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Mitogen-induced up-regulation of non-smooth muscle isoform of α-tropomyosin in rat aortic smooth muscle cells

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Abstract

Correlation between the expression of the α-tropomyosin isoforms and cell growth was investigated in rat aortic smooth muscle cells. The levels of exon 1a, exons 1a + 2a (smooth muscle type) and exons 1a + 2b (non-smooth muscle type) were determined by reverse transcription-polymerase chain reaction (RT–PCR). When the cells were cultured, the level of exons 1a + 2b transiently increased while reaching a maximum at 3–5 days. When the serum-deprived confluent cells were stimulated with 3–20% serum for 1.5 h, the level of exons 1a + 2b increased by about twofold. The 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) but not 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X) inhibited this up-regulation. Phorbol-12,13-dibutyrate (PDB) mimicked the effect of serum. The DNA synthesis as determined by the incorporation of 5-bromo-2′-deoxy-uridine (BrdU) was not enhanced by the 1.5 h stimulation with serum or phorbol ester. The up-regulation of non-smooth muscle isoform of α-tropomyosin occurred during G_0/G_1 transition before entering S phase. Protein phosphorylation is suggested to be involved in the up-regulation. However, the responsible kinase(s) remain to be elucidated.

Keywords: Differentiation; Protein kinase C; Atherosclerosis; Gene expression; α-tropomyosin; (Rat)

1. Introduction

There are at least two distinct states of smooth muscle cells, i.e., contractile and synthetic phenotypes (Owen, 1995; Ross, 1993; Thyberg et al., 1990). Phenotypic conversion from the differentiated contractile type to proliferative synthetic type is considered to be an important event regarding the formation of various vascular lesions including atherosclerosis. The expression patterns of proteins in smooth muscle cells change in accordance with phenotypic conversion. Several proteins including smooth muscle α-actin (Drew et al., 1991), smooth muscle myosin heavy chain (Aikawa et al., 1993; Miano et al., 1994), myosin light chain (Fisher et al., 1997; Nabeshima et al., 1987), SM-22 (Duband et al., 1993), calponin (Duband et al., 1993), caldesmon (Ueki et al., 1987) and smoothelin (van der Loop et al., 1996) are known to characterize the phenotypes. The α-tropomyosin is one of such markers for developmental differentiation in smooth muscle cells (Muthuchamy et al., 1993). Smooth muscle cells express several nonmuscle as well as smooth muscle isoforms of α-tropomyosin (Fatigati and Murphy, 1984; Holycross et al., 1992). The organization of the α-tropomyosin gene has already been determined in the rat (Lees-Miller and Helfman, 1991; Ruiz-Opazo and Nadal-Ginard, 1987; Wieczorek et al., 1988) and chicken (Forry-Schaudies and Hughes, 1991; Lemonnier et al., 1991). According to a review article (Lees-Miller and Helfman, 1991), five exons among total 15 exons are the constitutive exons that are included in all types of isoforms and other exons are alternatively spliced in a tissue-specific manner. Numbering of exons of rat tropomyosin gene in this article is as described (Lees-Miller and Helfman, 1991). Exons 2a and 2b are mutually exclusive exons (Smith et al., 1989). Exon 2b is spliced in mRNAs of all isoforms except for the smooth muscle isoform that contains exon 2a in place of exon 2b (see Fig. 1A). Alternative splicing is thus consid-

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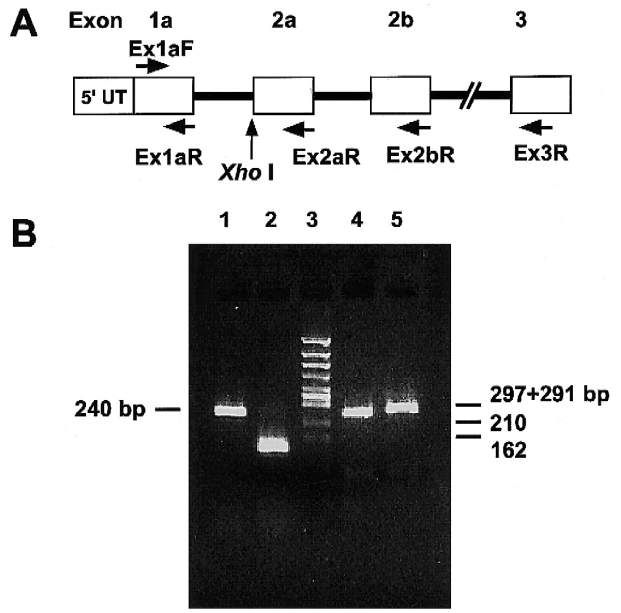

Fig. 1. Specificity of the RT–PCR analysis for the expression of α-tropomyosin isoforms. (A) Diagram of the rat α-tropomyosin gene organization and primers for the analysis of RT–PCR. The region of the four exons of the rat α-tropomyosin gene is shown. The boxes and thick lines represent the exons and introns, respectively. The exons are numbered according to (Lees-Miller and Helfman, 1991). 5′ UT, a 5′ untranslated region of exon 1a. Five primers are indicated by arrows at the corresponding regions on the gene. The sequences of the primers are 5′-ATG GAC GCC ATC AAG AAG AA-3′ (Ex1aF), 5′-CTG CTT GCT CCG GTC TTC CG-3′ (Ex1aR), 5′-CTT GGC GGC GGT CTC GTC GG-3′ (Ex2aR), 5′-ATC TGT GGC CTT TTT CTC CG-3′ (Ex2bR) and 5′-CCT TCT CAG CCT CCT CCA GC-3′ (Ex3R). (B) *Xho*I-digestion of RT–PCR products of exons 1a + 2a (lanes 1, 2) and exons 1a + 2b (lanes 4, 5). Sixty nanogram of the PCR products were treated with 6 unit *Xho*I for 3 h (lanes 2, 5). Lanes 1 and 4, *Xho*I-untreated PCR products; Lane 3, *Hinc*II-digested ϕX174 DNA as size markers. The expected size of the PCR products and the size of markers are indicated on the left and on the right, respectively.

ered to play an important role in the generation of tissue-specific isoforms (Wieczorek et al., 1988). However, the regulatory mechanism regarding the expression of non-smooth muscle and smooth muscle isoforms of the α-tropomyosin gene is still not well understood. It is not known whether or not the isoform selection of α-tropomyosin occurs during the phenotypic conversion of arterial smooth muscle.

In the present study, the expression of the α-tropomyosin isoforms was determined in cultured smooth muscle cells during the stimulation of proliferation with a serum supplement, by using the reverse transcription-polymerase chain reaction (RT–PCR). To investigate the regulatory mechanism of the selection between exons 2a and 2b in the rat α-tropomyosin, the effects of protein kinase inhibitors, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) and wortmannin were examined. To examine the growth state of smooth muscle cells, cellular DNA synthesis was evaluated by incorporation of 5-bromo-2′-deoxyuridine (BrdU).

2. Materials and methods

2.1. Rat aortic smooth muscle cells in primary culture

Smooth muscle cells were enzymatically dispersed from the aortic media of male Wistar rats (Yamamoto et al., 1983). The rats were sacrificed by ether according to the guidelines for Animal Experiment in Faculty of Medicine, Kyushu University. The cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics (Gibco, Rockville, MD, USA). The growth medium was changed every 2–3 days. When the cells reached confluence, they were treated in a serum-free medium for 2 days before the experiment. A high cell viability (> 99%) was maintained during each experimental procedure, as determined by the trypan blue exclusion test (Kobayashi et al., 1994).

2.2. Isolation of total RNA from cultured smooth muscle cells

The total RNA was isolated from the cultured smooth muscle cells or rat aortas, as previously described (Chomczynki and Sacchi, 1987) with minor modifications. In brief, cells grown on 60 mm diameter dish or rat aortas were lysed in 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. The cell lysate was made 0.2 M Na-acetate (pH 4.0) and was extracted twice in a mixture (50:49:1) of phenol, chloroform and isoamyl alcohol. Any possible contaminating genomic DNA was digested with RNase-free DNase (Promega, Madison, WI, USA) in the presence of RNase inhibitor (Toyobo, Osaka, Japan) at 37°C for 30 min. The concentration of final RNA sample was estimated by measuring absorbance at 260 nm.

2.3. RT–PCR analysis of α-tropomyosin splice isoforms

The primers used in the RT–PCR analysis were synthesized according to the sequence of the rat α-tropomyosin gene, as shown in Fig. 1 (Lees-Miller and Helfman, 1991; Wieczorek et al., 1988). The method of RT–PCR was as previously described (Nishimura et al., 1992). In brief, the first-strand cDNA was synthesized in the 20 μl reaction mixture, containing 200 units Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, USA), 1 μg total RNA, 0.5 mM each dNTPs (dATP, dCTP, dGTP, dTTP), 50 nM RT primer for α-tropomyosin (Ex3R), 50 nM RT primer for rat β-actin, 20 units of RNase inhibitor and 10 mM dithiothreitol. A 1 μl aliquot of the same RT product was then subjected to the PCR amplification of cDNA of exon 1a, exons 1a + 2a, exons 1a + 2b and β-actin separately. The PCR reaction for α-tropomyosin cDNA was composed of an initial 2 min denaturation at 94°C, the following 30-cycle amplification step with 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min

extension at 72°C, and the final additional 10 min extension step at 72°C. The primers used for the RT–PCR and the condition of PCR reaction for β-actin was as previously described (Nishimura et al., 1992). The PCR product was separated on 3% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide and recorded on Kodak Tri-X pan 400. The densities of bands were determined with Gel Plotting Macros of the NIH image version 1.61 (National Institute of Health, USA) after scanning the gel image on an Epson color scanner GT-9500. The levels of α-tropomyosin splice isoforms were compared as an arbitrary ratio of the density against that of β-actin product obtained from the corresponding RT product. The density of β-actin appeared to be unchanged in all experimental conditions in the present study (see Figs. 2A and 3A). The nucleotide sequences were determined by dideoxy-mediated chain termination method on an automated sequencer (Prism-310, Applied Biosystems, Foster City, CA, USA).

2.4. Stability of mRNA

The stability of mRNA was examined by inhibiting nascent RNA synthesis by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (te Poele et al., 1999). The 2-day serum starved confluent cells were either stimulated with 10% serum for 1.5 h or kept unstimulated, and then treated with 60 μM DRB. At each time point (0, 6, 12, 18 and 24 h) after treatment with DRB, the levels of exon 1a, exons 1a + 2a, exons 1a + 2b and β-actin were determined as described in Section 2.3. The time course of decay was expressed as a percentage of the level of mRNA observed at time 0.

2.5. Analysis of DNA synthesis of smooth muscle cells in primary culture

The DNA synthesis of smooth muscle cells was examined by the incorporation of BrdU (Boehringer Mannheim, Tokyo, Japan) in nuclear DNA (Ellwart and Dörmer, 1985). The cells were labeled with 10 μM BrdU in growth media for 1.5 h. In Fig. 7, when DNA synthesis was examined at 1.5 h after stimulation with serum or PBDu, BrdU was added to the media at the time of stimulation. When DNA synthesis was examined at 24 h after the stimulation, BrdU was included in the media for the last 1.5 h of stimulation. After labeling, the cells were fixed in 70% ethanol, 50 mM glycine (pH 2.0) at −20°C for 20 min. The amount of incorporated BrdU was determined by either immunofluorescence staining (Table 1) or enzyme immunoassay (Fig. 7). In both cases, a monoclonal anti-BrdU antibody (Boehringer Mannheim) was used as a primary antibody. In immunofluorescence staining, the immune complex was detected by fluorescein isothiocyanate-labeled secondary antibody (Boehringer Mannheim). The fluorescent image obtained with a fluorescence microscope (Axioskop, Zeiss, Germany) was photographed on Kodak Ektachrome 400. The fraction of the BrdU-incorporated nuclei in total nuclei was determined by counting 150–300 nuclei on photographs. In an enzyme immunoassay, horseradish peroxidase-labeled secondary antibody (Sigma, St. Louis, MO, USA) was used to detect the primary antibody. The cells were then incubated with 1 mM 2,2′-azino-di-3-ethyl-benzothiazoline-6-sulfonic acid (Sigma) and 0.03% hydrogen peroxide in 100 mM citric acid (pH 4.2) at room temperature for 60 min. Absorbance at 405 nm of the substrate solution was determined with a spectrophotometer (Lambda 11; Perkin Elmer, Norwalk, CT, USA). The background level of absorbance was determined in each measurement by omitting the primary antibody. The BrdU incorporation was assessed based on the net increase in the absorbance after subtracting the background level.

Table 1
Changes in DNA synthesis based on the culture day in rat aortic smooth muscle cells in primary culture

Culture day	BrdU-incorporated nuclei (%)
3	51.9 ± 5.5
5	40.9 ± 4.5
7	20.3 ± 7.7
Confluent	9.2 ± 4.3

The changes in the nuclear incorporation of BrdU based on the culture day were determined by immunofluorescence staining. The fluorescence image was photographed, and the fraction of the BrdU-incorporated nuclei in the total nuclei was determined by counting 150–300 nuclei in the photographs. Data are mean ± S.D. of four independent cell cultures.

2.6. Drugs and chemicals

Phorbol-12,13-dibutyrate (PDB) and DRB were purchased from Sigma. H-7 was purchased from Seikagaku (Tokyo, Japan). Wortmannin was purchased from Kyowa (Tokyo, Japan). 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X) and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB 203580) were purchased from Calbiochem (La Jolla, CA, USA), and 2′-amino-3′-methoxyflavone (PD 98059) was purchased from Biomol (Plymouth Meeting, PA, USA). Oligonucleotides for primers were synthesized by Sawady Technology (Tokyo, Japan).

2.7. Data analysis

All data are presented as the means ± standard deviation (S.D.). A statistical analysis of the data was performed using the unpaired Student's *t*-test. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Specificity of RT–PCR reaction

The specificity of RT–PCR reaction was confirmed as follows: (1) The size of the products: the size of the PCR product of exon 1a was approximately 114 base pairs as expected from the published sequence (Ruiz-Opazo and Nadal-Ginard, 1987). The size of product of exons 1a + 2a was approximately 240 base pairs and same as that of exons 1a + 2b (Fig. 1B). The intron of the 765 base pairs follows exon 1a. It was thus unlikely that the products of exons 1a + 2a and exons 1a + 2b contained this intron, indicating that the products originated from mRNA but not from genomic DNA or pre-mRNA. (2) The digestion of RT–PCR products with *Xho*I (Fig. 1B): among exons 1a, 2a and 2b, only exon 2a contained a unique site of *Xho*I (Ruiz-Opazo and Nadal-Ginard, 1987). As shown in Fig. 1B, *Xho*I cleaved only product of exons 1a + 2a and the size of digests was as expected (114 and 126 base pairs). The product of exons 1a + 2b was not digested by *Xho*I. (3) The nucleotide sequences: the DNA sequences of the products of exons 1a + 2a and exons 1a + 2b were determined to be identical to the published sequences (Wieczorek et al., 1988) (data not shown).

3.2. Changes in the expression of α-tropomyosin isoforms in cell culture

The isolated rat aortic tissue before the culture expressed both exons 1a + 2a and exons 1a + 2b (Fig. 2A). When smooth muscle cells were enzymatically dispersed and cultured, the cells proliferated rapidly during the 3–7 days and reached confluence at about day 10. While the cell culture was in progress, the level of exons 1a + 2b transiently increased reaching a maximum at 3–5 culture days (Fig. 2A). The level of exons 1a + 2b obtained at day 5 was 3.2 ± 0.5-fold of that seen before culture (Fig. 2B). The level of exons 1a + 2b thereafter decreased to a level slightly higher than that seen before culture (Fig. 2A,B). The 2-day serum-deprivation caused a further decrease in the level of exons 1a + 2b. However, this decrease was not statistically significant (Fig. 2B). No apparent changes were observed in the levels of exon 1a, exons 1a + 2a or β-actin (Fig. 2A,B).

In accordance with the transient increase in the level of exons 1a + 2b, DNA synthesis transiently increased (Table 1). Fraction of BrdU-incorporated nuclei for 1.5 h labeling reached maximum (51.9 ± 5.5%) on day 3. Thereafter, it decreased progressively to 40.9 ± 4.5 (day 5), 20.3 ± 7.7 (day 7) and 9.2 ± 4.3% (at confluence). Thus, the up-regulation of exons 1a + 2b apparently correlated with the increase in DNA synthesis.

3.3. Serum replenishment increased the expression of exon 2b

As shown in Fig. 3A, the serum-deprived rat aortic smooth muscle cells at confluence expressed both smooth muscle (exons 1a + 2a) and non-smooth muscle isoform (exons 1a + 2b) of α-tropomyosin (0% serum). When the serum-deprived cells were stimulated by replenishing the serum for 1.5 h, the level of exons 1a + 2b increased in a concentration-dependent manner (Fig. 3A). There were no apparent changes in the expression levels of exon 1a, exons 1a + 2a or β-actin. Fig. 3B summarizes the results of 6 independent measurements. The level of exons 1a + 2b was significantly increased by stimulation with 1% serum

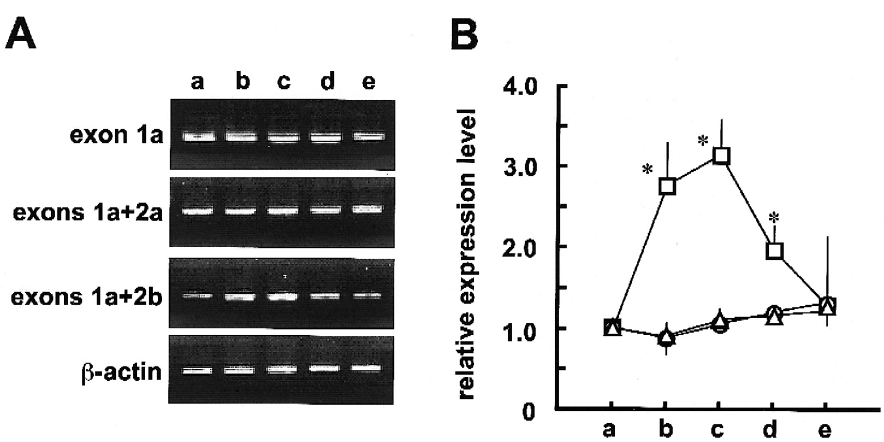

Fig. 2. Changes in the expression of α-tropomyosin in the cell culture. (A) A representative electrophoretograph of PCR products of exon 1a, exons 1a + 2a, exons 1a + 2b and β-actin obtained with the aortic tissue before culture (a) and the cultured rat aortic smooth muscle cells at day 3 (b), day 5 (c), confluence (d) and after 2-day serum-deprivation of the confluent culture (e). (B) Summary of the changes in the levels of exon 1a (○), exons 1a + 2a (△) and exons 1a + 2b (□). The levels of the α-tropomyosin exons in the cultured cells (b–e) were expressed as a ratio to that seen in the aortic tissue before culture (a). The data are the mean ± S.D. ($n = 3$). *, significantly different ($P < 0.05$) from the expression level seen in the aortic tissue.

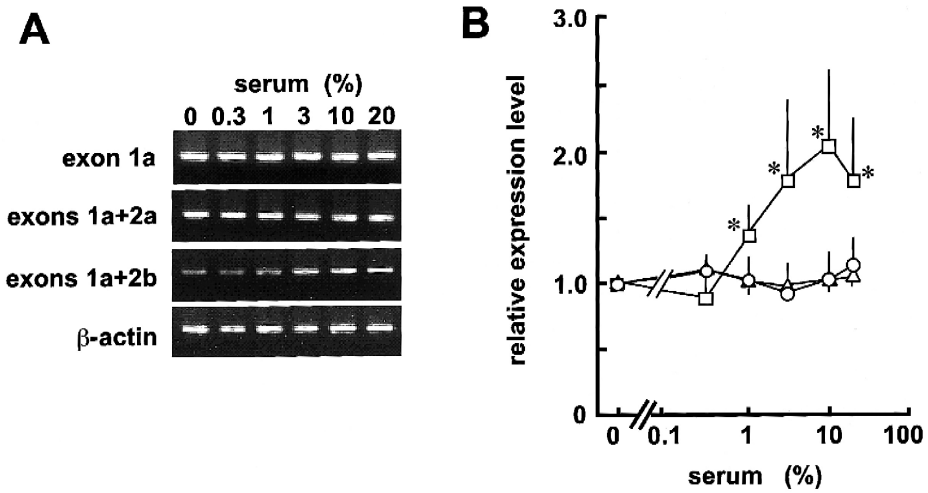

Fig. 3. Serum-induced up-regulation of the expression of exons 1a + 2b. (A) A representative electrophoretograph of the PCR products of exon 1a, exons 1a + 2a, exons 1a + 2b and β-actin. The 2-day serum-deprived confluent cells were stimulated with serum at the indicated concentrations for 1.5 h. (B) Summary of six independent experiments on the level of exon 1a (○), exons 1a + 2a (△) and exons 1a + 2b (□). The levels obtained after the serum stimulation were expressed as the ratio to the level obtained before the stimulation. The data are the mean ± S.D. *, significant increase ($P < 0.05$) compared to the level seen in the serum-deprived unstimulated cells.

(1.4 ± 0.2-fold of unstimulated cells). The increases in the expression level of exons 1a + 2b reached a maximum at 3% serum. The stimulation with 10% and 20% serum caused a similar enhancement to that seen with the 3% serum. The levels of exons 1a + 2b obtained with 3%, 10% and 20% serum were 1.8 ± 0.6-, 2.0 ± 0.6- and 1.8 ± 0.5-fold of that seen in the unstimulated cells ($n = 6$).

3.4. Effects of kinase inhibitors on serum-induced up-regulation of exon 2b

The treatment with H-7 20 min prior to and during stimulation with 3% serum inhibited the up-regulation of exons 1a + 2b (Fig. 4A). This inhibitory effect was concentration-dependent in the range between 0.1 μM and 10 μM, and the complete inhibition was observed at 1 μM. These concentrations of H-7 had no significant effects on the basal level of exons 1a + 2b without the stimulation by the serum. Treatment with H-7 had no effect on the levels of expression of exon 1a and exons 1a + 2a, both with and without stimulation by the serum (data not shown). Another protein kinase C inhibitor, GF 102930X had no significant effect on the up-regulation of exons 1a + 2b and the expression of exons 1a + 2b seen in the quiescent cells (Fig. 4B). GF 102930X had no effect on the levels of expression of exon 1a and exons 1a + 2a both with and without stimulation by the serum (data not shown).

We examined the effect of wortmannin, an inhibitor of phosphatidylinositol 3-kinase and myosin light chain kinase (Kapeller and Cantley, 1994; Yano et al., 1993) (Fig. 4C). The treatment with 10 μM wortmannin decreased the level of exons 1a + 2b from 1.8 ± 0.6- to 1.3 ± 0.4-fold of that seen in the unstimulated cells. However, the inhibition by wortmannin was not statistically significant and it was only partial even at 10 μM. These concentrations of wortmannin had effects neither on the basal level of exons 1a + 2b seen without the stimulation by the serum (Fig. 4C), nor the levels of exon 1a and exons 1a + 2a with or without stimulation by the serum (data not shown). The inhibition of mitogen activated protein kinase pathways by PD 98059 or SB 203580 had no significant effect on the up-regulation of exons 1a + 2b (data not shown).

3.5. Up-regulation of exon 2b by phorbol ester

Since H-7 completely inhibited the serum-induced up-regulation of exons 1a + 2b, we examined the effect of PDB on the level of α-tropomyosin isoforms. The stimulation with PDB for 1.5 h increased the expression level of exons 1a + 2b in a concentration-dependent manner within the range between 1 and 100 nM (Fig. 5). The maximum effect was obtained at 100 nM and the level of exons 1a + 2b was 2.1 ± 0.4-fold of that seen in the unstimulated cells ($n = 3$). No effect was observed on the levels of exon 1 and exons 1a + 2a (Fig. 5). Treatment with 10 μM H-7 inhibited the increase in the level of exons 1a + 2b induced by PDB (data not shown).

3.6. Stability of mRNA

The stability of mRNA was compared between the presence and absence of serum stimulation (Fig. 6). The serum starved confluent cells were either stimulated with 10% serum for 1.5 h or kept unstimulated as a control, and then treated with 60 μM DRB to inhibit nascent synthesis of mRNA. The level of β-actin mRNA slightly decreased to 80% by 24 h. The decay of β-actin mRNA seen in the presence of 10% serum did not significantly ($P > 0.05$)

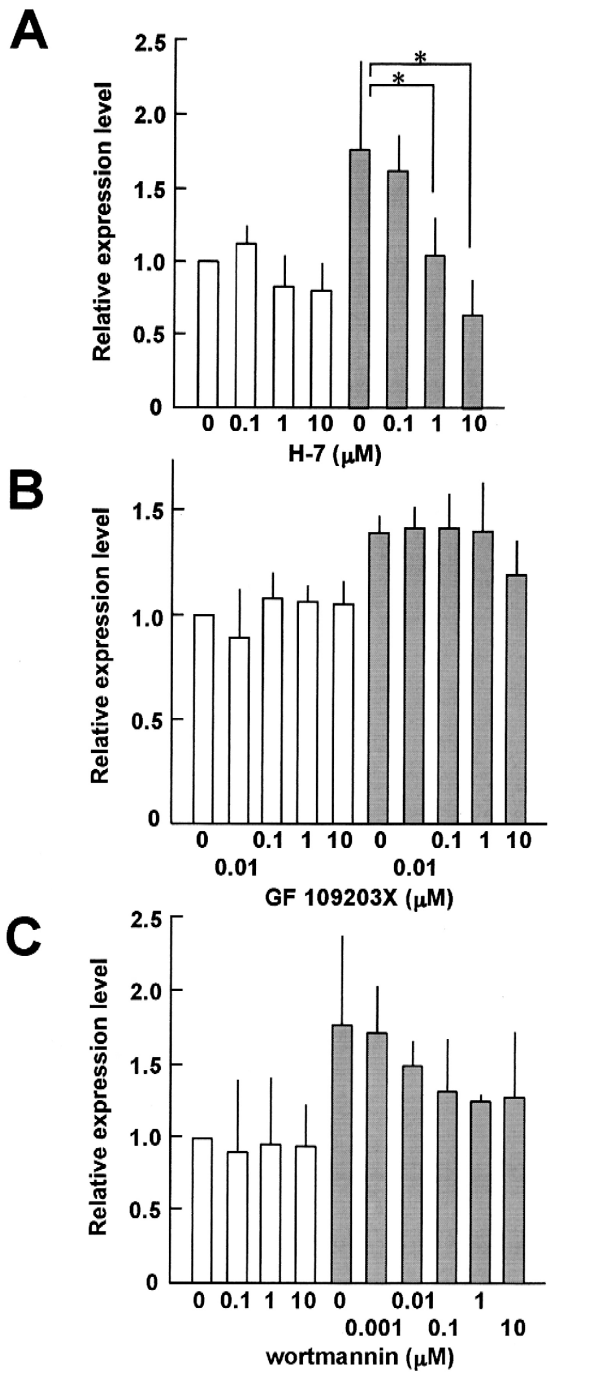

Fig. 4. Effects of kinase inhibitors on the up-regulation of exons 1a + 2b induced by 3% serum. The expressions of exons 1a + 2b in H-7 (A), GF 102930X (B) or wortmannin (C) -treated cells with (shaded columns) and without (open columns) the subsequent stimulation of 3% serum were analyzed as shown in Fig. 3. The serum-deprived cells were treated with kinase inhibitors 20 min before and during the 1.5 h stimulation with 3% serum. The data are the mean ± S.D. ($n = 4$–6). *, significant difference ($P < 0.05$).

differ from that seen in the absence of serum. On the other hand, the decay of exons 1a + 2b was faster than that of β-actin. The exons 1a + 2b decreased to 27.8 ± 9.3% at 24 h in the absence of serum stimulation, while it decreased to 40.5 ± 15.9% in the presence of 10% serum. There was no significant difference in the decay of exons 1a + 2b between the presence and absence of serum stimulation. The decay of exon 1a and exons 1a + 2a did not significantly differ from that seen with β-actin either in the presence or absence of serum, and there was no significant differences in their decay between the presence and absence of serum (data not shown).

3.7. DNA synthesis induced by serum repletion and phorbol ester

The DNA synthesis of cultured smooth muscle cells was examined during the stimulation with serum and PDB. As shown in Fig. 7, the serum-deprived cells at confluence showed a low level of the BrdU incorporation. The cells at confluence that had been continuously cultured in the growth media containing 10% serum showed a significantly higher level of BrdU incorporation than that seen after the 2-day serum-deprivation. Since the expression of exons 1a + 2b increased within 1.5 h after stimulation with serum or PDB, the BrdU incorporation was examined after 1.5 and 24 h stimulation. When the serum-deprived cells were stimulated with serum (Fig. 7A) or PDB (Fig. 7B) for 1.5 h, the BrdU incorporation did not differ from that obtained with the unstimulated cells. When the serum-deprived cells were stimulated with serum for 24 h, the BrdU incorporation increased in a concentration-dependent man-

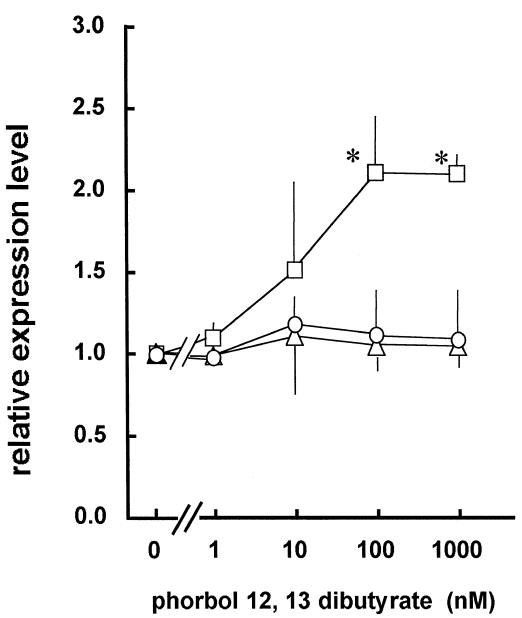

Fig. 5. Up-regulation of the expression of exons 1a + 2b induced by PDB. The 2-day serum-deprived confluent cells were stimulated with PDB for 1.5 h. The concentration-dependent effects of PDB on the level of expression of exon 1a (○), exons 1a + 2a (△) and exons 1a + 2b (□) were analyzed as shown in Fig. 3. The data are the mean ± S.D. ($n = 3$). *, significantly different ($P < 0.05$) from the expression level obtained with the serum-deprived unstimulated cells.

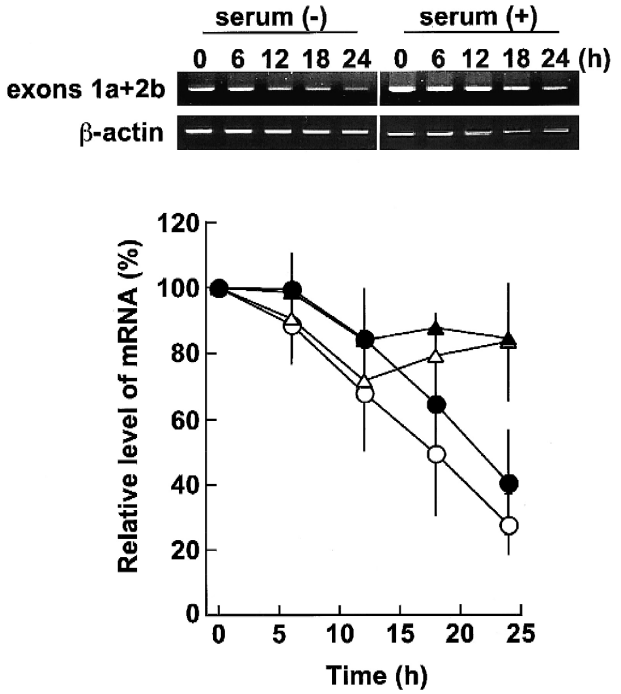

Fig. 6. Stability of transcript containing exons 1a + 2b. Representative photo and the summary of four independent experiment showing the changes in the expression level of exons 1a + 2b (○, ●) and β-actin (△, ▲) after treatment with 60 μM DRB in the cells unstimulated (○, △) and stimulated with 10% serum (●, ▲). Data are the mean ± S.E.M. ($n = 4$).

ner (Fig. 7A). Significant increases were observed with 3%, 10% and 20% serum and were about 1.5-, 3.5- and 4-fold of that obtained with the serum-deprived cells, respectively. Interestingly, the level of BrdU incorporation obtained with 24 h stimulation with 10% and 20% serum was higher than that obtained with the cells continuously cultured in the presence of the serum. On the other hand, the stimulation with PDB for 24 h had no significant effect on the BrdU incorporation (Fig. 7B).

4. Discussion

The RT–PCR analysis of expression of the α-tropomyosin isoforms revealed rat aorta in situ to express a non-smooth muscle isoform as well as a smooth muscle isoform. This observation was consistent with a report showing that the differentiated smooth muscle tissues express both smooth muscle type and non-smooth muscle type of α-tropomyosin (Fatigati and Murphy, 1984). In the case of gizzard smooth muscle (Kashiwada et al., 1997), in situ gizzard exclusively expresses smooth muscle type of α-tropomyosin, while the cells in primary culture rapidly converted α-tropomyosin isoforms to non-smooth muscle type within 1 day under serum-stimulated culture conditions, and the smooth muscle type disappeared. However, in the present study, the level of exons 1a + 2a in the cells at confluence was similar to those seen in rat aorta tissue, and the cells expressed both smooth muscle type and non-smooth muscle type α-tropomyosin. (Fig. 2). Thus, the rat aortic smooth muscle cells maintained their expression of the smooth muscle isoform of α-tropomyosin in primary culture.

The main finding of the present study is that the up-regulation of the non-smooth muscle isoform of α-tropomyosin took place in accordance with the transition from a quiescent state to a proliferative state of the rat aortic smooth muscle cells in primary culture. Although it is well known that the isoforms of α-tropomyosin alter in a tissue- and developmental-specific manner, there has so

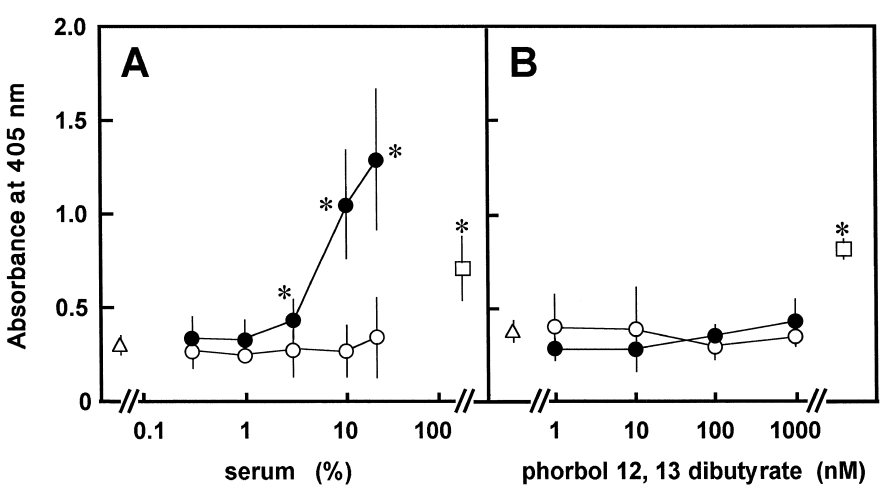

Fig. 7. DNA synthesis of the smooth muscle cells in primary culture during the stimulation with serum and PDB. The serum-deprived cells were stimulated with serum (A) or PBDu (B) for 1.5 (○) and 24 h (●) and labeled with 10 μM BrdU for 1.5 h. The incorporated BrdU was quantitatively determined by an enzyme immunoassay. △, the pre-stimulation levels of the BrdU incorporation obtained with the serum-deprived cells. □, the BrdU incorporation of the cells at confluence that were continuously cultured under normal growth conditions with 10% serum. The data are the mean ± S.D. ($n = 4$–5). *, significantly different ($P < 0.05$) from the level obtained with the serum-deprived unstimulated cells.

far been no report showing any alteration in the α-tropomyosin isoforms during the phenotypic conversion of arterial smooth muscle cells (Owen, 1995). Kashiwada et al. (1997) reported a similar conversion of α-tropomyosin isoforms in visceral smooth muscle cells in primary culture obtained from chicken gizzard.

The up-regulation of non-smooth muscle isoform observed during the progression of cell culture closely correlated with the transient increase in BrdU incorporation, indicating that the up-regulation correlated with the increase in the S phase population of the cell cycle. However, when serum-deprived quiescent cells were stimulated with serum or phorbol ester, the up-regulation of non-smooth muscle isoform of α-tropomyosin dissociated from the increase in DNA synthesis (Fig. 7). The stimulation with serum for 1.5 h did not increase the BrdU incorporation, while 24 h stimulation significantly increased the BrdU incorporation. Furthermore, PDB had no effect on the BrdU incorporation with either 1.5 or 24 h stimulation. This is consistent with the report that the serum-deprived rat aortic smooth muscle cells in the primary culture and subculture took 10–20 h to proceed to the S phase of the cell cycle after stimulation with the growth factors (Thyberg et al., 1990). Thus, these findings suggested that the exons 1a + 2b was up-regulated during G_0/G_1 transition but before the cells entered the S phase. The regulation of the G_0/G_1 transition is fundamentally distinct from that of G_1/S progression. The activation of mitogen activated protein kinase cascades, the up-regulation of cyclin D and down-regulation of cyclin-dependent kinase inhibitors such as $p27^{Kip1}$ are the early events which are considered to be related to the G_0/G_1 transition and the re-entrance into the cell cycle (Nishida and Gotoh, 1993; Sherr, 1993; Sherr and Roberts, 1995). These events are caused by competence growth factors. On the other hand, the activation of phosphatidylinositol 3-kinase and cyclinE and A-dependent kinases are considered to be related to the G_1/S progression and the DNA synthesis (Sherr and Roberts, 1995; Williams, 1989). As a consequence, our study suggests that the mechanism responsible for the up-regulation of exons 1a + 2b are related to those activated during the G_0/G_1 transition. The findings of the present study also indicate that the serum contained both competence and progression factors, while phorbol ester acted as a competence factor to the rat aortic smooth muscle cells in primary culture. These competence factors were thus suggested to play an essential role in the up-regulation of exons 1a + 2b. The competence factors in the serum that are responsible for the up-regulation of exons 1a + 2b of α-tropomyosin, however, remain to be identified.

Most mitogens elicit a cascade of biological responses in smooth muscle cells, including protein phosphorylation (Thyberg et al., 1990). The phosphorylation cascade involves many types of kinases such as protein kinase C, phosphatidylinositol 3-kinase, mitogen activated protein kinase (Hershenson et al., 1997; Kapeller and Cantley, 1994; Scott et al., 1996; Thyberg et al., 1990). Platelet-derived growth factor or fibroblast growth factor have been shown to activate protein kinase C (Williams, 1989). In the present study, the serum-induced increase in the level of exons 1a + 2b expression was completely inhibited by the treatment with H-7. Since it is well known that H-7 is not a specific inhibitor of protein kinase C, the inhibitory effect of H-7 on the up-regulation of exons 1a + 2b may be mediated by the inhibition of kinases other than protein kinase C. However, the effective concentrations of H-7 correlated with those required to inhibit protein kinase C (Hidaka et al., 1984) and phorbol ester mimicked the effect of serum repletion at the concentration range that activates protein kinase C (Nishizuka, 1984). These findings suggest protein kinase C to be the first candidate of kinase that is involved in the up-regulation of exons 1a + 2b. However, the finding that GF 109230X had no effect on the up-regulation of exons 1a + 2b is critical against the involvement of protein kinase C in the serum-induced up-regulation. We therefore suggested that the activation of protein kinase C (such as in case of PDB stimulation) is sufficient to induce up-regulation of exons 1a + 2b. However, the involvement of protein kinase C in the serum-induced up-regulation remains to be established. The involvement of phosphatidylinositol 3-kinase and mitogen activated protein kinase pathways is not likely because wortmannin, PD 98059 and SB 203580 had no significant effect on the up-regulation. As a consequence, protein phosphorylation is involved in the up-regulation of exons 1a + 2b, and the kinase(s) responsible for the up-regulation is considered to be activated during the G_0/G_1 transition. However, the identity of the kinases remains to be elucidated.

Alternative splicing is considered to play an important role in the regulation of the isoform conversion of α-tropomyosin (Lees-Miller and Helfman, 1991; Smith et al., 1989; Wieczorek et al., 1988). The exon 2a is exclusively used in smooth muscle cells, while exon 2b is used in other cell types (Lees-Miller and Helfman, 1991). The mechanism of the regulation of alternative splicing of α-tropomyosin is still under investigation. Several reports have suggested, however, that protein kinase C or related kinases are involved in the post-transcriptional regulation in other cases. The splicing of c-*fos* mRNA was inhibited by treatment with H-7 (Harbers and Hilz, 1991). Regarding human papillomavirus capsid protein L1, phorbol ester was found to increase the level of L1 transcript without increasing the transcriptional activity. It is thus possible that the splice site selection that generates exons 1a + 2b was facilitated in a protein kinase C-dependent manner in the growth-stimulated cells. However, in the present study, the level of exons 1a + 2a did not significantly change while the level of the exons 1a + 2b increased in response to growth stimulation. The alternative splicing may not be a mechanism for the up-regulation. It is also possible that the stability of the transcript containing exons 1a + 2b was specifically inhibited by the mitogenic stimulation, and

thereby caused the up-regulation of exons 1a + 2b. However, this possibility is excluded by the observation shown in Fig. 6. The decay of exons 1a + 2b after DRB treatment under the serum stimulation did not differ from that seen without stimulation. The precise mechanism of isoform conversion in response to growth stimulation remains to be elucidated.

In conclusion, in rat aortic smooth muscle cells in primary culture, the non-smooth muscle isoform of α-tropomyosin increased in the rapidly proliferating cells and also in response to stimulation with serum repletion of the serum-deprived quiescent cells. The present study demonstrated for the first time that the alteration of the α-tropomyosin isoforms occur during the phenotypic conversion of vascular smooth muscles. The up-regulation of non-smooth muscle isoform of α-tropomyosin was suggested to occur during the G_0/G_1 transition before entering the S phase of the cell cycle. It indicates that the competence factors but not progression factors play an essential role in the up-regulation of a non-smooth muscle isoform of α-tropomyosin. A complete inhibition by H-7 and the mimicking effect of phorbol ester suggested that protein kinase C or its related kinase is involved in the up-regulation of non-smooth muscle isoform of α-tropomyosin. Our findings therefore imply that the conversion from a contractile to synthetic phenotype takes place during the G_0/G_1 transition before DNA synthesis is activated.

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