Biochem. J. (1984) **221**, 907–910
Printed in Great Britain

Effect of calcium chelators on the Ca^{2+}-dependent luminescence of aequorin

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(*Received 27 February 1984/Accepted 23 May 1984*)

The luminescence of aequorin, a useful tool for studying intracellular Ca^{2+}, was recently found to be inhibited by the free EDTA and EGTA that are present in calcium buffers. In the present study we have examined the effect of the free forms of various chelators in the calibration of $[Ca^{2+}]$ with aequorin. Free EDTA and EGTA in low-ionic-strength solutions strongly inhibited the Ca^{2+}-triggered luminescence of aequorin, causing large errors in the calibration of $[Ca^{2+}]$ (approx. 2 pCa units), whereas in solutions containing 150mM-KCl, errors were relatively small (0.2–0.3pCa units). Citric acid in low-ionic-strength solutions and [(carbamoylmethyl)imino]diacetic acid in high-ionic-strength solutions showed no inhibition and did not cause detectable error in the calibration of $[Ca^{2+}]$, indicating that they are better chelators than EDTA and EGTA for use with aequorin.

The photoprotein aequorin (Shimomura *et al.*, 1962; Blinks *et al.*, 1976) emits light in aqueous solutions when a trace amount of Ca^{2+} is added, independently of the presence of O_2. Because of its sensitivity, specificity and harmlessness, aequorin has been extensively used in the studies of Ca^{2+} in various biological systems and has been especially successful in the research on intracellular Ca^{2+}.

EDTA and EGTA have been routinely used in the past to prepare calcium buffers for the calibration of aequorin luminescence. However, recent findings that they inhibit the luminescence, not only by chelating with Ca^{2+} but also by directly interacting with the molecules of aequorin (Shimomura & Shimomura, 1982; Ridgway & Snow, 1983), cast serious doubt on the use of these chelators and on the credibility of certain data previously obtained by using them.

The objective of the present study was to determine the extent of errors that would be caused when EDTA or EGTA was used and also to find suitable calcium buffers for calibrating aequorin luminescence.

Materials and methods

Chemicals

Aequorin (luminescence activity 4×10^{15} quanta/mg at 25°C) was obtained as previously described (Shimomura & Johnson, 1976). Ada, Bicine, EGTA, glycylglycine, Mops, NTA and Arsenazo III were purchased from Sigma Chemical Co. EDTA and citric acid (reagent grade) were from MCB Manufacturing Chemists, Norwood, OH, U.S.A.; KCl and NaOH were Suprapur grade manufactured by E. Merck, Darmstadt, Germany. Distilled deionized water with a resistance of 18MΩ was used throughout this work.

HCl used for washing ion-exchange resin was of reagent grade. For adjusting the pH of buffer solutions, reagent-grade HCl was distilled as follows. Conc. (37%, w/v) HCl (20ml) in a 100ml flask equipped with a silicone-rubber stopper and Teflon tubing (5mm internal diameter, 40cm long) through the stopper was carefully heated, and HCl gas evolved from the end of the tubing was adsorbed into 20ml of cold water contained in a Teflon bottle.

Procedures

All solutions were made and stored in bottles of Teflon or polycarbonate, and measured with Eppendorf pipettes. The measurements of light were done with a calibrated photomultiplier apparatus (Shimomura *et al.*, 1972). To initiate the luminescence reaction, 2ml of buffer solution to be tested was quickly injected into a polycarbonate test tube containing 2μl of aequorin solution that contained 10μM-EDTA. The injection was done with an all-plastic syringe equipped with a stainless-steel needle (14 gauge, with plastic hub) which was the only non-plastic substance that came in contact with the test solution.

Abbreviations used: Ada, [(carbamoylmethyl)imino]diacetic acid; Bicine, *NN*-bis-(2-hydroxyethyl)glycine; Mops, 4-morpholinepropanesulphonic acid; NTA, nitrilotriacetic acid.

Calcium buffers

The concentration of the free form of a chelator (not chelated with a metal ion) was kept constant irrespectively of the pCa values of the solutions tested, because only the free form appears to inhibit luminescence by directly interacting with aequorin (Shimomura & Shimomura, 1982). Thus calcium-buffer solutions to be tested (2ml each) having various pCa values were prepared by mixing the following six components in a 10ml Teflon beaker.

(1) A fixed amount of pH-buffer solution, i.e., 20 or 40μl of 100mM-Mops, pH7.0, to give a final concentration of 1mM or 2mM.

(2) A fixed amount of the calcium-free solution of a chelator, pH7.0, to give a final concentration of 1mM or 2mM.

(3) An amount of calcium acetate solution that would give the intended pCa value if alone diluted to 2ml with water. The addition of calcium acetate was omitted if the amount was less than 1/300 of the calcium in the calcium complex of the chelator to be used [see (4)].

(4) Calcium-complex solution of the chelator (CaL), pH 7.0, for which the amount to be used was calculated from the apparent stability constant ($K_{app.}$; see Table 1), the concentration of free chelator [L; see (2) above], and the concentration of free Ca^{2+} [see (3)] by the following equation:

$$K_{app.}[Ca^{2+}][L] = [CaL]$$

The apparent stability constant at pH7.0 was calculated as described by Schwarzenbach (1957) from the pK_a values of the chelator and the stability constant of the calcium complex, as described by Perrin & Dempsey (1974).

(5) When testing the effects of 150mM-KCl, 0.3ml of 1M-KCl was added.

(6) Water was added to make the total 2.0ml. The pH of the solution was confirmed to be 7.0$\pm$0.05 by measuring the pH of a separately made, identical mixture. Some examples of the composition of calcium buffers are shown in Table 2.

Preparation of Ca^{2+}-free solutions

No effort was made to remove contaminating

Table 1. *Stability constants (K) and apparent constant (K_{app}) of the calcium complexes of various chelators at 20–25°C*

Chelator	I	pK_a*	log K*†	log $K_{app.}$‡
Citric acid	0	6.40, 4.76	4.80 (3.07)	4.70
	0.15	5.62, 4.34	3.20	3.20
Ada§	0.1	6.60, 2.30	3.96	3.81
NTA	0	10.50, 3.07	7.60 (3.43)	4.10
	0.1	9.73, 2.50	6.41	3.68
EDTA	0	11.01, 6.32	11.00	6.92
	0.1	10.26, 6.16	10.58 (3.51)	7.25
EGTA§	0.1	9.46, 8.85	10.90 (5.33)	6.60

* Average values taken from Sillen & Martell (1964), except the values for Ada (Schwarzenbach *et al.*, 1955). The two highest values of pK_a are shown here and used in the calculations.

† The value of K are for the most ionized species. The values for the next most ionized species, when available, are shown in parentheses.

‡ Values at pH7.0 calculated from the values of pK_a and K.

§ pK_a and K at zero ionic strength ($I0$) were not available.

Table 2. *Composition of some examples of calcium buffers at pH7.0**

Chelator used (L)	[Free L]	KCl	CaL complex (logarithm of the molar concn.) necessary for pCa value of: 4	5	6	7	8	9	10
Citric acid	1mM	None	−2.30	−3.30	−4.30	−5.30	−6.30	−7.30	−8.30
Ada	2mM	150mM	−2.89	−3.89	−4.89	−5.89	−6.89	−7.89	−8.89
NTA	2mM	None	−2.60	−3.60	−4.60	−5.60	−6.60	−7.60	−8.60
EDTA	1mM	None		−1.08	−2.08	−3.08	−4.08	−5.08	−6.08
		150mM			−1.75	−2.75	−3.75	−4.75	−5.75
EGTA	1mM	150mM		−1.40	−2.40	−3.40	−4.40	−5.40	−6.40

* All buffers contained free L, CaL and free Ca^{2+} corresponding to the pCa value shown. In addition, all buffers other than citrate contained 2mM-Mops as pH buffer.

Ca^{2+} from the solutions of EDTA and EGTA. Contaminating Ca^{2+} in the solutions of other chelators and pH buffers was removed by two different methods, with nearly comparable results: (*a*) by passing the solution through a column of Chelex 100 as described by Blinks *et al.* (1978), and (*b*) by passing a solution (105mM) neutralized with NaOH through a column of cation-exchange resin (AG 50W-X8; 1.6cm × 15cm; Bio-Rad Laboratories) that had been thoroughly washed first with 0.1M-HCl, then with water. Acidic effluent that followed the first two column lengths of solution was collected in a Teflon bottle until the visible change of resin colour, caused by Na^+ exchange, reached the middle of the column. The acidic solution was neutralized with 5M-NaOH (Suprapur grade), then adjusted to 100mM by adding a small amount of water.

Preparation of the solutions of the calcium complex

Ca–EDTA complex and Ca–EGTA complex were prepared by titrating 100mM-chelator, pH7.0, containing a trace of Arsenazo III (indicator), with 105mM-calcium acetate. All other complexes were made by simply mixing equimolar amounts of chelator solution and calcium acetate solution. In all cases the pH was adjusted with 5M-NaOH as needed to maintain pH7.0, and finally the concentration was adjusted to 50mM by adding water. The solutions were made immediately before use to avoid precipitation of the complex.

Results and discussion

To examine the extent of inhibition caused by chelators, the intensities of aequorin luminescence were measured at various concentrations of free Ca^{2+} in the presence of a fixed concentration of the free form of a chelator plus widely varied concentrations of the calcium complex of the chelator. The calcium-complexed forms of chelators were not significantly inhibitory in comparison with the inhibition of free chelators; thus their influence was ignored.

The results are shown in Fig. 1. The low-ionic-strength solutions ($I < 0.005$) of pH-buffer reagents, i.e., Mops, glycylglycine and Bicine (not shown), and of a weak Ca^{2+} chelator, i.e., citric acid, resulted in curves that were nearly superimposable on each other for the range of $[Ca^{2+}]$ higher than 10nM (Fig. 1*a*). EDTA, a strong Ca^{2+} chelator, was strongly inhibitory and resulted in a curve that was offset almost 2 pCa units from the curves of other agents just mentioned. NTA was

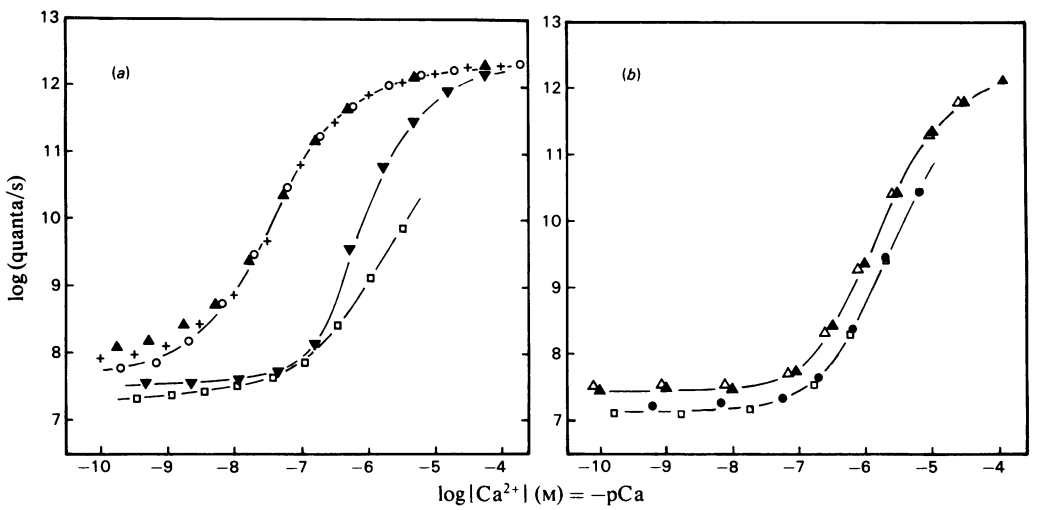

Fig. 1. *Relation between Ca^{2+} and the initial maximum intensity of aequorin luminescence at 23–25°C (a) in low-ionic-strength buffer ($I<0.005$) and (b) with 150mM-KCl added*

A 2μl portion of 10μM-aequorin solution containing 10μM-EDTA was mixed with 2ml of buffer solutions (pH7.0±0.05) containing the following chemicals in their free form (in addition to various concentrations of calcium complex when a chelator is included, and Ca^{2+}): 1mM-citrate (○), 1mM-Mops (▲), 1mM-glycylglycine (+), 1mM-EDTA plus 2mM-Mops (□), 1mM-EGTA plus 2mM-Mops (●), 2mM-NTA plus 2mM-Mops (▼), and 2mM-Ada plus 2mM-Mops (△). Ca^{2+} concentrations in Mops and glycylglycine, with no chelator, were set by dilution of calcium acetate. In preparing calcium buffers used in (*a*) the apparent stability constants at zero ionic strength were used without correction, except the value for citrate, which was adjusted to $10^{4.5}$ because of the exceptionally large concentration effect; in (*b*) the values at I0.1 were used without correction, except the value for citrate (I0.15).

clearly inhibitory, but the curve was somewhat atypical, possibly due to a slightly unmatched ratio of Ca and NTA in the Ca–NTA complex used, or for some other unknown reasons. As expected, the effect of EGTA was similar to that of EDTA, although the curve of EGTA could not be drawn, owing to the lack of stability-constant data and pK_a values at low ionic strength.

When tested in the presence of 150mM-KCl (to simulate intracellular conditions), the curves obtained with simple pH buffers, such as glycylglycine (not shown) and Mops, and weak Ca^{2+} chelators, such as citrate (not shown) and Ada, all coincided but shifted as a group to the high-$[Ca^{2+}]$ side, apparently due to the inhibition effect of the 150mM-KCl added (Fig. 1*b*). In contrast, the curve obtained with EDTA was very little affected by the addition of 150mM-KCl. Consequently the difference between the EDTA curve and the curves obtained with the four other buffers became only 0.2–0.3 pCa unit in the presence of 150mM-KCl. Data obtained with EGTA buffer were very close to the data obtained with EDTA, as expected.

In Fig. 1 the data points below about 10nM-Ca^{2+} and above 10μM-Ca^{2+} may have considerable errors in regard to the values of $[Ca^{2+}]$, because corrections were not made for various factors, such as the presence of 10nM-EDTA that originated from the aequorin sample, the contaminating calcium in the 'calcium-free' solutions (probably less than 10nM-Ca^{2+}), the contaminating calcium originating from KCl when 150mM-KCl was added (approx. 10nM-Ca^{2+}), the increases in the ionic strength caused by the large concentration of CaL required at Ca^{2+} concentrations higher than 10μM, and any inaccuracy in the ratio of calcium and chelator in CaL solutions. Such errors, however, would not affect the conclusions given below.

The present data indicate that calcium buffers made with EDTA and EGTA give large errors and are clearly unsuitable for use in the calibration of $[Ca^{2+}]$ by aequorin luminescence when the ionic strength of the buffer is low. When the buffers contain 150mM-KCl, the errors due to EDTA and EGTA become relatively small, i.e. 0.2–0.3 pCa unit in the presence of 1mM free chelator, a consequence resulting from the decreased sensitivity to Ca^{2+} by inhibition. Further decrease in the error at the expense of the sensitivity may be possible by the use of a still higher concentration of KCl or NaCl and also by the addition of Mg^{2+}, a known inhibitor.

In the application of aequorin, calcium buffers containing 20mM-total EGTA plus 150mM-KCl have often been used. The calcium-sensitivity of aequorin in such EGTA buffers should not be significantly different from the present data obtained with 1mM free EGTA, shown in Fig. 1(*b*). Note that, in the present data, the EGTA buffer of pCa 5.3 contained 20mM total EGTA (see Table 2). At pCa values over 5.3, the increase of free EGTA caused by maintaining 20mM total EGTA may increase inhibition slightly (Shimomura & Shimomura, 1982), but the luminescence intensity of aequorin also decreases at the same time, thus making its apparent effect insignificant.

Citrate buffer of low ionic strength and Ada buffer containing 150mM-KCl are both usable in the calibration of $[Ca^{2+}]$ with aequorin, although they are weak in buffering Ca^{2+}. In the presence of a high concentration of salt, however, citrate is not usable, owing to the decreased value of the stability constant of its calcium complex (Table 1).

The pK_a values of Ada and the stability constant of the Ca–Ada complex, both at zero ionic strength, were not available in the literature and therefore the curve was not plotted in Fig. 1(*a*). However, the apparent stability constant of Ca–Ada at pH7.0 was found to be $10^{4.0}$ by first plotting a curve using a tentative value of apparent stability constant, then shifting the curve horizontally to superimpose it with the curves of citrate, Mops and glycylglycine, assuming that Ada does not inhibit the aequorin reaction.

This work was aided in part by National Science Foundation Grant PCM-8201005, National Institutes of Health Grant GM 31314, and facilities provided by Friday Harbor Laboratories, University of Washington, and the Marine Biological Laboratory.

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