Novel Effects on the *Gonyaulax* Circadian System Produced by the Protein Kinase Inhibitor Staurosporine

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**Abstract** The protein kinase inhibitor staurosporine was found to cause a dramatic increase in the free-running period (FRP) of circadian rhythms in the dinoflagellate *Gonyaulax polyedra*, and its effect was similar when added at different phases of the circadian cycle. Chronic exposure to staurosporine lengthened the FRP by as much as 7 h without significantly affecting the amplitude or waveform of the bioluminescence rhythm. The effect on the length of the FRP occurred only above a threshold concentration, and it lasted for a limited number of cycles that depended on the dose of the drug. The FRP lengthening was not evident until 23 to 26 h after staurosporine addition, even though the drug entered *Gonyaulax* cells in 1 h or less. When tested in combination with bright light pulses, staurosporine was found to enhance both light-induced phase advances and delays, indicating that the drug acts on circadian phototransduction. At concentrations that alter the FRP and the response to light pulses, staurosporine appears to act on a small number of protein kinases, attenuating the activity of two individual protein kinases without affecting overall phosphate incorporation into proteins in vitro.

**Key words** dinoflagellates, bioluminescence, protein kinase inhibitor, staurosporine, free-running period, phase response curve

**INTRODUCTION**

The involvement of protein phosphorylation in circadian regulation in *Gonyaulax polyedra* has been inferred from effects of inhibitors of both protein kinases and phosphoprotein phosphatases. Such drugs have been demonstrated to alter both the free-running period (FRP) of the measured circadian rhythms and the phase-shifting response to light. The protein kinase inhibitors 6-dimethylaminopurine (6-DMAP) and roscovitine lengthened the FRP of the bioluminescence rhythm of *G. polyedra* and blocked phase shifting by light (Comolli et al., 1994; Hastings et al., 1997). Three different inhibitors of serine/threonine protein phosphatases—cantharidin, calyculin A, and okadaic acid—also had dramatic effects on the FRP and phase shifting by light, further implicating complex regulation by protein phosphorylation and dephosphorylation in circadian function (Comolli et al., 1996).

In this article, we characterize the unusual action of the protein kinase inhibitor staurosporine on the *Gonyaulax* circadian system and present evidence suggesting that it may alter circadian function by blocking only a small number of protein kinases.

**MATERIALS AND METHODS**

**Cell Culture and Circadian Rhythm Measurements**

*G. polyedra* strain 70 was cultured in f/2 supplemented sea water medium (Guillard and Ryther, 1962)
at 19°C under a 12-h light/12-h dark cycle (LD 12:12) using cool white fluorescent bulbs at an intensity of approximately 150 μE m⁻² sec⁻¹. For rhythm measurements, cultures were diluted to 5000 cells ml⁻¹ during the day phase, and 5 ml of cells were pipetted into vials. The following day at LD 0 (the dark-to-light transition), vials were transferred into constant dim white light (35 to 40 μE m⁻² sec⁻¹) at 19°C, and their spontaneous bioluminescent flashing, glow, and aggregation were measured with an automated photometer apparatus (Broda et al., 1986; Roenneberg et al., 1989). Staurosporine and K252b (LC Laboratories, San Diego) were prepared as 1-mM stock solutions, and K252a (a gift of J. Sheen) were prepared as 2 mM, both in DMSO; appropriate dilutions were made in DMSO. Additions of the drugs were made directly to the medium at the times indicated but always after the cells were transferred to constant conditions. In the case of drug pulses, cells were collected with a 20-μm Nitex filter (Tekto, Briarcliffe Manor, NY) at the time indicated as the end of the pulse, rinsed thoroughly, and resuspended in 5 ml of fresh f/2 medium. Raw data of the bioluminescent glow rhythm, displayed by the Chrono II program (Roenneberg and Taylor, in press), were smoothed by a running mean of 2.8 h. The FRPs of the recorded rhythms were calculated by regression analysis of 2 to 6 peak values of luminescence, depending on the dose of the drug, and the error represents standard deviation from the regression. The amplitudes of the glow rhythms for a particular experiment were plotted with the same relative units of light intensity.

For phase response curve (PRC) experiments involving light pulses, cells maintained in constant dim white light were given 3-h pulses of bright blue light (~120 μE m⁻² sec⁻¹) at 19°C from cool white light fluorescent bulbs wrapped in Rosco blue filter No. 80 (primary blue). Blue light pulses were given both to cells receiving no drug and to those treated with 20 nM staurosporine; the drug was added at the transition to constant light (LL 0), 30 h prior to the first light pulse. Phase shifts were calculated by comparison to a regression analysis of luminescence peaks from an appropriate control. Circadian times were calculated using the following formula: 1 circadian hour = FRP × 24⁻¹.

Kinase Assays

Gonyaulax cells grown in an LD cycle were diluted to 1 × 10⁴ cells ml⁻¹ in fresh f/2. Cells were harvested the next day at LD 2 or LD 14 by vacuum filtration onto Whatman filter paper, and 0.3 g wet weight of cells were lysed in a Mini Bead-Beater (Biospec Products, Bartlesville, OK) for 1 min in extraction buffer (50 mM Tris-Cl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 2 mM DTT, 200 μM PMSF, 1 mM benzamidine, 1 μM E-64, 1 μM 3,4-DCI). The extract was then centrifuged at 20,000 × g for 10 min at 4°C, and the supernatant was used for renaturation assays. The procedure was performed as described previously (Comolli et al., 1994) with the following modifications: a 12.5% minigel was loaded in duplicate, the gel was cut in half prior to the kinase assay, and the autophosphorylation reaction was performed using 15 μCi ml⁻¹ [γ-³²P]ATP in kinase buffer for 1.5 h in the absence or presence of 30 nM staurosporine. Gels were washed and treated for autoradiographic detection as described.

**RESULTS**

**Staurosporine Treatment Increases the Gonyaulax Free-Running Period**

When added to Gonyaulax cultures maintained in constant dim light, staurosporine at a concentration of 4 nM or greater caused a large increase in the FRP (Fig. 1A). The dose response of the FRP to staurosporine clearly was not linear, as is evident in a plot of the FRPs calculated from the raw bioluminescence data (Fig. 1B). Cells that received up to 3 nM staurosporine had FRPs of 23.0 to 23.5 h, identical to that of a control not receiving the drug or to those receiving DMSO carrier (data not shown), but cells treated with 4 nM staurosporine had FRPs near 27 h. Higher concentrations of staurosporine (between 5 and 40 nM) increased the FRP to a maximum of approximately 31 h. Thus, a dramatic difference in the FRP (4 h or more) occurs over a narrow dose range (3 to 4 nM), whereas additional increases in dose have a lesser effect.

Staurosporine at low effective concentrations did not have evident deleterious effects on G. polyedra; drug treatment either did not change or increased the amplitude of the glow rhythm relative to that of a control (Fig. 1A). Concentrations of the drug higher than 20 nM appeared to affect viability and were lethal above 40 nM. The bioluminescent flashing and aggregation rhythms were monitored during the same experiments and behaved the same as the bioluminescent glow rhythm (data not shown). Also, the FRP was increased regardless of the intensity or wavelength of...
background light; drug treatment in blue or red light, using filters as described in Roenneberg et al. (1988), had similar effects (data not shown).

The number of period-lengthened cycles was dependent on the staurosporine concentration (Fig. 2). Whereas the 28.1-h FRP caused by 11 nM staurosporine lasted for only two circadian cycles, 20 nM staurosporine caused the cells to exhibit a 29.4-h period for four complete cycles. After the staurosporine-induced effect had worn off, the FRP returned to that of cells not receiving the drug, thus indicating no drug after-effects. Because the longer period persisted for several cycles, staurosporine was interpreted to induce an increase in the FRP rather than simply a large phase delay.

A lag of approximately one circadian cycle between drug addition and the resulting FRP increase was evident from chronic staurosporine treatment. As displayed in Figs. 1A and 2, the first bioluminescent glow peaks of cells immediately following staurosporine addition had a phase nearly identical to that of a control not exposed to the drug, indicating that the FRP had not yet been altered; the increase in the FRP was seen only in subsequent circadian cycles. This effect could be expected if only one particular phase of the *Gonyaulax* circadian cycle was staurosporine sensitive. However, the lag is independent of the circadian phase of the drug addition; chronic addition of 20 nM staurosporine to different cultures every 3 h over a circadian cycle induced a longer FRP in each case, always after a lag (Fig. 3). By comparing the peak phases from the staurosporine additions at LL 43 and LL 46, it can be determined that the drug required between 23 and 26 h to cause an increase in the FRP. This experiment also demonstrates that the degree of drug-induced FRP lengthening varied only slightly (and not systematically) with the circadian phase of drug addition; the period length increased to 27.2 to 29.2 h (4.6 to 6.6 h longer than that of the control) regardless of the time in the cycle of staurosporine addition.

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**Figure 1.** Staurosporine lengthens the circadian free-running period (FRP) of the bioluminescent glow rhythm. (A) Staurosporine was added to the cultures at the final concentrations indicated 29 h after transfer to constant dim white light at 19°C (indicated by the arrow). The drug remained in the vials for the duration of the experiment. The raw data were smoothed by a 2.8-h running mean. (B) Chronic staurosporine treatment exhibits a threshold effect with concentration. The FRPs of the bioluminescent glow rhythms represented in Panel A were calculated by regression analysis of peak values.

**Figure 2.** The duration of the free-running period increase induced by chronic staurosporine treatment depends on the concentration. This phase plot represents the phase (in degrees, real time) of the peak of the bioluminescent glow rhythm during each measured circadian cycle. Vials exposed to 11 nM or 20 nM staurosporine 35 h after their transfer to constant dim white light (during the first full circadian cycle) are compared to a control not receiving staurosporine.
Pulses of Staurosporine Have Effects Similar to Chronic Exposures

To assess the rate of uptake of staurosporine, *Gonyaulax* cultures were given short (1- to 5-h) pulses of 25 nM staurosporine, after which the drug was removed from the medium by filtration and washing. Each short pulse of staurosporine induced a period lengthening of 9.6 to 9.9 h compared to a control not receiving treatment, and each displayed a lag prior to the FRP increase, as with chronic treatments (Fig. 4). Thus, staurosporine is able to enter cells rapidly but remains ineffective in increasing the FRP until more than 23 h later. The increase of the FRP resulting from such pulses of staurosporine was short-lived, lasting for only two circadian cycles, similar to a chronic exposure to a low dose of the drug. With short pulses of staurosporine, as in chronic exposure, the FRP increase and its duration did not vary systematically with the circadian phase of pulse application (data not shown).

Staurosporine Enhances the Phase Response to Light Pulses

We sought to determine whether the action of staurosporine on the *Gonyaulax* circadian system involved the light input pathway. Vials of cells maintained in constant dim white light were given 20 nM staurosporine followed by 3-h pulses of bright blue light starting 30 h later at 3-h intervals over a circadian cycle. The light pulse PRC generated in the presence of staurosporine was significantly different from that of cells not receiving the drug (Fig. 5), indicating that the drug influences the action of light on the *Gonyaulax* circadian system. A repeat of the experiment gave a similar result. Without staurosporine, cells exhibited a typical *Gonyaulax* PRC for light pulses of this intensity, with approximately 1-h delays in late subjective day and early subjective night and from 2- to 5-h phase advances in middle to late subjective night (Johnson and Hastings, 1989; Roenneberg and Taylor, 1994). Staurosporine had a greater effect on the delay portion of the PRC, such that the maximal delay induced by a light pulse increased from 1 to 6 circadian hours. The effect on phase advances was not as significant, although a 4-h increase was evident at the phase of maximal advances. Phase shifts from light pulses given during the dead zone of the PRC were not greatly affected. In effect, staurosporine changed the PRC of a light pulse of this intensity from a Type 1 to a Type 0 (Winfree, 1980).

The light pulse PRC and the FRP were altered by staurosporine in the same concentration range and with the same lag; because of the lag, 20 nM staurosporine given 3 h prior to light pulses did not enhance light-induced phase shifts (data not shown). No matter when given, 3 nM staurosporine was without effect. These observations are consistent with the drug effects on the FRP and PRC being due to the same action of this inhibitor.
K252a and K252b Can Block the Effects of Staurosporine

K252a and K252b are additional indolocarbazole protein kinase inhibitors related in structure to staurosporine but with slightly different specificities and efficacies (Rüegg and Burgess, 1989). These drugs also were tested for their ability to alter the circadian function of G. polyedra. Neither K252a nor K252b lengthened the FRP as did staurosporine (Table 1). The pattern, period, and amplitude of the bioluminescent glow rhythm was unaltered in cells exposed to 0.1 to 30 nM K252a, whereas 50 nM or greater killed the cells. K252b did have an effect on the FRP, but unlike staurosporine, this was a small, dose-dependent period shortening. Concentrations ranging from 50 to 400 nM K252b (the highest nonlethal dose) progressively decreased the FRP of the bioluminescent glow rhythm up to 1.5 h compared to that of a control not receiving the drug. The lethality of low drug concentrations indicates that K252a and K252b are able to enter cells to act on cellular kinases but do so at sublethal concentrations without altering the circadian system.

We next investigated whether the targets of K252a or K252b and staurosporine were overlapping by adding the inhibitors in combination. K252a (20 nM) or K252b (400 nM) added 3 h prior to staurosporine treatment greatly reduced the FRP increase caused by staurosporine (Fig. 6 A,B). The period did lengthen, but for only one circadian cycle before reverting to that of cells treated with K252a or K252b alone. This can be compared to treatment with 10 nM staurosporine alone (without K252a or K252b), which lengthened the period for three cycles. The interference of the actions of staurosporine and K252a or K252b suggests that the drugs have overlapping target specificities and might be interacting with the same binding sites.

In the converse experiment, when staurosporine treatment preceded K252a or K252b addition by 3 h, the luminescence rhythm was identical to a control receiving staurosporine only (data not shown). Thus, these drugs cannot readily displace staurosporine or reverse its effect. In summary, these experiments suggest that K252a and K252b affect the same processes as does staurosporine or that the uptake or modification of staurosporine is blocked by prior K252 treatment.

Staurosporine Inhibits Gonyaulax Protein Kinases in vitro

To examine the extent to which protein phosphorylation is perturbed by staurosporine in the concentration range (5 to 50 nM) where the circadian rhythm is affected, staurosporine was tested in in vitro protein kinase assays using G. polyedra extracts. Initial experiments demonstrated that staurosporine caused no detectable change in overall protein phosphorylation as measured by the in vitro incorporation of radio-labeled phosphate into endogenous Gonyaulax proteins or into the exogenous substrates myelin basic protein or histone H1 (data not shown).

Table 1. Protein kinase inhibitors K252a and K252b have little or no effect on the free-running period (FRP) of the luminescence rhythm.

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<td>FRP (nanomolar)</td>
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<td>350</td>
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Figure 5. Staurosporine enhances the phase response to light pulses. Three-hour pulses of blue light at 120 µE m⁻² sec⁻¹ were given at 3-h intervals (from LL 30 to LL 57) to cells maintained in dim white light, beginning at the circadian times indicated. A PRC was produced in the absence (solid line) and in the presence of 20 nM staurosporine (dashed line). Phase shifts were calculated by comparison of regression analyses for samples receiving only light pulses or light pulses and the drug to controls receiving no light pulses but the appropriate drug treatment. All phase shifts and times for light pulses are represented in circadian hours and, therefore, compensated for the period lengthening induced by staurosporine treatment.
The activities of individual protein kinases in the presence and absence of staurosporine were examined using an in situ renaturation assay. Of the seven protein kinases capable of autophosphorylation in this assay, the activities of at least two protein kinases (approximately 32 and 120 kDa) were greatly reduced in the presence of 30 nM staurosporine, whereas other measured kinases showed no effect (Fig. 7). Also, there was no discernible difference in the inhibition by staurosporine between extracts from cells at LD 2 (early day) or LD 14 (early night). Thus, there appear to be protein kinases that are sensitive to staurosporine at concentrations capable of causing an FRP increase, whereas several other protein kinases are unaffected at these concentrations.

**DISCUSSION**

The involvement of protein phosphorylation in circadian rhythm generation has been demonstrated in a number of organisms (Carre and Edmunds, 1992; Carter et al., 1991; Garceau et al., 1997; Ginty et al., 1993; Roberts et al., 1989, 1992; Zwartjes and Eskin, 1989). One of the most compelling instances is in the circadian system of *Drosophila melanogaster*, where two proteins essential for proper circadian function, PERIOD and TIMELESS, have been found to undergo progressive phosphorylation during a circadian cycle (Edery et al., 1994; Price et al., 1995; Zeng et al., 1996). PERIOD phosphorylation likely involves the product of the *Drosophila* gene *double-time*, a protein kinase...
homologous to casein kinase Iε, which recently has been shown to play a crucial role in circadian rhythmicity by interacting with and controlling the accumulation of PERIOD (Kloss et al., 1998; Price et al., 1998). Thus, double-time is the first protein kinase identified as being essential for generation of circadian rhythmicity. In this article, we use a protein kinase inhibitor to suggest that a protein kinase might also be necessary for the proper functioning of the circadian system of the dinoflagellate Gonyaulax polyedra.

Staurosporine is a potent agent for disturbing the Gonyaulax circadian system and does so in a manner that is distinct from other circadian-altering substances including other tested protein kinase inhibitors. Chronic exposure to nanomolar concentrations of staurosporine causes a period increase of more than 10 h, generating the longest FRP that has been recorded for Gonyaulax circadian rhythms. A surprising feature of the effect of staurosporine is that the bioluminescent glow rhythm remains robust with an unaltered waveform, whereas the FRP is lengthened. The same is true for the flashing and aggregation rhythms. By this measure, the drug is able to alter the FRP without significant effects on viability or metabolism. Its action also has no apparent phase dependence, indicating (but not proving) that staurosporine likely acts on the Gonyaulax circadian mechanism throughout the circadian cycle.

The relationship between the inhibitor concentration and the length of the FRP is a particularly unusual aspect of staurosporine treatment. The drug has no effect at 3 nM, but at 4 nM it lengthens the period significantly (Fig. 1B). This effect has been observed repeatedly, and whereas the actual “breakpoint” concentration has been found to vary between 2 and 9 nM in different experiments, the steep increase in period length with concentration always was observed. Indeed, the quantitative effect of a particular concentration of staurosporine was found to vary from one experiment to another; see Fig. 2 [11 nM] and Fig. 6 [10 nM].) Once the threshold concentration is exceeded, higher doses lengthen the FRP to a lesser extent but continue to increase the duration of the FRP change. The cause of this threshold effect is unclear, but it might be due to a cumulative effect of the inhibitor on sequential phosphorylation steps in a pathway leading to a highly phosphorylated end product. Another possibility is that there are several staurosporine-sensitive phosphorylation sites on a single target protein or that the drug is interfering with a synergistic interaction. There are numerous examples in which the phosphorylation of one residue may promote phosphorylation of other sites on the same protein (Chen et al., 1993; DePaoli-Roach, 1984; Flowtow and Roach, 1989; Miller and Kennedy, 1986). Regardless of the mechanism, the effects of the drug terminate when, by being metabolized or degraded, the effective intracellular staurosporine concentration drops below the threshold level. At this time, the FRP returns to that of untreated cells. The time necessary to drop below this threshold correlates with the amount of staurosporine added to the cells, as is evident in Fig. 2.

Another feature of the effect of staurosporine is the lag between the addition of staurosporine to the medium and the FRP increase. The lag is not due to slow uptake of the drug, because staurosporine remains effective even if given at a 1-h exposure. Staurosporine might need to be metabolized or modified intracellularly in a time-dependent manner to give a product that alters the FRP. Alternatively, it might bind to its target(s) rapidly but require time to produce downstream changes. For example, inhibition of a kinase might take some time to have a net effect on a target if it is dephosphorylated at a relatively slow rate. This delayed effect after drug treatment has been seen with other substances in Gonyaulax; it has been reported after the addition of creatine, which shortens the FRP (Roenneberg et al., 1988), and actinomycin D, which abolishes the bioluminescence rhythm, presumably by inhibiting RNA synthesis (Karakashian and Hastings, 1962).

Even though it often is referred to as specific for protein kinase C, staurosporine does inhibit many different protein kinases at nanomolar concentrations including cyclic AMP- and cyclic GMP-dependent kinases, MAP kinases, calcium/calmodulin-dependent kinases, Cdc2 kinases, and a number of tyrosine kinases (Rüegg and Burgess, 1989; Gschwendt et al., 1994). However, K252a and K252b, despite having structures and specificities similar to staurosporine and their ability to compete for the same binding sites as staurosporine on protein kinases (Herbert et al., 1990), do not affect circadian rhythms in a similar manner. Because of this and the lack of effects from numerous other kinase inhibitors tested, including those that affect the classes mentioned (Comolli et al., 1994), staurosporine may inhibit the relevant kinase(s) in a relatively specific manner. Furthermore, the in vitro phosphorylation studies suggest that the effects of staurosporine on the circadian system are not accompanied by a general reduction in cellular kinase activity (as is the case with 6-DMAP). Supporting this
hypothesis is the renaturation assay, which indicates that the autophosphorylation of at least two, but not all, detected protein kinases was significantly inhibited by 30 nM staurosporine. However, although these kinases may play a role in circadian function, the in vivo and in vitro potencies of staurosporine might not necessarily correspond, so their activities might not be related to the circadian mechanism.

Staurosporine and 6-DMAP differ markedly in their action on the Gonyaulax circadian system and on protein phosphorylation. The concentration of 6-DMAP necessary to modify circadian function is approximately 10^4 times higher than that of staurosporine, and at this concentration much of the kinase activity of the cell is inhibited. 6-DMAP lengthens the FRP by up to 4 h in a monotonic, dose-dependent fashion, whereas staurosporine causes a much larger effect but with a sharp concentration threshold, exhibiting cooperative-like behavior. 6-DMAP acts immediately when added to the medium and is able to induce 2- to 5-h delay phase shifts when given in pulses; these properties are not evident with staurosporine treatment. Finally, 6-DMAP can completely block phase shifts in response to light pulses, whereas staurosporine enhances this photic response (Fig. 5). The different actions of these two kinase inhibitors implies that protein phosphorylation plays numerous roles in regulation of the circadian system.

The action of staurosporine in altering the FRP and light pulse PRC shows the same sensitivity and time dependence, and indeed both might be due to the action of the drug on a single target. The FRP in constant light has been proposed to relate to the shape of the PRC (Pittendrigh and Daan, 1976). Although this does not hold true in all cases (Johnson, 1992), this “velocity response” hypothesis is based on the proposal that dim constant light affects the circadian clock by causing many small phase resets, both delays and advances. The FRP in constant light would be equal to the FRP in darkness plus advances and delays from dim light resets integrated over one circadian cycle. When applied to Gonyaulax, the large advance-to-delay ratio of the light pulse PRC would result in an FRP in constant light that is shorter than 24 h, as observed under the conditions used. However, because staurosporine enhances the delay portion of the Gonyaulax light pulse PRC, it should result in an increase in the FRP. This suggests a possible relationship between the effects of staurosporine on the light pulse PRC and the FRP of the Gonyaulax circadian system.

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REFERENCES


Roenneberg T and Taylor WR (in press) Data acquisition and analysis from *Gonyaulax polyedra*. Meth Enzymol.


