Inhibitors of Serine/Threonine Phosphoprotein Phosphatases Alter Circadian Properties in Gonyaulax polyedra

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Protein serine/threonine phosphatases were implicated in the regulation of circadian rhythmicity in the marine dinoflagellate Gonyaulax polyedra based on the effects of three inhibitors specific for protein phosphatases 1 and 2A (okadaic acid, calyculin A, and cantharidin). Chronic exposure to okadaic acid resulted in a significant period lengthening, whereas cantharidin and calyculin A caused large phase delays but no persistent effect on period. Short pulses of the phosphatase inhibitors resulted in phase delays that were greatest near subjective dawn. Unlike 6-dimethylaminopurine, a protein kinase inhibitor, okadaic acid, calyculin A, and cantharidin did not block light-induced phase shifts. The inhibitors tested also increased radiolabeled phosphate incorporation into Gonyaulax proteins in vivo and blocked protein phosphatase 1 and 2A activities in Gonyaulax extracts. This study indicates that protein dephosphorylation catalyzed by protein serine/threonine phosphatases is necessary for proper functioning of the circadian system.

Protein phosphorylation and dephosphorylation are involved in the regulation of many eukaryotic intracellular processes. The activities of protein kinases and phosphoprotein phosphatases alter protein function by covalent modification and coordinately provide molecular switches that regulate many diverse systems, such as photosynthesis and the determination of budding yeast mating type.

The circadian system controls physiological processes in numerous organisms, ranging from bacteria to humans, resulting in their fluctuation on a daily basis. The ubiquity of circadian systems with similar properties suggests that there may be a common underlying mechanism among diverse species. Recent studies implicate protein phosphorylation in circadian-controlled processes in different systems. These include Euglena (Carré and Edmunds 1993), higher plants (Carter et al., 1991), the marine gastropods Bulla gouldiana (Roberts et al., 1989, 1992) and Aplysia (Takahashi, 1992), and the mammalian suprachiasmatic nucleus (Ginty et al., 1993). Also, the Drosophila putative clock protein PER has been shown to be subject to phosphorylation at multiple sites in relation to a postulated feedback loop in the circadian cycle (Edery et al., 1994).

We have previously shown that a protein kinase inhibitor, 6-DMAP, effectively slows or stops the progression of the circadian oscillator of the marine dinoflagellate G. polyedra in a concentration-dependent manner and blocks light-induced phase shifting (Comolli et al., 1994). In the present study, the effects of several inhibitors of Ser/Thr phosphatase action provided further evidence for the role of protein phosphorylation in Gonyaulax circadian regulation.

Two broad groups of protein phosphatases, type 1 and type 2, are responsible for the dephosphorylation of phosphoserine or phosphothreonine in eukaryotes. Three structurally unrelated molecules, OKA, CAL, and CAN, are naturally occurring inhibitors that specifically affect the major Ser/Thr protein phosphatases PP1 and PP2A. OKA and CAN are 10- to 100-fold more effective in inhibiting PP2A than PP1 (Biololan and Takai, 1988), but CAN has a lower potency toward both enzymes (Li and Casida, 1992); CAL is equally effective in inhibiting both PP1 and PP2A (Ishihara et al., 1989). These inhibitors have been used to indicate that PP1 and PP2A have roles in many different cellular processes and to demonstrate that these phosphatases have a wide range of substrates (reviewed in Shenolikar, 1994; Hunter, 1995). The present results show that CAN, CAL, and OKA have significant effects on the circadian oscillator in Gonyaulax; these effects are correlated to an inhibition of PP1/PP2A and to an increase in the phosphorylation state of many proteins.

MATERIALS AND METHODS

Cell Culture and Rhythm Measurements

Gonyaulax polyedra cells (strain 70) were grown in f/2 modified seawater medium (Guillard and Ryther, 1962) and maintained at 19°C in a light/dark cycle (12 h of light and 12 h of dark; 150 μE m⁻² s⁻¹ cool-white fluorescent light). For measurements of circadian rhythms of bioluminescence and of cell aggregation, cells were diluted to 5 × 10⁵ cells/mL in fresh f/2 medium; at dawn (CT 0), vials with 5 mL of cells were placed in constant dim light (40 μE m⁻² s⁻¹, unless
otherwise specified) and temperature (19°C) on an automated measuring apparatus (Broda et al., 1986; Roenneberg and Morse, 1993). Recording of the measured rhythms began upon transfer of the cells to constant conditions. Phosphatase inhibitors were added to cells at the times indicated (at least 24 h after transfer of the cells to constant conditions). In the case of inhibitor pulses (in contrast to continuous treatments), cells were filtered on a 20-μm Nitex (Tetko, Briarcliffe Manor, NY) membrane at specified times after addition of the inhibitor to remove the drugs, rinsed thoroughly, and resuspended in fresh 1/2 medium. Blue light pulses of approximately 100 μE m⁻² s⁻¹ were performed at 19°C with cool-white light fluorescent bulbs wrapped in Rosco (Port Chester, NY) blue filter #80 (primary blue).

**In Vivo Phosphate Labeling**

Cells (10⁶/mL) growing in light/dark were filtered on Nitex, rinsed, and resuspended at 19°C for 12 h in fresh 1/2 plus antibiotics (600 μg/mL ampicillin, 15 μg/mL erythromycin, 15 μg/mL rifampicin). Cells were again filtered and resuspended in the same volume of sterile seawater and given 3 h for recovery. Carrier-free ³²P (20 μCi; DuPont-NEN) was added to 2-mL samples of cells (approximately 10⁶ cells/mL) for 1 min at room temperature, followed by a 1-min exposure to OKA or CAL (controls received no inhibitor). Cells were then harvested by centrifugation, washed in 20 mM Tris-Cl, pH 7.5, plus 150 mM NaCl, and then lysed in 75 μL of lysis buffer (10 mM Tris, pH 6.8, 50 mM DTT, 4% SDS [w/v], 12% glycerol [v/v]) by vortexing for 30 s after the addition of 0.2 g of 1-mm diameter zirconium beads. The samples were then boiled for 5 min, allowed to cool, and treated with 5 μL of RNase/DNase solution for 5 min. Each sample (10 μL each) was then loaded onto a 10% SDS-polyacrylamide gel, and electrophoresis (Bio-Rad Mini Protean II cell) was performed for 1 h at 200 V. Gels were Coomassie stained and destained to examine the amount of protein loaded, then dried and autoradiographed for 6 d with an intensifying screen using Kodak X-OMAT film.

**In Vitro Phosphorylase a Phosphatase Assay**

³²P-phosphorylase a was prepared and assays were performed using the GIBCO-BRL protein phosphatase assay system (catalog no. 3188SA). Cells to be extracted (1 L) were harvested by vacuum filtration onto Whatman no. 54 filter paper, scraped into tubes containing 2.5 g of 1-mm zirconium beads, and lysed for 1 min using the MiniBeadbeater (Biospec Products, Bartlesville, OK) in 1.2 mL of extraction buffer [50 mM Tris-Cl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 1 mM DTT, 500 μM PMSF, and 10 μM l-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butanone]. After centrifugation for 20 min at 27,000g at 4°C to pellet cell debris, the supernatant was diluted 1:200 in extraction buffer and used in phosphorylase assays. Diluted extract (10 μL) was pretreated with phosphatase inhibitors (except for controls) for 10 min before being assayed for phosphorylase activity. The assay mixture, consisting of the extract plus 10 μL of assay buffer (200 mM imidazole-HCl, pH 7.63, 1% 2-mercaptoethanol [v/v], 1 mM and EDTA, 10 mg/mL BSA) and 10 μL of ³²P-phosphorylase a, was incubated at 24°C for 10 min. The reactions were stopped by the addition of 90 μL of cold 20% TCA (w/v). The samples were stored on ice for 15 min and centrifuged for 2 min at 12,000g, and 100 μL of the supernatant were added to 5 mL of EcoScint A (National Diagnostic, Atlanta, GA) scintillation fluid to determine the amount of ³²P released from phosphorylase a. All samples were adjusted for ³²P released by a blank without added extract. Specific activity was calculated using total protein concentration in the added extract as determined by Bio-Rad protein assay.

**RESULTS**

**Effect of Phosphatase Inhibitors on the Gonyaulax Circadian Oscillator**

Chronically applied OKA lengthened the period of the *Gonyaulax* circadian clock, as indicated by the progression of the bioluminescence glow rhythm (Fig. 1A). The free-running period was increased to a maximum of approximately 29 h in a dose-dependent fashion (Fig. 1B). At higher concentrations, the amplitude of the glow rhythm was decreased, and levels above 3 μM were lethal.

In a similar protocol, CAN and CAL had effects on the *Gonyaulax* circadian clock that were significantly different from those of OKA. Each of these inhibitors caused relatively small changes in period (Fig. 1B) but induced a concentration-dependent phase delay, up to 24 h in the case of 600 nM CAN and as great as 20 h with 1 μM CAL (Fig. 1C). The effect of these drugs on phase rather than period may be due to the instability of CAN and CAL so that they act for only a short time. CAN and CAL added to medium only and left for 24 h under the experimental conditions were then without effect when cells were added (data not shown). The effects of OKA persisted, so similar tests on its stability were not carried out. The phosphatase inhibitors similarly affected the three measured circadian processes of *Gonyaulax*: bioluminescent glow, bioluminescent flashing, and aggregation with cells maintained in white, red, or blue light.

Shorter exposures (3-h pulses) of the phosphatase inhibitors were most effective in altering the bioluminescent glow rhythm during late subjective night and early subjective day. Pulses of 2 μM OKA, 700 nM CAL, or 250 nM CAN caused phase delays at all circadian times of drug addition except near subjective dusk (CT12), when OKA or CAL resulted in small phase advances and CAN had a much reduced effect (Fig. 2). OKA (2 μM) caused only small phase shifts (maximum delay 2 h), whereas 700 nM CAL or 250 nM CAN induced phase delays of greater magnitude (up to 10 h). These effects were also concentration dependent (data not shown).

The effects of OKA pulses on the rhythm were reversible; the free-running period of treated cells after removal of the drug was identical to that of a control. The small effect of OKA pulses were consistent with the temporary slowing of the circadian oscillator as seen with chronic additions. However, CAN or CAL treatment resulted in a slightly shortened circadian period when the drugs were removed.
Phosphatase inhibitors alter the *Gonyaulax polyedra* circadian system. **Figure 1.** Effects of OKA, CAL, and CAN on *Gonyaulax* circadian rhythmicity. Drugs at the concentrations specified were added to the cell cultures 26 h after the transfer to constant conditions. A, Raw data (smoothed by a 2.8-h running mean) of the bioluminescence glow rhythm with chronic OKA added at the concentrations indicated. The time of OKA addition is indicated by the arrow. The circadian free-running period (τ) of each vial, calculated by regression analysis of the glow peaks, is indicated on the right side of the figure. A reduction in amplitude of the main glow peak is accompanied by an increase in that of the minor peak; this is especially evident in the first cycle after drug addition. B, Concentration-dependent effects of chronic OKA, CAL, or CAN on circadian period, which was calculated by regression analysis of bioluminescence glow peaks. C, Concentration dependence of phase delays caused by continuous CAL (■) or CAN (○) treatment. Phase delays were calculated by comparing regression of the glow peaks to a control that received no drug addition.

from the medium. After exposure to CAN for 3 h, the period was approximately 1.2 h shorter than that of a control; after a 3-h pulse of CAL, the period was 0.8 h less than a control. The cause of these “aftereffects” is not known.

Effects of Inhibitors on Light-Induced Phase Shifting

In contrast to the kinase inhibitor 6-DMAP, which blocks light-induced phase shifting (Comolli et al., 1994), OKA and CAL (present in the medium only during the light pulse) do not (Fig. 3). Indeed, the phase shifts due to light pulses were actually greater at some circadian times, and the phases at which maximum shifts occur were different. Control cells phase-advanced a maximum of 5.1 h in response to a 3-h bright blue light pulse that began 39 h after transfer to constant dim white light. The magnitude of the maximal phase shift was increased to 8.3 h with 3 μM OKA present during the blue light pulse and to 10 h with 1 μM CAL. In addition, the phase at which the maximum phase shift occurred was earlier than the control in both cases. Light-induced phase shifts were also altered, but not blocked, by CAN (data not shown). Phosphatase inhibitors alter the phase and amplitude of the response to light pulses, so it may be inferred that the phosphorylation state...
of certain proteins is important in some aspect of the reception and/or transduction of light signals to the circadian oscillator.

Effects of the Inhibitors on *Gonyaulax* Ser/Thr Phosphatases

To determine the action of the inhibitors on *Gonyaulax* Ser/Thr phosphatases, OKA or CAL was added to the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effects of OKA and CAL on the phase-response curve to bright light pulses. Cells maintained in constant dim white light received a 3-h bright blue light pulse (100 μE m⁻² s⁻¹) beginning at the time indicated. Controls (○) received only a light pulse, whereas experimental cells were exposed to 3 μM OKA (●) or 1 μM CAL (☺) during the exposure to bright light. Inhibitors were added immediately before the light pulse, then removed by filtration after the 3-h exposure to light. Phase shifts were calculated by comparison to the appropriate control that received no drug or a 3-h pulse of OKA or CAL only.

The three structurally unrelated phosphatase inhibitors used in this study, OKA, CAL, and CAN, altered *Gonyaulax* circadian rhythms and blocked phosphorylase a phosphatase activity in vitro. The two inhibitors that were tested in vivo, CAL and OKA, inhibited protein dephosphorylation. These results support the conclusion that dephosphorylation of Ser/Thr residues of one or more substrates is necessary for the normal functioning of the circadian system. The phosphorylation of proteins by protein kinases, which has been implicated in the circadian system in *Gonyaulax* and other organisms, is normally a reversible reaction that necessitates dephosphorylation by phosphatases. The identification of the phosphoprotein phosphatases and their substrates that influence the circadian system is important in some aspect of the reception and/or transduction of light signals to the circadian oscillator.
Okadaic acid  
Cononadic acid  
Calphostin C  
Calybeacin A

**Figure 5.** OKA (●), CAL (○), and CAN (▲) block the phosphorylase phosphatase activity in *Gonyaulax* extracts. Diluted extracts were incubated with varying concentrations of the inhibitors for 10 min before the addition of radiolabeled phosphorylase a for 10 min at 24°C. The amount of radiolabeled phosphate released from the substrate was calculated and expressed as a percentage of the specific activity of the control (no inhibitor added).

The circadian oscillator will provide insight into the circadian mechanism and its action.

Although the phosphatase inhibitors used in this study are known to be highly specific for phosphatase classes 1 and 2A, the inhibitors did act somewhat differently on the circadian system. PP1 and PP2A are two closely related enzyme classes (40-50% homology), each of which is known to have several members in other systems (Chen et al., 1992; Arino et al., 1993). The specific effects of the inhibitors may differ between individual enzyme species, as is so with PP1 and PP2A (Bialajan and Takai, 1988; Li and Casida, 1992).

The circadian effects of OKA appeared to be tonic in nature, similar to those found with the protein kinase inhibitor 6-DMAP. Greater than 500 nM OKA caused significant period lengthening (Fig. 1A), apparently slowing the circadian mechanism. The relatively small phase changes resulting from pulses of OKA was consistent with a tonic effect (Fig. 2). At concentrations >100 nM, OKA was effective in blocking phosphatase activity, as measured in vivo by Pi incorporation (Fig. 4) and in vitro by dephosphorylation of radiolabeled phosphorylase a (Fig. 5). The circadian effects of OKA either require PP1/PP2A activity to be nearly completely inhibited or the phosphatase that elicits the OKA effects to be less sensitive to the inhibitor than is measured in the assays used in this study. Alternatively, OKA may not be able to affect all phosphatases in vivo. Radiolabeled OKA accumulates in membranes, where PP1 was more abundant than PP2A (Shenolikar, 1994), so it may significantly inhibit protein dephosphorylation without blocking all the active enzymes. In contrast to the increase of the *Gonyaulax* circadian period caused by OKA, *Bulla gouldiana* displayed no changes in circadian phase or period upon addition of OKA (Roberts et al., 1992). It may be that the effect of OKA is specific to the *Gonyaulax* circadian system.

CAL and CAN had pronounced effects on the *Gonyaulax* circadian mechanism that were different than those of OKA. Both inhibitors caused significant concentration-dependent phase delays when added either chronically or as a short pulse (Figs. 1C and 2; data not shown). The phase delays were induced at all circadian phases (Fig. 2), so these effects can also be interpreted as tonic in nature but of larger magnitude than those of OKA. CAL and CAN were probably not acting directly on a circadian mechanism component because transient phasic perturbation of the circadian oscillator should result in both phase advances and phase delays, such as occur in *Gonyaulax* with inhibitors of protein synthesis (Dunlap et al., 1980; Taylor et al., 1982).

The data indicate that 1 μM CAL caused greater than 20-h phase delays (Fig. 1C), inhibited phosphatases in vivo (Fig. 4), and completely blocked PP1/PP2A activity in vitro (Fig. 5). However, 100 nM CAL was also effective in causing phase delays and inhibiting phosphatases in vitro but did not appear to increase phosphorylation state in vivo. This may be due to a slow rate of entry into the cells during in vivo labeling.

CAN caused phase delays at concentrations that did not completely inhibit PP1/PP2A activity in vitro (Figs. 1C and 5). Nearly complete inhibition of PP1/PP2A activity was achieved at approximately 1 μM CAN. The specific in vitro assay for PP1/PP2A did not directly parallel the effects of the inhibitor on phase, so it is possible that CAN was acting on another cellular process. This could be a protein phosphatase able to affect the circadian oscillator but not able to dephosphorylate phosphorylase a.

*Gonyaulax* protein Ser/Thr phosphatases may differ from the four typical classes (see introduction), accounting for the variations in the in vivo and in vitro efficacies of CAN, CAL, and OKA. Dinoflagellates are distinct from other eukaryotes in numerous ways, including a lack of histones and an atypical cell division. On the other hand, there is evidence suggesting that the structure, and perhaps the function, of *Gonyaulax* PP1 may be similar to the enzyme characterized from many other organisms. We have isolated and sequenced a *Gonyaulax* cDNA (J. Comolli, unpublished data) whose putative protein sequence closely resembles that of the PP1 catalytic subunit from other species. Attempts to isolate a PP2A cDNA from *Gonyaulax* have thus far been unsuccessful, and it is possible that *Gonyaulax* may lack PP2A-like activity, as is true for another dinoflagellate, *Proorocentrum lima* (Boland et al., 1993).

PP1 and PP2A class enzymes have a wide and overlapping range of substrates involved in many intracellular processes (Bollen and Stalmans, 1992; Shenolikar, 1994). These include proteins involved in lipid and carbohydrate metabolism (Bollen et al., 1988; Clotet et al., 1995), protein synthesis (Redpath and Proud, 1990, 1991), and cell division (Bollen and Stalmans, 1992; Ludlow and Nelson, 1995). PP1 and PP2A have also been demonstrated to have a role in regulating gene expression (Sheen, 1993; Takeda et al., 1994; Mayerjaekel and Hemmings, 1995). This leaves...
many potential dephosphorylation event(s) that may be affected by OKA, CAN, or CAL in altering circadian functions. Hence, individual PP1/PP2A species must be examined to determine the timing and substrate specificity relevant to circadian function. Overall PP1/PP2A activity, measured by phosphorylase a dephosphorylation, does not vary on a circadian basis (data not shown).

It is likely that the *Gonyaulax* circadian system involves the phosphorylation and dephosphorylation of more than one protein substrate, so the interpretation of the actions of the inhibitors is complex. 6-DMAP, an inhibitor of protein kinases, lengthened the circadian period in a concentration-dependent manner, as did OKA. In addition, pulses of 6-DMAP caused phase delays at all circadian times of application, much like the effects of the phosphatase inhibitors. The action of chronically added phosphatase inhibitors was not counteracted by 6-DMAP (data not shown), suggesting that normal circadian function involves the phosphorylation and dephosphorylation of two or more substrates or that the inhibitors work in some manner other than control of the phosphorylation state of one particular substrate.

Despite the different amplitudes of their phase-response curves, OKA, CAN, and CAL all induced lesser phase delays and, in some cases, a small phase advance during late subjective day/early subjective night (near CT12; Fig. 2). By contrast, pulses of 6-DMAP were most effective in eliciting phase delays at that time. Thus, the shapes of the phase responses to kinase and phosphatase inhibitors appear to be inversely related. This suggests that there may be some antagonistic actions in the effects of the inhibitors on the circadian system.

Whereas the protein kinase inhibitor 6-DMAP completely blocked light-induced phase shifting in *Gonyaulax* (Comolli et al., 1994), the phosphatase inhibitors studied here did not. Light pulses caused substantial phase shifts in the presence of all three; OKA and CAL enhanced the phase advances caused by light pulses and altered the time of the maximum response. Phosphorylation of proteins also appears to be involved in light transduction in other systems. In maize, light-inducible gene expression has been shown to require PP1/PP2A activity (Scheen, 1993), and both red and blue light are capable of changing the phosphorylation state of proteins (Reymond et al., 1992; Harter et al., 1994). Also, light-induced resetting of a circadian pacemaker has been reported to involve stimulation of a cAMP-dependent protein kinase (Takahashi et al., 1993; Levine et al., 1994).

The details of these effects are not yet known; the key point for the present communication is that, although the kinase inhibitor 6-DMAP blocked light phase shifting, three phosphatase inhibitors studied did not. Thus, although 6-DMAP and OKA both lengthened the circadian period, they appeared to be working in an antagonistic manner on the *Gonyaulax* circadian phototransduction pathway. These results are consistent with a model in which phase shifting by light involves the action of a protein kinase. 6-DMAP would block the action of this kinase so that, in its presence, no phase shifts occur in response to light pulses. By preventing dephosphorylation, phosphatase inhibitors help maintain a high state of phosphorylation and thus increase the response to light.

This study highlights the importance of phosphorylation in the circadian mechanism and indicates that PP1/PP2A is an enzyme activity necessary for normal circadian function. Further definition of the kinases and phosphatases regulating the circadian clock and the determination of their relevant substrates are necessary to understand the scope of the involvement of protein phosphorylation in the circadian mechanism and its relationship to other cellular processes.

ACNOWLEDGMENTS

We are grateful to Drs. T. Wilson and T. Fagan for helpful comments and critical review of the manuscript.

Received November 15, 1995; accepted February 21, 1996.

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LITERATURE CITED


Phosphatase Inhibitors Alter the Gonyaulax polyedra Circadian System


