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Mechanism of activation of cyclic nucleotide phosphodiesterase: Requirement of the binding of four Ca^{2+} to calmodulin for activation*

(kinetic analysis/Ca^{2+} regulation/cooperative phenomenon/cellular control)

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ABSTRACT **Kinetic studies on the activation of cyclic nucleotide phosphodiesterase (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) as a function of calmodulin and Ca^{2+} concentrations have been carried out. A general approach to analyzing the mechanism of activation, which takes into consideration the various interactions among phosphodiesterase and calmodulin liganded with Ca^{2+} to differing degrees, is presented. The method is applicable to other calmodulin-regulated enzyme systems. Our kinetic analysis reveals that all four Ca^{2+} must be bound to calmodulin for the protein to form an activated complex with phosphodiesterase. The mechanistic and regulatory advantages of having four Ca^{2+} sites on calmodulin can be briefly stated as follows. (*i*) With the enzyme–calmodulin–Ca_4^{2+} complex as the dominant active species, the activation of phosphodiesterase as a function of Ca^{2+} concentration is highly cooperative. This phenomenon serves as an effective on/off switch for phosphodiesterase activation. (*ii*) At normal cellular levels of Ca^{2+} (<0.1 μM), phosphodiesterase and calmodulin do not form a complex. Thus, the distribution of calmodulin among its various target enzymes is reshuffled for each Ca^{2+} surge. (*iii*) The affinity between the enzyme and the fully liganded calmodulin (0.1–1 mM) is 10^4–10^5 times better than that in the absence of Ca^{2+} (≥10 μM). The tremendous increase in affinity can be achieved rather easily through a 10- to 20-fold increase in the affinity of Ca^{2+} for the enzyme–calmodulin complex in each of the four binding steps.**

Since its discovery by Cheung (1, 2) as a protein activator of mammalian cyclic nucleotide phosphodiesterase (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17), calmodulin has been shown to be capable of binding four Ca^{2+} and stimulating the activities of a number of enzymes (3, 4). A two-step mechanism has been proposed for the activation of phosphodiesterase (5) and other enzymes regulated by calmodulin (6, 7). In this mechanism, Ca^{2+} is first bound to calmodulin to form an activated complex which in turn combines with and activates the target enzyme. As a general, qualitative description, this two-step mechanism is sufficient; but it is inadequate as a model for quantitative analysis because the interactions among phosphodiesterase and the various forms of calmodulin which are liganded with Ca^{2+} to differing degrees are not taken into consideration. Several aspects of the activation process remain obscure. For instance, what is the dominant species of the Ca^{2+}–calmodulin complex that exerts the activating effect on the target enzyme? What are the mechanistic and regulatory advantages provided by having four Ca^{2+} sites on calmodulin?

To answer these questions, we have performed detailed kinetic experiments on the activation of phosphodiesterase as a function of calmodulin and Ca^{2+} concentrations. In this report, a general approach to the treatment of the kinetics of activation of phosphodiesterase and other calmodulin-mediated enzymes will be presented, evidence for the requirement of occupancy of all four Ca^{2+} sites on calmodulin for the activation will be shown, and the mechanistic and regulatory significance of having four Ca^{2+} sites on calmodulin will be discussed.

MATERIALS AND METHODS

Materials. Calmodulin and cyclic nucleotide phosphodiesterase from bovine brain, 95–100% pure, were prepared according to published procedures (8, 9). Rabbit muscle myokinase and beef heart lactic dehydrogenase were purchased from Sigma; rabbit muscle pyruvate kinase was from Boehringer Mannheim. Ultrapure $CaCl_2$ and ultrapure $MgSO_4$ were purchased from Ventron. Ultrapure KCl was the product of BDH Chemicals. All other chemicals were of reagent grade. The buffer and protein solutions were passed through a Chelex 100 column prior to use.

Methods. Initial velocity of phosphodiesterase was measured at 25°C in 50 mM Hepes, pH 7.0/5 mM $MgCl_2$/50 mM KCl/1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). The assay mixtures contained 1 nM phosphodiesterase, varying amounts of calmodulin and Ca^{2+}, and a saturating level of cyclic AMP (4 mM) in a final volume of 1 ml. They were allowed to react for 40 min; then 75 μl of solution containing 53.4 mM $MgSO_4$, 6.67 mM ATP, 26.7 mM phospho*enol*pyruvate, 2.67 mM NADH, 33 units of myokinase, 13 units of pyruvate kinase, 17 units of lactic dehydrogenase, 8 mM EGTA, and 66.7 mM Hepes (pH 7.0) was then added to each assay mixture and the reaction was allowed to proceed for 1 min. In this step, through the coupled reactions of myokinase, pyruvate kinase, and lactic dehydrogenase, each 1 mol of the product AMP led to the oxidation of 2 mol of NADH. The inclusion of excess EGTA reduced the activity of phosphodiesterase to the basal level during this 1-min period. The reaction was stopped by the addition of 100 μl 0.2 M EDTA. The calmodulin-activated activity was determined by taking the difference in absorbance at 340 nm between the samples and a control containing no calmodulin. Control reactions which contained calmodulin or phosphodiesterase alone or neither were routinely performed. Linearity of the assay as a function of time was established when the coupling enzymes and substrates were added at the onset of the reaction. The stepwise assay was used so that the conditions of the enzymatic reaction simulated

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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those of Ca^{2+} binding and molecular weight measurements. The concentrations of free Ca^{2+} in the EGTA-buffered solution were calculated by using an association constant of 1.1×10^6 M^{-1} for Ca^{2+}-EGTA determined under our experimental conditions. The value is comparable to that reported by Ogawa for other buffer systems (10).

RESULTS

Model for the Analysis of Kinetic Studies. Fig. 1 shows the model constructed for kinetic analysis according to the interactions between phosphodiesterase and the various forms of Ca^{2+}-calmodulin complexes. Although the phosphodiesterase is a dimeric enzyme, it is treated as a monomeric enzyme because no cooperative phenomenon was detectable in the binding of two molecules of calmodulin to the enzyme (11). The dissociation constants for the $CM{\cdot}Ca^{2+}$ complexes, K_1, K_2, K_3, and K_4, were determined by fluorescent techniques under conditions similar to the assay conditions (details to be published elsewhere). The values shown in the scheme are comparable to those reported by Crouch and Klee (12) at a somewhat lower level of Mg^{2+} (3 mM) and reveal similar initial positive cooperativity and subsequent negative cooperativity. The activation constant for $CM{\cdot}Ca_4^{2+}$, K_a, was determined by the activation of phosphodiesterase as a function of calmodulin concentration at a saturating level of Ca^{2+} and by measurement of the on and off rate constants of calmodulin. It should be noted that the scheme is intended to describe the degree of saturation of Ca^{2+} for calmodulin and enzyme–calmodulin complexes. Sequential binding of Ca^{2+} is not necessarily implied. Also, because a saturating level of cyclic AMP was used in our kinetic experiments, the symbol E actually represents the enzyme–substrate complex.

From this scheme, a rate equation for the activation of phosphodiesterase by calmodulin and Ca^{2+} can be derived. We shall consider, for the moment, only the case in which the fully liganded $E\text{–}CM\text{–}Ca_4^{2+}$ is the sole activated form:

$$\frac{\Delta v}{E_{total}} = \frac{\Delta k\,(E{\cdot}MC{\cdot}Ca_4^{2+})}{E + E{\cdot}CM + E{\cdot}CM{\cdot}Ca^{2+} + E{\cdot}CM{\cdot}Ca_2^{2+} + E{\cdot}CM{\cdot}Ca_3^{2+} + E{\cdot}CM{\cdot}Ca_4^{2+}}$$

in which Δv = activated activity minus basal activity and Δk = difference between the catalytic rate constants for the activated and nonactivated enzyme species. In order to simplify the analysis of kinetic data, it is necessary to impose the condition that $CM_{total} >> E_{total}$. With this condition, the following rate equation in the reciprocal form is obtained:

$$\frac{1}{\Delta v} = \frac{1}{\Delta v}\left(\frac{\phi_1}{\phi_2} + \frac{\phi_3 K_e}{\phi_2 CM_{total}}\right). \qquad [1]$$

In this equation, $\Delta v = \Delta k{\cdot}E_{total}$

$$\phi_1 = 1 + C/K_1' + C^2/K_1'K_2' + C^3/K_1'K_2'K_3' + C^4/K_1'K_2'K_3'K_4'$$

$$\phi_2 = C^4/K_1'K_2'K_3'K_4'$$

$$\phi_3 = 1 + C/K_1 + C^2/K_1K_2 + C^3/K_1K_2K_3 + C^4/K_1K_2K_3K_4$$

in which C is Ca^{2+} concentration.

When the free Ca^{2+} concentrations are maintained constant by the use of EGTA buffer, Eq. 1 predicts that a double reciprocal plot of $1/\Delta v$ vs. $1/CM_{total}$ will yield a straight line.

Initial rate measurements conducted over 500-fold variation of calmodulin concentration (20 nM–10 μM) and a 10-fold variation of Ca^{2+} concentrations (0.23–2.1 μM) yielded a family of linear lines in double reciprocal plots which appeared to intersect on the ordinate (Fig. 2; only part of the data shown). The linear plots are a support for the validity of Eq. 1 and the scheme in Fig. 1. The observed apparent common intersection point suggests that K_e, the apparent dissociation constant for the complex between is enzyme and unliganded calmodulin large (K_e > 10 μM) relative to the concentrations of calmodulin (up to 10 μM) used in these experiments. An alternative explanation is that $\phi_1 \simeq \Phi_2$ (see Eq. 1). We shall elaborate further on this point in the *Discussion*.

Determination of $E{\cdot}CM{\cdot}Ca_4^{2+}$ as the Activated Form. From Eq. 1, it can be shown that the slope, S, of the double reciprocal plot is given by

$$S = \phi_3 K_e/\Delta v{\cdot}\phi_2$$

$$= \phi_3 K_e K_1'K_2'K_3'K_4'/\Delta v{\cdot}C^4.$$

Rearrangement of the above equation in logarithmic form yields

$$\log(\phi_3/\Delta v{\cdot}S) = 4\log C - \log K_e K_1'K_2'K_3'K_4'. \qquad [2]$$

Thus, if all four Ca^{2+} sites must be filled in order for calmodulin to activate phosphodiesterase, a plot of $\log(\phi_3/\Delta v{\cdot}S)$ vs. $\log C$ should yield a slope of 4. ϕ_3 can be computed from the known values of K_1, K_2, K_3, and K_4 for any given free Ca^{2+} concentration. Furthermore, the intercept of such a plot at $\log C = 0$ should yield the value of $K_eK_1'K_2'K_3'K_4'$, which can be verified by the equilibrium relationship (see Fig. 1):

$$K_eK_1'K_2'K_3'K_4' = K_aK_1K_2K_3K_4$$

The $\log(\phi_3/\Delta v{\cdot}S)$ vs. $\log C$ plot is shown in Fig. 3. A slope of 4.03 ± 0.15 was obtained from this plot by the least squares method. The intercept obtained at $\log C = 0$ is $-\log(K_eK_1'K_2'K_3'K_4') = 30.5$, which is in good agreement with the value of $-\log(K_aK_1K_2K_3K_4) = 29.7$. The analysis strongly suggests that all four Ca^{2+} must be bound before calmodulin can activate phosphodiesterase. Alternatively, it may reflect a very high degree of cooperativity among the four Ca^{2+} sites when calmodulin combines with phosphodiesterase such that the con-

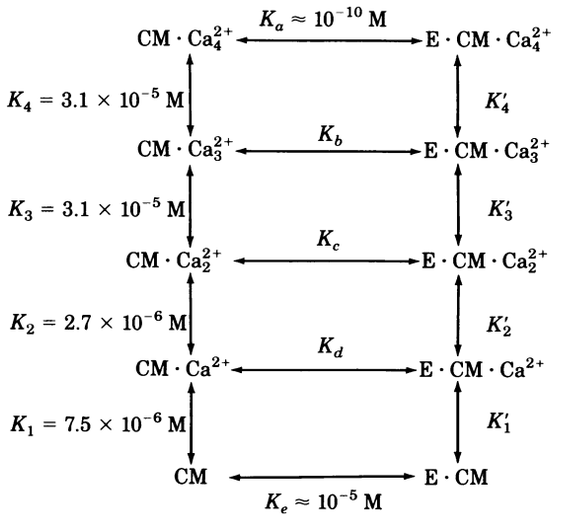

FIG. 1. Model for kinetic analysis. E, enzyme; CM, calmodulin.

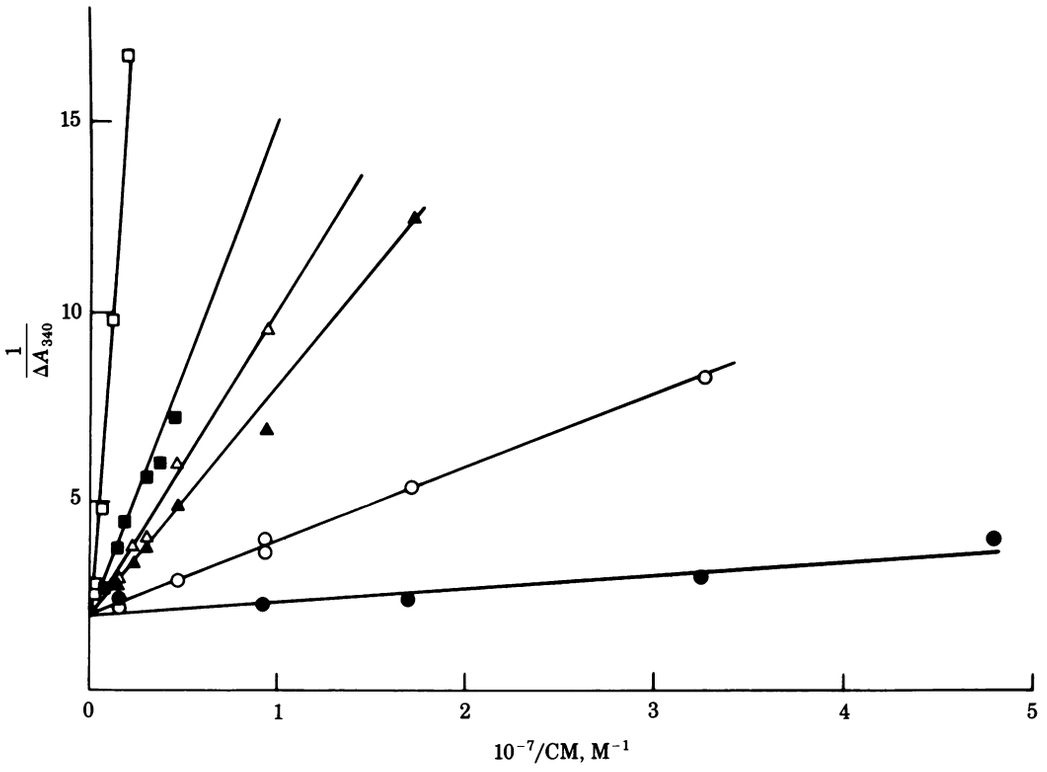

FIG. 2. Double reciprocal plot of the activation of phosphodiesterase by calmodulin at various fixed concentrations of free Ca^{2+}. The free Ca^{2+} concentrations are: □, 0.606 μM; ■, 0.910 μM; △, 1.03 μM; ▲, 1.11 μM; ○, 1.36 μM; and ●, 2.12 μM.

centrations of E·CM, E·CM·Ca^{2+}, E·CM·Ca_2^{2+}, and E·CM·Ca_3^{2+} are negligible relative to the concentration of E·CM·Ca_4^{2+}.

Activation of Phosphodiesterase as a Function of Ca^{2+} Concentration. The highly cooperative phenomenon of Ca^{2+}-dependent activation of phosphodiesterase by calmodulin is shown in Fig. 4. Similar percent activation vs. log Ca^{2+} concentration plots have been presented by others (5, 12–14). However, in our experiments, the concentrations of calmodulin were much higher than those of the enzyme such that the total calmodulin concentrations were equal to the free (not in combination with phosphodiesterase) calmodulin concentration. In fact, the same maximal activation was reached in each curve. Consequently, the data shown in Fig. 4 permit one to interpret the activation of phosphodiesterase in terms of free calmodulin and Ca^{2+} concentrations and to gain some insight into the regulation of phosphodiesterase and other calmodulin-mediated enzymes *in vivo*.

DISCUSSION

Our kinetic treatment of the activation of phosphodiesterase reveals that the binding of all four Ca^{2+} is required in order for

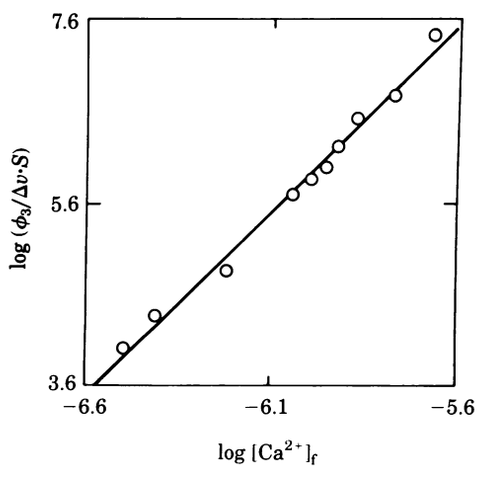

FIG. 3. $\log(\phi_3/\Delta v \cdot S)$ vs. log Ca^{2+} plotted according to Eq. 2. Δv is expressed in terms of $M \cdot min^{-1}$. The slope of this plot, which indicates the number of Ca^{2+} bound to the activated phosphodiesterase–calmodulin complex, was determined by the least squares method to be 4.03 ± 0.15. The intercept of this plot at log Ca^{2+} = 0, which is equivalent to $-\log(K_3K_1'K_2K_3K_4')$, is 30.5.

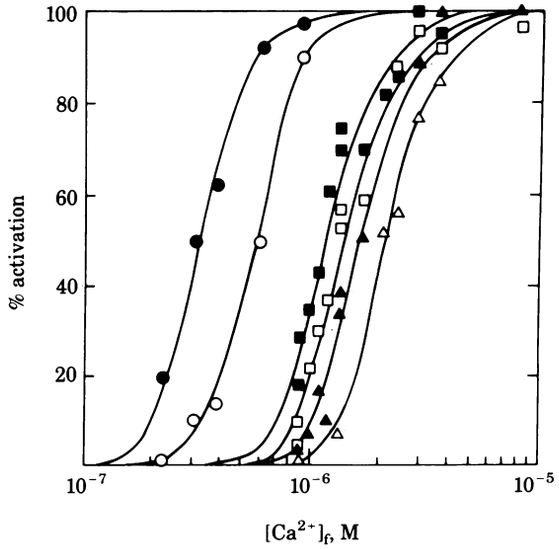

FIG. 4. Percentage activation of phosphodiesterase as a function of free Ca^{2+} concentration at different levels of calmodulin. The calmodulin concentrations are: △, 20.9 μM; ▲, 58.8 μM; □, 0.108 μM; ■, 0.216 μM; ○, 1.93 μM; and ●, 11.6 μM.

calmodulin to form an activated complex with the enzyme. This finding is supported by a slope of 4.03 ± 0.15 obtained from Fig. 3 and by the agreement between $K_eK_1'K_2'K_3'K_4'$ and $K_aK_1K_2K_3K_4$ determined separately from kinetic and binding experiments. Since Eqs. 1 and 2 were derived for the case in which $E{\cdot}CM{\cdot}Ca_4^{2+}$ is the only activated species, one may wonder whether the unsaturated forms—say, $E{\cdot}CM{\cdot}Ca_3^{2+}$,—are not activated. If we assume that both the $E{\cdot}CM{\cdot}Ca_3^{2+}$ and $E{\cdot}CM{\cdot}Ca_4^{2+}$ forms are fully activated, then ϕ_2 becomes $(C^3/K_1'K_2'K_3')(1 + C/K_4')$ and the slope in a log $(\phi_3/\Delta v{\cdot}S)$ vs. log C plot becomes $[3 + 4(C/K_4')]/[1 + (C/K_4')]$. This indicates that, when $[Ca^{2+}] \leqq K_4'$, the plot will be nonlinear and the slope will be considerably less than 4. For example, when $C = K_4'$, the slope is 3.5; when $C = K_4'/10$, the slope is 3.1. If $C = 10\ K_4'$, however, the slope would be 3.9. Because the lowest Ca^{2+} concentration used in our experiment was 0.23 μM, K_4' would have to be ≤10 μM (lower than K_4 by a factor of 3000) to give rise to this situation. In that case, equilibrium consideration dictates that K_b, which reflects the affinity between phosphodiesterase and $CM{\cdot}Ca_3^{2+}$, be 1/3000th of K_a (since $K_bK_4' = K_aK_4$). In other words, the affinity of phosphodiesterase for $CM{\cdot}Ca_3^{2+}$ is negligible compared with that for the fully liganded calmodulin. Clearly, whether or not the unsaturated $CM{\cdot}Ca^{2+}$ complexes can activate phosphodiesterase, $CM{\cdot}Ca_4^{2+}$ is the overwhelmingly dominant form as far as the activation of the enzyme is concerned.

Although the intersection of all the lines in Fig. 2 on the ordinate indicates that $K_e \geq 10$ μM, the situation can also be explained by a K_4' considerably smaller than the Ca^{2+} levels used in our kinetic studies. A small K_4' would make $\phi_2 = C^4/K_1'K_2'K_3'K_4'$ become the dominant term such that $\phi_1 = \phi_2$ (see Eq. 1) and the y intercept assumes a constant value of $1/\Delta v$. However, the estimated value of $K_e \geq 10$ μM is consistent with our molecular weight measurements by the airfuge technique (15). No complex formation between phosphodiesterase and calmodulin in the absence of Ca^{2+} could be detected at protein concentrations on the order of 1 μM (unpublished data).

The regulatory and mechanistic advantages of having four Ca^{2+} sites on calmodulin can be discussed on the basis of the Ca^{2+} activation curves shown in Fig. 4. It can be seen that phosphodiesterase can be fully activated from its basal level by a 10-fold increase in Ca^{2+} concentration. This sharp increase cannot be accomplished if calmodulin had only one Ca^{2+} binding site. The existence of four Ca^{2+} sites on calmodulin, therefore, provides a very effective on/off switch to activate or deactivate phosphodiesterase over a narrow range of Ca^{2+} concentration. It can also be seen from Fig. 4 that the Ca^{2+} level for 50% activation decreases with increasing calmodulin concentration. The implication is that, depending on the levels of calmodulin, the Ca^{2+} concentration needed for the activation of phosphodiesterase in different tissues may be quite different. In this regard, it is interesting to note that the transformation of chicken embryo fibroblasts by Rous sarcoma virus is accompanied by a marked increase in the cellular concentration of calmodulin (16). Another implication is that, at normal cellular Ca^{2+} concentration (<0.10 μM), phosphodiesterase and calmodulin do not form a complex because the highest known total cellular calmodulin concentration (in bovine brain) is about 10 μM (see Fig. 4, solid circles). It is likely that the distribution of calmodulin among its various target enzymes may be reshuffled for each Ca^{2+} surge, depending on the levels of the enzyme's substrates and other ions (e.g., Mg^{2+}) at that moment.

Another advantage of having four Ca^{2+} sites on calmodulin can be appreciated from the 10^4- to 10^5-fold increase in affinity between phosphodiesterase and calmodulin in the absence of Ca^{2+} (recall $K_e \geq 10$ μM) and at a saturating level of Ca^{2+} ($K_a = 10^{-10}$ M at 25°C, 10^{-9} M at 30°C). The tremendous increase in affinity can be achieved relatively easily through a 10- to 20-fold increase in Ca^{2+} affinity for the enzyme–calmodulin complex in each of the four binding steps. If there were only one Ca^{2+} site on calmodulin, such an increase would require a dissociation constant of Ca^{2+} for the $E{\cdot}CM{\cdot}Ca^{2+}$ complex on the order of 10^{-11} M. Such a tight binding would reduce the efficiency of the control process in terms of time. Thirty minutes or more would be needed to deactivate the enzyme completely because of the slow off/rate constant ($\approx 10^{-3}$ s^{-1}, based on an on rate constant for Ca^{2+} of 10^8 M^{-1} s^{-1}; $t_{1/2} \approx 12$ min). The very tight binding of $Cm{\cdot}Ca_4^{2+}$ to phosphodiesterase ($K_a = 10^{-9}$–10^{-10} M) is significant because it permits the enzyme to be fully activated at a free calmodulin concentration of 0.1–10 nM during a surge of Ca^{2+}—that is, practically every molecule of calmodulin is utilized.

It should be noted that the scheme (Fig. 1) described for cyclic nucleotide phosphodiesterase and the kinetic treatment presented in this report should be applicable to other calmodulin-regulated enzyme systems.

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