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Delay Model of the Circadian Pacemaker

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We present a simple and realistic model of the circadian pacemaker that can be interpreted in molecular terms. The model, which consists of a single time-delay differential equation, simulates the expression of a generic clock protein that inhibits its own expression through a feedback mechanism. Despite its simplicity, this model fulfils most of the necessary characteristics of a realistic representation of natural circadian clocks: robust and stable oscillations with circadian free-running periods, typical phase response curves and entrainment to environmental zeitgebers. The present model reduces the molecular mechanism necessary to sustain stable oscillations to its bare bones, suggesting that the essential factor is the time-delayed negative feedback of the oscillating protein on its own expression.

Introduction

Circadian rhythms are generated by biological clocks that seem to be ubiquitous in nature, ranging from periodic biochemical reactions in unicellular organisms to complex structures in the mammalian brain. One of the present goals of chronobiology is to understand which components of the circadian clock are necessary to generate and/or maintain overt rhythmicity and entrainment to environmental synchronizers (zeitgebers).

The purpose of this work is to develop a model of the circadian pacemaker that is simple, general, interpretable in molecular terms and biologically realistic. By "simple", we understand that it involves as few parameters as possible. "General" means that it is useful to understand the minimal requirements of a molecular clock, regardless of the particular system under study. Despite the simplicity requirement, we aim at a model that can be interpreted in molecular terms. Finally, we look for a biologically realistic model: one that reproduces experimental observations. In particular, we will study free-running oscillations, phase response curves, pulse entrainment and photoperiodic entrainment (Pittendrigh, 1981).

Recent research has helped to identify putative molecular mechanisms responsible for circadian oscillations in cyanobacteria (Golden *et al.*, 1998; Ishiura *et al.*, 1998), fungi (Crosthwaite *et al.*, 1997; Merrow *et al.*, 1997; Aronson *et al.*, 1994), flies (Darlington *et al.*, 1998) and mammals (Sangoram *et al.*, 1998; Gekakis *et al.*, 1998), among others. It is very significant that all these circadian systems share a general mechanism by which a (clock) gene might directly or indirectly regulate its own expression by means of a feedback loop (Dunlap, 1999).

Several mathematical and physical models have been proposed over the years in order to explain different features of circadian systems in general (Jewett and Kronauer, 1998; Nunes,

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1998) and of specific organisms in particular (Benedito-Silva, 1997; Pavlidis, 1981; Leloup and Goldbeter, 1998). Recently, a general delay model based on a molecular mechanism has been reported (Scheper *et al.*, 1999a, b). This model, which consists of two coupled differential equations, is based on the kinetics of synthesis and degradation of a clock protein and of its messenger RNA.

In this paper, we propose a simple time-delay model that consists of a single delay differential equation. Our model takes into account the expression of a clock protein, its degradation, and a time-delayed inhibition of this protein on its own expression. Furthermore, we have modelled different kinds of coupling to the environment through time-dependent variations of the relevant parameters of the model.

Materials and Methods

THE CLOCK

To develop our model, we start by assuming that the basic necessary steps of a molecular clock are the expression and degradation of a clock protein, together with a negative feedback of this protein on its own synthesis. Also, we assume that there is a significant timedelay between the beginning of transcription and the final effect of retro-inhibition. A detailed description of the multiple reactions involved is particular to each biological system; however, it is relevant to develop simple unifying models that include only the basic factors necessary for sustained circadian oscillations in biosystems.

Taking the previous considerations into account, we propose the following model for the molecular clock:

$$\frac{\mathrm{d}E(t)}{\mathrm{d}t} = K_e\, G(t-\delta) - K_d\, E(t), \qquad (1)$$

$$G(t-\delta) = \frac{1}{1+[E(t-\delta)/K_i]^n}, \qquad (2)$$

where t is the time, E represents the level of the mature clock protein and G stands for the level of activation of the gene. The first term in eqn (1) represents the overall kinetics of expression. K_e is the expression rate constant and δ represents a time delay. This term accounts for every reaction from the very beginning of the mRNA synthesis to the mature protein. The time delay means that the expression rate at a certain time is related to the degree of activation of the gene at a previous time, because of the delay imposed by the cumulative steps of transcription, translation, and other events like post translational modifications, transport through membranes, etc. In turn, G is related to the level of mature protein through eqn (2). This accounts for the nonlinear negative feedback of the mature protein on its own production. In this expression, K_i is the inhibition rate constant, and n is the Hill coefficient of inhibition. The second term of eqn (1) takes into account the degradation of the clock protein, K_d being the degradation rate constant.

RUNNING THE CLOCK

In order to integrate eqn (1) we need to establish the initial conditions; $E(t=0)$ and $G(t-\delta)$ in the range $0 < t < \delta$. We used $E(t=0) = 0$ and a constant value of $G(t-\delta) = 0$ in $(0, \delta)$, except when stated otherwise. The model was solved by numerical integration. All runs were performed using a fourth-order Runge–Kutta algorithm, with a 0.1 hr fixed step. The most relevant results were confirmed with a step of size 0.01 hr.

In most cases, we found that the system reached a stable oscillation regime in a few cycles. In order to determine the period of these oscillations, we discarded the first 200 h of simulation, in order to make sure that the transient phase was over. Periods were determined by means of periodogram estimation based on a standard fast Fourier transform algorithm. We considered the oscillatory output to be stable when the periodogram consisted of a single well-defined peak, after 200 h of integration.

COUPLING TO THE ENVIRONMENT

It has been shown that the environment might affect circadian oscillators through modulation of the synthesis and/or degradation of key molecular species (Dunlap, 1999; Hunter-Ensor *et al.*, 1996; Crosthwaite *et al.*, 1995, Albrecht *et al.*, 1997; Shigeyoshi *et al.*, 1997; Blasius *et al.*, 1999). Therefore, we chose to study the response of the system to time-dependent perturbations of the parameters K_e or K_d, which represent rate constants of expression and degradation, respectively.

PHASE RESPONSE CURVES (PRCs)

To study the effect of single-pulse perturbations to the clock, we obtained PRCs, as follows. First, the clock was run for 200 hr, to make sure that it reached the stable oscillatory regime. Then, a 1-hr pulse perturbation was applied. We considered four different possibilities: (1) stimulation of expression, by multiplying K_e by 10 for the duration of the pulse; (2) inhibition of expression, by reducing K_e to zero; (3) stimulation of degradation, by multiplying K_d by 10; (4) inhibition of degradation, by reducing K_d to zero. After the pulse, the model was run for another 100 hr. Finally, we calculated the phase shift from the difference in the maxima of E cycles (acrophases) between the perturbed system and a control one. This procedure was repeated 24 times by increasing in one circadian hour the time of application of the pulse. One circadian hour is defined as the free-running period divided by 24.

PULSE ENTRAINMENT

Pulse (i.e. non-parametric) entrainment was modelled with the same pulses used for PRC curves, by repeating them periodically. The system was considered entrained when it reached stable oscillations with a period that was equal to that of the perturbation. The details of the numerical simulations are the same as for the free-running oscillator, already described.

PHOTOPERIODIC ENTRAINMENT

Photoperiodic (i.e. parametric) entrainment was modelled as a stimulation of the expression by adding to K_e the positive half of a sinusoidal function of time. This resembles the natural variations in daylight cycles (see e.g., Fig. 9 in DeCoursey, 1989). The amplitude of the sinusoidal function was 9 times K_e, such that the maximum K_e was equal to that of the pulse mode of entrainment. The perturbed clock was run in the same way as the unperturbed one, and entrainment was assessed as in the pulse mode case described above.

Results

FREE RUNNING SYSTEM

In Fig. 1(a), we show the time dependency of the clock protein level, $E(t)$, for the parameter values of Table 1. It is easy to see that the system shows stable oscillations with a circadian period of 22.9 hr. Variations of the parameters around the values of Table 1 result in variations of the period around 24 hr. Figure 1(b) shows alternative plots of the very robust limit cycle to which the clock converges after a relatively short transient period. Fixed points of eqn (1) (i.e. E values such that $dE/dt = 0$) are easily obtained by equating eqn (1) to zero. These fixed points showed to be very unstable, with a 1% or even smaller perturbations on the value of E or G being enough to disturb the system and drive it to a very robust oscillating regime (data not shown). Furthermore, these oscillations were very robust with respect to variations on the initial conditions. We ran simulations with different initial conditions (values of $E(t = 0)$ and $G(t - \delta)$ for $0 \leqslant t \leqslant \delta$) and found that, for the same parameter values, only the phase of the oscillations change.

The period and amplitude of the oscillations of $E(t)$ depend on the clock parameters. In Table 2, we show the sensitivity of period and amplitude to small parameter variations around the values of Table 1. It is clear from this table that the time delay δ is the most important parameter of the model, regarding the determination of the period; small variations of δ result in large variations of the period. Table 2 shows that the amplitude is in general more sensitive than the period, and the effects of the five parameters of the model are comparable.

A more complete picture of the effects of parameter variation can be found in Fig. 2. In this figure, we show the effects of varying each of the parameters at a time, while keeping the others at the values of Table 1. Figure 2 shows that there is a minimal threshold value of δ necessary to produce oscillations. As from this threshold value, the amplitude of the oscillation rises and reaches a saturation value, but the period increases with an almost linear trend. This reflects the high dependency of the period on the time-delay of expression. The period was found not be very sensitive to K_e, although changes in this parameter significantly affected the amplitude of the oscillation. There is a minimal K_e needed in order to sustain oscillations, and from there on, there is an exponential increase of the amplitude. The

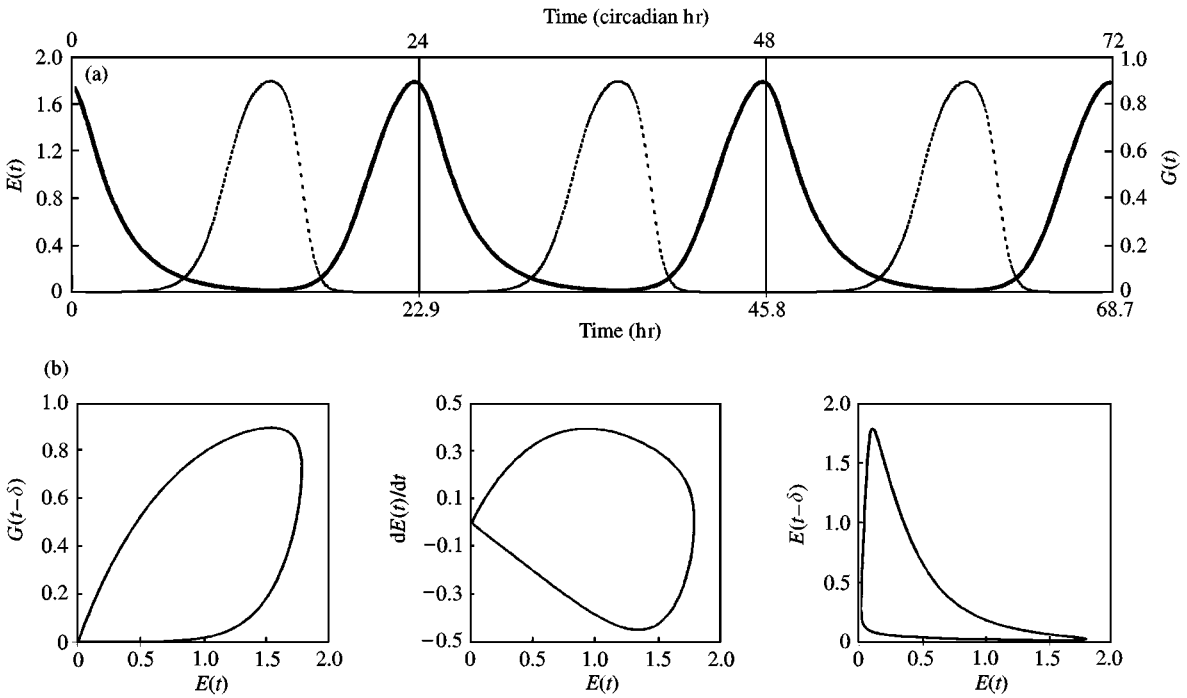

FIG. 1. (a) Free-running oscillation of the model with the parameter values of Table 1. Protein level $E(t)$ (——) and activated gene level $G(t)$ (- - - -) are shown. The free-running period is 22.9 hr (bottom axis), which, by definition, corresponds to 24 circadian hr (top axis). (b) Three different ways to display the robust limit cycle.

TABLE 1

Model Parameters. Parameter values used for the basic simulation of the circadian system. See text for definitions

Parameter	Value
K_d	0.4
K_e	1
K_i	0.04
n	2.5
δ	8

TABLE 2

Sensitivity to parameter variations

Parameter	Period sensitivity	Amplitude sensitivity
K_d	− 0.24	0.46
K_e	0.05	0.87
K_i	− 0.05	0.14
n	0.18	0.99
δ	0.83	1.27

Sensitivities of the period and amplitude of the stable oscillations to changes in each of the parameters around the values of Table 1. For a given variable, x, and parameter, p, the sensitivity is defined as $S = p/x\ \mathrm{d}x/\mathrm{d}p = \mathrm{d}\ln(x)/\mathrm{d}\ln(p)$. Defined in this way, S is a dimensionless quantity and, therefore, it can be used to compare the effects of different parameters. Note that a positive (negative) sensitivity means that the variable increases (decreases) with an increase of the parameter.

period is not very sensitive to changes in K_i. It is somewhat more sensitive to changes in K_d. The amplitude was found to be quite sensitive for these two parameters, with a definite range where significant oscillations can occur. As for n, we found a minimal threshold for sustained oscillations, as from which the amplitude increases up to a saturation value. The period is not very sensitive to this parameter. This shows that a Hill coefficient $n > 1$ is required in order to produce oscillations, which can be interpreted in terms of interaction (cooperative binding) between the E monomers.

The simplicity of our model allowed us to perform a linear stability analysis around the

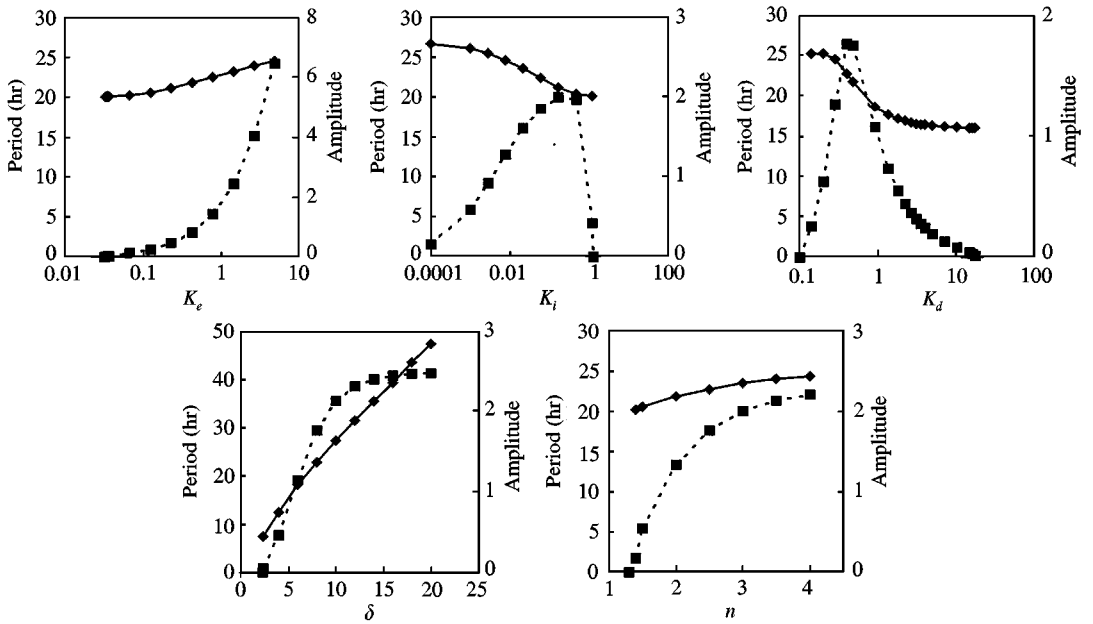

FIG. 2. Effects of changing the parameters on the period (——) and amplitude (- - -) of $E(t)$ oscillations. Parameters were changed one at a time while keeping the others at the values of Table 1.

TABLE 3
Linear Stability Analysis

Parameter	Condition
K_d	> 0.11
K_e	> 0.03
K_i	< 1.21
n	> 1.39
δ	> 2.33

For each parameter, the condition required for sustained oscillations was obtained by performing a linear stability analysis. One parameter is changed at a time, while the others are kept constant at the values of Table 1. Compare with the numerical results shown in Fig. 2.

fixed stationary point of eqn (1) (Mackey, 1997; Murray, 1993). Such an analysis allows the calculation of the region of parameter space where the fixed point becomes unstable. In Table 3, we show the critical values of the parameters, obtained using Mackey's equation (Mackey, 1997)

$$\tau = \frac{\cos^{-1}[\gamma/S]}{\sqrt{S^2 - \gamma^2}},$$

where, in terms of the parameters of our model.

$$\tau = K_d\,\delta,$$

$$\gamma = 1,$$

$$S = \frac{-n}{1 + [K_i/E_{ss}]^n},$$

with E_{ss} being the steady-state value of the E variable at the fixed point. As expected, the results of Table 3 are in perfect agreement with the numerical simulations shown in Fig. 2.

PHASE RESPONSE CURVES

In Fig. 3(a), we show the PRCs for stimulation and inhibition of K_e, along with the relevant variable $G(t - \delta)$ [see eqn (1)]. We set the origin of circadian time ($CT = 0$) at the maximum of $G(t - \delta)$. Note that the PRCs show a definite dead zone, between $CT = 6$ and 12. The early part of the subjective night (defined as $12 < CT < 24$) exhibits phase delays in response to the stimulus, while the late part shows phase advances. Inhibition shows opposite effects.

In Fig. 3(b), we show the PRCs for stimulation and inhibition of K_d, along with $E(t)$ [see eqn (1)].

We set $CT = 0$ at the maximum of $E(t)$. These PRCs show roughly the same properties as the K_e ones of Fig. 3(a). In contrast to K_e PRCs, however, K_d PRCs show zones of low response, rather than absolute dead zones.

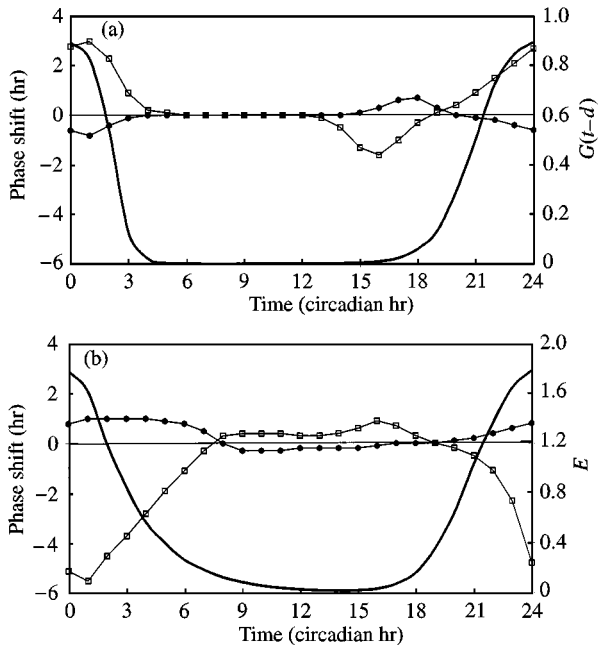

FIG. 3. Phase response curves (PRCs). (a) Stimulation (□-□-□) and inhibition (●-●-●) of K_e. The origin of the circadian day was arbitrarily placed at the maximum of $G(t-\delta)$ (——). (b) Stimulation (□-□-□) and inhibition (●-●-●) of K_d. The origin of the circadian day was arbitrarily placed at the maximum of $E(t)$ (——).

PULSE ENTRAINMENT

In Fig. 4, we show the effects of periodic pulse perturbations on the model clock. Figure 4(a) shows a representative simulation of the effect of periodic light pulses, modelled as pulse stimulations of K_e. It is clear that periodic K_e stimulations induce a steady entrainment of the simulated pacemaker after a few entraining cycles (data not shown). The same behaviour is found for the other three ways of modelling the periodic light pulses (i.e. inhibition of K_e, stimulation and inhibition of K_d). Figure 4(b) shows the range of entrainment corresponding to the four conditions tested. It should be noted that the range of entrainment for K_e stimulation resembles that of photic synchronization in nature.

PHOTOPERIODIC ENTRAINMENT

Since K_e stimulations seem to be the most representative of pulse entrainment, we chose to use this parameter to simulate parametric synchronization. Figure 5(a) shows a representative simulation of entrainment to photoperiodic stimulations of K_e. Note the phase-locked relationship between the zeitgeber cycle, $K_e(t)$, and the entrained $E(t)$ cycle. It should be noted that the amplitude of the oscillation in E yields similar values to the one found for pulse entrainment. In Fig. 5(b), we show the range of entrainment. This is similar to that found for pulse

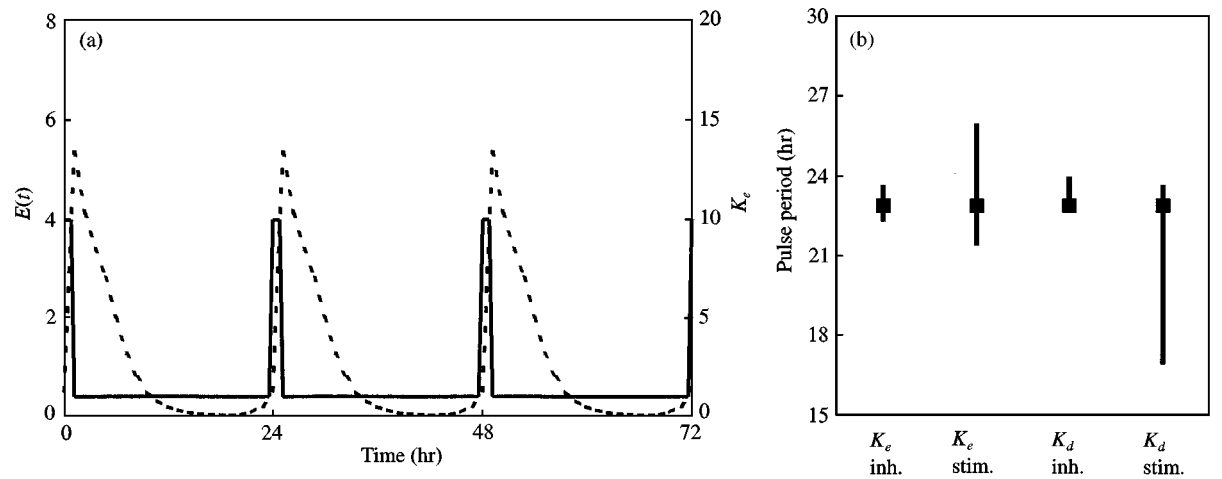

FIG. 4. Pulse entrainment. (a) Representative time plot showing a periodic pulse stimulation of K_e (——) and its effect on $E(t)$ (- - -). (b) Range of entrainment for the four different ways used to simulate the perturbation. The free-running period is also shown (■-■-■).

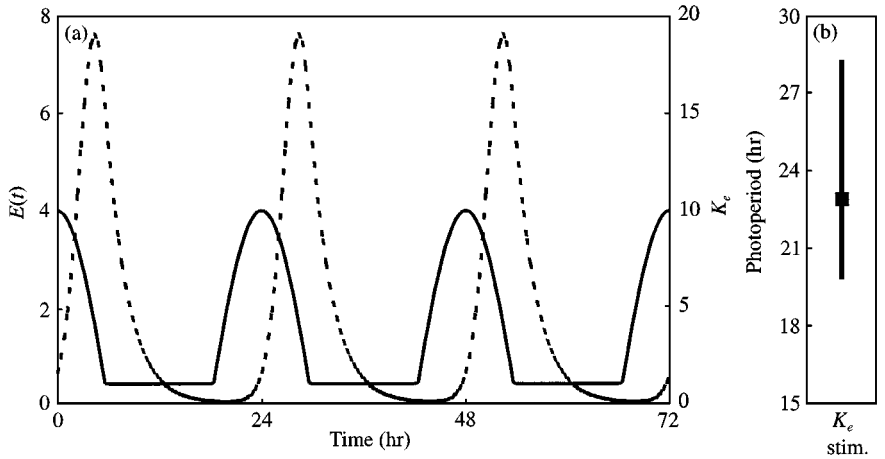

FIG. 5. Photoperiodic entrainment. (a) Representative time plot showing semi-sinusoidal increases of K_e (——) and the resulting entrained $E(t)$ (- - -). (b) Range of entrainment for semi-sinusoidal K_e stimulation. The free-running period is also shown (■–■–■).

entrainment, as can be seen by comparing Fig. 5(a) with Fig. 4(a).

Discussion

We have presented a model of a molecular circadian pacemaker that consists of a single delay differential equation. The model is inspired by known molecular mechanisms, and it includes the expression and degradation of a clock protein that inhibits its own expression. We have performed extensive numerical simulation using this model and we found that it fulfils most of the necessary characteristics of a realistic representation of natural circadian clocks (Pittendrigh & Daan, 1976). It shows robust and stable cycles under constant conditions with a circadian period (Fig. 1). In addition, with appropriate parameters, 24-hr rhythms are easy to achieve (Fig. 2). Moreover, these free-running rhythms can be entrained to external cycles by either daily pulses or complete periodic zeitgebers (Figs 4 and 5, respectively). The basis of pulse entrainment can be found in the phase response curves of the oscillator, which we have modelled as pulse perturbations of the pacemaker, by means of stimulation or inhibition of relevant parameters (Fig. 3). PRCs can be obtained that resemble those found in response to light for most circadian behaviours.

Current evidence suggests that the core of the molecular clock consists of negative feedback loops by which a protein inhibits, directly or indirectly, its own synthesis (Dunlap, 1999). Several clock components have been identified in different biological systems whose dynamics might fit into the general scheme of our model. The general assumption for this model is that clock genes will feedback negatively into their own expression mechanism, such as was found for Neurospora (Crosthwaite *et al.*, 1997; Merrow *et al.*, 1997; Aronson *et al.*, 1994), Drosophila (Darlington *et al.*, 1998), and mice (Sangoram *et al.*, 1998; Gekakis *et al.*, 1998). Although our model deals only with the simple case in which the same gene represses its own induction, more elements may take part in this process. In Drosophila, for example, a Per–Tim complex, which is induced by the expression of Clock, represses Clock induction by a transcriptional feedback mechanism, homologous to the one that appears to be functional in mice. In fungi, the frq gene, whose expression is tightly regulated by wc-1 and wc-2, plays these roles.

According to the present model, the retro-inhibition must occur with a delay, in order to ensure that a circadian period is achieved. This delay turns out to be the single most important parameter of the model (see Fig. 2). In nature, there might be many ways of obtaining the necessary

delay. For example, it is possible that before the gene inhibition factors enter the nucleus, they might need to accumulate in the cytoplasm beyond a certain threshold. In addition, relevant proteins might need to be activated in order to act as transcriptional regulators. This activation might occur by several mechanisms, including homo- or heterodimerization (e.g. Per–Tim interactions), and phosphorylation mechanisms, such as Per in Drosophila (Edery *et al*., 1994).

Let us now consider the coupling response of the clock to the environment. We modelled clock–environment coupling in four different ways: stimulation or inhibition of either expression (K_e) or degradation (K_d). Single pulses were applied to obtain model PRCs that include advances, delays and dead zones, resembling what is usually found in biological systems (Pittendrigh, 1981). To model non-parametric and parametric modes of entrainment, we applied periodic pulses and smooth periodic perturbations, respectively. Entrainment occurs within a limited range around the circadian period of the free-running model. Outside this range of entrainment, we found no synchronization.

Of the four ways that we used to perturb our model oscillator, stimulation of K_e seems to be the one that more closely resembles experimental observations. It has been found that frq levels in Neurospora and Per levels in Drosophila and mice change after light pulses that cause phase shifts in overt circadian rhythms (Albrecht *et al*., 1997; Crosthwaite *et al*., 1995; Lee *et al*., 1996; Shigeyoshi *et al*., 1997). This is consistent with the PRCs obtained with our model when we applied pulse stimulations to K_e (Fig. 3). The ranges of entrainment for all situations tested (Figs 4 and 5) seem to fit with the normal values found in the literature, especially for K_e stimulation. Interestingly, the range of entrainment was found to be slightly asymmetrical around the free running circadian period of the system, which can be predicted from the corresponding PRCs. Even though in the present simulations K_e stimulations seem to be the preferred coupling method, we should note that any of the other ways we considered can also be changed in order to generate good simulations of natural PRCs (data not shown). The nature of the changes in the parameters and their relationship to specific biochemical reactions remains to be solved.

Let us now compare our model with another closely related recent model (Scheper *et al*., 1999a, b). In contrast with ours, their model takes explicitly into account the kinetics of mRNA synthesis and degradation. As a result, they have two coupled delay differential equations. In our model, we only consider protein synthesis and degradation, together with a time-delayed inhibition of the clock protein. This leads to a single delay differential equation, with fewer parameters, which makes the mathematical and computational study, as well as its interpretation, much simpler. It can be regarded that mRNA synthesis and degradation steps are included implicitly in our model, together with any intermediate step of the protein maturation sequence. It is interesting to note that whereas we needed an 8 hr delay to obtain circadian periods, in Scheper's model the delay was of about 4 hr. The reason for this is that we are adding the mRNA-related steps to the mechanisms responsible for the delay, rather than considering them apart.

We would like to note that it would be very simple and straightforward to model a system with a second protein whose expression is under the control of the level of the clock protein, E. This kind of situation has been found in real circadian systems such as the control of a vasopressin output from the mammalian circadian clock (Jin *et al*., 1999). Obviously, this consideration would increase the number of parameters to be included, and would unnecessarily complicate the model.

To summarize, we have presented a simple and realistic model of the circadian pacemaker that can be interpreted in molecular terms. Despite its simplicity, this model fulfils most of the necessary characteristics of a realistic representation of natural circadian clocks: circadian free-running periods, typical phase response curves, and entrainment to environmental zeitgebers. The present model reduces the molecular mechanism necessary to sustain stable oscillations to its bare bones, suggesting that the essential factor is the time-delayed negative feedback of the oscillating protein on its own expression.

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