PROTEIN BINDING OF CALCIUM USING ^{45}Ca WITH EGTA BUFFERS AND MYOSIN AS A MODEL

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1. Introduction

The various anomalies which occur using ^{45}Ca, with ^{40}Ca/EGTA binding, led us to examine the properties of this isotope relative to complexing with the protein, myosin. Normal isotope dilution procedures [1], where the unlabeled ligand acts as a competitive inhibitor with respect to the labeled ligand, can be used with ^{45}Ca + ^{40}Ca protein binding, but cannot be used accurately with the ^{45}Ca + ^{40}Ca/EGTA system to determine mol of bound Ca/mol of protein. First, the chelator, EGTA, decreases the availability of ^{45}Ca in the dialysate for protein binding so that only free ^{45}Ca in the dialysate is accessible to the protein; therefore, ^{45}Ca in the dialysate does not represent free Ca and thus the isotope dilution method which is based on the binding of free Ca [1] cannot be used accurately with the ^{45}Ca + ^{40}Ca/EGTA system. Second, binding in such a system is based on the assumption that the specific activity of the free Ca pool is equal to that of the total Ca pool. This assumption is incorrect because the affinity constant of $^{45}Ca \cdot$ EGTA is greater than that of $^{40}Ca \cdot$ EGTA, due to the isotope effect of ^{45}Ca [2–4]. Third, the possibility exists that chelators could bind to proteins, such as myosin [5], and thus not allow the protein to express true binding characteristics because of subsequent incorrect Ca values. It is concluded that analyses of mol Ca bound/mol of protein, using the ^{45}Ca + ^{40}Ca/EGTA system can be more correctly assessed using tracer techniques [6]; with this technique it is necessary to retain a constant specific activity but alter the free Ca values by varying the EGTA concentration. The isotope, ^{45}Ca in the dialysate at time zero represents known concentrations of total Ca. However, the other errors discussed above continue to occur even with use of tracer techniques. The binding of ^{45}Ca can be most accurately analyzed by using a system free of divalent cations ($<10^{-7}$ M), with no chelators present.

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2. Materials and methods

Myosin was prepared from rabbit skeletal muscle as described in previous reports [7]. Protein concentration was determined according to the method of Lowry [8] or the use of the biuret method [9]. Aliquots of myosin (7–8 mg/ml) were added to individual bags (1 ml) and predialyzed in 0.2 M Tris/maleate, pH 6.5 or 7.4, 0.1 M KCl, 1 mM EDTA, and 1 mM DTT. The second and third dialysis contained the same except no EDTA was added. (Exhaustive dialysis with larger concentrations of chelators could lead to light chain dissociation [10].) Prior to dialysis the water and buffer were treated with Chelex-100 to remove divalent metal contaminants. The amount of contaminating Ca^{2+} and Mg^{2+} in the protein was assessed as described earlier using atomic absorption on the supernatant following an EGTA extraction [10]. Contaminating Ca^{2+} was less than 10^{-6} M and Mg^{2+} less than 10^{-7} M. The chelator, EGTA, was prepared as the free acid and buffered with KOH. For preparing a stock solution of Ca^{2+}, $CaCO_3$ (AR grade) was heated to 120°C for 4 h, cooled in a desiccator under vacuum and the desired amount dissolved in 0.01 M HCl, and neutralized with KOH.

For the dialysis equilibrium in assessing mol of bound Ca^{2+}, 1 ml of protein (7–8 mg/ml) previously predialyzed, was again dialyzed in 100 ml of buffer, i.e., 0.2 M Tris/maleate, pH 7.5, 0.1 M KCl, and 5 μCi ^{45}Ca. Free Ca^{2+} concentrations were varied from 10^{-7} to 10^{-5} M. EGTA was varied from 5×10^{-6} to 5×10^{-5} M as indicated in the figures. A high concentration of Tris/maleate was used to buffer the release of H^+ from EGTA in the reaction, the pH remained constant throughout the reaction. KCl (0.1 M) was used to prevent the Donnan effect [11]. Ionic concentration was kept minimal to restrict competition for Ca^{2+} binding sites on EGTA and myosin; an ionic strength of 0.3 M was necessary to keep myosin in solution. Myosin was not analyzed at high Ca^{2+} concentrations, i.e., greater than 10^{-5} M because high EGTA concentrations resulted in biphasic binding curves. All buffers and water were rapidly filtered through Chelex-100 to remove divalent metal contaminants. After a dialysis of 48 h (4°C) samples from both inside and outside the bag were removed, an equal volume of H_2O_2 was added, and the mixture heated in closed vials for 4 h at 55°C; 10 ml of scintillation fluid was added and the samples analyzed in a scintillation counter for assessing isotope. Duplicate samples (0.1 ml) of the protein solution inside the dialysis bags were taken for determination of protein concentration by the Bio-Rad Assay (Bio-Rad Laboratories, Bulletin 1051 (1977)). If myosin concentration was not greater than 8 mg/ml and not subject to large ionic changes during dialysis the protein concentration changed little.

Free Ca^{2+} was calculated using the computer program by Perrin and Sayce [12], where the cumulative logarithm of the six dissociation constants of ionized EGTA and Ca · EGTA complexes were taken into consideration as described by Bulos and Sacktor [13]. These free Ca^{2+} values were similar to those obtained using the computer program by Botts et al. [14]. Such calculated free Ca^{2+} values were similar to those values obtained using a K_{eq} constant for the Ca + EGTA/Ca · EGTA equation for a defined buffer system as described by Ogawa [15] and applied to myosin [7]. Analyses of Scatchard plots were also computer programmed [10].

3. Results and discussion

The isotope, ^{45}Ca, is used as a radioactive tracer to detect and quantitatively determine the moles of this metal bound to proteins such as myosin. Although free Ca^{2+} is theoretically calculated from the Ca + EGTA/Ca · EGTA equilibrium, nevertheless binding is based on the measurement of protein bound ^{45}Ca. When ^{45}Ca is mixed with ^{40}Ca in a system buffered with EGTA, ^{45}Ca is proportional to the total pool (fig.1). The free Ca^{2+} pool depends on the concentration of Ca + EGTA and ionic conditions, and is represented by the K_{eq} of the Ca/EGTA equation (fig.1). Experimental and theoretical data have been thoroughly evaluated for this system in the absence of ^{45}Ca [16]. However, in the presence of ^{45}Ca, where both the isotope and the free Ca^{2+} pool are constant (fig.1A,B), an increase in EGTA (fig.1B) requires an increase in total ^{40}Ca (fig.1B); thus two different specific activities, each representing a constant free Ca^{2+} pool (10^{-6} M), are obtained (fig.1A,B). An increase in EGTA at a constant ^{45}Ca concentration requires an increase in the ratio of $^{40}Ca/^{45}Ca$ to attain a specific free Ca concentration, thus the specific activity of both pools are lowered. Fig.2 shows the amount of Ca added to increasing EGTA concentrations at a constant ^{45}Ca concentration to attain approximately 10^{-6} M free Ca^{2+}. If the concentration of ^{45}Ca is fixed with these varying conditions the specific activity of both the free and total pool decrease proportional to increases in Ca.

If Ca binding in the presence of EGTA is based on the isotope dilution Eqn. 1, with no additional corrections, then one assumes that all of the

$$\eta = \frac{\dfrac{(\text{cpm}_{\text{bound}}/\text{ml})}{(\text{cpm}_{\text{dialysate}}/\text{ml})} \times (\text{free } ^{40}\text{Ca (mol/ml)})}{\text{Myosin (mol)}}$$

^{45}Ca in the dialysate is available to myosin for binding. If this is true then increases in both EGTA and ^{40}Ca concentration at constant free ^{40}Ca and ^{45}Ca values, causes alterations in the apparent binding affinity of myosin for Ca (fig.3). With an increase in EGTA there is a corresponding decrease in $^{45}Ca_{bound}$ because EGTA decreases the availability of ^{45}Ca in the dialysate. An increase in the $^{40}Ca/^{45}Ca$ ratio causes a dilution of the isotope and thus also results in a decrease in $^{45}Ca_{bound}$.

When myosin was dialyzed in the $^{45}Ca + ^{40}Ca$/EGTA system, varying binding affinity values were obtained when analyses were based on the isotope

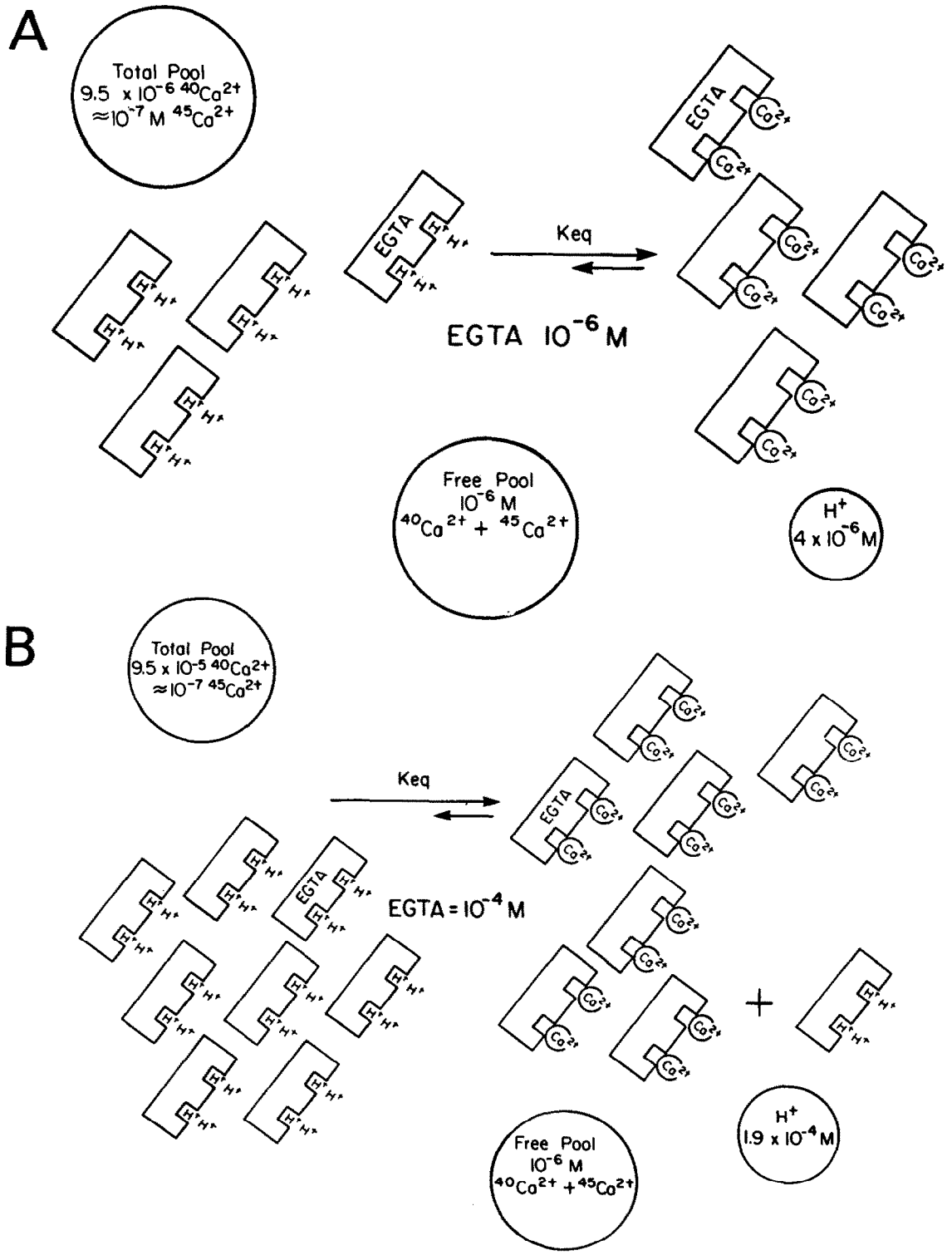

Fig.1. Diagrammatic representation of the ^{40}Ca + ^{45}Ca/EGTA buffer at low (A) and elevated (B) EGTA concentrations. Free Ca^{2+} pool is regulated by EGTA concentration and the K_{eq} of the reaction. The specific activity of the free Ca^{2+} pool is the same as the total Ca^{2+} pool, however, the specific activity of ^{45}Ca is regulated by the concentration of ^{40}Ca.

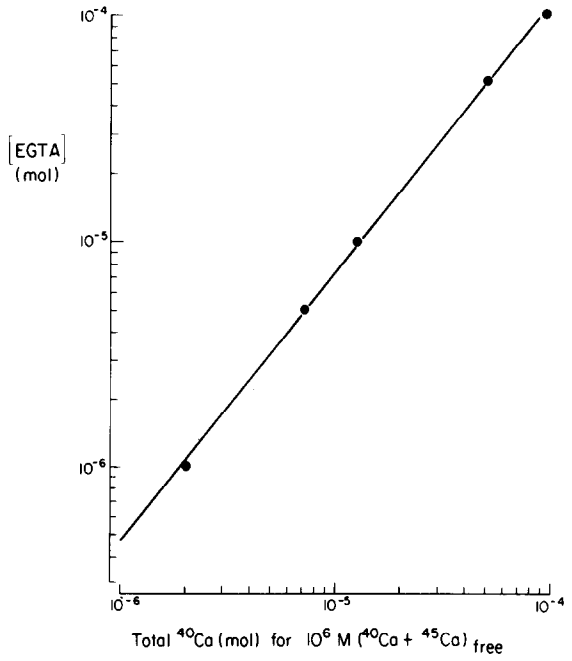

Fig.2. The concentration of ^{40}Ca required at increasing EGTA concentrations to attain 10^{-6} M free Ca^{2+} at constant ^{45}Ca concentrations is shown. The pH is 7.4.

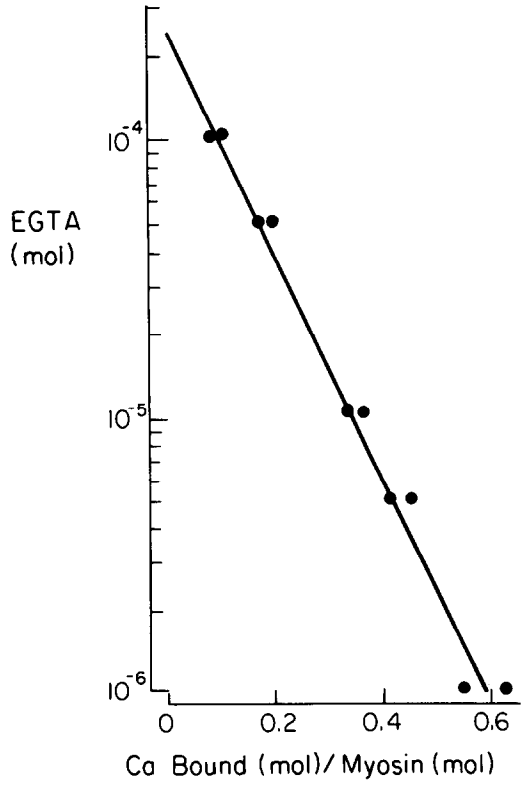

Fig.3. The moles of Ca^{2+} bound as assessed by ^{45}Ca at decreasing EGTA concentrations at a constant 10^{-6} M free Ca^{2+} and constant ^{45}Ca concentrations are shown. The pH is 7.4. Calculations were based on isotope dilution techniques where ^{45}Ca in the dialysate was considered proportional to free Ca.

dilution Eqn. 1; these values depended on the concentration of EGTA (fig.4). The apparent Ca^{2+} binding affinity values at pH 7.4 are the following: for no EGTA the value is $5 \times 10^5\ M^{-1}$; for 5×10^{-6} M EGTA the value is $3.2 \times 10^5\ M^{-1}$; for 10^{-5} M EGTA the value is $2.2 \times 10^5\ M^{-1}$; and for 5×10^{-5} M EGTA the value is $1.0 \times 10^5\ M^{-1}$ for ^{45}Ca at 5 μCi/100 ml. The data are consistent with a decrease in Ca^{2+} binding in

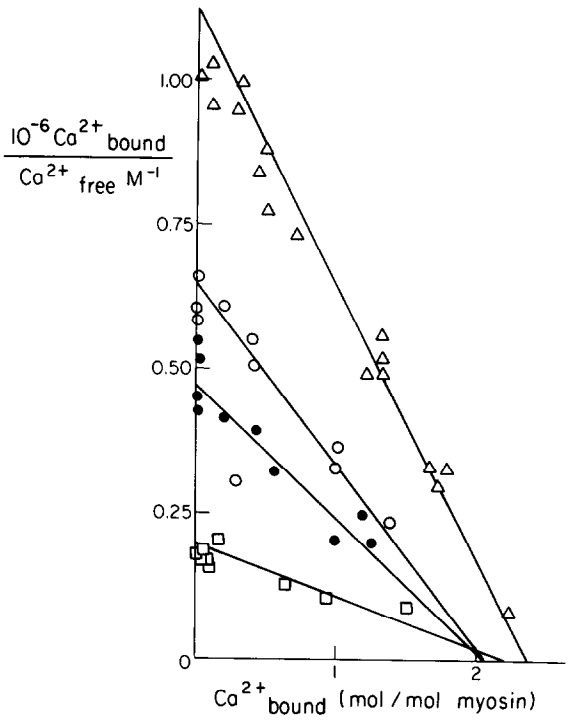

Fig.4. Scatchard plots of mol Ca^{2+} bound/mol of myosin/mol of free Ca^{2+} as a function of mol Ca^{2+} bound/mol of myosin. Binding is based on the degree of complexing at constant ^{45}Ca. Free Ca^{2+} is calculated as described in Section 2. Increasing EGTA concentration gives dilution of the free Ca^{2+} pool by exaggerating the difference between the total and free Ca^{2+} pools as diagrammed in Fig.1. △—△, no EGTA; ○—○, 5×10^{-6} M EGTA; ●—●, 10^{-5} M EGTA; □—□, 5×10^{-5} M EGTA. The pH is 7.4. Calculations were based on isotope dilution techniques [1] where ^{45}Ca in the dialysate was considered proportional to free Ca.

the low Ca^{2+} concentration ranges with an increase in EGTA and where analyses are based on the isotope dilution equation; for this equation one assumes that the ^{45}Ca in the dialysate represents free Ca. Even if accurate adjustments could be made for this assumption, considerable error would still remain because the affinity constant of $^{45}Ca \cdot$ EGTA is greater than that of $^{40}Ca \cdot$ EGTA [2–4]; due to this fact the specific activity of ^{45}Ca will be higher in the total pool than in the free one. Since the isotope, ^{45}Ca, has five additional neutrons as compared to ^{40}Ca its vibrational energy as zero point energy is less and thus the rate of dissociation is slower. Such a variance in mass, affects the thermodynamics and kinetics of a reaction as has been shown for isotopes of Ca^{2+} [2–4]. The octahedral solvation of ^{40}Ca and ^{45}Ca are not the same; there are also variances in competition for EGTA. The isotope, ^{45}Ca, combined with EGTA will give a 10–30% isotope effect on the overall Ca/EGTA system [2–4].

Fig.1–4 demonstrate that the ^{45}Ca in the dialysate does not represent free Ca and that the isotope dilution equation cannot be applied to the $^{45}Ca + {}^{40}Ca$/EGTA system. However, ^{45}Ca binding in the presence of EGTA can be analyzed at a constant $^{40}Ca/^{45}Ca$ ratio. In such a system the ^{45}Ca in the dialysate at time zero would represent total Ca and the following equation would be applied:

$$\eta = \frac{(\text{cpm}_{\text{bound}}/\text{ml})}{(\text{cpm}_{\text{dialysate}}/\text{ml})_{T_0}} \times \frac{(\text{total } ^{40}Ca \text{ (mol/ml)})}{\text{Myosin (mol)}}$$

When this is applied to the Scatchard equation [17], $A = {}^{40}Ca_{\text{free}}$ for η/A. When myosin was analyzed at increasing EGTA concentrations but a constant $^{40}Ca/^{45}Ca$ ratio, and data were treated according to this equation, a Ca binding affinity value of $\sim 1.2 \times 10^7$ M^{-1} was obtained for myosin at pH 6.5. When similar analyses were carried out at pH 7.4, the Ca binding affinity values were nearly twice as high as that obtained at pH 6.5.* The apparent binding affinity values based on tracer techniques (fig.5) are more like those reported in literature where methods other than ^{45}Ca were employed for assessing Ca binding [18–21]. All of these values were considerably higher than those where analyses were based on ^{45}Ca [22–26].

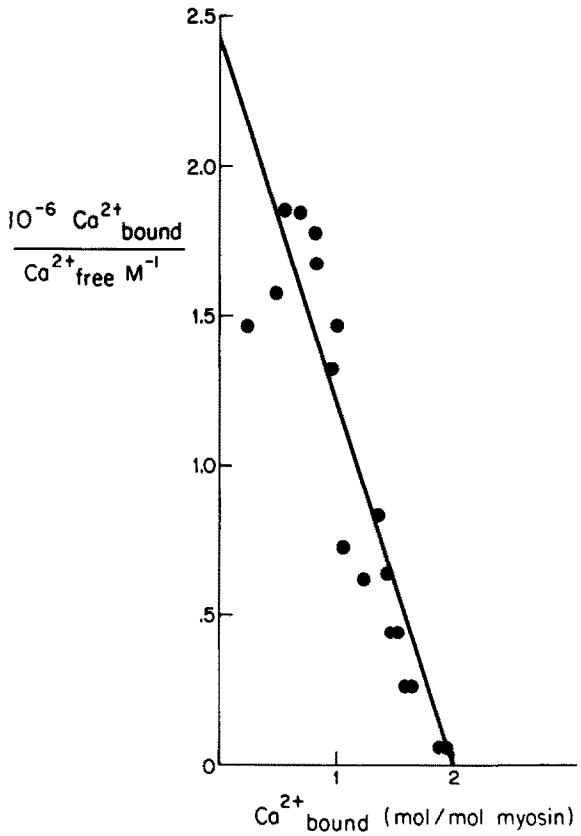

Fig.5. Myosin (5 mg/ml) binding was carried out at pH 6.5; EGTA was varied from 0.5×10^{-3} M to 0.1×10^{-4} M. Total Ca was constant (1.0×10^{-5} M) and ^{45}Ca was 5 μCi/100 ml. Calculations were based on tracer techniques [6] where ^{45}Ca in the dialysate was considered proportional to the total Ca. The data are from two separate experiments.

Myosin may be best analyzed with $^{40}Ca + {}^{45}Ca$ in the absence of EGTA. With this system one does not need to be concerned with additional isotope effects of ^{45}Ca with EGTA [2–4], the possibility of chelators binding to proteins such as myosin [5], and other artifacts which could occur with EGTA such as disparate pH effects [27].

* The pH effects on the Ca binding properties of myosin will be discussed in later reports

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