THE SIGNIFICANCE OF EGTA TITRATIONS IN MUSCLE MECHANICS EXPERIMENTS

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Abstract

Ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, or EGTA, is a synthetic, tetraprotic acid widely used in biological experiments that require precise regulation of calcium (Ca2+) levels. EGTA contains four carboxylic acid and two amine groups that can deprotonate and donate lone pairs to form stable complexes with metal ions, exhibiting a particularly high affinity for Ca2+ over other cations such as Mg2+, Fe2+, or Zn2+. This makes EGTA an effective chelating agent for maintaining low, stable free Ca2+ concentrations that mimic cytosolic conditions. Its binding capacity is pH-dependent which dictates the number of deprotonated carboxyl groups and available donor sites on the molecule. Optimal binding occurs around physiological pH (~7) when sufficient negatively charged donor sites are exposed and can strongly bind Ca2+. In muscle mechanics experiments, EGTA is especially valuable for studying Ca2+-mediated contraction, as muscle activation relies on Ca2+ binding to troponin C to initiate cross-bridge cycling. By using EGTA-buffered solutions with controlled amounts of free Ca2+, a relationship between Ca2+ concentration and generated muscle force can be determined in skinned muscle fiber experiments. EGTA stock solutions prepared with MOPS as a pH buffer and KCl and dH2O to simulate physiological conditions can be titrated with known Ca2+ solutions to confirm EGTA concentration as it possesses a 1:1 molar binding ratio with Ca2+. This is pivotal in muscle mechanics experiments, such as k_{tr} analysis, where calcium concentrations must be carefully manipulated to observe muscle function at various biological conditions such as relaxing and activating conditions.

Methods

Preparing EGTA stock solution (250 mL; 0.2 M)

- 19.0175 g of EGTA powder was mixed in 100 mL of dH_2O; initial amount of dH_2O is inconsequential; dH_2O can be added later to achieve the correct volume.
- 100 mL of 1M KOH slowly added to bring pH to 7.5 - 8.0, allowing the EGTA to dissolve
- dH_2O added until solution reached 250 mL
- Solution was sealed and refrigerated at 32° C to prevent degradation.

Performing the titration with [0.1M] Ca2+

- 25 uL of 2M MOPS, 8.2 mL of dH_2O, 1.8 mL of 1M KCl, and 200 uL of EGTA stock combined with a stir bar; pH of solution is recorded.
- Drops of 1 M and 0.1 M KOH added until a pH range of 7.5 - 8.0 is reached.
- 200 uL of 0.1M Ca2+ added; again, drops of 1 M and 0.1 M KOH added until the pH returns to 7.5 - 8.0.
- Pipette is filled with 200 uL of 0.1 M Ca2+; drops of about 10 uL at a time added until the pH levels out (there is no change in pH for 3 consecutive drops); if pH does not stabilize after 200 uL, more Ca2+ may be used.
- pH is recorded after every drop; when pH levels out, the titration is complete, and analysis can begin.

Results

EGTA Structure

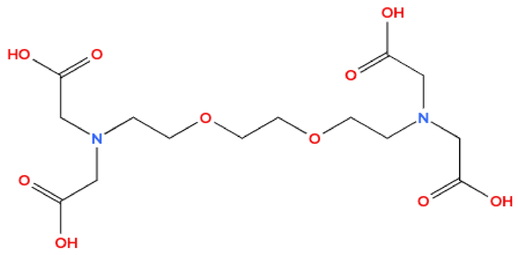

Figure 1. (Above) Structure of an EGTA molecule, showing 4 carboxylic acid groups and two amine groups.

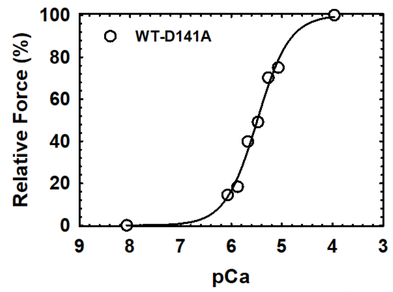

Figure 2. (Above) Figure of relative force-pCa curve produced by k_{TR} analysis.

Results

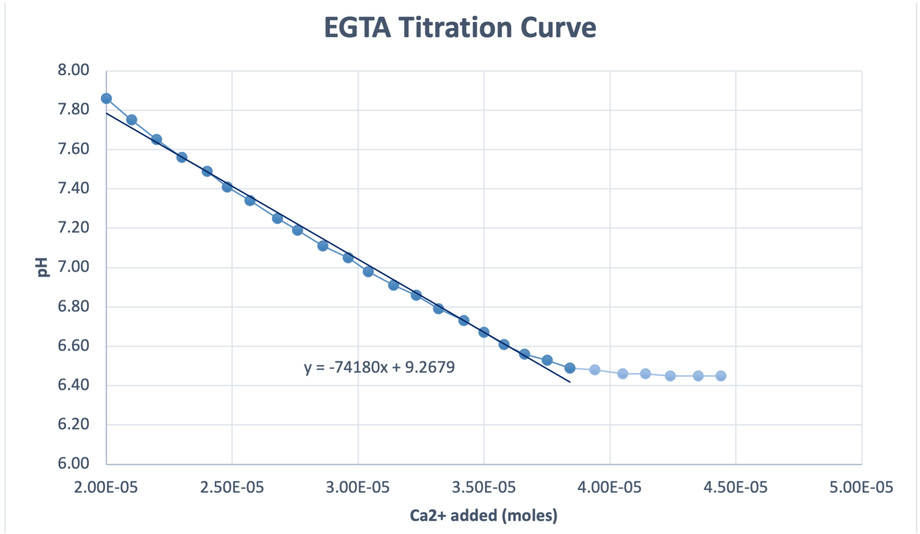

Figure 3. (Above) Titration curve of Ca2+ standard with EGTA, showing pH change (y-axis) as moles of Ca2+ are added (x-axis). A linear regression was fitted to the early portion of the curve prior to the equivalence region where Ca2+ and EGTA demonstrate linear binding.

The data obtained by the titration of EGTA with Ca2+ was created into a graph, visually showing the initial linear decrease in the solution's pH as moles of Ca2+ were added. The concentration of EGTA stock was determined by finding the intersection point between the plateau region and the linear regression line of the pre-equivalence region. This point corresponded to where the moles of Ca2+ and moles of EGTA were equal in the solution. This value was then divided by the volume of EGTA in the solution (200uL) to calculate the concentration of the EGTA, yielding an EGTA concentration of 0.190 M.

Discussion

The stock EGTA concentration had to be accurately determined prior to conducting muscle fiber experiments, which was done by performing a titration using Ca2+ standard. Given that Ca2+ and EGTA exhibit a 1:1 binding ratio, a strong linear stoichiometric EGTA-Ca2+ relationship is seen by its titration curve (fig. 3) up until a plateau point is reached where the EGTA became saturated and can no longer bind any more Ca2+. The intersection point between the two regions is key for calculating the concentration of the EGTA as this denotes the moles of Ca2+ and equivocally the moles of EGTA present. In muscle mechanics experiments, the EGTA concentration directly determines the free Ca2+ levels in experimental solutions. Muscle force is highly sensitive to any changes in Ca2+, so any inaccuracies in EGTA concentration can alter the levels of free calcium present, thus impacting the efficacy of the relaxation and activation solutions and ultimately the force produced by the muscle fibers. Ensuring the precise concentration of the EGTA stock solution via titration methods prevents against variation or errors in Ca2+ buffering and improves the validity of muscle mechanics results.

Conclusion

In conclusion, titrating EGTA with Ca2+ is an essential step in the preparation of biological experimental solutions, specifically in muscle force experiments, since muscle contraction is a Ca2+-sensitive mechanism. Therefore, titrations to determine EGTA concentration need to be done correctly to ensure that the free Ca2+ concentration can be accurately determined. The structure of EGTA encourages a strong specificity for Ca2+ binding and allows for a 1:1 molar ratio between the two. Graphically, this is demonstrated by the linear segment of a titration curve, and a plateau segment indicating that the EGTA is saturated with Ca2+ (fig. 3). Analysis of these curves allow for the determination of the moles of EGTA in solution, which can then be used to calculate EGTA concentration.

Next Steps

- The verified EGTA stock will be incorporated into pCa-buffered activation and relaxation solutions ranging from pCa 8 (low Ca2+, below physiological range) to pCa 4 (high Ca2+, beyond physiological range).
- Papillary muscle fibers from WT and mutated hearts will be incubated at 30° C under either beta-adrenergic stimulation (PKA) or under drug incubation (myosin inhibitors: Mavacamten or Aficamten).
- Fibers will be tested to investigate Ca2+ sensitivity, force production rates of tension redevelopment, and sinusoidal stiffness under each condition.
- Data will be used cooperatively with Western blots and echocardiographs.