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SIMILARITY IN Ca^{2+}-INDUCED CHANGES BETWEEN TROPONIN-C AND PROTEIN ACTIVATOR OF 3′:5′-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND THEIR TRYPTIC FRAGMENTS

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Summary

1. The Ca^{2+}-dependent protein activator of 3′:5′-cyclic nucleotide phosphodiesterase (3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) has been isolated from bovine brain and its properties have been compared with those of troponin-C. It has been shown that bovine brain does not contain troponin-C, but only the protein activator of phosphodiesterase. Its molecular weight is 16 500 as compared with 18 000 for troponin-C.

2. Similarly to troponin-C, calcium has a pronounced effect on the rate of cleavage of protein activator by trypsin and on the obtained peptide pattern. In the presence of 0.1 mM $CaCl_2$ two large peptides are formed, essentially resistant to further splitting, whereas upon removal of calcium by chelators a fast cleavage of protein activator into small peptides takes place.

3. Similarly to troponin-C, tyrosine fluorescence intensity of protein activator is markedly enhanced in the presence of Ca^{2+}. The apparent binding constant for Ca^{2+} calculated from the transition midpoint of fluorescence changes is about $1 \cdot 10^7$ M^{-1}. During digestion of protein activator with trypsin in the presence of $CaCl_2$ essentially no change of fluorescence intensity takes place and the subsequent decrease upon removal of calcium is reversible.

4. Two large peptides that accumulate during digestion by trypsin in the presence of Ca^{2+} of both troponin-C and protein activator have been isolated and their properties have been compared with those of corresponding parent molecules. One of the peptides obtained from both proteins retains the ability of the intact molecular to change the mobility on the polyacrylamide gel electrophoresis in the presence of urea depending on concentration of Ca^{2+} and to interact with the inhibitory component of troponin in the presence of Ca^{2+}.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl-ether)*N,N′*-tetraacetic acid; SDS, sodium dodecyl sulphate; TN-C, calcium binding component of troponin; TN-I, inhibitory component of troponin.

This peptide also shows Ca^{2+}-dependent fluorescence changes, characteristic for parent molecule.

5. On the basis of similarity between the two proteins and their tryptic fragments it is suggested that in the presence of Ca^{2+} protein activator is cleaved in the same area as troponin-C. The peptide obtained from protein activator, which shows Ca^{2+}-dependent changes characteristic for intact molecules, corresponds to the troponin-C peptide containing calcium binding sites 3 and 4. All these results furnish new pieces of evidence for a pronounced structural similarity between troponin-C and protein activator of cyclic nucleotide phosphodiesterase.

Introduction

Recently a Ca^{2+}-dependent protein activator of 3′:5′-cyclic nucleotide phosphodiesterase (3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) was isolated from brain and heart and its structural similarity to troponin-C was shown [1—6]. Troponin-C (TN-C) is an 18 000-dalton subunit of troponin which neutralizes inhibition of the Mg^{2+}-stimulated ATPase of vertebrate skeletal-muscle actomyosin by a second troponin subunit (TN-I) [7]. It has been shown that calcium induces conformational changes in the TN-C molecule [8] and has a pronounced effect on the cleavage of this protein by trypsin [9]. TN-C digested in the presence of calcium retains the Ca^{2+}-dependent conformational changes of the native molecule; i.e. the reversible decrease of intrinsic tyrosine fluorescence and α-helix content upon removal of bound Ca^{2+} by calcium chelators such as EGTA.

TN-C binds 4 mol of calcium [10] and contains four regions each containing a Ca^{2+}-binding site [11]. The cleavage of TN-C by trypsin in the presence of calcium yields two peptides. One of them containing calcium binding sites 1 and 2, and called in our previous work [9] C_2 peptide, is referred to in this paper as peptide I; the other, containing sites 3 and 4, and called previously C_3 peptide, is referred to as peptide II. In view of the similarity between TN-C and protein activator of cyclic nucleotide phosphodiesterase (referred to throughout this paper as protein activator), the effect of Ca^{2+} on trypsin digestion and on subsequent Ca^{2+}-dependent conformational changes of protein activator was investigated in the present work. The peptides accumulating during tryptic digestion of both TN-C and protein activator were isolated and their properties were analysed. The results show that calcium has the same effect on the cleavage of both proteins by trypsin and that Ca^{2+}-induced conformational changes characteristic for both native proteins are retained in one of the peptides, furnishing further evidence for the pronounced structural similarity between TN-C and protein activator.

Materials and Methods

Protein activator was prepared from bovine brain according to Watterson et al. [6]. Its activity was kindly examined by Dr. J. Wang. Troponin components were obtained as previously described [12]. In order to isolate tryptic fragments, TN-C or protein activator were digested with trypsin added at 1 : 50 to

1 : 100 (w/w) ratio in the presence of 0.1 mM $CaCl_2$. The digestion mixture was subjected to a preparative urea-gel electrophoresis in slabs in the presence of 1 mM $CaCl_2$. The areas occupied by the peptides were cut out, the peptides were eluted, lyophilized, dissolved in a small volume and subjected to another gel electrophoresis in the presence of 2 mM EGTA. The peptides were eluted, lyophilized, dissolved in a small volume and purified from any low molecular weight impurities originating from polyacrylamide on Sephadex G-25 column. The purity of all proteins was checked with the use of SDS- and urea-polyacrylamide gel electrophoresis. Urea gel electrophoresis was performed on slabs made from 8% polyacrylamide gel with 80 mM glycine, 20 mM Tris, pH 8.5 in the presence of 5 M urea. SDS gel electrophoresis was performed on slabs made from 15% polyacrylamide gel with 0.1% SDS, 100 mM phosphate buffer, pH 7.2. Gels were stained with Coomassie Brilliant Blue. For details of the electrophoresis see refs. 9 and 12. Fluorescence was measured using a Perkin Elmer Model MPF-2L fluorescence spectrophotometer.

Results

Digestion of protein activator with trypsin

Protein activator migrates on SDS polyacrylamide gel electrophoresis slightly faster than TN-C (Fig. 3, samples 1—3). A molecular weight of about 16 500 was obtained from calibration of the gels as compared to 18 000 for TN-C. As in the case of TN-C, the digestion of protein activator with trypsin is markedly effected by calcium, both with respect to its time-course and the resulting peptide pattern. In the presence of EGTA a rapid splitting of the protein activator takes place. Only at low trypsin to substrate ratio (1 : 500) it is possible to detect two peptides at the first stage of digestion (Fig. 1, sample 6), which are subsequently degraded to small fragments not visible on the gel. In 0.1 mM $CaCl_2$ a 10-fold increase of concentration of trypsin is required to achieve a slow cleavage yielding two peptides which are resistant to further splitting (Fig. 1, samples 1—5).

Fluorescence changes of protein activator

Similarly to TN-C [8,9] the intensity of tyrosine fluorescence of the protein activator is Ca^{2+}-dependent. Figure 2A presents the changes in the fluorescence intensity as the function of the concentration of free Ca^{2+}. The apparent binding constant of Ca^{2+} to protein activator calculated from the transition midpoint of the fluorescence changes is about $1 \cdot 10^7\ M^{-1}$. The decrease of fluorescence intensity of protein activator upon removal of Ca^{2+} with EGTA is about 50%. On the other hand, 2 M guanidine-HCl causes in the presence of 0.1 mM $CaCl_2$ only about a 35% decrease of fluorescence intensity in contrast to the approximate 70% decrease in the presence of EGTA (Fig. 2B, zero time), again indicating a protective effect of calcium on the structure of protein activator.

During digestion of TN-C in the presence of Ca^{2+} there is only a small drop of tyrosine fluorescence, and its decrease upon subsequent addition of EGTA is reversible [9]. Fig. 2B shows that protein activator behaves in the same way. There is virtually no decrease of fluorescence intensity after cleavage of the molecule (the latter checked in the control sample with the use of gel electropho-

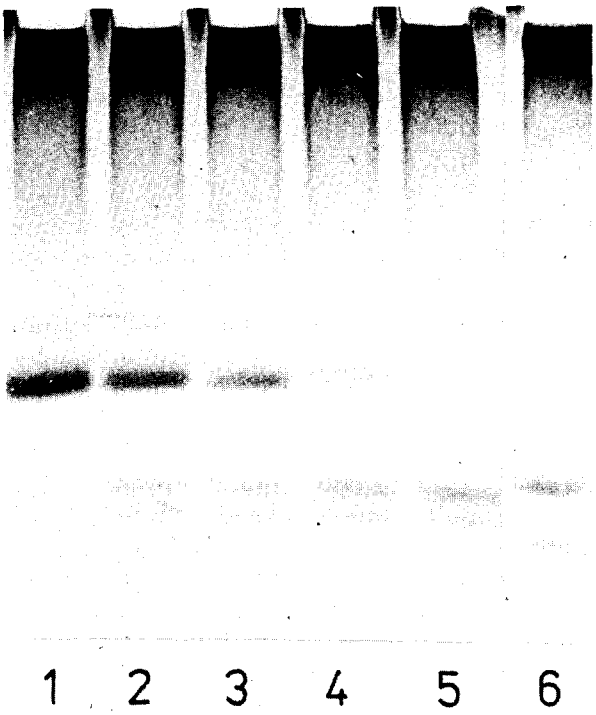

Fig. 1. Digestion of protein activator with trypsin. Protein activator (1 mg/ml) dissolved in 20 mM NH_4HCO_3, pH 8.2, was digested at 20°C with trypsin added at 1 : 50 (w :w) ratio in the presence of 0.1 mM $CaCl_2$ for 5 min (1), 10 min (2), 15 min (3), 30 min (4) and 60 min (5), or with trypsin added at 1 : 500 (w : w) ratio in the presence of 1 mM EGTA for 5 min (6). Electrophoresis was performed on 15% polyacrylamide slabs in the presence of 0.1% SDS [9].

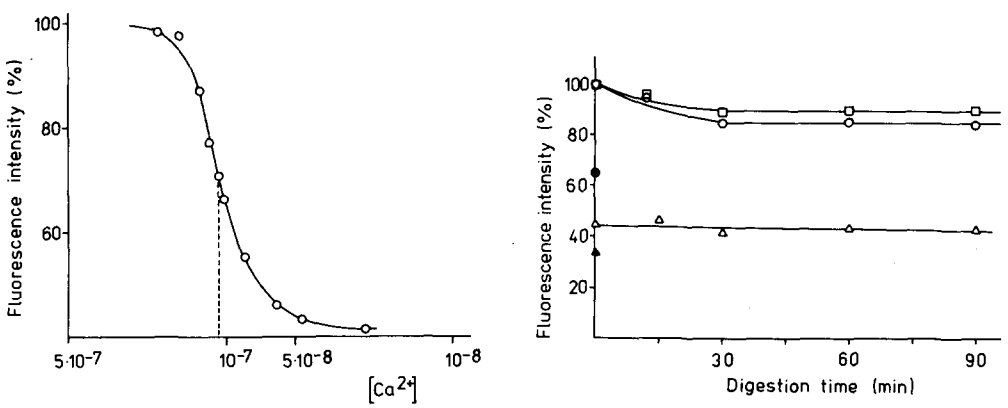

Fig. 2. Tyrosine fluorescence of protein activator. A. Changes in the fluorescence intensity as the function of free Ca^{2+} concentration. Fluorescence of protein activator (0.2 mg/ml) was measured in a medium containing 2 mM CaEDTA buffer with free Ca^{2+} concentration varying from 10^{-9} to 10^{-5} M and 50 mM HEPES, pH 7.3. For calculation of free Ca^{2+} concentrations the stability constants from Sillen and Martell [13] were used. B. Changes in the fluorescence intensity during tryptic digestion. Protein activator (1 mg/ml) was digested with trypsin (1 : 50, w : w) in the presence of 0.1 mM $CaCl_2$ and 20 mM NH_4HCO_3, pH 8.2. At the time intervals indicated in abscissa samples were withdrawn and the reaction was stopped by addition of soya-bean trypsin inhibitor. After 10-fold dilution fluorescence was measured directly (○), after addition of 0.2 mM EGTA (△), and after subsequent addition of 0.3 mM $CaCl_2$ (□). Fluorescence of the original protein activator in the presence of 2 M guanidine-HCl and 0.1 mM $CaCl_2$ (●) or 1 mM EGTA (▲). Ordinate, relative fluorescence intensity in percent fluorescence of protein activator saturated with calcium. Excitation wavelength 280 nm, emission wavelength 305 nm.

resis), and similar Ca^{2+}-dependent reversible changes in the fluorescence intensity can be obtained with the digest.

When the digestion is performed in the presence of EGTA the ability to increase fluorescence intensity upon subsequent addition of Ca^{2+} is lost.

Peptides from TN-C and protein activator

The two peptides accumulating during digestion of both TN-C and protein activator in the presence of Ca^{2+} have been isolated by preparative urea-gel electrophoresis and desalted on Sephadex G-25 columns. Their mobilities on SDS gels are presented in Fig. 3 (samples 4—6). The peptides obtained from protein activator have electrophoretic mobilities that are different from those of the corresponding TN-C peptides obtained under similar conditions, clearly indicating the difference in the molecular weight.

The exact point of cleavage of protein activator is not known at present. However, in view of the pronounced similarity between the two proteins, the peptide from protein activator which shows the same properties in urea gel (see below) as peptide II from TN-C is referred to also as peptide II. Peptide II from TN-C reveals the same Ca^{2+}-induced spectral changes as the native TN-C molecule, as measured by the changes of circular dichroism and fluorescence intensity. Moreover, the apparent binding constant of Ca^{2+} to peptide II, calculated from the transition midpoint of fluorescence changes (about $3 \cdot 10^7\ M^{-1}$), is the same as that for the intact TN-C molecule [14]. Determination of fluorescence

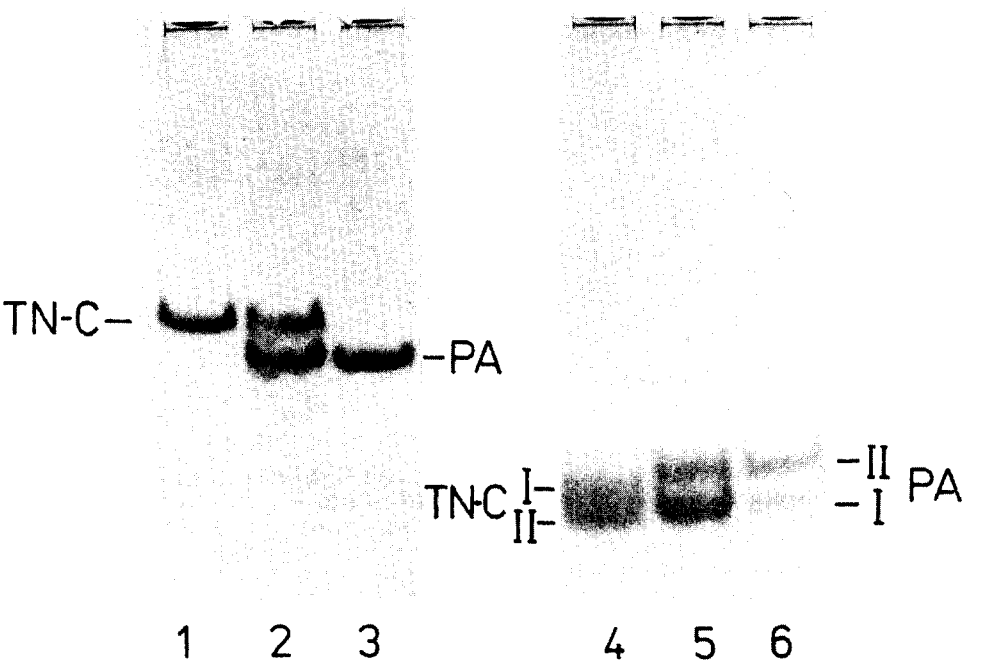

Fig. 3. Comparison of the mobilities of intact proteins and their tryptic fragments. Samples 1 and 2, TN-C; samples 2 and 3, protein activator (PA); samples 4 and 5, peptides I and II from TN-C; samples 5 and 6, peptides I and II from protein activator. SDS gel electrophoresis on 15% polyacrylamide slabs.

intensity of peptides obtained from protein activator, performed in this work, indicates that one of the peptides contains the tyrosine residue. Similarly to TN-C, only peptide II exhibits Ca^{2+}-dependent changes in the tyrosine fluorescence characteristic for native protein activator.

Mobility of proteins and peptides in urea gel and their interaction with TN-I

In Fig. 4 the mobility of the peptides I and II in the urea gel in the presence and absence of Ca^{2+} and their ability to interact with TN-I is compared with that of intact proteins. Again, a striking similarity between protein activator and TN-C has been found. TN-C moves in urea gel much faster in the presence of calcium than in its absence [12]. Moreover, in the presence of Ca^{2+} a complex between TN-I and TN-C can be observed in the urea gel. (Fig. 4A, samples 1, 4 and 7). Protein activator reveals similar features (Fig. 4B, samples 1, 4 and 7) as also recently reported by Amphlett et al. [15]. The mobility of protein activator in the presence of Ca^{2+} is slightly higher than that of TN-C (compare sample 4 in Fig. 4A with that in Fig. 4B). On the other hand, the complex between TN-I and protein activator has distinctly slower mobility than that of TN-I · TN-C complex (compare sample 7 in Fig. 4A with that in Fig. 4B). In the absence of calcium both proteins move with approximately the same mobility (Figs. 4A and 4B, sample 1).

Peptide II from TN-C preserves the properties of the original molecule, i.e. reveals on urea gel much higher mobility in the presence of Ca^{2+} and also interacts with TN-I (Fig. 4A, samples 3, 6 and 9). Similarly to peptide II from TN-C, the corresponding peptide from protein activator moves faster in urea gel in the presence of Ca^{2+} than in its absence and also interacts with TN-I (Fig. 4B, samples 3, 6 and 9), although its affinity seems to be weaker than that of peptide II from TN-C.

Peptide I from TN-C does not show any Ca^{2+}-dependent changes in mobility on urea gel (Fig. 4A, samples 2 and 5), in contrast to some changes found in case of peptide I from protein activator (Fig. 4B, samples 2 and 5). In both cases, however, peptide I does not interact with TN-I (Fig. 4A and 4B, sample 8).

The lack of TN-C in brain

Fine and co-workers reported recently [16] that brain tissue contains TN-C. The evidence was based on the mobility increase of the isolated brain protein and its ability to interact with TN-I in the presence of Ca^{2+}. Moreover, this protein could replace TN-C in the troponin · tropomyosin complex in conferring Ca^{2+}-sensitivity to the actin-myosin interaction. The recent results of Amphlett et al. [15], as well as those of the present work, show, however, that all these properties are shared by both TN-C and protein activator. In view of these results we have investigated whether brain tissue indeed contains both proteins. An 8 M urea extract from bovine brain homogenate has been subjected to preparative urea gel electrophoresis in the presence of EGTA and the rapidly migrating band eluted. This protein separates into two bands on urea gel in the presence of calcium (Fig. 5, sample 1). One of those bands contains S-100 protein [17] which does not interact with TN-I. The protein present in the second band forms a complex with TN-I with the mobility characteristic for the pro-

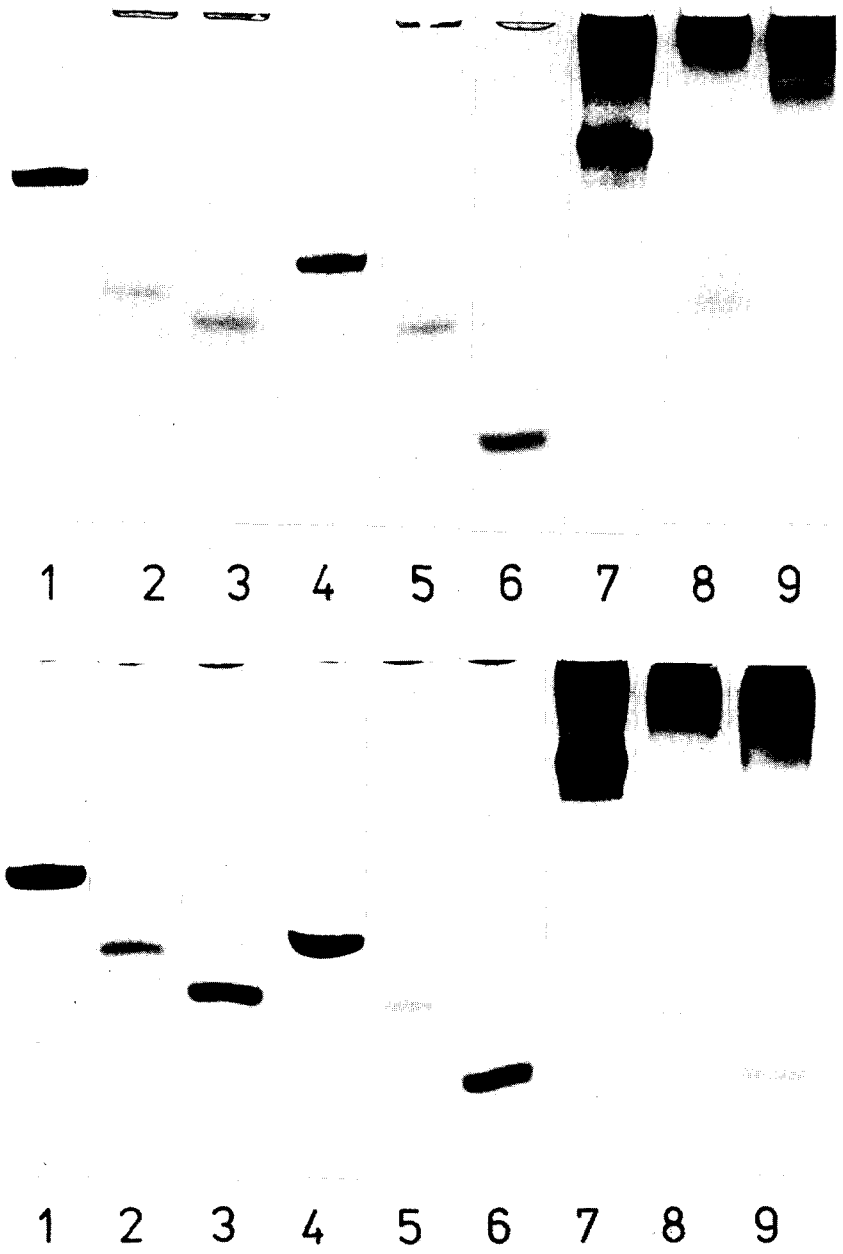

Fig. 4. Urea gel electrophoresis of TN-C and its tryptic fragments (A) and protein activator and its tryptic fragments (B). Samples 1, 4, 7, native proteins; samples 2, 5, 8, peptide I; samples 3, 6, 9, peptide II. TN-I was added to samples 7—9. Electrophoresis was performed on 8% polyacrylamide slabs in the presence of 5 M urea. 1.0 mM EGTA was added to samples 1—3 and 0.1 mM $CaCl_2$ to samples 4—9.

tein activator · TN-I complex, and not for the TN-C · TN-I complex (Fig. 5, samples 2—4). Also the mobility of that protein in SDS gel corresponds exactly to that of the protein activator but not to TN-C (Fig. 5, samples 5—7). Moreover, this protein, after elution, activates cyclic nucleotide phosphodiesterase activity

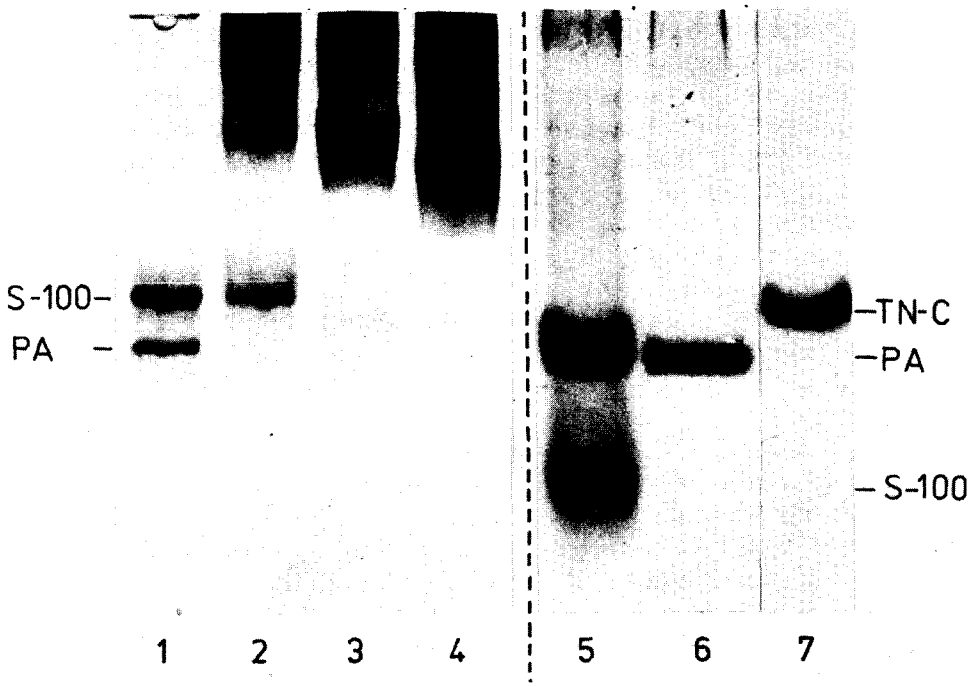

Fig. 5. The identification of fast-moving acidic proteins in brain homogenate. Urea gel electrophoresis in the presence of 0.1 mM $CaCl_2$ (samples 1—4), SDS gel electrophoresis (samples 5—7). 8 M urea extract from brain homogenate (20 mg protein) was subjected to preparative urea gel electrophoresis in the presence of EGTA. The protein material which formed a fast-moving band was eluted and run in both urea gel in the presence of calcium (samples 1 and 2) and in SDS gel electrophoresis (sample 5). Samples 3 and 6 contained protein activator, samples 4 and 7 contained TN-C. Samples 2, 3, 4 contained TN-I in addition. S-100, S-100-protein from brain; PA, protein activator; TN-C, troponin-C.

in the presence of calcium. As it was shown by Wang et al. [3] this specific property of protein activator is not shared by TN-C.

Discussion

It has been recently shown that a protein activator of the cyclic nucleotide phosphodiesterase exhibits several common features with TN-C [5,6]. There is a striking similarity in the amino acid composition, especially the absence of tryptophan and the high phenylalanine to tyrosine ratio, although the peptide map for the two proteins is different [5,6]. Also, the mass of the protein activator is smaller. Contrary to some workers [5,6] who reported that protein activator has the same mobility on SDS gel as TN-C, our results, as well as those of Lin et al. [2], clearly show that the protein activator moves faster and its molecular weight corresponds to about 16 500.

The results of the present work furnish several new pieces of evidence for a close structural similarity between protein activator and TN-C. Both proteins exhibit a similar susceptibility to trypsin degradation. Recent studies have

indicated that Ca^{2+} stabilizes the structure of TN-C against the action of trypsin [9]. A similar protective effect of Ca^{2+} on the protein activator, first observed by Ho et al. [4] in respect to the changes in the biological activity of this protein, has been demonstrated in this work. In the presence of Ca^{2+} each protein yields two tryptic fragments resistant to further cleavage. One of these two peptides (called in this paper peptide II) retains several properties characteristic for the corresponding intact protein.

Protein activator, as TN-C, binds 4 mol of calcium [1,2,6] and its similarity to TN-C implies that it too contains four regions, each containing one Ca^{2+}-binding site. One of the TN-C tyrosine residues (Tyr 109) [18] is located in a loop forming calcium binding site 3. Recent studies indicate that regardless of the method of splitting of TN-C molecule the fragments that contain binding site 3 show Ca^{2+}-induced spectral changes [19]. Judging from the amino acid sequence determined for a part of protein activator molecule [6] one of its two tyrosines seems to be located in the same position as in the TN-C molecule. Thus, the Ca^{2+}-dependent spectral changes of protein activator and its fragment II showed in this work strongly suggest exactly the same configuration in the region around calcium binding site 3 for both proteins.

The peptide II from TN-C with sites 3 and 4 also contains the site of interaction with TN-I. Weaker interaction of the analogous peptides from protein activator with TN-I is in accord with some differences between the two proteins in their ability to restore Ca^{2+}-sensitivity to the system containing actomyosin, tropomyosin and TN-I (cf. ref. 15).

The value of the apparent binding constant for calcium obtained in this work for protein activator, about $1 \cdot 10^7\ M^{-1}$, based on changes in the tyrosine fluorescence, is of the same order of magnitude as that derived from direct measurements of binding of Ca^{2+} [1,2,6]. Preliminary determinations performed in this work show that the binding constant of calcium in peptide II from protein activator calculated also from the transition midpoint of the fluorescence changes is virtually the same as that in the original protein activator molecule. The Ca^{2+}-binding constant for protein activator seems to be slightly lower than the corresponding binding constant for TN-C and its fragment II [19]. This is consistent with the recent observations of Amphlett et al. [15] who showed that the troponin · tropomyosin complex in which TN-C was replaced by protein activator needed slightly higher concentration of calcium for activation of the actomyosin ATPase.

All the results of this work clearly indicate that TN-C and protein activator [3] together with other low molecular weight proteins, e.g., parvalbumins, soluble sarcoplasmic calcium binding proteins and myosin light chains, form a group of homologous proteins originating from the same ancestor by gene duplications.

In view of the recent suggestions that various non-muscle tissues contain TN-C an attempt was made to see if brain tissue contains both protein activator and TN-C. Utilizing the approach developed in this work to distinguish between these two proteins we were unable to find TN-C in whole brain homogenate. Moreover, our preliminary results suggest that only striated muscle contain TN-C, whereas all other tissues as adrenal medulla [20], platelets [21] and even smooth muscle (chicken gizzard) [22] where recently a TN-C-like protein was

found, contain only protein activator of the phosphodiesterase. Whether the role of this protein is only connected with the metabolism of cyclic nucleotides or is directly related to some process similar to the regulation of the contraction in muscle remains to be solved.

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The suggestions made in this paper concerning the identity of the area of splitting of TN-C and protein activator by trypsin obtained recently a direct experimental proof. The determinations (Walsh, M., Stevens, F.C., Kuźnicki, J. and Drabikowski, W. (1978) J. Biol. Chem., submitted) show that the tryptic cleavage of protein activator in the presence of Ca^{2+} takes places at Lys(77)-Asp(78) peptide bond, homologous to the Lys(84)-Glu(85) peptide bond in TN-C.

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