TROPONIN C LIKE PROTEIN OF BLOOD PLATELETS

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1. Introduction

Numerous physiological and biochemical processes of the animal cell are mediated through the availability of Ca ions in the μM concentration range. Ca^{2+} regulation is usually triggered by its action on specific Ca^{2+} binding proteins. Troponin C (TNC) of skeletal muscle is a typical example of this kind of protein. This highly acidic component of the tropomyosin-troponin system binds Ca^{2+} with high affinity and confers Ca^{2+} sensitivity to actomyosin [1]. Most recently it has been established that the Ca^{2+}-dependent protein activator of cyclic nucleotide metabolism (PA) [2,3] in brain and heart tissues is also a TNC-like protein [4–7]. TNC and PA although clearly distinguishable from each other have quite a few common properties and probably evolved from a common ancestor [4–7].

In blood platelets, the availability of Ca^{2+} is a prerequisite for several steps of haemostatic activation [8]. The contractile activity of platelet-rich plasma clots [9] as well as 'natural' platelet actomyosin also possesses Ca^{2+} sensitivity [10]. Although the existence of platelet TNC was assumed several years ago [11] so far no TNC-like protein has been isolated from these cells. The aim of the present study was to identify and isolate a TNC-like protein from bovine platelets and to establish if it is equivalent to brain PA or to muscle TNC.

2. Materials and methods

Platelets were obtained from citrated bovine blood by differential centrifugation. The isolation of rabbit skeletal muscle TNC and troponin I (TNI) was carried out according to Drabikowski et al. [12]. Highly purified PA and activator deficient cyclic nucleotide phosphodiesterase were prepared from bovine brain [5]. Cyclic AMP phosphodiesterase activity was determined in the presence of 0.1 mM Ca^{2+} by a two-stage assay [5] measuring the phosphate liberated [13] in the second stage. Alkaline urea (AU) [14] and sodium dodecylsulfate (SDS) [15] polyacrylamide gel electrophoresis (PAGE) were performed in 8.0% and 12.5% gels, respectively. Preparative AU PAGE was carried out on a Poly-prep 200 (Buchler) equipment. The following reference proteins were used for molecular weight determination by SDS PAGE: TNI, TNC, myoglobin and cytochrom *c* (Boehringer). Absorbance spectrum was recorded on a Unicam SP 8000 spectrophotometer. Fluorescence was measured using a Perkin–Elmer MPF-2L spectrofluorometer. The apparent Ca^{2+} binding constant was calculated by transition midpoint analysis of the fluorescence change upon addition of Ca^{2+} [16]. Protein concentration was estimated by Lowry's procedure or UV absorbance measurement.

3. Results and discussion

For the detection of TNC-like protein in platelet homogenate we took the advantage of AU PAGE. In this system if Ca^{2+} is removed the highly negatively charged TNC moves far ahead of the bulk of the other proteins. If both Ca^{2+} and a TNC-binding protein, e.g., TNI are present a complex with slower mobility is formed and the band corresponding to TNC disappears [14]. This kind of approach has been

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successfully applied to the detection of TNC-like protein in brain tissue [17]. In whole platelet homogenate if EGTA was added two fast moving bands could be detected by AU PAGE (fig.1). In the presence of Ca^{2+} one of them, like TNC in muscle or brain homogenate, disappeared while the other remained, even if muscle TNI was included (not shown). The latter band probably represents the light chains of platelet myosin which in alkaline urea gel move together and with higher mobility than any of the light chains of skeletal muscle myosin [18], even slightly faster than TNC [19].

The protein corresponding to the band with TNC-like behaviour was isolated, partially characterized and compared to skeletal muscle TNC and to the PA of brain. To get rid of myosin components and some other proteins platelets were treated with acetone then extracted by 1 M KCl. The extract was exhaustively dialyzed against 0.5 mM DTT solution (pH 7.0), and the precipitate which formed was removed by centrifugation. In the supernatant, although still a crude mixture of proteins, only one fast moving band – the one with the ability of forming a complex in the presence of Ca^{2+} – could be detected (fig.1). Further purification by preparative AU PAGE resulted in a highly purified protein. A single band was seen when it was subjected to analytical AU and SDS PAGE (fig.2).

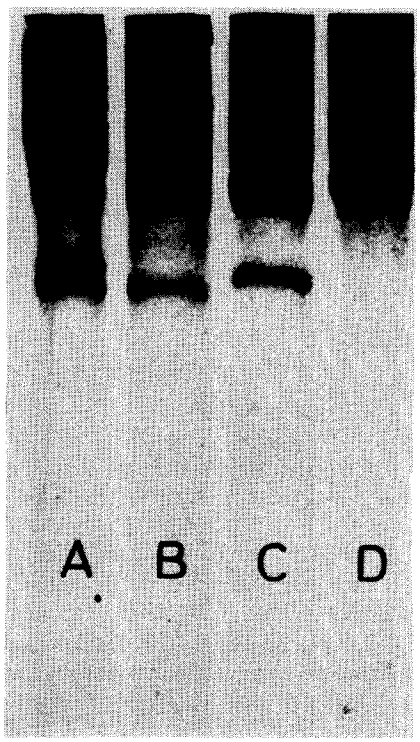

Fig.1. Alkaline urea polyacrylamide gel electrophoresis of platelet homogenate (A, B) and 1 M KCl extract of platelet acetone powder (C, D). 5 mM EGTA (A, C) or 1 mM $CaCl_2$ (B, D) were added to the samples and 400 μg (A, B) or 200 μg (C, D) protein was applied onto the gels. The homogenate was prepared as described by Fine et al. [17] for brain tissue.

This protein shared the common properties of TNC-like proteins. Like TNC [16] and PA [7] it exhibited a fluorescence emission spectrum (maximum at 306 nm) characteristic of tyrosine peptides, and the removal of Ca^{2+} resulted in a decrease of fluorescence intensity. It possessed an absorbance spectrum common for polypeptides with a high phenylalanine : tyrosine ratio and as other TNC-like proteins [4,5] showed considerable vibrational structure in the region of 250 to 280 nm (peaks were seen at 253, 258, 264, 268 and 275 nm, $E_{1\ mg/ml,\ 275\ nm} = 0.18$). The electrophoretic behaviour of the platelet protein in alkaline urea gel again resembled TNC [14,20] and brain PA [6,7]. Its mobility corresponded to that of other TNC-like proteins, was increased by Ca^{2+} and if TNI was also included complex formation occurred (fig.2). These findings clearly prove that a TNC-like protein does exist in platelets, but still leave open the question whether muscle TNC or PA is the equivalent of it.

Drabikowski et al. [7] have shown that in AU PAGE the complex of brain PA and TNI migrated significantly slower than the TNC–TNI complex. The complex of platelet protein and TNI moved with the same mobility as PA–TNI (fig.2a). The molecular weight of platelet protein was found to be 16 500 by SDS PAGE. It co-migrated with brain PA but not with TNC (18 000) (fig.2b). The binding constant of platelet protein for Ca^{2+} was estimated to be 4.2×10^6 M^{-1} by fluorescence titration. The respective values based on the same fluorescence method for brain PA and TNC are 1×10^7 M^{-1} and 5×10^7 M^{-1} [7]. Finally, in the presence of Ca^{2+} the TNC-like protein of platelets activated activator deficient brain phosphodiesterase in a manner identical to brain PA while TNC in the same concentration was without effect (fig.3). To sum up, in certain characteristics platelet TNC-like protein clearly differs from TNC but is

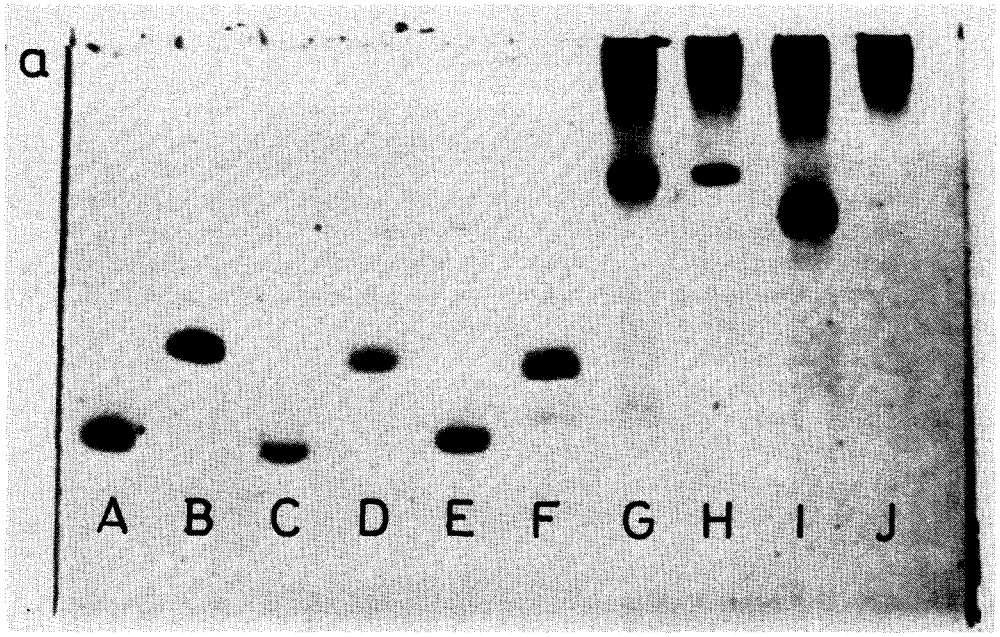

Fig.2a

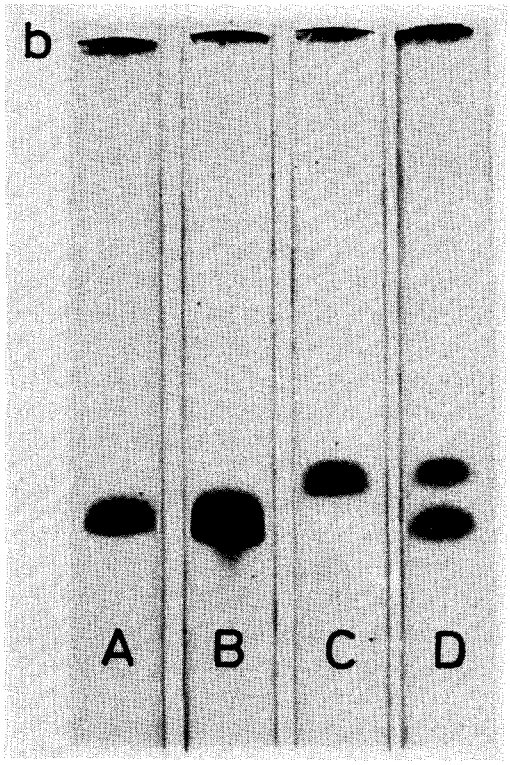

Fig.2b

indistinguishable from brain PA, i.e., the protein we isolated has to be considered as the platelet protein activator of cyclic nucleotide metabolism.

In the presence of Ca^{2+} PA stimulates the activity of cyclic nucleotide phosphodiesterase [21,22] and solubilized adenyl cyclase [23,24]. It probably plays a key regulatory role in the cyclic nucleotide metabolism. Changes of cyclic nucleotide metabolism and increase in intracellular Ca^{2+} concentration are deeply involved in platelet functions such as shape change, aggregation and release reaction [8,25] and PA may provide a link of high significance between the two mechanisms. Smoake et al. [26] have demonstrated a

Fig.2. Electrophoretic behaviour of the TNC-like protein from platelets in alkaline urea (a) and SDS (b) polyacrylamide gel. (a): 1 mM $CaCl_2$ or 5 mM EGTA were included into the samples. 3 μg brain PA, 1 μg platelet protein, 3 μg TNC and 10 μg TNI were applied onto the gels. (A) Brain PA + Ca^{2+}; (B) brain PA + EGTA; (C) platelet protein + Ca^{2+}; (D) platelet protein + EGTA; (E) TNC + Ca^{2+}; (F) TNC + EGTA; (G) brain PA + TNI + Ca^{2+}; (H) platelet protein + TNI + Ca^{2+}; (I) TNC + TNI + Ca^{2+}; (J) TNI + Ca^{2+}. (b): (A) platelet protein 5 μg; (B) platelet protein 5 μg + brain PA 5 μg; (C) TNC 3 μg; (D) TNC 2 μg + platelet protein 4 μg.

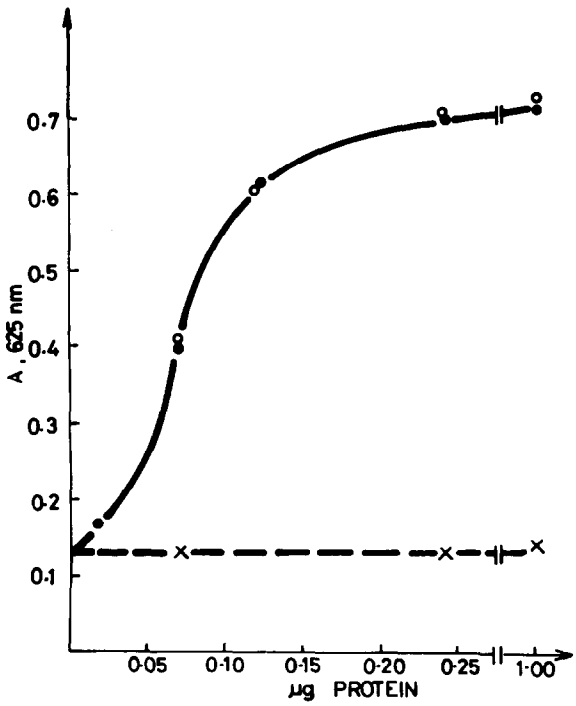

Fig.3. Effect of TNC-like protein from platelets, brain PA and TNC on the activity of activator deficient brain phosphodiesterase. Platelet protein (•——•); brain PA (○——○); TNC (X- - - X). Activity was measured on 0.07 units [6] of the enzyme. The amount of different TNC-like proteins in the assay mixture is indicated on the abscissa.

hitherto unidentified factor in platelet homogenate that in the presence of Ca^{2+} activated cyclic nucleotide phosphodiesterase. The TNC-like protein isolated and characterized in this study is in all probability the entity responsible for the effect observed by those authors.

In an earlier paper Cohen et al have provided some evidence for an actin-linked Ca^{2+} regulatory mechanism in platelet actomyosin [11]. More recently, however, a myosin-connected Ca^{2+} regulation was verified in this system [19]. Here, we showed that beside PA no other TNC-like protein exists in platelets. It is interesting to note that in a reconstituted skeletal muscle actomyosin system brain [6] as well as testis [27] PA exerted TNC-like activity conferring Ca^{2+} sensitivity to it. A similar activity of platelet PA in in vivo conditions can be also surmised although during a conventional actomyosin purification procedure it does not copurify with the actomyosin complex [19].

Acknowledgements

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