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EFFECT OF pH ON GRAMICIDIN-MEDIATED CHANGES IN LIGHTSCATTERING, ION UPTAKE, AND ATP EXCHANGE IN DIGITONIN PARTICLES

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SUMMARY

1. In the presence of a cation and ATP or succinate, gramicidin initiates a pH-dependent decrease in light scattering and proton release by digitonin-treated inner membrane preparations of rat liver mitochondria. The responses are greatest at pH 6.4, and decrease markedly as the pH is raised or lowered. Superimposed upon the initial proton release is a slower, linear loss of H^+ that depends upon the extra-/intramitochondrial proton concentration ratio.

2. At low pH, K^+ is more effective than Na^+ in yielding greater gramicidin-mediated light scattering changes in the presence of ATP, as well as a higher rate of respiration with succinate as substrate. At pH 8.0, however, the rate of respiration is more rapid with Na^+ than K^+.

3. Ionic balance studies indicate that the ATP-mediated swelling of digitonin particles results from an uptake of Na^+ (or K^+) and retention of P_i, the P_i being formed from the gramicidin-stimulated ATPase.

4. The translocation of ATP is increased by gramicidin, but only in the presence of 5 mM Na^+ (or K^+).

5. The results are explained in terms of a gramicidin-mediated increase in membrane permeability to H^+ and monovalent cations, which leads to a pH-dependent activation of ATPase activity and an increased rate of ATP translocation.

INTRODUCTION

It is generally known that the antibiotic gramicidin increases the permeability of both artificial and natural membranes toward alkali cations[1] and protons[2]. The limited proton permeability of membranes induced by gramicidin distinguishes it from valinomycin, which increases the cation conductance of membranes[3], and from uncouplers, which enhance the proton permeability[4,5]. Evidence has been presented by various groups that the primary action of uncouplers and transport-mediating antibiotics is on anion uptake[6], cation uptake[1,7–9], or on proton conductance[3,10–12]. The object of this report is to compare the effects of Na^+ and K^+ on gramicidin-mediated light scattering changes in digitonin particles, and examine more closely the role of ATP in this process.

METHODS

The studies were carried out using digitonin-treated inner membrane preparations from rat liver mitochondria prepared by the method of HOPPEL AND COOPER[13,14]. These particles, which show normal oxidative phosphorylation capacity, were frozen in 0.3 M sucrose and 15 % anhydrous dimethyl sulfoxide at —80° until used.

Light scattering, H^+ concentration, and respiration were measured simultaneously as previously described[15] with a fluorophotometer built in the electronic instruments shop at the Johnson Research Foundation. Measurements were performed at room temperature in a quartz cell containing 10 ml mixture and stirred magnetically.

The exchange of endogenous ATP with extra-mitochondrial ATP was measured in [^{14}C]ATP prelabeled digitonin particles at 5–10°, as previously described[14]. Briefly, the exchange was initiated by 0.5 mM unlabeled Tris–ATP (pH 7.4) and 15–20 sec later stopped with 50 μM atractyloside. After centrifuging 45 sec at 13500 rev./min in an Eppendorf microcentrifuge, the ^{14}C label appearing in the supernatant was counted by liquid scintillation.

Gramicidin was obtained from Nutritional Biochemicals Corporation. Rotenone was donated by S.B. Penick Company, while K^+-atractyloside was kindly provided by Dr. A. Bruni, Padova, Italy.

RESULTS

Effect of pH

The effect of pH was measured on gramicidin-mediated light scattering and respiration by digitonin particles suspended in 0.1 M sucrose–mannitol. (Preliminary studies showed that lowering the osmolarity to 0.1 M yielded the greatest light scat-

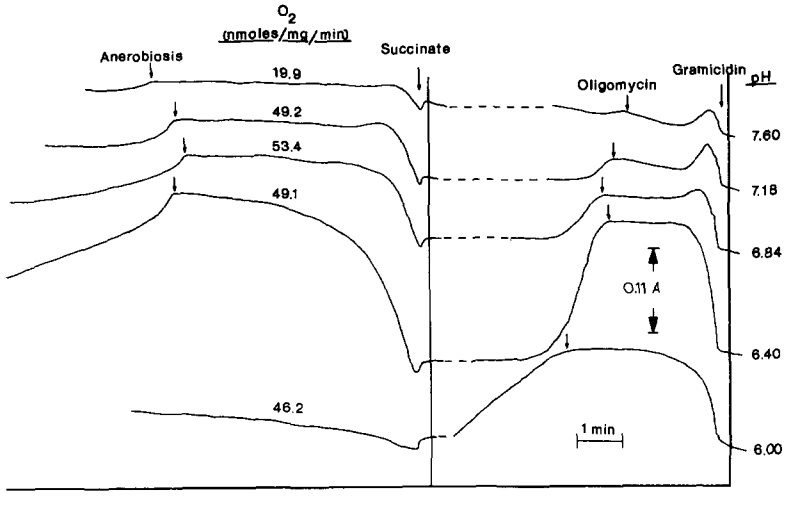

Fig. 1. Effect of pH on gramicidin-mediated light scattering changes and respiration in digitonin particles supported by ATP or succinate. Digitonin particles (9.0 mg) were added to 0.1 M sucrose–mannitol, 2.5 mM Tris–ATP at the indicated pH, and 5 mM KCl, in a total volume of 10.0 ml. Additions were: 1 μg gramicidin; 10 μg oligomycin; and 5 mM Tris–succinate. Before adding gramicidin or succinate, the pH was adjusted as indicated with saturated Tris. An upward deflection indicates a decrease in light scattering.

tering responses, proton flux, and respiration.) In the presence of 5 mM K^+, Fig. 1 shows that the ATP-induced as well as the succinate-induced light scattering responses are greatest at pH 6.4, and decrease markedly as the pH is raised or lowered. Above pH 6.4, the ATP-induced light scattering responses show a spontaneous swelling and shrinking cycle that becomes more marked as the pH is increased. Oligomycin reverses the ATP-induced light scattering responses, and anaerobiosis similarly reverses the succinate-induced responses. The respiratory rate (Fig. 1, in parenthesis) bears little or no relationship to the extent of succinate-induced light scattering changes as a function of pH. For example, between pH 6.0 and 6.8 the respiratory rate shows little change, while the light scattering response first increases markedly, then declines.

Fig. 2A is illustrative of the proton release that accompanies the decrease in light scattering after addition of gramicidin to digitonin particles incubated with 5 mM K^+, Tris–ATP (pH 6.4) and $3 \cdot 10^{-5}$ M rotenone. The inclusion of rotenone in this experiment slightly reduced the extent of proton release, suggesting that the particles contained a certain amount of endogenous subtrate. The initial release of protons in the presence of K^+ amounts to 25 nmoles H^+/mg protein, and corresponds well with the rapid decrease in light scattering. The omission of K^+ (Fig. 2B) results in a rapid uptake of 27 nmoles H^+/mg, and only a slight change in light scattering. In either case, however, there follows a slower linear release of protons amounting

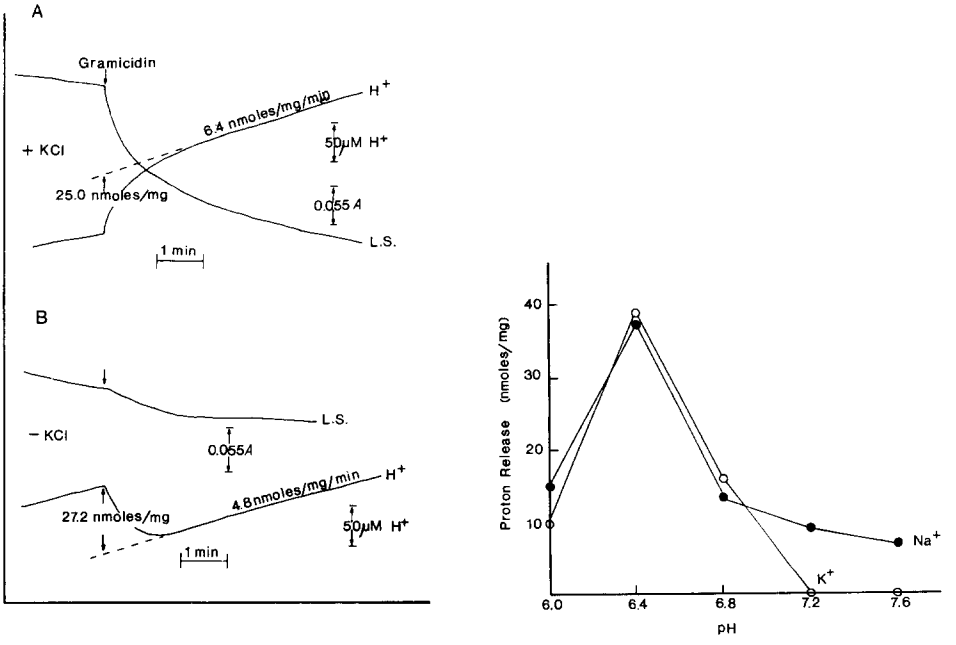

Fig. 2. Effect of K^+ on gramicidin-mediated light scattering and proton changes supported by ATP. Digitonin particles (32 mg) were added to 0.3 M sucrose–mannitol, 10 mM Tris–ATP (pH 6.4), $3 \cdot 10^{-5}$ M rotenone, and where indicated, 5 mM KCl. Additions were: 1.0 μg gramicidin. An upward deflection indicates a decrease in light scattering (L.S.) and uptake of protons.

Fig. 3. ATP-supported proton release in digitonin particles initiated by gramicidin. Incubation conditions are as described in Fig. 1. Proton release is measured as the rapid loss of protons following addition of gramicidin, as described in Fig. 2A.

to 6.4 nmoles/mg per min in the presence of K^+ and 4.8 nmoles/mg per min in the absence of K^+.

The dependency of the direction of proton flux on the cation may be ascribed to the preference of gramicidin in inducing a cation–cation exchange over a cation–proton exchange[3]. Thus, in the absence of K^+, the exchange of external H^+ for internal K^+ is preferred, leading to an uptake of H^+.

Fig. 3 represents H^+ electrode measurements, showing the extent of the initial release of protons following addition of gramicidin to particles incubated with 5 mM Na^+ or 5 mM K^+. As the pH is raised, there is a pronounced increase in the gramicidin-mediated uptake of protons that reaches a maximum at pH 6.4, corresponding to the maximal light scattering response. Increasing the pH beyond 6.4 greatly reduces the initial release of H^+ into the medium. Fig. 3 shows that the release of protons is rather independent of the type of monovalent cation present.

Table I shows the effect of pH on the linear rate of proton loss preceeding and accompanying the addition of gramicidin to digitonin particles. At pH 6.0, protons are taken up before and after gramicidin is added, but as the pH is raised, the decreased external proton pressure results in an increasingly greater loss of protons. The greatest stimulation of the proton flux by gramicidin is observed at low pH, and when the pH is raised to 7.6, there is no further effect of gramicidin.

TABLE I

EFFECT OF GRAMICIDIN ON PROTON RELEASE IN DIGITONIN PARTICLES AS A FUNCTION OF pH

Measurements of proton flux were obtained from the experiment reported in Fig. 1. Rates are expressed as nmoles H^+/min per mg, as described in Fig. 2. Negative (−) values indicate proton uptake.

Additions	*pH*: 6.0	6.4	6.8	7.2	7.6
None	−2.7	7.8	29.7	74.0	89.0
Gramicidin	−6.4	20.8	46.5	82.9	89.0

Efficacy of Na^+ vs. K^+

WENNER AND HACKNEY[15] demonstrated that K^+ is more effective than Na^+ in bringing about gramicidin-mediated light scattering changes in mouse liver mitochondria, and have proposed that a higher energy requirement is necessary to drive the uptake of Na^+ than K^+. This point was examined in digitonin-treated inner membrane preparations incubated with gramicidin at different pH and a low ATP concentration. Fig. 4 represents light scattering results using 0.8 mM ATP in place of the 2.5 mM ATP normally employed. Addition of gramicidin causes a shrinkage of the particles that is more pronounced in the presence of K^+ than Na^+, and is greatest at pH 7.0. Subsequent addition of 5 mM succinate causes a swelling that is greatest in the presence of K^+ and at pH 7.0.

The more marked effects of K^+ than Na^+ at pH 6.4 are also apparent in the higher respiratory rate with K^+ as the cation and with succinate as substrate (Table II). As the pH is increased to 8.0, however, the difference in the respiration becomes less pronounced, and at pH 8.0 Na^+ yields a greater respiratory rate than K^+.

Uptake of Na^+, P_i, and adenine nucleotides induced by gramicidin

The pH-dependent decrease in light scattering upon addition of gramicidin to digitonin particles incubated with ATP implied a net uptake of ions, yet proton traces revealed a release of H^+. To better understand the ionic balance under these conditions, the gramicidin-mediated uptake of Na^+, ATP, and P_i was measured, and is

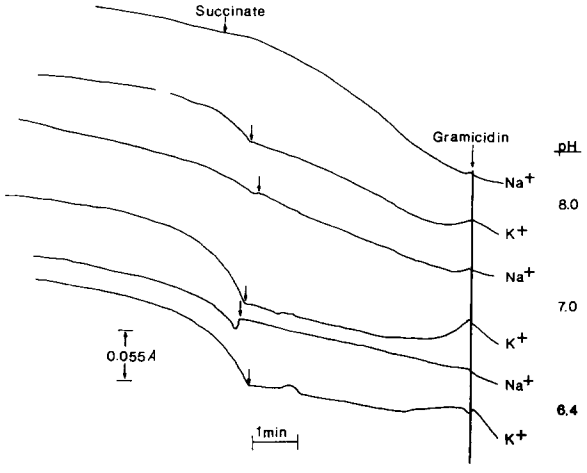

Fig. 4. Effect of Na^+ and K^+ on gramicidin-mediated light scattering as a function of pH. Digitonin particles (10.0 mg) were added to 0.3 M sucrose–mannitol containing 0.8 mM Tris–ATP, 20 μl rotenone (saturated solution), and 5 mM NaCl or KCl. Additions were: 1 μg gramicidin; 10 μg oligomycin; 1.0 mM Tris–succinate. The pH was adjusted to 6.4, 7.0, or 8.0 with saturated Tris. An upward deflection indicates a decrease in light scattering.

TABLE II

EFFECT OF K^+ AND Na^+ ON RESPIRATION IN DIGITONIN PARTICLES AS A FUNCTION OF pH

Digitonin particles (8.9 mg) were incubated in 0.3 M sucrose–mannitol, 1 mM Tris–succinate, 1 μg gramicidin, and, where indicated, 5 mM K^+ or Na^+. The pH was adjusted with saturated Tris. Respiration was determined polarographically.

Additions	*pH*: 6.4	7.0	8.0
	nmoles O_2/min per mg		
None	6.3	8.6	12.0
K^+	10.2	16.2	16.0
Na^+	6.3	12.6	19.1

presented in Table III. The method used to separate mitochondria from medium, that of centrifugal filtration through a layer of silicone oil[16], allows an indication of ionic balance at a precise time; in this case, 2 min after adding gramicidin. Whole mitochondria were used in place of digitonin particles, as the silicone oil (Dow 550, spec. gravity 1.065) blocked the passage of the lighter particles. The values in the table represent the difference in the ionic content of mitochondria incubated with gramicidin *vs.* those incubated under identical conditions without gramicidin. Table III shows that there is a comparatively large uptake of Na^+ and P_i that reaches

TABLE III

EFFECT OF GRAMICIDIN ON ION UPTAKE IN RAT LIVER MITOCHONDRIA AS A FUNCTION OF pH

Rat liver mitochondria (6.5 mg/ml) were incubated, with shaking, at 25° in 0.3 M sucrose; 5 mM Tris–(*N*-morpholino)ethane sulfonic acid (pH 6.0 or 6.4) or 5 mM Tris-*N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (pH 7.2 or 7.6); 5 mM NaCl; ^{22}Na (0.8 μC/ml); 2.5 mM Tris–ATP (pH as indicated); in a total volume of 1.0 ml. After 30 sec, 0.5 μg gramicidin was added; the reaction was stopped 2 min later by rapidly cooling to 0°, followed by centrifugal filtration through silicone oil at 25000 rev./min for 2 min in a Spinco SW-50L rotor. ^{22}Na was measured in a Unilux II scintillation counter, using glass discs in a mixture of toluene and 0.4 % 2,5-diphenyl-oxazole. Assays for ATP, ADP, AMP, and P_i were performed as described[21] on the neutralized pellet and supernatant. All values are corrected for adherent extramitochondrial fluid by [^{14}C]-dextran, mol. wt. 60000–90000, and expressed as the difference between gramicidin-treated and untreated samples.

		pH:	6.0	6.4	7.2	7.6
			nmoles/mg			
Mitochondria	Na^+		67	70	42	25
	P_i		95	131	48	28
	Adenine nucleotides		−6.7	−6.4	−3.8	−6.8
			nmoles/ml			
Supernatant	P_i		110	440	460	360

a maximum at pH 6.4, and decreases as the pH is increased to 7.6. Rather surprisingly, the level of adenine nucleotides is somewhat reduced after addition of gramicidin. The gramicidin-induced loss of nucleotides represents a decrease from 22 to 16 nmoles/mg. The endogenous level of adenine nucleotides in rat liver mitochondria is about 15 nmoles/mg (ref. 14), and the loss may thus represent a removal or hydrolysis of external ATP taken up prior to the addition of gramicidin. Inasmuch as phosphate was not originally present, it is likely that gramicidin induced an uptake of ATP, which was hydrolyzed to ADP and P_i by the gramicidin-stimulated ATPase, the ADP being exchanged for external ATP. The activation of the ATPase by gramicidin was supported by measuring the total P_i content of the supernatant and mitochondria.

TABLE IV

EFFECT OF Na^+ AND GRAMICIDIN ON ATP EXCHANGE IN DIGITONIN PARTICLES

Freshly prepared digitonin particles (2.44 mg protein) labeled with [^{14}C]ATP, were added to 0.3 M sucrose containing 1 mM Tris–*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (pH 7.4) and the exchange initiated by 0.5 mM Tris–ADP or ATP (pH 7.4). Other additions were: 0.5 μg gramicidin; 5 mM NaCl. Total volume, 1.0 ml. The exchange was measured at 10°, and stopped at 15 sec with 50 μM atractyloside.

Additions	*Exchange* (%)
ATP	18.9
ATP, gramicidin	16.9
ATP, Na^+	24.6
ATP, Na^+, gramicidin	40.9
ADP	36.2

Raising the pH from 6.0 to 6.4 stimulated the ATPase activity from 710 to 1280 nmoles/ml, and at pH 7.6 the total phosphate declined to 540 nmoles/ml.

Gramicidin and ATP translocation

It has been demonstrated that the uncoupling agents *m*-chlorocarbonyl cyanide phenylhydrazone or dinitrophenol, or a cation permeability-inducing agent such as valinomycin, will stimulate the translocation of ATP, and not affect the exchange of ADP[16]. If the observed decrease in light scattering in the presence of gramicidin is due to a greater uptake of the ATP^{4-} anion and Na^+ (or K^+) cation, this should be expressed as an increased rate of binding of ATP^{4-}. Table IV shows the results of an experiment designed to determine whether gramicidin increased the ATP exchange in digitonin particles. The results show that under conditions similar to those employed in light scattering studies, gramicidin stimulated the exchange of ATP in particles containing 5 mM Na^+, but not in the absence of Na^+. Experiments not reported here show no effect of gramicidin on the exchange of ADP. Furthermore, K^+ can substitute equally well for Na^+ in the gramicidin-stimulated ATP exchange under these experimental conditions.

DISCUSSION

The demonstration that pH has similar effects on gramicidin-mediated light scattering changes, whether induced by ATP or by succinate, suggests that a common mechanism must be present. In the following discussion, two properties of gramicidin will be employed to explain the results; (1) the finding of CHAPPELL AND CROFTS[2,17] and HENDERSON *et al.*[3] that the transport-inducing antibiotics, particularly gramicidin and valinomycin, increase the passive permeability of natural and artificial membranes to protons and monovalent cations, and (2) that gramicidin, like valinomycin[16] and CCCP (refs. 14, 18), increases the mitochondrial ATP exchange, the ATP being exchanged intact and converted to ADP and P_i after the translocation[19].

The increased permeability to protons and cations by gramicidin results in a stimulation of ATPase activity. It is proposed that, provided the intramitochondrial phosphate potential is sufficiently high, and exogenous ATP and cations are present to diffuse inward down a concentration gradient, this leads to a decrease in light scattering. The coupling of the hydrolysis of ATP and a more rapid binding, or exchange, of ATP to the membrane in the presence of gramicidin and Na^+ (or K^+) leads to a passive uptake of the ATP anion, and accounts for the release of protons and "uptake" of P_i. It should be noted that inorganic phosphate *per se* is not taken up, but results from the stimulation of ATPase activity by gramicidin[17,20], with the ADP thus formed being back exchanged for external ATP. Under conditions of adenine nucleotide flux in fresh rat liver mitochondria, a diffusion-dependent uptake of ATP occurs at a concentration of 0.5–1.0 mM (ref. 21). The level of ATP found to increase light scattering in digitonin particles was 1.5 mM or higher, which is in reasonably good agreement. If the concentration of either ATP or cation is too low, no influx of the counter-ion will occur, and swelling will not take place.

The decrease in light scattering as the pH is raised above 6.4 is considered to be a consequence of the increased permeability to H^+ in the absence of gramicidin as the intra-/extra-mitochondrial proton gradient becomes greater. The more rapid loss

of protons serves to counterbalance the influx of cations which is not compensated for by ATP or succinate anion influx. Rossi *et al.*[22], using valinomycin-treated rat liver mitochondria, have shown that an increase in pH increases the H^+ release, and decreases the uptake of K^+ and succinate. The reduced intramitochondrial succinate concentration at pH above 7.0–7.5 observed by Rossi *et al.*[22] may be responsible for the drop in respiration (Fig. 1) at pH 7.6.

According to the scheme outlined above, oligomycin would prevent the gramicidin-mediated increase in ATPase activity, thus preventing the initial burst of H^+ release, leading to a retardation of the inward movement of exogenous ATP^{4-}. Although oligomycin does not reduce the rate of exchange of ATP in the uncoupled system[14,16], the lack of an external/internal ATP concentration gradient would inhibit the net uptake of ATP, and thus decrease swelling.

The succinate anion, which yields much the same light scattering changes as a function of pH as the ATP anion, would affect proton flux by translocating protons outward *via* the electron transport chain across the membrane[23]. Although the maintenance of the phosphorylation potential by the substrate is stressed, the importance of the anionic charge is underscored by the fact that 5 mM $succinate^{2-}$ gave the same degree of light scattering change as did 2.5 mM ATP^{4-}.

Efficacy of Na^+ and K^+

The observation by Wenner and Hackney[15] that gramicidin yields a greater light scattering change in the presence of K^+ than Na^+ is confirmed, and extended to include respiration with succinate as substrate. The explanation that a higher "energy flux" is necessary to drive the uptake of Na^+ than K^+ (ref. 15), can now be interpreted in terms of attractive forces between negative membrane sites and the cation[25,26]. In this interpretation, the mitochondrial membrane is depicted as containing anionic sites that interact with monovalent cations to different extents, depending upon (1) the field strength of these sites, and (2) the free mobility of the cation. The cationic preference would depend upon the magnitude of the loss in free energy involved in the cation–site interaction. At low pH, between 6 and 7, the negative sites on the mitochondrial membrane are effectively screened by protons, the effect being to weaken the sites. This forces the selectivity pattern towards the lyotropic or hydrated series ($K^+ > Na^+$), where the dominant factor is the mobility of the free ion. As the pH is raised, the selectivity begins to shift towards those monovalent cations having the highest center of charge ($Na^+ > K^+$), due to the greater field strength of the exposed negative sites. This is particularly evident in the respiration studies, where at high pH the respiration rate with Na^+ is higher than with K^+. In the light scattering studies, K^+ consistently yields a greater effect than Na^+, but the results are complicated by the reduction of the light scattering changes at high pH, which obscures a possible Na^+–K^+ crossover.

At low external ATP levels (0.8 mM), the greater shrinkage of the particles in the presence of K^+ is thought to be due to the loss of adenine nucleotides and accompanying K^+. The K^+_{int} is more easily exchanged with K^+_{ext} than with Na^+_{ext}.

Gramicidin and ATP exchange

The cation-dependent stimulation of the ATP exchange by gramicidin is not unexpected, in view of its ability to enhance proton and cation permeability. Pfaff

AND KLINGENBERG[16] have postulated that the higher rate of exchange diffusion of ATP in the presence of uncoupler is due to the collapse of the membrane potential, and a similar mechanism may be invoked for gramicidin. The fact that PFAFF AND KLINGENBERG[16] did not observe a stimulation of the ATP exchange with valinomycin until decimolar levels of KCl were reached may be due to the different effects of gramicidin and valinomycin on H^+ uptake. CARAFOLI *et al.*[24] have shown that gramicidin is three times more effective than valinomycin in inducing proton uptake. HENDERSON *et al.*[3] have concluded that gramicidin can moderately increase the permeability to protons in natural and artificial membrane systems, but valinomycin renders the membrane permeable only to cations. The greater effectiveness of gramicidin, then, resides in its ability to reduce the membrane potential, allowing a higher rate of exchange diffusion of the exogenous ATP^{4-} molecule against a less negative potential.

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