



## Activation of the Liver Glycogen Phosphorylase by $\text{Ca}^{2+}$ Oscillations: a Theoretical Study

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Cytosolic calcium plays a crucial role as a second messenger in cellular signalling. Various cell types, including hepatocytes, display  $\text{Ca}^{2+}$  oscillations when stimulated by an extracellular signal. However, the biological relevance of this temporal organization remains unclear. In this paper, we investigate theoretically the effect of  $\text{Ca}^{2+}$  oscillations on a particular example of cell regulation: the phosphorylation–dephosphorylation cycle controlling the activation of glycogen phosphorylase in hepatocytes. By modelling periodic sinusoidal variations in the intracellular  $\text{Ca}^{2+}$  concentration, we show that  $\text{Ca}^{2+}$  oscillations reduce the threshold for the activation of the enzyme. Furthermore, as the activation of a given enzyme depends on the kinetics of its phosphorylation–dephosphorylation cycle, specificity can be encoded by the oscillation frequency. Finally, using a model for signal-induced  $\text{Ca}^{2+}$  oscillations based on  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, we show that realistic  $\text{Ca}^{2+}$  oscillations can potentiate the response to a hormonal stimulation. These results indicate that  $\text{Ca}^{2+}$  oscillations in hepatocytes could contribute to increase the efficiency and specificity of cellular signalling, as shown experimentally for gene expression in lymphocytes (Dolmetsch *et al.*, 1998).

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### Introduction

Intracellular calcium is a primary regulator in a variety of cell functions. In several cell types,  $\text{Ca}^{2+}$  signalling displays a specific spatiotemporal pattern. When oscillations of cytosolic  $\text{Ca}^{2+}$  occur, they are often associated with the propagation of  $\text{Ca}^{2+}$  waves within the cytosol (Berridge, 1997). In this paper, we focus on the possible implication of the temporal pattern of  $\text{Ca}^{2+}$  signalling for the physiological response of the cell. Spikes or oscillations of cytosolic  $\text{Ca}^{2+}$  appear either spontaneously or after stimulation by hormones or neurotransmitters. The regula-

tion of  $\text{Ca}^{2+}$ -dependent mechanisms could be encoded in such temporal patterns. Interestingly enough, in nearly all cell types the frequency of  $\text{Ca}^{2+}$  oscillations increases with the level of stimulation (Woods *et al.*, 1986; Berridge, 1997) suggesting that the level of stimulation could be encoded in the frequency of  $\text{Ca}^{2+}$  oscillations. The question thus arises as to how the  $\text{Ca}^{2+}$ -dependent mechanisms could be sensitive to the frequency of  $\text{Ca}^{2+}$  oscillations.

In this context, the possibility of frequency encoding by  $\text{Ca}^{2+}$  oscillations has been investigated theoretically in a few prototypic systems. It has been shown that protein phosphorylation through a  $\text{Ca}^{2+}$ -activated kinase provides a plausible mechanism for the encoding of external

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stimulation in terms of the frequency of signal-induced  $\text{Ca}^{2+}$  oscillations (Goldbeter *et al.*, 1990; Dupont & Goldbeter, 1992). Such a frequency encoding, reflected by the fact that the average level of phosphorylated protein increases with the frequency of repetitive  $\text{Ca}^{2+}$  spikes, was, however, shown to require precise kinetic conditions. In a similar manner, Meyer & Stryer (1991) have shown that the  $\text{Ca}^{2+}$ -activated sequential phosphorylation of a protein can behave as a "spike counter", providing a digital encoder of  $\text{Ca}^{2+}$  oscillations.

Moreover, recent experimental findings strengthen the hypothesis of a functional role for  $\text{Ca}^{2+}$  oscillations. Indeed, a molecular mechanism underlying the decoding of the information mediated by high-frequency  $\text{Ca}^{2+}$  oscillations has been established in the particular case of  $\text{Ca}^{2+}$ /calmodulin kinase II (CaM kinase II). By immobilizing the CaM kinase II and subjecting it to pulses of  $\text{Ca}^{2+}$  of variable amplitude and frequency, De Koninck & Schulman (1998) have shown that the autonomous activity of CaM kinase II is highly sensitive to the temporal pattern of  $\text{Ca}^{2+}$  spikes, as predicted by theoretical models (Hanson *et al.*, 1994; Michelson & Schulman, 1994; Dosemeci & Albers, 1996; Prank *et al.*, 1998). In addition, it has been demonstrated that, on a much slower time-scale,  $\text{Ca}^{2+}$  oscillations increase the efficiency and specificity of gene expression. It was shown indeed that oscillations can reduce the effective  $\text{Ca}^{2+}$  threshold and that the expression of various transcription factors can be selectively stimulated by appropriate frequencies of  $\text{Ca}^{2+}$  oscillations (Dolmetsch *et al.*, 1998; Llopis *et al.*, 1998).

Here, we extend a previous, rather abstract, investigation of frequency encoding of  $\text{Ca}^{2+}$  oscillations through protein phosphorylation (Dupont & Goldbeter, 1992), by considering a well-known system in which the physiological response can be mediated by  $\text{Ca}^{2+}$  oscillations, namely the case of glycogen breakdown in liver cells. We indeed explore theoretically the possible role of  $\text{Ca}^{2+}$  oscillations in the regulation of a phosphorylation–dephosphorylation cycle involved in glycogen degradation by glycogen phosphorylase in hepatocytes (for review see Bollen *et al.*, 1998). This process plays a vital role in the regulation of glycaemia, providing glucose for

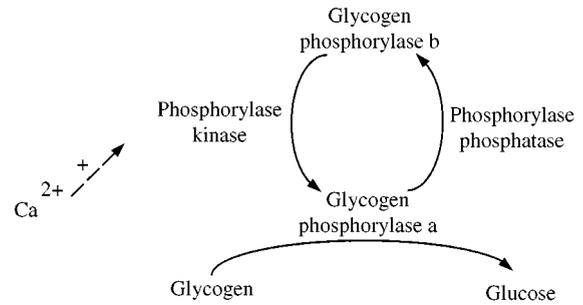


FIG. 1. Scheme of the phosphorylation–dephosphorylation cycle considered for the control of glycogen degradation. The system involves glycogen phosphorylase and its converter enzymes. The activation of phosphorylase kinase by  $\text{Ca}^{2+}$  is taken into account.

the organism between feeding. Hormones control hepatic glycogen metabolism through transmembrane signalling pathways dependent on cAMP and/or  $\text{Ca}^{2+}$ . In particular, glycogenolysis can be promoted by hormones, like vasopressin, acting primarily through the phosphoinositide signalling pathway (Kraus-Friedmann & Feng, 1996) and leading to intracellular  $\text{Ca}^{2+}$  mobilization. The corresponding rise in the level of cytosolic  $\text{Ca}^{2+}$  affects the dynamics of phosphorylase kinase, activating glycogen phosphorylase which controls glycogen degradation (see Fig. 1). On the other hand, it has been shown that repetitive  $\text{Ca}^{2+}$  spikes occur in hepatocytes in the presence of vasopressin (Woods *et al.*, 1986). Therefore, the control of glycogen phosphorylase by  $\text{Ca}^{2+}$  provides a prototypic example to study the impact of  $\text{Ca}^{2+}$  oscillations on cellular regulation.

### Model for $\text{Ca}^{2+}$ Control of Glycogen Phosphorylase

The function of glycogen phosphorylase is to govern glycogen degradation. The enzyme acts as a sensor of blood glucose level, liberating glucose from stored glycogen as needed. The dynamics of the  $\text{Ca}^{2+}$ -associated phosphorylation–dephosphorylation cycle involving glycogen phosphorylase is illustrated in Fig. 1. Glycogen phosphorylase is converted from the inactive *b*-form into the active *a*-form by phosphorylase kinase, and inactivated by a phosphatase. Phosphorylase kinase is a hexadecamer composed of four different subunits ( $\alpha_4\beta_4\gamma_4\delta_4$ ). The  $\delta$  subunit

is identical to calmodulin and mediates the  $\text{Ca}^{2+}$ -sensitivity of phosphorylase kinase (Cohen *et al.*, 1978).

The minimal model we use for the phosphorylation–dephosphorylation cycle is based on the bicyclic cascade model proposed by Cárdenas & Golbeter (1996) for the control of glycogen phosphorylase and glycogen synthase by glucose. This model has proven to be consistent with experimental findings concerning the sequential changes in the activity of glycogen phosphorylase and glycogen synthase observed following the addition of suprathreshold amounts of glucose. From the original model, we have only retained the equation pertaining to the dynamics of the phosphorylation–dephosphorylation cycle controlling the activation of glycogen phosphorylase. We have extended the model to take into account the activation of glycogen phosphorylase by cytosolic  $\text{Ca}^{2+}$  ( $Z$ ). The balance equation governing the time evolution of the fraction of active glycogen phosphorylase ( $Pha$ ) is given by

$$\dot{Pha} = V_1(Z) \frac{1 - Pha}{K_1(Z) + 1 - Pha} - \frac{V_{M2}(1 + \alpha \text{Glc}/(K_{a1} + \text{Glc})) Pha}{K_2/(1 + \text{Glc}/K_{a2}) + Pha}, \quad (1)$$

where  $\text{Glc}$  represents the intracellular concentration of glucose. All maximum rates and Michaelis constants for the converter enzymes are normalized by division by the total concentration of glycogen phosphorylase. The glucose dependency in eqn (1) arises from the fact that the binding of glucose to the active site of the enzyme makes it more susceptible to inactivation by dephosphorylation (Stalmans *et al.*, 1987). Thus, glucose is assumed to act on both the maximal velocity and the Michaelis constant of the phosphatase. The sensitivity of phosphorylase phosphatase to glucose allows the glycogen phosphorylase to act as a glucose sensor. We focus here on the activation of glycogen phosphorylase by  $\text{Ca}^{2+}$  ( $Z$ ). We assume a constant cAMP level, so that the rates of phosphorylation of glycogen phosphorylase kinase by the cAMP-dependent kinases are taken as constant parameters. Moreover, we consider here a situation where the

glucose level is constant and low (10 mM), promoting the activation of the glycogen phosphorylase. Concerning the  $\text{Ca}^{2+}$  dependency of the phosphorylase kinase, it has been shown experimentally (Doorneweerd *et al.*, 1982) that  $\text{Ca}^{2+}$  stimulates phosphorylase kinase activity by increasing its maximum rate and lowering its Michaelis constant for phosphorylase b. In this model, we assume that  $\text{Ca}^{2+}$  activates the phosphorylase kinase (of maximum rate  $V_{M1}$  and normalized Michaelis constant  $K_1$ ) by decreasing the  $K_m$  of the enzyme, with an activation constant  $K_{a6}$ , and further activates the enzyme by enhancing its maximum rate by a multiplicative factor  $\gamma$ , with an activation constant  $K_{a5}$ . In addition, there is a  $\text{Ca}^{2+}$ -independent term in the expression for  $V_{M1}$  because some basal activity of the liver phosphorylase kinase is still observed in the absence of  $\text{Ca}^{2+}$ :

$$V_1 = V_{M1} \left( 1 + \gamma \frac{Z^4}{K_{a5}^4 + Z^4} \right), \quad K_1 = \frac{K_1^1}{1 + Z^4/K_{a6}^4}. \quad (2)$$

As calmodulin is involved, we assume that the activation of phosphorylase kinase by  $\text{Ca}^{2+}$  is a cooperative process (Klee & Vanaman, 1982). The activation constants ( $K_{a5}$  and  $K_{a6}$ ) were chosen to be  $0.5 \mu\text{M}$  in agreement with the values observed in crude rat liver fractions (Khoo & Steinberg, 1975; Vandenheede *et al.*, 1977). In view of the regulatory role exerted by  $\text{Ca}^{2+}$  in physiological conditions, such values seem plausible, even if lower values have been reported for the purified enzyme (Chrisman & Jordan, 1982).

### Effect of Calcium Oscillations on Glycogen Phosphorylase Activity

Our initial approach is to compare the activation of glycogen phosphorylase by  $\text{Ca}^{2+}$  oscillations relative to the activation obtained by a stimulation with a constant level of cytosolic  $\text{Ca}^{2+}$  ( $Z$ ) of the same average value. To this end, we first have to determine the response of the model to a stimulation by a sustained  $\text{Ca}^{2+}$  level. This corresponds to the stable steady-state values for  $Pha$  as a function of  $Z$ . The solution can be obtained analytically and is defined by  $\dot{Pha} = 0$

with  $0 \leq Pha \leq 1$  [eqns (1) and (2)]. The result is shown in Fig. 3(a). It can be seen that the relation between the fraction of active phosphorylase and the cytosolic  $Ca^{2+}$  concentration has a steep sigmoidal nature. This result is a direct consequence of the saturation of the converter enzymes by their substrates, leading to a phenomenon known as “zero-order ultrasensitivity” (Goldbeter & Koshland, 1981), and of the cooperativity in the kinase activation by  $Ca^{2+}$  (Dupont & Goldbeter, 1992). Interestingly, in the case of muscle glycogen phosphorylase (Meinke *et al.*, 1986), “zero-order ultrasensitivity” has indeed been demonstrated experimentally.

To study the effects of  $Ca^{2+}$  oscillations on the dynamics of the phosphorylation–dephosphorylation loop, we investigate the effects of sinusoidal  $Ca^{2+}$  oscillations on the phosphorylase a levels. Thus, we describe the level of cytosolic  $Ca^{2+}$  as a function of time  $t$  as:

$$Z = A + B \sin(2\pi vt), \quad (3)$$

where  $v$  stands for the frequency of the sinusoidal variation of  $Z$  and the parameters  $A$  and  $B$  are chosen to match the observed amplitudes of  $Ca^{2+}$  oscillations in hepatocytes ( $A = 0.3 \mu\text{M}$ ,  $B = 0.25 \mu\text{M}$ ). Although sinusoidal-shaped oscillations do not resemble the oscillations observed experimentally, their temporal characteristics are easily controlled through the different parameters. This allows a clearer analysis of the impact of the pattern of  $[Ca^{2+}]_i$  oscillations on the dynamics of the phosphorylation–dephosphorylation cycle. It has been shown experimentally (Woods *et al.*, 1986) that the frequency of the oscillations increases with the level of stimulation while their amplitude remains approximately constant. To test whether the activation of glycogen phosphorylase could be sensitive to the frequency of  $Ca^{2+}$  oscillations,  $v$ , we have compared the phosphorylase a levels averaged over one period of the sinusoidal oscillation,  $\langle Pha \rangle$  for different values of  $v$  with the steady-state value of  $Pha$  corresponding to the mean value  $Z = A$ . The  $\langle Pha \rangle$  levels are obtained by numerical integration of eqn (1) with  $Z$  given by eqn (3). The time series obtained for increasing values of the  $Ca^{2+}$  oscillations frequency,  $v$  and the corresponding  $\langle Pha \rangle$  are shown in Fig. 2(a) and (b), respectively.

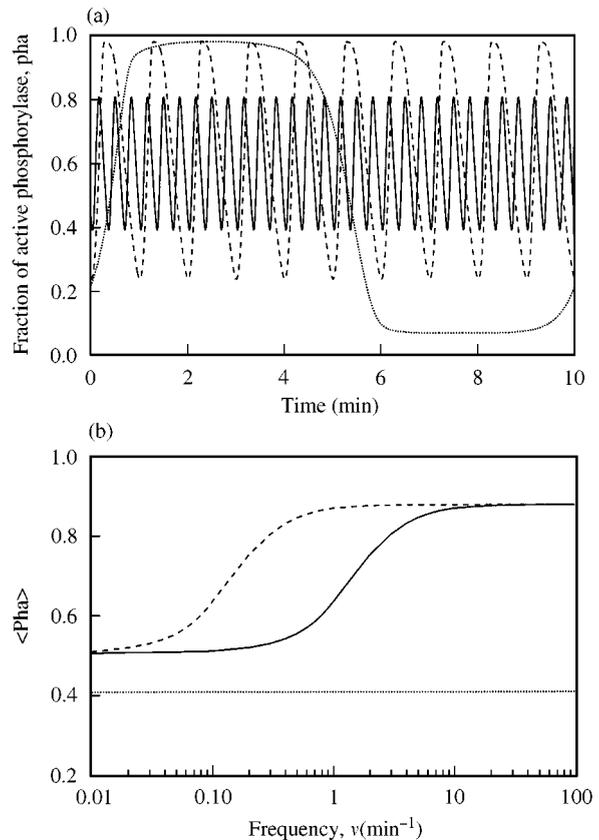


FIG. 2. Effect of a stimulation by a sustained elevation of cytosolic  $Ca^{2+}$  concentration and by sinusoidal  $Ca^{2+}$  oscillations on phosphorylase a levels,  $Pha$ . Panel (a) shows the phosphorylase a levels time series for increasing values of the  $Ca^{2+}$  oscillations frequency,  $v$ , obtained by numerical integration of eqn (1) with  $Z$  given by eqn (3) with  $A = 300 \text{ nM}$  and  $B = 250 \text{ nM}$ . The values of the parameters are  $Glc = 10 \text{ mM}$ ,  $K_1^1 = 0.1$ ,  $K_2 = 0.2$ ,  $K_{a1} = K_{a2} = 10 \text{ mM}$ ,  $K_{a5} = K_{a6} = 0.5 \mu\text{M}$ ,  $\alpha = \gamma = 9$ ,  $V_{M1} = 1.5 \text{ min}^{-1}$ ,  $V_{M2} = 0.6 \text{ min}^{-1}$ . Using the same parameters as in (a): (.....)  $v = 0.1 \text{ min}^{-1}$ , (---)  $v = 1 \text{ min}^{-1}$ , (—)  $v = 3 \text{ min}^{-1}$ . (b) Shows the frequency sensitivity of the phosphorylation–dephosphorylation cycle, obtained by evaluating the phosphorylase a levels averaged over one period of oscillation,  $\langle Pha \rangle$  as a function of  $v$  (—). The  $Pha$  levels obtained with a stimulation by an equivalent sustained  $Ca^{2+}$  level ( $Z = 350 \text{ nM}$ ) are shown for comparison (.....). The ---- shows the effect of a ten-fold decrease in the maximum rates  $V_{M1}$  and  $V_{M2}$  on the frequency response of the system: steady state ( $Z = 300 \text{ nM}$ ), .....; oscillations ( $V_{M1} = 1.5$ ,  $V_{M2} = 0.6$ ), —; oscillations ( $V_{M1} = 0.15$ ,  $V_{M2} = 0.06$ ), ----.

At low frequency [dotted curve in Fig. 2(a)], the phosphorylation–dephosphorylation dynamics is faster than the kinetics of  $Ca^{2+}$  oscillations, thus the kinase and the phosphatase can proceed so rapidly that the system stays close to its steady state. Even in this situation, the average phosphorylase a levels are higher than those obtained

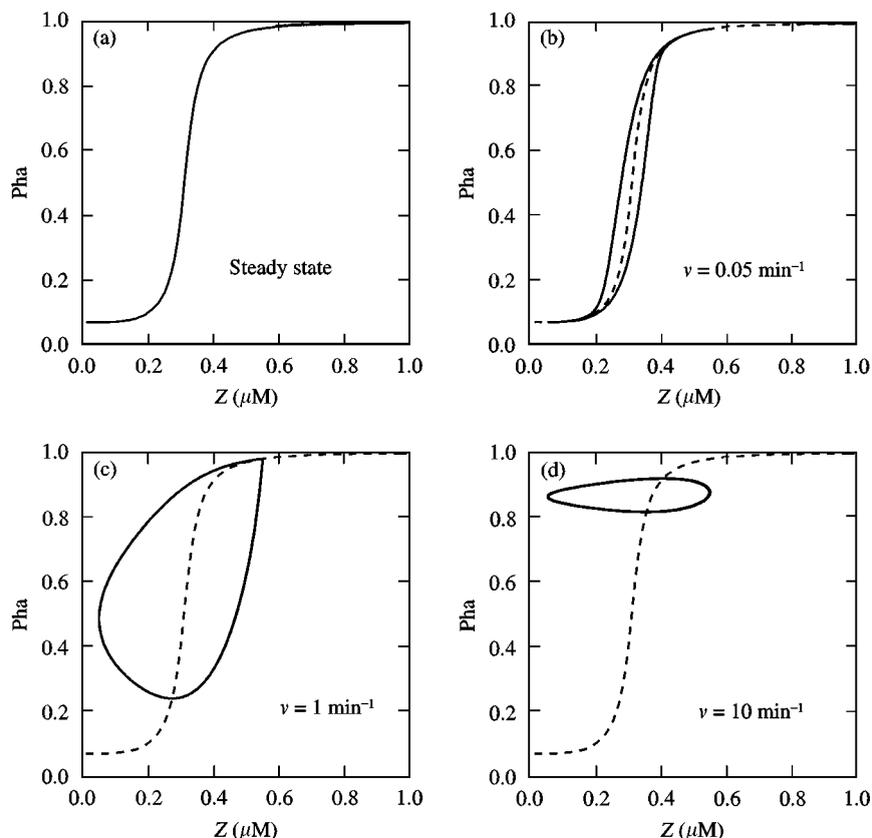


FIG. 3. Dynamics of the phosphorylation–dephosphorylation cycle in response to stimulation by a sustained elevation of cytosolic  $\text{Ca}^{2+}$  concentration or by sinusoidal  $\text{Ca}^{2+}$  oscillations. The — shown in (a) gives the stable steady-state values for  $Pha$  as a function of  $Z$ . The values of the parameters are  $Glc = 10$  mM,  $K_1^1 = 0.1$ ,  $K_2 = 0.2$ ,  $K_{a1} = K_{a2} = 10$  mM,  $K_{a5} = K_{a6} = 0.5$   $\mu\text{M}$ ,  $\alpha = \gamma = 9$ ,  $V_{M1} = 1.5$   $\text{min}^{-1}$ ,  $V_{M2} = 0.6$   $\text{min}^{-1}$ . The closed curves in (b)–(d) are projections, for increasing values of the  $\text{Ca}^{2+}$  oscillations frequency  $\nu$ , of the time series obtained by numerical integration of eqn (1) with  $Z$  given by eqn (3) (same parameter values as in Fig. 2). For increasing  $\nu$  the system moves away from its steady-state values (shown in ----) and the progressive accumulation of phosphorylase a can be observed.

with a stimulation by an equivalent sustained  $\text{Ca}^{2+}$  level of  $0.3$   $\mu\text{M}$  [compare solid and dotted curves in Fig. 3(b)]. This effect is a direct consequence of the steep sigmoidal nature of the relation between the fraction of active phosphorylase and the cytosolic  $\text{Ca}^{2+}$  concentration. Indeed, the latter relation allows the increase in  $\text{Ca}^{2+}$  concentration occurring during the half-period when  $Z$  is above its mean value ( $A = 0.3$   $\mu\text{M}$ ) to switch the system from a situation in which most of the phosphorylase is unphosphorylated into a situation in which this enzyme is largely phosphorylated.

As the frequency of  $\text{Ca}^{2+}$  oscillations increases, the kinetics of the phosphorylation–dephosphorylation cycle starts to play a substantial role. The change in  $\text{Ca}^{2+}$  concentration becomes

faster than the change in phosphorylation. The instantaneous fraction of phosphorylated protein is not able to reach its steady-state value. This is illustrated in Fig. 3(b)–(d) where the time series have been plotted in the  $(Pha, [\text{Ca}^{2+}]_i)$  plane for different values of  $\nu$ . Moreover, the kinetics of the converter enzymes allows on its own a further increase of the  $\langle Pha \rangle$  levels. This increase is induced by the fact that the maximum rate of the phosphorylase activation by the kinase is higher than the maximum rate of inactivation by the phosphatase. Therefore, significant dephosphorylation of glycogen phosphorylase is only possible when the time interval between two successive  $\text{Ca}^{2+}$  spikes is large enough. At higher frequency, dephosphorylation between successive  $\text{Ca}^{2+}$  spikes is not complete and higher  $\langle Pha \rangle$

levels can be maintained. This dependence of  $\langle Pha \rangle$  levels on the frequency of  $Ca^{2+}$  oscillations could allow a dependence of the physiological response of the cell on the sole frequency of  $Ca^{2+}$  oscillations, a phenomenon that we refer to as “pure frequency encoding”.

We have also investigated the effect of the maximum rates of the phosphorylase kinase and phosphatase on the relation between  $\langle Pha \rangle$  and the frequency of  $Ca^{2+}$  oscillations. We have thus varied the parameters  $V_{M1}$  and  $V_{M2}$  while keeping their ratio constant, so that the steady-state values for  $Pha$  as a function of  $Z$  are unchanged. The corresponding result is illustrated in Fig. 2(b) where the dashed curve shows the effect of a ten-fold decrease of the maximum rates  $V_{M1}$  and  $V_{M2}$  on the frequency response of the system. As expected intuitively, at low frequencies of  $Ca^{2+}$  oscillations, the difference between the response of the system to a constant  $Ca^{2+}$  increase and the response to an oscillating  $Ca^{2+}$  is larger when the kinase and phosphatase are slower. Again, this slow enzymatic kinetics implies that  $Pha$  does not adjust to its steady-state value. Because the kinase is faster than the phosphatase,  $Pha$  remains phosphorylated most of the time and, in consequence, the  $\langle Pha \rangle$  levels start to increase at lower frequencies in Fig. 2(b). This suggests that processes characterized by different intrinsic time-scales are sensitive to different ranges of frequencies of  $Ca^{2+}$  oscillations.

### Potential of Hormonal Stimulation of Glycogenolysis by $Ca^{2+}$ Oscillations

As mentioned above,  $Ca^{2+}$  oscillations that occur in physiological conditions in hepatocytes are not sinusoidally shaped. Furthermore, it is interesting to study the impact of  $Ca^{2+}$  oscillations in the physiological context of hormone-induced glycogenolysis. Indeed, the level of hormonal stimulation affects both the frequency of  $Ca^{2+}$  oscillations and the mean level of  $Ca^{2+}$ . Here, we use a theoretical approach to investigate if realistic  $Ca^{2+}$  oscillations confer a signalling advantage in the hormonal stimulation of glycogenolysis.

In hepatocytes, repetitive  $Ca^{2+}$  spikes can be obtained by the application of  $Ca^{2+}$ —mobilizing agonists, acting through the phosphoinositide

signalling pathway (Woods *et al.*, 1986). Each transient rises within 3 s from basal  $Ca^{2+}$  ( $\sim 100$  nM) levels to a peak of at least 600 nM and has a duration of approximately 7 s. The oscillation period varies, from 0.3 to 4 min, depending on the agonist concentration. In these conditions, a rise both in the frequency of  $Ca^{2+}$  oscillations and in the average  $Ca^{2+}$  concentration is observed. Several models have been proposed to explain the underlying mechanism of these simple periodic oscillations (for review, see Sneyd *et al.*, 1995; Dupont, 1999). In this study, we have chosen to use the minimal model originally proposed by Goldbeter *et al.* (1990) which assumes that the oscillations are caused by the interplay between two releasable pools of  $Ca^{2+}$ , one sensitive to the  $Ins(1, 4, 5)P_3$  and the other activated by  $Ca^{2+}$  (see Fig. 4). Even if this model does not represent accurately the exact physiological mechanism underlying  $Ca^{2+}$  oscillations in hepatocytes (Thomas *et al.*, 1996), it provides a realistic  $Ca^{2+}$  spike generator. In addition, we have checked that the results presented here below remain qualitatively unchanged when using another model for  $Ca^{2+}$  oscillations. This is intuitively obvious as there is no feedback of phosphorylase kinase activity on  $Ca^{2+}$  oscillations.

In the presence of an external stimulation, the steady release of  $Ca^{2+}$  from the  $Ins(1, 4, 5)P_3$ -sensitive pool induces repetitive  $Ca^{2+}$  spikes through the self-amplified release of  $Ca^{2+}$  from the  $Ca^{2+}$ -sensitive pool. This positive feedback loop by which cytosolic  $Ca^{2+}$  activates  $Ca^{2+}$

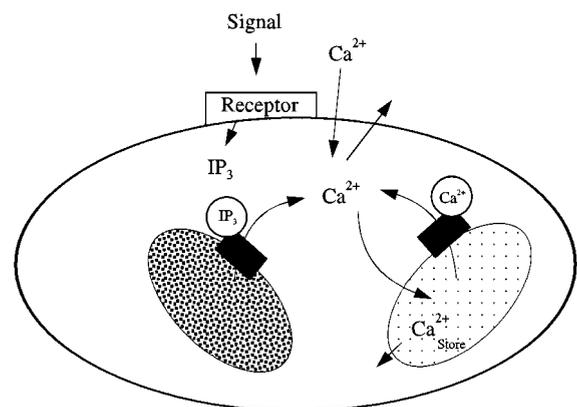


FIG. 4. Schematic representation of the two-pool model for simple  $Ca^{2+}$  oscillations. Solid arrows denote  $Ca^{2+}$  fluxes (see text for details).

release from intracellular stores is known as  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR). The model is formulated into two evolution equations for the two variables of the system, the cytosolic  $\text{Ca}^{2+}$  ( $Z$ ) and the free  $\text{Ca}^{2+}$  concentration in the  $\text{Ca}^{2+}$ -sensitive pool ( $Y$ ):

$$\frac{dZ}{dt} = V_{in} - V_{2i} + V_{3i} + k_f Y - kZ, \quad (4)$$

$$\frac{dY}{dt} = V_{2i} - V_{3i} - k_f Y, \quad (5)$$

where

$$V_{in} = v_0 + v_1 \beta, \quad (6)$$

$$V_{2i} = V_{M2i} \frac{Z^n}{K_{2i} + Z^n}, \quad (7)$$

$$V_{3i} = V_{M3i} \frac{Y^m}{K_{Ri} + Y^m} \frac{Z^p}{K_{Ai} + Z^p}. \quad (8)$$

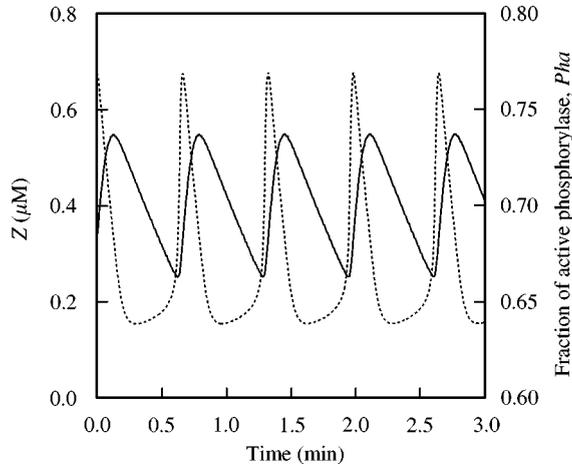


FIG. 5. Cytosolic  $\text{Ca}^{2+}$  concentration,  $Z$  (.....) and phosphorylase levels,  $Pha$  (—) time courses at a given level of stimulation corresponding to  $\beta = 0.3$ . The curves are obtained by numerical integration of eqns (4), (5) and (1). The values of the parameters are  $Glc = 10 \text{ mM}$ ,  $K_1^1 = 0.1$ ,  $K_2 = 0.2$ ,  $K_{a1} = K_{a2} = 10 \text{ mM}$ ,  $K_{a5} = K_{a6} = 0.5 \text{ } \mu\text{M}$ ,  $\alpha = \gamma = 9$ ,  $V_{M1} = 1.5 \text{ min}^{-1}$ ,  $V_{M2} = 0.6 \text{ min}^{-1}$ ,  $k = 10 \text{ min}^{-1}$ ,  $k_f = 0.7 \text{ min}^{-1}$ ,  $n = m = 2$ ,  $p = 4$ ,  $v_0 = 1 \text{ } \mu\text{M min}^{-1}$ ,  $v_1 = 5.7 \text{ } \mu\text{M min}^{-1}$ ,  $V_{M2i} = 30 \text{ } \mu\text{M min}^{-1}$ ,  $V_{M3i} = 325 \text{ } \mu\text{M min}^{-1}$ ,  $K_{2i} = 0.5 \text{ } \mu\text{M}$ ,  $K_{Ri} = 1.7 \text{ } \mu\text{M}$ ,  $K_{Ai} = 0.46 \text{ } \mu\text{M}$ .

The entry of  $\text{Ca}^{2+}$  in the cytosol,  $V_{in}$ , is constant and is the sum of the influx  $v_0$  of extracellular  $\text{Ca}^{2+}$  ions and of the  $Ins(1, 4, 5)P_3$ -stimulated  $\text{Ca}^{2+}$  release  $v_1 \beta$  [where  $0 < \beta < 1$  is the degree of saturation of the  $Ins(1, 4, 5)P_3$  receptor];  $V_{2i}$  and  $V_{3i}$  are, respectively, the rate of pumping into and release from the  $\text{Ca}^{2+}$ -sensitive store; these latter processes are described by Hill functions with maximum rates  $V_{M2i}$  and  $V_{M3i}$ , threshold constants  $K_{2i}$  and  $K_{Ri}$  and cooperativity coefficients  $n$  and  $m$ ; the CICR activation process is characterized by a cooperativity coefficient  $p$  and a threshold constant  $K_{Ai}$ . The model for control of phosphorylase activity by hormone-induced  $\text{Ca}^{2+}$  oscillations is constituted by combining eqns (4), (5) with eqn (1). Figure 5 shows the time courses of cytosolic  $\text{Ca}^{2+}$ ,  $Z$ , and of the fraction of active phosphorylase,  $Pha$ , at a given level of stimulation  $\beta = 0.3$ , obtained by numerical integration of the equations of the model. In the presence of incremental concentrations of  $\text{Ca}^{2+}$ -mobilizing agonist, the increase in  $Ins(1, 4, 5)P_3$  leads to a rise in the stimulation parameter  $\beta$  and, subsequently, to a higher frequency of  $\text{Ca}^{2+}$  oscillations and an increased mean  $\text{Ca}^{2+}$  level  $\langle Z \rangle$ .

We have examined if this frequency encoding is more efficient than the encoding based on varying the amplitude of a steady level of cytosolic  $\text{Ca}^{2+}$ . For this purpose, we have numerically evaluated the phosphorylase levels averaged over one period of the  $\text{Ca}^{2+}$  oscillation,  $\langle Pha \rangle$ , for increasing values of  $\beta$ . The result is illustrated in Fig. 6(a) where the response of the model to a stimulation by a sustained cytosolic  $\text{Ca}^{2+}$  concentration is also shown for comparison. It is clear that in the range of  $\beta$  values producing oscillations ( $0.1 < \beta < 0.46$ ) the average fraction of phosphorylated phosphorylase is larger than in the corresponding steady state situation. Figure 6(b) which is based on the same results as Fig. 6(a), provides a comparison between the  $\langle Pha \rangle$  induced by a constant (dashed line) and that by an oscillating level of  $\text{Ca}^{2+}$  (solid line) as a function of the average  $\text{Ca}^{2+}$ . It should be noted that as periodic  $\text{Ca}^{2+}$  spikes can only be obtained in a limited range of  $\beta$  values,  $\langle Pha \rangle$  values are only computed in the corresponding  $\langle Z \rangle$  range. Outside this domain,  $\langle Pha \rangle$  corresponds to steady-state values of  $Pha$ . For all

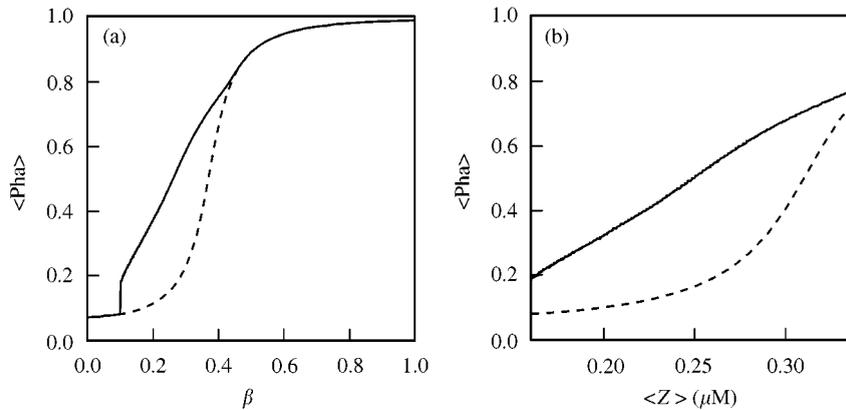


FIG. 6. Potentiation of a hormonal stimulation by  $Ca^{2+}$  oscillations. In the presence of incremental concentrations of hormone, the increase in  $Ins(1, 4, 5)P_3$  leads to a rise in the stimulation parameter  $\beta$  and, subsequently, to a higher frequency of  $Ca^{2+}$  oscillations and an increased mean  $Ca^{2+}$  level  $\langle Z \rangle$ . We compare this frequency encoding to the encoding based on varying the amplitude of a steady level of cytosolic  $Ca^{2+}$ . Panel (a) shows the phosphorylase levels averaged over one period of the  $Ca^{2+}$  oscillations,  $\langle Pha \rangle$ , for increasing values of the stimulation parameter  $\beta$  (—) and the  $\langle Pha \rangle$  levels obtained with a stimulation by an equivalent sustained cytosolic  $Ca^{2+}$  concentration [corresponding to the steady-state values of eqns (4) and (5), given by  $(v_0 + v_1\beta)/k$ ] (---). Periodic  $Ca^{2+}$  spikes can only be obtained in a bounded range of  $\beta$  values. For all values of  $\beta$  where periodic  $Ca^{2+}$  oscillations occur, the  $\langle Pha \rangle$  levels are higher than those obtained with an equivalent stimulation by a steady cytosolic  $Ca^{2+}$  concentration. Panel (b) shows the  $\langle Pha \rangle$  values in the limited  $\langle Z \rangle$  range where periodic  $Ca^{2+}$  spikes are obtained. Except for  $\beta$ , the values of the parameters are the same as in Fig. 2: —,  $Ca^{2+}$  oscillations; ---, steady state.

values of  $\langle Z \rangle$  where periodic  $Ca^{2+}$  oscillations exist, the  $\langle Pha \rangle$  levels are higher than those obtained with an equivalent stimulation by a steady cytosolic  $Ca^{2+}$  concentration. This clearly demonstrates that frequency coding based on  $Ca^{2+}$  oscillations potentiates the cell response to a hormonal stimulation.

### Discussion

Our minimal model for the control of glycogen phosphorylase activity by  $Ca^{2+}$  suggests that  $Ca^{2+}$  oscillations could play an important role in the regulation of glycogenolysis in hepatocytes. Indeed, we have first shown by simulating sinusoidal variations of the level of cytosolic  $Ca^{2+}$ ,  $Z$ , that a given level of active phosphorylase can be induced by lower average  $Ca^{2+}$  levels when  $Ca^{2+}$  oscillates. In other words, oscillations decrease the effective  $Ca^{2+}$  threshold for the activation of glycogen phosphorylase. In that respect, we recover the experimental results obtained by Dolmetsch *et al.* (1998) as to the expression of transcription factors in lymphocytes. It would thus be highly interesting to investigate if similar experimental techniques could be used to measure the effect of  $Ca^{2+}$  oscillations on glycogenolysis.

In addition to this effect, a further modulation of the  $\langle Pha \rangle$  levels can be induced when the frequency of the oscillations reaches a similar time-scale as the maximum rate of the converter enzymes involved in the phosphorylation–dephosphorylation cycle. This dependence of  $\langle Pha \rangle$  levels on the oscillations frequency could allow “pure frequency encoding” (i.e. coding by an increase in  $Ca^{2+}$  spiking frequency without modification of the mean  $Ca^{2+}$  level). This kinetic effect leads to substantial regulation of  $\langle Pha \rangle$  levels for a large (ten-fold) variation in the oscillation frequency [see Fig. 2(b)]. In physiological conditions, the  $[Ca^{2+}]_i$  oscillation frequency does vary in such a range in hepatocytes (Woods *et al.*, 1986), between 0.3 and 4  $min^{-1}$ . Nevertheless, as shown in Fig. 2(b), substantial regulation by “pure frequency encoding” occurs in a definite frequency interval requiring a fine tuning of the kinetic parameters of the phosphorylation–dephosphorylation cycle. In this context, it is remarkable that our results, based on an already tested model (Cárdenas & Golbeter, 1996), show that the interval of frequencies of  $Ca^{2+}$  oscillations providing a sizable modulation of the activation of glycogen phosphorylase lies precisely in the physiological range. Therefore, “pure frequency encoding” is

likely to play a substantial role in the regulation of glycogenolysis.

Another interesting feature of the present model concerns the fact that the level of activity of the phosphorylase kinase oscillates in phase with  $\text{Ca}^{2+}$  oscillations. That the latter assumption is realistic from the point of view of the intrinsic time-scales of phosphorylase kinase activation is corroborated by experimental observations on pancreatic acinar cells (Craske *et al.*, 1990). In this cell type indeed, it has been shown that calmodulin translocation into the nucleoplasm by a  $\text{Ca}^{2+}$ -CaM-dependent pathway allows the calmodulin concentration to oscillate in synchrony with  $\text{Ca}^{2+}$  spikes in the apical region. As the  $\delta$  subunit of phosphorylase kinase is identical to calmodulin and mediates the  $\text{Ca}^{2+}$  sensitivity of the enzyme, the variations in the level of activity of the phosphorylase kinase could indeed follow the hormone-induced  $\text{Ca}^{2+}$  spikes.

Furthermore, using a model for realistic  $\text{Ca}^{2+}$  oscillations based on the mechanism of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR), we have explored the impact of  $\text{Ca}^{2+}$  oscillations on the cell response to hormonal stimulation. An increase in the stimulation parameter ( $\beta$ ) induces a higher frequency of  $\text{Ca}^{2+}$  spiking and also a larger mean value for the  $\text{Ca}^{2+}$  concentration. In these conditions, it appears that both effects are involved in the increase in the average fraction of phosphorylated phosphorylase occurring after a rise in stimulation in hepatocytes. Thus, in addition to avoiding potential damage to the cell and increasing the robustness in signal detection at low levels of stimulation (Rapp *et al.*, 1981; Rapp, 1987),  $\text{Ca}^{2+}$  oscillations seem to optimize the effect of hormonal stimulation.

In conclusion, this theoretical study suggests that  $\text{Ca}^{2+}$  oscillations could play a functional role in the regulation of glycogenolysis at the single-cell level. Interestingly, recent experimental findings plead in favour of this hypothesis, also at the multicellular level. The intracellular  $\text{Ca}^{2+}$  waves recently observed in intact livers perfused with vasopressin have indeed been shown to allow the coordination of the glycogenolytic response at the level of the whole organ (Eugenin *et al.*, 1998).

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