Photic Stimulation of Intracellular cAMP-Dependent Protein Kinase in *Paramecium*

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The cellular concentration of *Paramecium* protein kinase A (PKA) exhibits a bi-modal pattern in a 12-h light and 12-h dark cycle, with peaks around midday and immediately after dusk, in contrast to that measured in constant darkness (DD) which exhibits a mono-phasic pattern with a trough after subjective midday and a peak around subjective midnight. The bi-modal increases in PKA concentration caused by the onset (dawn) and offset (dusk) of illumination might contribute discrete phase-advances of the motility rhythm in *P. multimicronucleatum*. Fluctuation of PKA concentrations in DD is significantly related to that of frequency of changing moving-direction, on which PKA concentrations elevated during illumination may be additively observed to cause the bi-modal pattern.

KEYWORDS: Circadian motility rhythm; Frequency of avoiding reaction; PKA; Swimming velocity; *Paramecium*

Introduction

In the ciliate protozoan, *Paramecium*, extracellularly added cAMP is known to stimulate powerful and frequent ciliary beating, consequently propelling the cells fast and straight (Nakaoka and Ooi, 1985; Bonini et al., 1986; Bonini and Nelson, 1988). This effect of cAMP is mediated by protein kinase A (PKA), the C-subunit of which uniquely phosphorylates a specific protein in dynein, one group of microtubule-based motor proteins in the cilia (Walczak and Nelson, 1993). We have measured a convincing circadian rhythm [traverse frequency (=TF) rhythm] in swimming behavior in a population of *P. multimicronucleatum* (Hasegawa et al., 1984) and shown that illumination encourages fast straight swimming of individuals (Hasegawa and Tanakadate, 1984). Recent results suggest that individual cells swim faster and straighter during the day (and subjective day), when the resting membrane potential is more deeply hyperpolarized and internal cAMP concentration is higher, than at night (and subjective night), when the resting membrane potential becomes shallower and cAMP concentration lower (Hasegawa et al., submitted). This suggests that photic signals to activate ciliary beating are transferred through the cAMP-dependent signal transduction pathway, during which elevated concentrations of both cAMP and PKA should be detectable.

In the present study, we show that activities of PKA in *P. multimicronucleatum* exhibit (1) a bi-modal pattern with peaks around midday and 4-h after the offset of light in a 12-h light and 12-h dark cycle (LD, 12:12), and (2) in constant darkness (DD) clearly diminish during subjective day, subsequently exhibiting a mono-phasic pattern with a trough around subjective midday and a peak around subjective midnight. The mono-phasic pattern in DD parallels the circadian fluctuation of the frequency of changing direction (the frequency of avoiding reaction, F_{AR}, Naitoh, 1968). The bi-modal increases of PKA concentrations immediately following dawn and dusk may be related to the discrete phase-advances after dawn and dusk observed in the motility rhythm in *P. multimicronucleatum* (Hasegawa and Tanakadate, 1984).

Materials and Methods

Stock culture of *P. multimicronucleatum* acycler were grown in axenic medium (Fok and Allen, 1990) under LD (12:12;L=1,000 lux at the surface of incubation bottle, with a fluorescent lamp as the light source) at 20°C.

Every 3 h, cells in early stationary phase in LD or DD were condensed from about 100 m*l* of cell suspension to 300 μ*l* by centrifugation at 150 × g for 30 sec. The condensed cells were then added to 300 μ*l* of a homogenization buffer containing 100 mM 4-(2-hydroxyethl)-1-piperazineethanesulfonic acid (Hepes), 500 mM sucrose, 4 mM EDTA, 4 mM [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), 0.6 mM phenylmethylsulphonyl

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fluoride (PMSF), 0.6 mM $N\alpha$-p-tosyl-L-arginine methyl ester (TAME), 0.4 µg/ml leupeptin, 20 µg/ml pepstatin A, and 0.4 unit/ml aprotinin (pH7.5). Cells were dissociated by passage through the barrel of a 1-ml plastic syringe held tightly against the flat bottom of an acrylate tube. 100 µl was then frozen for measuring protein concentrations and the remainder (~500 µl) for measuring concentrations of PKA at −20°C. After gentle thawing, total protein concentrations in the 100 µl samples were measured using a protein assay kit (Bio-Rad Protein Assay, Bio-Rad, Hercules, Ca 94547, U.S.A.), and used to adjust the 500 µl-preparations to 2 mg protein/ml. The latter were then centrifuged at 300,000 × g_{av} for 60 min and 250 µl of the resulting supernatant was taken for PKA measurement.

The active C-subunit of *Paramecium* PKA, which dissociates from the R-subunit on binding with cAMP, is known to share extensive sequence similarities with all eukaryotic PKAs (Taylor, 1989; Hochstrasser and Nelson, 1989). Recently, glial fibrillary acidic protein (GFAP), the intermediate filament component of astroglial cells, was found to serve as a potent and specific substrate for PKA (Inagaki et al., 1990). We measured activities of the C-subunit from the cyclically sampled supernatants to estimate PKA activities in the preparations, using a commercial kit (NRPK assay kit, MBL Co., Nagoya). This measurement is based upon the phosphorylation of serine in a synthesized peptide by the C-subunit of PKA (Inagaki et al., 1990), and subsequent binding of the phosphorylated serine to a monoclonal antibody specific for the phosphorylated peptide. The synthetic peptide sequence, RRRVTS*AARRS, corresponds to residues 3-13 of GFAP (the asterisk in the sequence denoting the serine to be phosphorylated). PKA activities thus measured were determined by comparison with a calibration curve obtained using a commercially available PKA (Sigma, U.S.A.), and expressed in µmol ($µmol \cdot min^{-1} \cdot mg \times protein^{-1}$).

Results

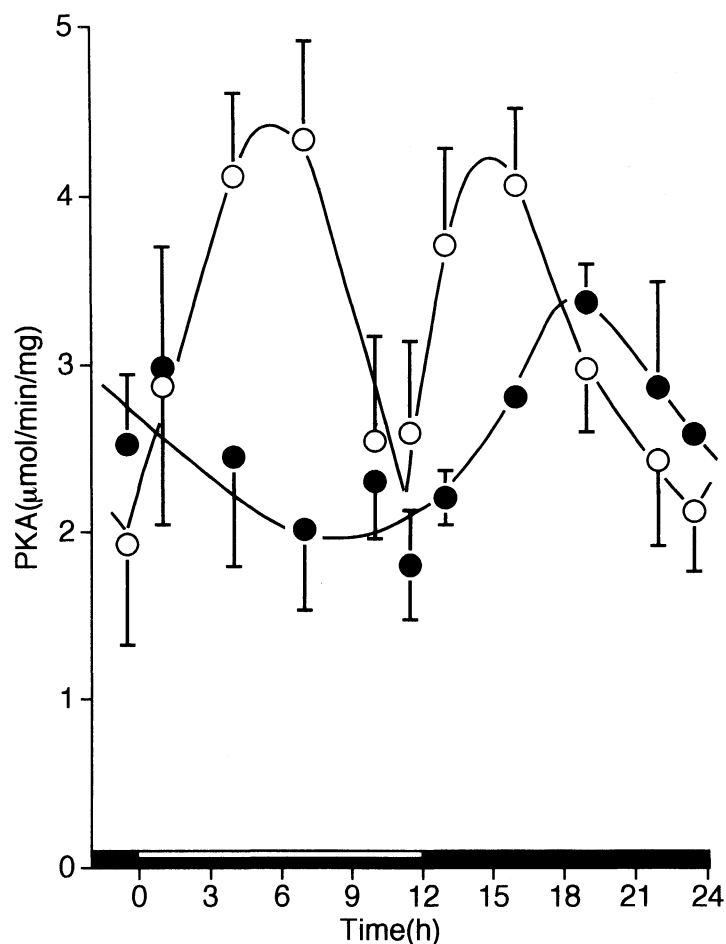In LD, PKA activity rises after dawn, reaching a peak around midday, followed by a decline until dusk (Fig.

Fig. 1   Fluctuations of the C subunit concentration of PKA in *P. multimicronucleatum* in LD (○) and DD (●). Mean ±SD (n=3). Black bars on the abscissa denote dark time in LD and DD.

1). Immediately after dusk, PKA begins to rise again, quickly reaching a peak around 4 h after dusk, then gradually declining towards dawn. The pattern is thus a bi-modal. In DD, PKA activity clearly diminishes during subjective day, producing a mono-phasic pattern with a trough around subjective midday and a peak around subjective midnight.

We have previously demonstrated that forward swimming velocity (V) and frequency of changing moving-direction (F_{AR}) fluctuate inversely with each other in populations of *P. multimicronucleatum* (Hasegawa and Tanakadate, 1984), as illustrated in Fig. 2A: light prominently encourages V and suppresses F_{AR}. The PKA activ-
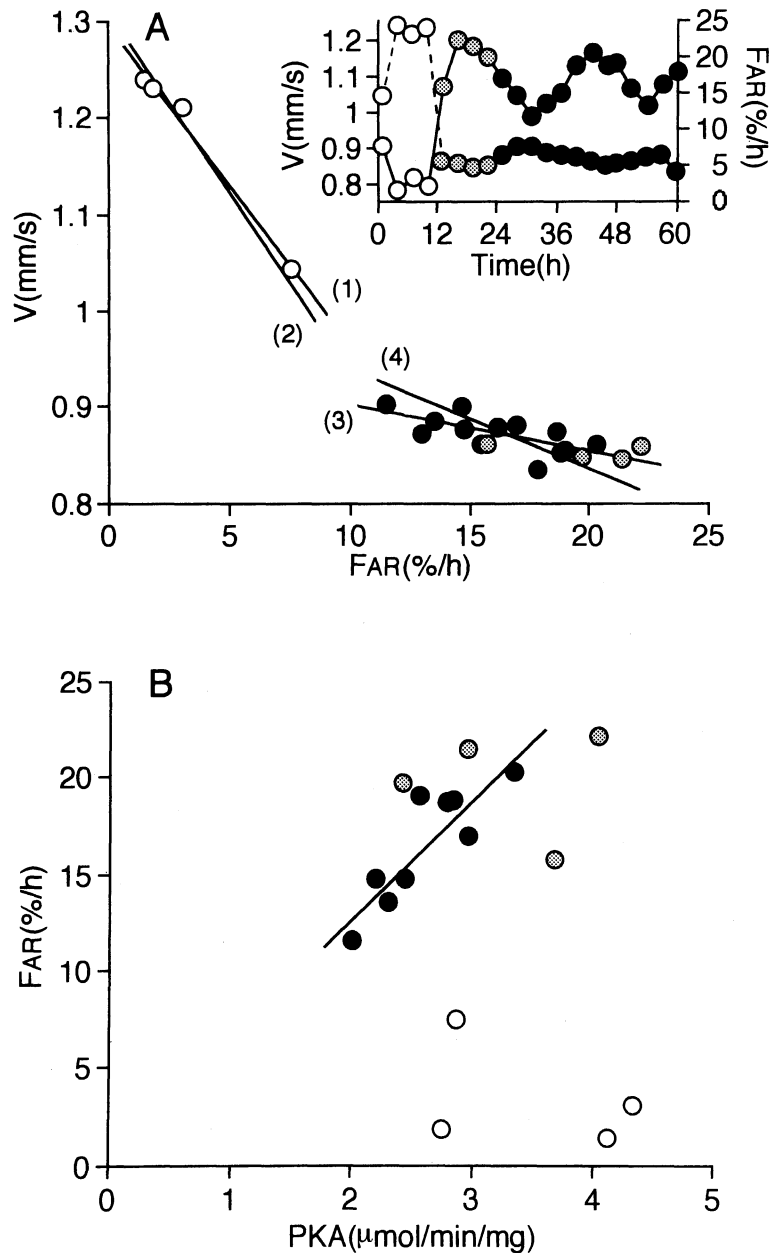

Fig. 2 Relationship between turning frequency (F_{AR}) and swimming velocity (V), and PKA concentrations and F_{AR} in *P. multimicronucleatum*. (A) F_{AR} vs V, both of which were analyzed (Hasegawa and Tanakadate, 1984) from the widths of electric pulses recorded when images of *Paramecium* cells traversed underneath a photosensor placed on the surface of the CRT display in a computerized close-up photoamplifier system (Hasegawa and Tanakadate, 1984). Open circles denote data observed during the L phase of LD, gray toned circles those observed in the D phase of LD, and black circles those in DD. Regression lines: in the L phase of LD (open circles), $y = -3.29 \times 10^{-2} x + 1.29$ for regression of V on F_{AR} (1) and $y = -3.38 \times 10^{-2} x + 1.30$ for regression of F_{AR} on V (2) (r=0.99); and in the D of LD (gray toned circles) and DD (black circles), $y = -4.58 \times 10^{-3} x + 0.95$ for regression of V on F_{AR} in DD (3) and $y = -1.14 \times 10^{-2} x + 1.06$ for regression of F_{AR} on V (4) (r=0.70). Inset: fluctuation of V and F_{AR} in the population of *P. multimicronucleatum* (data Hasegawa and Tanakadate, 1984). Dotted line for V. (B) PKA concentration vs F_{AR}. Regression line for data in DD, $y=6.12x+0.46$ (r=0.87). Symbols as for (A).

ities observed in initial DD subsequent to LD, fluctuate significantly in parallel with F_{AR}s during the same period. It is therefore likely that PKA (at least during initial DD) basically functions to regulate F_{AR}. In LD, PKA activities in the D phase quickly approach the DD regression line, while those in the L phase are markedly different (Fig. 2B).

Discussion

The activities of *Paramecium* PKA exhibit a bi-modal pattern in LD, with peaks appearing around midday and immediately after dusk, contrasting with the mono-phasic pattern in DD, with a trough around subjective midday and a peak around subjective midnight. The bi-modal pattern of PKA activities in LD can be explained by postulating a triggering effect of discrete changes in light intensity (i.e., dawn and dusk), elevating PKA activity, with PKA subsequently undergoing gradual degradation during ensuing constant light intensities (day or night). Using a phase-plane analysis of the phase angles of motility (TF) rhythms in LD contrasted with those in initial DD, we have previously shown that dawn and dusk transiently accelerate the TF rhythm with gradual deceleration during day and night, resulting in a bi-modal modification of phase angles (Hasegawa and Tanakadate, 1984). That is, the bi-modal pattern of PKA activities apparently coincides with phase angle modification of the TF rhythm by the LD regimen.

Recently we have shown that the cells swim (i) faster and straighter during the day, when cAMP concentration is higher and cGMP concentration is lower, and (ii) slowly with frequent turns at night, when cAMP concentration is lower and cGMP concentration is higher (Hasegawa et al., 1995). It appeared that the ratio of cGMP to cAMP significantly oscillates with a trough around midday and a peak around midnight, well mimicking the circadian fluctuations of resting membrane potential in LD, which is hyperpolarized during the day and becomes shallower at night (Hasegawa et al., 1995). The elevated concentrations of both cAMP and PKA during the day in LD presumably tend to an increase in the amount of dissolved C-subunits of PKA. Since one of the functions of *Paramecium* PKA is to phosphorylate a specific protein in dynein (Walczak and Nelson, 1993), orienting ciliary beat direction to effectively propel the cell fast and straight, the elevation of PKA activity would explain the fast and straight swimming of *P. multimicronucleatum* during the day. At night, cGMP concentrations are relatively higher than cAMP concentrations (Hasegawa et al., 1995). A more recent study has demonstrated that cGMP-dependent protein kinase (PKG) also phosphorylates a specific protein in dynein (Ann et al., 1995). There is therefore a possibility that the functioning of PKA activities elevated immediately after dusk is sidelined as PKG activities begins to dominate.

Photic stimulation causing a phase-shift of mammalian circadian pacemakers in the suprachiasmatic nucleus (SCN) of the hypothalamus (Inoue and Kawamura, 1979) is known to activate immunodetectable amount of c-fos within the SCN (Ginty et al., 1993), for which phosphorylation of a cAMP-related element (CREB) by PKA (and CaM-kinase II) is required (Sheng and Greenberg, 1990). It will therefore be interesting to clarify whether PKA is also a second messenger of photic signals leading to stimulation of c-fos transcription in *Paramecium*.

Acknowledgment

Abbreviations used:

CaM-kinase II, calmodulin-kinase II; cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; CREB, cAMP-related element; C-subunit, catalytic subunit; DD, constant darkness; F_{AR}, frequency of avoiding reaction; LD, light-dark cycle; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; SCN, suprachiasmatic nucleus; TF, traverse frequency ($\times 10^3$ cells/hour); V, swimming velocity.

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