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THE EFFECT OF PHYSIOLOGICALLY OCCURRING CATIONS UPON AEQUORIN LIGHT EMISSION

DETERMINATION OF THE BINDING CONSTANTS

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SUMMARY

1. The effect of K^+, Na^+, Mg^{2+} and pH upon the rate of aequorin utilization has been investigated in the presence of Ca^{2+}.

2. The aequorin light emission in a medium simulating the in vivo cationic conditions for barnacle muscle fibres indicates that two Ca^{2+} are apparently involved in this process for free calcium concentrations higher than approx. 10^{-5} M. However, for free calcium concentrations lower than 10^{-6} M, the intensity of light emitted by aequorin shows a steeper dependency upon $[Ca^{2+}]$ than the square low relationship, indicating that a third Ca^{2+} should be involved in the process of aequorin light emission, as it has been previously predicted (Moisescu, D. G., Ashley, C. C. and Campbell, A. K. (1975) Biochim. Biophys. Acta 396, 133–140).

3. The inhibitory effect of physiologically occurring cations upon the aequorin light emission can be explained by the cooperative action of two cations, competing with Ca^{2+} for the reactive sites on aequorin.

4. At a given concentration, Na^+ was found to have a stronger inhibitory effect upon the aequoring light emission than K^+.

5. The experiments indicate a strong interaction between Na^+ and K^+ in this inhibitory process, since for a given total concentration of monovalent cations, a mixture containing both Na^+ and K^+ has a larger inhibitory effect on the aequorin light response than solutions containing either Na^+ or K^+ alone.

6. All other interactions between K^+, Na^+, H^+ and Mg^{2+} appear to be weak.

7. The reaction schemes used for the explanation of these and other published results on aequorin (Moisescu, D. G., Ashley, C. C. and Campbell, A. K. (1975) Biochim. Biophys. Acta 396, 133–140 and Blinks, J. R. (1973) Eur. J. Cardiol. 1, 135–142) are described, and the 'absolute' binding constants of all physiologically occurring cations for aequorin have been determined.

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8. Based on these parameters one can make accurate quantitative predictions for the aequoring light response under a variety of ionic conditions, and this suggests that it is possible to determine absolute free calcium concentrations providing that the ionic composition of the solutions is known, and that the relative rate of aequorin utilization is higher than 0.005.

INTRODUCTION

In recent years there has been a considerable interest in the use of calcium-sensitive indicators for the detection of free calcium changes in biological systems [1–3]. In particular, the calcium-sensitive photoproteins aequorin and obelin have been employed to detect calcium changes in cells, especially those associated with the processes of excitation and contraction [2, 4]. Recently, some details concerning the apparent calcium stoichiometry of these photoproteins for the process of light emission has been presented [5]. In this paper the interaction of aequorin with other cations of physiological importance has been investigated in more detail. The influence of these ions upon the process of aequorin light emission can be explained by a competition for the aequorin calcium-binding sites, and on this basis, the absolute binding constants for these cations has been determined.

MATERIALS AND METHODS

Aequorin was extracted from the hydromedusa *Aequorea forskalea*, purified as described elsewhere (see refs. 6 and 7) and lyophilized. Before each experiment aequorin was disolved in 2 M NaCl 'Specpure' solution, which decreases substantially the apparent sensitivity of aequorin for Ca^{2+} (Fig. 3, ref. 5). Small aliquots (30 or 90 nl) of this solution containing approx. 0.1–1 mM aequorin were then added as previously described [5] to the testing solutions (1 ml, if not otherwise specified). The mixing times were of the order of 0.1 s. From the exponential decay of light emission (Fig. 1), an accurate estimate of the overall rate of aequorin utilization can be made. This value was divided by the maximal rate of utilization of the photoprotein, obtained for saturating Ca^{2+} concentration, and expressed on the ordinate of the figures in percent. The chemicals employed were of 'Specpure' grade (Johnson Matthey, London) or equivalent, except for $CaCl_2$ which was 'Analar' grade and

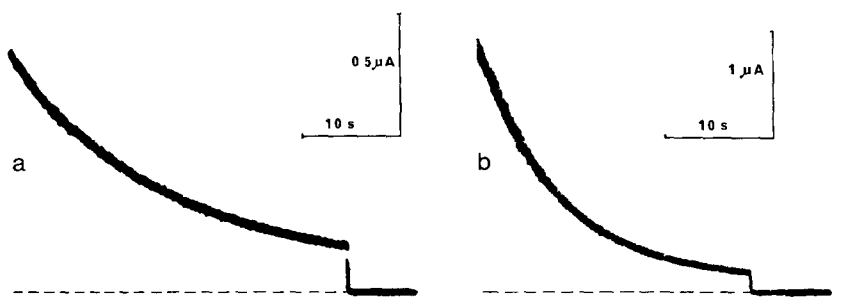

Fig. 1. See opposite page for legend.

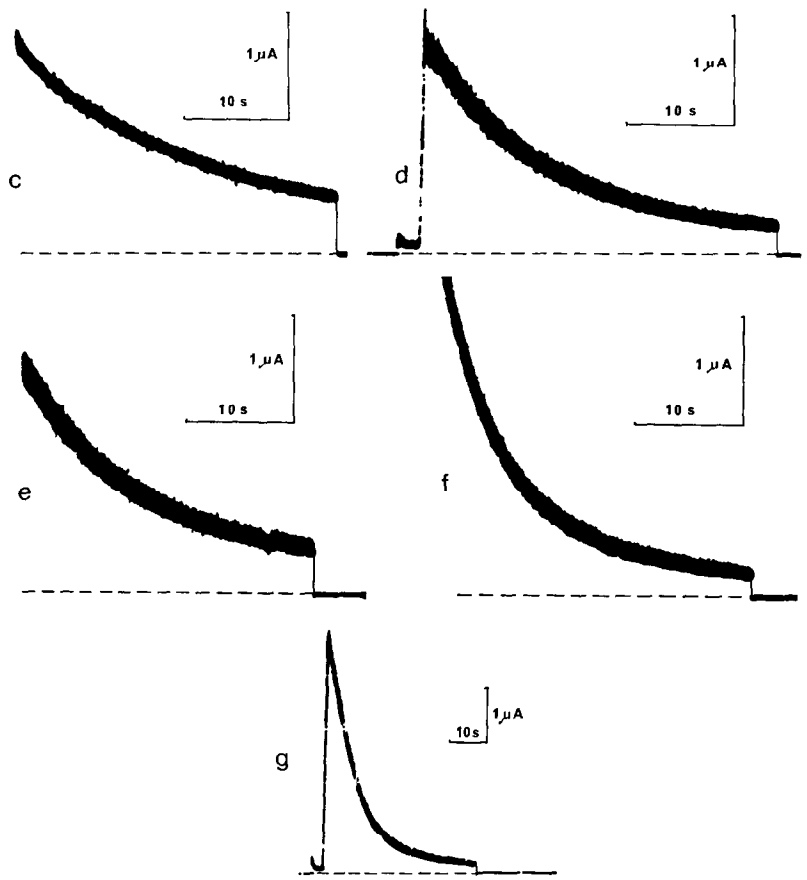

Fig. 1. Records showing the process of aequorin utilization, indicated by the decay in the intensity of emitted light, in the presence of 20 μM unbuffered Ca^{2+} and: (a) 1 M NaCl, pH 7.10; (b) 1 M KCl, pH 7.10; (c) 0.5 M NaCl, 0.5 M KCl, pH 7.10; (d) 1 M KCl, pH 5.00; (e) 0.5 M NaCl, 1.8 mM $MgCl_2$, pH 7.10; (f) 0.5 M KCl, 1.8 mM $MgCl_2$, pH 7.10; (g) 0.25 M NaCl, 0.25 M KCl, pH 7.10. All solutions contained in addition 50 mM TES buffer (pK 7.50 at 20 °C) adjusted with KOH. The amount of aequorin solution added by the procedure described in ref. 5 was approx. 30 nl for traces a, b and approx. 60 nl for c–g. Photomultiplier output filtred with 15 ms time constant in a–c and 2 ms in d–g. The dotted lines in all traces represent the zero line for the current. The background current in these experiments was about 90 nA, and can be observed in the records d and g immediately before the sudden increase in the light intensity, following the addition of the photoprotein. This value should be added to the zero line in order to determine the apparent rate constants for the aequorin utilization. Temperature 23 °C. Maximal rate of aequorin utilization: 1.5 s^{-1} (see also ref. 5). Theoretically predicted rate constants for the traces a–g are mentioned in Table II.

TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid) which was obtained from either Calbiochem or Serva and whose calcium contamination was experimentally determined by flame photometry. In the experiments where the effects of Na^+, K^+, H^+ and Mg^{2+} upon aequorin light emission were investigated, a standard condition of 10 μM activating Ca^{2+} and 50 mM TES was used (see figure legends for details), such that pH was adequately buffered and the added free calcium concentration was much higher than the contamination level.

RESULTS

In previous work [5] we have shown that the process of light emission from aequorin is strongly inhibited by the presence of monovalent cations in the activating solutions. Here this effect is investigated in more detail and in Figs. 1a and 1b the experimental traces illustrate the influence of 1 M NaCl and 1 M KCl, respectively, upon the decay of aequorin light emission in the presence of 20 μM unbuffered Ca^{2+} (see legend). It is clear that of the two ions, sodium has a greater inhibitory effect at a given concentration, and this is reflected by both the intensity of light emission and by the rate constant of the photoprotein utilization (Eqn. 2, ref. 5). The fact that sodium is more effective than potassium at a given concentration in inhibiting the light emission from aequorin is again emphasized by the results presented in Fig. 2, where a more extensive range of sodium and potassium concentrations has been investigated. The inhibitory curve for Na^+ is displaced to lower concentrations compared to the curve for K^+; 50 % inhibition occurring at approx. 0.1 M NaCl compared to approx. 0.18 M KCl. Another interesting effect can be deduced from Fig. 1c, where the experimental record has been obtained in the presence of 0.5 M NaCl plus 0.5 M KCl, and the same pH and $[Ca^{2+}]$ as for the traces a and b in Fig. 1. The rate of aequorin utilization shown by trace c is smaller than either of the rate constants observed in 1 M NaCl or 1 M KCl, and this implies a strong interaction between Na^+ and K^+ in inhibiting the Ca^{2+}-induced light emission from aequorin. An experiment showing directly the decrease in the apparent sensitivity of aequorin

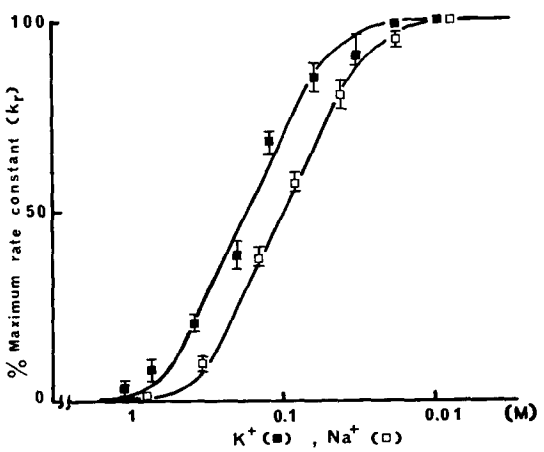

Fig. 2. Semi-logarithmic plot of the apparent relative rate (k_f) of the aequorin utilization as function of $[Na^+]$ (□), and $[K^+]$ (■) in solutions containing 10 μM unbuffered Ca^{2+}. The pH of all solutions has been adjusted to 7.10±0.01 with NaOH for the Na^+ curve and with KOH for the K^+ curve. All solutions contained 50 mM TES buffer with the exception of those for the last point of each curve around 0.01 M, which had only 25 mM. The solutions also contained Cl^-. The experimental points represent the average values from three separate aequorin utilization curves obtained for a given concentration of Na^+ or K^+. The scatter of the results is indicated by vertical bars. Temperature 23 °C. The solid curves were drawn to fit the experimental results using Eqn. A12 and the following apparent affinity constants: $(K_{Na}^{app})^2 \ll K_{NaNa}^{app}$, $K_{NaNa}^{app} = 100\ M^{-2}$; $(K_K^{app})^2 \ll K_{KK}^{app}$, $K_{KK}^{app} = 30\ M^{-2}$. The concentrations of K^+ or Na^+ required to neutralize 50 mM TES at pH 7.10 have been taken into consideration.

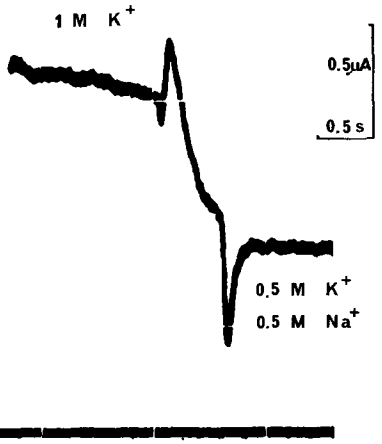

Fig. 3. The change in the light intensity and in the rate of aequorin utilization following the addition of 0.5 ml (1 M NaCl, 50 mM TES buffer, pH 7.10, 20 μM unbuffered Ca^{2+}) to 0.5 ml of a solution in which aequorin has been previously added, and which contained 1 M KCl, 50 mM TES, pH 7.10 20 μM unbuffered Ca^{2+}. The lower trace represents the zero current line. The background current was 100 nA. The mixing time for this kind of experiment was longer than usual since the Na^+ solution was added from a pipette. Time constant for the photomultiplier filter: 15 ms. Temperature 23 °C.

for Ca^{2+}, when K^+ is partly replaced by Na^+ is presented in Fig. 3. Here, the aequorin light response is indicated in the first part of the trace in the presence of 1 M KCl. Subsequently, an equal volume of 1 M NaCl (having the same pH and $[Ca^{2+}]$ as the 1 M KCl solution) is added to the aequorin mixture, which produces a decrease in the intensity of the light emission and an associated increase in the decay time. In control experiments, where equal volumes of 1 M KCl are added to the mixture instead of sodium, there is no decrease in the light intensity. Frequently, a small increase in the light intensity (but no appreciable change in the decay time) is observed, and this may be due to the change in the volume and concentration of the aequorin solution which consequently would change the geometry of the light source. This result is in complete agreement with the results shown in Figs. 1a–1c.

The results presented in Figs. 4a and 4b illustrate the effect of changing $[Mg^{2+}]$ and $[H^+]$, respectively, upon the rate of aequorin utilization. The ionic conditions chosen were such that the ionic strength of the activating solutions did not significantly change by adding $MgCl_2$, or by changing the pH in the activating solutions. In these particular experiments (Figs. 4a and 4b), neither curve achieves 100 % maximum rate constant for aequorin utilization at this temperature and free calcium concentration (10 μM), even when Mg^{2+} and H^+ concentrations are low, because of the presence of relatively high concentrations of K^+ (see legend and compare with Fig. 2). Both curves in Figs. 4a and 4b show a pronounced S-shape when plotted semilogarithmically against pH or pMg ($-\log [Mg^{2+}]$), and the rise from 9 to 91 % of their maximal values takes place over less than two logarithmic units, implying [5] that in each case at least two ions are cooperatively involved in this kind of inhibitory process.

The interaction between the different species of cations which occur physiologically, apart of that between K^+ and Na^+, seems to be relatively weak, since all

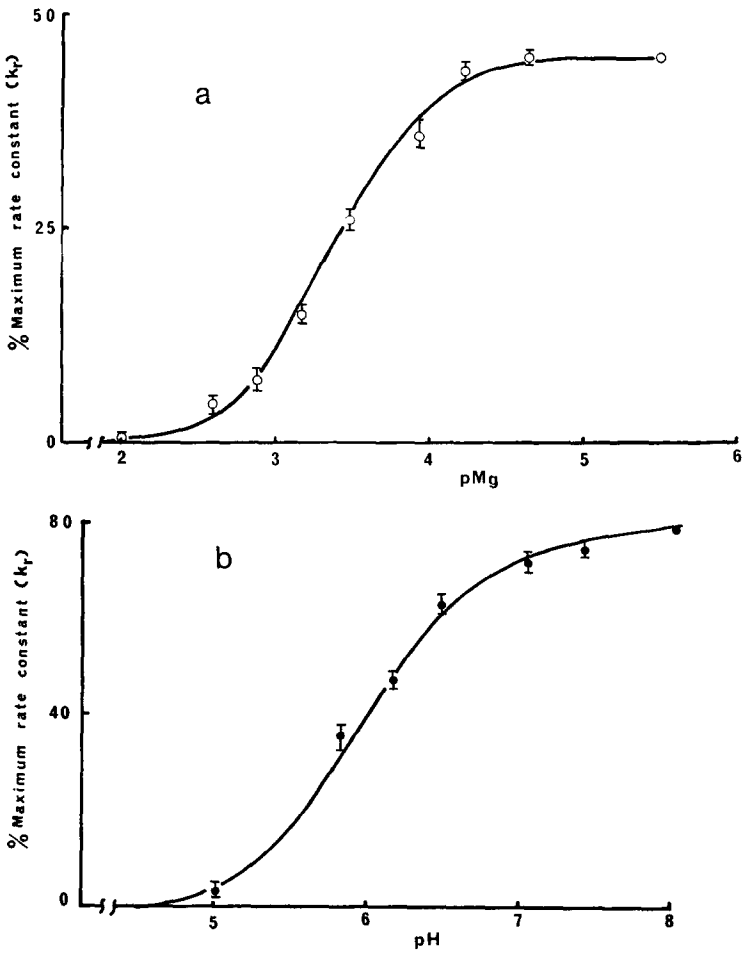

Fig. 4. Relative rate of aequorin utilization (k_r) in the presence of 10 μM unbuffered Ca^{2+}, as function of pMg (= $-\log[Mg^{2+}]$) (a) and pH (b). The solutions for a had 217 mM K^+, 50 mM TES (pH 7.10), 200 mM Cl^-, and various concentrations of $MgCl_2$. The solutions for b had 87 mM K^+, 50 mM TES, and different concentrations of Cl^- to produce the desired pH value. The experimental points (○) and (●) represent the average values from three independent experimental records. The vertical bars indicate the scatter of the results. The solid curves were drawn to fit the experimental results using Eqn. A12, and the following apparent affinity constants: $K_{Mg}^{app} = 1.14 \cdot 10^3$ M^{-1}, $K_{MgMg}^{app} = 3 \cdot 10^6$ M^{-2}; $K_H^{app} = 8.96 \cdot 10^5$ M^{-1}, $K_{HH}^{app} = 1.5 \cdot 10^{11}$ M^{-2}. Temperature 23 °C. The concentration of K^+ required to neutralize 50 mM TES at a given pH value has been taken into consideration.

results obtained in solutions containing a mixture of cations (e.g. Figs. 1d–1g, and 5) can be explained by considering separately the inhibitory effect due to each species of cations (see Table II).

It is also important to note that in a medium containing only very low Na^+ and K^+ concentrations (< 1 mM), the increase of pH up to a value of 10 resulted in a continuous increase in the apparent affinity constants of aequorin for Ca^{2+}, so that aequorin was at this pH maximally activated even in the presence of 10^{-7} M Ca^{2+}.

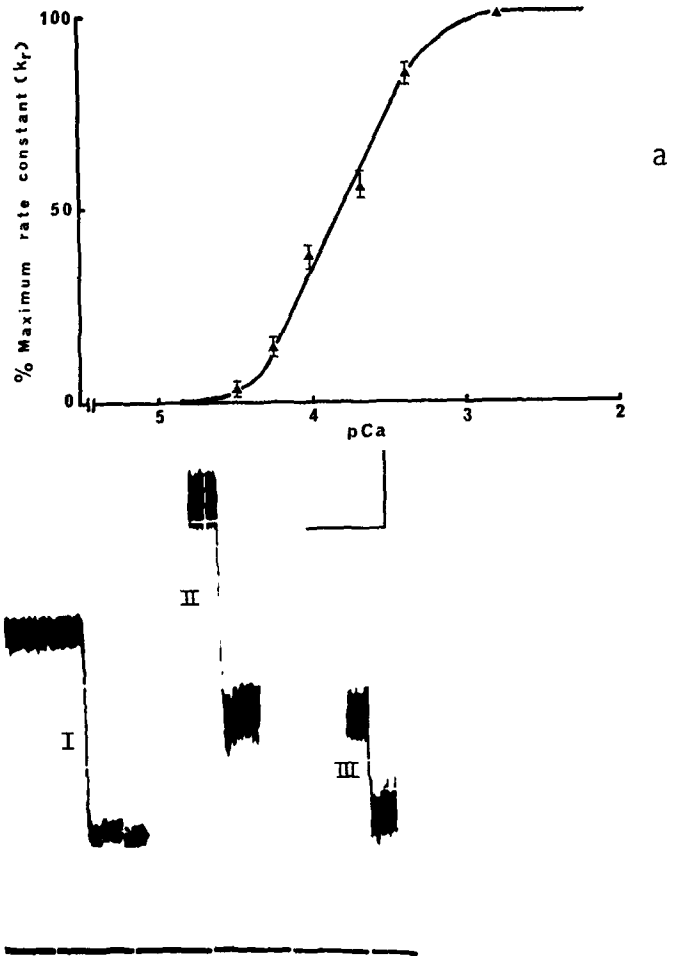

Fig. 5 (a) Relation of aequorin luminiscence indicated by the relative rate of photoprotein utilization, to pCa (= $-\log [Ca^{2+}]$) in a medium containing the following final concentrations (mM): K^+, 155; Na^+, 20; Mg^{2+}, 5; TES, 50 (pH 7.10±0.01); $Cl^- \cong 170$, to which were added various amounts of $CaCl_2$. Temperature 23 °C. The experimental points are average values for three independent measurements at a given $[Ca^{2+}]$ and the vertical bars indicate the scatter of the results. The solid line represents the predicted curve based on Eqn. A8 and on the values of the parameters indicated in Table I (see also the text). (b) Aequorin light response in a medium similar to that in Fig. 5a, in which $[Ca^{2+}]$ has been gradually decreased from pCa 6.38 to 6.78 by using Ca EGTA buffers. The apparent affinity constant of Ca^{2+} to EGTA in the presence of 5 mM $[Mg^{2+}]$ and pH 7.10 was considered $4.9 \cdot 10^6$ M^{-1} at 20 °C (see ref. 18). In the 2 ml of the initial solution (see the legend for Fig. 5a), 6 mM Cl^- have been replaced by 1 mM $EGTA^{2-}$ plus 2 mM Ca $EGTA^{2-}$ (pCa 6.38), and the total magnesium concentration was 5.2 mM in order to maintain a free magnesium concentration of 5 mM [18]. The aequorin concentration in this solution was approx. 0.5 μM. The first drop in the light intensity (I) was due to the addition of 10 μl of a solution containing 100 mM EGTA, 23.7 mM $MgCl_2$ adjusted with NaOH to pH 7.10 (5 mM Mg^{2+}, [18]) which caused a pCa increase of 0.176 unit. The second drop in the light intensity (II) was due to a further addition of 10 μl of the 100 mM EGTA solution (5 mM Mg^{2+}) and this resulted in a further increase of pCa by 0.125 unit; and the third drop (III) was caused by an additional 10 μl of the 100 mM EGTA solution (5 mM Mg^{2+}) which again increased the pCa by 0.097 unit. The pH of the solution did not change by more than 0.01 unit during the whole experiment. The light intensity drop was proportional to the free calcium concentration drop to the power 2.5 for I; 2.3 for II and 2.7 for III. Calibration bars: horizontal: 16 s, vertical: 0.2 μA for I and 50 nA for II and III. The base line represents the zero current line. The dark current was 11 nA. Time constant for the photomultiplier filter: 15 ms.

It was interesting to investigate the relationship between $[Ca^{2+}]$ and the aequorin light response in a medium whose cationic composition was similar to that of the invertebrate muscle fibres in which aequorin has been originally used [6]. The sodium and potassium concentrations were taken as the total internal concentration of these ions in barnacle muscle fibres, i.e. 20 and 155 mM, respectively [8], while the free magnesium concentration used was 5 mM [9]. The chosen pH was 7.10, similar to that of large fibres from the crab *Maia* [10]. The results presented in Fig. 5a show the dependency of the relative rate of aequorin utilization upon pCa ($-\log [Ca^{2+}]$) for Ca^{2+} concentrations higher than 10^{-5} M. The points obtained experimentally can be accurately explained by assuming that aequorin apparently involves two Ca^{2+} per functional unit over this pCa range (see Discussion). Since in biological systems aequorin is supposed to operate far from saturation, it was important to reinvestigate the relationship between $[Ca^{2+}]$ and the intensity of light emission for free calcium concentrations in the range 10^{-6}–10^{-7} M. For this purpose we have used in our medium 3 mM CaEGTA buffer ($[Ca^{2+}] < 10^{-6}$ M; EGTA = ethanedioxy-bis(ethylamine)tetraacetic acid), and we have followed directly the decrease in the light intensity after further additions of known amounts of free EGTA. A typical result is presented in Fig. 5b, and it was obtained by gradually decreasing the ratio of CaEGTA/$EGTA_{free}$ (i.e. of the free calcium concentration) from 2 up to 0.8 under otherwise constant conditions (see legend). One can observe that here, the drop in the emitted light is higher than the square of the drop in $[Ca^{2+}]$. This implies that under these circumstances a third Ca^{2+} appears to be involved. The affinity constant associated with this ion should be of the order of 10^6–10^7 M^{-1} and this is 3–4 orders of magnitude higher than the inverse of Ca^{2+} concentration corresponding to 50 % maximum activation in this medium (Fig. 5a).

It is noteworthy that previous measurements of aequorin light emission in this pCa range [5, 12, 13] failed to put in evidence a third Ca^{2+}, which, however, has been observed with obelin [5], and which was expected with aequorin for very low free calcium concentrations [5, 11]. This effect can be easily explained by the influence of the cationic conditions in the medium upon aequorin, indicating that like the activation curve between 1 and 100 % maximal activation, the apparent affinity constant for this Ca^{2+} would also be shifted by the different cationic conditions in the testing solutions.

DISCUSSION

As we have shown above, the intensity of light emitted by aequorin is not a simple function of free calcium concentration, but it also depends upon the ionic conditions in the medium. In order to understand fully the results obtained with aequorin in biological systems one has to find a quantitative explanation for both the present and previously published results with aequorin in vitro.

In Appendix are derived the relationships which follow from the reaction schemes described below, in which Na^+, K^+, H^+ and Mg^{2+} compete with Ca^{2+} for the reactive sites on aequorin (Aeq):

$$\begin{array}{l}
Ca^{2+} + \text{Aeq} \overset{K}{\rightleftharpoons} \text{Ca-Aeq} \\
\text{Ca-Aeq} + Ca^{2+} \overset{K'}{\rightleftharpoons} Ca_2\text{-Aeq} \\
Ca_2\text{-Aeq} + Ca^{2+} \overset{K''}{\rightleftharpoons} Ca_3\text{-Aeq} \xrightarrow{k_2} X \xrightarrow{k_3} \\
Y^* \xrightarrow{k_4} Y + h\nu
\end{array} \qquad (1)$$

$$\begin{array}{l}
K^+ \overset{K_{NaK}}{\rightleftharpoons} \text{KNa-Aeq} \\
+ \\
Na^+ + \text{Aeq} \overset{K_{Na}}{\rightleftharpoons} \text{Na-Aeq} \\
+ \\
Na^+ \overset{K_{NaNa}}{\rightleftharpoons} Na_2\text{-Aeq}
\end{array} \qquad (2)$$

$$\begin{array}{l}
K^+ \overset{K'_{NaK}}{\rightleftharpoons} \text{KNaCa-Aeq} \\
+ \\
Na^+ + \text{Ca-Aeq} \overset{K'_{Na}}{\rightleftharpoons} \text{NaCa-Aeq} \\
+ \\
Na^+ \overset{K'_{NaNa}}{\rightleftharpoons} Na_2\text{Ca-Aeq}
\end{array} \qquad (3)$$

$$\begin{array}{l}
Na^+ \overset{K_{KNa}}{\rightleftharpoons} \text{NaK-Aeq} \\
+ \\
K^+ + \text{Aeq} \overset{K_{K}}{\rightleftharpoons} \text{K-Aeq} \\
+ \\
K^+ \overset{K_{KK}}{\rightleftharpoons} K_2\text{-Aeq}
\end{array} \qquad (4)$$

$$\begin{array}{l}
Na^+ \overset{K'_{KNa}}{\rightleftharpoons} \text{NaKCa-Aeq} \\
+ \\
K^+ + \text{Ca-Aeq} \overset{K'_{K}}{\rightleftharpoons} \text{KCa-Aeq} \\
+ \\
K^+ \overset{K'_{KK}}{\rightleftharpoons} K_2\text{Ca-Aeq}
\end{array} \qquad (5)$$

$$\begin{array}{l}
Mg^{2+} + \text{Aeq} \overset{K_{Mg}}{\rightleftharpoons} \text{Mg-Aeq} \\
+ \\
Mg^{2+} \overset{K_{MgMg}}{\rightleftharpoons} Mg_2\text{-Aeq}
\end{array} \qquad (6)$$

$$\begin{array}{l}
Mg^{2+} + \text{Ca-Aeq} \overset{K'_{Mg}}{\rightleftharpoons} \text{MgCa-Aeq} \\
+ \\
Mg^{2+} \overset{K'_{MgMg}}{\rightleftharpoons} Mg_2\text{Ca-Aeq}
\end{array} \qquad (7)$$

$$\begin{array}{l}
H^+ + \text{Aeq} \overset{K_{H}}{\rightleftharpoons} \text{H-Aeq} \\
+ \\
H^+ \overset{K_{HH}}{\rightleftharpoons} H_2\text{-Aeq}
\end{array} \qquad (8)$$

$$\begin{array}{l} H^+ + \text{Ca-Aeq} \overset{K'_H}{\rightleftharpoons} \text{HCa-Aeq} \\ \qquad\qquad + \\ \qquad\qquad H^+ \overset{K'_{HH}}{\rightleftharpoons} H_2\text{Ca-Aeq} \end{array} \tag{9}$$

$$\begin{aligned} &[\text{Aeq}] + [\text{Ca-Aeq}] + [\text{Ca}_2\text{-Aeq}] + [\text{Ca}_3\text{-Aeq}] + [\text{X}] + [\text{Y}] + [\text{Y}^*] + [\text{Na-Aeq}] \\ &\quad + [\text{Na}_2\text{-Aeq}] + [\text{NaCa-Aeq}] + [\text{Na}_2\text{Ca-Aeq}] + [\text{K-Aeq}] + [\text{K}_2\text{-Aeq}] + [\text{KCa-Aeq}] \\ &\quad + [\text{K}_2\text{Ca-Aeq}] + [\text{KNa-Aeq}] + [\text{KNaCa-Aeq}] + [\text{NaK-Aeq}] + [\text{NaKCa-Aeq}] \\ &\quad + [\text{Mg-Aeq}] + [\text{Mg}_2\text{-Aeq}] + [\text{MgCa-Aeq}] + [\text{Mg}_2\text{Ca-Aeq}] + [\text{H-Aeq}] \\ &\quad + [\text{H}_2\text{-Aeq}] + [\text{HCa-Aeq}] + [\text{H}_2\text{Ca-Aeq}] = [\text{Aeq}]_0 \end{aligned} \tag{10}$$

where $[\text{Aeq}]_0$ represents the initial concentration of aequorin. Here K, K', K'', represent the absolute binding constants of the three Ca^{2+} to aequorin in a consecutive reaction scheme (see Appendix); k_2, k_3, k_4 are the rate constants in the kinetic reaction scheme proposed by Hastings et al. [15] for the process of light emission from aequorin, and K_{X_i}, K'_{X_i}, $K_{X_iX_j}$, $K'_{X_iX_j}$ $(i, j = 1, 2, 3, 4$ where $X_1 = Na$, $X_2 = K$, $X_3 = Mg$, $X_4 = H)$ represent the equilibrium constants of the respective cations with aequorin. We have used explicitly three Ca^{2+} in Eqn. 1 since this number is required for explaining both the results presented in Fig. 5b and the biochemical measurements of Shimomura and Johnson [11]. The reaction steps in which the Ca^{2+} are involved were found to equilibrate much faster than $1/k_3$ (0.01 s at 20 °C) and k_4 is of the order of 10^8–10^9 s^{-1}, so that the rate of aequorin utilization would be controlled by the step k_2 [15]. The other steps in which H^+, K^+, Na^+ and Mg^{2+} bind to aequorin are also considered to equilibrate rapidly, and the aequorin products containing either of these ions are assumed to be inactive in emitting light. We have assumed a competitive inhibition between Ca^{2+} and the monovalent cations Na^+ and K^+, since the inhibitory effect shown by these ions on the process of light emission from aequorin cannot be explained by a change in the ionic strength. Thus for identical ionic strength values, Na^+ has a more pronounced inhibitory effect than K^+ and a combination of Na^+ plus K^+ is even more effective than either of them alone. It is of importance to mention here that the maximum rate constant for aequorin utilization is not significantly affected by the ionic strength over the range indicated in Fig. 2 (see Fig. 2, ref. 14, Figs. 1a and 3 in ref. 5).

Furthermore we had to consider a cooperative participation of minimum two cations per aequorin molecule (Eqns. 2–9) in order to explain the steepness of the inhibitory curves in Figs. 2 and 4 (see ref. 5). The observed interaction between Na^+ and K^+ (Figs. 1a–1c and 3) has been expressed in Eqns. 2–5, and all the other interactions between K^+, Na^+, Mg^{2+}, and H^+ have been here neglected ($K_{X_iX_j} \ll K_{X_iX_i}$, $K'_{X_iX_j} \ll K'_{X_iX_i}$ if $(i,j) \neq (1, 2), (2, 1)$) based on results such as those in Figs. 1d–1g. However, it was necessary to consider a strong interaction between the first Ca^{2+} and K^+, Na^+, Mg^{2+} and H^+ (Eqns. 3, 5, 7 and 9), since otherwise only the apparent affinity constant of the first Ca^{2+} would be expected to shift under various cationic conditions, but not the Ca^{2+} activation curve between 1 and 100 % which is only determined by the last two Ca^{2+} (Appendix). All the Ca^{2+} activation curves so far investigated (Figs. 5a, 6 and 7) can be very well fitted by an equation of the following type:

$$\text{Relative response} = \beta^2 \cdot [Ca^{2+}]^2 (1 + \beta^2 \cdot [Ca^{2+}]^2)^{-1} \tag{11}$$

where β is the inverse of the free calcium concentration corresponding to 50 % maximum activation, and this strongly suggest that under these experimental conditions only two Ca^{2+} are apparently involved in the process of aequorin light emission. As most of the results in this paper are obtained over a pCa range in which only the last two Ca^{2+} are involved, we limit our investigations mainly on the apparent affinity constants of these Ca^{2+}.

The observation that the apparent sensitivity of aequorin for Ca^{2+} changes with pH in a medium containing very low concentrations of K^+, Na^+ and Mg^{2+} indicates that all determinations are necessarily made in the presence of significantly high proton concentrations if pH < 10, so that for all physiologically important determinations,

$$[HCa\text{-}Aeq]+[H_2Ca\text{-}Aeq] \gg [Ca\text{-}Aeq] \tag{12}$$

The parameter k_r, which indicates the apparent degree of activation for aequorin (Eqn. A2 in the Appendix) can be obtained either from the rate constant associated with the process of aequorin utilization ($k_2 \cdot k_r$), or from the initial value of the light intensity ($\gamma \cdot k_2 \cdot k_r \cdot [Aeq]_0$). In the latter case one should know the precise aequorin concentration, and how the quantum yield γ is influenced by the ionic conditions in the medium. We have generally avoided these additional difficulties by determining the value of k_r from the rate constant of aequorin utilization.

Based on Eqns. 1–10 and on the experimental curves presented in Figs. 1c, 2 and 4 one can calculate the values of the parameters indicated in Table I (see Appendix). We have chosen to express these aequorin constants as fractions of the product $K' \cdot K''$ (see Eqn. 1) in order to facilitate the calculation of the apparent affinity constant of the last two Ca^{2+} for aequorin under any ionic conditions.

It is of importance to test the validity of our assumptions and to compare other experimental curves for the aequorin light response obtained under a variety of ionic conditions, with the theoretical predictions based on the Eqns. 1–10 and on the absolute affinity constants in Table I. Should we start first with the results shown in Figs. 1a, 1b, 1d–1g. Considering that for 23 °C, k_2 has a value of 1.5 s^{-1}, one can predict the value of the relative rate of aequorin utilization, k_r (Eqn. A11 in Ap-

TABLE I

THE ABSOLUTE AFFINITY CONSTANTS FOR AEQUORIN AT 20 °C

Cation	Affinity constant(s)	Value	Units	Observations
Ca^{2+}	K^2	$> 10^4\ K'K''$		
	$K' \cdot K''$	$> 10^{15}$	M^{-2}	$K' \ll K''$
H^+	$K'_H/K' \cdot K''$	$1.12 \cdot 10^{-4}$	M	
	$K'_H \cdot K'_{HH}/K' \cdot K''$	18.7	–	$K'_{HH} = 1.67 \cdot 10^5\ M^{-1}$
Mg^{2+}	$K'_{Mg}/K' \cdot K''$	$3 \cdot 10^{-7}$	M	
	$K'_{Mg} \cdot K'_{MgMg}/K' \cdot K''$	$7.9 \cdot 10^{-4}$	–	$K'_{MgMg} = 2.63 \cdot 10^3\ M^{-1}$
K^+	$K'_K \cdot K'_{KK}/K' \cdot K''$	$3.27 \cdot 10^{-9}$	–	$K'_K \ll K'_{KK}$
Na^+	$K'_{Na} \cdot K'_{NaNa}/K' \cdot K''$	$1.1 \cdot 10^{-8}$	–	$K'_{Na} \ll K'_{NaNa}$
K^+, Na^+	$(K'_{Na} \cdot K'_{NaK}+K'_K \cdot K'_{KNa})/K' \cdot K''$	$3.3 \cdot 10^{-8}$	–	

TABLE II

OBSERVED AND PREDICTED RATE CONSTANTS FOR AEQUORIN UTILIZATION ($k_2 \cdot k_r$)

The value for k_2 was 1.5 s^{-1} (see legend Fig. 1). The rate constant measured from Fig. 1c has been used for the calculation of the parameters in Table I (see Appendix), and therefore was not included in this table.

Fig. 1	Observed rate constants (s^{-1})	Predicted rate constants * (s^{-1})
a	0.06	0.05
b	0.14	0.158
d	0.085	0.09
e	0.084	0.092
f	0.14	0.137
g	0.155	0.17

* It has been also taken into consideration the amount of K^+ required to neutralize TES at pH 7.10.

pendix) for each particular case. The calculated values for the rate constants ($k_2 \cdot k_r$, Eqn. A2) associated with the decay in the light emission are listed together with the observed values in Table II, and one can see that these values are very similar.

Based on the values of the parameters indicated in Table I one can calculate $K' \cdot K''/\delta$ in Eqn. A10 as $4.65 \cdot 10^7$ M^{-2} for the medium simulating the cationic conditions in vivo for barnacle and in Fig. 5a we have plotted the curve predicted by Eqn. A10 independent of the experimental results. One can observe that the agreement between the "predicted" curve and the "observed" results is very good indeed. In this medium the photoprotein operates far from saturation for free calcium concentrations lower than 10^{-5} M. Considering that the resting $[Ca^{2+}]$ in barnacle muscle fibres is under 10^{-6} M [12, 16], it follows from Eqns. A6 and A10 and Fig. 5b that the rate constant for aequorin utilization in resting state at 10 °C is even smaller than initially estimated [17], yielding a half-time of several weeks. On the other hand, the results from Fig. 5b might lead to a higher number than two for the Ca^{2+} involved in producing tension in barnacle [17]. One can show that a pH change in this solution from 7.1 to 6.5 would only produce a minor change (<0.3 %) in the aequorin light intensity for $k_r > 0.005$. This indicates that if $[Mg^{2+}]$ remains constant, the aequorin light emission curve will follow a Ca^{2+} transient, even if the pH did change in the mentioned range. However, a change in the intracellular $[Mg^{2+}]$ during the Ca^{2+} transient will be strongly reflected by the aequorin light response.

It is important to use the values in Table I to predict other published experimental observations. In Fig. 6 the experimental points are the measured relative rate constants for aequorin utilization at different free calcium concentrations, using CaEGTA buffers in Figs. 6a and 6b and unbuffered calcium solutions in the presence of a high Na^+ concentration in Fig. 6c (see ref. 5). Since the affinity constant of Ca^{2+} to EGTA was found to be approx. 25 % smaller [18] than that previously considered for the conditions in Figs. 6a and 6b, the experimental points have been shifted by 0.1 pCa unit in comparison with Fig. 1a in ref. 5. The solid lines in Figs. 6a and 6b represent the predicted curves for the aequorin response in the respective cationic

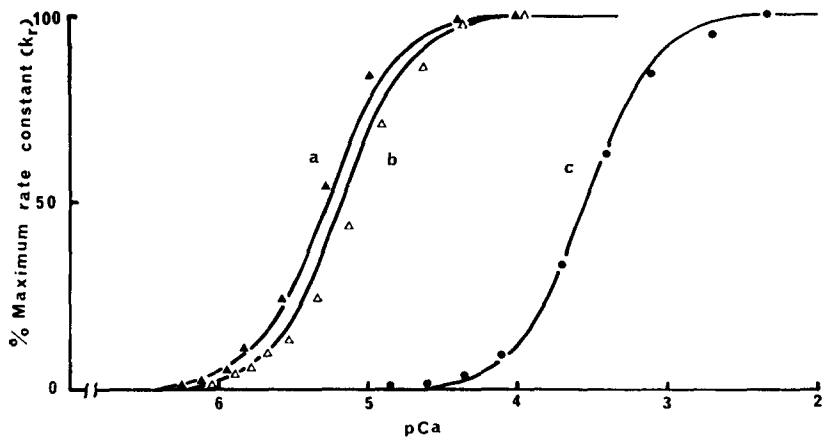

Fig. 6. Experimental data (▲, △, ●) from ref. 5, and theoretical predictions (solid lines) for the relative rate of aequorin utilization as function of pCa under different ionic conditions: (a) 83 mM K^+, 20 mM Ca EGTA buffer, 10 mM TES (pH 7.10±0.01); (b) 83 mM K^+, 20 mM Ca EGTA buffer, 10 mM TES (pH 6.80±0.01); (c) 2.83 M Na^+, 17 mM K^+, 50 mM TES (pH 7.10±0.01). The experimental data obtained in the presence of EGTA have been translated by 0.1 pCa unit in comparison with those in Fig. 1a from ref. 5 (see text for details). The predicted continuous curves have been drawn according to Eqn. A10 with the values of $K' \cdot K''/\delta$ ($3.2 \cdot 10^{10}$ M^{-2} for a; $2.45 \cdot 10^{10}$ M^{-2} for b; $1.11 \cdot 10^{7}$ M^{-2} for c) calculated from Table I. The concentrations of K^+ necessary to neutralize TES at pH 7.10 have been taken into consideration.

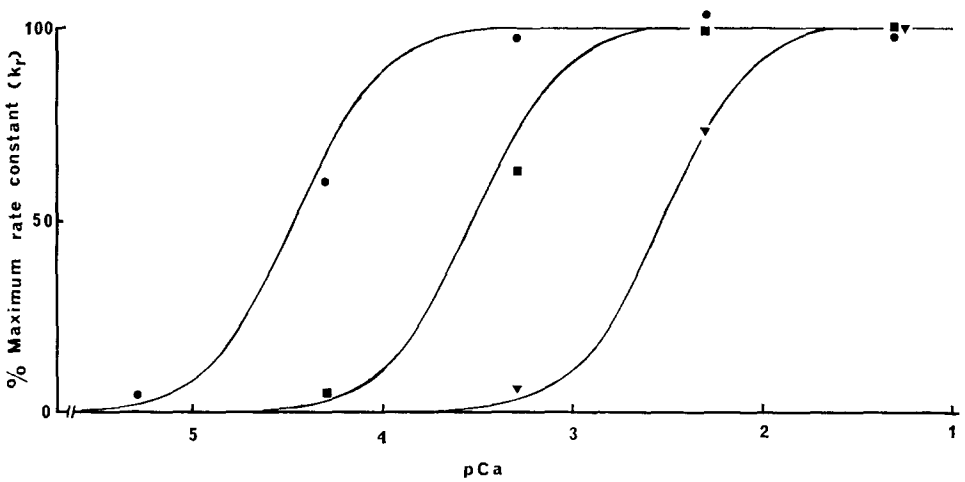

Fig. 7. Relative rate of aequorin utilization as function of pCa observed by Blinks [14] in the presence of 1 mM (●), 10 mM (■), and 100 mM (▼) Mg^{2+}, and the corresponding theoretical curves (continuous lines) predicted by Eqn. A10 for $K' \cdot K''/\delta$ ($9.17 \cdot 10^{8}$ M^{-2} for (●), $1.22 \cdot 10^{7}$ M^{-2} for (■), $1.26 \cdot 10^{5}$ M^{-2} for (▼)) calculated from Table I. Although the pH of the solutions was not known, one can calculate that if the pH values were higher than 6, then the experimental results would be shifted by maximum 0.025 pCa unit.

environment, and they approach closely the experimental observations. The agreement between the predictions and the experiments supports the suggestions that EGTA has no unexpected effect upon the photoprotein [5], and that the affinity constant of Ca^{2+} to EGTA in these solutions is closer to that measured by Schwarzenbach [19] rather than that reported by Ogawa [20].

The marked decrease of the aequorin sensitivity for Ca^{2+} in the presence of 2.83 M Na^+ [5] is very well predicted by the continuous line in Fig. 6c, which was calculated according to Eqns. A10 and A11.

In Fig. 7 are plotted the experimental results reported by Blinks [14] in the presence of significant free magnesium concentrations so that the lack of knowledge of the exact pH will not significantly affect the predictions as long as the actual value was higher than 6. The agreement between our predicted curves (the continuous lines in Fig. 7) and the experimental data of Blinks [14] is surprisingly good, proving that the affinity constants and the stoichiometry of cations in the process of aequorin light emission are not significantly different from those described in the present paper, although the aequorin purification procedures employed by Blinks [14] were more extensive.

The observations presented here, which show that Na^+ and K^+ are not identical in their effect upon the aequorin light emission might provide a clue for the explanation of some interesting results obtained on giant axons injected with aequorin and stimulated electrically [13].

The results presented in this paper indicate that the inhibitory effect of physiologically occurring cations can be simply interpreted on the basis of competitivity with calcium for the aequorin reacting sites. For conditions far from saturation, and for constant $[Mg^{2+}]$, $[K^+]$, $[Na^+]$, and pH, the aequorin light emission is proportional to $[Ca^{2+}]^2$, as long as the free calcium concentration is higher than the apparent affinity constant of the first Ca^{2+}, K_{app} in Eqn. A6. For lower $[Ca^{2+}]$ the relationship shifts gradually towards a cubic law (Fig. 5b).

This approach permits to make accurate predictions as to the expected behaviour of aequorin light emission under different ionic conditions and should enable even the predictions of absolute free calcium concentration changes for $k_r > 0.005$, if the cationic composition of the environment is precisely known.

Recently, Kretsinger [21] has put forward the hypothesis that all proteins which are Ca^{2+} modulated should contain homologous regions of known spatial configuration, where Ca^{2+} are bound and inversely, that all proteins which contain such regions should be Ca^{2+} modulated. Among these proteins are both aequorin and the regulatory proteins of the muscular contraction. In this context it is interesting to mention that the relationship between the relative isometric tension response and $[Ca^{2+}]$ is influenced in a very similar way by the monovalent metallic cations [22], pH and $[Mg^{2+}]$ [23, 24] as the Ca^{2+}-induced aequorin light response. This observation might reinforce the likelihood that Kretsinger's hypothesis is correct, and if so, it would predict that in all other Ca^{2+}-regulated proteins, the monovalent cations such as Na^+ and K^+ would play a more direct role in inhibiting the respective Ca^{2+}-modulated process than is usually considered.

APPENDIX

Deduction of formulae

It was considered that the rate-limiting step in the reaction schemes 1–9 is the step k_2, so that all the reaction steps in which the cations are involved can be always assumed in steady state for the kind of experiments described in this paper. We have assumed that the cations react in a consecutive kind of scheme (Eqns. 1–9) since this kind of cooperativity is most general from a mathematical point of view for steady-state conditions, including all the other reaction schemes which involve cooperatively the same number of cations (see e.g. refs. 24 and 25).

From Eqn. 1 it follows directly that the intensity of light emitted by aequorin, L, is given by the expression:

$$L = \gamma \cdot k_2 \cdot [\mathrm{Ca_3\text{-}Aeq}] \tag{A1}$$

where γ is the quantum yield. Based on Eqns. 1–9 one can express $[Ca_3\text{-Aeq}]$ as a function of time, t, and of the cationic concentrations of Ca^{2+}, Mg^{2+}, Na^+, K^+ and H^+:

$$L = \gamma \cdot k_2 \cdot k_r \cdot [\mathrm{Aeq}]_0 \cdot \exp(-k_r \cdot k_2 \cdot t) \tag{A2}$$

where

$$k_r = K \cdot K' \cdot K'' \cdot [Ca^{2+}]^3 \cdot \{1+K \cdot [Ca^{2+}](1+\delta)+K \cdot K' \cdot [Ca^{2+}]^2+ \\ +K \cdot K' \cdot K'' \cdot [Ca^{2+}]^3+\alpha\}^{-1} \tag{A3}$$

and

$$\alpha = (K_{Na} \cdot K_{NaK}+K_K \cdot K_{KNa}) \cdot [Na^+] \cdot [K^+]+ \sum_{i=1}^{4} K_{X_i} \cdot [X_i](1+K_{X_iX_i} \cdot [X_i]) \tag{A4}$$

$$\delta = (K'_{Na} \cdot K'_{NaK}+K'_K \cdot K'_{KNa}) \cdot [Na^+] \cdot [K^+]+ \sum_{i=1}^{4} K'_{X_i} \cdot [X_i](1+K'_{X_iX_i} \cdot [X_i]) \tag{A5}$$

where X_i, K'_{X_i}, K_{X_i}, $K_{X_iX_i}$, $K'_{X_iX_i}$ have the same meaning as in Discussion. Dividing both the nominator and the denominator in the right part of Eqn. A3 by the product $(1+\alpha)(1+\delta)$, and substituting $K \cdot (1+\alpha)^{-1}$ with K_{app} and $K' \cdot (1+\delta)^{-1}$ with K'_{app}, one obtains the following expression for the relative rate constant, k_r:

$$k_r = K_{app} \cdot K'_{app} \cdot K''[Ca^{2+}]^3/(1+K_{app} \cdot [Ca^{2+}]+K_{app} \cdot K'_{app} \cdot [Ca^{2+}]^2 \\ +K_{app} \cdot K'_{app} \cdot K'' \cdot [Ca^{2+}]^3) \tag{A6}$$

Taking into account that all the Ca^{2+} activating curves for aequorin in the range 0.5–100 % are very well fitted by a relationship of the type shown by Eqn. 11 (Figs. 5–7) it is important to see under which conditions Eqn. A6 reduces to Eqn. 11 for $k_r > 0.005$. One can show that if

$$K_{app} > 100\,(K'_{app} \cdot K'')^{\frac{1}{2}} \quad \text{and if} \quad K'_{app} < 0.01\,K'' \tag{A7}$$

then for $k_r > 0.005$ one can approximate Eqn. A6 with

$$k_r \cong K'_{app} \cdot K'' \cdot [Ca^{2+}]^2/(1+K'_{app} \cdot K'' \cdot [Ca^{2+}]^2) \tag{A8}$$

which is of the same kind as Eqn. 11. One can directly observe that if were was no

competition between the second Ca^{2+} and the other cations X_i ($i = 1, 2, 3, 4$), then $\delta = 0$ and the activation curve for $k_r > 0.005$ could not be shifted by the different cationic conditions in the medium. On the other hand, if no competition exists between the first Ca^{2+} and the other physiologically occurring cations for aequorin, then $\alpha = 0$, and the affinity constant of the first Ca^{2+} will not change under various ionic conditions in the medium. If so, it will be difficult to explain why not all the three Ca^{2+} have been previously identified in the experiments measuring the aequorin light intensities over the same pCa range as in Fig. 5b.

From Eqn. 12 in Discussion it follows directly that the concentration of Ca-Aeq is negligible small in comparison with the sum of all the other aequorin forms X_iCa-Aeq, X_iX_jCa-Aeq (see Eqn. 10) in any medium of physiological interest, and this is equivalent to say that:

$$\delta \gg 1 \tag{A9}$$

Therefore Eqn. A8 can be written as:

$$k_r \cong (K' \cdot K''/\delta) \cdot [Ca^{2+}]^2 \cdot \{1+(K' \cdot K''/\delta) \cdot [Ca^{2+}]^2\}^{-1} \tag{A10}$$

If the values of K'_{X_i}, $K'_{X_iX_j}$ were known, one could determine the value of δ for any cationic concentrations, and thus one could predict the whole curve for $k_r < 0.005$ as a function of $[Ca^{2+}]$ for these conditions. By dividing both the nominator and denominator in the right term of Eqn. A10 by $K' \cdot K'' \cdot [Ca^{2+}]^2/\delta$, and taking into consideration the detailed expression for δ (Eqn. A5), it follows:

$$k_r \cong \{1+(K'_K \cdot K'_{KNa}+K'_{Na} \cdot K'_{NaK})[Na^+] \cdot [K^+] \cdot [Ca^{2+}]^{-2}/K' \cdot K''+\sum_{i=1}^{4}(K'_{X_i}/K' \cdot K'') \cdot [X_i] \cdot [Ca^{2+}]^{-2}+\sum_{i=1}^{4}(K'_{X_i} \cdot K'_{X_iX_i}/K' \cdot K'') \cdot [X_i]^2 \cdot [Ca^{2+}]^{-2}\}^{-1} \tag{A11}$$

If the solutions contain either Na^+ or K^+ as for Figs. 2 and 4, then it is more convenient to express k_r as a function of the apparent affinity constants $K_{X_i}^{app}$, $K_{X_iX_i}^{app}$:

$$k_r \cong K' \cdot K'' \cdot [Ca^{2+}]^2 \cdot \{\varepsilon_i \cdot (1+K_{X_i}^{app} \cdot [X_i]+K_{X_iX_i}^{app} \cdot [X_i]^2)\}^{-1} \tag{A12}$$

where $K_{X_i}^{app} = K'_{X_i}/\varepsilon_i$ and $K_{X_iX_i}^{app} = K'_{X_i} \cdot K'_{X_iX_i}/\varepsilon_i$. The expression for ε_i can be simply generalized if the solutions contain either Na^+ or K^+, as is the case for the results in Figs. 2 and 4.

$$\varepsilon_i = K' \cdot K'' \cdot [Ca^{2+}]^2+\delta-K'_{X_i} \cdot [X_i] \cdot (1+K'_{X_iX_i} \cdot [X_i]) \tag{A13}$$

The experimental curves presented in Figs. 1c, 2 and 4 were fitted by the Eqns. A11 or A13, resulting a system of nine equations with nine unknowns, from which were calculated the values of the parameters indicated in Table I. We have chosen to express these aequorin constants as a fraction of the product of the absolute Ca^{2+} affinity constants $K' \cdot K''$, in order to facilitate the calculation of the apparent affinity constant of Ca^{2+} for aequorin, $K' \cdot K''/\delta$ (Eqn. A10) for any ionic conditions, and thus to calculate the Ca^{2+} activation curve for $k_r > 0.005$.

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