Acta Protozool. (2001) 40: 311 - 315

ACTA
PROTOZOOLOGICA

Short Communication

Light Regulation of Protein Phosphorylation in *Blepharisma japonicum*

Hanna FABCZAK, Bożena GROSZYŃSKA and Stanisław FABCZAK

Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warszawa, Poland

Summary. We demonstrate alterations in protein phosphorylation levels in light-exposed *Blepharisma* using immunological and densitometric methods. The examinations show that cell illumination elicited a two-fold enhancement of phosphorylation of a 46 kDa protein and a marked decrease in the phosphorylation of a 28 kDa protein over that measured in dark-adapted cells. The observed light-dependent changes in the phosphorylation levels of both proteins were entirely reversible. In contrast, no alteration in protein phosphorylation was detected in cells treated by external potassium, which depolarizes the cell membrane. These observations suggest that both the 28 kDa and 46 kDa proteins might be involved in the light transducing mechanism, which results in the photophobic response of *Blepharisma.*

Key words: *Blepharisma japonicum,* ciliate, phosphorylation and dephosphorylation, phosphoserine, phosphoprotein, photophobic response, phototransduction.

INTRODUCTION

The ciliate *Blepharisma japonicum* possess a photoreceptor system that renders the cells capable of avoiding light. The pigment granules located just beneath the plasma membrane, containing pink-colored quinones - blepharismin, are believed to function as the photosensor units of the cellular light-sensitive system (Giese 1973, Tao *et al.* 1994, Maeda *et al*. 1997, Song 1997a, Matsuoka *et al*. 2000). On account of this feature, the ciliates exhibit photodispersal as they tend to move predominantly toward shady or dark regions (Mast 1906, Fabczak H. 2000a). The light-avoiding behavior of *Blepharisma japonicum* results largely from their motile behavior (step-up photophobic response) to a sudden increase in light intensity (Kraml and Marwan 1983). During prolonged intense illumination, an increase in forward movement (positive photokinesis) and a marked elongation of the cell body also occur (Kraml and Marwan 1983, Ishida *et al.* 1989). The cell photophobic response consists of a delayed cessation of forward swimming, a period of backward movement (ciliary reversal) followed by restoration of forward swimming, usually in an altered direction (Fabczak H. *et al.* 1993, Fabczak S. *et al.* 1993). The ciliary reversal during photophobic response is mediated by a Ca^{2+}/K^{+} action potential elicited by the light-induced receptor potential (Fabczak S. *et al.* 1993). The photoreceptor system in *Blepharisma japonicum* seems to be coupled to membrane potential and photomotile changes *via* a sensory transduction pathway (Fabczak H. *et al.* 1993,

Address for correspondence: Hanna Fabczak, Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, ul. Pasteura 3, 02-093 Warszawa, Poland; Fax: (4822) 8225342; E-mail: hannafab@nencki.gov.pl

Song 1997b). It involves, as is the case in invertebrate or vertebrate photoreceptor cells (Rayer *et al.* 1990), cGMP and inositol trisphosphate as the possible second messengers (Fabczak H. *et al.* 1993, 1998, 1999; Fabczak H. 2000b).

One of the most important mechanisms in the regulation of cellular metabolism or cell function is the process of protein phosphorylation (Greengard 1978, Cohen 1982, Bünemann and Hosey 1999, Dickman and Yarden 1999, Graves and Krebs 1999). In the photoreceptor rod of vertebrate retina, there is evidence that phosphorylated proteins may participate in the regulation of visual function. Of particular relevance are proteins that exhibit light-dependent changes in the extent of their phosphorylation (Polans *et al.* 1979, Hermolin *et al.* 1982, Lee *et al.* 1984, Hamm 1990). In the present study using immunological and densitometer methods, we demonstrate that protein phosphorylation or dephosphorylation occurs in the ciliate when light is applied. These observations indicate that the light-dependent processes of protein phosphorylation may be involved in the control of the photophobic behavior of *Blepharisma japonicum*.

MATERIALS AND METHODS

Cell culture

Cells of *Blepharisma japonicum* were grown in 100 ml glass vessels containing Pringsheim solution (pH 7.0) at room temperature in darkness (Fabczak H. *et al.* 1993). The cells were fed with axenically cultured cells of *Tetrahymena pyriformis*. Prior to experiments, the cells were starved for at least 24 h before the organisms were collected, washed in an excess of fresh culture medium and subsequently used for assays after a further 12 h incubation in fresh culture medium in darkness.

Cell stimulation

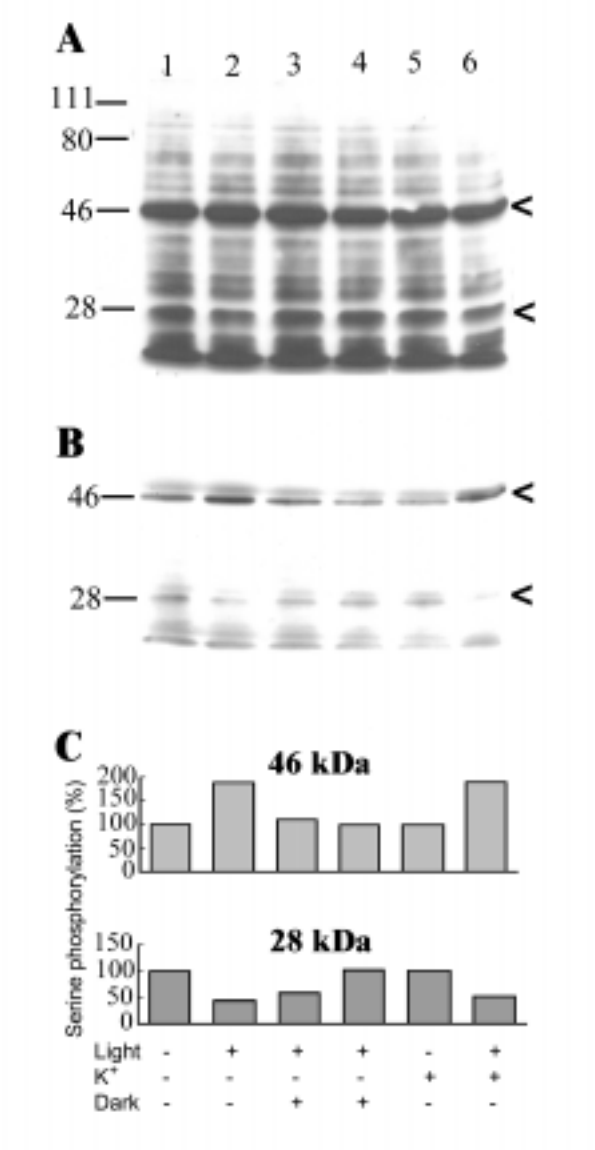
Before each experiment, 200 µl samples of dark-adapted cells were first left at rest for about 10 min to avoid mechanical disturbances. They were subsequently exposed to light or ionic stimulation. The cell samples were illuminated for 2 s with light afforded by a 150 W fiberoptic light source (MLW, Germany) equipped with an electromagnetic programmable shutter (model 22-841, Ealing Electro-Optics, England). Ionic stimulation was performed by cell incubation for 2 s in external 4 mM K^+ solution prepared by gentle addition of the proper amount 0.1 M KCl to the cell suspension.

Immunoblotting assay

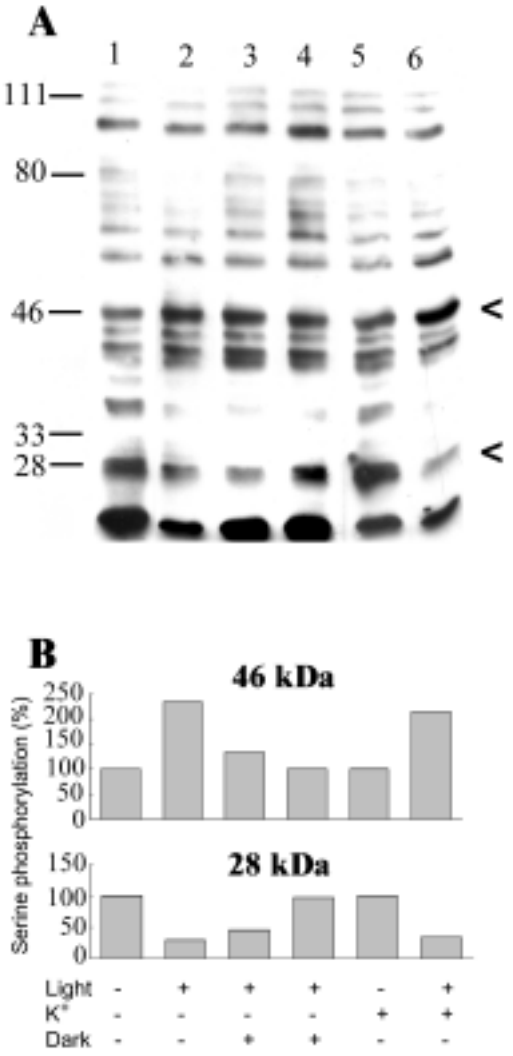
In order to quantify protein phosphorylation levels, samples of control (dark-adapted) cells and cells following treatment with light or potassium ions were immediately mixed after exposure with sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 1.0 mM EDTA and 62.5 mM Tris at pH 6.8 (Laemmli 1970) supplemented with protease or phosphatase inhibitors (50 mM NaF, 2 mM phenylmethylsulfonylfluoride, 1 µM okadaic acid, 10 µg/ml aprotinin or 50 µM leupeptin) and then boiled for 5 min. The equal amounts of cell proteins (30 µg) were separated on 10% SDS-polyacrylamide gels (SDS-PAGE) with the use of the Hoefer System (Amersham, USA) and transferred to nitrocellulose filters for 1 h at 100 V in transfer buffer consisting of 192 mM glycine, 20% methanol and 25 mM Tris at pH 8.3 (Towbin *at al.* 1979). For detection of phosphoserine-containing proteins, the blots were incubated in blocking solution composed of 2% BSA in TBS (50 mM NaCl, 25 mM Tris, pH 8.0) and 0.05% Tween-20 (BSA-Tris-Tween) for 2 h at room temperature. After the blocking procedure, the blots were incubated with the primary antibody (Alexis, Switzerland) against phosphoserine overnight at a concentration of 0.05 µg/ml in 0.3% BSA-TBS-Tween at 4 ^{0}C. The nitrocellulose filters were finally washed several times in TBS with 0.05% Tween (TBS-Tween). After washing, the blots were treated for 1h with anti-mouse IgG-horseradish peroxidase conjugates at a dilution of 1:10 000 (Calbiochem) prepared in the blocking buffer. Immunoreactive bands were developed using an Amersham Enhanced Chemiluminescence (ECL) detection system. Their intensities were quantified using a molecular dynamics personal laser densitometer and ImageQuant software. Molecular weights of polypeptides were determined based on their relative electrophoretic mobilities using prestained molecular weight standards (BioRad). In a control set of experiments, incubation with primary antibodies was omitted. The concentration of cell proteins in individual samples was estimated by a method reported elsewhere (Bradford 1976).

RESULTS AND DISCUSSION

Detection of phosphorylated proteins in cell samples were made using three different monoclonal antibodies, Pser-7F12, Pser-4A9 and Pser-1C8, which may specifically recognize phosphorylated serine epitopes. Two of the applied antibodies, Pser-7F12 and Pser-4A9, revealed marked protein phosphorylation levels of similar patterns (Figs 1 A; 2 A), while the Pser-1C8 antibody indicated a lower number of phosphoproteins (Fig. 3 A), possibly due to lower amino acid sequence specificity. It is well known that antibody specificity depends on phosphorylation of the amino acid and on the surrounding amino acid sequence. If one of these two criteria is unfulfilled, the antibody will not detect the phosphorylation site. Most labeled cell proteins were unaffected by illumination, except for proteins of a molecular weight of about 46 kDa (Figs 1 A, B; 2 A) and proteins below 28 kDa (Figs 1 A, B; 2 A; 3 A). The increased phosphorylation of the 46 kDa protein on illumination (Figs 1 A; 2 A and lanes 1, 2) lasts up to 180 s after illumination (Figs 1 A, B; 2 A and lanes 1 to 4). In the



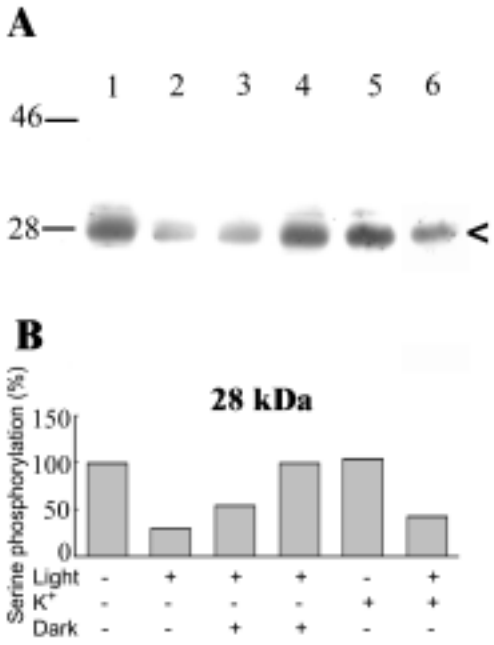
Figs 1 A-C. Detection of light-induced protein phosphorylation in *Blepharisma* by monoclonal antibody, Pser-7F12**. Lane 1:** cells adapted to darkness (control); **Lane 2:** cells exposed to light for 2 s; **Lanes 3, 4:** cells exposed to light for 2 s and then kept in darkness for 180 s and 300 s, respectively; **Lane 5:** dark adapted cells stimulated by 4 mM K^+ for 2 s; **Lane 6:** dark-adapted cells stimulated by 4 mM K^+ and subsequently exposed to light for 2 s. The arrows indicate 46 kDa and 28 kDa proteins, of which phosphorylation levels were altered when illuminated. Immunoblot exposure to X-ray film for 10 s (**A)** and 2 s (**B**); **C** - quantification of protein phosphorylation intensity of bands from **B**. The phosphorylation values found for control cells were defined as 100%



Figs 2 A, B. Detection of protein phosphorylation by light in *Blepharisma* by monoclonal antibody, Pser-4A9. **A -** 30 s exposure of immunoblot to X-ray film. **B** -quantification of serine phosphorylation of 46 kDa and 28 kDa proteins. Other details as in Fig. 1

dark, the most extensively labeled phosphoprotein had an apparent molecular weight of 28 kDa (Figs 1 A, B; 2 A; 3 A and lane 1) and the phosphorylation level of this protein was markedly reduced by illumination (Figs 1 A, B; 2 A; 3 A and lane 2). The light-induced dephosphorylation of 28 kDa protein was entirely reversible, as progressive phosphorylation of the protein was observed in dark-adapted cells for 180 s (Figs 1 A, B; 2 A; 3 A and lane 3) and after about 300 s the phosphorylation level reached the level observed in control cells (Figs 1 A, B; 2 A; 3 A and lane 4).

To test whether phosphorylation and dephosphorylation of cellular proteins in *Blepharisma* is specifically induced by light or is simply elicited by cell membrane



Figs 3 A, B. Detection of light-induced protein phosphorylation in *Blepharisma* by monoclonal antibody, Pser-1C8. **A -** 20 min. exposure of immunoblot to X-ray film. **B -** quantification of serine phosphorylation of the 28 kDa protein. Other details as in Fig. 1

depolarization, the effect of ionic stimulation was examined. These experiments indicate that the phosphorylation level of proteins of 28 kDa and 46 kDa was unaffected by membrane depolarization compared to cell samples exposed to light (Figs 1 A, B; 2 A; 3 A and lane 5). The pattern of protein phosphorylation in cell samples, which were first treated with K^+ and subsequently exposed to light (Figs 1 A, B; 2 A; 3 A and lane 6), was similar to that obtained for cells that were only illuminated (Figs 1 A; 2 A; 3 A and lane 2).

The results of semi-quantitative analysis of protein phosphorylation indicated that the level of phosphorylation of 46 kDa proteins by 2 s illumination caused a two-fold increase over the values found in dark-adapted cells (Figs 1 C; 2 B). In the case of the 28 kDa polypeptide, the level of phosphorylation is lower in all cell samples exposed to light. The phosphorylation levels for both these proteins returned to the control levels after about 300 s of cell incubation under dark conditions (Figs 1 C, 2 B, 3 B).

It has been shown that phosphorylation and dephosphorylation of cellular proteins play a crucial role in the regulation of various sensory transduction pathways (Greengard 1978, Cohen 1982, Bünemann and Hosey 1999, Dickman and Yarden 1999, Graves and Krebs 1999). In the visual system, light induces phosphorylation of photoreceptors (rhodopsin) by a protein kinase (Bownds *et al.* 1972, Kühn 1974, Frank *et al.* 1973). The higher light intensity causes a marked dephosphorylation of proteins of low molecular weight in intact rods (Polans *et al.* 1979, Lee *et al.* 1984, Bownds and Brewer 1986). In the present study, we showed that light is capable of influencing the level of protein phosphorylation in *Blepharisma japonicum*, as it takes place in the photoreceptor cells of vertebrates. In this ciliate, however, the proteins being phosphorylated or dephosphorylated by light have not yet been identified. It was recently shown that the photosensory pigment blepharismin in *Blepharisma japonicum* is associated with a 200 kDa membrane protein (Matsuoka *et al.* 2000). It is likely that the polypeptide of molecular weight of 46 kDa, which underwent highly specific phosphorylation on serine after light stimulation, is associated with the 200 kDa complex photoreceptor protein or it simply represents a fragment of the high molecular weight protein resulting from digestion by endogenous cellular proteases during detergent solubilization (Matsuoka *et al.* 2000). Further investigations are necessary regarding the identification of this protein and elucidation of the mechanism that governs the observed protein phosphorylation and dephosphorylation by light in protozoan ciliate *Blepharisma*.

Acknowledgements. This work was supported in part by grant no. 6P04C-057-18 from the Committee for Scientific Research and the statutory funding for the Nencki Institute of Experimental Biology.

REFERENCES

Binder B. M., Brewer E., Bownds M. D. (1989) Stimulation of protein phosphorylation in frog rod outer segments by protein kinase activators. *J. Biol. Chem.* **264:** 8857-8864

Bownds M. D., Brewer E. (1986) Changes in protein phosphorylations and nucleotide triphosphates during phototransduction. In: The Molecular Mechanism of Photoreception. (Ed. H. Stieves), Springer Verlag, New York Inc., New York, 159-169

Bownds M. D., Bawes J., Miller J., Stahlman M. (1972) Phosphorylation of frog photoreceptor membranes induced by light. *Nature New Biol.* **237:** 125-127

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254

Bünemann M., Hosey M. M. (1999) G-protein coupled receptor kinases as modulators of G-protein signaling. *J. Physiol.* **517:** 5-23

Cohen P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **296:** 613-620

Dickman M. B., Yarden O. (1999) Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Gen. Biol.* **26:** 99-117

Fabczak H. (2000a) Protozoa as model system for studies of sensory light transduction: photophobic response in the ciliate *Stentor* and *Blepharisma*. *Acta Protozool.* **39:** 171-181

Fabczak H. (2000b) Contribution of the phosphoinositide-dependent signal pathway to photo motility in *Blepharisma*. *J. Photochem. Photobiol*. *B.* **55:**120-127

Fabczak H., Tao N., Fabczak S., Song P.-S. (1993) Photosensory transduction in ciliates. IV Modulation of the photomovement response of *Blepharisma japonicum* by cGMP. *Photochem. Photobiol*. **57:** 889-892

Fabczak H., Walerczyk M., Fabczak S. (1998) Identification of protein homologous to inositol trisphosphate receptor in ciliate *Blepharisma*. *Acta Protozool*. **37:** 209-213

Fabczak H., Walerczyk M., Groszyńska B., Fabczak S. (1999) Light induces inositol trisphosphate elevation in *Blepharisma japonicum*. *Photochem. Photobiol.* **69:** 254-258

Fabczak S., Fabczak H., Song P.-S. (1993) Photosensory transduction in ciliates. III. The temporal relation between membrane potentials and photomototile responses in *Blepharisma japonicum. Photochem. Photobiol.* **57:** 872-876

Frank R. N., Cavanagh H. D., Kenyon K. R. (1973) Light stimulated phosphorylation of bovine visual pigments by adenosine triphosphate. *J. Biol. Chem.* **248:** 596-609

Giese A. C. (1973) *Blepharisma.* The Biology of a Light-sensitive Protozoan. Stanford Univ. Press, Stanford

Grave J. D., Krebs E. G. (1999) Protein phosphorylation and signal transduction. *Pharmacol. Ther*. **82:** 111-121

Greengard P. (1978) Phosphorylated proteins as physiological effectors. *Science* **199:** 146-152

Hamm H. (1990) Regulation by light of cyclic nucleotide-dependent protein kinases and their substrates in frog rod outer segments. *J. Gen. Physiol.* **95:** 545-567

Hermolin J., Karell M. A., Hamm H. E., Bownds M. D. (1982) Calcium and cyclic GMP regulation of light-sensitive protein phosphorylation in frog photoreceptor membranes. *J. Gen. Physiol.* **79:** 633-655

Ishida M., Shigenaka Y., Taneda K. (1989) Studies on the mechanism of cell elongation in *Blepharisma japonicum.* I. Physiological mechanism how light stimulation evokes cell elongation. *Europ. J. Physiol.* **25:** 182-186

Kraml M., Marwan W. (1983) Photomovement responses of the heterotrichous ciliate *Blepharisma japonicum. Photochem. Photobiol.* **37:** 313-319

Kühn H. (1974) Light dependent-phosphorylation of rhodopsin in living frogs. *Nature (Lond).* **250:** 588-590

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277:** 680-685

Lee R. H., Brown B. M., Lolley R. N. (1984) Light-induced dephosphorylation of a 33K protein in rod outer segments of rat retina. *Biochemistry* **23:** 1972-1977

Matsuoka T., Tokumori D., Kotsuki H., Ishida M., Matsushita M., Kimura S., Itoh T., Checcucci G. (2000) Analyses of structure of photoreceptor organelle and blepharismin-associated protein in unicellular eukaryote *Blepharisma. Photochem. Photobiol***. 72:** 709-713

Maeda M., Haoki H., Matsuoka T., Kato Y., Kotsuki H., Utsumi K., Tanaka T. (1997) Blepharismin 1-5, novel photoreceptor from the unicellular organism *Blepharisma japonicum. Tetrahedron Lett.* **38:** 7411-7414

Mast S. O. (1906) Light reactions in lower organisms. 1. *Stentor coeruleus. J. Exp. Biol.* **3:** 359-399

Polans A. S., Hermolin J., Bownds M. D. (1979) Light-induced dephosphorylation of two proteins in frog rod outer segments. *J. Gen Physiol.***74:** 595-613

Rayer B., Naynert M., Stieve H., (1990) Phototransduction; Different mechanisms in vertebrates and invertebrates. *J. Photochem. Photobiol*. *Part B*. **7:** 107-148

Song P.-S., (1997a). Light signal transduction in ciliate *Stentor* and *Blepharisma*. 1. Structure and function of the photoreceptors. In: Biophysics of Photoreception Molecular and Phototransductive Events (Ed. C. Taddei-Ferretti), World Sci., Singapore, New Jersey, London, Hong Kong, 48-66

Song P.-S., (1997b). Light signal transduction in ciliate *Stentor* and *Blepharisma*. 2. Transduction mechanism. In: Biophysics of Photoreception Molecular and Phototransductive Events (Ed. C. Taddei-Ferretti), World Sci., Singapore, New Jersey, London, Hong Kong, 76-82

Tao N., Diforce L., Romanowski M., Meza-Keuthen S., Song P.-S., Furuya M. (1994) *Stentor* and *Blepharisma* photoreceptors: structure and function. *Acta Protozool.* **33:** 199-211

Towbin H., Staehelin T., Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:** 4350-4354

Received and accepted on 10th October, 2001