Caldesmon, a calmodulin-binding, F actin-interacting protein, is present in aorta, uterus and platelets

Ritsu Kakiuchi, Makoto Inui*, Kouichi Morimoto, Keiko Kanda, Kenji Sobue and Shiro Kakiuchi

Department of Neurochemistry and Neuropharmacology, Institute of Higher Nervous Activity, Osaka University Medical School, Nakanoshima, Kita-ku, Osaka 530, Japan

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Caldesmon, a protein originally found in chicken gizzard, was concluded also to be present in bovine aorta, uterus, and human platelets by demonstration of a protein with the following properties: (a) Ca^{2+}-dependent calmodulin-binding; (b) binding to F actin in such way that the binding was broken on Ca^{2+}-dependent binding of calmodulin; (c) cross-reactivity in immune blotting procedures with affinity-purified antibody against gizzard caldesmon; (d) similar subunit M_r-values on SDS-gel to those of gizzard caldesmon. Like gizzard caldesmon, platelet caldesmon was composed of two polypeptide bands of M_r 150000 and 147000, but caldesmon in aorta and uterus gave a single band of M_r 150000. A polypeptide of M_r 165000 that was immunologically distinct from caldesmon but, like caldesmon, bound to calmodulin and F actin in a flip-flop fashion, was also demonstrated in aorta and uterus.

Calmodulin *Calmodulin-binding protein* *Actin binding* *Smooth muscle contraction* *Platelet contractile protein* *Flip-flop mechanism*

1. INTRODUCTION

Calmodulin is thought to be a mediator of the regulating action of Ca^{2+} on smooth muscle contraction in vertebrates, although its mechanism of action is not known in detail and is controversial (for reviews, see [1,2]). We recently discovered a major calmodulin-binding protein, caldesmon, in chicken gizzard smooth muscle that could bind to F actin filaments [3,4]. About 70% of the total calmodulin in gizzard muscle can be bound to the caldesmon [3]. The binding of caldesmon to F actin was abolished by Ca^{2+}-dependent binding of calmodulin to caldesmon [4]. Thus, the binding of caldesmon to calmodulin and F actin can alternate depending upon the concentration of Ca^{2+} (flip-flop binding). Caldesmon did not produce gel formation or shortening of actin filaments, nor did it influence re-annealing of fragmented actin filaments [5,6], and therefore differed from other proteins interacting with actin such as gelsolin [7], actinogelin [8], fragmin [9], and villin [10]. Caldesmon is thought to regulate the function of actin filaments without altering their state of polymerization-depolymerization. Subsequent experiments in our laboratory using gizzard contractile proteins have provided evidence that the Ca^{2+}- and calmodulin-dependent association of caldesmon with F actin regulates the filamin-induced gelation of actin filaments [1] and the actin–myosin interaction measured by super-precipitation and ATPase activity [11]. On the basis of these and other results, we have proposed

* On leave of absence from the First Department of Internal Medicine, Osaka University Medical School, Japan

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline (20 mM sodium phosphate buffer, pH 7.4, and 150 mM NaCl)

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a dual mechanism consisting of phosphorylation and dephosphorylation of two of the myosin light chains and a caldesmon-linked flip-flop switch for the control of the gizzard smooth muscle contractile system [11]. It therefore seemed of particular interest to see if caldesmon was present in other types of smooth muscle. Here, we examined whether it is present in bovine aorta, uterus, and human platelets. Platelets were also examined as they are similar to smooth muscle in containing relatively large amounts of contractile proteins and undergo a series of contractile events upon stimulation [12].

2. MATERIALS AND METHODS

2.1. *Preparation of calmodulin-binding protein fraction*

A calmodulin-binding protein fraction was prepared from bovine aorta, uterus, and human platelets by the procedure developed for preparation of caldesmon from chicken gizzard [4], with a slight modification. The following procedures were carried out at 4°C. Aorta and uterus (10 g each) were homogenized in a Polytron PT 10 homogenizer (Kinematica, Switzerland) (three 15-s operations at an output setting of 7) with 3 volumes of buffer I consisted of 0.3 M KCl, 2 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM imidazole–HCl (pH 6.9), 0.25 mM phenylmethyl-sulfonyl fluoride, 0.1 mM diisopropyl fluorophosphate, and 1 μg/ml of leupeptin. Washed human platelets (2 ml packed volume) prepared as described in [13] were suspended in 3 volumes of buffer I and disrupted by sonication for five 5-s periods in a Sonifier Model W-185 (Branson-Sonic Power) at an output setting of 5. The homogenate of each tissue was centrifuged for 30 min at 50000 × g. The supernatant was adjusted to 60% saturation of ammonium sulfate and the resulting precipitate was collected by centrifugation and dissolved in 4–5 ml of buffer II (20 mM Tris–HCl, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM EGTA and 0.6 M KCl). The solution was clarified by centrifugation for 30 min at 105000 × g, and the resulting clear supernatant (about 3–4 ml) was applied to a column (45 × 2 cm) of Sepharose 4B which had been equilibrated with buffer II. Material was eluted with buffer II and a fraction corresponding to M_r 100000–500000 was collected. This fraction was dialyzed overnight against buffer III (20 mM Tris–HCl, pH 7.5, 0.1 mM dithiothreitol, and 100 mM KCl) plus 0.1 mM EGTA. The solution was made 0.2 mM in Ca^{2+} by adding $CaCl_2$ and then clarified by centrifugation for 60 min at 105000 × g. The clear supernatant was subjected to affinity column chromatography on calmodulin-Sepharose. The column (3 × 1.2 cm) was washed with buffer III plus 0.2 mM $CaCl_2$, and then the calmodulin-binding protein fraction was eluted with buffer III plus 1 mM EGTA.

2.2. *Other methods and materials*

Antiserum to chicken gizzard caldesmon was produced in rabbits. Caldesmon (1 mg) in 0.5 ml of PBS was emulsified with an equal volume of complete Freund's adjuvant and injected into the footpads of a rabbit. Two additional injections (0.5 mg protein with incomplete Freund's adjuvant) were given on days 28 and 42, and the rabbit was bled on day 56. The anti-caldesmon IgG fraction was prepared from the serum by ammonium sulfate precipitation (48% saturation) and then affinity chromatography on caldesmon-Sepharose 4B, as will be described in detail elsewhere. Calmodulin was prepared from bovine brain as in [15]. Calmodulin-Sepharose 4B was prepared as in [16]. Caldesmon [4], myosin light chain kinase [17], filamin [18], myosin [19], actin [20], and α-actinin [21] were prepared from chicken gizzard smooth muscle. F actin was prepared from G actin as in [6]. The sources of commercial materials used in this work were as follows: Diisopropyl fluorophosphate, and phenylmethylsulfonyl fluoride from Sigma, leupeptin from the Peptide Institute (Osaka), carrier free ^{125}I (100 mCi/ml) from Amersham International, and peroxidase-labelled goat anti-rabbit IgG from Miles Immunochemicals. The concentration of protein was determined as in [22].

3. RESULTS

In a ring test, the anti-caldesmon serum obtained in this study formed a precipitating complex at 1:10000 dilution in PBS; and in an Ouchterlony immunodiffusion test, it gave a single precipitin band against gizzard caldesmon, but not against myosin light chain kinase, α-actinin, filamin or

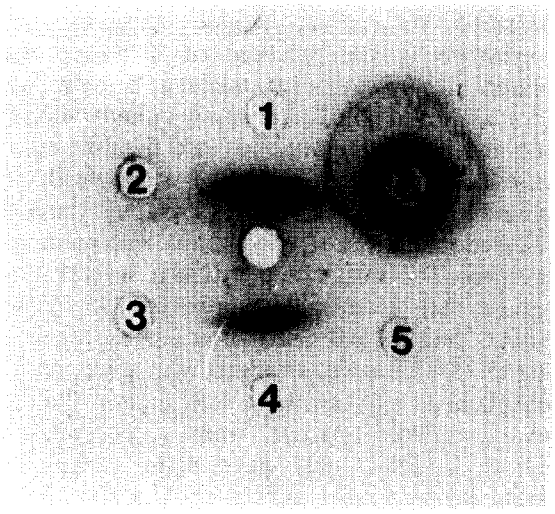

Fig.1. Ouchterlony immunodiffusion test of anti-caldesmon serum. Each well received 8 μl of protein sample: Center well, 1:5 dilution of anti-caldesmon serum in PBS; well 1, caldesmon (1.5 mg/ml); 2, myosin light chain kinase (1.2 mg/ml); 3, α-actinin (1.3 mg/ml); 4, caldesmon (0.75 mg/ml); 5, filamin (1.5 mg/ml); 6, myosin (1.6 mg/ml). Protein precipitation lines were stained with Coomassie brilliant blue.

myosin, all of which were purified from chicken gizzard (fig.1).

To determine if proteins related to caldesmon are present in tissues other than gizzard, we prepared a calmodulin-binding protein fraction from each of bovine aorta, uterus, and human platelets by affinity column chromatography on calmodulin-Sepharose. The method was a modification of that used for preparation of the caldesmon-containing fraction from chicken gizzard [3]. The proteins in these fractions were

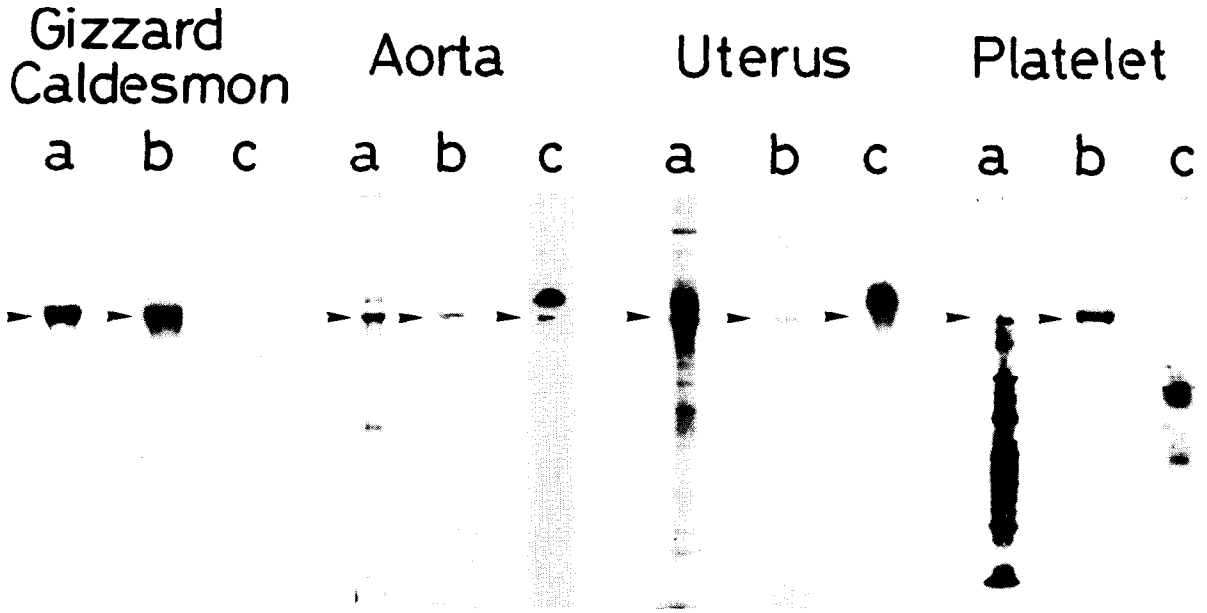

Fig.2. Detection of caldesmon in aorta, uterus, and platelets. Calmodulin-binding protein fractions from bovine aorta, uterus, and human platelets were subjected to SDS–polyacrylamide gel electrophoresis (7.5% polyacrylamide) as in [12]. The proteins separated on the gels were then detected in 3 ways: by protein staining with Coomassie brilliant blue (lane a); by immune blotting and then the peroxidase reaction (lane b); and by the gel overlay method and then autoradiography of bound ^{125}I-calmodulin (lane c). The immune blotting procedure was essentially as in [23]. Caldesmon or caldesmon-related proteins in the gels were labelled with affinity-purified anti-caldesmon IgG bound to peroxidase-labelled goat anti-rabbit IgG. The latter protein was then located by the peroxidase reaction with diaminobenzidine and H_2O_2. The method in [24] was used for the gel overlay technique. ^{125}I-Calmodulin was bound to the calmodulin-binding proteins separated on the gel and detected by autoradiography. ^{125}I-Calmodulin (spec. act. 6.8×10^5 cpm/μg) was prepared by a lactoperoxidase method [25]. The following amounts of proteins were loaded per slot: purified caldesmon (gizzard), 4 μg; aorta, 3 μg; uterus, 5 μg; platelets, 15 μg.

separated on polyacrylamide gels by electrophoresis in the presence of SDS and then located in 3 ways: by staining protein with Coomassie blue (fig.2, lane a), by immune blotting with anti-caldesmon antibody and then the peroxidase reaction (lane b), and by gel overlay with ^{125}I-calmodulin and then autoradiography (lane c). Caldesmon purified from gizzard was used as a reference. The aorta and uterus gave several bands stained with Coomassie, those of M_r 165000, 150000 and 100000 being most intense (lane a). The 150000 band was strongest with aorta, but the 165000 band was strongest with uterus. Platelets gave a different pattern from aorta or uterus smooth muscles: they gave many polypeptide bands, because more platelet protein was applied to the gel slot, and of these bands, those of M_r 150000, 100000, 80000, 60000 and 45000 were predominant.

The immune blotting technique revealed the presence of a band of polypeptide of M_r 150000, coincident with that of gizzard caldesmon, in all tissue samples examined (lane b). A doublet form of caldesmon of M_r 150000 and 147000 was detected in gizzard (see also [6]) and platelets, whereas a single band of M_r 150000 was obtained with aorta and uterus. In addition, the colour intensities of the 150000 bands from aorta and uterus observed on immune blotting (lane b) were much weaker than suggested from their staining intensities with Coomassie blue (lane a) when compared with those of gizzard and platelet proteins. Caldesmon could not be detected by the gel overlay method, presumably because this protein did not recover a native configuration capable of binding to calmodulin after exposure to SDS (submitted). This was confirmed in this study with gizzard caldesmon and platelet protein (fig.2 lane c). However, the 150000 proteins of aorta and uterus were detected by ^{125}I-calmodulin autoradiography, although the intensities of their bands on autoradiography were much weaker than those expected from their Coomassie blue-stained counterparts relative to the 165000 bands. Other bands

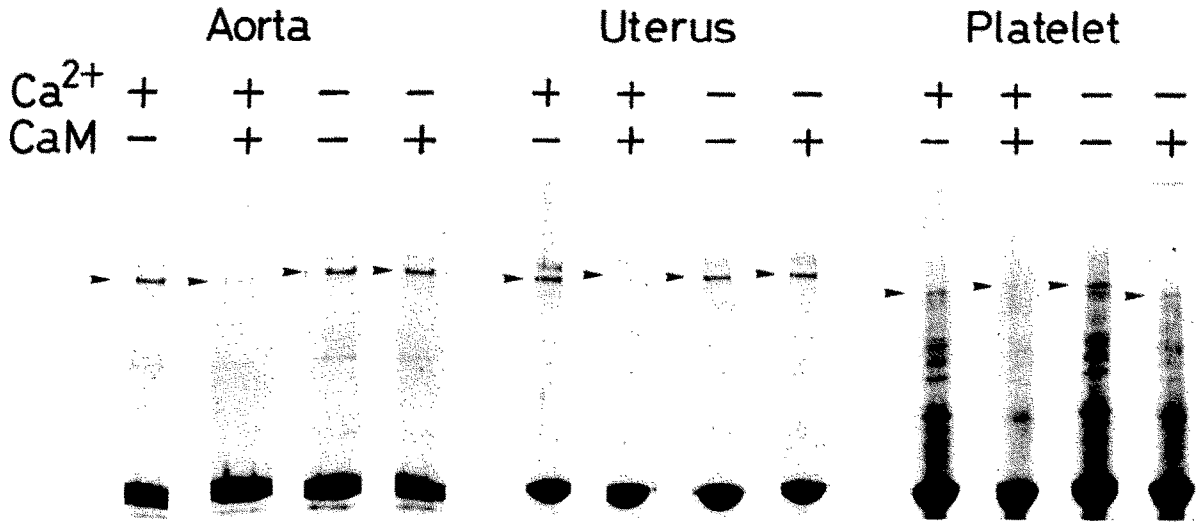

Fig.3. Binding of proteins to F actin or to calmodulin in a flip-flop fashion depending upon the concentration of Ca^{2+}. Calmodulin-binding protein fractions from aorta (25 μg protein), uterus (37.5 μg protein) and platelets (50 μg protein) were incubated with 250 μg of gizzard F actin at 30°C for 30 min in medium consisting of 20 mM Tris–HCl (pH 7.5), 0.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol and 0.1 mM ATP in the presence or absence of 50 μg of calmodulin in a total volume of 250 μl. Either 0.1 mM $CaCl_2$ or 1 mM EGTA, as indicated in the figure, was added. After incubation, mixtures were centrifuged at 30°C for 30 min at 105000 × g. The resulting pellets were dissolved in 250 μl of 2% SDS, 100 mM 2-mercaptoethanol and 50% glycerol and the solutions were heated in boiling water for 3 min and then subjected to SDS–polyacrylamide gel electrophoresis in the buffer system in [14]. The protein bands on the gels were detected by Coomassie blue staining (shown here) and immunoblotting procedures (not shown). CaM, calmodulin.

were also detected by the gel overlay method. Thus, on autoradiography, M_r 165000 was predominant for aorta and uterus and 90000 and 60000 were the major bands for platelets.

Next we examined whether these 150000 polypeptides (or 150000 plus 147000 doublet for platelets) could bind to F actin in such a way that the binding was abolished by the Ca^{2+}-dependent binding of calmodulin to the polypeptides (flip-flop binding). As shown in fig.3 (Coomassie blue staining), these polypeptides cosedimented with F actin, and addition of calmodulin in the presence, but not in the absence, of Ca^{2+} eliminated the association of the polypeptides with F actin. Essentially the same results were obtained by the immunoblotting method (not shown). Fig.3 shows several F actin-binding polypeptides other than the caldesmon polypeptides. These include the 165000 polypeptide from aorta and uterus, and 100000, 80000 and 60000 polypeptides from platelets. The results also show that all of them except the 60000 polypeptide exhibited binding to F actin or to calmodulin in a flip-flop fashion depending upon the concentration of Ca^{2+}.

4. DISCUSSION

In searching for caldesmon in tissues other than chicken gizzard, we used multiple criteria for this protein, because polypeptides possessing similar properties were found near the bands of caldesmon on SDS-gels. For instance, a 135000 polypeptide, which is probably myosin light chain kinase [15], and a 165000 polypeptide (fig.2 and 3) were shown to bind to calmodulin and F actin in a flip-flop fashion. However, these polypeptides could be distinguished immunologically from caldesmon (fig.1 and 2). Therefore, the criteria we adopted for identification of caldesmon were as follows:

(i) It should be a Ca^{2+}-dependent calmodulin-binding protein with similar subunit M_r-values to gizzard caldesmon on SDS-gel.

(ii) It should be an F actin binding protein and its binding to F actin should be eliminated by the Ca^{2+}-dependent binding of calmodulin.

(iii) It should cross-react immunologically with antibody raised against gizzard caldesmon.

Here, we found proteins that fulfilled all these criteria in bovine aorta, uterus, and human platelets. However, small differences were seen among these proteins. Caldesmon in gizzard and platelets was composed of two subunit polypeptides with M_r of 150000 and 147000, but caldesmon in aorta and uterus gave a single 150000 polypeptide on SDS-gel. It is uncertain whether these proteins have homo-dimeric structures. Another difference was that the proteins of aorta and uterus were detectable by the gel overlay method, although very weakly, while those of gizzard and platelets could not be detected by this method. Therefore, we propose to name the protein in aorta and uterus s-caldesmon and that in gizzard and platelets d-caldesmon.

The Ca^{2+}-dependent regulation by calmodulin of the function of actin filaments is mediated by caldesmon (see section 1). Thus, caldesmon has been proposed to be an important regulator in the smooth muscle contractile device [9]. The present results demonstrating the presence of caldesmon in the aorta, uterus and platelets are compatible with this view. Moreover, we recently found by an immunocytochemical technique with antibody to gizzard caldesmon that the indirect immunofluorescence coincided with the cellular stress fibers and ruffles of cultured fibroblast cells (Yahara, I. and Kakiuchi, S., unpublished). These findings suggest the interesting possibility that caldesmon or related proteins may also be involved in the control of the contractile devices of non-muscle cells.

REFERENCES

[1] Hartshorne, D.J. and Siemankowski, R.F. (1981) Annu. Rev. Physiol. 43, 519–530.

[2] Ebashi, S., Nonomura, Y., Nakamura, S., Nakasone, H. and Kohama, K. (1982) Fed. Proc. FASEB 41, 2863–2867.

[3] Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981) Biochem. Internat. 2, 469–476.

[4] Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981) Proc. Natl. Acad. Sci. USA 78, 5652–5655.

[5] Maruyama, K., Sobue, K. and Kakiuchi, S. (1982) in: Calmodulin and Intracellular Ca^{2+}-Receptors, pp.183–188, Plenum, New York.

[6] Sobue, K., Morimoto, K., Kanda, K., Maruyama, K. and Kakiuchi, S. (1982) FEBS Lett. 138, 289–292.

[7] Yin, H.L. and Stossel, T.P. (1979) Nature 281, 583–586.

[8] Mimura, N. and Asano, A. (1979) Nature 282, 44–48.

[9] Hasegawa, T., Takahashi, S., Hayashi, H. and Hatano, S. (1980) Biochemistry 19, 2677–2683.
[10] Bretcher, A. and Weber, K. (1980) Cell 20, 839–847.
[11] Sobue, K., Morimoto, K., Inui, M., Kanda, K. and Kakiuchi, S. (1982) Biomed. Res. 3, 188–196.
[12] Stossel, T.P. (1978) Annu. Rev. Med. 29, 427–457.
[13] Tsujinaka, T., Shiba, E., Kambayashi, J. and Kōsaki, G. (1983) Biochem. Internat. 6, 71–80.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Kakiuchi, S., Sobue, K., Yamazaki, R., Kambayashi, J., Sakon, M. and Kōsaki, G. (1981) FEBS Lett. 126, 203–207.
[16] Klee, C.B. and Krinks, M.H. (1978) Biochemistry 17, 120–126.
[17] Sobue, K., Morimoto, K., Kanda, K., Fukunaga, K., Miyamoto, E. and Kakiuchi, S. (1982) Biochem. Internat. 5, 503–510.
[18] Shizuta, Y., Shizuta, H., Gallo, M., Davies, P., Pastan, I. and Lewis, M.S. (1976) J. Biol. Chem. 251, 6562–6567.
[19] Ebashi, S. (1976) J. Biochem. 79, 229–231.
[20] Strzelecka-Golaszewska, H., Prochniewicz, E., Nowak, E., Zmorzynski, S. and Drabikowski, W. (1980) Eur. J. Biochem. 104, 41–52.
[21] Feramisco, J.R. and Burridge, K. (1980) J. Biol. Chem. 255, 1194–1199.
[22] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[24] Carlin, R.K., Grab, D.J. and Siekevitz, P. (1980) Ann. NY Acad. Sci. 356, 73–74.
[25] Graf, E., Filoteo, A.G. and Penniston, J.T. (1980) Arch. Biochem. Biophys. 203, 719–726.