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Protein phosphorylation driven by intracellular calcium oscillations: A kinetic analysis

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Abstract

Given the ubiquitous nature of signal-induced Ca^{2+} oscillations, the question arises as to how cellular responses are affected by repetitive Ca^{2+} spikes. Among these responses, we focus on those involving protein phosphorylation. We examine, by numerical simulations of a theoretical model, the situation where a protein is phosphorylated by a Ca^{2+}-activated kinase and dephosphorylated by a phosphatase. This reversible phosphorylation system is coupled to a mechanism generating cytosolic Ca^{2+} oscillations; for definiteness, this oscillatory mechanism is based on the process of Ca^{2+}-induced Ca^{2+} release. The analysis shows that the average fraction of phosphorylated protein increases with the frequency of repetitive Ca^{2+} spikes; the latter frequency generally rises with the extent of external stimulation. Protein phosphorylation therefore provides a mechanism for the encoding of the external stimulation in terms of the frequency of signal-induced Ca^{2+} oscillations. Such a frequency encoding requires precise kinetic conditions on the Michaelis–Menten constants of the kinase and phosphatase, their maximal rates, and the degree of cooperativity in kinase activation by Ca^{2+}. In particular, the most efficient encoding of Ca^{2+} oscillations based on protein phosphorylation occurs in conditions of zero-order ultrasensitivity, when the kinase and phosphatase are saturated by their protein substrate. The kinetic analysis uncovers a wide variety of temporal patterns of phosphorylation that could be driven by signal-induced Ca^{2+} oscillations.

Keywords: Calcium regulation; Kinase; Calmodulin; Frequency coding; Biochemical oscillations

1. Introduction

Oscillations in cytosolic Ca^{2+} concentration have been observed in many cell types either spontaneously or in response to an extracellular agonist. In the latter case, this process only occurs in an appropriate range of external stimulation and is characterized by periods which are generally of the order of seconds to minutes [1–6]. In most cases, the frequency of the oscillations increases with the level of external stimulation, thus leading to the transformation of an analogue signal (the agonist concentration) into a digital one (the frequency of repetitive Ca^{2+} spikes). Frequency-encoded signals present the advantage of being more resistant to noise [7,8]. Moreover, pulsatile Ca^{2+} signalling allows for the

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avoidance of desensitization or other deleterious effects that could result from a high steady-state level of intracellular Ca^{2+} [2,9,10].

Besides the circumvention of noxious effects, cytosolic Ca^{2+} oscillations could also play a more positive role. Their ubiquity, as well as the observation that their frequency varies with external stimulation, raise the possibility that secondary messengers could be frequency-encoded. Thus, the simultaneous measurement of intracellular Ca^{2+} concentration and hormone secretion in somatotrope pituitary cells shows that the amount of growth hormone released correlates with the frequency of Ca^{2+} oscillations [11]. The same kind of phenomenon had already been described by Rapp and Berridge [12] for blowfly salivary glands where an increase in 5-hydroxytryptamine concentration enhances both the rate of secretion and the frequency of Ca^{2+} oscillations. A further example that suggests a physiological role for Ca^{2+} oscillations is that of pancreatic acinar cells where maximal secretion occurs at intermediate levels of acetylcholine [13] or cholecystokinin [14] producing oscillations in cytosolic Ca^{2+}.

What could be the physiological link between the cellular response and the frequency of Ca^{2+} oscillations? A most likely mechanism would involve the reversible phosphorylation of proteins playing a key role in cellular activity [15,16]. In the following, we consider a protein substrate that becomes active once it is phosphorylated by a kinase controlled by cytosolic Ca^{2+}; the phosphorylated substrate can then be dephosphorylated by a phosphatase. Activation of the kinase by Ca^{2+} could either be direct as in the case of protein kinase C (PKC) [17], or mediated by calmodulin [18]. Our analysis establishes that such a system allows for the frequency encoding of agonist-induced Ca^{2+} oscillations, but only in precise kinetic conditions.

As shown in a preliminary analysis [16,19], one of the conditions required for efficient frequency encoding of Ca^{2+} oscillations pertains to the degree of saturation of the kinase and phosphatase by their respective substrates. Here, we extend these results by determining the effect of additional factors such as the maximal rates of the converter enzymes, the threshold value for kinase activation by Ca^{2+}, and the degree of cooperativity characterizing the latter regulatory process. The present study allows one to predict a wide variety of dynamic patterns of phosphorylation that can be driven by Ca^{2+} oscillations.

Although we resort to a specific model based on Ca^{2+}-induced Ca^{2+} release to generate Ca^{2+} oscillations [16,20,21], the results obtained as to frequency encoding based on protein phosphorylation are largely independent of the model used to induce repetitive Ca^{2+} spikes.

2. Model for phosphorylation driven by Ca^{2+} oscillations

2.1 Model for Ca^{2+} oscillations based on Ca^{2+}-induced Ca^{2+} release

The net effect of agonist binding to its extracellular membrane receptor is the synthesis of inositol 1,4,5-trisphosphate ($InsP_3$) which mobilizes Ca^{2+} from internal $InsP_3$-sensitive pools. This rise in cytosolic Ca^{2+} in turn leads, through a so-called Ca^{2+}-induced Ca^{2+} release mechanism [1,2,22–25], to the release of Ca^{2+} from an $InsP_3$-insensitive, Ca^{2+}-sensitive pool. This model, schematized in Fig. 1, contains only two variables,

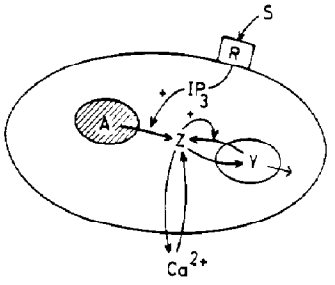

Fig. 1. Model for signal-induced Ca^{2+} oscillations. The stimulus (S) acting on a cell surface receptor (R) triggers the synthesis of $InsP_3$; the latter intracellular messenger elicits the release of Ca^{2+} from an $InsP_3$-sensitive store (A) at a rate proportional to the saturation function (β) of the $InsP_3$ receptor. Cytosolic Ca^{2+} (Z) is pumped into an $InsP_3$-insensitive intracellular store; Ca^{2+} in the latter store (Y) is released into the cytosol in a process activated by cytosolic Ca^{2+}. This feedback gives rise to Ca^{2+} oscillations [16,20,21]. Other arrows refer to a possible leak of Y into Z, and to the exchange of Ca^{2+} between the cytosol and the extracellular medium (see text).

Z and Y, which denote the concentration of free Ca^{2+} in the cytosol and in the $InsP_3$-insensitive pool, respectively. The model is a minimal one as the level of stimulation is considered as a parameter, β, measuring the saturation function of the $InsP_3$ receptor at the surface of the $InsP_3$-sensitive Ca^{2+} pool.

This model can generate repetitive spiking for appropriate parameter values, in a precise range of stimulation level bounded by two critical values of β. The latter property is in accordance with experimental results in most cell types; moreover, the theoretical predictions agree with the observation that the frequency of Ca^{2+} oscillations increases with agonist concentration and with the level of extracellular Ca^{2+} [3–6,16,20,21]. Support for a mechanism of Ca^{2+} oscillations based on Ca^{2+}-induced Ca^{2+} release has been obtained in a number of cell types [1,2,22–25].

Other models [26,27] ascribe the periodic variation of Ca^{2+} to an oscillating level of $InsP_3$; these models involve activation of $InsP_3$ synthesis by Ca^{2+} [26] or inactivation of the $InsP_3$-producing pathway [27]. The former type of mechanism is supported by experiments performed in some cell types [28]. The use of one of these models instead of that based on Ca^{2+}-induced Ca^{2+} release would not significantly influence the results of the analysis presented below.

The time evolution of the two variables in the model based on Ca^{2+}-induced Ca^{2+} release is governed by the following kinetic equations:

$$\begin{aligned} \frac{dZ}{dt} &= v_0 + v_1\beta - v_2 + v_3 + k_f Y - kZ \\ \frac{dY}{dt} &= v_2 - v_3 - k_f Y \end{aligned} \tag{1}$$

with:

$$v_2 = v_{M2}\frac{Z^n}{K_P^n + Z^n},$$

$$v_3 = v_{M3}\frac{Y^m}{K_R^m + Y^m}\cdot\frac{Z^p}{K_A^p + Z^p}$$

In these equations, v_0 and $v_1\beta$ represent the constant inputs of Ca^{2+} from the extracellular medium and from the $InsP_3$-sensitive pool; v_{M2} and v_{M3} are the maximum rates of pumping and release into and from an $InsP_3$-insensitive pool, while K_P, K_R and K_A are threshold constants for pumping, release and activation. The possible cooperative nature of these processes is characterized by the Hill coefficients n, m and p. Finally, k_f measures the passive leak of Y into Z, while k relates to the transport of Ca^{2+} from the cytosol into the extracellular medium. All parameters and concentrations are defined with respect to the total cell volume.

2.2 Protein phosphorylation

Reversible phosphorylation can be incorporated into the model by taking into account a protein substrate (whose total amount is denoted by W_T), which can exist both in a phosphorylated form (W^*) and in a dephosphorylated form (W); the interconversion between the two forms is catalysed by a kinase and a phosphatase. Assuming Michaelis–Menten kinetics for these converter enzymes and defining W^* as the fraction of phosphorylated protein ($W^* = [W^*]/[W_T]$), the equation governing the time evolution of the fraction of phosphorylated protein can be written as follows (see ref. [29]):

$$\frac{dW^*}{dt} = \left(\frac{v_P}{W_T}\right) \times \left\{\left(\frac{v_K}{v_P}\right)\frac{1 - W^*}{K_1 + 1 - W^*} - \frac{W^*}{K_2 + W^*}\right\} \tag{2}$$

In this equation, v_K and v_P denote the effective maximal rates of the kinase and phosphatase; $K_1 = K_{M1}/W_T$ and $K_2 = K_{M2}/W_T$, where K_{M1} and K_{M2} are the Michaelis–Menten constants of the two enzymes.

2.3 Coupling of protein phosphorylation with Ca^{2+} oscillations

Interplay of phosphorylation, governed by eq. (2), and Ca^{2+} oscillations, generated by eqs. (1), arises when assuming that the kinase is activated

by cytosolic Ca^{2+} (Z). Thus, the expression for the kinase reaction rate, v_K, takes on the general form of an equation of the Hill type:

$$v_K = v_{MK} \frac{Z^q}{K_a^q + Z^q} \tag{3}$$

where v_{MK} is the maximal rate of the kinase, and K_a denotes the threshold constant for activation of this enzyme by cytosolic Ca^{2+}; the Hill coefficient q allows for positive cooperativity ($q > 1$) in the activation process.

Strictly speaking, eq. (3) defines a situation where the protein kinase is directly activated by cytosolic Ca^{2+}, as could be the case for PKC. It seems, however, that PKC might be fully active at resting levels of Ca^{2+} in the presence of its synergistic agent, diacylglycerol, which is produced concomitantly with $InsP_3$ [17]. A more relevant activation of the protein kinase by Ca^{2+} would involve calmodulin. The formation of the Ca^{2+}-calmodulin complex would require an additional step; however, because of the rapid binding of Ca^{2+} to calmodulin and of the excess of calmodulin in comparison with the kinase, eq. (3) could, in a first approximation, also apply to calmodulin-dependent kinases.

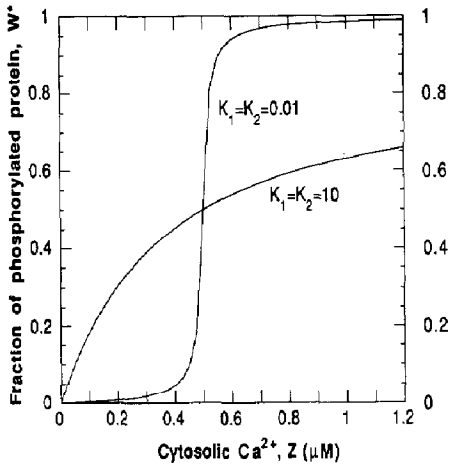

Fig. 2. Fraction of phosphorylated protein as a function of Ca^{2+} level in the absence of Ca^{2+} oscillations. It is assumed that a kinase activated by cytosolic Ca^{2+} phosphorylates a protein W into the form W^*. If both the kinase and the phosphatase are saturated by their substrate ($K_1 = K_2 = 0.01$), the transition in the fraction of protein phosphorylated at steady state (W^*) as a function of Ca^{2+} is very sharp; this effect is know as "zero-order ultrasensitivity" [29,31]. In contrast, the curve is much smoother when both enzymes are acting in the region of first order kinetics ($K_1 = K_2 = 10$). The curves yield the steady-state value of W^* as a function of Z in eqs. (2) to (3) (see refs. [29] and [31]). For both curves, $W_T = 10\ \mu M$, $v_{MK} = 5\ \mu M\ min^{-1}$, $v_P = 1\ \mu M\ min^{-1}$, $K_a = 2\ \mu M$ and $q = 1$.

3. Protein phosphorylation as a function of Ca^{2+} in the absence of oscillations

Before studying the dynamics of the system defined by eqs. (1)–(3), it is useful to consider the behavior of the phosphorylation system at steady state, in the absence of Ca^{2+} oscillations, as a function of the level of cytosolic Ca^{2+}. The steady-state behavior of a reversible covalent modification system has already been examined by Goldbeter and Koshland [29] who showed that when the modifying enzymes operate outside the region of first-order kinetics (i.e. when they become saturated by their substrate), the relationship between the fraction of modified protein (W^*) and the ratio of effective, maximum velocities of the converter enzymes acquires a steep, sigmoidal nature. The steepness of the transition curve can exceed that of allosteric enzymes characterized by Hill coefficients larger than 10 [29,30]. The value of the ratio v_K/v_P in which $W^* = 0.5$ is given [31] by the expression [4]:

$$\frac{v_K}{v_P} = \frac{1 + 2K_1}{1 + 2K_2} \tag{4}$$

Relation (4) provides a simple way of locating the threshold of the transition curve. Thus, when $K_1 = K_2$, the threshold is always located in $v_K/v_P = 1$. This phenomenon of steep sigmoidal dependence of W^* on v_K/v_P, referred to as "zero-order ultrasensitivity", has now been demonstrated in several experimental systems such as isocitrate dehydrogenase [32] and glycogen phosphorylase [33].

The above, steady state results can readily be extended to situations where the kinase is activated by cytosolic Ca^{2+}, i.e. when v_K is given by eq. (3). Then, the ratio v_K/v_P becomes a function of Ca^{2+} concentration. In Fig. 2, the fraction

of phosphorylated protein is plotted as a function of Ca^{2+} concentration for two different values of the normalized Michaelis–Menten constant K_1 in a system in which $K_1 = K_2$. For large values of K_1 (e.g., 10), the curve is shallow. But at enzyme saturation ($K_1 = 0.01$), a tiny increase in Ca^{2+} concentration in the appropriate range induces a change from largely unmodified protein to largely modified protein. For the parameter values considered in Fig. 2, the threshold is located at $Z = 0.5$ μM which, as expected from eqs. (3–4), corresponds to $v_K/v_P = 1$. Zero-order ultrasensitivity thus provides a simple, robust mechanism for the cell machinery to be sensitive to small changes in effector concentration.

4. Protein phosphorylation driven by Ca^{2+} oscillations

The Ca^{2+} level is seen to oscillate in a large number of cells [1–6]; it is thus of general interest to examine the consequences of an oscillating level of this second messenger on cellular activities and, in particular, on protein phosphorylation. To this end, let us consider the full system of eqs. (1) to (3), whose expected behavior is schematized in Fig. 3. In the course of sustained Ca^{2+} oscillations, the amount of phosphorylated protein should vary periodically; each Ca^{2+} spike indeed activates the kinase, while the phosphatase becomes the most active during the silent phase that separates successive spikes. As we shall see, however, significant periodic variation in the level of phosphorylated protein requires appropriate conditions on the kinetic properties of the converter enzymes and on the activation of the kinase by cytosolic Ca^{2+}.

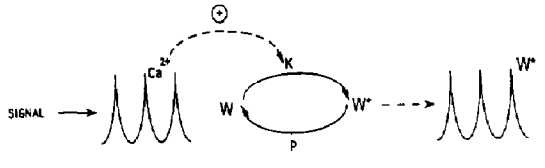

Fig. 3. Protein phosphorylation driven by Ca^{2+} oscillations. Schematized is a protein W phosphorylated by a kinase K into the form W^*; the latter form is dephosphorylated by a phosphatase P. If kinase K is activated by Ca^{2+}, oscillations in cytosolic Ca^{2+} would likely be accompanied by periodic variations in the level of phosphorylated substrate.

4.1 Effect of the Michaelis–Menten constants of kinase and phosphatase

The first condition pertains to the Michaelis–Menten constants of the converter enzymes. Saturation of these enzymes by their protein substrate favors the occurrence of large-amplitude changes in protein phosphorylation. In the course of Ca^{2+} oscillations (Fig. 4a), the fraction of phosphorylated protein indeed varies over a much larger range if both the kinase and phosphatase are acting in their region of zero order kinetics (compare panels b and c in Fig. 4). This is of course a natural consequence of the results shown in Fig. 2; in the case of a steep sigmoidal curve ($K_1 = K_2 = 0.01$), W^* can indeed vary from nearly zero to one during each Ca^{2+} spike. In other words, although the ratio v_K/v_P undergoes large variations from below to above the threshold in the course of Ca^{2+} oscillations for all values of $K_1 = K_2$, only the case of zero order ultrasensitivity can transform these variations into significant changes in the level of phosphorylated protein. Importantly, both situations do not only induce different dynamics, but also different average levels of phosphorylated protein: for the parameter values considered in Fig. 4, $\langle W^* \rangle = 0.4$ for $K_1 = K_2 = 0.01$ (panel b), and 0.5 for $K_1 = K_2 = 10$ (panel c).

4.2 Effect of maximal phosphorylation and dephosphorylation rates

Even in the case of zero-order ultrasensitivity, phosphorylation in the course of Ca^{2+} oscillations depends on the maximal rates of the kinase and phosphatase. We have seen in Section 3 that the value of the ratio v_K/v_P determines the level of phosphorylated protein at steady state, i.e. at a stationary level of Ca^{2+}. Let us call W^*_{stat} this reference value of the fraction of phosphorylated protein. If the phosphorylation dynamics is faster than the kinetics of Ca^{2+} oscillations, the kinase and phosphatase reactions will proceed so rapidly that the system at each moment will reach the

value W^*_{stat} corresponding to the instantaneous value of the ratio v_K/v_P. In contrast, for small values of the maximal rates v_{MK} and v_P, the change in Ca^{2+} concentration will be faster than the changes in phosphorylation: the instantaneous fraction of phosphorylated protein will not have the time to reach the value W^*_{stat} predicted by the steady-state curve. The different situations

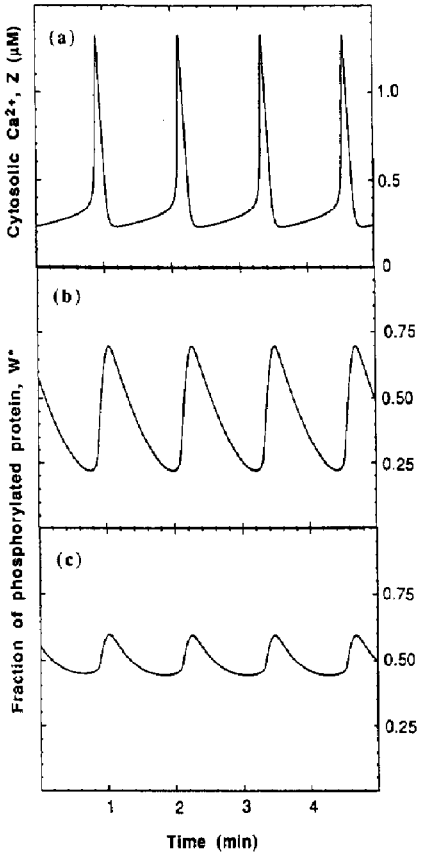

Fig. 4. Protein phosphorylation driven by Ca^{2+} oscillations in the presence and absence of zero-order ultrasensitivity. Sustained oscillations in cytosolic Ca^{2+} are shown in panel (a) These spikes generate oscillations in the fraction of phosphorylated protein, W^*, in the system defined by eqs. (1) to (3). The amplitude of these oscillations is quite large in conditions of zero-order ultrasensitivity, i.e. when the kinase and phosphatase are saturated by their protein substrates (panel b), while the range of variation of W^* becomes much smaller in the opposite case (panel c). The curves are generated by numerical integration of eqs. (1) to (3) with $v_0 = 1\ \mu M\ min^{-1}$, $v_1\beta = 2.7\ \mu M\ min^{-1}$, $v_{M2} = 65\ \mu M\ min^{-1}$, $v_{M3} = 500\ \mu M\ min^{-1}$, $K_R = 2\ \mu M$, $K_P = 1\ \mu M$, $K_a = 0.9\ \mu M$, $k = 10\ min^{-1}$, $k_f = 1\ min^{-1}$, $n = m = 2$, $p = 4$, $W_T = 1\ \mu M$, $v_{MK} = 20\ \mu M\ min^{-1}$, $v_P = 2.5\ \mu M\ min^{-1}$, $K_a = 2.5\ \mu M$, $q = 1$. Moreover, $K_1 = K_2 = 0.01$ for panel (b) and $K_1 = K_2 = 10$ for panel (c).

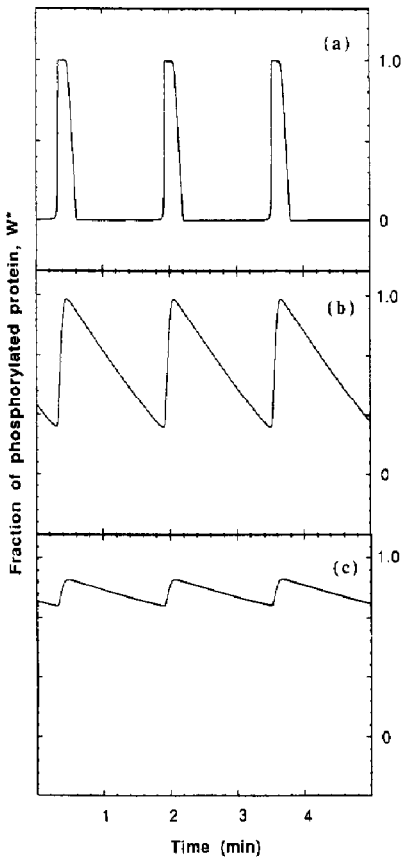

Fig. 5. Protein phosphorylation as a function of the maximal rates of kinase (v_{MK}) and phosphatase (v_P) at constant ratio (v_{MK}/v_P). When v_{MK} and v_P are very large, the fraction of modified substrate switches from nearly zero to one at each Ca^{2+} spike (panel a). Decreasing both velocities leads to a decreased amplitude in the oscillations in W^* (panel b). Finally, when the kinase and phosphatase are very slow in comparison with the Ca^{2+} kinetics, the level of phosphorylated protein undergoes small periodic variations around the mean value predicted by the steady state situation (panel c). Parameter values are the same as in Fig. 4(b), except that $v_1\beta = 2.4\ \mu M\ min^{-1}$, $K_a = 1\ \mu M$ and $q = 4$. Moreover $v_{MK}/v_P = 20/0.89$, with $v_{MK} = 2$ mM min^{-1} for panel (a), $v_{MK} = 100\ \mu M\ min^{-1}$ for panel (b) and $v_{MK} = 20\ \mu M\ min^{-1}$ for panel (c).

are illustrated in Fig. 5 where the periodic variation in W^* is shown at different values of v_{MK}, holding the ratio v_{MK}/v_P constant. If v_{MK} and v_P are both larger than W_T/T, where W_T denotes the total concentration of protein substrate and T the period of Ca^{2+} oscillations, then W^* will switch from nearly zero to one at each Ca^{2+} spike (Fig. 5a). For decreasing values of v_{MK} and v_P, the amplitude of the oscillations in W^* will diminish (Fig. 5b) and will eventually take on the

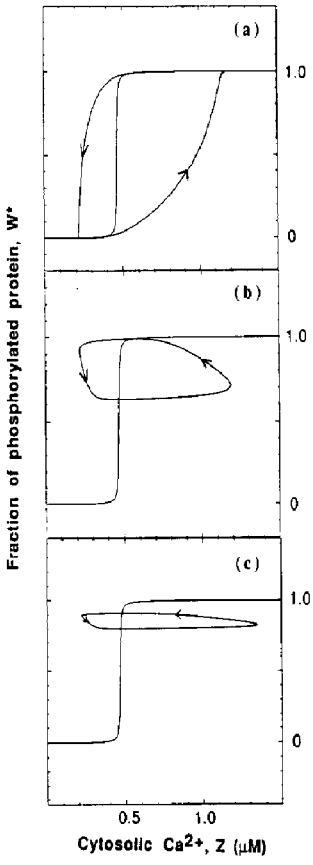

Fig. 6. Visualization in the $Z-W^*$ plane of the effects of the maximum enzyme velocities. The sigmoidal curve represents the steady-state value of W^* as a function of Z in eqs. (2)–(3). At the right of the threshold, net phosphorylation of the substrate occurs, while net dephosphorylation occurs on the left. During one period of Ca^{2+} oscillations, the Ca^{2+} spike gives rise to an increase in W^* followed by a decrease during the interspike interval; the dynamics consequently describes a closed cycle in the $Z-W^*$ plane. The curves (a)–(c) correspond to the data shown in the corresponding panels of Fig. 5. Decreasing the maximal rates of the converter enzymes leads to cycles of diminishing amplitude (compare panels a, b and c).

form of tiny oscillations (Fig. 5c). The asymmetric shape of the oscillations in W^* in panels (b) and (c) is due to the fact that in all cases the ratio v_{MK}/v_P considered is larger than unity.

The distinction between the various cases becomes clearer when resorting to the representation used in Fig. 6. There, the steady-state solution curve of eqs. (2–3), yielding W^* as a function of Z, is plotted together with the dynamical evolution of the two variables in the (Z, W^*) plane. Each point at the right of the sigmoidal curve represents a situation where net phosphorylation will occur: Z is larger than the threshold value, so that the kinase is more active than the phosphatase. On the other hand, when Z decreases and reaches a point at the left of the threshold, net dephosphorylation will occur. The direction of movement during oscillations of Z is indicated by the arrows. In Fig. 6a, v_{MK} and v_P are very large, and thus W^* switches from nearly zero to one in the course of Ca^{2+} oscillations. Decreasing v_{MK} and v_P, while keeping the same ratio of maximal rates, leads to a decreased dephosphorylation when the point representing the system is located to the left of the threshold (Fig. 6b). Further diminution in the amplitude of the cycle in the (Z, W^*) plane is observed when the modification rates become very small (Fig. 6c).

Again, these different values for the kinetic parameters v_{MK} and v_P, at a given ratio v_{MK}/v_P, not only induce different temporal patterns for the fraction of phosphorylated protein, but also lead to different average fractions of modified substrate. In Fig. 6, $\langle W^* \rangle$ is equal to 0.137, 0.825 and 0.861 in cases (a), (b) and (c), respectively.

How are these results related to the steady-state solution of eqs. (2)–(3)? Given that a link between W^* and v_K/v_P exists in the steady-state situation, one would think that there must be some similar link between $\langle W^* \rangle$ and $\langle v_K \rangle/v_P$, where the brackets mean that the relevant quantity is averaged over one period of Ca^{2+} oscillations. Since there is no way of obtaining an analytical expression for the mean value of v_K given by eq. (3), we have determined $\langle v_K \rangle$ by numerical simulations: for the values of v_{MK}/v_P considered in Figs. 5 and 6, the average ratio $\langle v_K \rangle/v_P$ always equals 1.060 (this quantity remains of course similar for cases (a), (b) and (c) since Ca^{2+} oscillations and the ratio v_{MK}/v_P remain unchanged). Introducing this value in eq. (2), one obtains a hypothetical value of 0.862 for the steady state fraction W^*. As expected, the effective value of $\langle W^* \rangle$ determined numerically approaches this value when v_{MK} and v_P decrease.

The time required to reach the asymptotic oscillatory regime from a situation where the entire substrate is unmodified also depends on

the absolute values of v_{MK} and v_P. This time increases in a nearly linear manner with v_{MK} and v_P, at constant ratio of the maximal rates. For very large velocities, this time interval reaches a minimum value beyond which it does not decrease further: as soon as v_{MK} and v_P are large enough to allow for variations in the fraction of phosphorylated protein from nearly zero to one, the asymptotic regime will be established during the first Ca^{2+} spike (see, e.g., Figs. 5a and 6a).

A dynamical representation of the evolution towards the asymptotic regime in the case of small values of v_{MK} and v_P is given in Fig. 7 where the value of the ratio v_{MK}/v_P is the same as in Figs. 5 and 6. Starting from an entirely unmodified substrate, some net phosphorylation occurs each time the Ca^{2+} concentration increases. However, during the interspike interval, dephosphorylation of nearly the same amount of protein occurs. The fact that the net increase in W^* is quite small prolongs the time needed to reach the asymptotic regime.

An interesting situation arises when the phosphatase is much slower than the Ca^{2+}-activated kinase. In that case, an increase in the stimulation level, and hence in the frequency of Ca^{2+} oscillations, leads to a decrease in the time required to reach the asymptotic pattern of periodic protein phosphorylation (Fig. 8). During the transient phase, a marked difference in the instantaneous level of W^* exists at two distinct levels of stimulation.

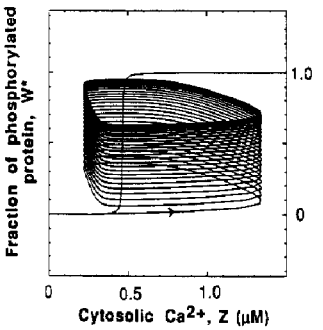

Fig. 7. Evolution of the level of phosphorylated protein towards the asymptotic oscillatory regime. Starting from no phosphorylated protein, a significant fraction of substrate is modified at each Ca^{2+} spike; dephosphorylation of a large part of this quantity occurs during the interspike interval. The net effect of each cycle is the modification of a small amount of protein, which results in raising the next cycle somewhat higher. This process repeats until the system reaches a situation in which the amount of protein phosphorylated during the spike is exactly balanced by the quantity dephosphorylated between two successive spikes. The curve is obtained by numerical integration of eqs. (1)–(3), starting from $W^* = 0$. Parameters values are the same as in Fig. 5 with $v_{MK} = 60$ μM min^{-1} and $v_P = 2.67$ μM min^{-1}.

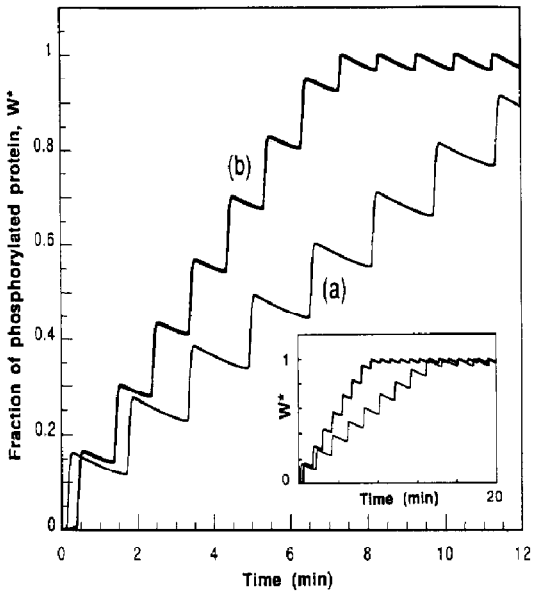

Fig. 8. Transient patterns of phosphorylation induced by distinct levels of stimulation. When the phosphatase is much slower than the kinase, the time taken to reach the asymptotic oscillatory pattern of protein phosphorylation decreases when the degree of stimulation (measured by β) increases. Parameters for Ca^{2+} oscillations are the same as in Fig. 5 with $v_1\beta = 2.4$ μM min^{-1} for curve (a) and 3 μM min^{-1} for curve (b). For the phosphorylation dynamics, $v_{MK} = 25$ μM min^{-1}, $v_P = 0.5$ μM min^{-1} while the other parameter values are the same as in Fig. 5.

4.3 Effect of cooperativity in kinase activation by Ca^{2+}

Another factor that influences periodic protein phosphorylation is the degree of cooperativity in kinase activation by Ca^{2+}. The question of the role of such a cooperativity arises naturally as one of the best-known Ca^{2+} requiring activator, calmodulin, is characterized by large Hill coefficients for Ca^{2+} binding [18].

The effect of cooperativity can be tested by changing the value ascribed to parameter q in eq. (3). An increase in q generally leads, however, to

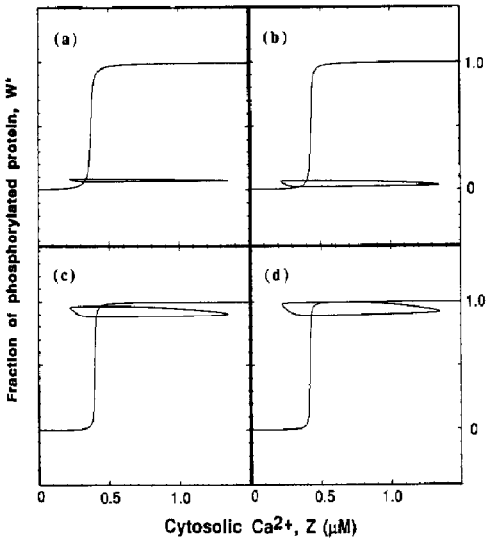

Fig. 9. Phosphorylation as a function of the degree of cooperativity in kinase activation by Ca^{2+}. To analyze the pure effect of cooperativity in kinase activation by Ca^{2+}, the value of the threshold constant for activation (K_a) has been adapted in such a way that the threshold value remains in $Z = 0.45\ \mu M$ in each case (see text). In the absence of any cooperativity, i.e. when $q = 1$ (a), or with $q = 2$ (b), the cycle is very flat, which means that the system remains nearly insensitive to Ca^{2+} changes and no significant phosphorylation occurs. Hill coefficients equal to 3 (c) or 4 (d) allow for substantial substrate modification. Parameter values are the same as in Fig. 5, with $v_{MK} = 20\ \mu M\ min^{-1}$, $v_P = 0.89\ \mu M\ min^{-1}$ and $K_a = 8$, 2, 1.1 and 1 (in μM) in panels (a)–(d), respectively.

shifting to the right the curve yielding W^* as a function of Z (eqs. 2–3) at a fixed value of K_a. Thus, to analyze the pure effect of cooperativity, K_a has been decreased when increasing q so that in all cases considered in Fig. 9 the threshold in which $W^* = 0.5$ is reached for the same Ca^{2+} concentration, namely, $Z = 0.45\ \mu M$.

The effect of cooperativity is most significant at small values of v_{MK} and v_P. As shown in Fig. 9, it then appears that a higher cooperativity in kinase activation allows for a slightly larger variation of W^* with Ca^{2+} as well as for a shift in the mean level of W^*. For the parameter values considered, when $q = 1$ (Fig. 9a), the cycle performed by the system in the Z–W^* plane is very flat, reflecting the fact that W^* is unable to "follow" the Ca^{2+} spikes in a significant manner. The corresponding average value of W^* ($\langle W^* \rangle$) is equal to 0.08. Increasing q up to 2 (Fig. 9b) does not significantly improve the situation. With a Hill coefficient equal to 3 (Fig. 9c), the cycle is broader and the mean fraction of phosphorylated protein rises up to 0.94. This value still augments when $q = 4$ (Fig. 9d). Thus, it appears that cooperativity in kinase activation by Ca^{2+} of the order of that observed experimentally [18] allows the system to be sensitive to fast variations in the cytosolic Ca^{2+} level at small or intermediate velocities of the converter enzymes.

The situation is rather different for large values of v_{MK} and v_P. In that case indeed, the average value of $\langle W^* \rangle$ only shows a slight dependence on the Hill coefficient for kinase activation, since W^* switches from nearly zero to one during each Ca^{2+} spike (see Fig. 6a).

5. Frequency encoding of Ca^{2+} oscillations based on protein phosphorylation

One purpose of this study is to examine whether Ca^{2+} oscillations triggered by external stimulation can be efficiently encoded in terms of frequency on the basis of protein phosphorylation controlled by cytosolic Ca^{2+}. The theoretical approach has shown so far that for the fraction of phosphorylated protein to vary over a significant range, conditions must be met with regard to at least two points. First, saturation of the converter enzymes is needed. Second, v_{MK} and v_P have to be sufficiently large to be able to "feel" the rapid changes in the Ca^{2+} level; this condition can, to some extent, be relaxed if the activation of the kinase by cytosolic Ca^{2+} possesses a cooperative nature.

The question now arises as to how the average level of phosphorylated protein, $\langle W^* \rangle$, varies with the frequency of Ca^{2+} oscillations. To address this question, we have computed $\langle W^* \rangle$ for different levels of external stimulation measured by β (see Section 2). In a previous analysis [16,19], such a study was carried out in the absence of cooperativity in kinase activation by Ca^{2+}. The results in the presence of cooperativity ($q = 4$) are shown in Fig. 10. In conditions of zero-order ultrasensitivity ($K_1 = K_2 = 0.01$), $\langle W^* \rangle$ varies from nearly zero to one in the domain of β

values inducing Ca^{2+} oscillations; the resulting curve is nevertheless smoother than the corresponding sigmoidal curve obtained for W^* as a function of Z when the level of cytosolic Ca^{2+} remains constant (Fig. 2).

In contrast, when the enzymes operate in the first-order kinetic region ($K_1 = K_2 = 10$ in Fig. 10), $\langle W^* \rangle$ presents only weak changes for various agonist concentrations. Thus, efficient frequency encoding through protein phosphorylation only occurs when the kinase activated by cytosolic Ca^{2+} and the phosphatase are saturated by their substrate; this is a natural consequence of the increased responsiveness of the phosphorylation cycle to variations in the Ca^{2+} level in condition of zero-order ultrasensitivity. A similar conclusion is reached in the absence of cooperativity in kinase activation, i.e. when $q = 1$, although the sigmoidal relationship between $\langle W^* \rangle$ and stimulation level is then less steep [16,19].

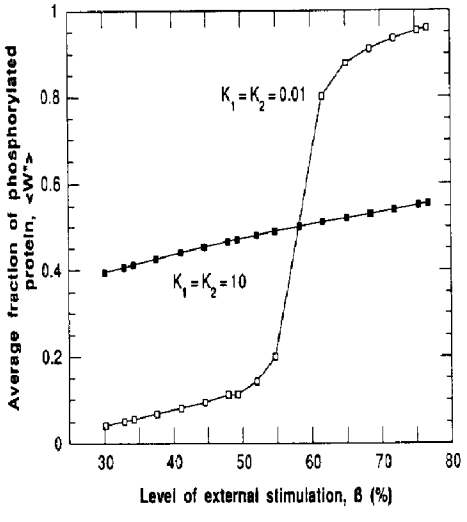

Fig. 10. Frequency encoding of Ca^{2+} oscillations based on protein phosphorylation. Shown is the variation of the mean fraction of a protein phosphorylated by a Ca^{2+}-dependent kinase as a function of the stimulus level in the model for signal-induced Ca^{2+} oscillations. Data (points) are obtained by numerical integration of eqs. (1)–(3) for different values of the level of stimulation, β. For the set of parameter values considered, repetitive Ca^{2+} spiking occurs for $29.4 \leq \beta \leq 76.7\%$ with $v_1 = 7.3\ \mu M\ min^{-1}$. In this range, the frequency of Ca^{2+} oscillations varies from 0.44 to 3.01 min^{-1}. Other parameter values are the same as in Fig. 5 except for $v_{MK} = 100\ \mu M\ min^{-1}$, $v_P = 20\ \mu M\ min^{-1}$ and $K_a = 2\ \mu M$.

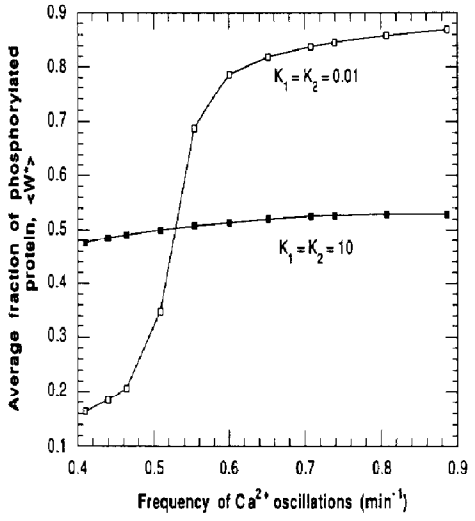

Fig. 11. "Pure" frequency encoding of Ca^{2+} oscillations through protein phosphorylation. The frequency of Ca^{2+} oscillations is raised by decreasing the threshold constant K_R characterizing the release of Ca^{2+} from the $InsP_3$-insensitive pool. As this parameter does not affect the mean Ca^{2+} level ($\langle Z \rangle = 0.34\ \mu M$ for all situations considered), in contrast with the effect of β considered in Fig. 10, this curve reflects a "pure" frequency-encoding. Parameter values are the same as in Fig. 5 with $v_{MK} = 60\ \mu M\ min^{-1}$ and $v_P = 2.67\ \mu M\ min^{-1}$. The data are generated for a range of values limited to those that produce oscillations of nearly constant amplitude in Z, i.e. $1.2\ \mu M \leq K_R \leq 2.9\ \mu M$.

Not taken so far into account is the fact that an increase in stimulation (β) induces not only a higher frequency of Ca^{2+} spiking, but also a larger mean value for the Ca^{2+} concentration. As can be seen from eqs. (1), the latter is indeed equal to the (unstable) steady-state value, i.e. $Z_0 = (v_0 + v_1\beta)/k$. Which of these two effects plays a major role in enhancing the mean level of phosphorylated protein in the course of Ca^{2+} oscillations? One way of addressing this issue is provided by a property of the model based on Ca^{2+}-induced Ca^{2+} release. The frequency of Ca^{2+} oscillations in this model changes, without affecting the mean Ca^{2+} level, with the threshold constant K_R characterizing the release of Ca^{2+} from the $InsP_3$-insensitive pool [16,20]. The pure effect of frequency can thus be determined by altering the value of K_R in eqs. (1). The results shown in Fig. 11 indicate that $\langle W^* \rangle$ may vary over a nearly complete range when the frequency of Ca^{2+} transients is changed by increasing K_R;

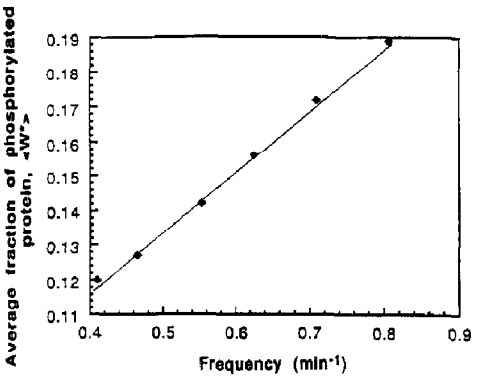

Fig. 12. Absence of efficient frequency encoding of Ca^{2+} oscillations at very large kinase and phosphatase velocities. As W^* switches from nearly zero to one at each Ca^{2+} spike, the average value of phosphorylated substrate becomes a quasi-linear function of the oscillation frequency. The resulting range of average values of W^* is, however, very small, due to the fact that the interspike interval is always much larger than the half-width of the Ca^{2+} spike in this model. Parameter values are the same as in Fig. 11 except for $v_{MK} = 2$ mM min^{-1}, $v_P = 89\ \mu M\ min^{-1}$, $K_a = 0.85\ \mu M$.

once again, however, significant variations occur only in the case of zero-order ultrasensitivity (compare the two curves of Fig. 11). Another prerequisite for efficient frequency encoding in the case where the frequency is changed by altering K_R, is the cooperative activation ($q = 3$ or 4) of the kinase by cytosolic Ca^{2+}. Since the average value of Z remains constant in that case, the sharp dependence of $\langle W^* \rangle$ as a function of frequency in Fig. 11 illustrates the possibility of a "pure frequency encoding".

The above results have been obtained with intermediate values for the maximal velocities of both kinase and phosphatase. As indicated in Section 4.2, the case of very slow converter enzymes does not differ significantly from the steady-state situation [29,31].

On the other hand, a completely different situation is reflected by the quasi-linear relationship between $\langle W^* \rangle$ and the frequency of Ca^{2+} oscillations in the case where the maximal velocities for kinase and phosphatase are very large (Fig. 12). Since W^* goes from nearly zero to one during each Ca^{2+} spike (see Figs. 5a and 6a), the average value of modified substrate is a direct function of the number of spikes over a given time interval when the half-width of each Ca^{2+} spike remains roughly independent of the stimulation, as is the case in both the present model [16,21] and the experiments [3,34]. As the time during which Ca^{2+} concentration is high remains brief when compared with the interspike interval over the whole range of stimulation levels, the range of variations of $\langle W^* \rangle$ remains limited. It thus appears that effective frequency encoding is favored by the closeness between the time scales enhancing Ca^{2+} oscillations and protein phosphorylation.

6. Discussion

The above analysis shows that protein phosphorylation provides a plausible molecular mechanism for the encoding of an extracellular signal in terms of the frequency of the repetitive Ca^{2+} spikes that it generates. As in a preliminary analysis of frequency encoding of Ca^{2+} oscillations based on protein phosphorylation [16,19], a model for Ca^{2+} oscillations based on Ca^{2+}-induced Ca^{2+} release has been used for definiteness as Ca^{2+} spike generator. The occurrence of Ca^{2+}-induced Ca^{2+} release in a number of cells is supported by an increasing number of experiments [22–25]. The results obtained here are, however, largely insensitive to the model used to generate Ca^{2+} oscillations.

In the phosphorylation process considered, a target protein is phosphorylated by a kinase activated by cytosolic Ca^{2+}; dephosphorylation by a phosphatase independent of cytosolic Ca^{2+} reverses this modification. It is assumed that the cellular response to external stimulation is related to the amount of protein phosphorylated by the Ca^{2+}-dependent kinase. The precise relation is probably complex, given the existence of numerous steps between Ca^{2+} elevation and physiological responses; examples of the latter could include secretion [9,11,12,13] or ooplasmic segregation [35].

Results similar to those reported here would of course be obtained, *mutatis mutandis*, if the phosphatase were controlled by Ca^{2+} concentra-

tion, with a kinase unaffected by the level of the latter messenger. An example of such a situation is encountered in nerve cells with calcineurin, a Ca^{2+}-activated phosphatase [36] or in muscle cells with protein phosphatase 2B [37]. On the other hand, enzymes other than kinases or phosphatases could be involved in the transduction of extracellular signals based on intracellular Ca^{2+} variations. Thus, proteases such as calpain can be activated by Ca^{2+} [38]. Interestingly, such Ca^{2+}-activated proteases could, in turn, promote phosphorylation in producing active catalytic fragments of some protein kinases [39,40].

The question arises as to the nature of the kinases and phosphatases that might be controlled by Ca^{2+} oscillations. The multifunctional Ca^{2+}/calmodulin kinase [41] is one attractive candidate because of its action on numerous substrates, but many other Ca^{2+}-activated kinases exist [41,42]. Thus Ca^{2+} oscillations could be particularly effective in hepatocytes where Ca^{2+} is involved in the activation of phosphorylase kinase which plays a crucial role in glycogen metabolism [34,37,43]. Some phosphorylations controlled by Ca^{2+} oscillations may in turn feed back on the mechanism of Ca^{2+} spike generation through modulation of the effect of $InsP_3$; such a possibility is suggested by the observation that the $InsP_3$ receptor can be phosphorylated by a Ca^{2+}-activated kinase [44]. The occurrence of complex oscillations in Ca^{2+} resulting from such a regulation is currently being investigated [21], but falls outside the scope of the present analysis.

The results reported here indicate that protein phosphorylation can serve as a basis for a "pure" frequency encoding if several kinetic conditions are fulfilled. First, both the kinase and the phosphatase have to be saturated by their protein substrate, thus allowing for zero-order ultrasensitivity. This phenomenon, whose occurrence was first predicted theoretically [29], has been demonstrated in several experimental systems [32,33] in steady-state conditions.

The increase in the average fraction of phosphorylated protein with the level of external stimulation (Fig. 10) arises as a combination of two effects since the increased level of agonist produces both a larger frequency of Ca^{2+} oscillations and a higher mean Ca^{2+} concentration. The latter effect of course also enhances protein modification, as shown by the steady-state dependence of protein phosphorylation on Ca^{2+} (see Fig. 2).

To observe a "pure" frequency encoding, we have varied the frequency of Ca^{2+} oscillations by changing a parameter (K_R) that leaves unaltered the mean Ca^{2+} level (Fig. 11). To obtain efficient frequency encoding in such conditions, two additional assumptions have to be made concerning the kinetics of phosphorylation. First, the kinase activation by cytosolic Ca^{2+} has to be characterized by large Hill coefficients, of up to 3 or 4. This condition is probably fulfilled at least for physiological processes where calmodulin is involved [18]. Second, the kinase and phosphatase have to operate on a comparable time scale as Ca^{2+} spikes. When the converter enzymes are too slow, the system responds to the mean level of cytosolic Ca^{2+} and takes no profit of oscillatory Ca^{2+} signals. Conversely, when the converter enzymes act very rapidly, the response follows each Ca^{2+} spike so closely that the mean level of phosphorylation varies only slightly with the frequency of Ca^{2+} oscillations (Fig. 12).

That the average value of phosphorylated protein ($\langle W^* \rangle$) as well as the time required to reach the asymptotic oscillatory regime depend on the maximal velocities of kinase and phosphatase at constant ratio v_{MK}/v_P could provide mechanisms of "selective differential stimulation" [34]. Such a differential modulation of the response could not be attained solely by controlling the amplitude of the Ca^{2+} signal.

The rapidity of evolution towards the asymptotic oscillatory regime may also vary for a given set of kinase and phosphatase at different levels of stimulation. The enhancement in evolution rate seen in Fig. 8 results, as for frequency encoding, from the mixed effect of a rise in β on both the frequency of oscillations and the mean level of cytosolic Ca^{2+}. Indeed, a more rapid evolution to higher steady-state levels of phosphorylated protein would also be seen in the absence of oscillations if the increase in kinase activity were produced by a step increase in Ca^{2+} level (see Fig. 2a in ref. [31]).

It is doubtful that a unique mechanism of

transduction of Ca^{2+} oscillations prevails in all cell types. Another mechanism through which the cellular activity could take some advantage of an oscillating level of Ca^{2+} has been proposed by Meyer and Stryer [45]. The proposed mechanism, called "solitary spike detector" is assumed to be sensitive to the number of spikes rather than to the frequency of Ca^{2+} oscillations. It is also based on reversible protein phosphorylation. Different sites on the kinase are activated at different Ca^{2+} levels (i.e. either during the spike, or during the silent phase) and the phosphorylation sequence is ordered, thus allowing the system to distinguish between various Ca^{2+} profiles. Such a model would require more complex kinetic conditions than the model proposed in the present study.

Some oscillatory cellular responses have been observed following stimulation by agonists inducing Ca^{2+} oscillations. Thus, these oscillations are associated with the periodic release of growth hormone in pituitary somatotropes [11]. As a result, target cells could be stimulated by the hormone in a periodic manner; the advantages provided by Ca^{2+} oscillations would then rely on the avoidance of desensitization to the hormonal signal in target cells [10]. Since Ca^{2+} is known to control secretory processes, it is plausible that periodic protein phosphorylation associated with Ca^{2+} oscillations is involved in periodic hormone secretion. Whether periodic protein phosphorylation plays a significant role in this and other cellular responses associated with Ca^{2+} oscillations remains to be established.

In several cell types, Ca^{2+} signalling takes the form of propagating waves. These spatio-temporal patterns also possess physiological significance as exemplified by ooplasmic segregation in ascidian oocytes [35], secretion in pancreatic acinar cells [13], waves of contractile motion in eggs [46] or cardiac cells [47], or long-range signaling between glial cells within the brain [48]. The present analysis could be extended to include spatial propagation of the Ca^{2+} signal [21]. Through the control of kinases or phosphatases, the spatially inhomogeneous, oscillating patterns of Ca^{2+} ions could transform into propagating waves of protein phosphorylation within the cell.

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