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ROLE OF CYCLIC GMP IN THE MEDIATION OF CIRCADIAN RHYTHMICITY OF THE ADENYLATE CYCLASE–CYCLIC AMP–PHOSPHODIESTERASE SYSTEM IN *EUGLENA**

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Abstract—Cyclic AMP (cAMP) and cyclic GMP (cGMP) are two second messengers that have been proposed to act as a dualistic system in biological regulation. To determine if cGMP plays a role in the mediation of circadian rhythmicity of the adenylate cyclase (AC)–cAMP–phosphodiesterase (PDE) system in the achlorophyllous ZC mutant of the unicellular flagellate *Euglena*, the levels of cAMP and cGMP were monitored in synchronized cell populations, and the effects of the cGMP analog 8-bromo-cGMP (8-Br-cGMP) and the cGMP inhibitor 6-anilinoquinoline-5,8-quinone (LY 83583) on the activity of AC and PDE, as well as on the level of cAMP, were measured *in vivo*. A bimodal, 24-hr rhythm of cGMP content was found in both dividing and nondividing cultures in either a 12-hr:12-hr light–dark cycle or constant darkness. The peaks and troughs of the cGMP rhythm occurred 2 hr in advance of those of the cAMP rhythm that has been reported previously. The addition of 8-Br-cGMP at different circadian times increased the cAMP level *in vivo* by two to five times, whereas LY 83583 reduced the amplitude of the cAMP rhythm so that it disappeared. The effects of 8-Br-cGMP on the activity of AC and PDE were circadian phase-dependent and consistent with the changes in cAMP content. These findings suggest that cGMP may serve as an upstream effector that mediates the cAMP oscillation by regulation of the AC–cAMP–PDE system.

Circadian changes in levels of the adenylate cyclase (AC‡)–cAMP–phosphodiesterase (PDE) system have been reported recently in cultures of the achlorophyllous ZC mutant of *Euglena gracilis* [1, 2]. Analysis of the results indicates that both AC and PDE are themselves oscillatory and are responsible for the generation of the circadian rhythm of cAMP content. Since all elements of this system appear to be under control of an endogenous pacemaker, it would be interesting to determine how they are regulated by upstream factors. Preliminary studies have shown that effectors such as calcium, calmodulin, and cGMP may play a role in mediating all these rhythmicities (Tong J and Edmunds LN, unpublished observation).

The "Yin Yang" or dualism hypothesis proposed about 20 years ago provided a theory of biological regulation through opposing actions of cGMP and cAMP [3]. According to this theory, reciprocal changes in the levels of the two cyclic nucleotides should bring about a maximum expression of the mitogenic signal. An interesting observation was that an increase in tissue cGMP levels was accompanied by either a decrease, an increase, or no change in cAMP concentration [4]. The question then arises as to the origin of the cAMP variation, and whether these changes in cGMP/cAMP ratios are stochastic, or whether, as the hypothesis posits, cGMP may serve as a positive mediator. In fact, the two cyclic nucleotide concentrations in the bacterium *Escherichia coli* have been observed to fluctuate independently during the exponential and stationary growth phases so that the ratios change continuously [5]. In this paper, we report that a cGMP rhythm similar to that of cAMP exists in *Euglena*, and that it is this oscillation in cGMP that brings about phase-related changes of the cAMP level by modulating the activities of AC and PDE, which thus may represent a model of a dualistic, regulatory system.

MATERIALS AND METHODS

Materials. [^{3}H]cAMP (20 Ci/mmol) and [α-^{32}]ATP (10 Ci/mmol) were obtained from the Amersham Corp. cAMP, GTP, 8-bromo-cGMP (8-Br-cGMP), 6-anilinoquinoline-5,8-quinone (LY-83583), phosphoenol pyruvate, pyruvate kinase and snake (*Ophiophagus hannah*) venom were provided by the

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‡ Abbreviations: DD, continuous darkness; LD, light–dark cycle; LD: *x*, *y*, a repetitive light–dark cycle comprising *x* hours of light and *y* hours of dark; CT, circadian time (CT 00 indicates the phase-point of a free-running rhythm that has been normalized to 24 hr that corresponds to the onset of light in a reference LD: 12, 12 cycle; the onset of cell division in a dividing culture occurs at approximately CT 12); CDC, cell division cycle; AC, adenylate cyclase; 8-Br-cGMP, 8-bromo-cGMP; LY-83583, 6-anilinoquinoline-5,8-bromo-cGMP; LY-83583, 6-anilinoquinoline-5,8-quinone; and PDE, phosphodiesterase.

Sigma Chemical Co. The cAMP and cGMP radioimmunoassay kits were provided by the Amersham Corp. The achlorophyllous ZC mutant was obtained from Dr. R. Calvayrac (Laboratoire des Membranes Biologiques, Université Paris VII, France). It was derived from the wild type Z strain of *E. gracilis* Klebs by action of 2.5×10^{-5} M diuron (DCMU) in a 33 mM lactate medium (pH 3.5) under illumination and anoxia [6].

Cell culture. Cells were grown at 16.5 ± 0.5° on a modified Cramer and Myers' medium supplemented with vitamins B_1 and B_{12} and containing ethanol (0.1%, v/v) as the carbon source [7]. Illumination (3000 lx) was provided by clock-programmed, cool-white fluorescent lamps as light–dark cycles (LD: 12, 12). Cell number was monitored every 2 hr by a miniaturized fraction collector and a Coulter Electronic Particle Counter [8], and samples were taken from either dividing cultures or those having reached the stationary phase of growth.

Preparation of cell extracts. Cells were pelleted at 4° by centrifugation for 10 min at 7700 *g* and then resuspended in 2 mL of 50 mM Tris (pH 7.8) at a concentration of 3×10^6 cells/mL. Each sample was sonicated for three intervals of 20 sec (MSE sonicator set on high power at amplitude 4). The crude sonicate was centrifuged for 10 min at 39,000 *g*. The resulting supernatant fraction was kept for assay of PDE, and the pellet was resuspended in 3 mL Tris (50 mM, pH 7.8) for the AC assay. For cAMP and cGMP assays, the 7700 *g* pellet was extracted in trichloroacetic acid at 7.5% (w/v) final concentration for 20 min. After centrifugation (10 min, 39,000 *g*), the supernatant was extracted five times with equal volumes of water-saturated diethyl ether, the ether phase being eliminated by boiling it off in a water bath.

Assays of AC and PDE activities. The AC assay was performed as described by Alvarez and Daniels [9], in which a one-step method was used for the separation of cAMP from other nucleotides on polypropylene columns of neutral aluminum oxide. The final concentrations of the components of the incubation medium were 0.5 mM (0.1 μCi) [α-^{32}P]-ATP, 1 mM $MgSO_4$, 40 mM Tris–HCl buffer (pH 7.4), 2 mM cAMP, 0.1 mM GTP, 20 mM phosphoenol pyruvate, and 6 units of pyruvate kinase. The activity of PDE was measured by separating the [^{3}H]adenosine formed from [^{3}H]3′,5′-AMP and [^{3}H]5′-AMP by precipitation with an anion exchange resin, and then counting with a liquid scintillation spectrometer [10]. The incubation mixture for the PDE assay (final concentration) consisted of 25 pM [^{3}H]-labeled cAMP (2.2×10^6 cpm), 5 mM $MgCl_2$, 40 mM Tris (pH 8.0), 3.75 mM mercaptoethanol, 0.15 μM cAMP, and 10 μL of snake venom (1 mg/1 mL). Incubation time for both enzymes was 10 min at 31°. Enzyme activities were expressed as picomoles cAMP formed or hydrolyzed per minute per 10^6 cells.

Assays of cAMP and cGMP content. Both cAMP and cGMP were measured using the RIA kits from Amersham according to the manufacturer's instructions.

In vivo *experiments.* Culture aliquots taken at the circadian times (CT 04, CT 10, CT 16, CT 22) that corresponded to the peaks and troughs of the AC and PDE rhythms, which had been determined previously [2], were incubated with the cGMP analog 8-Br-cGMP or the guanylate cyclase inhibitor LY-83583. (Control experiments confirmed that addition of 10 pmol 8-Br-cAMP to culture aliquots elevated the assayable intracellular cGMP by 77% after 2 hr; the level of this analog was relatively stable over a 3-day time span, having a half-life of about 24 hr. Similarly, addition of LY83583 was demonstrated to lower cellular cGMP in control culture aliquots.) Samples from the experimental cultures were incubated for 2 hr with a 10 μM concentration of either chemical under the same conditions as those used for the controls. At the end of the incubation, both experimental and control samples were extracted and assayed for AC, PDE, and cAMP.

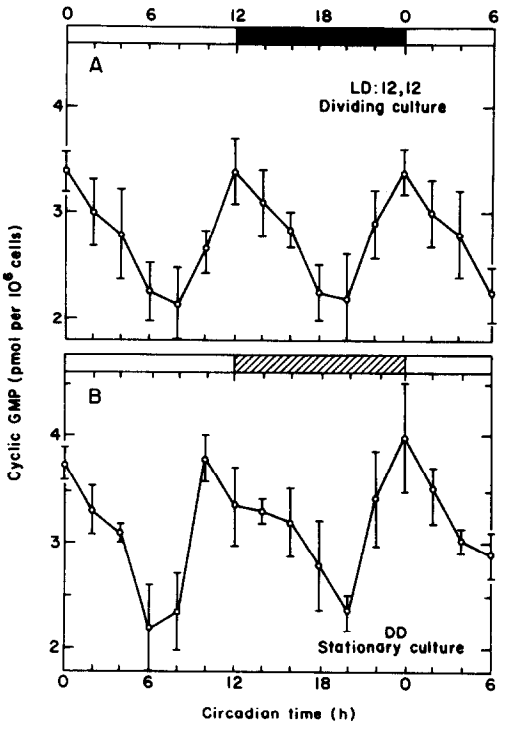

Fig. 1. Circadian rhythms of cGMP in dividing (A) and nondividing (B) cultures of the achlorophyllous ZC mutant of *E. gracilis* Klebs (strain Z), grown in LD:12,12 (A) and DD (B) at 16.5° on a mineral medium supplemented with ethanol (0.1%). The results shown are averages of two independent experiments with duplicate assays; error bars indicate the range of the values. The hatched bar in B indicates subjective night in order to facilitate comparison with A.

RESULTS

Oscillation of cGMP content in dividing and nondividing cultures. Under the LD: 12, 12 light–dark schedule, a bimodal, 24-hr oscillation of cGMP content in synchronously dividing cells was observed (Fig. 1A). Two peaks were found, at CT 00 and CT 12 (that is, at the beginning of the light and the

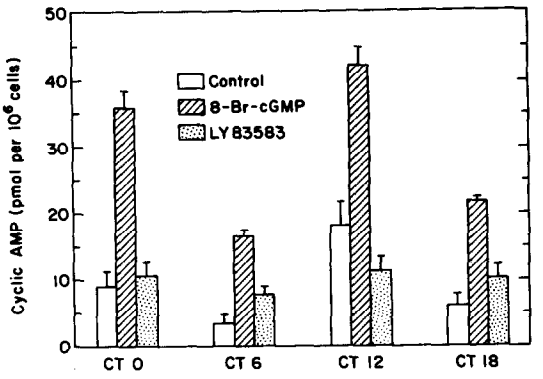

Fig. 2. *In vivo* effects of 8-Br-cGMP (10 μM) and LY 83583 (10 μM) on cAMP content in the ZC mutant of *Euglena*. Cells were sampled at different circadian times and were incubated for 2 hr with or without either of the chemicals before the cAMP assays, which were carried out at the indicated time points. Mean values of duplicate assays on replicate culture aliquots are shown; error bars indicate one half of the range of values.

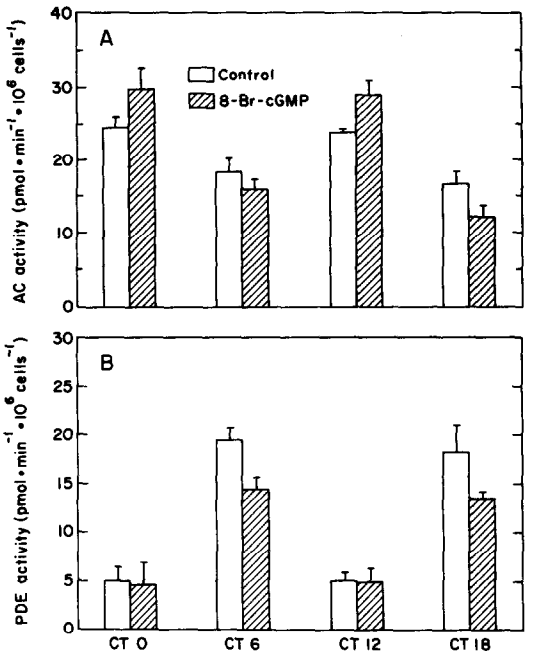

Fig. 3. *In vivo* effects of 8-Br-cGMP on AC activity (A) and PDE activity (B) in *Euglena*. Cells from different circadian times were incubated with 10 μM 8-Br-cGMP for 2 hr, and triplicate samples were assayed for the activities of both enzymes. Mean values are shown; error bars indicate one half the range. CT 00 and CT 12 corresponded to the peaks, and CT 06 and CT 18 to the troughs, of the AC rhythm as previously reported; the rhythm of PDE has an inverse phase relationship [2].

dark intervals), and troughs at CT 06–08 and CT 18–20. In these LD-synchronized cultures, cell division was confined primarily to the dark phase. After the cells were transferred from LD: 12, 12 to constant darkness (DD) shortly before they had stopped dividing as they entered the stationary phase, the bimodal circadian changes of cGMP content still persisted (Fig. 1B), with approximately the same pattern as that observed in dividing cultures. The range of the oscillation in nondividing cultures appeared to be slightly greater than that in dividing cells.

In vivo *effects of 8-Br-cGMP and LY-83583 on cAMP content.* After a 2-hr incubation of cells with 8-Br-cGMP, the cAMP content at the four circadian times that were examined increased by factors of 3.93 (CT 00), 4.67 (CT 06), 2.24 (CT 12), and 3.40 (CT 18), as shown in Fig. 2. When LY-83583 was added to the incubation medium, cAMP content showed no changes at CT 00, a decrease at CT 12, and a small increase at CT 06 and CT 18. While the new oscillatory pattern of cAMP with 8-Br-cGMP remained approximately the same, the amplitude of the rhythm after the addition of LY-83583 was so reduced that the rhythmicity of cAMP content disappeared.

In vivo *effects of 8-Br-cGMP on AC and PDE activities.* Incubation of culture samples with 10 μM 8-Br-cGMP resulted in an increase of AC activity in aliquots taken at CT 00 (22%) and CT 12 (23%). In contrast, in samples taken at CT 06 and CT 18, the activity of AC was reduced by about 16 and 32% (Fig. 3A). The effect of 8-Br-cAMP on PDE activity exhibited a similar circadian phase dependency, with decreases occurring at CT 06 (35%) and CT 18 (37%), while there were no changes in activities at CT 00 and CT 12 (Fig. 3B).

DISCUSSION

Circadian oscillations of cGMP have been reported recently in various organisms, including the bread mold *Neurospora crassa* [11] and the duckweed *Lemna paucicostata* [12], as well as in mammalian cells [13] and the body fluids of humans [14]. Patterns of the cGMP oscillations usually differed from those of cAMP rhythms in the same system. In an earlier report [1], we demonstrated a bimodal circadian variation of cAMP in *E. gracilis*, which was autonomously oscillatory, persisting in DD. In this study, cGMP content also was found to exhibit a bimodal, 24-hr fluctuation, but with a lower amplitude and a 2-hr difference in phase. The two peaks of the cGMP rhythm appeared at CT 00–02 and CT 12–14, and the two troughs at CT 06 and CT 20 (Fig. 1). When compared with the cAMP rhythm under the same experimental conditions, the peaks and troughs of the cGMP rhythm were found to occur 2 hr earlier. This difference in phase is interesting because in *in vivo* experiments 2 hr was required for cAMP to be elevated to its maximum level by the addition of cGMP (data not shown), which might be indicative of a mutual regulatory relationship between these two rhythms.

To resolve the question whether the cGMP rhythm might be dependent on the imposed LD cycle, or upon the transit of the cells through the different stages of their cell division cycle (CDC), or both, measurements were performed on cultures that had

been transferred to DD and that had entered the stationary phase of growth (Fig. 1B). Under these constant conditions, the free-running rhythm of cGMP content persisted with a phasing identical to that observed in LD-synchronized, dividing cultures (Fig. 1A). Thus, the cGMP rhythm in *Euglena* can be uncoupled from the driving force of the CDC and seems also to be under the control of an endogenous circadian pacemaker.

The concentration of cGMP in most tissues is generally several orders lower than that of cAMP, usually from 1/10 to 1/50 [4, 11, 15, 16]. In *Euglena*, however, the ratio of cGMP/cAMP content is only 1/3 to 1/8, depending upon whether the culture is dividing or nondividing. The reason for this difference is not known, nor is the mechanism whereby the cGMP rhythm is generated. Since the metabolism of cGMP is controlled for the most part by guanylate cyclase and cGMP-specific PDE, one would expect that these two enzymes could be responsible for the fluctuation.

It has been thought that the cAMP and cGMP systems represent two independent pathways controlling similar or different cell functions, and that the interactions between the two systems occur mainly at the level of degradation of the nucleotides [17]. *In vitro*, when the concentration of exogenous cGMP in the incubation medium was in the range of 1–100 μM, PDE activity in *Euglena* was inhibited by about 37% (Tong J and Edmunds LN, unpublished data). This inhibitory effect still existed *in vivo*, but only in cells sampled at CT 06 and CT 18 (Fig. 3B). On the other hand, the same dose of cGMP (10 μM) stimulated the activity of AC at CT 00 and CT 12 but not at CT 06 and CT 18 (Fig. 3A). These results indicated that, at least in *Euglena*, the regulation of cAMP level by cGMP can take place not only at the stage of degradation, but also through cAMP synthetic processes.

It is quite interesting to find that the effects of cGMP on AC and PDE *in vivo* are circadian phase dependent. It appears that PDE was sensitive to inhibition by cGMP only at CT 06 or CT 18 (Fig. 3B), whereas the activation of AC occurred in antiphase (Fig. 3A). Considering the fact that the activity of AC was high at CT 02 and CT 12 and low at CT 08 and CT 20, exactly opposite to that of PDE [2], it is likely that the phase-specific actions of cGMP may result from a difference in sensitivity of the enzymes during times at which cGMP functions. For example, identical amounts of cGMP, which could reduce PDE activity by about 1/3 at CT 06 and CT 18 when the activity was high (Fig. 3B), were ineffective at times when PDE activity was low.

The precise mechanism for this time-dependent sensitivity is as yet unknown. In some organisms, cGMP can enhance cAMP-PDE and stimulate the hydrolysis of cAMP, and thus act as a positive allosteric effector [18, 19]. In the present study, however, 8-Br-cGMP was found to inhibit PDE activity and raise the cAMP level *in vivo* (Figs. 2 and 3B). This divergent effect might be accounted for by the fact that there exist several types of PDE isoenzyme whose predominant type(s) is diversely distributed among different organisms [20]. (Indeed, if multiple forms of PDE were present in the soluble fraction of *Euglena*, the effects of 8-Br-cGMP that we observed might be even more pronounced if specific forms were assayed.) Since the PDE activity in *Euglena* was inhibited by the cGMP analog, we suggest that a cGMP-inhibited, "low K_m" cAMP-PDE prevails in this unicellular organism. cGMP also has been reported to be involved in the accumulation of cAMP in other organisms [21].

The effect of cGMP on the circadian rhythm of cAMP is not fully understood at present. LY-83583, an inhibitor of guanylate cyclase, could reduce the amplitude of the cAMP rhythm (Fig. 2), a finding that suggests a specific role for cGMP in the generation of the cAMP variation. This action of cGMP is supported by the observations that the cGMP rhythm leads that of cAMP by 2 hr and that cGMP exerts circadian phase-dependent modulations of the activities of AC and PDE. On the basis of results recently obtained with *Euglena*, we have hypothesized [22] that cAMP acts downstream from the oscillator and that the cAMP oscillation is an essential component of the signaling pathway for the control of the CDC by the circadian clock. Theoretically, cGMP should be able to modify the cAMP signaling pathway by affecting the AC–cAMP–PDE system, and, therefore, to modulate the processes that regulate the CDC. Contrary to the temporary phase-shifting effect found for pulses of cAMP pulses on the CDC but not on the rhythm itself [22], cGMP seems to permanently perturb the oscillator that underlies CDC rhythmicity (Tong J and Edmunds LN, data not shown). Obviously, this effect of cGMP would have to be mediated independently of the AC–cAMP–PDE system. The mechanisms involved in the modulatory effect of cGMP on circadian rhythms of cell function may include variations in cGMP-dependent protein kinase,* membrane depolarization, protein synthesis, and the opening of Na^+ and Ca^{2+} channels, among others [17, 23].

In summary, we conclude that cGMP is an effector with multiple pathways in cellular function in *E. gracilis*. It mediates the cAMP oscillation from upstream by regulating changes in AC and PDE activities. It also down-regulates CDC through mechanisms other than the AC–cAMP–PDE system. The exact nature of the regulatory pathway whereby this second messenger exerts its control remains to be elucidated.

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