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LIGHT-INDUCED pH CHANGES IN SUB-BACTERIAL PARTICLES OF *HALOBACTERIUM HALOBIUM*

EFFECTS OF IONOPHORES

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Summary

The kinetics of light-induced acidification and of the subsequent dark-induced alkalization in suspensions of sub-bacterial particles of *Halobacterium halobium* may be expressed as the sum of two exponentials, indicating two processes (Eisenbach, M., Bakker, E.P., Korenstein, R. and Caplan, S.R. (1976) FEBS Lett. 71, 228—232).

We studied the effects of carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone, nigericin, gramicidin D, valinomycin, and monactin on the extents and the rate constants of the two processes. The various ionophores affected the two processes differently and in general the slower process was more sensitive to their presence. Valinomycin and monactin had relatively minor effects, apparently due to the high ionic strength of the suspension. When an artificial membrane potential was created in the dark, the light-induced acidification was preceded by a transient alkalization as is usually observed in intact cells.

These results are discussed in the light of a suggested model accounting for the two processes (Caplan, S.R., Eisenbach, M., Cooper, S., Garty, H., Klemperer, G. and Bakker, E.P. (1977) in Bioenergetics of Membranes (Packer, L., Papageorgiou, G.C. and Trebst, A., eds.), pp. 101—114, Elsevier/North-Holland Biomedical Press, Amsterdam), taking into account the different selectivities of the ionophores applied.

Introduction

Bacteriorhodopsin located in the purple membrane of *Halobacterium halobium* acts as a light-driven proton pump which ejects protons from the cell

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone; $TPMP^+$, triphenylmethylphosphonium.

upon illumination [1]. The proton electrochemical potential difference formed is used for ATP synthesis [2,3], sodium extrusion [4,5] and amino acid uptake [6,7].

Similar to whole cells, but simpler, are sub-bacterial particles prepared from intact bacteria by sonication [5,6,8]. The kinetics of the light-induced pH changes taking place in such particles and in proteoliposomes containing bacteriorhodopsin was analyzed and found to fit a sum of two exponentials, indicating two simultaneous processes [9,10]. Recently, a model was developed suggesting that only one of the two processes is actually proton transport, while the other reflects dissociation-association from the bacteriorhodopsin-lipid complex [10,11]. In this communication we use a group of ionophores of different ionic selectivities as a tool for the examination of the effect of ion permeability and proton motive force on the two processes.

Materials and Methods

Growth of *H. halobium* strain M-1 and preparation and characterization of sub-bacterial particles were performed as described [5]. The pH of a suspension of particles was measured in a thermostatted vessel using a Radiometer (Copenhagen) pH meter (type 64) connected to a high speed recorder (Varian A-25 with a response time of 0.5 s) and a combined pH electrode (Radiometer type GK 2321^c). The vessel was illuminated by a slide projector through either an OG 517 nm 'cut-off' filter (Schott) or a 580 nm interference (Baird Atomic) filter, the light intensities being at most 350 W/cm^2 (which is too small to bring about sensible accumulation of the 412 nm photointermediate [11]).

In order to avoid leakage of protons through the Na^+/H^+ antiport the sub-bacterial particles were prepared and suspended in 4 M KCl. However, in some cases particles prepared in 4 M NaCl were used. In these experiments the light intensity was 45 W/cm^2, which is too low to drive Na^+/H^+ exchange (cf. Table I in ref. 5).

Soybean lecithin proteoliposomes were prepared by sonication as described by Bakker et al. [12], asolectin was purified by the method of Kagawa and Racker [13], and purple membrane fragments were isolated as described by Oesterhelt and Stoeckenius [14].

Accumulation of ^{3}H-labelled $TPMP^+$ was measured by the filtration method. Unlabelled $TPMP^+$ (final concentration $3 \cdot 10^{-5}$ M) was added to suspensions of sub-bacterial particles prepared in 4 M KCl (4 mg protein/ml). After 20 min incubation ^{3}H-labelled $TPMP^+$ was added (final concentration 0.3 μCi/ml, 10^{-6} M) and the particles were allowed to incubate in the dark for an additional 20 min. Samples (50 μl) were then filtered through cellulose acetate Millipore filters (0.5 μm) and washed twice with 2 ml 4 M KCl containing $3 \cdot 10^{-5}$ M unlabelled $TPMP^+$. Aliquots of unfiltered suspension were sampled as well.

Lysed particles were prepared as follows: A suspension of particles was diluted 10-fold with water and after 30 min incubation spun down and resuspended in the original volume of 4 M salt solution.

FCCP, gramicidin D, soybean lecithin, and valinomycin were obtained from Sigma Chemical Co.; $TPMP^+Br^-$ from K&K Laboratories; nigericin from Eli Lilly and Co.; monactin was a gift from Mrs. Y. Shahak, The Weizmann Insti-

tute of Science, Rehovot; ^{3}H-labelled TPMP$^+$ (114 Ci/mol) was a gift from Dr. R. Kaback, Roche Institute of Molecular Biology, Nutley, N.J., U.S.A.

Results

Effects of FCCP

As shown previously [9], the kinetics of proton release from sub-bacterial particles upon illumination, i.e. during the 'on' reaction, and their passive diffusion in the following 'off' reaction, are composed of two distinct first-order processes (denoted 'rapid' and 'slow'). A proton conductor, such as FCCP, is expected to increase the membrane permeability for protons and hence to decrease the extent of proton extrusion in the light. In order to see whether the increase in proton permeability affects the two processes differently, we examined the effect of FCCP on the light-induced pH changes. Table I summarizes the various kinetic parameters measured at two light intensities in the presence and absence of FCCP. As expected, FCCP reduced the extents of the 'on' and 'off' processes, but did not seem to distinguish between the rapid and slow phases. On the other hand, the rate constant of the slow process (k_2) turned out to be much more sensitive to FCCP than that of the fast one (k_1). The effects of FCCP on the rate constants are due to the fact that these rate constants are apparent values, i.e., they contain contributions from passive leaks. Increasing the leaks reduces k_{on} and increases k_{off}. These effects are more pronounced at low light intensities, where the rate of proton pumping by bacteriorhodopsin is commensurate with that of their leakage. The relatively small effect of FCCP on the kinetics of the fast process suggests that most of the influx of protons induced by this uncoupler occurs on the time scale of the slow one, although a significant inflow must nevertheless occur during the fast process since its extent is reduced markedly upon addition of FCCP.

Effects of nigericin

Nigericin is a polyalcohol-polyether carboxylic acid which usually exchanges H^+ for K^+ in a 1 : 1 ratio [15]. However, it was shown that in the concentration range above 1 μM this ionophore can mediate net transfer of charge by carrying K^+ electrogenically [16]. Fig. 1 shows the effects of nigericin on the kinetic parameters of the pH changes.

Different effects of nigericin on the rate constants were observed at concentrations below and above 0.5 μM. Below this concentration k_1 (on and off) and k_2 (off) were hardly affected by nigericin; however, k_2 (on) decreased with increasing concentration. Above this concentration, all rate constants increased markedly.

The total extent of the light-induced pH change decreases upon addition of nigericin and this effect is much more pronounced at concentrations higher than 0.5 μM (Figs. 1C and D). The kinetic analysis shows that nigericin mainly affects the slow process, and at low concentrations the extent of the rapid process is insensitive to it. At concentrations higher than 3 μM the two phases could not be distinguished, since the extent of the rapid process was very small. As will be discussed below these effects can be explained by the different modes of action of nigericin at low and high concentrations.

TABLE I

EFFECTS OF FCCP ON THE KINETIC PARAMETERS OF THE LIGHT-INDUCED pH CHANGES

Sub-bacterial particles (3.5 mg protein) prepared in either 4 M KCl or 4 M NaCl were suspended in 2.5 ml of their preparation medium. The pH was brought to 7.5 and after pre-illumination the kinetics were followed at 25°C as described under Methods. KCl particles were illuminated at a light intensity of 350 W/m^2 and NaCl particles at 45 W/m^2 only (to avoid interference by the sodium/proton antiport [5]). Increasing amounts of FCCP were added from alcoholic solution, the amount of alcohol present in the suspension being at most 0.1% (v/v). The light-induced pH changes were analyzed according to ref. 9.

Ion composition and experimental conditions		'on' Process				'off' Process			
		Extents (nmol H^+/mg)		Rate constants (s^{-1})		Extents (nmol H^+/mg)		Rate constants (s^{-1})	
		Rapid	Slow	k_1	k_2	Rapid	Slow	k_2	k_2
	Control	1.8 ± 0.1	2.1 ± 0.1	0.27 ± 0.02	0.09 ± 0.02	1.3 ± 0.02	2.0 ± 0.02	0.31 ± 0.02	0.045 ± 0.015
4 M NaCl	4 μM FCCP	0.9 ± 0.1	0.9 ± 0.1	0.22 ± 0.03	0.012 ± 0.002	0.37	0.74	0.46 ± 0.05	0.10 ± 0.02
I = 45 W/m^2	125 μM FCCP	0	0	—	—	0	0	—	—
4 M KCl	Control	5.5 ± 1.0	15.5 ± 1.0	0.20 ± 0.01	0.03 ± 0.002	5.5 ± 0.2	12.0 ± 0.5	0.18 ± 0.02	0.010 ± 0.001
I = 350 W/m^2	100 μM FCCP	2.0 ± 0.3	6.0 ± 0.5	0.26 ± 0.03	0.013 ± 0.003	2.0 ± 0.1	5.5 ± 0.5	0.23 ± 0.03	0.015 ± 0.001

TABLE II

THE EFFECTS OF K^+ CARRIERS ON PROTON TRANSLOCATION BY BACTERIORHODOPSIN

The kinetics of the light-induced pH changes were followed under the conditions described in the legend to Table I in KCl- and NaCl-loaded particles, with and without valinomycin or monactin. In NaCl particles the two phases could not be distinguished in the presence of valinomycin during the 'on' process.

Ion composition (inside and outside the particles)	'on' Process				'off' Process			
	Extent (nmol H^+/mg)		Rate constants (s^{-1})		Extent (nmol H^+/mg)		Rate constants (s^{-1})	
	Rapid	Slow	k_1	k_2	Rapid	Slow	k_1	k_2
4 M KCl	2.3 ± 0.6	4.0 ± 0.7	0.18 ± 0.01	0.045 ± 0.006	2.3 ± 0.3	3.1 ± 0.1	0.17 ± 0.01	0.014 ± 0.003
+ 1 μM valinomycin	2.3 ± 0.2	3.7 ± 0.3	0.3 ± 0.05	0.06 ± 0.01	2.5 ± 0.1	3.7 ± 0.6	0.15 ± 0.01	0.020 ± 0.003
3 M KCl	1.8 ± 0.2	2.4 ± 0.1	0.13 ± 0.02	0.011 ± 0.005	0.85 ± 0.1	2.7 ± 0.3	0.27 ± 0.03	0.027 ± 0.003
+ 1 μM valinomycin	1.9 ± 0.1	2.1 ± 0.2	0.18 ± 0.03	0.010 ± 0.003	1.0 ± 0.05	2.4 ± 0.2	0.18 ± 0.02	0.040 ± 0.005
4 M NaCl + 160 mM KCl	1.6 ± 0.2	2.8 ± 0.1	0.27 ± 0.03	0.12 ± 0.01	2.2 ± 0.1	1.3 ± 0.2	0.22 ± 0.03	0.056 ± 0.005
+ 1 μM valinomycin				0.18 ± 0.02	2.4 ± 0.1	1.8 ± 0.1	0.27 ± 0.05	0.081 ± 0.007
4 M KCl	6 ± 1	11 ± 1	0.17 ± 0.03	0.025 ± 0.001	5.0 ± 0.5	10.5 ± 1.0	0.16 ± 0.02	0.010 ± 0.001
+ 0.2 μM monactin	6 ± 1	15 ± 1	0.25	0.050 ± 0.005	6.5 ± 1.0	12.5 ± 1.0	0.20 ± 0.02	0.016 ± 0.001

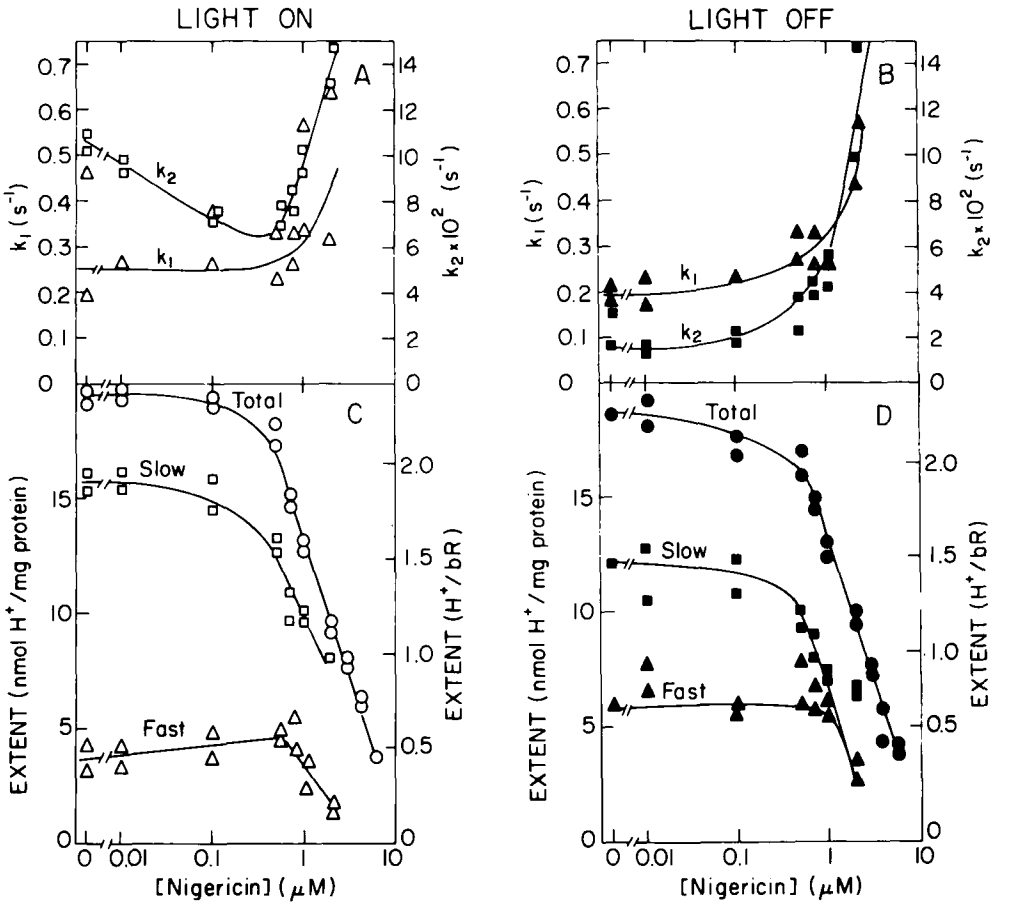

Fig. 1. Effects of nigericin on the kinetic parameters of the pH changes. The experiment was carried out and analyzed as described in the legend to Table I. KCl-loaded particles were used and the light intensity was 350 W/m^2. △, ▲, fast process; □, ■, slow process; ○, ●, total extents. The open and closed symbols stand for the 'on' and 'off' processes respectively.

A decrease in the light-induced pH change upon addition of nigericin to sub-bacterial particles has previously been observed by Kanner and Racker [8]. On the other hand, addition of nigericin to intact cells caused a marked increase in the magnitude of the pH change [17,18]. The difference between the two systems is due to the fact that intact cells, unlike sub-bacterial particles, are characterized by a large transmembrane gradient of K^+. As discussed by Bakker et al. [18], addition of nigericin in the dark stimulates an efflux of K^+ (exchanged for H^+) and decreases the internal pH. Thus more protons can be extruded upon illumination. Indeed it was found that adding nigericin to intact cells in the dark causes a marked alkalization [17,18]. On the other hand, adding nigericin to a sub-bacterial suspension in the dark does not drive K^+/H^+ exchange, since the two ions are at equilibrium across the membrane (in fact we did not observe dark alkalization). Thus the dominant effect here is leakage of protons through the ionophore site and the total extent decreases on illumination.

Effects of monactin, valinomycin, and gramicidin D

In order to see whether changes in the permeability to co-ions (accompanying the protons) affects the two phases differently, we used the K^+ carriers monactin and valinomycin. Table II shows the effects of these ionophores on the kinetic parameters under different conditions. It turned out that both of them had quite a limited effect on the kinetics of proton translocation. Although the effects were relatively small, it appears that valinomycin slightly increased the rate constants of the slow process, irrespective of the ion composition, and did not influence the extents, while monactin increased both the extents and the rate constants. In both cases, the fast process seemed to be less affected than the slow one.

The small effect of valinomycin on the pH changes is in agreement with the observation that valinomycin hardly affects the rate of $^{86}Rb/Rb$ exchange [19] or the kinetics of pH change [18] in intact cells, but seems to be in disagreement with the observations of Lanyi and coworkers on amino acid transport [6,20]. Garty and Caplan [19] speculated that the limited activity of valinomycin generally observed in *H. halobium* has to do with the high ionic strength of the growth medium of these microorganisms. To check this hypothesis, we turned to bacteriorhodopsin-containing proteoliposomes, since these can be prepared at various salt concentrations [9,21].

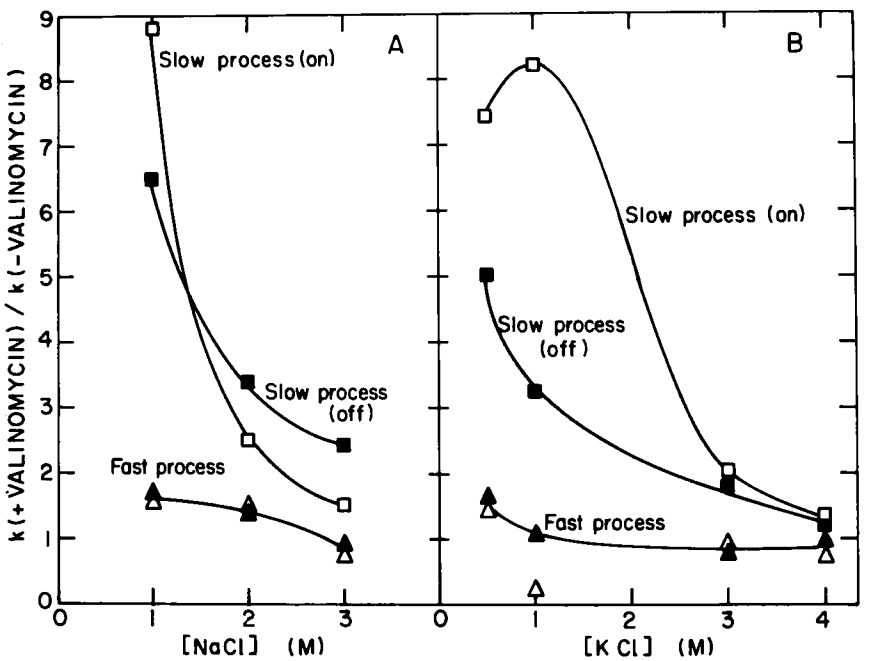

Fig. 2. The effects of valinomycin on the rate constants in proteoliposomes. Liposomes containing 12 μM bacteriorhodopsin and 5 mg soybean lecithin in total volume of 3 ml were prepared as described under Methods in the presence of: (A) 0.1 M KCl plus various NaCl concentrations; (B) Various KCl concentrations. Under all conditions the pH was 7.5 and the temperature was 25° C. The light-induced pH changes were followed at 350 W/m^2 with and without 1 μM valinomycin. The relative increase in the rate constants is plotted vs. the salt concentration. △, ▲, fast process; □, ■, slow process. The open and closed symbols stand for the 'on' and 'off' processes, respectively.

Fig. 2 shows the relative increase in rate constants induced by 1 μM valinomycin in proteoliposomes prepared in 0.1 M KCl plus increasing NaCl concentrations (Fig. 2A), or increasing KCl concentrations alone (Fig. 2B). Valinomycin mainly affected the rate constants of the slow process, causing at most a 1.5-fold increase in the rate constants of the rapid process. The effect of valinomycin on the rate constants of the slow process turned out to be salt-dependent. In 1 M KCl a more than 8-fold increase in k_2 (on) was observed upon addition of 1 μM valinomycin, but increasing the concentration of KCl to 4 M reduced the effect to 1.25-fold only (Fig. 2B). Similar results were obtained with proteoliposomes prepared in increasing concentrations of NaCl in the presence of a constant concentration of K^+ (Fig. 2A). Hence the inactivation of valinomycin is due to the increase in ionic strength rather than the high K^+ concentration. For 0.5 M KCl the increase in k_2 (on) observed was only 7.5-fold (Fig. 2B), and at lower salt concentrations the effect was even smaller (not shown). This is in agreement with the observations of Läuger [22], who showed that the conductance of a lipid bilayer in the presence of valinomycin increases on increasing the concentration of K^+, reaching saturation at about 1 M KCl. The decrease observed in valinomycin activity at higher ionic strength explains the results reported above as well as results reported previously [18,19,23].

In contrast to the small effects of valinomycin and monactin, gramicidin was found to increase both the rate and the extent of the light-induced pH changes markedly even at a concentration as low as 0.1 nM. Unfortunately, the kinetics of proton translocation in the presence of this ionophore turned out to be complex (i.e., not describable as a sum of two exponentials). This is probably because it acts as a channel not only for K^+ and Na^+, but also for protons [24].

Light-induced pH changes in the presence of a pre-existing electrical potential difference

We examined the effects of valinomycin and gramicidin D in the presence of a diffusion potential (negative inside). This was induced by diluting KCl-loaded particles with 4 M NaCl. As in the case of intact cells [18,25], the transmembrane potential difference can be estimated from the distribution of the hydrophobic cation $TPMP^+$. This was measured in the dark, in KCl-loaded sub-bacterial particles diluted with either 4 M KCl or 4 M NaCl, and in lysed particles. A 1.2-fold dilution of the particles in NaCl gave rise to an accumulation factor of 30 (i.e. $[TPMP^+]_{in}/[TPMP^+]_{out} = 30$). This indicates the formation of a diffusion potential with negative inside polarity. Such a potential may be the result of Na^+ influx through an electrogenic Na^+/H^+ antiport [10,20] and/or passive peak K^+ leak [19]. The accumulation factor could be reduced to 7 by addition of 20 nM gramicidin, and the same value was obtained when dilution of the particles was performed with 4 M KCl. A 7-fold accumulation of $TPMP^+$ was observed in lysed particles as well. We conclude that this represents $TPMP^+$ associated with the membrane, and not a residual dark potential as in intact cells [18,25].

The amount of $TPMP^+$ associated with lysed particles was found to increase on increasing the concentration of $TPMP^+$ in the medium. This means that the absorption of $TPMP^+$ reflects partition of the lipophilic cation between the

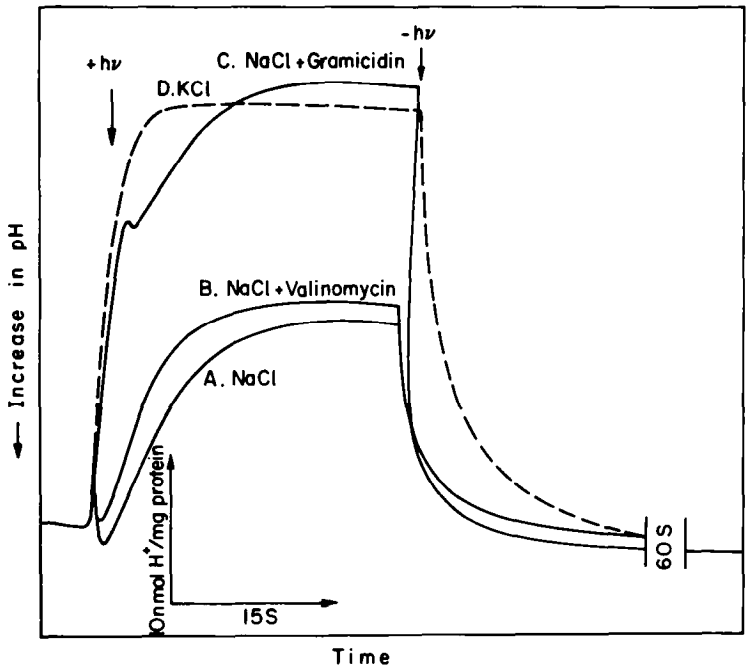

Fig. 3. The kinetics of the light-induced pH changes in the presence of a concentration gradient. Subbacterial particles (3.5 mg protein) prepared in 4 M KCl were suspended in 2.5 ml 4 M KCl at pH 7.5 and 25°C. After an equilibration period of 15 min the following substances were added: (A) 1 ml 4 M NaCl; (B) 1 ml 4 M NaCl plus 1 μM valinomycin; (C) 1 ml 4 M NaCl plus 20 nM gramicidin; (D) 1 ml 4 M KCl. The light-induced pH changes were followed as described under Methods at 350 W/m^2.

two phases, or binding under conditions such that the binding sites are far from saturated. In both cases the amount of $TPMP^+$ in the membrane would be expected to increase with increasing concentration of $TPMP^+$ inside the particles, i.e. with increasing electrical potential difference. Thus only a semiquantitative estimation of the membrane potential can be obtained from its accumulation factor.

Fig. 3 shows the light-induced pH changes observed in particles diluted with KCl or NaCl. It appears that the presence of a dark potential difference markedly reduces both the rate and the extent of proton extrusion. With 20 nM gramicidin, which was shown to abolish the diffusion potential, the initial rate and extent increased to the values observed with KCl-diluted particles. On the other hand, 1 μM valinomycin hardly affected the kinetics. In addition, the dark potential difference appears to bring about a transient light-induced inflow of protons, quite similar to that usually observed in intact cells [1,3,26]. According to Bogomolni et al. [17], the initial transient inflow of protons in intact cells is in fact due to a pre-existing dark potential difference with negative inside polarity.

Our results indicate that a small dark potential difference may indeed be associated with light-induced alkalization.

TABLE III

THE RELATIVE EFFECTS OF IONOPHORES ON THE KINETIC PARAMETERS

The relative effects of each ionophore on the kinetic parameters were calculated from the data shown in the previous figures and tables, and expressed as percentage, i.e. 100 times (value in the presence of ionophore)/(value in the absence of ionophore).

Ionophore	'on' Process				'off' Process			
	Extent		Rate constant		Extent		Rate constant	
	Rapid	Slow	k_1	k_2	Rapid	Slow	k_2	k_2
100 μM FCCP in 4 M KCl (particles) (I = 350 W/m2)	40	40	130	40	40	50	130	150
4 μM FCCP in 4 M NaCl (particles) (I = 45 W/m^2)	50	40	80	10	30	40	140	210
0.1 μM nigericin in 4 M KCl (particles)	100	100	100	70	100	100	100	120
2 μM nigericin in 4 M KCl (particles)	40	50	140	150	50	50	270	750
0.2 μM monactin in 4 M KCl (particles)	100	140	150	200	130	120	120	160
1 μM valinomycin in 4 M KCl (particles)	100	100	170	140	110	120	90	140
1 μM valinomycin in 1 M KCl (liposomes)	100	110	160	870	90	110	170	650

Discussion

The ionophores examined in this communication are in principle classifiable into two groups: (i) ionophores which more or less selectively mediate an electrogenic flow of cations, such as gramicidin, valinomycin, monactin, and nigericin at high concentrations; and (ii) ionophores which mediate a flow of protons, either in an electrogenic manner, such as FCCP, or in an electroneutral manner, such as nigericin at low concentrations. As discussed above, the effect of proton conductors would be expected to reduce the apparent rate constant of proton release from the cell and to increase the apparent rate of their uptake in the dark. The extent of the light-induced pH changes may be reduced or not, depending on the relative magnitude of the leak introduced by the uncoupler. As discussed previously [11,27], proton translocation by purple membrane is essentially electroneutral and its rate is limited by the permeability of the membrane to co- and counterions. Thus, increasing the membrane permeability to ions by addition of valinomycin, monactin or gramicidin should speed up the rate of the light-induced pH changes, while the extent may or may not be increased. Table III summarizes the relative changes in the kinetic parameters brought about by the different ionophores used. As can be seen, the observed effects agree with the above prediction (within the experimental range of error), and the only exception occurs in the case of 2 μM nigericin (probably because the fluxes of H^+ and K^+ are both increased in a non-stoichiometric way).

It has recently been suggested [10,11] that only the slow phase represents translocation of protons from the cell interior, while the rapid phase is due to dissociation of protons from the lipid-protein complex as a result of light-induced pK shifts. On this basis it is expected that ionophores which increase the membrane permeability to protons and other ions should mainly affect the rate constant of the slow phase. Table III shows that k_2 is indeed more sensitive to the presence of ionophores than k_1, especially when large changes in the rate constants are caused (i.e. FCCP at low light intensity or valinomycin in liposomes). The slight dependence of the rapid phase on the presence of ionophore may possibly be due to the fact that k_1 is only an apparent rate constant as a consequence of the back leakage of protons, and the effect of ionophores on the leak indirectly affects the kinetics of this phase as well. In addition, one should not rule out the possibility of minor effects of the ionophores on the membrane other than increases in ion permeability.

Although all the ionophores used affected the kinetic parameters, some of them (e.g. monactin, valinomycin, and FCCP at high light intensities) had quite a limited effect in sub-bacterial particles as compared to their effects in proteoliposomes (Bakker, E.P. and Caplan, S.R., unpublished results) or in other systems. A possible reason for low activity of these ionophores, in contrast to the high activity of gramicidin D, may be the high viscosity of the bacterial cell membrane [28]. As suggested previously [6], the highly viscous membrane will hinder the effectiveness of carriers like monactin or FCCP, but will not affect channels such as gramicidin. In this view, the effect of salt may be to decrease membrane fluidity by the salting out of water. An alternative explanation of the salt-dependent effect of valinomycin may be the influence of ionic strength

on the hydrogen bonds determining the structure of this ionophore (Ovchinnikov, Y.A., personal communication).

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