a high concentration of gramicidin increases proton permeability and collapses $\Delta\tilde{\mu}_H$ (Rottenberg et al., 1971). These observations, which appeared to be compatible with the explanation of uncoupling by the chemiosmotic theory, were assumed to confirm the suggestion that the gramicidins uncouple by the collapse of $\Delta\tilde{\mu}_H$. However, more recently, several lines of evidence suggested that $\Delta\tilde{\mu}_H$ might not be the only intermediate in the synthesis of ATP by the H^+-ATPase [reviewed in Rottenberg (1985)]. This led to a more rigorous examination of the relationship between uncoupling and the collapse of $\Delta\tilde{\mu}_H$. In mitochondria, valinomycin and high concentrations of potassium led to the collapse of $\Delta\psi$ in parallel to the inhibition of phosphorylation and stimulation of static head respiration. If gramicidin uncoupled by the same mechanism, it was expected to find a similar dependence of phosphorylation and respiration on $\Delta\tilde{\mu}_H$ as for valinomycin. However, even in high concentrations of sodium or potassium, uncoupling by gramicidin is not associated with as large a reduction of $\Delta\tilde{\mu}_H$ as valinomycin (Rottenberg & Hashimoto, 1986). We have previously identified several uncouplers which uncouple oxidative phos-

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phorylation without significant effect on $\Delta\tilde{\mu}_H$, which we call "decouplers". We concluded that gramicidin, even in the presence of high salt concentration, works partially as a classical uncoupler and partially as a decoupler. Only when $\Delta\tilde{\mu}_H$ was generated by ATP did gramicidin collapse $\Delta\tilde{\mu}_H$ at low concentration in parallel with the stimulation of ATPase activity.

In chloroplasts, the characteristics of gramicidin uncoupling were identical with other decouplers; e.g., uncoupling of photophosphorylation occurs at low concentrations in which there is no significant effect on $\Delta\tilde{\mu}_H$ (Pick et al., 1987). Moreover [as found for fatty acids in mitochondria (Rottenberg & Steiner-Mordoch, 1986)], gramicidin in low concentrations does not uncouple ATP synthesis when the latter is driven by artificially generated $\Delta\tilde{\mu}_H$. Additionally, gramicidin is a more potent inhibitor of photosystem II driven phosphorylation than photosystem I driven phosphorylation (Pick et al., 1987). These findings suggested to us that the gramicidin uncoupling of oxidative phosphorylation and photophosphorylation does not depend on the formation of a cation-conducting dimer channel and it is not due to intrinsic uncoupling of the ATPase either. Rather, it appears that gramicidin interferes directly with proton transfer between redox and the ATPase pumps.

To further investigate the mechanism of uncoupling by gramicidin in mitochondria, we decided to extend our previous studies in two directions (a) to compare the effect of gramicidin on phosphorylation, respiration, ATPase, and $\Delta\tilde{\mu}_H$ without external sodium or potassium in an effort to dissociate the cation uptake effect from the decoupling effect and (b) to compare the effects of gramicidin (Gram) with two gramicidin derivatives, desformylgramicidin (desfor) and des(formylvalyl)gramicidin (desval). Desformylgramicidin is a derivative in which the formyl group at the head of the peptide was removed. Des(formylvalyl)gramicidin is further truncated by removal of the valine group. The ability to form head to head dimer channels should be greatly attenuated in these derivatives, and if the uncoupling depends on the ion conductance of the head to head dimmer, we do not expect these derivatives to uncouple oxidative phosphorylation. The experiments described below show that decoupling by gramicidin and the derivatives is comparable and is not associated with reduction of $\Delta\tilde{\mu}_H$. Thus, the decoupling activity of the gramicidins at low concentration and in the absence of salt does not require the formation of channels and is not due to facilitation of cation transport across the membrane. In contrast, the truncated derivatives are orders of magnitude less effective than gramicidin in enhancing passive $K^+(SCN)^-$ uptake and oxidation-dependent ^{86}Rb uptake at low salt concentrations, which suggests that this process is due to the head to head "channel" structure as is the case in artificial phospholipid bilayers. It appears that there are specific interactions of the gramicidins with the H^+-ATPase which lead to decoupling.

EXPERIMENTAL PROCEDURES

Preparation of Mitochondria. Rat liver mitochondria were prepared by conventional differential centrifugation, as described previously (Rottenberg & Hashimoto, 1986).

Synthesis of Gramicidin Derivatives. Gramicidin D from Sigma Chemical Co. (St. Louis), consisting of approximately 80% gramicidin A, 5% gramicidin B, and 15% gramicidin C, was deformylated by treatment for 1 h at 40 °C in 2 N HCl in anhydrous methanol/dioxane (50:50) and was purified by ion-exchange chromatography twice on AGMP-50 resin (Bio-Rad) and by molecular sieve chromatography on Sephadex LH-20 (Pharmacia), as previously described (Weiss & Koeppe, 1985). A portion of the desformylgramicidin was treated with phenyl isothiocyanate followed by HCl to remove the N-terminal amino acid and was purified, first on LH-20 and then twice on AGMP-50 (Weiss & Koeppe, 1985). The desfor and desval products were assayed by reversed-phase HPLC (Koeppe & Weiss, 1981) and were shown to be devoid of unreacted (formylated) gramicidin at the limits of detection (approximately 0.5%).

Measurement of K^+ Transport. The measurement of the rate of passive uptake of KSCN by mitochondria follows the procedure described by Beavis and Garlid (1987). The rate of swelling of mitochondria was followed by measuring light absorption at 520 nm. The medium was composed of 60 mM KSCN, 5 mM Tris-HCl, and 1 mM EGTA, pH 7.4. Mitochondria was added (0.1 mg of protein/mL) and the uptake started by the addition of gramicidin or its derivatives. Rates of K^+ uptake were estimated from the initial rate of swelling. The rate of Rb^+ transport was measured from ^{86}Rb uptake, as described previously (Rottenberg, 1979).

Measurement of Membrane Potential. Membrane potential was estimated from the distribution of [^{3}H]tetraphenylphosphonium (TPP^+) and corrected for binding, essentially as described previously (Rottenberg, 1984).

Measurement of ΔpH. ΔpH was estimated from the distribution of [^{14}C]DMO, as described previously (Rottenberg, 1979).

Measurement of Matrix Volume. Matrix volume was estimated from the distribution of [^{14}C]sucrose and ^3H_2O, as described previously (Rottenberg, 1979).

Measurement of the Rate of ATP Hydrolysis. ATP hydrolysis was measured enzymatically, as described previously (Pullman et al., 1960).

Measurement of the Rate of ATP Synthesis. ATP synthesis was estimated from the incorporation of [^{32}P]P_i into [^{32}P]ATP, as described previously (Shahak, 1982). The rate of respiration was measured by a polarographic oxygen electrode. Protein concentration was determined by the biuret method (Szarkowska & Klingenberg, 1963). Gramicidin D, Tris–ATP, Tris–ADP, and all other fine chemicals were obtained from Sigma. All other reagents were of analytical grade. Gramicidin and their derivatives were dissolved in ethanol (1 mg/mL). A series of dilution in ethanol were prepared daily from stock solutions. The diluted solutions (1–100 μg/mL) were added directly to the mitochondrial suspension to obtain the final concentrations.

RESULTS

Cation Channel Conductance of Gramicidin Derivatives. To assess the concentration dependence of the enhancement of cation conductance by Gram, desfor, and desval, we utilized an assay which allows estimation of the rate of potassium transport from the rate of mitochondrial swelling in a hypotonic solution of KSCN (Beavis & Garlid, 1987). The permeable SCN^- anion creates a diffusion potential that drives K^+ uptake at a rate limited by the permeability of potassium. Salt uptake is accompanied by swelling, from which the rate of uptake is estimated. Figure 1 shows the dependence of the rate of K^+ uptake on gramicidin concentration. As expected, gramicidin is a much more potent channel former than either derivative. Removal of the formyl group (desfor), which is expected to prevent a head to head hydrogen-bonding dimer, resulted in a 2 order of magnitude reduction in K^+ transport. Desval, which is a shorter peptide than desfor, is approximately twice as active but still much less active than Gram. The K^+ conductance by the truncated derivatives is not due to contamination by gramicidin. Further purification of desfor did

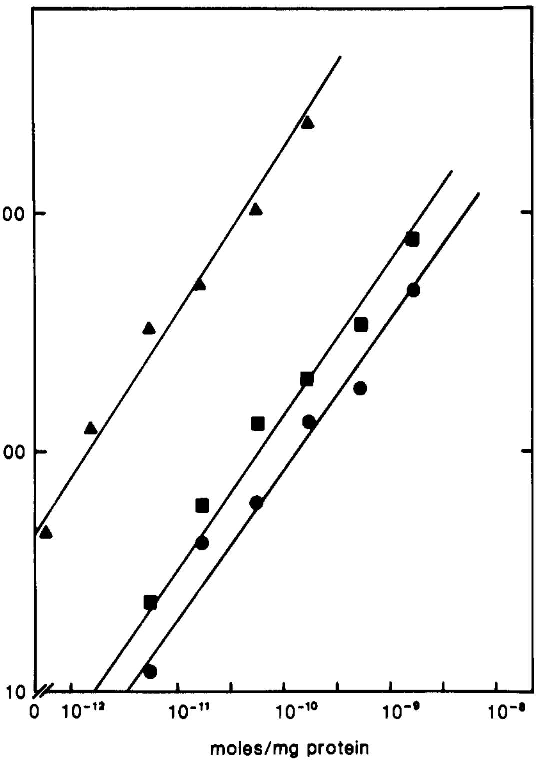

FIGURE 1: Concentration dependence of KSCN uptake induced by gramicidin (▲), desformylgramicidin (●), and des(formylvalyl)-gramicidin (■). The rate of passive KSCN uptake by rat liver mitochondria was estimated from the rate of swelling. Swelling was followed spectrophotometrically at 520 nm. The medium was 60 mM KCNS, 5 mM Tris-HCl, and 1 mM Tris-EGTA, pH 7.4. Mitochondria were added at 0.1 mg of protein/mL, and the transport was initiated by the addition of the indicated concentration of the various gramicidins. All experiments were preformed in parallel on the same mitochondrial preparation. The results are the average of three preparations.

not result in reduction of K^+ conductance. Moreover at high salt concentration, the truncated derivative induced macroscopic fluxes that approach gramicidin (Rottenberg & Koeppe, 1989). Thus, their effects appear to be a genuine activity of the truncated derivatives. The residual enhancement of K^+ transport by the truncated peptides does not necessarily indicate limited capacity for head to head dimer formation (channel). It is possible that an alternative ion-conducting form may be responsible for the residual activity (Durkin et al., 1987). Nevertheless, if uncoupling by gramicidin depends on cation conductance, we expect these derivatives to be 2 orders of magnitude less active than gramicidin.

Effect of Gramicidin Derivatives on Oxidative Phosphorylation. Figure 2 shows the uncoupling of oxidative phosphorylation, and the effect on membrane potential by Gram (A), desfor (B), and desval (C). The experiments shown in Figure 2 were carried out in a medium which did not contain added sodium or potassium salts. As we showed previously, the inclusion of sodium and/or potassium salts in the medium leads to considerable reductions of $\Delta\psi$, even at relatively low concentrations of gramicidin (Rottenberg & Hashimoto, 1986; Rottenberg, 1973). However, when these cations are eliminated from the medium, there is little effect on membrane potential up to about 50 pmol of gramicidin/mg of protein. Moreover, the slight decline of $\Delta\psi$ over this range is accompanied by a parallel increase in ΔpH (Figure 6), so that the value of $\Delta\tilde{\mu}_H$ is unchanged. Yet at this range more than 50% uncoupling of oxidative phosphorylation is observed. Higher concentrations of gramicidin lead to further uncoupling, which is associated with a decline of $\Delta\tilde{\mu}_H$. Thus, in (Na^+, K^+)-free medium, it is possible to separate decoupling (i.e., uncoupling without collapse of $\Delta\tilde{\mu}_H$), which is observed at low concentrations of gramicidin, from uncoupling, which is observed at high concentrations. As Figure 2B,C shows desfor and desval are almost equal to gramicidin in their potency as decouplers; i.e., they partially uncouple oxidative phosphorylation without effect on $\Delta\psi$ (or ΔpH, not shown). They are, however, less effective as uncouplers in low-salt medium, since their effects at high concentration on $\Delta\tilde{\mu}_H$ and oxidative phosphorylation are much less pronounced. Thus, with these derivatives, the separation between the decoupling activity and uncoupling activity in (Na^+, K^+)-free medium is even more effective than in the case of gramicidin. Under the conditions of Figure 2, we could not detect induction of swelling (indicator of massive salt uptake) by any of the gramicidins. Comparing the results of Figures 1 and 2, we can conclude that decoupling of oxidative phosphorylation by low concentrations of gramicidin and its derivatives does not depend upon the activity of the cation-conducting dimer channel. Moreover, since $\Delta\tilde{\mu}_H$ is not reduced significantly at the decoupling range, the effects on phosphorylation and respiration could not be the result of increased proton permeability.

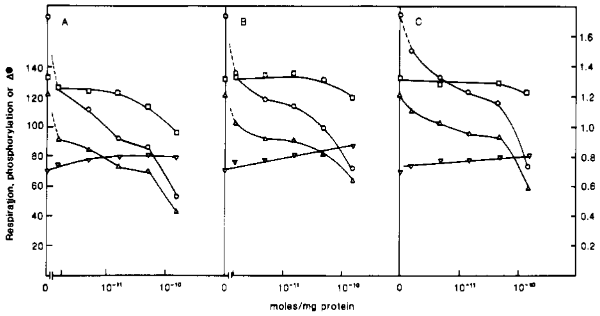

FIGURE 2: Concentration dependence of the uncoupling of oxidative phosphorylation in (Na^+, K^+)-free medium induced by gramicidin (A), desformylgramicidin (B), and des(formylvalyl)gramicidin (C). The rates of phosphorylation (△) and oxidation (▽), the membrane potential (□), and the calculated P:O ratio (○) are shown. Medium was composed of 0.2 M sucrose, 5 mM Tris-phosphate, 5 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM Tris-ADP, and 5 mM Tris-succinate. Rotenone was 2 μM with the indicated concentration of gramicidin. All experiments were performed, as described under Experimental Procedures, in parallel on the same mitochondrial preparation. Results are the average of three preparations.

Effect of Gramicidins on Static Head Respiration. Another important measure of uncoupling is the stimulation of static head ("state 4") respiration. Classical uncoupling by ionophores such as valinomycin collapses the proton electrochemical potential in parallel with the stimulation of state 4 respiration and the reduction of respiratory control. Figure 3 shows that in a (Na^+, K^+)-free medium the stimulation of state 4 respiration (or the reduction of respiratory control) is not associated with a significant reduction of $\Delta\tilde{\mu}_H$ at low concentrations. The slight reduction observed with gramicidin is associated with a slight increase in ΔpH (Figure 6), while desfor and desval have no effect on either $\Delta\psi$ or ΔpH at low concentration. As in Figure 2, high concentrations of Gram and desval lead to further stimulation which is associated with a reduction of $\Delta\psi$ (uncoupling). We could not detect any swelling in these experiments either. Thus, the stimulation of state 4 respiration at low concentrations is not associated with cation conductance. At low concentrations, the potency of the derivatives was only slightly less pronounced than that of gramicidin, indicating that the decoupling does not depend on channel formation. However, at higher concentrations, the uncoupling of gramicidin is much more pronounced and is associated with a larger reduction of $\Delta\psi$.

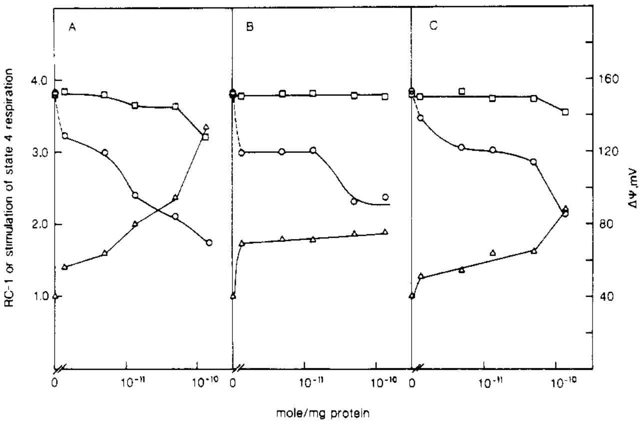

FIGURE 3: Concentration dependence of the uncoupling of respiration in (Na^+, K^+)-free medium by gramicidin (A), desformylgramicidin (B), and des(formylvalyl)gramicidin (C). Procedures for the determination of respiration (△) and membrane potential (□) are the same as in Figure 2. The medium was the same medium as in Figure 2 except that only 50 μM Tris–ADP was added to the oxygen electrode chamber (twice) and the respiratory control ratio (○) and state 4 respiration were estimated from the rates of state 3 and state 4. Experiments were preformed in parallel on the same batch of mitochondria. Results are the average of three preparations.

Under the same conditions as in the experiments of Figure 3, we measured uptake of ^{86}Rb (at trace amounts of RbCl). Figure 4A shows the gramicidin-induced uptake of ^{86}Rb in low-salt medium. The results are similar to those of Figure 1. Gramicidin is much more potent in stimulation of Rb uptake than desval, and the latter is more potent than desfor. In Figure 4A, gramicidin, at high concentrations, does not further stimulate Rb uptake. This effect is the result of the depletion of the external Rb (and hence an increase of $\Delta\tilde{\mu}_{Rb}$) at these concentrations. The slopes of the curves in Figure 4A appear to be steeper with the less active derivatives. This effect is due to an increase in the depletion of the external Rb as the activity of the channel is increased. To correct this effect, we normalized the uptake to the ^{86}Rb concentration in the medium after 1 min of uptake. This is shown in Figure 4B, which shows the Rb concentration ratio as a function of the gramicidin concentrations. Plotted this way, the slopes appear equal. It is observed that gramicidin is 50-fold more effective than desfor in stimulating ^{86}Rb uptake (similar to the difference observed in the KSCN diffusion experiments, Figure 1). Desval appears somewhat more potent in this process, approximately 5-fold more active than desfor and 10-fold less active than gramicidin, which agrees with their relative effects on respiration at high concentration.

Effect of Gramicidins on the Activity of H^+-ATPase. Figure 5 shows the effect of Gram (A), desfor (B), and desval (C) on the rate of ATP hydrolysis and membrane potential generated by the H^+-ATPase pump in the absence of alkali salts. The effect of gramicidin on the H^+-ATPase is different in several respects from the effect on respiration. Gramicidin strongly collapses $\Delta\psi$ generated by the ATPase, even in the absence of added salt. Even more remarkable is the effect on ΔpH. While gramicidin enhances the ΔpH generated by the respiratory chain, as expected from its effect on enhanced cation uptake, it very strongly collapses ΔpH when the latter is generated by the H^+-ATPase (Figure 6). This specific effect suggests a specific interaction of gramicidin with the H^+-ATPase. Desfor and desval behave somewhat differently. In the absence of salt (as was observed in state 4, Figure 3), there is no significant effect on $\Delta\psi$, while there is considerable stimulation of ATPase activity. The stimulation of ATPase by the truncated derivatives is considerably more pronounced than that of the respiratory chain.

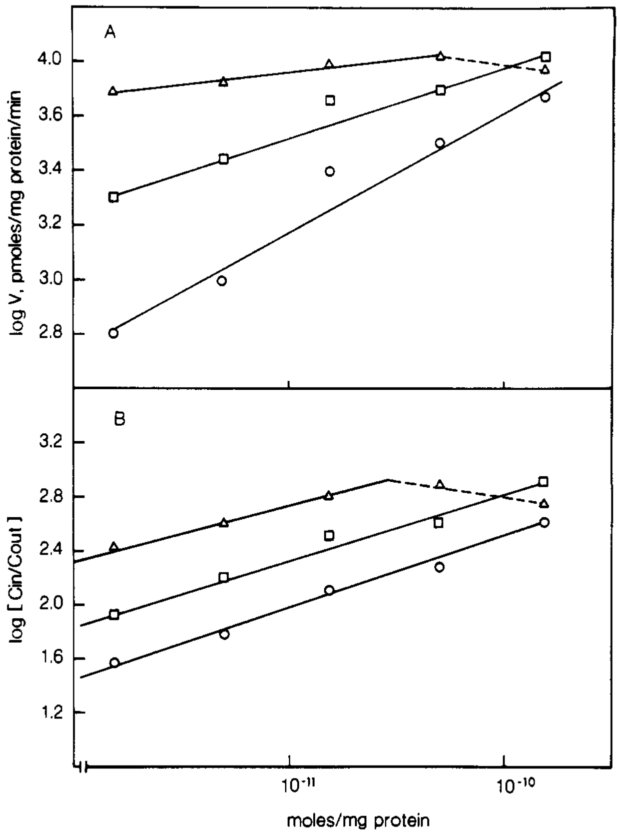

FIGURE 4: Stimulation of respiration-driven ^{86}Rb uptake by gramicidin. Conditions are the same as in Figure 3, except for the addition of a trace concentration of RbCl together with ^{86}Rb. Mitochondria (2.8 mg of protein/mL) were incubated in the assay medium with the indicated concentration of gramicidin. After 1-min incubation, the mitochondria were separated by rapid centrifugation and the pellet and supernatant samples counted for ^{86}Rb, as described before (Rottenberg, 1979). Panel A shows the pellet accumulation of ^{86}Rb (corrected for trapped medium). Panel B shows the intramitochondrial:extramitochondrial ratio of ^{86}Rb. Results are the average of three experiments [gramicidin (△); desformylgramicidin (○); des(formylvalyl)gramicidin (□).

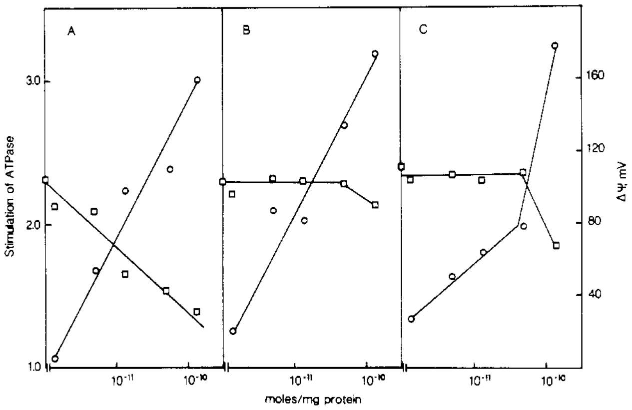

FIGURE 5: Concentration dependence of the uncoupling of H^+-ATPase by gramicidin (A), desformylgramicidin (B), and des(formylvalyl)-gramicidin (C). Membrane potential (□) was determined as in Figure 2. ATPase rates (○) were determined enzymatically, as described under Experimental Procedures. The medium was the same as in Figure 2, except that Tris–ADP was omitted and replaced with 2 mM Tris–ATP. The average rate of ATP hydrolysis in the absence of gramicidin was 72 nmol (mg of protein)$^{-1}$ min^{-1}.

DISCUSSION

Gramicidin Channel in Rat Liver Mitochondria. Gramicidin may exist in several dimeric complexes. It is generally

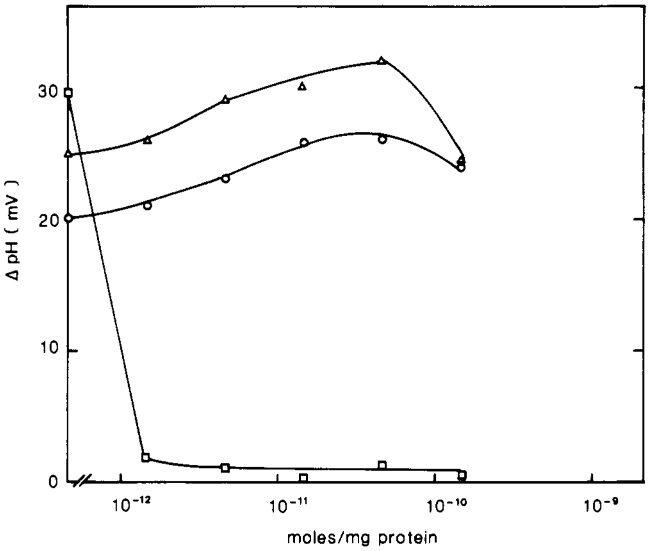

FIGURE 6: Effect of gramicidin on ΔpH in (Na^+, K^+)-free medium. ΔpH was measured from the distribution of [^{14}C]DMO, as described (Rottenberg, 1979). Medium for state 3 (○) was as in Figure 2; medium for state 4 (△) was as in Figure 3 (without ADP); medium for ATPase (□) was as in Figure 5. Results are the average of three separate preparations. $\Delta\psi$ was measured in parallel and is shown in Figures 2, 3, and 5.

believed that the major cation-conducting channel in phospholipid bilayers is the head to head single-stranded $\beta^{6.3}$-helix structure, generally referred to as the "Channel" (Urry, 1971; Weinstein et al., 1980; Urry et al., 1983). Another structure, referred to as "Pore", was recently crystallized and analyzed by X-ray diffraction to very high resolution (Wallace & Ravikumar, 1988). This double-helix antiparallel structure, which predominates in organic solvents (Veatch et al., 1974), is believed to be important in gramicidin incorporation into bilayers. However, it is generally assumed that this structure contributes only marginally to cation conductance in lipid bilayers. On the basis of these notions, we have planned this study to examine the relationships between cation conductance and uncoupling. We expected that the truncated gramicidins, desformylgramicidin and des(formylvalyl)gramicidin, would not conduct cations and thus allow a better separation of the uncoupling effect from the cation conductance effect. This expectation is based on the fact that the formyl groups at the head of the peptide are essential for hydrogen bonding in the head to head structure that holds the dimer together. When passive KSCN transport was assayed, the derivatives were indeed orders of magnitudes less active than gramicidin, as expected if the "Channel" form is the major conducting species. Also, when respiration-driven transport of ^{86}Rb was induced in low-salt medium, gramicidin was orders of magnitude more potent than the derivatives, suggesting that under these conditions, too, the channel form is the dominant conducting form. However, the fact that the truncated derivatives also conduct K^+ and Rb^+, and in particular the fairly potent activity of des(formylvalyl)gramicidin, suggests that other structures may contribute to the conductance in this case (Durkin et al., 1987). Indeed, we found that at high salt concentrations, the conductance of the derivatives is further enhanced, approaching the conductance of gramicidin itself (Rottenberg & Koeppe, 1989). The slopes of the curves in Figures 1 and 4, which relate cation transport to peptide concentration, are less than 1 for both the gramicidin and the truncated derivatives. The original observation of Tosteson et al. (1968) of a slope of 2 (indicating dimer formation), though often cited, has not been widely observed. Apparently, the slope depends on the lipid composition (Goodall, 1970), and it is quite possible that a large fraction of the gramicidin exists in the form of other (nonconducting) complexes (e.g., protein–gramicidin complexes) which could reduce the slope to the observed values.

Uncoupling of Oxidative Phosphorylation. The experiments of this study clearly demonstrate that it is possible to separate the decoupling effect of gramicidin from the effect on cation translocation. Of course, as expected, it is possible to enhance uncoupling by addition of high concentrations of alkali salts, which collapse the membrane potential. However, in contrast to valinomycin (Hofer & Pressman, 1966; Rottenberg, 1970), gramicidin will also uncouple in a medium devoid of alkali cations and without any effect on $\Delta\tilde{\mu}_H$ (decoupling). This activity is separated from the potential-dependent uncoupling, which in low-salt medium is significant only at high gramicidin concentrations. The separation is even better in the truncated gramicidins. Because the derivatives are less effective in inducing cation transport (in low salt), they cause less reduction of $\Delta\mu_H$. Since neither ΔpH nor $\Delta\psi$ is reduced by the truncated derivatives at the uncoupling range, it is clear that proton permeability is not increased and cannot be the cause of the uncoupling. Moreover, even the slight uncoupling that is observed at high concentrations appears to result from enhanced cation uptake since ΔpH was not reduced. Indeed, because of the high pH of the mitochondrial suspension and the low selectivity of gramicidin, proton uptake is never a factor in uncoupling of mitochondria by gramicidin. The situation is quite different in chloroplasts, where the internal pH is very low and the pH gradient is very high and gramicidin under some conditions, but not generally, uncouples by increasing proton permeability (Pick et al., 1987).

Interaction with the ATPase. The specific effect of gramicidin on the activity and the H^+ gradient generated by the ATPase is a strong indication for specific interactions. Gramicidin at low concentrations collapses the membrane potential generated by the ATPase, even in the absence of salt, in contrast to its effect on the membrane potential generated by oxidation. Even more unexpected is the effect on ΔpH. The reduction of membrane potential by gramicidin (or valinomycin) is usually associated with an increase of ΔpH. This is because the reduced potential (due to the inward cation current) stimulates proton pumping further, resulting in increased ΔpH (Figure 6). The fact that in the absence of salt, when generated by H^+-ATPase, both ΔpH and $\Delta\psi$ are collapsed and ΔpH even more quickly than $\Delta\psi$ indicates that gramicidin conducts protons and not cations under these conditions. Considering the weak selectivity of the gramicidin channel and the concentration of external sodium (which even up to 50 mM did not affect this phenomenon), it is expected that at external pH 7.4 the Na current will predominate. The fact that the proton current dominates only when protons are generated by ATPase suggests that gramicidin is able to accept protons directly from the ATPase or at least from a localized domain which is adjacent to the ATPases. A similar specific access of gramicidin to protons, which appears to be localized in an intramembranal domain, was observed in chloroplasts (Theg & Junge, 1983).

Mechanism of Decoupling by Gramicidin. The strong effect of gramicidin on proton pumping by the H^+-ATPase is no doubt related to its decoupling of oxidative phosphorylation. One possible mechanism, which was suggested to explain the action of other decouplers (Pietrobon et al., 1987), is that gramicidin uncouples H^+ translocation from ATP hydrolysis (or synthesis) intrinsically within the ATPase H^+ pump. However, this explanation is not compatible with our findings that in chloroplasts low concentrations of gramicidin, which inhibit light-driven ATP synthesis, either by photosystem II or by photosystem I, are without effect on artificial cyclic photophosphorylation in which protons are pumped by an artificial redox H^+ mediator (pyocyanine) (Pick et al., 1987). Moreover, photophosphorylation driven by photosystem II is much more sensitive to gramicidin than photosystem I. Also, in submitochondrial particles, gramicidin has a relatively weak effect on ATP synthesis when the latter is induced by artificially imposed $\Delta\tilde{\mu}_H$ (not shown). Hence, it appears that it is the direct proton transfer from redox pumps to H^+-ATPase which is short-circuited by gramicidin. This model could explain the specific effect of gramicidin on the ATPase, the inhibition of phosphorylation driven by the redox pump, the stimulation of respiration, and the lack of inhibition of phosphorylation driven by artificially generated $\Delta\tilde{\mu}_H$. It is not clear as yet how gramicidin short-circuits the intermembranal proton current. An attractive hypothesis is offered by the recent observation that monomers of gramicidin derivatives may also form the $\beta^{6.3}$-helix, thus creating half a channel (Killian et al., 1988). This half-channel may have direct access to protons in the intramembranal pathway, which is presumably associated with the DCCD binding protein of the H^+-ATPase (Rottenberg, 1988). Alternatively, the release of intramembranal protons may be related to the recently discovered effects of gramicidin on phospholipid structure (Killian & de Kruijff, 1987). Thus, gramicidin may modify the lipid–protein interactions which are needed for intramembranal occlusion of protons.

While the release of occluded intramembrane protons does not require transmembrane channel formation, the gramicidin-induced collapse of $\Delta\tilde{\mu}_H$, when generated by the H^+-ATPase, appears to require the formation of the head to head dimer channel. Hence, even though the truncated derivatives are almost as effective as gramicidin in their decoupling activities, they are not able (at low salt concentrations) to conduct protons across the membrane and collapse the $\Delta\tilde{\mu}_H$ generated by H^+-ATPase (Figure 5).

In conclusion, our study demonstrates that decoupling of oxidative phosphorylation by the gramicidins does not depend on transmembrane cation transport and the collapse of $\Delta\tilde{\mu}_H$. The decoupling appears to be due to interaction with the H^+-ATPase and the gramicidins' ability to release occluded intramembranal protons.

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Registry No. ATP, 56-65-5; ATPase, 9000-83-3; gramicidin D, 1393-88-0; desformylgramicidin, 74505-27-4; des(formylvalyl)-gramicidin, 71388-81-3.

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