Changes in Ca²⁺ Signaling and Contractile Protein Isoforms in Smooth Muscle Cells from Guinea Pig Ileum during Culture

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Abstract

Single smooth muscle cells (SMCs) isolated from guinea pig ileum using collagenase and papain were cultured on laminin-coated dishes in MEM containing fetal calf serum. Temporal changes in intracellular calcium ion concentration in response to carbachol and to ATP were investigated using fluo-3/AM and fluorescence microscopy. It was observed that carbachol caused an increased intracellular calcium ion in freshly isolated single SMCs but a reduced or negative response of cultured SMCs before confluence. On the other hand, ATP was observed to cause an increase in the calcium ion content of SMCs throughout the culture. SDS-PAGE and Western blot analyses revealed changes in the expression of contractile proteins as follows. *l*-Caldesmon and non-muscle type myosin heavy chain (NMHC) (considered to be marker molecules for dedifferentiation in smooth muscle cells) and non-muscle type tropomyosin were not observed in freshly isolated single SMCs. *l*-Caldesmon and NMHC appeared in the cultured SMCs within 2 days and the tropomyosin isoform was observed 6 days following seeding. Simultaneously, smooth muscle type myosin heavy chain (SMHC) decreased strikingly and the 41 kDa tropomyosin monomer was lost. The content of α -actin decreased gradually to a minimum on day 6 when non-muscle type tropomyosin appeared, and the cells began to proliferate rapidly. These results suggest that the loss of contractility in cultured smooth muscle cells is more closely related to changes in contractile protein profiles than to receptor-mediated signal transduction and that in addition to NMHC and *l*-caldesmon, non-muscle type tropomyosin may be useful as a marker molecule for de-differentiation of smooth muscle cells.

Key words: intracellular Ca²⁺, caldesmon, myosin heavy chain, tropomyosin, actin

Introduction

The contraction mechanism of smooth muscle cells (SMCs) has been poorly understood compared with that of striated muscle cells. In order to gain information about the contraction

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mechanism in SMCs, we compared differences in the expression of contractile proteins such as myosin heavy chain, actin and tropomyosin between freshly isolated single SMCs and cultured SMCs. The results showed that the loss of contractility in cultured SMCs was closely related to changes in protein profiles from smooth muscle type to non-muscle type (Iijima *et al.*, 1998). SMCs show remarkable phenotypic changes during culture, and while diversity of protein isoforms has been reported, the physiological role of each isoform is not yet clear (Adelstein *et al.*, 1996; Lehman *et al.*, 1996; Smillie, 1996). In addition to these proteins, *h*-caldesmon and *l*-caldesmon are considered respectively to be a regulatory protein in smooth muscle contraction (Nomura and Sobue, 1987) and a marker molecule for dedifferentiation (Ueki *et al.*, 1987). Therefore, an investigation of the temporal change in these isoforms may provide information on their roles, which may be related not only to contractility but also to morphology and proliferation.

In this paper, we report temporal changes in the intracellular calcium ion concentration in response to agonists and correlate these with the changes in the isoforms of both caldesmon and contractile proteins. The results are discussed in terms of the contraction mechanism of SMCs.

Materials and Methods

Materials

Male guinea pigs (Hartley) weighing 250–500 g were used to obtain SMCs for these experiments. Carbamylcholine chloride (carbachol) and ATP were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fluo-3/AM from Bio-Rad Co. (Hercules, CA, USA), cremophor EL from Nacalai Tesque Inc. (Kyoto, Japan), and ionomycin from Calbiochem Co. (La Jolla, CA, USA). Anti- α -smooth muscle actin IgG, anti- β -actin IgG, anti-myosin heavy chain (smooth muscle), anti-caldesmon IgG and anti-*l*-caldesmon IgG were obtained from Sigma Co. (St Louis, MO, USA), anti-myosin heavy chain (non-muscle) from Biochemical Technologies (Stoughton, MA, USA), and anti-tropomyosin from Chemical International Inc. (Temecula, CA, USA). Goat anti-rabbit IgG was obtained from Bio-Rad Co., and goat anti-mouse IgG from American Qualex Co. (San Clemente, CA, USA). All other chemicals were commercial products of the highest available grade of purity.

Isolation of SMCs and cell culture

SMCs were isolated from guinea pig longitudinal ileum and cultured as described in a previous paper (Iijima *et al.*, 1999). Briefly, male guinea pigs were killed by a blow to the neck and single SMCs were prepared from segments of the longitudinal ileum. The isolated cells were suspended in Eagle's Minimum Essential Medium (MEM) containing 20% fetal calf serum (FCS; Intergen Co.), 15 μ g/ml of gentamicin (Boehringer Mannheim Co.), 1 μ g/ml of minocycline (Sigma Co.), 2 μ g/ml of amphotericin B (Boehringer Mannheim Co.) and 50 μ g/ml of ampicillin (Sigma Co.). The cell suspension containing 7×10^3 cells/cm² was seeded on a 60 mm diameter laminin-coated dish, and kept at 37°C under humidified conditions with 95% air and 5% CO₂. Two days after seeding, the culture medium was replaced with MEM containing

10% FCS, and the medium changed every 3 days until cells were used for experiments. Cells were harvested at intervals by using trypsin-EDTA solution. Counts of cell density were made using a haemocytometer under a phase contrast microscope.

SDS-PAGE and Western blotting

Smooth muscle cells were treated with 0.1% SDS and heated for 3 min in a boiling water bath. Proteins in each cell extract were electrophoretically separated by SDS-PAGE performed according to Laemmli (1970). Western blotting of polyacrylamide gel onto nitrocellulose sheet was carried out according to the method of Towbin *et al.* (1979).

Measurement of $[Ca^{2+}]_i$

Intracellular calcium ion concentration was measured as follows. Cultured cells were incubated with 5 μ M fluo-3/AM in Tyrode Hepes buffer at 37°C for 1 hr. Then the cells were washed three times by Tyrode Hepes buffer. Fluorescence images of fluo-3 were collected using an ARGUS-HiSCA image analyzer set to record the fluorescence emission at greater than 515 nm with an excitation wavelength of 488 nm.

Results

Growth characteristics of SMCs in culture

The growth curve and morphology of SMCs in primary culture are shown in Figs. 1-A and B. The curve shows that the cell number in each culture dish decreased 2 days after seeding to about 20% of the SMCs initially seeded being attached to the laminin coat on the dish. The cell number began increasing about 4 days after seeding. It appeared that the rate of increase in cell number was maximal from 6 to 8 days after starting the primary culture. Isolated SMCs were spindle-shaped with a length of about 200 μ m. On day 3, the cells appeared to be more ribbon-shaped and then began to form a network-like structure. On day 8, the cells began to appear elongate with some cells becoming spindle-shaped. By day 16, the cultured SMCs had become confluent and exhibited a hill and valley pattern.

Effects of carbachol and ATP on calcium response in SMCs

An increased intracellular $[Ca^{++}]$ has been observed in response to the application of carbachol in both single and cultured SMCs (Iijima *et al.*, 1998). However, there has been no report of a temporal change in $[Ca^{++}]_i$ during the culture of SMCs. Furthermore, it has been reported that both acetylcholine (ACh) and ATP caused an increase in $[Ca^{++}]_i$ in smooth muscle but with different effects on contraction (Ozaki *et al.*, 1992). Therefore, the effects of both carbachol and ATP on $[Ca^{++}]_i$ in SMCs during culture were investigated. The result is shown in Fig. 2. A clear difference was observed between the temporal changes in $[Ca^{++}]_i$ in response to carbachol compared with that in response to ATP. The cells responded to ATP with an increase in cytosolic calcium ion throughout the culture. On the other hand, SMCs had lost any response to carbachol 2 days after seeding. The response to carbachol then recovered gradually

Fig. 1. Growth characteristics of the smooth muscle cells in primary culture. A. Growth curve of cultured smooth muscle cells. Isolated cells were grown in MEM on laminin-coated dishes. Cells were harvested on the indicated day after seeding by using trypsin-EDTA solution and counted in a haemocytometer using a phase contrast microscope. Each value represents the mean from three dishes. Error bars indicate the standard deviation of the mean. B. Phase-contrast micrographs of the cultured smooth muscle cells. The micrographs were taken at days 0, 3, 8, and 16 of culture.

Fig. 2. Intracellular Ca²⁺ concentration of smooth muscle cells in response to carbachol (solid bars) or ATP (hatched bars) during culture. The cultured cells were incubated with 5 μ M fluo-3/AM in Tyrode Hepes Buffer and rinsed three times. After washing, carbachol or ATP was applied to the cells, and fluorescence images of fluo-3 were recorded. The fluorescence emission greater than 515 nm at an excitation wave length of 488 nm was measured. SMCs that were responsive to the agonists were defined as those cells in which the intensity of fluo-3 fluorescence increased to more than 30% of the baseline level within 30 seconds after the application of the agonist. The total number of SMCs that responded to ionomycin and of SMCs that responded to carbachol or ATP in three experiments ranged from 26 to 88 and 9 to 73, respectively.

to the same extent as seen in freshly isolated SMCs by day 16 of culture when the cells were growing confluent.

Changes in protein profiles during culture

Differences in protein profiles were observed between isolated and cultured SMCs. During culture, the cells became attached to the laminin coating of the culture dishes, then proliferated over this surface and exhibited a number of morphological changes. So, an investigation of the possible changes in contractile or regulatory proteins, which are involved in adhesion, and in the morphological changes during culture, could provide information about the role of isoforms in relation to contractility. We focused in particular on caldesmon in addition to myosin heavy chain, actin and tropomyosin, which have been studied previously (Iijima *et al.*, 1998).

The protein isoforms in extracts of SMCs prepared after different periods in culture were separated using 10% SDS-PAGE. Comparisons of the protein bands at the different stages provided evidence of changes during culture. As shown in Fig. 3, two notable changes were observed. On day 2 the 41 kDa tropomyosin monomer was lost, and a number of new protein bands observed. On day 6, the protein with an apparent molecular mass of 140 kDa was lost and a protein with molecular mass of 74 kDa appeared. These proteins with molecular masses of 140 kDa and 74 kDa were considered to be *h*-caldesmon and *l*-caldesmon respectively.

Fig. 3. Protein profile of longitudinal smooth muscle cells on SDS-PAGE. Smooth muscle cells were treated with 0.1% SDS and heated for 3 min in a boiling water bath. Proteins in each cell extract were separated by SDS-PAGE (10% acrylamid gel). The amount of protein loaded was 9.0 μ g for each lane. Note the loss of 41 kDa band for tropomyosin at day 2. On day 6, also note the loss of the 140 kDa band that is considered to be due to *h*-caldesmon and the appearance of the 74 kDa band considered that due to *l*-caldesmon.

Change in caldesmon and myosin heavy chain isoforms during culture

Both caldesmon and myosin heavy chain are regarded as marker molecules for differentiation. Therefore, it was considered to be significant to investigate the temporal change in these isoforms during culture. Immunoblot analysis was carried out, and the results are shown in Fig. 4-A (caldesmon) and 4-B (myosin heavy chain). For analysis of *h*-caldesmon and *l*-caldesmon, antibodies reactive to both isoforms and only to *l*-caldesmon were used respectively, because the former antibody reacted slightly with *l*-caldesmon in the cultured SMCs from the guinea pig ileum. As shown in Fig. 4-A-a, the content of *h*-caldesmon on day 3 was less than 10% of that observed in freshly isolated SMCs, and was absent 6 days after seeding. However, *h*-caldesmon was present again by the time the cells were growing confluent at day 16. *l*-Caldesmon began to be observed 2 days after seeding and increased strikingly between day 6 and day 9 (Fig. 4-A-b). The content decreased to about 50% of the maximum by the time the cells had become confluent at day 16.

The content of smooth muscle type myosin heavy chain (SMHC) decreased rapidly to about 10% of that in freshly isolated SMCs after the onset of culture, and increased reaching about 50% of the maximum demonstrated by freshly isolated SMCs by day 16 when the cells were postconfluent. Non-muscle type myosin heavy chain (NMHC) appeared within 2 days of seeding and increased gradually, then more rapidly from day 6, but decreased a little in the postconfluent cells. These patterns of temporal change of SMHC and NMHC are similar to those of *h*- and *l*- caldesmon.

Fig. 4. Temporal changes in abundance of caldesmon and myosin heavy chain isoforms in primary culture. Proteins in the cell extracts were separated by 10% SDS-PAGE and examined by immunoblot analysis. Total protein load for each lane was 9.0 μ g. The relative amount of each isoform was estimated from the blot densities and expressed as the ratio to the maximum. A-a: Immunoblot reacted with anti-*h*-caldesmon. A-b: Immunoblot reacted with anti-*l*-caldesmon. B-a: Immunoblot reacted with anti-smooth muscle cell type myosin heavy chain. B-b: Immunoblot reacted with anti-non muscle cell type myosin heavy chain.

Fig. 5. Temporal changes in abundance of tropomyosin isoforms in primary culture. Proteins in the cell extracts were separated by 10% SDS-PAGE and examined by immunoblot analysis. Total protein load for each lane was 9.0 μ g. The relative amount of each isoform was estimated from the blot densities and expressed as the ratio to the maximum. a. Immunoblot reacted with anti-tropomyosin. The molecular mass of the tropomyosin is 39 kDa. b. Immunoblot reacted with anti-tropomyosin. The molecular mass of the tropomyosin is 32 kDa.

Analysis of tropomyosin isoforms

In isolated SMCs, tropomyosin isoforms with molecular mass of 41 kDa and 39 kDa were observed, but the former was lost in the cultured cells. Non-muscle cell tropomyosins have been reported to consist of monomers with a molecular mass of about 30 kDa, which is smaller than that of muscle tropomyosin. From our studies on the temporal change in isoforms of myosin heavy chain and caldesmon described above, it was expected that at least one of the tropomyosin isoforms in the cultured cells would show a molecular mass of about 30 kDa. In order to examine the changes in tropomyosin isoforms during culture, immunoblot analysis was performed using antibodies reactive to the tropomyosin of the cultured SMCs, which also reacted with the 39 kDa isoform of tropomyosin. The result is shown in Fig. 5. The tropomyosin monomer of molecular mass of 41 kDa was present in the freshly isolated SMCs, but was lost within 2 days of seeding (Fig. 3). On the other hand, the 39 kDa monomer was present in both uncultured and cultured cells. It decreased to about 40% of the level in isolated SMCs on day 3 before returning to levels on day 6 similar to that found before culture. The content of the 39 kDa monomer in postconfluent cells was 2 fold higher than that in uncultured

Fig. 6. Temporal changes in abundance of actin isoforms in primary culture. Proteins in the cell extracts were separated by 10% SDS-PAGE and examined by immunoblot analysis. Total protein load for each lane was 9.0 μ g. The relative amount of each isoform was estimated from the blot densities and expressed as the ratio to the maximum. a. Immunoblot reacted with anti- α -SM actin. b. Immunoblot reacted with anti- β -actin.

cells. The 32 kDa isoform of tropomyosin was not observed in freshly isolated SMCs but appeared during culture. It is of note that this isoform increased strikingly on day 6, when the SMCs began to proliferate.

Changes in actin isoforms

Actin is an important protein not only for contraction but also for cell motility and determination of cell structure. It has been reported that α -SM-, β - and γ -actin isoforms are localized in SMCs (Lehman *et al.*, 1996), but the role of each of these isoform is not clear. Previously, we had reported that α -SM actin was predominant in freshly isolated single SMCs while β -actin was the major isoform in cultured SMCs (Iijima *et al.*, 1998). However, this observation did not give any information about the role of each isoform. Therefore, temporal changes in the relative occurrence of the isoforms were investigated. As shown in Fig. 6, the content of α -SM actin declined gradually to a minimum level (about 30% of the maximum) 6 days after seeding, when the SMCs are beginning to proliferate rapidly. The level of this isomer recovered in amount by day 9. On the other hand, β -actin increased during culture with a content which was 2 fold higher in cultured SMCs than in freshly isolated SMCs.

Discussion

In SMCs from the guinea pig ileum, differences in intracellular calcium ion concentration in response to ATP and carbachol were observed during culture. Acetylcholine receptors are reported to be regulated in response to serum and cell growth (Olson *et al.*, 1983), and selective restoration of calcium coupling to muscarinic M₃ receptors in cultured myocytes has recently been reported (Mitchell *et al.*, 2000). So, it is possible that in the present study the presence of fetal calf serum caused a decrease in carbachol receptors during culture. Furthermore, it has been reported that ATP raises $[Ca^{2+}]_i$ via different P₂ receptor subtypes in freshly isolated and cultured aortic myocytes (Pacaud *et al.*, 1995). Therefore, we consider that the difference in response of SMCs to ATP and carbachol during culture are probably due to changes in receptor expression. A detailed study is required on calcium signaling and contraction in SMCs of the guinea pig ileum to explain these differences.

Isoforms of proteins that participate in smooth muscle contraction have been extensively investigated by many authors as described below, but the role of each isoform has not yet been defined. We had reported the difference in profiles of expression of contractile proteins between freshly isolated single SMCs and cultured SMCs, and suggested a relationship between contractility and these isoforms (Iijima *et al.*, 1998). However, we were unable to draw conclusions about the role played by each protein isoform in smooth muscle contraction. From the present results, the isoforms can be divided into three groups. The first is the group of protein isoforms in which a striking change was observed on day 2 after seeding, by which time the SMCs had attached to the laminin matrix. The results suggest that these protein isoforms (caldesmon, myosin heavy chain and tropomyosin) are related to the adhesion of SMCs to the matrix. Furthermore, it was also remarkable that the relative contents of myosin heavy chain and tropomyosin in the cultured cells on day 2 and 3 were lower than those in the freshly isolated SMCs. During this period, the SMCs exhibited morphological changes from spindle shaped cells, and then to ribbon shaped cells that were able to join together into a network structure. Therefore, we suggest that myosin heavy chain and tropomyosin are correlated with these morphological changes observed in the SMCs. The second is the group of isoforms in which a similar striking change was observed within 2 days of seeding, but where the apparent content of isoforms did not return within 16 days. Although more detailed studies are required, it is suggested that these play a regulatory role in contraction. The third group was the isoforms in which a marked change in the isoform content was observed on day 6, when the cells began to proliferate rapidly. It is probable that these protein isoforms are related to SMC proliferation. The role of each of the protein isoforms in these three groups will be further discussed below.

It is reported that *h*-caldesmon is a regulatory protein in the contraction of SMCs while *l*-caldesmon has a role in the organization of the actin cytoskeleton (Nomura and Sobue, 1987; Sobue and Sellers, 1991). In addition, *h*-caldesmon and *l*-caldesmon are considered to be good markers for differentiation and dedifferentiation, respectively (Ueki *et al.*, 1987). However, their physiological roles have not yet been defined. Our result showing changes in the content of these two caldesmon isoforms suggests that the changes in their expression is correlated with phenotypic modulation, and that *h*-caldesmon and *l*-caldesmon play important roles in

contraction and proliferation respectively. More detailed consideration of the correlation between changes in these isoforms during culture with those of tropomyosin as described below provide further insight into the possible role of each caldesmon isoform.

Myosin heavy chain isoforms (SM1, SM2 and NMHC) have been noted as molecular markers of vascular development and atherosclerosis (Aikawa *et al.*, 1993; Kawamoto and Adelstein, 1987; Rovner *et al.*, 1986). In addition, NMHC has been suggested to play a role during meiosis and secretion in non-muscle cells (Kelly *et al.*, 1995; Ludowyke *et al.*, 1989). However, the role of each of the myosin heavy chain isoforms in SMCs is poorly defined. The results in the present study suggested that SM1 and/or SM2 keep SMCs spindle-shaped and contractile. On the other hand, NMHC may be related to the shape, adhesive capacity and proliferative ability of the SMCs. It is also interesting that the temporal changes in the content of *h*-caldesmon and *l*-caldesmon described above were similar to those for the content of smooth muscle type and non-muscle myosin heavy chain isoforms. Both of those isoforms are considered to be marker molecules for differentiation. Therefore, the coincident changes in expression of these protein isoforms may be related. More detailed studies such as a distinction between SM1 and SM2, and between NMHC-A and NMHC-B together with an investigation of the phosphorylation of these isoforms should give information about the role of each isoform.

The changes in the tropomyosin isoforms during culture are of particular interest. There was no correlation between the appearance or disappearance of the two isoforms in the cultured SMCs, which had molecular masses of 39 kDa and 32 kDa respectively. The two tropomyosin monomers in the freshly isolated SMCs with molecular masses of 41 kDa and 39 kDa are considered to be α - and β -tropomyosin respectively. This is because only the 39 kDa monomer reacts with the antibody derived from chicken gizzard tropomyosin which is composed of both α - and β -tropomyosin monomers with molecular masses of 44 kDa and 38 kDa respectively. Tropomyosin is considered to be a coiled-coil homo- or hetero- dimer (Jancsó and Graceffa, 1991). In addition, it is reported that the molecular mass of tropomyosin in non-muscle cells is about 30 kDa (Côté, 1983). So, the tropomyosin monomer with molecular mass of 32 kDa in the present study may be one of the non-muscle type tropomyosin isoforms. If tropomyosin in the cultured SMCs is composed of 39 kDa and 32 kDa monomers, then considerable change in protein conformation could occur in the SMCs, and result in an effect on the interaction of actin and myosin. It is yet to be resolved how these monomers form a dimer. The decrease in α -SM actin content together with this probable change in tropomyosin conformation, actin filaments would lose stability and result in the morphological changes seen in the cultured SMCs in the proliferative stage. It has been proposed that the essential role of tropomyosin was cooperative regulation of smooth muscle thin filament activity by caldesmon (Sobue and Sellers, 1991; Marston and Redwood, 1993). However the physiological role of tropomyosin in SMCs is not yet clear (Smillie, 1996). Although more detailed studies are required, our results suggested that the 41 kDa tropomyosin monomer plays an important role in contraction. Regarding the interaction between tropomyosin and caldesmon, coincident expression of α -tropomyosin and caldesmon isoforms in SMCs has been reported (Kashiwada *et al.*, 1997). While the present result on tropomyosin is similar, studies at the mRNA level and on the relationship between caldesmon isoforms are required before the role of tropomyosin isoforms can be understood.

The present study also suggests that tropomyosin is a better marker molecule for differentiation, because the tropomyosin monomer of 41 kDa was observed only in the freshly isolated SMCs and was lost as the SMCs were maintained in culture.

A relationship has been proposed between the expression of α -SM Actin and cyto-differentiation (Owens *et al.*, 1986; Clowes *et al.*, 1988; Campbell *et al.*, 1989). In addition there has also been a report that post-transcriptional control of α -SM actin expression is linked to the organization of actin filaments in SMCs (Nomura *et al.*, 1992). Furthermore, α -SM actin was found around the contractile apparatus while β -actin was in the dense body (North *et al.*, 1994). Together with these reports, our results suggest that the decrease in α -SM actin content in the cultured SMCs is related to proliferation and loss of contractility.

In SMCs that are growing confluent, the protein profiles apparently returned to those of freshly isolated SMCs except that the 41 kDa monomer is still absent. It is of interest to know if both protein profiles and contractility return or not when the cells are grown post-confluent. Regarding the culture medium, the conditions applied in the present study resulted in dedifferentiation of the SMCs. If culture conditions were achieved which kept the SMCs in their differentiated state as reported for other SMCs (Bowers and Dahm, 1993; Kashiwada *et al.*, 1997; Ma *et al.*, 1998; Halayko *et al.*, 1999), protein isoform changes during culture may be expected to be different from those in this study. The differences would provide important information about the role of each isoform of the contractile proteins and of the contraction mechanism of SMCs.

With further studies of SMCs in different culture media and a correlation with their contractility under these different conditions, insight may be gained into the molecular basis of contractility and differentiation in SMCs.

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