Troponin C-Like Proteins (Calmodulins) from Mammalian Smooth Muscle and Other Tissues

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1. An acidic protein with properties similar to those of troponin C from rabbit skeletal muscle has been shown to be present in bovine and rabbit smooth muscles, chicken gizzard and rabbit liver, kidney and lung. 2. A simple new method involving the use of organic solvents is described for the purification of the troponin C-like proteins from various tissues. 3. The troponin C-like proteins can be distinguished from rabbit skeletal-muscle troponin C by their electrophoretic behaviour on polyacrylamide gels at pH 8.3 in the presence and absence of Ca^{2+} . The troponin C-like proteins have been shown to form complexes with rabbit skeletal-muscle troponin I that migrate on electrophoresis in polyacrylamide gels. 4. Behaviour on electrophoresis, amino acid analysis and the patterns of CNBr digests on polyacrylamide gels indicate that the troponin C-like proteins from bovine uterus and aorta, rabbit uterus, and liver and chicken gizzard are very similar to, if not identical with, bovine brain modulator protein. 5. With bovine cardiac muscle the organic-solvent method yields a preparation consisting of roughly similar amounts of troponin C and troponin C-like protein. 6. By the isotope-dilution technique, troponin C-like protein has been shown to represent 0.42% of the total protein in rabbit uterus. 7. In homogenates of smooth muscle, rabbit lung, kidney and brain, the troponin C-like proteins form a complex with other protein (or proteins) that requires Ca^{2+} for its formation and that is not dissociated in 9 M-urea.

The question whether a troponin-like system is involved in the regulation of contraction in vertebrate smooth muscle is still not resolved. Carsten (1971) and Sparrow & van Brockxmeer (1972) presented evidence suggesting that this tissue contained a system similar to that present in skeletal muscle, whereas other investigators (Bremel, 1974; Sobieszek & Bremel, 1975; Aksoy *et al.*, 1976) have concluded that smooth muscle does not contain a troponin system. Ebashi *et al.* (1975) and Mikawa *et al.* (1977), on the other hand, maintain that the contraction of gizzard smooth muscle is regulated by a troponin system that is different from that found in skeletal muscle.

Previous studies from this laboratory (Head *et al.*, 1977a) provided evidence that smooth muscle from rabbit uterus, ox aorta and chicken gizzard contain an acidic protein of apparent mol.wt. about 18000 that migrated rapidly to the anode when extracts of whole muscle, made in 6 M-urea/5 mM-EGTA, pH 8.6, were subjected to polyacrylamide-gel electrophoresis in this buffer. If the EGTA in the buffer was replaced by 1 mM- CaCl_2 , the acidic protein band disappeared, presumably because it formed a complex with some other components in the extract, and consequently migrated much more slowly. This behaviour is very similar to that observed with similar extracts of whole skeletal muscle and that was shown to be due to the

presence of a Ca^{2+} -dependent complex of troponin C and troponin I that is stable in 6 M-urea (Head & Perry, 1974). In view of these observations, an affinity-chromatographic technique using fast-skeletal-muscle troponin I linked to Sepharose was used to isolate a troponin C-like protein from uterus muscle (Head *et al.*, 1977a) and gizzard (Head *et al.*, 1977b). This protein was shown to possess properties similar to those of troponin C of skeletal muscle, but it also exhibited certain other properties (Grand *et al.*, 1977) that suggested that it might be similar to the modulator protein isolated from bovine brain (Watterson *et al.*, 1976; Stevens *et al.*, 1976). The latter protein has also been shown to behave like skeletal-muscle troponin C in restoring Ca^{2+} sensitivity to actomyosin adenosine triphosphatase systems in the presence of tropomyosin and other components of the troponin complex (Fine *et al.*, 1975; Amphlett *et al.*, 1976).

The present paper describes a new method for the isolation of the troponin C-like protein from smooth muscle, which can also be used for the isolation of a similar protein from other tissues. Study of the troponin C-like protein isolated by this method from bovine smooth muscle and other tissues confirms that it is probably very similar to, if not identical with, the modulator protein isolated from bovine brain.

Materials and Methods

Preparation of troponin C-like proteins

Bovine uterus, aorta, heart, brain and pig stomach were obtained from the local abattoir immediately after slaughter. Uterus, liver, brain, lung, kidney and skeletal muscle from New Zealand White rabbits were excised immediately after death. Chicken gizzards were obtained in a frozen state from Whitehouse Poultry, Rubery, Birmingham B45, U.K. Tissues were either used immediately or stored frozen at -20°C until required. Methods 1 and 2 below were carried out at 21°C .

Method 1, using affinity chromatography. Troponin C-like protein was prepared from rabbit uterus by a slight modification of the method of Head *et al.* (1977a). Tissue (20g) was homogenized with a Waring blender in 10vol. of 9M-urea/75mM-Tris adjusted to pH 8.0 with 1M-HCl/1mM- CaCl_2 /15mM-2-mercaptoethanol, centrifuged at 23 000g for 20min and the supernatant applied to a column (3cm $\times$ 10cm) of DEAE-cellulose equilibrated with the same buffer. After washing with the buffer until protein was no longer eluted, about 10–20% of the total protein was held on the column. Almost all of this bound protein was eluted by the application of the buffer to which 0.5M-KCl had been added. The fraction eluted by the latter buffer was then applied directly to an affinity column (1.5cm $\times$ 10cm) of 20mg of rabbit skeletal-muscle troponin I coupled to 20g of Sepharose 4B, which was equilibrated against 9M-urea/75mM-Tris/HCl buffer (pH 8.0)/1mM- CaCl_2 . Bound protein was eluted with the same buffer in which the 1mM- CaCl_2 was replaced by 10mM-EGTA, dialysed exhaustively against water and freeze-dried. Average yields were approx. 2mg of protein from 40g of tissue.

Method 2, using organic solvents. Tissue (100g) was homogenized in 700ml of 9M-urea/75mM-Tris adjusted to pH 8.0 with 1.0M-HCl/1mM- CaCl_2 /15mM-2-mercaptoethanol with a Waring blender, centrifuged at 23 000g for 20min and then filtered through glass wool. Bovine uterus and aorta were rehomogenized in 200ml of urea buffer, the solution centrifuged again for 20min at 23 000g and the supernatants combined. In the case of the other tissues this second extraction was not necessary, as virtually complete solubilization was achieved with one homogenization.

Ethanol was added slowly, with vigorous stirring, to a final concentration of 55%. Alternatively, methanol was added to a final concentration of 50%. After 15min the solution was centrifuged at 4000g for 10min and the precipitate discarded. Ethanol or methanol was added to the supernatant to a concentration of 80% and the precipitate sedimented by centrifugation at 4000g for 10min. The precipitate was redissolved in approx. 50ml of 9M-urea/75mM-

Tris/HCl (pH 8.0)/1mM- CaCl_2 /15mM-2-mercaptoethanol, and dialysed against the same buffer and applied to a column (3cm $\times$ 10cm) of DEAE-cellulose equilibrated in the same buffer. Protein was eluted with a gradient of KCl from 0 to 0.35M, and 10ml fractions collected (Fig. 1). Troponin C-like protein was detected by electrophoresing 50 μ l portions on polyacrylamide gels at pH 8.3 in the presence of 6M-urea. The fractions (eluting in the conductivity range 6.5–8.5 mmho) that contained the troponin C-like protein were combined, dialysed against water and freeze-dried.

The freeze-dried protein was dissolved in 3ml of 6M-urea and chromatographed on a column (2.2cm $\times$ 120cm) of Sephadex G-100 equilibrated with 20mM- NH_4HCO_3 (pH 7.9) and eluted with the same buffer. Troponin C-like protein with a high A_{215}/A_{280} was shown by electrophoresis to be present in the more retarded of the two peaks of protein eluted (Fig. 2). Fractions containing troponin C-like protein were combined, dialysed against water and freeze-dried. The method usually yielded 10–15mg of troponin C-like proteins from 100g of tissues other than striated muscle (Table 1). There was no noticeable difference in yield or purity of the proteins obtained in the methods using ethanol or methanol.

Fig. 1. Chromatography on DEAE-cellulose of the protein precipitated from bovine uterus homogenates by 80% ethanol

The column (3cm $\times$ 10cm) was equilibrated and developed with 9M-urea/75mM-Tris adjusted to pH 8.0 with 1M-HCl/15mM-2-mercaptoethanol. Bound protein was eluted with a gradient of 0–0.35M-KCl, and 10ml fractions were collected. Troponin C-like protein was detected by the electrophoresis of 50 μ l portions on polyacrylamide gels at pH 8.3. The fractions containing troponin C-like protein were eluted in the region represented by the bar and were pooled. —, A_{280} ; ----, conductivity.

Fig. 2. Chromatography of partially purified troponin C-like protein from bovine uterus on Sephadex G-100

The protein was applied to a column (2.2 cm $\times$ 120 cm) of Sephadex G-100 equilibrated with 20 mM- NH_4HCO_3 and eluted with the same buffer; 5 ml fractions were collected. Fractions under the bar were pooled. —, A_{280} ; ----, A_{215} .

Preparation of other proteins

Rabbit fast-skeletal-muscle troponin I and troponin C were prepared by the method of Ebashi *et al.* (1971). Bovine brain modulator protein, prepared by the method of Watterson *et al.* (1976), was a gift from Mrs. Heather Cole (Department of Biochemistry, University of Birmingham).

Preparation of affinity column

Troponin I was coupled to Sepharose 4B by the method of March *et al.* (1974) as described by Head *et al.* (1977a).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in 14 mM-Tris/90 mM-glycine, pH 8.3, either in the presence or

Table 1. Yields of troponin C-like protein from muscle and non-muscle tissues obtained by the organic-solvent method

All preparations were carried out on 50–120 g of tissue. With the exception of the preparation from rabbit skeletal muscle, which consisted entirely of troponin C, and that from cardiac muscle, which was about 40% troponin C, all other preparations consisted of modulator protein only.

Species	Tissue	No. of preparations	Average yield (mg/100 g of tissue)
Ox	Uterus	12	11
Ox	Aorta	1	6
Ox	Brain	2	17
Ox	Cardiac muscle	1	7
Pig	Stomach	1	5
Chicken	Gizzard	1	18
Rabbit	Skeletal muscle	2	42
Rabbit	Liver	1	14

absence of 6 M-urea was carried out by the method described by Head & Perry (1974). Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was by the method of Weber & Osborn (1969), either in 0.1 M-sodium phosphate buffer, pH 7.0, or 0.1 M-Tris/0.1 M-Bicine [*N,N*-bis-(2-hydroxyethyl)glycine] (pH 8.3).

For electrophoresis of whole-tissue homogenates, 5 g of rabbit brain, liver, kidney, lung and bovine uterus were homogenized in 30 ml of 9 M-urea/75 mM-Tris/HCl (pH 8.0)/1 mM- CaCl_2 /15 mM-2-mercaptoethanol, centrifuged at 80000g for 30 min and filtered through glass wool. Samples (50 μ l) were run on polyacrylamide gels at pH 8.3 in 6 M-urea in the presence of 1 mM- CaCl_2 or 5 mM-EGTA.

Cyanogen bromide cleavage

Protein (1 mg) was dissolved in 200 μ l of 70% formic acid and a 100-fold excess of CNBr over methionine residues added. After 24 h the protein was freeze-dried.

Carbamoylmethylation

Troponin C-like protein (about 5 mg) from rabbit uterus and bovine uterus and rabbit skeletal-muscle troponin C were labelled with iodo[^{14}C]acetamide by the method described by Wilkinson *et al.* (1972) for carbomethylation.

Amino acid analysis

Duplicate samples of proteins were hydrolysed in 6 M-HCl for 24 h and 72 h as described by Wilkinson *et al.* (1972)

Proteins were analysed for trimethyl-lysine by using 'system C' described by Kuehl & Adelstein (1969).

Phosphodiesterase activation

The stimulation of bovine brain phosphodiesterase activity by bovine uterus troponin C-like protein was assayed by the method of Watterson *et al.* (1976). Bovine brain phosphodiesterase was prepared by the affinity-chromatographic method of Watterson & Vanaman (1976).

Results

Preparation methods

Although the troponin I affinity-column method has been shown to be very effective for the preparation of troponin C-like protein from relatively small samples of certain smooth muscles (Head *et al.*, 1977a), it was less suitable for use with large quantities of tissue. With some tissues, e.g. bovine aorta, no troponin C-like protein could be isolated by this method. The method using organic solvents was therefore developed to prepare troponin C-like proteins from bovine uterus, rabbit uterus, bovine aorta, bovine brain, rabbit liver, bovine cardiac muscle, rabbit skeletal muscle and chicken gizzard. Unless otherwise stated, all the studies reported here were carried out on troponin C-like proteins isolated by the organic-solvent method. In each case the protein migrated as a single band on polyacrylamide-

gel electrophoresis of at least 70 μ g in the presence of 0.1% sodium dodecyl sulphate/0.1 M-sodium phosphate buffer, pH 7.0, with a mobility similar to that of rabbit skeletal-muscle troponin C. Better resolutions of the two types of Ca^{2+} -binding proteins on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate could be obtained in 0.1 M-Tris/0.1 M-Bicine buffer, pH 8.3. Under these conditions the troponin C-like protein from smooth muscle and non-muscle tissues migrated slightly faster than rabbit fast-skeletal-muscle troponin C.

Effect of Ca^{2+} on the electrophoretic mobility of troponin C-like proteins

On electrophoresis in polyacrylamide gel in 14 mM-Tris/90 mM-glycine, pH 8.3, the troponin C-like proteins migrated as a single band when up to 50 μ g was applied. In the presence of Ca^{2+} the mobility of fast-skeletal-muscle troponin C is increased (Head & Perry, 1974), whereas all the troponin C-like proteins tested showed a decrease in mobility in the presence of Ca^{2+} and could be distinguished from skeletal-muscle troponin C by this test (Fig. 3). The effect of Ca^{2+} on the mobility of the troponin C-like proteins was identical with that obtained with bovine brain modulator protein under similar conditions (Amphlett *et al.*, 1976) (Fig. 3). This suggests that the troponin C-like protein of

Fig. 3. Electrophoresis of troponin C and troponin C-like proteins in the presence and absence of Ca^{2+} . Electrophoresis was carried out in polyacrylamide gels in 14 mM-Tris/90 mM-glycine (pH 8.3); 20 μ g of protein was applied in each case; 1 mM- CaCl_2 or 5 mM-EGTA was added to the sample as indicated. The major bands in (i) and (j) are due to cardiac-muscle modulator protein; arrows indicate the position of cardiac troponin C. O, Origin. (a) Rabbit fast-skeletal-muscle troponin C + Ca^{2+} ; (b) rabbit fast-skeletal-muscle troponin C + EGTA; (c) bovine brain modulator protein + Ca^{2+} ; (d) bovine brain modulator protein + EGTA; (e) bovine uterus troponin C-like protein + Ca^{2+} ; (f) bovine uterus troponin C-like protein + EGTA; (g) chicken gizzard troponin C-like protein + Ca^{2+} ; (h) chicken gizzard troponin C-like protein + EGTA; (i) ox cardiac troponin C + Ca^{2+} ; (j) ox cardiac troponin C + EGTA.

smooth muscle is more like the modulator protein found in non-muscle tissues. The behaviour of troponin C-like proteins in the presence and absence of Ca^{2+} on electrophoresis in 6M-urea, pH 8.3, was indistinguishable from that of skeletal-muscle troponin C.

Complex formation of troponin C-like proteins with fast-skeletal-muscle troponin I

Fast-skeletal-muscle troponin C forms a complex with fast-skeletal-muscle troponin I in 6M-urea, pH 8.6, in the presence of Ca^{2+} , that has 50% of the mobility of troponin C and that is dissociated when the bivalent cation is removed (Head & Perry, 1974).

Fig. 4. Electrophoresis of troponin C-like proteins with fast-skeletal-muscle troponin I in high urea concentrations
Conditions of electrophoresis were as for Fig. 3, with 1 mM- CaCl_2 and 7M-urea added to the Tris/glycine buffer, pH 8.3. Rabbit fast-skeletal-muscle troponin I (20 μg) was applied in all cases, together with troponin C-like proteins (20 μg) where indicated. O, Origin. Complexes are indicated by an arrow. (a) Skeletal-muscle troponin I; (b) skeletal-muscle troponin I + ox aorta troponin C-like protein; (c) skeletal-muscle troponin I + chicken gizzard troponin C-like protein; (d) skeletal-muscle troponin I + cow uterus troponin C-like protein; (e) skeletal-muscle troponin I + rabbit fast-skeletal-muscle troponin C (the arrow indicates the troponin I-troponin C complex); (f) skeletal-muscle troponin I + bovine brain modulator protein.

Fig. 5. Complex-formation of troponin C-like proteins in the absence of urea

Conditions were as for Fig. 4, but without 7M-urea. Rabbit fast-skeletal-muscle troponin I (20 μg) was applied in all cases, together with troponin C-like proteins (20 μg) where indicated. O, Origin. (a) Skeletal-muscle troponin I and bovine brain modulator protein; (b) troponin I and troponin C from rabbit fast skeletal muscle; (c) skeletal-muscle troponin I and cow uterus troponin C-like protein; (d) skeletal-muscle troponin I and chicken gizzard troponin C-like protein; (e) skeletal-muscle troponin I and cow aorta troponin C-like protein.

Fig. 6. Complex-formation of troponin C-like proteins in whole homogenates of non-muscle tissues

Homogenates were made as described in the Materials and Methods section and either 50 μl applied (+ Ca^{2+}) or 50 μl + 5 μl of 100 mM-EGTA (+EGTA). Samples were run on polyacrylamide gels containing 6M-urea/14 mM-Tris/90 mM-glycine (pH 8.3). The arrows indicate troponin C-like protein. O, Origin. (a) Rabbit brain extract + Ca^{2+} ; (b) rabbit brain extract + EGTA; (c) rabbit lung extract + Ca^{2+} ; (d) rabbit lung extract + EGTA; (e) rabbit kidney extract + Ca^{2+} ; (f) rabbit kidney extract + EGTA; (g) rabbit liver extract + Ca^{2+} ; (h) rabbit liver extract + EGTA; (i) bovine uterus extract + Ca^{2+} ; (j) bovine uterus extract + EGTA.

This property was invariably obtained with fast-skeletal-muscle troponin C in 6M-urea. Although complexes of similar mobility were obtained with the troponin C-like proteins in urea concentrations up to approx. 5M, after storage or in the presence of higher urea concentrations the troponin C-like proteins from smooth muscle and non-muscle tissues did not form a Ca^{2+} -dependent complex with fast-skeletal-muscle troponin I (Fig. 4). In the absence of urea at pH 8.3 the troponin C-like proteins invariably formed a Ca^{2+} -dependent complex with fast-skeletal-muscle troponin I. The complex possessed a slightly lower mobility than that formed by fast-skeletal-muscle troponin C (Figs. 5 and 6).

Primary-structural differences between the troponin C-like proteins and fast-skeletal-muscle troponin C

The electrophoretic studies suggested that the troponin C-like proteins were not identical with fast-skeletal-muscle troponin C. The patterns obtained on electrophoresis of CNBr digests of troponin C-like proteins from smooth muscle of cow and chicken, from bovine brain, and from rabbit liver were very similar, but different from, digests of troponin C from rabbit fast-skeletal and cardiac muscles.

The amino acid analyses of the troponin C-like proteins were very similar among themselves (Table 2). They resembled that of fast-skeletal-muscle troponin C, but a few marked differences, e.g. in threonine and serine content, are apparent that indicate non-identity. Uterus troponin C-like protein contained less than 0.5 mol of carboxymethylated cysteine per mol. As preliminary sequence studies have failed to reveal the presence of a cysteine residue in bovine troponin C-like protein, it is probable that this residue is absent from this protein.

Vanaman *et al.* (1976) identified an unusual amino acid present in the troponin C-like protein from bovine brain as trimethyl-lysine. In the present study we have found that the amount of this amino acid in troponin C-like proteins from rabbit liver, bovine uterus, bovine aorta and chicken gizzard is approx. 1 mol/mol. Analysis of the bovine cardiac-muscle preparation showed the presence of small amounts of trimethyl-lysine and histidine, the amount being considerably less than 1 mol/mol. This is probably accounted for by the presence of significant amounts of cardiac-muscle troponin C in the troponin C-like protein preparation obtained from heart. Troponin C prepared from rabbit skeletal muscle by the organic-solvent method described did not contain detectable amounts of trimethyl-lysine.

Presence of troponin C-like protein in striated muscle

The evidence presented strongly suggests that the organic-solvent method isolates a troponin C-like protein from smooth muscle that is not identical with the striated-muscle-type troponin C, but very similar

to the widely distributed modulator protein. This protein has been isolated from cardiac muscle (Teo *et al.*, 1973), which is also known to contain troponin C. The question arises as to whether striated muscle in general contains significant amounts of the modulator protein as well as troponin C. Troponin C prepared from cardiac muscle by the organic-solvent method was therefore examined by electrophoresis at pH 8.3 with or without Ca^{2+} . Two bands could be distinguished in the presence and absence of Ca^{2+} (Fig. 3). The slower band obtained in the absence of Ca^{2+} was presumed to represent modulator protein and on average accounted for about 60% of the total protein. Troponin C prepared from rabbit skeletal muscle, either by the standard procedures (see Head *et al.*, 1977a) or by the organic-solvent method, did not contain modulator protein as judged by this test.

Determination of the amount of troponin C-like protein in rabbit uterus

The observation that the troponin C-like protein migrated as a fast band clear of other components on electrophoresis of whole extracts of uterus muscle in 8M-urea, pH 7.8, in the presence of 5 mM-EGTA, indicated that the isotope-dilution technique could be used to determine the amount of troponin C-like protein in smooth muscle as described by Head & Perry (1974). A sample of troponin C isolated from uterus by affinity chromatography was carbamoylmethylated with iodo[^{14}C]acetamide. Although amino acid analysis and sequence studies do not indicate the presence of cysteine residues in smooth-muscle troponin C-like protein, significant amounts of covalently bound radioactivity were incorporated at pH 8.2, presumably due to carbamoylmethylation of histidine residues. Known amounts of radioactively labelled troponin C were added to a homogenate containing a known amount of muscle, and from the dilution of radioactivity in the troponin C-like protein band obtained on electrophoresis the troponin C-like-protein content of the muscle was determined. From five determinations an average value of $0.42\% \pm 0.046$ of the total protein was obtained for the troponin C-like-protein content of rabbit uterus.

Cyclic phosphodiesterase activity of uterus troponin C-like protein

The effect of the bovine uterus troponin C-like protein on the activity of a preparation of bovine brain phosphodiesterase (fully stimulated activity, 5.3 nmol of 3':5'-cyclic AMP hydrolysed/min at 30°C; basal unstimulated activity 0.5 nmol of 3':5'-cyclic AMP/min) was studied. Significant activation was obtained on addition of 0.1 μg of uterus troponin C-like protein. Full activation was obtained either with 5 μg of uterus troponin C-like protein or 5 μg of bovine brain-modulator protein. Similar results were

Table 2. *Amino acid analyses of troponin C-like proteins*

With the exception of the proteins from rabbit skeletal muscle and bovine brain, all proteins are troponin C-like proteins. Analyses were calculated on the basis of a mol.wt. of 16500. One preparation was analysed in each case. Abbreviations used: Me₃Lys, trimethyl-lysine; n.d., not determined.

	Rabbit fast-skeletal-muscle troponin C*	Bovine brain modulator protein†	Rabbit uterus‡	Bovine uterus	Chicken gizzard	Bovine aorta	Rabbit liver
Asp	22	24	22.8	23.4	23.2	23.7	25.2
Thr	6	12	12.3	10.6	10.3	10.8	10.5
Ser	7	5	10.2	7.2	5.8	6.3	5.7
Glu	31	29	29.6	27.2	26.5	27.4	27.8
Pro	1	2	3.0	3.8	2.9	3.3	1.1
Gly	13	12	16.6	12.9	11.3	11.8	10.9
Ala	13	12	13.1	10.3	10.3	10.5	10.5
Val	7	8	6.9	7.0	7.1	6.9	6.9
Met	9	10	8.4	5.8	7.0	6.7	6.6
Ile	10	8	6.6	6.8	7.1	7.1	7.3
Leu	9	10	9.2	9.1	9.4	9.0	9.9
Tyr	2	2	2.2	2.5	2.6	2.7	2.5
Phe	10	8	7.5	6.8	7.2	7.2	7.3
His	1	1	1.7	1.7	1.7	1.5	2.1
Lys	9	8	10.5	6.7	7.8	6.3	7.0
Arg	7	7	5.4	6.4	6.2	6.2	5.4
Me ₃ Lys	—	1	n.d.	0.7	0.7	0.7	0.8
Trp	—	—	—	—	—	—	—
Cys	1	—	n.d.	—	n.d.	n.d.	n.d.

* Collins (1974).

† Watterson *et al.* (1976).

‡ Head *et al.* (1977a).

obtained on two different preparations of uterus troponin C-like protein.

Component with which troponin C-like protein is associated in smooth muscle and non-muscle tissues

It was demonstrated previously that on electrophoresis of smooth-muscle extracts in 8M-urea in the presence of Ca²⁺ at pH 7.8 the band corresponding to the fast troponin C-like protein was not visible, presumably because the troponin C-like protein formed a complex with some other component or components in the whole-muscle homogenate, as has been demonstrated for skeletal muscle (Head & Perry, 1974). It was not possible, however, to identify the complex in the poorly resolved electrophoresis pattern obtained with smooth-muscle extracts. Radioactive troponin C-like protein from uterus as used for the isotope-dilution studies was therefore added to a sample of homogenate in 6M-urea/25mM-Tris/80mM-glycine (pH 8.6)/1 mM-CaCl₂ and electrophoresed on a 10% (w/v) polyacrylamide gel prepared in the same buffer. The distribution of radioactivity was then determined in slices of the gel. A typical distribution is illustrated in Fig. 7. Usually about 10–20% of the total radioactivity was located in the position where troponin C migrated, but the bulk was located

at the origin, implying that the troponin C-like protein was present as a Ca²⁺-dependent complex with other protein (or proteins) that, either due to its size or more likely to its charge, did not migrate into the gel under these conditions. In this respect the protein or proteins with which troponin C formed a complex differed from troponin I of skeletal and cardiac muscle, the complexes of which migrated with 50 and 37% respectively of the electrophoretic mobilities of the troponin C components of these muscles. On addition of 5mM-EGTA to the homogenate, virtually all the radioactivity was located in the band containing troponin C-like protein.

The results of electrophoresis of homogenates of a number of tissues in the presence and absence of Ca²⁺ are shown in Fig. 6. In the absence of Ca²⁺, troponin C-like protein could be seen as the fastest moving band in brain, kidney, lung and liver extracts. In the presence of Ca²⁺ the band corresponding to the troponin C-like protein was not visible and was presumably present as part of a complex that does not move into the gel under these conditions.

Discussion

The organic-solvent method presents a relatively simple procedure of wide application for the isolation

Fig. 7. Effect of Ca^{2+} on the electrophoretic migration of troponin C-like protein in homogenates of rabbit uterus

The Figure is a diagrammatic representation of the patterns obtained on electrophoresis carried out on 10% polyacrylamide gel containing 25 mM-Tris/80 mM-glycine, pH 8.6. Uterus (2 g) homogenized in 10 vol. of 8 M-urea/50 mM-Tris adjusted to pH 7.8 with 1 M-HCl/15 mM-2-mercaptoethanol/1 mM- CaCl_2 . ^{14}C -labelled carboxymethylated rabbit fast-skeletal-muscle troponin C ($50 \mu\text{g}$ in $60 \mu\text{l}$) was added to 5 ml of homogenate. One portion was dialysed against 8 M-urea/50 mM-Tris/HCl buffer (pH 8.0)/15 mM-2-mercaptoethanol/1 mM- CaCl_2 ; on electrophoresis 1 ml of the solution on a slab gel gave the distribution of radioactivity shown in (a). Another portion was dialysed against the same solution in which the 1 mM- CaCl_2 had been replaced by 10 mM-EGTA; on electrophoresis 1 ml of this solution gave the distribution of radioactivity shown in (b). After electrophoresis the gels were stained, the bands (hatched in diagram) were cut out, and ^{14}C determinations carried out as indicated in the Materials and Methods section. The numbers beside the shaded areas in the Figure refer to the total ^{14}C radioactivity (c.p.m.) in those areas.

of troponin C-like proteins from a range of tissues. Initial homogenization in high urea concentration and subsequent treatment with organic solvents produces rapid inactivation of endogenous proteinases, which can be a problem in isolation of troponin components from smooth muscle and other tissues. Previous studies (Amphlett *et al.*, 1976) suggest that treatment with strong urea does not appear to modify the biological properties of the modulator protein from brain in any significant manner. The troponin C-like proteins isolated from other tissues likewise retain their biological activity after exposure to urea and organic solvents.

The yields obtained for bovine brain modulator protein prepared by this method are higher than those obtained by more conventional means [15–20 mg/100 g of tissue compared with 7–10 mg/100 g of tissue obtained by Watterson *et al.* (1976)]. The organic-solvent method does, however, suffer from the slight

disadvantage that if it is scaled up to deal with very much larger amounts of tissue, problems may be presented by centrifugation of relatively large volumes.

The Ca^{2+} -binding protein from smooth muscle isolated by this method differs from skeletal troponin C in the following properties: (1) the effect of Ca^{2+} on mobility on electrophoresis at pH 8.6; (2) the electrophoretic mobility and stability to high urea concentration of the Ca^{2+} -dependent complex formed with fast-skeletal-muscle troponin I; (3) its amino acid composition; (4) its phosphodiesterase-activating ability. In all these properties the Ca^{2+} -binding protein from smooth muscle closely resembles the modulator protein of bovine brain. A protein of similar properties is widely distributed in other mammalian tissues. Appreciable amounts of the protein can be obtained from liver by the organic-solvent procedure, whereas attempts by other workers to prepare the modulator protein from the tissue have been less successful (T. C. Vanaman, personal communication). It seems probable that large amounts of the troponin C-like protein also occur in mammalian kidney and lung. These findings are in good agreement with the work of Cheung *et al.* (1975), who have shown that a protein similar to bovine brain modulator protein is present in a variety of mammalian tissues.

Since the present work was completed, Dabrowska *et al.* (1978) have reported the isolation of Ca^{2+} -binding protein from chicken gizzard smooth muscle that is very similar to the bovine brain modulator protein and that they consider to be an essential component of myosin light-chain kinase of that tissue.

In striated muscle the bulk of Ca^{2+} -binding protein consists of troponin C, which is bound to the myofibril. In cardiac muscle the proportion of modulator protein present in the preparation of Ca^{2+} -binding protein obtained by the organic-solvent method was larger than expected in view of the amount of troponin C known to be present in this tissue (Head & Perry, 1974). It seems that with the organic-solvent method, modulator protein is purified to some extent in preference to troponin C. Skeletal muscle also contains modulator protein in relatively small amounts and located in the sarcoplasm (A. C. Nairn & S. V. Perry, unpublished work; Yagi *et al.*, 1978). It is not normally detected in the preparation of troponin C made from this tissue either by the organic-solvent method or by the usual methods used for the preparation of troponin C. Although the bulk of the Ca^{2+} -binding protein in smooth muscle is very similar to, if not identical with, the bovine brain modulator protein, it is possible that a small amount of troponin C of the striated-muscle type is also present in this tissue. The results obtained so far suggest that the amount of troponin C present may well be related to the amount of actomyosin-type adenosine triphosphatase present in a tissue. Thus

when skeletal, cardiac, smooth muscle and non-muscle tissues are compared, the ratio of troponin C to modulator protein changes from a trace of modulator and virtually all troponin C in fast-skeletal to virtually all modulator in non-muscle tissue.

The isotope-dilution technique, which will not discriminate between striated-muscle troponin C and modulator protein, indicates that the total amount of troponin C-like protein in uterus falls within the range found in striated muscle. This varies from 0.7 to 0.4% of the total protein, depending on the type of muscle (Head & Perry, 1974).

In the presence of Ca^{2+} , the whole of the modulator protein in all the tissues studied formed a complex with some other substance, or substances, probably protein in nature. Calcium was essential for the formation of the complex, and in fresh tissue extracts at least, the interaction was stable to high urea concentrations. It would appear that the complex obtained by the interaction of the modulator protein with troponin I from fast skeletal muscle is less stable to urea.

The interaction of modulator protein with the other component(s) of the tissue is very similar to the interaction of skeletal-muscle troponin C with skeletal-muscle troponin I. The protein with which the smooth-muscle modulator protein is associated in the cell, however, must be different from skeletal-muscle troponin I, as the complex formed has a lower mobility on electrophoresis at pH 8.3 than that formed by the modulator protein with skeletal troponin I. The amount of the protein that forms a complex with the modulator protein in the cell must be appreciable if it interacts at an equimolar ratio, for there is no free modulator protein in tissue extracts electrophoresed in the presence of Ca^{2+} . The nature of the substance that forms a complex with modulator protein can only be a matter for speculation at the moment. It may be a basic protein, possibly analogous to the troponin I or troponin T of striated muscle. Alternatively it may consist of a complex of proteins such as cyclic phosphodiesterase and modulator-binding protein, as isolated by Wang & Desai (1977), both of which have been shown to interact with the modulator protein.

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