EFFECT OF IONOPHORIC COMPOUNDS ON AQUEOUS SUSPENSIONS OF PURPLE MEMBRANE

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1. Introduction

The purple retinal-protein complex, bacteriorhodopsin, functions in *Halobacterium halobium* as a light dependent proton pump [1]. It is organized in the cell membrane in patches designated as 'purple membrane' (PM) which can be isolated [2]. When PM is reincorporated into phospholipid vesicles it is able to transpose protons on illumination [3]. In aqueous suspensions of PM rapid sequence of spectral changes can be detected by flash spectroscopy which are associated with cyclic proton release and uptake [4]. Continuous illumination of PM, suspended in salt solution saturated with ether, establishes a steady state in which most of the purple complex is present in the unprotonated form absorbing maximally at 412 nm [5]. The protons released cause a measurable acidification of the medium as long as the illumination lasts. In pure aqueous suspension only a minute fraction of the purple complex is converted to the '412 nm complex' and correspondingly also the extent of proton release is very small.

The present report deals with a so-far unobserved response of PM to certain ionophoric substances. It is shown that valinomycin and beauvericin, when added to aqueous suspensions of PM, mimic in many respects the effect of ether-salt mixtures. They enhance on illumination the formation of the '412 nm-complex' with concomitant acidification of the medium.

2. Materials and methods

2.1. *Methods*

For growth of *H. halobium* and isolation of PM the method of Oesterhelt and Stoeckenius [2] was used with slight modifications. Light-induced proton release was assayed at 22°C in a water jacketed cell with temperature control as described by Racker and Stoeckenius [3]. A slide projector equipped with a 500 W tungsten lamp was used for illumination of the cell. The intensity of the light, after emerging from the cell, was 3.0×10^5 ergs cm^{-2} s^{-1}. A slide projector with a 150 W lamp was used as a source for actinic light to induce spectral changes. The intensity of the light beams, after passing a Corning No. 3486 filter, was 1.5×10^5 ergs cm^{-2} s^{-1}. The measurements were carried out in a Cary model 118 spectrophotometer. The guard filters, Corning 9782 and 3966, were placed in front of the photomultiplier tube to protect against scattered light.

2.2. *Definition of concepts and units*

2.2.1. $\triangle H_L^+$

Increment in the proton concentration of the assay medium (g equiv. H^+/mol bacteriorhodopsin) elicited by continuous illumination.

2.2.2. $\Delta A_L^{x\ nm}$

Increment in the absorbance of bacterorhodopsin (A cm^{-1}/μmol bacteriorhodopsin/ml) at the wavelength indicated, elicited by continuous illumination with actinic light.

2.3. *Materials*

Valinomycin and carbonylcyanide–trifluoromethoxyphenylhydrazone (FCCP) were the product of Sigma Chemical Co., St Louis, Mo., USA A23187 and beauvericin were a gift of Elli Lilly and Co., Indianopolis, Ind., USA and nigericin was donated by Dr N. Shavit, Ben-Gurion University of Negev, Beer-Sheva, Israel.

3. Results

The effect of ionophores on the reversible photolysis of the purple complex was studied in aqueous suspensions of PM. From the results shown in fig.1 it is apparent that in the absence of ionophores illumination of a suspension of PM in KCl caused very little acidification of the medium or change in the absorbance at 412 nm. Preincubation of the system with valinomycin enhanced considerably the above changes. Beauvericin, which like valinomycin is an uncharged depsipeptide but of smaller ring size [6], was ineffective alone. However, in combination with valinomycin it acted as a synergist. The uncoupler, FCCP, decreased slightly at high concentrations the effect of the ionophores. Two other ionophores bearing one negative charge, nigericin and A23187, were also tried (not shown) but had no effect on the photocycle. Figure 2

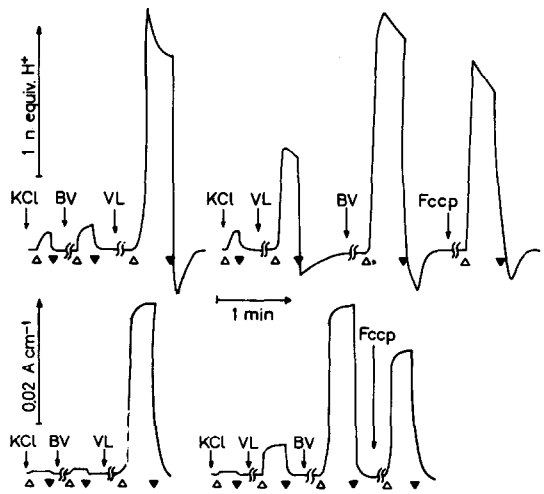

Fig.1. Effect of valinomycin and beauvericin on light-induced acidification and absorbance changes. The 'basal assay medium' contained in total vol. 1.0 ml, 50 μl PM. The latter is equivalent to 3.7 nmol bacteriorhodopsin determined spectrophotometrically (taking the millimolar absorption coefficient at 570 nm as 63 mM^{-1} cm^{-1}). The pH was adjusted to 6.5 by approx. 40 nmol. KOH. The following additions were made to the basal assay medium at the time indicated by an arrow: 100 mM KCl; 10 μg valinomycin (VL); 4 μg beauvericin (BV); 6 μg FCCP. For other conditions see under Materials and methods. (△ and ▼) Start and end of illumination. (−∫∫−) Five minutes of incubation before subsequent light–dark cycle. Original traces redrawn after correcting for changes in the buffer capacity.

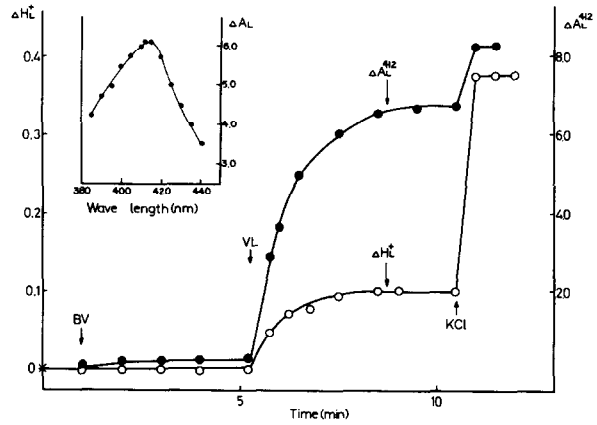

Fig.2. Time course of interaction between valinomycin and PM. To the basal assay medium (total vol. 1.0 ml) at the times indicated by an arrow the following additions were made: 4 μg beauvericin (BV): 10 μg valinomycin (VL): 100 mM KCl. $\triangle H_L^+$ and $\triangle A_L^{412\,nm}$ were assayed, at the time intervals shown. For definition of $\triangle H_L^+$ and $\triangle A_L^{412\,nm}$ see under Materials and methods. Insert: Spectrum of the intermediate formed by illuminating a suspension of PM with actinic light and monitoring $\triangle A_L$ between 390 nm and 440 nm. Basal assay medium supplemented with 10 μg valinomycin and 4 μg beauvericin (total vol. 1.0 ml) was used. For other details see Materials and methods.

shows the time-course of interaction between valinomycin and PM. In order to enhance the extent of changes, PM was preincubated with beauvericin before the addition of valinomycin. The interaction is reflected by a gradual increase in the extent of proton release and absorbance-increment at 412 nm ($\triangle H_L^+$ and $\triangle A_L^{412nm}$, respectively). As seen from fig.2, both $\triangle H_L^+$ and $\triangle A_L^{412nm}$ reached a plateau simultaneously (approx. after 5 min). Omission of beauvericin had no effect on the kinetics of interaction, only on the magnitude of $\triangle H_L^+$ and $\triangle A_L^{412nm}$. In the phase of the plateau, addition of KCl caused an instantaneous large increment of $\triangle H_L^+$ but had relatively little effect on $\triangle A_L^{412nm}$. The spectrum of the intermediate which is responsible for the rise in the absorbance at 412 nm was obtained by illuminating PM, preincubated with the two ionophores, with actinic light (> 510 nm) and monitoring $\triangle A_L$ as a funtion of the wavelength of the measuring beams. As shown in the insert to fig.2, the spectrum which was obtained is similar to that published for the '412 nm complex' [5]. In addition to KCl also some other salts were found to influence the photocycle

Table 1
The effect of salts on the light-induced proton release

Salt used for titration	K_m (mM)[a]	H_L^+ max[b]
None	–	0.1
NaCl	3.0	0.4
KCl	3.0	0.4
$MgCl_2$	2.0	0.7
$CaCl_2$	0.4	0.6

[a]Salt concentration yielding half maximum $\triangle H_L^+$
[b]The plateau reached by titrating with the respective salt

1.0 ml of the basal assay medium was incubated at 22°C for 10 min. with 10 μg of valinomycin and 4 μg of beauvericin and titrated with the respective salt.

in the presence of the two ionophores. The salts listed in table 1 had very little effect on $\triangle A_L^{412\ nm}$ (not shown) but enhanced considerably $\triangle H_L^+$. A higher plateau was reached with respect to $\triangle H_L^+$ by titrating the assay system with $MgCl_2$ or $CaCl_2$ than with NaCl or KCl. There was no preference for K^+-ions over Na^+ but the K_m for Ca^{2+} was found to be considerably lower than for Mg^{2+}. The stoichiometry of the PM–ionophore interaction was also determined (not shown). For this purpose the basal assay medium containing a fixed amount of PM and also 100 mM KCl was titrated either with valinomycin or with beauvericin. The concentration of the ionophore which produced maximum $\triangle H_L^+$ was related to the amount of bacteriorhodopsin present. A ratio of 1.3 molecules of valinomycin per molecule of bacteriorhodopsin was found whether the titration followed preincubation with an excess of beauvericin or not. The corresponding ratio for beauvericin was 0.9. In this latter case preincubation with an excess of valinomycin was obligatory since beauvericin alone had no significant effect on $\triangle H_L^+$.

4. Discussion

Release of one proton per molecule of bacteriorhodopsin has been achieved by flash-illumination of aqueous PM suspensions [7]. Under steadystate conditions, in salt ether, the proton yields were, however, very small [5]. In contradistinction, in the presence of certain ionophores and of divalent cations yields as high as 0.7 proton/molecule bacteriorhodopsin can be attained. Apparently the ionophores, like the organic solvents [5] act by stabilizing the '412 nm complex', which is the deprotonated form of the purple complex. The observed slow kinetics of the bacteriorhodopsin–ionophore interaction may be a reflection of a time-consuming rearrangement of the ionophore in the lipid layer which surrounds the pigment in the purple patches [8]. It cannot be decided at present whether the stoichiometry of near to 1:1 in the interaction between bacteriorhodopsin and the ionophores is an indication for the existance of specific binding sites. It is also premature to make generalizations on structure–function relationships based on the finding that among the ionophores tested up to now the active compounds (valinomycin and beauvericin) bear no charge while the two ineffective ones (nigericin and A23187) bear a negative charge [6]. The salts, unlike the ionophores, increased much more $\triangle H_L^+$ than $\triangle A_L^{412nm}$. Therefore, their main action probably is not on the photolysis per se, but rather on the release of the photodissociated protons into the aqueous phase. The order of effectiveness of the cations to enhance $\triangle H_L^+$ is not related to the known discriminatory ability of valinomycin and of beauvericin [6,9] for cations. Because of the greater effectiveness of divalent cations, electrostatic neutralization is likely to be involved in the salt effect. The preference observed for Ca^{2+}-ions recalls the role played by this cation in membranal phenomena involving aggregation, fusion and phase separation [10]. The release of the protons into the medium could be facilitated by formation of channels through aggregation of PM patches. Light-induced extrusion of protons from closed vesicles seems to be unlikely because of the low sensitivity of the proton-release to FCCP.

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