Stimulation of Cation Transport in Mitochondria by Gramicidin and Truncated Derivatives†

Hagai Rottenberg*,‡ and Roger E. Koeppe, II§

Department of Pathology, Hahnemann University, Philadelphia, Pennsylvania 19102, and Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701

Received November 7, 1988; Revised Manuscript Received February 3, 1989

ABSTRACT: Gramicidin and the truncated derivatives desformylgramicidin (desfor) and des(formylvalyl)gramicidin (desval) stimulate monovalent cation transport in rat liver mitochondria. Cation fluxes were compared indirectly from the effect of cations on the membrane potential at steady state (state 4) or from the associated stimulation of electron transport. Rb^+ transport was measured directly from the uptake of ^{86}Rb. The truncated gramicidins show enhanced selectivity for K^+ and Rb^+ when compared to gramicidin. Moreover, the pattern of selectivity within the alkali cation series is altered, i.e., $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ for desfor and desval as compared to $Cs^+ > Rb^+ > K^+ = Na^+ > Li^+$ for gramicidin. The cation fluxes through the truncated derivatives are more strongly dependent on the cation concentration. The presence of high concentrations of permeating cation enhances the transport of other cations through the truncated derivative channels, suggesting that cations are required for stabilizing the channel structure. In high concentrations of KCl, desfor and desval are nearly as effective as gramicidin in collapsing the mitochondrial membrane potential, and, consequently, in the uncoupling of oxidative phosphorylation and enhancement of ATP hydrolysis. Preliminary experiments with liposomes show that ^{86}Rb exchange is stimulated by desfor and desval almost to the same extent as gramicidin. These results strongly suggest that the truncated gramicidins form a novel conducting channel which differs from the gramicidin head-to-head, single-stranded $\beta^{6.3}$-helical dimer ("channel") in its conductance characteristic and its structure. On the basis of the secondary structure of the truncated derivatives, we suggest that the antiparallel double-stranded helix dimer ("pore") is a likely alternative structure for this novel channel.

The linear gramicidins are short-peptide antibiotics produced by *Bacillus brevis*. When incorporated into bilayer lipid membranes, they form a dimeric channel which conducts alkali cations [reviewed in Andersen (1984), Cornell (1987), and Urry (1985)]. The first demonstration that the linear gramicidins induce cation transport in biological membranes came from studies of rat liver mitochondria in which substrate oxidation, in the presence of gramicidin, induced massive alkali cation uptake and swelling (Pressman, 1965; Chappel & Crofts, 1965). It was shown later that gramicidin allows equilibration of both Na^+ and K^+ across the mitochondrial membrane in a process driven by the electrochemical gradient of these ions (Rottenberg, 1973). Studies with other membrane systems led to the suggestion that gramicidin opens a "pore" or a "channel" which allows free diffusion of alkali cations across the membrane (Hladky & Haydon, 1970). The structures of gramicidin dimers and the mechanism of ion transport by the gramicidin channel in artificial phospholipid membranes have been studied extensively over the last 2 decades (see reviews cited above). It is now accepted that the structure of the ion-conducting gramicidin channel is a head-to-head dimer of single-stranded $\beta^{6.3}$-helices as first proposed by Urry et al. (1971). However, a second dimeric structure ("pore"), an antiparallel double helix, exists in organic solvents (Veatch et al., 1974) and may be also capable of forming a conducting channel (Wallace & Ravikumar, 1988; Sung & Jordan, 1988).

We have recently examined in detail the uncoupling by gramicidin of ATP synthesis in mitochondria and chloroplasts (Rottenberg & Hashimoto, 1986; Pick et al., 1987). Our results suggested to us that the uncoupling is not due entirely to cation or proton transport across the membranes. In order to separate the uncoupling activity due to cation transport from the uncoupling which is unrelated to the transport (decoupling), we have synthesized truncated derivatives in which the formyl headgroup was removed. These derivatives are not expected to form head-to-head dimers and thus should be devoid of cation transport activity. In the low-salt medium, in which we conducted our investigation of the uncoupling of oxidative phosphorylation by gramicidin and its derivatives, respiration-induced Rb^+ transport and the reduction of membrane potential were indeed much more pronounced with gramicidin than with the truncated derivatives (Rottenberg & Koeppe, 1989). However, these derivatives were not totally devoid of conductance activity. In experiments of passive diffusion of KSCN, gramicidin was 100-fold more potent than desformylgramicidin, but only 50-fold more potent than des(formylvalyl)gramicidin. Moreover, in experiments of respiration-driven Rb^+ transport, in low-salt medium, the difference in activity between gramicidin and des(formylvalyl)gramicidin was much smaller, amounting only to 10-fold. We have verified that these activities of the derivatives are not due to contamination by gramicidin since further purification of desformylgramicidin did not result in reduction of activity. Thus, these activities appear to be a genuine ion conductance by the derivatives. Since the removal of the formyl group from the "head" of the peptide should prevent

† Supported by NIH Grants GM-28173 (to H.R.) and GM-34968 (to R.E.K.).

* Address correspondence to this author at the Department of Pathology, Hahnemann University, MS 435, Broad and Vine, Philadelphia, PA 19102.

‡ Hahnemann University.

§ University of Arkansas.

0006-2960/89/0428-4361$01.50/0

the formation of the head-to-head dimer, it appears that this activity is due to the formation of an alternative channel structure (Durkin et al., 1987).

In this study, we compared the alkali cation conductance of gramicidin and the truncated derivatives in rat liver mitochondria in order to characterize the ion transport process by this novel conducting structure.

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 were added (0.1 mg of protein/mL), and the uptake was 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). The standard error in these measurements is about 5%.

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). The standard error in these measurements is ±3 mV.

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

To compare the alkali cation conductance of gramicidin, desformylgramicidin (desfor), and des(formylvalyl)gramicidin (desval) in rat liver mitochondrial membranes, we employed two indirect, but highly sensitive assays, namely, the reduction of static head (state 4) membrane potential and the stimulation of static head respiration. Both assays depend on the generation of a large negative membrane potential by the mitochondrial redox H^+ pumps. Substrate oxidation induces electrogenic H^+ transport out of the matrix which leads to the generation of a large negative membrane potential. The magnitude of the potential that is generated by proton pumping depends on the passive cation (and proton) conductance of the membrane. Incorporating a cation channel and the addition of cations externally induces an inward-directed cation current which short-circuits the potential and reduces its magnitude. Thus, the reduction of $\Delta\Psi$ is approximately proportional to channel conductance. Since the magnitude of $\Delta\tilde{\mu}_H$ controls the rate of the redox H^+ pumps, a reduction of $\Delta\Psi$ by the cation channel conductance leads to stimulation of proton pumping by the redox pumps, and hence, an increased rate of respiration. Thus, the rate of respiration is also approximately proportional to the cation conductance (Nicholls, 1982).

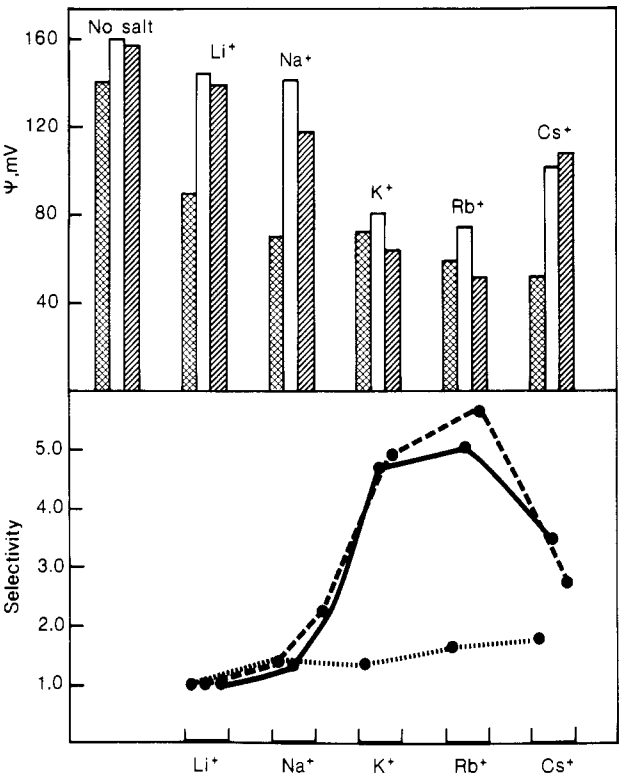

FIGURE 1: Reduction of membrane potential by gramicidin, desfor, and desval: dependence on alkali cations. Mitochondria (2.8 mg/mL) were incubated in a medium composed of 0.2 M sucrose, 5 mM Tris–phosphate, 5 mM $MgCl_2$, 5 mM Tris–succinate, 2 μM rotenone, and a 50 mM sample of the indicated alkali cation Cl^- salt. [^{3}H]TPP^+ (1 μM) was added for the estimation of $\Delta\Psi$, and 100 ng/mg protein of gramicidin (cross-hatched bars), desformylgramicidin (open bars), and des(formylvalyl)gramicidin (hatched bars) were added to induce cation uptake and reduction of $\Delta\Psi$. The top panel shows the potentials measured at steady state (3-min incubation). The bottom panel shows the selectivity of the channels for cations relative to Li^+ as derived from the results shown in the top panel. The selectivity is calculated as the reduction of membrane potential by an alkali cation divided by the reduction of membrane potential obtained with Li^+.

Figure 1 (top panel) shows the effects of Li^+, Na^+, K^+, Rb^+, and Cs^+ chloride salts (50 mM) on static head membrane potential in the presence of 100 ng/mg of protein of gramicidin, desfor, and desval. The membrane potential in the absence of added peptides was 160 mV. As observed previously (Rottenberg & Koeppe, 1989), even in the absence of added alkali salts, gramicidin, at this concentration, significantly reduced the magnitude of $\Delta\Psi$. This could be due to residual potassium, ammonium, and sodium ions, which are carried over by the mitochondrial preparations. Addition of 50 mM alkali salts reduced the potential further. The ef-

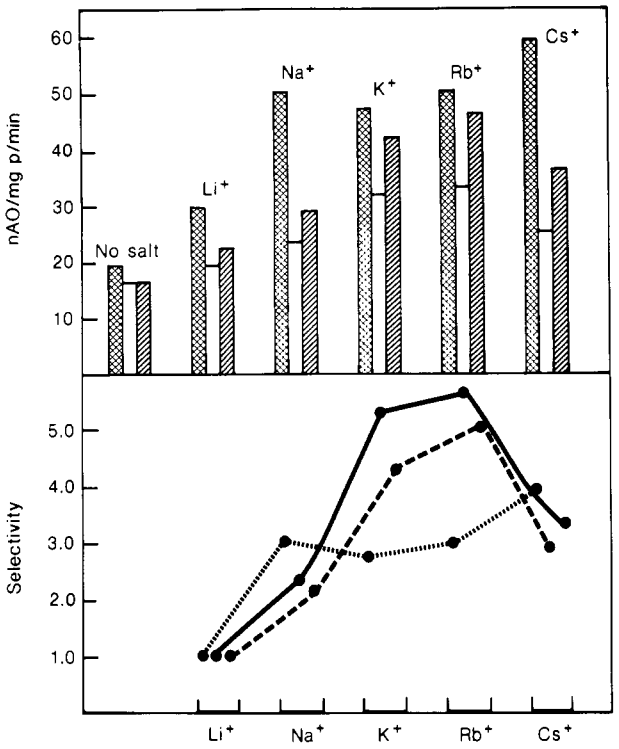

FIGURE 2: Stimulation of the rate of respiration by gramicidin, desfor, and desval: dependence on alkali cations. Medium was the same as in Figure 1. Mitochondria (1 mg/mL) were added to the oxygen electrode chamber followed by rotenone (2 μM) and Tris–succinate (5 mM) and then 100 ng/mg of the indicated peptide, and finally a 50 mM aliquot of the indicated salt was added. The top panel shows the rate of respiration measured at steady state. The bottom panel shows the selectivity of the channel for the cations relative to Li^+. The selectivity is calculated as the stimulation of respiration by an alkali cation divided by the stimulation obtained with Li^+. Symbols are the same as in Figure 1.

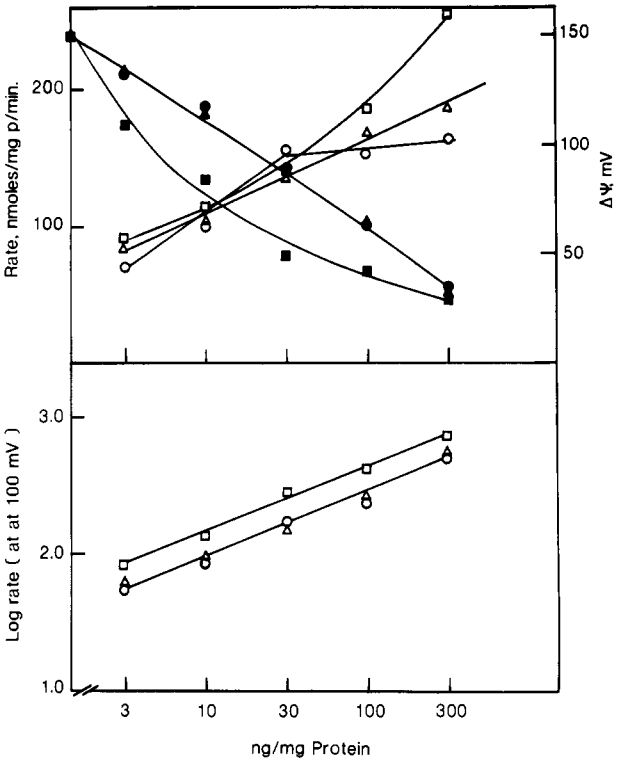

FIGURE 3: Stimulation of Rb^+ transport and the collapse of membrane potential by gramicidin, desfor, and desval in RbCl medium: dependence on peptide concentration. Conditions are as in Figure 1, except that RbCl (50 mM) and ^{86}Rb were added to the basic medium. Membrane potential was estimated from the distribution of [^{3}H]TPP$^+$ and the rate of Rb^+ uptake from the accumulation of ^{86}Rb. The top panel shows the measured membrane potential (closed symbols) and Rb^+ transport (open symbols) for gramicidin (□, ■), desfor (△, ▲), and desval (○, ●). The bottom panel shows the normalized rates of transport at constant potential (100 mV), as calculated from the data shown at the top.

fectiveness of the cations follows the established selectivity of the gramicidin channel ($Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$) with one exception: a reversal of K^+ and Na^+. This deviation, which was observed in the respiration assay as well (see below), is probably due to the fact that there is a high concentration of K^+ in the mitochondrial matrix (Rottenberg, 1969), which greatly reduces $\Delta\tilde{\mu}_K$. The low magnitude of selectivity, which corresponds roughly to the mobilities of the cations in water, is due to the very fast conductance of the channel which, in nonsaturating cation concentrations, is rate limited by aqueous diffusion (Andersen, 1984). Figure 1 shows that the truncated derivatives, desfor and desval, also conduct alkali cations. However, both the pattern of the selectivity and the magnitude are quite different from gramicidin. Desfor hardly conducts Li^+ and Na^+, the conductivity is greatly increased with K^+ and even more with Rb^+, but it is reduced with Cs^+. Desval is similar to desfor in its pattern, but even more pronounced in its magnitude of selectivity. This is best illustrated in the lower panel of Figure 1 which shows the selectivity, relative to Li^+, for these compounds. For gramicidin, the conductivity gradually increased from Li^+ to Cs^+, showing a maximal selectivity of less than 2. Desfor and desval also show low selectivity for Na^+ but much more enhanced selectivity for K^+ and Rb^+ (approaching a Rb^+:Li^+ ratio of 6) and falling off with Cs^+. Thus, the results of these experiments confirm that these derivatives are capable of forming a channel with very high fluxes (K^+ and Rb^+ fluxes of desval are comparable to gramicidin). Moreover, the different pattern of the selectivity strongly suggests that the structure of this channel is radically different from that of gramicidin (see Discussion).

Figure 2 shows a parallel experiment in which the effect of 50 mM alkali salts on the rate of respiration in the presence of gramicidin, desfor, and desval was estimated. The results are very similar to the results of Figure 1. With gramicidin, there is a gradual increase of respiration from Li^+ to Cs^+. The truncated derivative shows enhanced selectivity for K^+ and Rb^+. Some quantitative differences are observed; however, considering that factors other than $\Delta\Psi$ (e.g., internal pH, substrate concentration, matrix volume) may affect the rate of respiration, the agreement is excellent and essentially confirms the validity of the membrane potential measurements. Similar experiments at lower cation concentrations (10 mM) gave similar selectivity patterns. However, because the effects are much smaller, the errors in these experiments are considerably larger.

While the previous experiments strongly suggest that the derivatives form a channel with high alkali cation fluxes, it is important to demonstrate this process by a more direct measurement. This is shown in Figure 3 (top) which shows the effect of increasing concentrations of gramicidin, desfor, and desval on ^{86}Rb uptake. It is observed that all three compounds reduce $\Delta\Psi$ and induce ^{86}Rb uptake at 50 mM RbCl. In this experiment, the derivatives are somewhat less active than gramicidin in reducing $\Delta\Psi$ but are almost as potent in inducing ^{86}Rb transport. Since the magnitude of $\Delta\Psi$ strongly affects the rate of uptake, we normalized the results to Rb^+ uptake that would be obtained at constant potential (100 mV) assuming a linear relationship between the rate of uptake and $\Delta\Psi$ (i.e., $V_{100} = V \times 100/\Delta\Psi$). Plotted this way, the Rb^+ fluxes of the derivatives appear to be of equal magnitude,

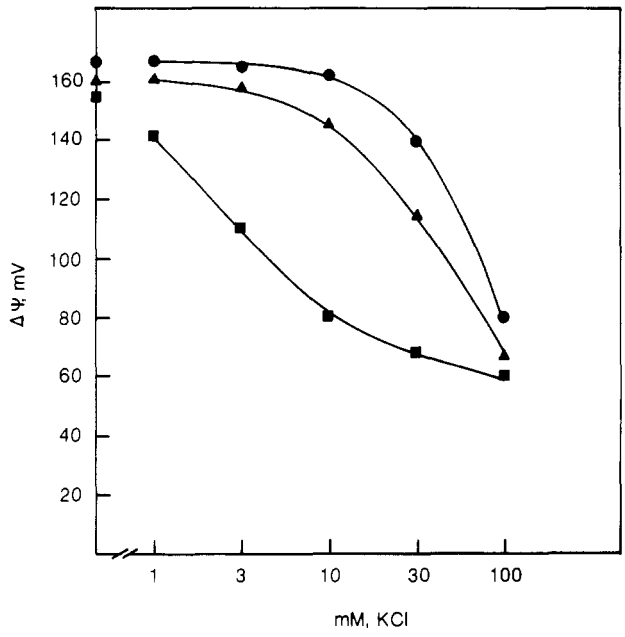

FIGURE 4: Reduction of membrane potential by gramicidin, desfor, and desval: dependence on KCl concentration. Medium is the same as in Figure 1, except for the indicated KCl concentration. Gramicidin (■), desformylgramicidin (▲), and des(formylvalyl)gramicidin (●) were added to 100 ng/mg of protein.

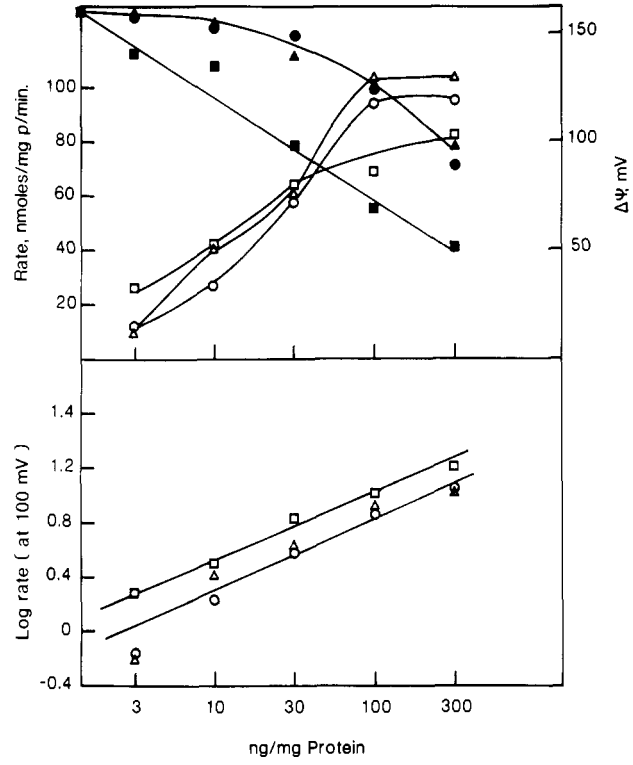

FIGURE 5: Stimulation of Rb^+ transport and the collapse of $\Delta\Psi$ by gramicidin, desfor, and desval in KCl medium: dependence on peptide concentration. Medium, experimental conditions, and symbols are the same as in Figure 3, except that RbCl concentration was only 1 mM and 50 mM KCl was added.

which is about half as much as gramicidin. The slopes of the curves in Figure 3, 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 a 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. The experiments shown in Figures 1–3 were conducted in relatively high salt concentrations (50 mM). In our previous study (Rottenberg & Koeppe, 1989), we showed that in low-salt medium, with only a trace concentration of RbCl, ^{86}Rb uptake, although significant, is much slower with the derivatives than with gramicidin. The difference between these experiments [Figure 4 of our previous study (Rottenberg & Koeppe, 1989) and Figure 3 of this study] suggests that the transport through the channel of the derivatives is dependent on high concentrations of cations. Figure 4 shows the dependence on KCl concentration of the effects of gramicidin, desfor, and desval on $\Delta\Psi$. It is clearly seen that at low KCl concentrations the derivatives are relatively ineffective when compared to gramicidin, while at high KCl concentrations their effects approach that of gramicidin. Clearly then, the effectiveness of the derivatives depends on high cation concentrations. We have tested the effect of nonpenetrating cations (e.g., Tris and TEA) on ^{86}Rb transport and found no effect (not shown). Thus, it appears that only high concentrations of cations that penetrate the channel (such as Rb^+ or K^+) are effective in stimulating transport. The question is whether this is an unusually strong dependence on the concentration of the transported cation, which could result from the mechanism of single-file conductance (Hille & Schwarz, 1978), or whether a penetrating cation can stabilize the channel such that the transport of other cations, even if present at low concentration, is enhanced. To answer this question, we measured the uptake of $^{86}Rb^+$, at 1 mM concentration in the presence of 50 mM KCl (Figure 5, top). It is observed that under these conditions the derivatives actually conduct Rb^+ at higher rates than gramicidin. This paradoxical result is due to the fact that the membrane potential (and thus $\Delta\tilde{\mu}_{Rb}$) is lower with gramicidin. To correct for this effect, we normalized the rates to a constant driving force of 100 mV, assuming ohmic conductance. This is shown in Figure 5, bottom. At constant force, the derivatives show equal activity, which is only slightly less effective than gramicidin in stimulating Rb^+ transport. The difference in the activities of the derivatives and gramicidin in this experiment is similar to that observed in RbCl medium (Figure 3, bottom). If the requirement for high salt was due to strong concentration dependence, we expect KCl to inhibit Rb^+ transport rather than to stimulate it. Thus, this experiment suggests that the requirement for high cation concentrations is due to a stabilizing effect on the conducting complex and not an unusual concentration dependence of the transport process. Cs^+ was also effective, but to a lesser extent (not shown). Hence, it appears that the effectiveness in stabilizing the channel correlated with the effectiveness of cation conductance. It is interesting that although there is a considerable difference in the activity between desfor and desval at low salt (Rottenberg & Koeppe, 1989), the differences disappear in high salt. This suggests that the formation of the desfor channel is more strongly dependent on cations than desval. If the derivatives are capable of conducting K^+ and Rb^+ at high salt concentrations at rates approaching those of gramicidin, we expect them to be nearly as effective as gramicidin in cation-dependent uncoupling of oxidative phosphorylation. We have shown recently that in the absence of added alkali cations, gramicidins and the truncated derivatives partially uncouple oxidative phosphorylation without collapsing $\Delta\tilde{\mu}_H$ (Rottenberg & Koeppe, 1989). We show here that in the presence of a *high* concentration of KCl the derivatives further uncouple oxidative phosphorylation, a process which is associated with the collapse of $\Delta\tilde{\mu}_H$.

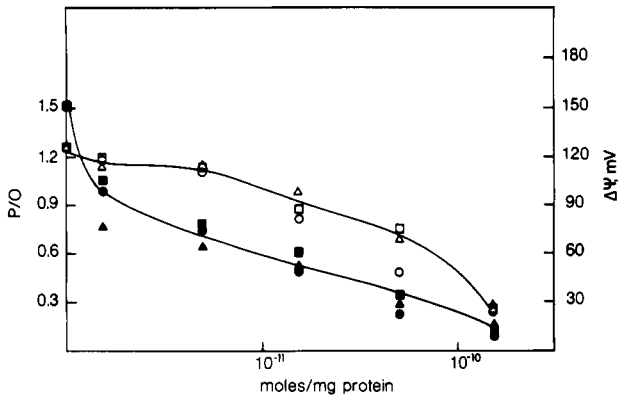

FIGURE 6: Uncoupling of oxidative phosphorylation and reduction of $\Delta\Psi$ by gramicidin, desfor, and desval in KCl medium: dependence on peptide concentration. Medium is the same as in Figure 1, except for the addition of 1 mM Tris–ADP and 50 mM KCl. [^{3}H]TPP was added for measurement of $\Delta\Psi$, [^{32}P]P_i was added for parallel measurement of the rate of ATP synthesis, and respiration was measured polarographically. Open symbols show the measured $\Delta\Psi$; closed symbols show the P:O ratio calculated from the measured rates of phosphorylation and respiration. Symbols are the same as in Figure 5.

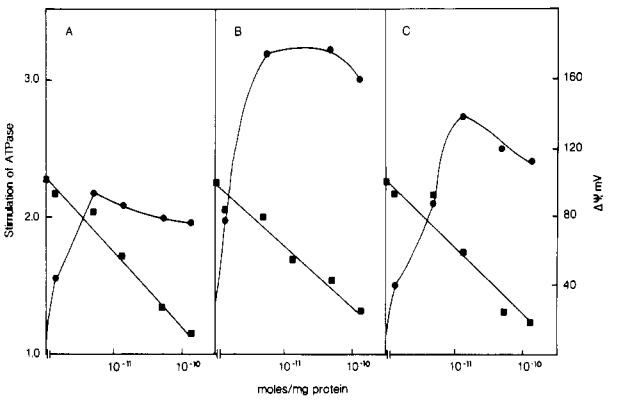

FIGURE 7: Concentration dependence of the uncoupling of the H^+-ATPase by gramicidin (A), desformylgramicidin (B), and des(formylvalyl)gramicidin (C): membrane potentials (■) were determined from [^{3}H]TPP^+ distribution. ATPase rates (●) were determined enzymatically (Pulman et al., 1960). Medium was the same as in Figure 6, except that Tris–succinate was omitted and Tris–ADP was replaced with 2 mM Tris–ATP.

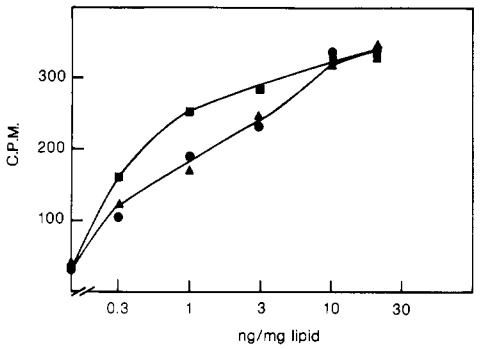

FIGURE 8: Concentration dependence of ^{86}Rb–Rb exchange by gramicidin, desfor, and desval in liposomes. Azolectin multilamellar liposomes were prepared in medium composed of 100 mM RbCl and 50 mM Tris-HCl (pH 7.0) containing ^{86}Rb. Liposomes were washed by centrifugation (20000g, 15 min) 3 times with 100 mM KCl and 50 mM Tris-HCl (pH 7.0). The release of ^{86}Rb was measured after incubation for 5 min in 100 mM RbCl and 50 mM Tris-HCl (pH 7.0) and centrifugation (18000g, 5 min). The concentrations of gramicidin (■), desfor (▲), and desval (●) are indicated.

Figure 6 shows the effect of gramicidin, desfor, and desval on membrane potential and phosphorylation efficiency (P/O) in mitochondria. The collapse of the membrane potential depends on the presence of potassium and hence results from stimulation of potassium conductance. As was shown above (Figures 1 and 4), there is not much difference between gramicidin and the truncated derivatives in their potency in collapsing the membrane potential at *high* KCl concentrations. Similarly, their potency in uncoupling of oxidative phosphorylation (i.e., reduction of the P:O ratio) is also approximately the same. Thus, in the presence of high concentrations of alkali cations, the uncoupling of oxidative phosphorylation by gramicidin and the truncated derivatives is correlated with the collapse of $\Delta\Psi$, as was observed for valinomycin (Rottenberg, 1970) and in accordance with the predictions of the chemiosmotic hypothesis (Mitchell, 1966). The fact that the dependence on the collapse of $\Delta\tilde{\mu}_H$ appears to be steeper for gramicidin than valinomycin (Rottenberg & Hashimoto, 1986) is now fully resolved as a combination of two effects, a partial decoupling effect, which is independent of cation transport (Rottenberg & Koeppe, 1989), and a classical, valinomycin-like, uncoupling effect which depends on the cation-induced collapse of $\Delta\Psi$. Further support for this conclusion is derived from Figure 7 which shows the effect of gramicidin, desformylgramicidin, and des(formylvalyl)gramicidin on the membrane potential generated by H^+-ATPase and the induced stimulation of ATP hydrolysis in KCl medium. Again, no major difference is observed between gramicidin and the truncated derivatives. Thus, when the potential is generated either by respiration (H^+ redox pump) or by H^+-ATPase and in the presence of KCl, the truncated derivatives are as active as gramicidin. Under the conditions of Figures 6 and 7, gramicidin and the truncated derivative induced massive swelling of comparable magnitude (not shown). This indicates that the uncoupling, under these conditions, is the result of the induced K^+ transport.

To test whether the cation conductance of the truncated derivatives can also be observed in artificial phospholipid bilayers, we prepared multilamellar liposomes from soybean lipids (Azolectin) in which we trapped ^{86}Rb. After washing the liposomes of external ^{86}Rb, we measured the amount of released ^{86}Rb after a 5-min incubation in 100 mM RbCl (^{86}Rb–Rb exchange) as a function of the peptide concentration (Figure 8). It is observed that the derivatives are about half as active as gramicidin in stimulating the exchange. This is similar to the results of Figures 3 and 5 and indicates that channel formation also occurs in these artificial phospholipid membranes. In the experiment shown in Figure 8, the stimulation of ^{86}Rb exchange at all concentrations of gramicidin and the derivatives was complete after the first measurement (no incubation, 5 min of centrifugation). This suggests that the exchange for each vesicle which contains an active channel proceeds to completion before the first measurement is taken. Therefore, the concentration dependence (Figure 8) shows the number of vesicles with active channels rather than the rate of exchange through the channel. Nevertheless, the results demonstrate that the truncated derivatives form active channels that quickly exchange ^{86}Rb.

Discussion

The experiments presented in this study demonstrate that the truncated gramicidin derivatives desformylgramicidin and des(formylvalyl)gramicidin are capable of forming ion-conducting channels. Under favorable conditions (high concentrations of Rb^+ or K^+ salts), their macroscopic fluxes approach the flux of the gramicidin channel. The requirement for permeating cations, the greatly enhanced selectivity for K^+

and Rb^+, and the different selectivity pattern, i.e., $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ as against $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$, suggest a different channel structure. It is possible that the enhanced selectivity of the derivatives is due to a significantly lower conductivity of the channel. In such a case, the cation transport through the channel would become rate limiting, rather than the diffusion rate in the unstirred water layer, as is the case for gramicidin at low cation concentrations (Andersen, 1984). How then can one explain the fact that the macroscopic fluxes of K^+ and Rb^+ with the derivatives approach that of gramicidin? This may be due to a significantly longer lifetime of these channels. The combination of significantly lower conductance with a longer lifetime could explain the observed enhanced selectivity, together with high macroscopic fluxes. Similarly, the fact that the pattern of selectivity is different, with maximal flux with Rb^+ rather than Cs^+, indicates a radically different transport mechanism. This may be related to the requirement of high concentrations of penetrating cations to stabilize the channel structure. If a cation must always reside in the channel, then the transport of larger cations, such as Cs^+, may be impeded. There is little doubt that the cation conductance of these channels is not due to the head-to-head, single-stranded $\beta^{6.3}$-helix structure. First, this complex, which is held by only six head-to-head hydrogen bonds, would be greatly weakened without the formyl headgroup, which contributes two of these bonds. Second, electrostatic repulsion between the charges of the amino headgroups would counteract the weak attraction of the remaining hydrogen bonds, and finally, the polar nature of the charged headgroup would tend to anchor the heads at the surface of the membrane away from the hydrophobic core where the head-to-head interaction must occur. Of course, our data do not provide us with any evidence concerning the structure of the conducting channel. However, of the various alternative gramicidin structures which are known to exist, the antiparallel double helix (Wallace & Ravikumar, 1988) is probably the most reasonable candidate for an alternative channel structure. This complex, which predominates in polar organic solvents such as ethanol (Veatch et al., 1974), is held together by a large number of hydrogen bonds (28) involving the entire peptide but not the formyl headgroups (Wallace & Ravikumar, 1988). Hence, removal of the formyl group should not reduce the complex stability. Moreover, since the headgroups in this putative channel should reside at the surface of the membrane, the removal of the formyl group, which increases the polarity of the headgroup, should greatly stabilize this structure in the membrane. While it is not clear why the conductance of this channel should depend, to such an extent, on the presence of a high concentration of cations, it is perhaps related to the fact that the structure of the cation-containing dimer (Wallace & Ravikumar, 1988) is very different from the structure of the uncomplexed dimer (Langs, 1988). If cations are necessary to stabilize the channel, it is possible that the detailed mechanism of ion migration through this channel is different from gramicidin, resulting in an altered selectivity pattern and enhanced selectivity for Rb^+ and K^+. One puzzling observation for which we could not offer a satisfactory explanation, at present, is the fact that in passive diffusion experiments in rat liver mitochondria (Rottenberg & Koeppe, 1989), even though conducted at high KSCN concentration, the activity of the derivatives is up to 2 order of magnitudes less than that of gramicidin. This difference is not due to the different anions (SCN^- vs Cl^-) since KSCN was as effective as KCl in experiments similar to those shown in Figures 1 and 2 (not shown). Another important difference, however, is that in the experiments shown in this study, the transport is driven by a membrane potential which is generated by ion pumps. It is possible that the specific pump-induced potential profile and charge distribution in the membrane contribute to the stability of these structures. Further experiments are necessary to resolve this problem.

There is no available information on the conductance properties of these derivatives from single-channel measurements in planar lipid membranes. Although there are references to the presumed nonconductance of desformylgramicidin [cf. Killian et al. (1988)], we could not find any published experimental evidence for this assertion. Previous studies with a very bulky *negatively* charged headgroup (Bamberg et al., 1978) are not very relevant to the properties of these derivatives. Our preliminary experiments with the negatively charged Azolectin liposomes, which show that ^{86}Rb exchange is stimulated by desformylgramicidin and des(formylvayl)gramicidin, suggest that it should be possible to observe single-channel conductance with these derivatives in negatively charged phospholipid membranes.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; Li, 7439-93-2; Na, 7440-23-5; K, 7440-09-7; Rb, 7440-17-7; Cs, 7440-46-2; gramicidin D, 1393-88-0.

References

Andersen, O. S. (1984) *Annu. Rev. Physiol. 46*, 531–548.

Bamberg, E., Apell, H. J., Alpes, H., Gross, E., Morell, J. L., Jarbaugh, J. F., Janko, K., & Lauger, P. (1978) *Fed. Proc. 37*, 2633–2638.

Beavis, A. D., & Garlid, K. D. (1987) *J. Biol. Chem. 262*, 15085–15093.

Chappel, J. B., & Crofts, A. R. (1965) *Biochem. J. 95*, 393.

Cornell, B. (1987) *J. Bioenerg. Biomembr. 19*, 655–676.

Durkin, J. T., Andersen, O. S., Heitz, F., Trudelle, Y., & Koeppe, R. E., II (1987) *Biophys. J. 51*, 451a.

Goodall, M. C. (1970) *Biochim. Biophys. Acta 219*, 471–478.

Hilleband & Schwarz, W. (1978) *J. Gen. Physiol. 72*, 409–442.

Hladky, S. B., & Haydon, D. A. (1970) *Nature (London) 225*, 451–453.

Killian, J. A., Prasad, K. U., Haims, D., & Urry, D. W. (1988) *Biochemistry 27*, 4848–4855.

Koeppe, R. E., II, & Weiss, L. B. (1981) *J. Chromatogr. 208*, 414–418.

Langs, D. A. (1988) *Science 241*, 188–191.

Mitchell, P. (1966) *Chemiosmotic coupling in oxidative and photosynthetic phosphorylation*, Glyn Research Laboratories, Bodmin, Cornwall, England.

Nicholls, D. G. (1982) *Bioenergetics*, Academic Press, London.

Pick, U., Weiss, M., & Rottenberg, H. (1987) *Biochemistry 26*, 8295–8302.

Pressman, B. C. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 1076–1083.

Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 2322.

Rottenberg, H. (1970) *Eur. J. Biochem. 15*, 22–28.

Rottenberg, H. (1973) *J. Membr. Biol. 11*, 117–137.

Rottenberg, H. (1979) *Methods Enzymol. 55*, 547–579.

Rottenberg, H. (1984) *J. Membr. Biol. 81*, 127–138.

Rottenberg, H., & Solomon, A. K. (1969) *Biochim. Biophys. Acta 193*, 48–57.

Rottenberg, H., & Hashimoto, K. (1986) *Biochemistry 25*, 1747–1755.

Rottenberg, H., & Koeppe, R. E., II (1989) *Biochemistry* (preceding paper in this issue).

Shahak, Y. (1982) *Plant Physiol. 70*, 87–91.

Sung, S. S., & Jordan, P. C. (1988) *Biophys. J. 54*, 519–526.
Szarkowska, L., & Klingenberg, M. (1963) *Biochem. Z. 338*, 674–695.
Tosteson, D. C., Andreoli, T. E., Tieffenbory, M., & Cook, P. (1968) *J. Gen. Physiol. 51*, 373–384.
Urry, D. W. (1985) in *The Enzymes of Biological Membranes* (Martonosi, N. A., Ed.) Vol. 1, pp 229–257, Plenum Press, New York.
Urry, D. W., Goodal, M. C., Glickson, J. D., & Mayers, D. F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672.
Veatch, W. R., Fossel, E. T., & Blout, E. R. (1974) *Biochemistry 13*, 5249.
Wallace, B. A., & Ravikumar, K. (1988) *Science 241*, 182–187.
Weiss, L. B., & Koeppe, R. E. II (1985) *Int. J. Pept. Protein Res. 26*, 305–310.

Uncoupling of Oxidative Phosphorylation: Different Effects of Lipophilic Weak Acids and Electrogenic Ionophores on the Kinetics of ATP Synthesis†

Akemi Matsuno-Yagi and Youssef Hatefi*
Division of Biochemistry, Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, California 92037

Received October 18, 1988; Revised Manuscript Received February 21, 1989

ABSTRACT: Previous studies from this laboratory have shown that the kinetics of ATP synthesis by bovine heart submitochondrial particles (SMP) are modulated by the coupled rate of respiration between two extremes of V_{max} and apparent K_m's for ADP and P_i [Matsuno-Yagi, A., & Hatefi, Y. (1986) *J. Biol. Chem. 261*, 14031–14038; Hekman, C., Matsuno-Yagi, A., & Hatefi, Y. (1988) *Biochemistry 27*, 7559–7565]. Thus, with ADP as the variable substrate, ATP synthesis occurred with V_{max} = 200 nmol of ATP min^{-1} (mg of protein)$^{-1}$ at 30 °C and an apparent K_m^{ADP} = 2–4 μM at low rates of respiration, and with V_{max} = 11 000 nmol of ATP min^{-1} (mg of protein)$^{-1}$ at 30 °C and an apparent K_m^{ADP} = 120–160 μM at high rates of respiration. At intermediate respiration rates, it was necessary to introduce a third intermediate K_m^{ADP} for best fit of the kinetic data, indicating that transition from one kinetic extreme to the other is not abrupt and involves intermediate kinetic states of the ATP synthase complexes. The present paper shows that uncouplers affect the kinetics of ATP synthesis by SMP in two ways. When used at moderate concentrations, electrogenic ionophores such as gramicidin D or valinomycin plus nigericin decreased the V_{max} for ATP synthesis without changing the contributions of the low, intermediate, and high K_m^{ADP} to the overall rate of ATP synthesis. By contrast, potent lipophilic weak acid uncouplers, such as FCCP, CCCP, S-13, and SF6847, decreased V_{max} and converted the kinetics of ATP synthesis toward high K_m^{ADP}. Similar results were obtained when P_i was the variable substrate, or when the energy-linked reaction studied was ATP-driven reverse electron transfer from succinate to NAD, with NAD as the variable substrate. When the ATP synthase complexes of SMP were fractionally inactivated by dicyclohexylcarbodiimide, and as a result the kinetics of ATP synthesis by these particles were converted to the high-K_m mode, then partial uncoupling of oxidative phosphorylation by FCCP resulted in large increases in the apparent K_m for ADP and P_i. These results have been interpreted as follows. In the absence of uncouplers, increases in the apparent K_m^{ADP} and $K_m^{P_i}$ are associated with increased rates of coupled respiration and increased rates of proton flux through the ATP synthase complexes. Lipophilic weak acid uncouplers, but not gramicidin D and valinomycin plus nigericin when used at moderate uncoupling concentrations, react with the ATP synthase complexes and increase slippage in the coupling mechanism within the enzyme complex. As a result, uncoupled proton flux through the ATP synthase complex increases and results in increased apparent K_m values for ADP and P_i even though the rate of ATP synthesis decreases. A similar interpretation applies to the uncoupler-induced increase in the apparent K_m^{NAD} during ATP-driven reverse electron transfer from succinate to NAD. This interpretation is also consistent with the very high apparent K_m^{ADP} and $K_m^{P_i}$ obtained when SMP containing fractionally inactivated ATP synthases were partially uncoupled by FCCP. In these SMP preparations, the remaining, active ATP synthase complexes turn over very rapidly during oxidative phosphorylation [Matsuno-Yagi, A., & Hatefi, Y. (1988) *Biochemistry 27*, 335–340]. Partial uncoupling by a lipophilic weak acid, such as FCCP, further increases the proton flux through these active ATP synthases via the slip mechanism, thus resulting in very high apparent K_m values for ADP and P_i.

The mechanism of protonic energy transfer in oxidative and photosynthetic phosphorylation has remained a matter of debate since the advent of the chemiosmotic hypothesis in 1966 (Mitchell, 1966). The fact that energy coupling can take place in accordance with the chemiosmotic hypothesis via bulk to bulk $\Delta\tilde{\mu}_{H^+}$[1] has been amply demonstrated [see, for example, Racker and Stoeckenius (1974), Thayer and Hinkle (1975a,b),

† Supported by U.S. Public Health Service Grant DK08126. This is Publication No. 5595-BCR from the Research Institute of Scripps Clinic, La Jolla, CA.
* To whom correspondence should be addressed.

[1] Abbreviations: SMP, submitochondrial particle(s); $\Delta\psi$, membrane potential; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical potential of protons; F_oF_1, ATP synthase complex; F_o, membrane sector of F_oF_1; CF_o–CF_1, chloroplast F_oF_1; DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; S-13, 2,5-dichloro-3-*tert*-butyl-4'-nitrosalicylanilide; SF6847, (3,5-di-*tert*-butyl-4-hydroxybenzylidene)-malononitrile; I_{50}, uncoupler concentration required for 50% uncoupling.

0006-2960/89/0428-4367$01.50/0