THE EFFECT OF LIGHT ON THE PERMEABILITY OF A MYXOMYCETE

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Received 24 April 1972

1. Introduction

Light is required to induce the gametic sporulation of suitably starved plasmodia of the coenocyte *Physarum polycephalum* [1]. Light also strongly and rapidly inhibits submerged culture growth of this organism [1, 2] suggesting the possibility that the primary light-dependent reactions assumed to initiate the sequence of differentiation reactions is present in at least these two stages of the life cycle. Previously, long term metabolic changes, for example, transient increases in plasmodial ATP and in a glycogen-like polysaccharide as well as a sustained and large increase in the pH of the culture medium were observed [3]. The present results strongly suggest that the light-dependent metabolism supports specific cellular proton uptake accompanied by release of K^+ into the medium. A technique which utilizes gramicidin S* for preliminary characterization of this light-dependent metabolism effecting proton transport is presented. A possible basis of the latter change will be reported here for microplasmodia in relation to the recent observation of the light-induced calcium deposition in vacuoles and mitochondria of plasmodia [4].

* *Abbreviations:*
GS, gramicidin S; EGTA, ethyleneglycol-bis (β-aminoethyl ether)-*N*,*N*'-tetracetic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; *N*-ethylmaleimide, NEM.

2. Methods

Cultures (microplasmodia) were grown in submerged culture on the semi-defined medium as previously described [1]. Microplasmodia are prepared for measurement by low speed centrifugation followed by washing three times with 10-fold the pellet volumes of 0.15 M sucrose–10 mM KCl each time with final resuspension in the same medium, 50 ml per ml packed pellet. The microplasmodial suspension is transferred to a 500 ml erlenmyer flask and incubated at 23° as for growth. For pH measurement, 10.0 ml aliquots are transferred to a 20 ml beaker, vigorously stirred and aerated. The pH of the unbuffered suspension was adjusted to approx. pH 6.3, made to 30 mM with KCl and pH changes monitored with a Radiometer type PHM 26 pH meter and combination electrode (GK 2322C) the output signal being read out on a Heath Co. #EUW-20H servo recorder. Additions were then made as needed, followed finally by a series of GS additions, each lasting 3 min (GS assay). The suspension was illuminated as required by two parallel pairs of 5W "cool white" General Electric fluorescent lamps continuously after initiation unless noted. When suspensions were pretreated with GS, the antibiotic was added to give 5 μg/ml, aerated for 30 sec, the suspension centrifuged for 30 sec, the supernatant discarded and the microplasmodial pellet resuspended in the sucrose–KCl medium for 3-fold washing and final suspension to give the original microplasmodial density (usually approx. 0.38 mg protein/ml) and incubated as before the pretreatment. The time elapsed between addition of GS and the

resuspension for the first washing was 1.5 min. The GS pretreatment removes little or no protein but releases about 50% of the yellow pigments. All pH experiments were performed in dim room light. Protein was measured by a modified Lowry method [5] and the yellow pigments as previously described [1]. Double glass-distilled water was used for all procedures except for growing the cultures. GS (gramicidin J), gramicidin D, NEM and EGTA were obtained from Sigma, Triton X-100 from Rohm and Hass, CCCP and valinomycin from California Biochemicals. Nigericin was kindly supplied by Dr. B. Pressman.

3. Results

3.1. *Alkalinization of the medium by plasmodial lysis*

The previous results [2] indicated that the action of light on sensitized plasmodia produced marked alkalinization of the culture medium. Whether this was net intracellular production and release or a proton exchange reaction with the medium was not clear. To gain a practical measurement of the pH of the plasmodial contents various permeability relaxing and lytic agents were tested, some of which are listed in table 1. All reagents effected alkalinization of the medium to similar but somewhat varying degrees. The sonication effect when NaCl replaces KCl is approximately the same as that of GS (KCl) and will be discussed in a later report. Although Triton X-100 effects complete lysis, GS, a non-ionophore detergent agent [6–8], essentially does not release plasmodial protein or other macromolecules under the present conditions and, as will be shown, allows partial reversibility of its effect. In addition the partial release obtained with GS is additive with Triton X-100. GS which enhances membrane permeability to cations does show a kinetic preference for K^+, voltage-dependent conductance and a strong dependence on surface change when tested on artificial lipid bilayer membranes [9, 10]. Nystatin also allows a smaller but much more rapid release. Brij 35 (0.12%) effects no release.

Table 1
pH changes induced in plasmodial suspensions by various agents.

Exp. no.	Releasing agent	ΔpH/3 min
1	Triton X-100, 0.060%	+1.13
	+GS, 5 μg/ml	+0.03
		+1.16, net
2	GS, 15 μg/ml	0.99
	+Triton X-100, 0.06%	0.12
		1.11, net
3	GS, 5 μg/ml	+0.30
	+Triton X-100, 0.06%	+0.79
		+1.09, net
4	NEM, 1 mM	+1.46
	+GS, 15 μg/ml	−0.12
	+Triton X-100, 0.06%	−0.03
		+1.31, net
5	Sonication, 10 sec	+0.75
	+GS, 15 μg/ml	+0.04
	+Triton X-100, 0.06%	+0.06
		+0.85, net

Plasmodia were prepared as described in Methods and pH changes recorded for sequential addition of agents listed for each experiment. In exp. 5 the vessel was momentarily removed for sonication. Sonication was accomplished with a Branson model W-185-C Sonifier equipped with a microhorn delivering approx. 15 W at 20 KHz. Plasmodial protein, 3.7 mg/ml. Suspensions initially pH 6.28–6.30.

3.2. *Effect of light on alkalinization*

Further study showed that the net proton uptake promoted by GS is quite light sensitive. Table 2 shows the effect of illumination time and of the KCl concentration on the net release of protons into the medium and on the net uptake induced by GS. The course of the reaction is approximately biphasic showing an initial increase followed by a decrease which is also affected by the KCl concentration (pt. B). When the sensitivity of proton uptake to GS is determined by sequential stepped increases in the GS concentration, the profile obtained for dark and light-treated plasmodia is seen plotted in fig. 1A. After illumination for 15 min, the Triton X-100 results (legend) emphasize the marked net reduction of plasmodial proton uptake capacity as already seen in table 2 (e.g. A II). However, as seen in table 2, a transient increase either in net proton uptake or in sensitivity of uptake occurs during the early phase of the light treatment. It was observed in separate experiments that by applying light after addition of GS, the course of release was greatly modified. These

Table 2
Effect of illumination time and [KCl] on net proton uptake.

Illumination (min)	ΔpH/3 min	+GS (10 μg/ml)
A. Min	Exp. I	Exp. II
0 (Dark)	0.40	0.55
0.50	0.55	–
3.0	0.82	0.64
15	0.58	0.22

KCl (mM)	ΔpH	
	+Light/15 min	+GS (10 μg/ml)/3 min
B.		
10	−0.42	0.25 (0.36)
30	−0.27	0.58 (0.40)
100	−0.16	0.51 (0.53)

Washed plasmodia were prepared as described in Methods or with the indicated [KCl] (pt. B) and treated with GS added after 15 min of illumination (pts. A and B). Illumination was for 15 min (prior to GS addition) except as noted. In pt. B parenthetic values represent release by GS in the dark. Plasmodial protein, 3.6 mg/ml.

observations consistent with the effect of light on proton uptake were developed into the GS pretreatment procedure described in Methods. The following data are a preliminary characterization of the GS-sensitized light-dependent proton uptake. Typical results are seen in fig. 1B as a function of illumination time prior to the addition of GS. A marked increase in sensitivity to GS develops rapidly in response to illumination with a small net increase in proton uptake with time (open vs. closed circles; also see table 1A). The pretreated plasmodia typically show somewhat less dark sensitivity to GS than the untreated plasmodia (not shown; compare fig. 1A). Also notable is the shift in shape of the uptake curve from sigmoid to hyperboloid suggesting the release of a membrane permeability control characteristic by light. The GS pretreatment thus appears to partially relax plasmodial control of proton uptake and to allow a more sensitive expression of light-sensitive reactions observed in the untreated cells. This response is then observable after the partial release of the opposing permeability controls in the growing (vegetative) plasmodium.

In the growing state, the initial light-dependent physiological response is expressed as an inhibition of growth rather than as the induction of sporulation. This effect is rapidly reversible in the dark. Further and more quantitative evidence of this reversibility or dark quenching reaction is strikingly shown by alternating ("chopping") the light period with a dark period (fig. 2). As little as a 0.1 cycle, or about 8 msec, dark interruption allows a measurable decay in the light response when near saturation intensity (5 min continuous illumination, see fig. 1) is used. Total illumination times were the same in all cases. The length of dark time required for detectable decay indicated the presence of a quenching dark metabolic reaction, presumably a measure of reverse proton transport across the plasma membrane, rather than a deficiency in illumination.

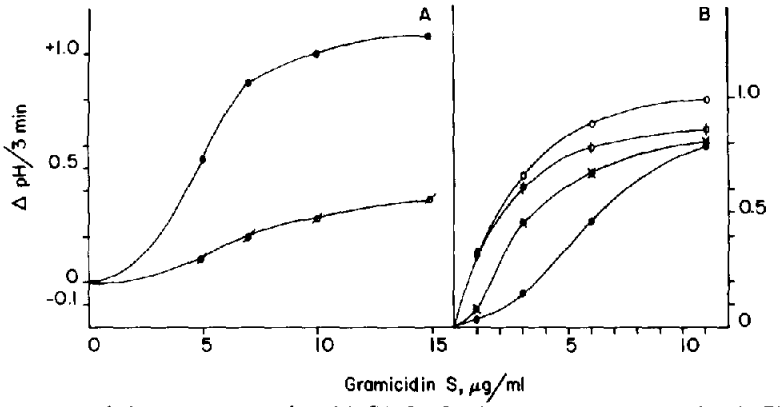

Fig. 1. Effect of light on plasmodial net proton uptake with GS. See Methods for experimental details. Plasmodial protein, 3.9 mg/ml. A) Untreated plasmodia. Plasmodia illuminated 15 min before treatment with GS. (•–•–•) Dark; (ϕ–ϕ–ϕ) illuminated 15 min. Triton X-100 change, Δ pH/3 min, upon addition of 0.06% Triton 3 min after last GS addition: +0.12; illuminated 15 min, +0.07. B) GS-pretreated plasmodia. Plasmodia were prepared and pretreated with GS (see Methods) before illumination and final GS treatment. (•–•–•) Dark; (o–o–o) illuminated 5.0 min; (ϕ–ϕ–ϕ) illuminated 2.0 min (⊠–⊠–⊠) illuminated 0.50 min.

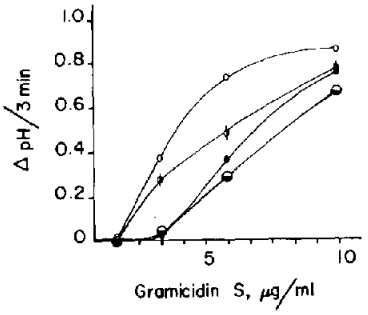

Fig. 2. Effect of light-dark cycles on net proton uptake. Plasmodia pretreated with GS (see Methods). Light source, masked fluorescent lamp, incident intensity, 5×10^4 ergs/cm^2/sec. (●–●–●) dark; (o–o–o) light, 5 min (unchopped) pre GS; (ø–ø–ø) light, chopped at dark-light cycles of 39 msec each-total chopping time, 10.0 min pre GS; (ϕ–ϕ–ϕ) light, chopped at dark-light cycle of 7.8 msec dark/78 msec, total chopping time, 5.5 min pre GS. Plasmodial protein, 3.5 mg/ml.

Isolated spectral bands (e.g. 375 mm, 425 mm) are quite effective in replacing white light. At, for example, 375 mm or 425 mm a full response is obtained with 400 erg/cm^2-sec under otherwise standard conditions for GS-mediated proton uptake. A complete action spectrum is being determined.

3.3. *Cation specificity*

An obviously important element in the light response is the ionic specificity upon which the proton uptake depends. To determine the cation specificity, plasmodia washed and prepared in media containing K^+, choline$^+$ or Na^+ were treated with gramicidin D and washed. This ionophore allows the exchange of the internal and external monovalent cations with little specificity [7, 14, 15]. The sensitivity to GS was determined in the dark and light as shown in fig. 3. Two general conclusions may be drawn: i) K^+, Na^+ or choline$^+$ as an external ion (without gramicidin D) is qualitatively sufficient for the light-stimulated proton uptake; ii) of these ions, only K^+ can satisfactorily serve as the internal ion for the light-dependent response. The effect of valinomycin also supports this dual ion (i.e. K^+–H^+) specificity (fig. 4A). The dark response is greatly enhanced at GS concentrations of 3 μg/ml and higher but not at 1 μg/ml. Correspondingly the light response is enhanced through the entire concentration range. Both in the dark and in the light the response is hyperboloid and the net H^+ uptake

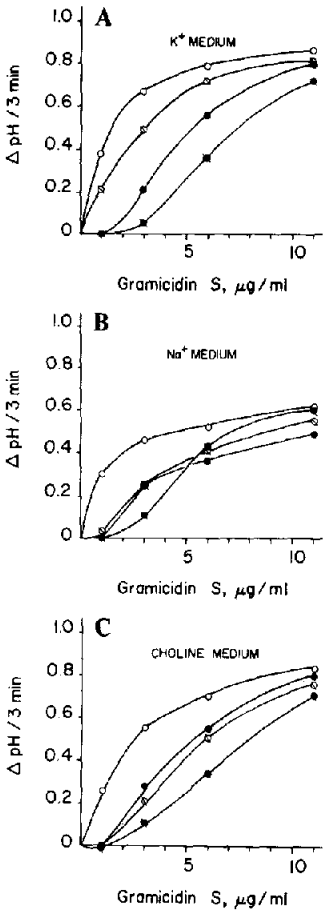

Fig. 3. Dependence of the light stimulation on K^+. GS-pretreated plasmodia (grown in KCl) were washed with NaCl or choline choride, treated with gramicidin D (20 μg/ml) for 5 min as indicated, washed and suspended in 10 mM KCl–150 mM sucrose as indicated, for GS assay (see Methods) after treatments described below. A) KCl-washed plasmodia: (●–●–●) dark (no gramicidin D treatment); (o–o–o) illuminated 5 min (no gramicidin D treatment); (ϕ–ϕ–ϕ) dark, gramicidin D-treated; (ϕ–ϕ–ϕ) illuminated 5 min, gramicidin D treated. B) NaCl-washed plasmodia: (●–●–●) dark (no gramicidin D treatment); (o–o–o) illuminated 5 min (no gramicidin D treatment); (ϕ–ϕ–ϕ) dark (gramicidin D pretreated); (ϕ–ϕ–ϕ) illuminate 5 min (gramicidin D pretreated). C) Choline Cl washed plasmodia: (●–●–●) dark (no gramicidin D treatment); (o–o–o) illuminated 5 min (no gramicidin D treatment); (ϕ–ϕ–ϕ) dark (gramicidin D treated); (ϕ–ϕ–ϕ) illuminated 5 min (gramicidin D treated). Plasmodial protein, 0.4 mg/ml.

substantially elevated over the controls. This strong valinomycin response again indicates the specificity [7] of the light reaction for K^+, an apparent coupled relation between K^+ and H^+ movements and again

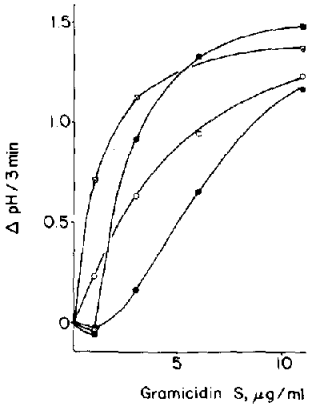

Fig. 4. A) Effect of valinomycin on GS-stimulated proton uptake. Plasmodia GS pretreated and assayed with GS assay (see Methods) after treatments described below. Plasmodial protein, 0.4 mg/ml. (●–●–●) Dark; (○–○–○) illuminated 5 min; (▣–▣–▣) +10 μg/ml valinomycin in dark 3 min (–ΔpH 0.18/3 min); (⊠–⊠–⊠) +10 μg/ml valinomycin in dark 3 min (–ΔpH 0.21/3 min) followed by 5 min illumination.

the specific effect of light in sensitizing this reaction.

3.4. *Effects of uncouplers and nigericin*

Since the effects of GS on the plasmodial (plasma membrane) permeability seemed to be at least to relax the control of proton transport, and that of light to enhance this, the effect of uncouplers was studied. Fig. 4B demonstrates the effect of CCCP. Although proton uptake is enhanced in the dark, the sigmoid characterisitic is retained while in the light marked stimulation of the normal hyperbolic response occurs without stimulation of net uptake. The resulting increased proton uptake from the medium (enhancing the GS sensitivity) implies the release of proton permeability control by CCCP [11–13] in the GS-treated plasmodia. 2,4-Dinitrophenol at 30 μM gives a similar response.

Of particular interest is the different reactivity of nigericin (fig. 4C). Nigericin appears, in mitochondria and erythrocytes, to promote a 1:1 H^+–K^+ exchange [16, 18] and at higher concentrations to effect a K^+-dependent uncoupling under specific [K^+] and substrate conditions [17]. The initial and very rapid response to nigericin (before GS) is a proton uptake (see fig. 4C legend) which implies a release of K^+ to accomodate the net internal and external K^+ and H^+ ratios. The effect of nigericin reflects in terms of

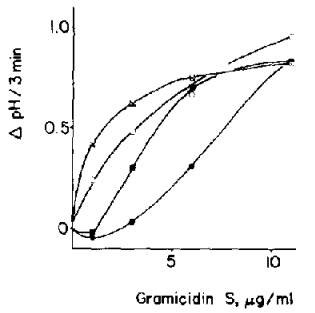

Fig. 4. B) Stimulation of proton uptake by CCCP. Plasmodia pretreated with GS (see Methods). CCCP (1 μM) added 1 min before addition of GS. (●–●–●) Dark; (○–○–○) illuminated 5 min; (▣–▣–▣) dark, +1 μM CCCP for 1 min; (⊠–⊠–⊠) dark +1 μM CCCP for 1 min followed by 5 min illumination. All experiments terminated followed by GS assay. Plasmodial protein, 0.4 mg/ml.

these ratios and a 1:1 exchange mechanism two responses: first, the dark stimulation indicates that the plasma membrane, minimally, is naturally limiting but that the concentration potential is present. Secondly, lack of a substantial effect on the light reaction indicates that the H^+–K^+ exchange can be driven no further by light energy and that the nigericin limitation may be similar to that of the natural membranes in light (plus GS) and tends to support the mutual dependence of H^+ and K^+ movements under the assay conditions. The large uncoupler effect of CCCP is not apparent.

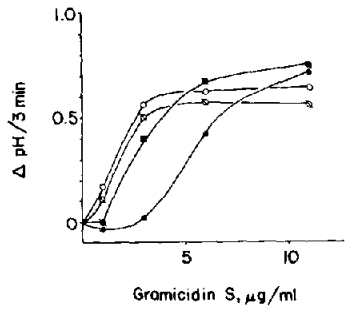

Fig. 4. C) Effect of nigericin on the GS-stimulated proton uptake. Plasmodia pretreated with GS, treated as described below and assayed with GS (see Methods). (●–●–●) Dark; (○–○–○) illuminated 5 min; (◔–◔–◔) in dark, +20 μg/ml (2.8×10^{-5} M) nigericin for 3 min; (◔–◔–◔) in dark, +20 μg/ml (2.8×10^{-5} M) nigericin for 3 min followed by illumination for 5 min.

3.5. *Ouabain sensitivity*

The light-sensitive transport features described do not appear to include a ouabain-sensitive component. In a 150 mM sucrose medium containing 10 mM each NaCl and KCl, 0.3 mM ouabain somewhat suppressed, in parallel, the sensitivity to GS both in the dark and in the light without affecting the net proton uptake or the relative sensitivities. The suppression is readily explained by the inclusion of NaCl in the system normally containing KCl only. Thus the ouabain-sensitive Na^+–K^+ ATPase found in many tissues and organisms does not appear to participate directly in the activity assayed.

4. Discussion

The foregoing results indicate that GS relaxes the cation permeability of at least the plasmodial plasma membrane to protons and K^+. Light greatly enhances this relaxation. The light stimulated component has at least two ion specificities: it is dependent externally on H^+ and internally on K^+. Results from titration of whole cells with GS (no pretreatment) indicate the same effect of light i.e. an induced permeability increase to protons; implying that the plasma membrane is an important part of the responding system. This sensitive light-dependent proton transport is quenched rapidly in the dark, readily detectable within 8 msec dark/78 msec total cycle. The effect of GS in sensitizing the plasma membrane to light-induced ion movements is particularly interesting in view of its voltage-sensitive induction of ion permeabilities in artificial lipid bilayers [9] and the recent findings of Changeux et al. [19, 20] that the tyrocidines, a group of antibiotics closely related to GS, specifically depolarize the electroplax membrane. In addition it has also been demonstrated, as a control model [21], that with the use of an artificial chromophore inhibitor of the electroplax membrane receptor, depolarization of this normally electrically excitable membrane can be controlled by light.

Wherever the photosensitive site is localized in *Physarum*, the foregoing data suggest that a basically similar mechanism may exist in this organism. The *Physarum* plasma membrane is reported [22] to display rapid and reversible changes in its transmembrane potential, or excitability. A preliminary report [23], suggests that this system functions in the light-induced sexual morphogenesis of this myxomycete where light-dependent calcium uptake of mitochondria and vacuoles has been detected by electron microscopy [4]. A subsequent report (Daniel and Eustace, in preparation) will describe the effect of calcium on this system.

Acknowledgement

This study was supported in part by the National Institute of Health General Research Support Grant No. S01-RR05690-01.

References

[1] J.W. Daniel and H.H. Baldwin, in: Methods in Cell Physiology, Vol. 1 (Academic Press, New York, 1964) p. 9.
[2] J.W. Daniel and J. Eustace, manuscript in preparation.
[3] J.W. Daniel, in: Cell Synchrony-Studies in Biosynthetic Regulation (Academic Press, New York, 1966) p. 117.
[4] J.W. Daniel and U. Järlfors, Tissue and Cell (1972) in press.
[5] O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[7] B.C. Pressman Proc. Natl. Acad. Sci. U.S. 53 (1965) 1076
[8] E.G. Finer, H. Hauser and D. Chapman, in: Inhibitors: Tools in Cell Research, 20th Colloquium of the Gesellschaft für Biologische Chemie (Springer-Verlag, 1969) p. 368.
[9] M.C. Goodall, Biochim. Biophys. Acta 219 (1970) 471.
[10] M.C. Goodall, Nature 225 (1970) 1257.
[11] P. Mitchell and J. Moyle, Biochem. J. 104 (1967) 588.
[12] E. Carafoli and C.S. Rossi, in: Mitochondria, Structure Function, FEBS Symposium 17 (1969) 353.
[13] S.J.D. Karlish, N. Shavit, and M. Avron, European J. Biochem. 9 (1969) 291.
[14] J.B. Chappell and A.R. Crofts, in: Biochim. Biophys. Library 7 (1966) 293, eds. J.M. Tager, S. Papa, E. Quagliariello, and E.C. Slater (Elsevier Publishing Co., Amsterdam).
[15] J.B. Chappell and A.R. Crofts, Biochem. J. 95 (1965) 393.
[16] B.C. Pressman, E.J. Harris, W.S. Jagger and J.H. Johnson, Proc. Natl. Acad. Sci. U.S. 58 (1967) 1949.
[17] S.M.F. Ferguson, S. Estrada-O, and H.A. Lardy, J. Biol. Chem. 246 (1971) 5645.
[18] P.J.F. Henderson J.D. McGivan and J.B. Chappell. Biochem. J. 111 (1969) 521.
[19] T.R. Podleski and J.P. Changeux, Nature 221 (1969) 541.
[20] M. Kasai, T.R. Podleski and J.P. Changeux, FEBS Letters 7 (1970) 13.
[21] W.J. Deal, B.F. Erlanger and D. Nachmansohn, Proc. Natl. Acad. Sci. U.S. 64 (1969) 1230.
[22] D.M. Miller, J.D. Anderson and B.C. Abbott, Comp. Biochem. Physiol. 27 (1968) 633.
[23] J.W. Daniel, The First International Mycological Congress. Exeter, England 1971. Abstracts, p. 22.