The biological clock is a timing mechanism used by eucaryotic cells to control the rates of some biochemical and physiological processes. The cell can thereby alter the rate of these reactions to match predictable daily changes in the environment such as the duration of the light cycle. Even though attempts have been made to study the biological clock in numerous systems, the molecular mechanism(s) used by cells to generate 24 h rhythms in such processes as cell division or photosynthesis has not yet been elucidated. It is generally accepted that the clock can be described as a limit cycle consisting of several steps (27). It has been determined that protein synthesis affects the expression of the clock (22), and membrane properties may also be involved (4, 18).

One approach that has not been used to study the biological clock is the identification of the coupling or transducing steps that connect the biological clock to the processes controlled by the clock. Steps connecting the various rhythmic processes to the clock must exist because it has been shown that although there are rhythms in photosynthetic rate and the timing of mitosis, neither of these processes are part of the actual clock. Disruption of the cell division or photosynthesis rhythms does not stop other clock-controlled reactions in Euglena (11, 13). Theoretically, it might be possible to trace the transducing steps backward for a process controlled by the clock, and thus approach and possibly identify the step(s) interfacing with the clock. This approach might prove useful if the transducing steps for several rhythms in the same organism are examined and compared, or if the transducing steps for several different organisms were compared. Collectively, such information might help identify potential biochemical components of the biological clock.

An attempt to identify the transducing steps linking the rhythm in oxygen evolution to the biological clock was begun in Euglena gracilis after a population of cells was discovered in which the light reactions were uncoupled from the clock (13). Instead of a peak in the rate of oxygen evolution in the middle of the light portion of the light/dark cycle, as was observed in control cells, the rate of oxygen evolution increased steadily throughout the day and only decreased after the cells were exposed to darkness. These results were interpreted to mean that in this population of cells, the biological clock no longer regulated the rate of oxygen evolution in the second half of the light cycle. The uncoupling of the oxygen evolution rhythm from the clock was mimicked in control cells by exposure to calmodulin antagonists (12). This observation suggested that calmodulin participated in the steps connecting the photosynthetic reactions to the biological clock and promoted a further examination of the role of calcium in the transducing steps. This report discusses evidence for the involvement of both extracellular and intracellular calcium in the coupling steps. Evidence was also found implicating both the IP₃¹ and diacylglycerol signaling systems in the coupling steps.

MATERIALS AND METHODS

Culture Conditions

Euglena gracilis, Klebs strain Z, was procured from the American Type Culture Collection (culture No. e12716, Rockville, MD) and grown as previously reported (11). Cultures were exposed to a 10 h light, 14 h dark cycle. The fluence rate at culture height was 300 μmol m⁻² s⁻¹ (20-W General Electric cool-white fluorescent tubes). The cell density used for the exposure of inhibitors was 7 × 10⁵ cells mL⁻¹. When the culture density was greater than this, cultures were diluted

¹ Abbreviations: IP₃, inositol-1,4,5-trisphosphate; PIP₂, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; PdBu, phorbol 12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; TMB-8, 8-diethylamino)octyl-3,4,5-trimethoxybenzoate.
with fresh growth medium, but cultures were never used if the density was not characteristic of an exponentially growing culture (less than 10⁷ cells mL⁻¹).

Experimental Design

Inhibitors were added at the time of transition from dark to light. Cells were removed from the culture and adjusted to a density of 7 × 10⁶ cells mL⁻¹. Inhibitors were added to culture tubes (25 × 150 mm) containing 20 mL of cells and aerated. The rate of whole cell oxygen evolution was measured each hour using a YSI oxygen monitor as previously described (10). Values plotted represent the mean of four to seven replicate experiments ± SD. Each experiment consisted of single hourly measurements.

Inhibitors

Stock solutions of most agents were made in DMSO and frozen in aliquots. The final concentration of DMSO in the culture medium was 0.1% (v/v). Nifedipine, verapamil, myo-inositol, PbBu, phorbol 13-monooacetate, PMA, acridine orange, and acridine-9-carboxylic acid were purchased from Sigma. scyllo-Inositol and TMB-8 were purchased from Calbiochem (San Diego, CA). CGP-28392 was a gift from Ciba-Geigy Corp. (Summit, NJ) and BAY-K 8644 was a gift from Miles laboratories, Inc. (New Haven, CT). UV-inactivated nifedipine was made as previously described (15). Each inhibitor was tested at 10, 50, 100, and 200 µM final concentration. The lowest concentration that caused the maximal observed response was used as the working concentration.

Chloroplast Isolation

Chloroplasts were isolated and the rate of whole chain electron flow was measured as previously described (14).

RESULTS

Effect of Ca²⁺ Channel Blockers on the Photosynthesis Rhythm

Figure 1A shows the pattern for oxygen evolution in untreated cells throughout the 10 h of light in a light/dark cycle. The rate steadily increased throughout the light portion of the cycle and reached a peak in the fifth hour. After that, the rate decreased. When cells were exposed to either 50 µM of the calcium channel antagonist nifedipine (Fig. 1B) (24) or verapamil (Fig. 1C) (9), the rate increased throughout the entire light portion of the cycle. The effect of nifedipine on the rate of oxygen evolution was reversed in three ways. When cells were exposed to the UV-inactivated isomer of nifedipine (Fig. 1D) (14), the cells displayed rhythmic activity in the rate of oxygen evolution. The effect of nifedipine was also reversed by simultaneous exposure of cells to 1 mM CaCl₂ (Fig. 1E), a treatment that has been reported to render the Ca²⁺ channels less sensitive to the channel blockers (9). Lastly, simultaneous exposure of cells to nifedipine and the nifedipine analog BAY-K 8644, which was reported to be a Ca²⁺ channel agonist that opens Ca²⁺ channels (1) reversed the effect of nifedipine (Fig. 1G). Bay-K 8644 alone had no effect on the rhythmicity (Fig. 1F) nor did the Ca²⁺ channel agonist CGP-28392 (data not shown). Exposure of cells to LaCl₃ (Fig. 1H), reported to be an antagonist of Ca²⁺ at the external side the plasma membrane (5, 17), caused the same response as exposure to Ca²⁺ channel blockers.

Effect of Altering Intracellular and Extracellular Ca²⁺

Exposure to the intracellular Ca²⁺ antagonist TMB-8 (16) also caused an uncoupling of the rhythm (Fig. 2A). TMB-8, by altering the level of intracellular Ca²⁺, may prevent the activation of calmodulin and thus uncouple the photosynthetic reactions from the clock (12). Calcium ionophores like A23187 have been used in many systems as a way to elevate intracellular Ca²⁺. Exposure of cells to 40 µM A23187 in the presence of 180 µM, the concentration of Ca²⁺ in the growth medium, had no effect on the expression of the rhythm (Fig. 2B) suggesting that either the ionophore did not cause Ca²⁺ entry in these cells, or that cells were able to compensate for the increased Ca²⁺ concentration by sequestering or removing the additional Ca²⁺. It is unlikely that the ionophore was inactive in these cells because simultaneous exposure of cells to LaCl₃ and A23187 reversed the effect of the La³⁺ (Fig. 2C).

Depriving cells of extracellular Ca²⁺ by exposure to EGTA (Fig. 2D) gave identical results to exposure to Ca²⁺ channel blockers, i.e. the oxygen evolution rhythm was uncoupled from the clock.

Involvement of the Inositol Phosphate Signaling System

The previously reported observation that Ca²⁺ and calmodulin were involved in coupling the photosynthetic light reactions to the biological clock (12) suggested that the inositol trisphosphate signaling system might also be involved in these steps. IP3 is hydrolyzed from the plasma membrane lipid phosphatidylinositol (2, 3). The IP3 acts as a signaling molecule causing Ca²⁺ to be released from intracellular stores. The free Ca²⁺ would then act as a ligand activating other molecules such as calmodulin. DAG, which remains in the membrane after the release of the IP3, has been reported to stimulate membrane-associated kinase C (2, 3). The IP3 and DAG signaling systems can both be perturbed in a number of ways. It is now possible to inhibit or activate several different steps in these signaling pathways, thus allowing their involvement in any process to be examined. Figure 3 is a summary diagram indicating the suspected site of action for the various classes of antagonists and agonists used in this study.

LiCl has been reported to inhibit one of the phosphatases converting IP3 to myo-inositol (25). myo-Inositol is used in the regeneration of phosphatidylinositol in the plasma membrane (3). Exposure of cells to 100 µM LiCl resulted in an uncoupling of the oxygen evolution rhythm from the biological clock (Fig. 4A) similar to that observed with the channel blockers. It has been reported that the effect(s) of LiCl can be reversed by the simultaneous exposure of cells to myo-inositol. The exogenous myo-inositol serves the cell as a source of inositol to replace that lost when the phosphatase is inhibited (26). When cells were exposed to both 100 µM myo-inositol and 100 µM LiCl, the rhythm in the rate of oxygen evolution was again observed (Fig. 4B), indicating a reversal of the Li⁺
effect in this system. myo-Inositol alone did not affect the expression of the rhythm (Fig. 4C). scyllo-Inositol is an isomer of myo-inositol that is not recognized by cells (26). When cells were exposed simultaneously to 100 μM scyllo-inositol and 100 μM LiCl, the blockage caused by the LiCl was not over-

Figure 1. Photosynthetic rate versus time in the presence of calcium channel agonists and antagonists. Cells were grown for 6 d in cycles of 10 h light, 14 h dark. The rate of oxygen evolution was measured each hour during the 10 h light portion of the cycle. Values plotted are the means of four to seven replicate experiments ± so. Chemical agents were added at the onset of light. A, Control containing 0.1% DMSO (v/v); B, 50 μM nifedipine; C, 50 μM verapamil; D, 50 μM UV-inactivated nifedipine; E, 1 mM CaCl₂ and 50 μM nifedipine; F, 50 μM BAY-K 8644; G, 50 μM BAY-K 8644 and 50 μM nifedipine; H, 200 μM LaCl₃.

Involvement of the DAG Signaling System

It has been reported that phorbol esters can mimic the action of DAG by activating or stimulating membrane-associated kinase C. The oxygen evolution rhythm was not affected by the presence of 50 or 100 μM phorbol 12-myristate 13-acetate (PMA, Fig. 4G), or phorbol 12,13 dibutyrate (PdBu, Fig. 3H), both reported to activate kinase C (6). Phorbol 13-monoacetate, an inactive phorbol, was used as a control and also had no effect on the coupling of the oxygen evolution rhythms to the clock (Fig. 4I). The lack of an effect after exposure to the phorbol esters can be interpreted in two ways. First, it could mean that activation of kinase C is not part of the coupling steps connecting this rhythm to the biological clock. Alternatively, if the coupling of the light reactions to the biological clock involves activation of kinase C, and kinase C is already activated, then phorbol esters would have little effect on the system. Evidence for the involvement of kinase C was obtained first by exposing cells to 10 μM acridine orange, which has been reported to be an inhibitor of kinase C (8). The rhythm in oxygen evolution was completely inhibited by acridine orange (Fig. 4I), but was not inhibited by 10 μM acridine-9 carboxylic acid, an analog of acridine orange which does not inhibit kinase C (Fig. 4K). The effect on the cells of acridine orange was different from the typical pattern of uncoupling in which the rate of oxygen evolution steadily increased throughout the light. Instead, the rate of oxygen evolution was relatively constant throughout the light portion of the cycle at a rate approximately fourfold lower than observed with other chemical agents. The possibility that the acridine orange was inhibiting one or more
steps in the light reactions, instead of affecting the coupling steps, was examined by exposing isolated chloroplasts to the inhibitor. The rate of whole-chain electron flow in both whole cells and isolated chloroplasts has been shown to be rhythmic (14). Therefore, the effect of acridine orange on the rate of whole chain electron flow (H₂O to methyl viologen) was measured at the beginning and middle of the light portion of the cycle. Table I indicates that the rate of electron flow in the presence of 10 μM acridine orange was not different from the control rate (Student t-test, 95% confidence limits) at either time of the cycle. Acridine orange contains a tertiary amine which can act as a weak base. Acridine orange should enter the Euglena cells in the unpronated form at pH 6.5, the pH of the growth medium. It is unlikely that the uncoupling of the light reactions is the result of pH change caused by the presence of acridine orange. It has been previously reported that 8 mM NH₄Cl, which enters the Euglena cells and acts as a base, does not disrupt rhythmic expression of the light reactions (14). Therefore, the lack of rhythmicity in the presence of acridine orange cannot be attributed to an inhibition
of the light reactions. Instead, it appears that activation of kinase C might also play a role in the transducing steps for this rhythm. The effect of acridine orange was reversed when cells were first exposed to 50 μM of the phorbol ester PMA (Fig. 4L).

**DISCUSSION**

The role of Ca^{2+} in the functioning of the biological clock is not well characterized. Calmodulin inhibitors caused phase shifts of the clock-controlled conidiation rhythm of *Neurospora*, as did various other agents that affected calcium concentration, such as EGTA (20, 21). In *E. gracilis*, exposure of cells to calmodulin inhibitors resulted in phase shifts of the cell division rhythm, indicating a direct effect on the timing mechanism of the clock (7). Inhibitors of calmodulin did not phase shift the *Euglena* photosynthesis rhythm, but did uncouple the photosynthetic reactions from the precise timing control dictated by the biological clock (12). The lack of phase shifting by the calmodulin inhibitors suggested that there may be more than one biological clock in *Euglena*, with a calmodulin-dependent clock controlling the process of cell division, and a second calmodulin-independent clock controlling the photosynthetic light reactions. While the clock controlling the light reactions does not use calmodulin directly in the clock steps, calmodulin is involved in the transducing steps. It is the long-range goal of this research to identify the steps coupling the biological clock to the photosynthetic light reactions. From previous work (12), it appeared that calmodulin, and therefore Ca^{2+}, were part of the transducing steps. The question then became what was the source of the calcium used? Two possibilities were that (a) extracellular calcium was required and entered the cell either by a Ca^{2+} transport system or by a Ca^{2+} channel or (b) Ca^{2+} was released from intracellular stores such as the endoplasmic reticulum.

From the results presented in Figures 1, 2, and 4, some preliminary statements can be made concerning the Ca^{2+}-dependent steps involved in the transducing mechanism. As previously reported, the light reactions were uncoupled from the clock when cells were exposed to the calmodulin inhibitors trifluoperazine or W-7, but were not uncoupled when W-5, an isomer of W-7 with little affinity for calmodulin, was used (12). It can now be reported that for expression of the clock-controlled rhythm in oxygen evolution, two calcium-related situations must be met. First, extracellular calcium must be present. Chelation of the Ca^{2+} with EGTA or displacement with La^{3+} appeared to uncouple the light reactions from the clock. Resuspension of cells in Ca^{2+}-free medium did not affect the expression of the photosynthesis rhythm (data not shown). Such resuspension should not, however, remove Ca^{2+} already bound to the surface of the membrane as EGTA or La^{3+} would be expected to do. When the Ca^{2+} channels were blocked with nifedipine or verapamil, the rhythmic control of the light reactions was altered, suggesting that extracellular Ca^{2+} was required for the coupling of these reactions to the clock. Elevating the Ca^{2+} level, either through the use of a Ca^{2+} channel agonist, such as BAY-K 8644 or a Ca^{2+} ionophore like A23187, did not disrupt the clock control over photosynthesis. Thus, while Ca^{2+} deprivation uncoupled the light reactions from the clock, elevation of intracellular Ca^{2+} did not. The cells may be capable of sequestering or binding extra Ca^{2+} resulting from either of these treatments.

In addition to the requirement for extracellular Ca^{2+}, when the IP_{3} branch of the phosphoinositid signaling pathway was perturbed, the rhythm was uncoupled suggesting that Ca^{2+} from intracellular stores was also a requirement for the coupling of the rhythm to the clock. Both neomycin and LiCl, which block the IP_{3} pathway at different steps, caused the same type of response as did exposure of cells to the intracellular Ca^{2+} antagonist TMB-8. The LiCl response was reversed by simultaneous exposure to myo-inositol. LiCl has been reported to inhibit IP phosphatase, thus inhibiting the regeneration of inositol from IP. Simultaneous exposure to myo-inositol would be expected to override the inhibition caused by Li^{+}.

The coupling of the light reactions to the biological clock may involve the IP_{3}/Ca^{2+} signaling system. The leaflet movement rhythm reported for *Samanea saman* is phase shifted by pulses of red and blue light (23). It has also been reported that the turnover of inositolphospholipid was light-stimulated in *Samanea* (19). The observation that the IP_{3} system was light sensitive suggests that this signaling system could either be part of the clock mechanism or could be part of the transducing steps linking the clock to the environmental light signals used to set the timing of the clock. Whether there are two or more separate roles for the IP_{3} system in coordinating clock control over rhythmic processes in the cell will be determined in future studies.

The possible involvement of the DAG branch of the signaling system, in addition to the IP_{3} branch, indicates the complex nature of the transducing steps. Inhibition of the membrane-associated protein kinase C with acridine orange resulted in an uncoupling of the oxygen evolution rhythm from the clock, implying that one or more of the transducing steps may be dependent on phosphorylation.

The general response of cells when exposed to chemical agents that alter the availability of extra- and intracellular Ca^{2+}, or alter the IP_{3} or DAG pathways, was a continuous increase in the rate of O_{2} evolution throughout the light portion of the cycle. These observations were interpreted as representing an uncoupling of the photosynthetic light reactions from the biological clock. While there are several pos-
sible alternative interpretations, uncoupling was chosen for the following reasons. It is unlikely that the uncoupling pattern shown in Figures 1, 2, and 4 represent either a delay in the phase of the rhythm or a transient inhibition in the expression of the rhythm. Antagonists that cause uncoupling, such as nifedipine (Fig. 1B) or LiCl (Fig. 4A), do not exert their effect when cells were exposed to the appropriate agonist (Fig. 1, E and G; Fig. 4B). Each antagonist and agonist used in combination had different sites of action. The establishment of the expected response in the presence of an antagonist and agonist indicated that the clock itself was still operating and maintaining a correct phase reference, i.e. the clock was not phase shifted or inhibited. Second, two extensive studies have been reported on the uncoupling of the light reactions from the biological clock. The presence of two different calmodulin antagonists caused the same response, i.e. a continuous increase in the rate of O2 evolution throughout the light portion of the light/dark cycle (12). When cells were followed for four consecutive days, the normal rhythm in O2 evolution was not observed (12), which argues against a phase delay or a transient inhibition in the expression of the rhythm. In addition, a naturally occurring population of *Euglena gracilis* was isolated in which the photosynthetic reactions were not controlled by or coupled to the biological clock but were, instead, influenced only by environmental parameters (13). The pattern of O2 evolution versus time was identical to the pattern in the presence of Ca2+ antagonists. Collectively, these observations strongly suggest that perturbation of Ca2+ levels or disruption of the IP3/DAG pathways uncouples the light reactions from the biological clock.

Current examination of the cellular shape change rhythm in *Euglena* (11) has indicated that the IP3 system may also be part of the coupling steps linking that rhythm to the biological clock (our unpublished results). While the specific order of coupling steps has not yet been identified, it is now clear that closer examination of the IP3 and DAG signaling systems may reveal the nature of the transducing process.

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LITERATURE CITED