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ION EXCHANGE PROPERTIES OF THE CALCIUM RECEPTOR SITE OF TROPONIN

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SUMMARY

1. The exchangeability of troponin-bound $^{45}Ca^{2+}$ with various cations was investigated, using a gel filtration technique.

2. Bound $^{45}Ca^{2+}$ was completely exchangeable with non-radioactive Ca^{2+} and partially exchangeable with Cd^{2+}, Sr^{2+}, Pb^{2+}, and Mn^{2+}, in the order given. There was no significant exchange with Mg^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+}. The affinity of divalent cations for the receptor site of troponin was closely related to ionic radius, with 1 Å (Ca^{2+}) providing the most favorable fit.

3. The trivalent lanthanide ions, La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, and Dy^{3+} could partially displace $^{45}Ca^{2+}$ from troponin but the affinity of these ions for the receptor site of troponin was nevertheless lower than that of Ca^{2+} despite their similarity to Ca^{2+} in size and chemical properties.

4. Elevation of the ionic strength to 0.5 with KCl or NaCl caused no dissociation of Ca^{2+} from troponin.

INTRODUCTION

There is now considerable evidence in support of the hypothesis that the initial reaction in the mechanochemical transduction mechanism in striated muscle is the binding of Ca^{2+} to troponin (see refs. 1–3, for reviews). Troponin is localized on the actin filament[4] where, in conjunction with tropomyosin, it confers Ca^{2+} sensitivity on the actomyosin system[5]. From the studies of WEBER AND HERZ[6] which established the essential role of Ca^{2+} as the activator of myofibrillar contraction it could be inferred that Ca^{2+} should bind to the myofibril with an apparent stability constant of about $1 \cdot 10^6$ M^{-1}. The subsequent investigations of FUCHS AND BRIGGS[7] and EBASHI *et al.*[8] with purified troponin provided values for the Ca^{2+}-troponin stability constant ranging from $0.9 \cdot 10^6$–$2.4 \cdot 10^6$ M^{-1}. Troponin appears to be the only myofibrillar protein which binds Ca^{2+} so strongly[7].

If troponin is indeed the physiological Ca^{2+} receptor it must be capable of discriminating between Ca^{2+} and a number of other cations, such as Mg^{2+} and the alkali metal ions, which are present in the muscle cell in much higher concentration. In a recent communication, FUCHS *et al.*[9] reported that several divalent cations,

* The publication of this article was affected by delays due to the British postal strike.

including Mg^{2+}, Zn^{2+}, and Mn^{2+}, had little or no ability to exchange with $^{45}Ca^{2+}$ bound to troponin. With regard to non-biological cations it had previously been shown that Sr^{2+} could activate the contractile system[20] and bind to troponin[8], although with lower affinity than Ca^{2+}.

BIRNBAUM *et al.*[11] have recently suggested that the trivalent lanthanide ions should be useful as probes of Ca^{2+} binding sites on proteins. The rationale for this proposal was that, (1) the ionic radii of the lanthanides (0.85–1.06 Å) fall in the same range as that of Ca^{2+} (0.99 Å) and, (2) both the alkaline earth and lanthanide ions bind electrostatically to the same types of ligands. FUCHS *et al.*[9] had noted that La^{3+} had relatively little effect on Ca^{2+} binding to troponin but not enough data were obtained to draw any quantitative conclusions. A preference for Ca^{2+} over La^{3+} would imply that simple electrostatic factors are not of primary importance in determining the cation selectivity of troponin. In view of our experimental results and the proposal of BIRNBAUM *et al.*[11] it appeared worthwhile to extend these investigations of ion exchange properties in an attempt to gain further information about the physicochemical features of the Ca^{2+} receptor site.

METHODS

The relative affinity of various cations for troponin was measured by means of the gel filtration technique used in the previous study[9]. This method is similar in principle to that used by other workers[12,13] for studying ion binding and ion exchange. Disposable columns (0.7 cm × 15 cm) obtained from Bio-Rad Laboratories were filled with Bio-Gel P-10 which had been previously equilibrated with a solution containing 50 mM KCl, 10 mM imidazole (pH 7.0) and added cation as indicated. 0.5 ml of $^{45}Ca^{2+}$-labelled troponin (2–4 mg protein) was applied to the column and eluted with the same solvent with which the gel had been equilibrated. The appearance of $^{45}Ca^{2+}$ in the void volume with the protein was taken as evidence of a Ca^{2+}-troponin complex whereas a retardation of $^{45}Ca^{2+}$ was considered to be indicative of an exchange between troponin-bound Ca^{2+} and added cation. In general it took about 10 min for the protein to pass through the column. The percent Ca^{2+} which exchanged was estimated on the basis of the amount of radioactivity in the void volume relative to the total amount of radioactivity eluted from the column. Elutions were carried out at room temperature.

No attempt was made beforehand to remove the intrinsic Ca^{2+} of troponin (25–30 nmoles/mg), virtually all of which is exchangeable with tracer[7,8]. However, to minimize contamination with unlabelled Ca^{2+} the gels, columns, and all glassware used for preparing solutions were exhaustively washed with EDTA and deionized water. Only plastic disposable pipets were used for transfer of protein and eluant solutions.

The divalent cations were added as chlorides (except for Pb $(NO_3)_2$) and were reagent grade products obtained from Fisher Scientific Co. The lanthanides were obtained as chloride salts from K and K laboratories, Plainview, New York.

Protein was determined by the method of LOWRY *et al.*[14] and radioactivity was measured with a Beckman LS-100 liquid scintillation spectrometer.

Troponin was prepared from rabbit muscle essentially as described by YASUI *et al.*[15], with 1 mM dithiothreitol being present in all preparative solutions.

RESULTS

Effect of divalent cations

As shown in Fig. 1, if $^{45}Ca^{2+}$-labelled troponin was passed through a column equilibrated with the standard buffer solution but without added polyvalent cation essentially all of the radioactivity appeared in the void volume with the protein. In addition to illustrating the tight binding of Ca^{2+} to troponin this result showed that the $^{40}Ca^{2+}$ contamination in the column was too low to displace any $^{45}Ca^{2+}$ from the troponin and could be safely disregarded.

The ability of the alkaline earth cations, at 0.1 mM concentration, to exchange with bound $^{45}Ca^{2+}$ is illustrated in Fig. 2. In the presence of $^{40}Ca^{2+}$ virtually all of the $^{45}Ca^{2+}$ was displaced from the troponin whereas in the presence of Sr^{2+} slightly less than half of the added $^{45}Ca^{2+}$ remained bound to the protein in its passage through the column. Both Mg^{2+} and Ba^{2+} failed to displace any significant amount of $^{45}Ca^{2+}$. Thus the observed affinity sequence obtained with this method, $Ca^{2+} > Sr^{2+} > Mg^{2+}$, Ba^{2+}, is consistent with other results based on enzymatic activity[10] and ion binding[8,9].

The results of a typical series of measurements with 10 divalent cations are shown in Table I. In addition to Ca^{2+} and Sr^{2+}, only Cd^{2+}, Pb^{2+}, and, to a lesser extent, Mn^{2+}, could displace significant amounts of $^{45}Ca^{2+}$ from troponin. It might be supposed that the effects of heavy metal cations such as Cd^{2+} and Pb^{2+} were due to denaturation of the troponin. However, if these cations were removed from the troponin by binding to EDTA followed by dialysis the protein exhibited normal Ca^{2+} sensitizing activity. Hence, it is assumed that the results in Table I represent a true ion exchange rather than a structural modification of the troponin. The ineffectiveness of Mg^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} is in accord with previous results from this laboratory[9].

There is no apparent relationship between the chemical properties of the divalent cation and the ability to exchange with the troponin-bound $^{45}Ca^{2+}$. Thus Ca^{2+} and Sr^{2+} on the one hand, and Cd^{2+} and Pb^{2+} on the other, are quite different in terms of ligand affinities and the types of bonds they form. More suggestive is the relation obtained when the data are plotted as a function of ionic radius (Fig. 3). It would appear that the receptor site of troponin can accommodate divalent cations having radii between 0.08 and 1.20 Å, with 1 Å providing the most favorable fit.

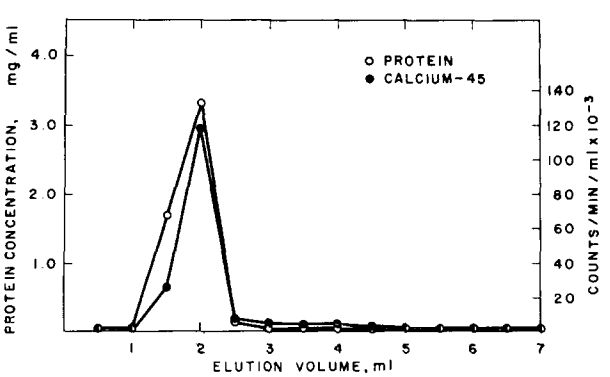

Fig. 1. Elution of $^{45}Ca^{2+}$ and troponin from Bio-Gel P-10 column equilibrated with 50 mM KCl, 10 mM imidazole (pH 7.0). See text for experimental details.

Effect of lanthanide ions

In the light of the above results, the recent paper of BIRNBAUM *et al.*[11] suggesting the use of lanthanides as Ca^{2+} substitutes at electrostatic binding sites of proteins was of great interest. The lanthanide ions bind strongly to the same anionic ligands (*e.g.* $-COO^-$) as Ca^{2+} and are very similar to Ca^{2+} in size. Thus, Nd^{3+}, with an ionic radius (0.995 Å) almost identical to that of Ca^{2+} but a higher charge density, might be expected to bind to the receptor site of troponin even more strongly than Ca^{2+}.

Fig. 4 illustrates the results of a series of measurements made with 5 lanthanides ranging in ionic radius from 0.908 Å (Dy^{3+}) to 1.06 Å (La^{3+}). In these experiments the added cation was at a concentration of 0.02 mM since it was found that the lanthanides tended to cause precipitation of protein when present at concentrations of 0.1–1 mM. Also shown for comparison are the amounts of $^{45}Ca^{2+}$ displaced by equivalent

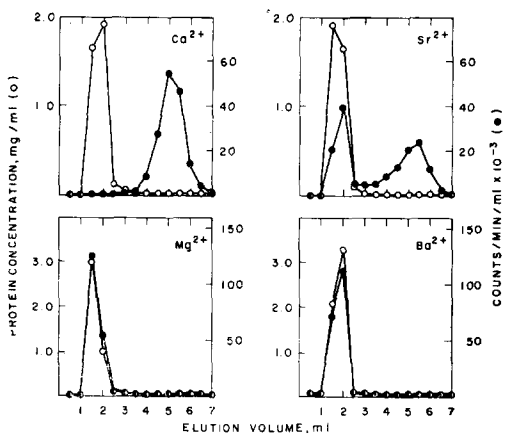

Fig. 2. Elution of $^{45}Ca^{2+}$ and troponin from Bio-Gel P-10 columns equilibrated with 50 mM KCl, 10 mM imidazole (pH 7.0) and 0.1 mM alkaline earth cation, as indicated. See text for experimental details.

TABLE I

EXCHANGE OF TROPONIN-BOUND $^{45}Ca^{2+}$ IN THE PRESENCE OF DIVALENT CATIONS, ALL AT CONCENTRATION OF 0.1 mM

See text for additional experimental details.

Cation	$^{45}Ca^{2+}$ *exchanged* (%)
Ca^{2+}	98
Cd^{2+}	72
Sr^{2+}	57
Pb^{2+}	45
Mn^{2+}	35
Co^{2+}	12
Ni^{2+}	10
Ba^{2+}	10
Mg^{2+}	8
Zn^{2+}	6

concentration of Ca^{2+}, Cd^{2+}, and Sr^{2+}. The lanthanides could displace about 25–35 % of the bound $^{45}Ca^{2+}$ from troponin. There did not appear to be any significant differences among the cations tested. The same concentrations of Cd^{2+} and Sr^{2+} could displace 40 and 29 % of the bound $^{45}Ca^{2+}$, respectively. Although more data are needed for quantitative conclusions it appears that the lanthanide ions lie somewhere between Cd^{2+} and Sr^{2+} in their affinity for the Ca^{2+} receptor site. There is no evidence that they surpass Ca^{2+} in affinity, despite their high charge-to-radius ratio.

Higher concentrations of lanthanide could cause further displacement of Ca^{2+} from troponin but this effect could not be readily demonstrated by the gel filtration technique since, as noted above, the solubility of troponin was reduced by lanthanide concentrations in excess of approx. 0.1 mM. If sufficient $LaCl_3$ (2 mM) was added to a solution of $^{45}Ca^{2+}$-labelled troponin to precipitate most of the troponin over 90 % of the $^{45}Ca^{2+}$ was found in the soluble phase (Table II). This result is consistent with the assumption at La^{3+} can exchange with tightly bound Ca^{2+} provided the $[La^{3+}]/[Ca^{2+}]$ ratio is sufficiently high. Denaturation of the protein was considered unlikely since the troponin re-dissolved without loss of biological activity upon removal of the La^{3+} with EDTA. Several divalent heavy metals can also precipitate troponin, in some cases even more effectively than the lanthanides. As an example, also shown in Table II, 2 mM $ZnCl_2$ caused almost complete precipitation of troponin. However, in contrast to La^{3+} precipitation, about 80 % of the $^{45}Ca^{2+}$ remained bound to the protein. Precipitation most likely results from cation binding at non-specific anionic sites.

Effect of monovalent cations

Small increases in ionic strength close to the physiological level are known to cause an increase in the Ca^{2+} concentration required for activation of myofibrillar

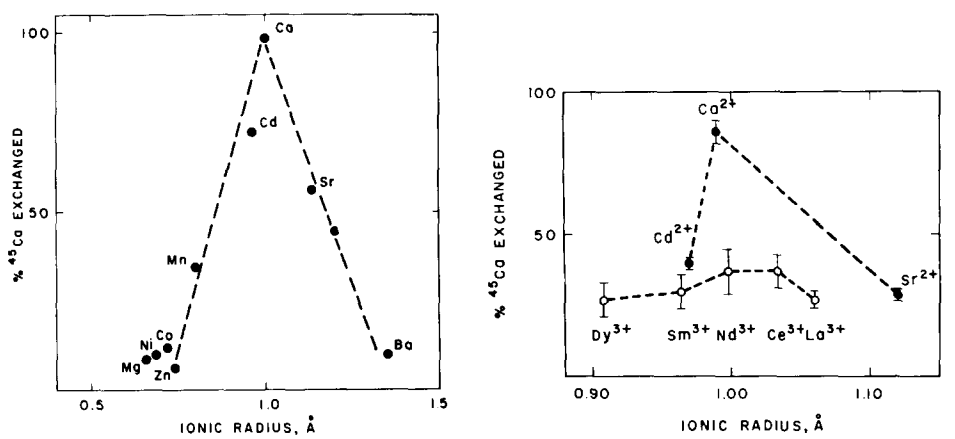

Fig. 3. The percent bound $^{45}Ca^{2+}$ exchanged as a function of ionic radius of divalent cation. Values of ionic radii were taken from ref. 16. (● between Sr and Ba is Pb.)

Fig. 4. The percent bound $^{45}Ca^{2+}$ exchanged as a function of ionic radius for 5 lanthanide ions (○) and 3 divalent cations (●), as indicated, all at 0.02 mM. Experimental conditions otherwise as in Fig. 1. Each point is mean of 3 experiments, with vertical bars indicating standard error of mean. See text for other experimental details.

ATPase[6]. In preliminary experiments[17] designed to test whether this effect is mediated through the Ca^{2+} receptor site of troponin it was found that similar small increases in ionic strength had no effect on the Ca^{2+}–troponin stability constant. In an extension of this work the results in Fig. 5 show that a 10-fold increase in ionic strength produced with either KCl or NaCl had no evident effect on the strength of the Ca^{2+}–troponin bonds. Thus, there do not appear to be significant electrostatic interactions between anionic sites which bind Ca^{2+} and monovalent cations, even when the size of the monovalent ion (Na^+ radius, 0.95 Å) is very close to that of Ca^{2+}.

DISCUSSION

At neutral pH troponin binds Ca^{2+} with an apparent stability constant of about $1 \cdot 10^6$ M^{-1}. From observations on the effect of pH on Ca^{2+} binding[9] it can be inferred that the absolute stability constant must be at least $1 \cdot 10^7$ M^{-1}. Values of this magnitude almost certainly require that the Ca^{2+} binds to troponin through some form of polydentate chelation[18]. Since the alkaline earth cations are attracted to highly electronegative ligands it seems reasonable to assume that the chelation of Ca^{2+} to troponin is effected through carboxyl groups of glutamic and aspartic acid. However, there is a considerable increase in Ca^{2+}–troponin stability in a pH range (7.0–8.5) where all carboxyl groups are presumably in the dissociated form[9]. Therefore, it seems

TABLE II

EFFECT OF EXCESS La^{3+} AND Zn^{2+} ON SOLUBILITY AND BOUND $^{45}Ca^{2+}$ OF TROPONIN

Solutions contained 2.20 mg/ml troponin, 10 mM imidazole (pH 7.0), 0.2 μC/ml $^{45}Ca^{2+}$, in volume of 2 ml. Following additions as indicated precipitates were removed by centrifugation and supernatants analyzed for protein and $^{45}Ca^{2+}$.

Additions	*Soluble protein (mg/ml)*	*Soluble $^{45}Ca^{2+}$ (counts/min)*	*Protein soluble (%)*	*$^{45}Ca^{2+}$ soluble (%)*
None	2.20	155 115	100	100
2 mM $LaCl_3$	0.37	143 600	17	93
2 mM $ZnCl_2$	0.10	35 193	5	23

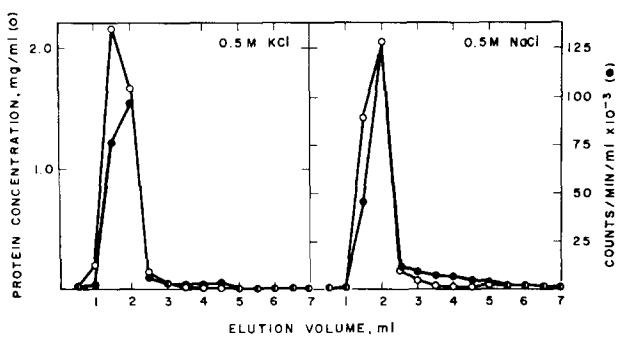

Fig. 5. Elution of $^{45}Ca^{2+}$ and troponin from Bio-Gel P-10 columns equilibrated with 10 mM imidazole (pH 7.0) and 0.5 M KCl or 0.5 M NaCl, as indicated. See text for experimental details.

quite likely that nitrogen ligands, perhaps imidazole and/or α-amino groups[18], are also present at the chelation site. More direct experimental evidence is needed to substantiate this point.

On the basis of the present investigation divalent cations which can bind to the receptor site are $Ca^{2+} > Cd^{2+} > Sr^{2+} > Pb^{2+} > Mn^{2+}$, given in the apparent order of affinity. Binding of Sr^{2+} has been demonstrated in other studies[8,10]. Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, and Ba^{2+} were essentially excluded from the receptor site. The cations which do bind to troponin have no obvious chemical similarities. Ca^{2+}, Sr^{2+}, and Mn^{2+} are related in that they prefer oxygen as an electron donor and tend to form bonds which are largely ionic in character[19]. On the other hand Cd^{2+} and Pb^{2+} would be expected to prefer nitrogen and sulfur as election donors and form bonds with a larger covalent component. It is conceivable that although several cations can bind at the same site they do not all form the same type complex[19]. The Sr^{2+}–troponin complex is known to have biological activity[8,10]. It would be of interest to find out whether the Cd^{2+}, Pb^{2+}, and Mn^{2+} complexes also exhibit activity*.

The ability of a divalent cation to exchange with troponin-bound Ca^{2+} is seemingly related to its ionic radius. The cations with the highest affinity for troponin, Ca^{2+} and Cd^{2+}, have radii of 0.99 and 0.97 Å, respectively. Ca^{2+} was also displaced by cations with slightly smaller (Mn^{2+}, 0.80 Å) or slightly larger radii (Sr^{2+}, 1.12 Å; Pb^{2+}, 1.20 Å). A qualitatively similar pattern has also been observed with another myofibrillar protein, G-actin[20,21]. It should be pointed out that the actual values for Ca^{2+} exchange are subject to some uncertainty since metal ion hydrolysis and complexation with buffer were not taken into account. However, a more quantitative analysis of free cation concentrations would probably not invalidate the general relation observed between ionic radii and ability to bind to the receptor site of troponin.

In the absence of detailed structural information one can only speculate about the physicochemical basis of such size recognition. One possible approach may be derived from BRIGHT'S[22] investigation of β-methylaspartase, in which case affinity for divalent metals is also related a to certain optimal size. This author suggests that the reduced affinity for small cations (Mg^{2+}, Ni^{2+}) is due to repulsive forces between adjacent anionic ligands. On the other hand, the large cations (Sr^{2+}), having a less ordered water of hydration would show a lower entropy difference on binding to the protein. Since entropy increase is the main driving force for chelation[23] any reduction in this parameter would appear as a lower stability constant. While these factors may be of importance it is likely, considering the greater selectivity of troponin compared to β-methylaspartase, that other factors, probably steric in nature, will have to be taken into account.

At this point it is of interest to consider the results with the trivalent lanthanide ions. The five lanthanides tested all had about the same capacity to exchange with troponin-bound Ca^{2+}. Their relative affinity for the receptor site was apparently less than that of Ca^{2+}, being in the same approximate range as that of Cd^{2+} and Sr^{2+}. Here again, some uncertainty arises from the possibility of lanthanide hydrolysis, although it would appear from the literature[24,25] that the lanthanides are coordinated mainly to water at neutral pH. In any case, it is well known[26] that carboxyl groups

* D. J. HARTSHORNE (personal communication) has obtained data showing that Cd^{2+}, but not Mn^{2+}, can activate the ATPase activity of "natural" actomyosin.

which can form chelate rings compete very favorably with solvent ligands (H_2O, OH^-) in the coordination sphere of the lanthanide ion. Simple chelating agents invariably bind lanthanides more strongly than Ca^{2+}. Considering, as an example, cations of identical size, Ca^{2+} and Nd^{3+}, the EDTA complexes have log K values of 10.6 and 16.6, respectively (ref. 23, p. 572). This general pattern has also been observed in some biological systems as well. MELA[27] has shown that even at extremely low concentrations ($1 \cdot 10^{-6}$ M) lanthanides interact strongly with the Ca^{2+} transport system of mitochondria. More recently DARNALL AND BIRNBAUM[28] demonstrated that 1 mM $NdCl_3$ was more than twice as effective as 10 mM $CaCl_2$ in activating the conversion of trypsinogen to trypsin at neutral pH. The evidence presented in this report suggests that the physiological receptor site of troponin differs from the systems just mentioned in that Ca^{2+} is bound more strongly than the highly charged lanthanides despite close similarities in ionic radii and ligand affinity. Such evidence would support the view that in addition to charge and radius considerations more subtle structural factors must also be involved in the cation selectivity of troponin.

Further studies along these lines may prove rewarding with regard to the function of troponin. Correlation of physicochemical properties and biological activity of a variety of metal–troponin complexes might provide valuable information about the type of structural change which triggers muscle contraction.

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