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Mechanism of Down-Regulation of L-Type Ca^{2+} Channel in the Proliferating Smooth Muscle Cells of Rat Aorta

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Abstract The mechanism of down-regulation of L-type Ca^{2+} channel (L-VOC) was investigated in rat aortic smooth muscle cells in primary culture. On culture days 3–5, the cells actively incorporated the 5-bromo-2′-deoxy-uridine (BrdU), and did not respond to K^+ depolarization nor express α_{1C} subunit of L-VOC. At confluence on day 8, BrdU incorporation decreased, and the cells up-regulated α_{1C} subunit mRNA, expressed α_{1C} subunit protein at cell periphery, and responded to K^+ depolarization. Treating the proliferating cells on day 3 with serum-free media or 10 μM PD98059, a MAP kinase kinase inhibitor, for 2 days induced the expression of α_{1C} subunit protein and the responsiveness to K^+ depolarization. However, the serum starvation, but not PD98059, decreased the BrdU incorporation and increased the α_{1C} subunit mRNA. It is concluded that the expression of L-VOC is substantially suppressed in the proliferating cells due to two mechanisms; a MAP kinase-mediated post-transcriptional down-regulation and the transcriptional down-regulation by additional mitogenic signals. J. Cell. Biochem. 87: 242–251, 2002.

Key words: L-type Ca^{2+} channel; smooth muscle cells; mitogen-activated protein kinase; $[Ca^{2+}]_i$

The vascular smooth muscle cells (VSMCs) can be reversibly converted between two distinct phenotypes, referred to as synthetic and contractile phenotypes [Owen, 1995; Thyberg, 1996; Sartore et al., 1999]. The VSMCs in the vasculature of adult animals are considered to demonstrate a differentiated contractile phenotype, and the conversion to a de-differentiated synthetic phenotype is a critical step in the onset of proliferative vascular disease such as artherosclerosis [Owen, 1995]. The extensive interest thus has been focused on the mechanism of phenotypic modulation of VSMCs. The contractile phenotype is characterized by the expression of proteins related to contractile function including smooth muscle α-actin, smooth muscle myosin, smooth muscle α-tropomyosin, and calponin, while the synthetic phenotype is characterized by the loss of these marker proteins [Owen, 1995; Sartore et al., 1999].

The voltage-operated Ca^{2+} channel (VOC) forms a major Ca^{2+} entry pathway in the smooth muscle cells and plays a crucial role in maintaining the smooth muscle contraction in response to depolarization of the plasma membrane in the differentiated VSMCs. Among the six types of VOCs, L- and T-type VOCs (L-VOC and T-VOC, respectively) are the major functional VOCs expressed in the VSMCs [Akaike et al., 1989; Birnbaumer et al., 1994], and P-/Q-type VOC was also reported to be expressed [Hansen et al., 2000]. In rat aortic VSMCs in primary culture, some cells express only one type of VOCs, and

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others express both types in the various ratio [Akaike et al., 1989; Kuga et al., 1990; Hirakawa et al., 1995]. We previously demonstrated that the majority of the non-proliferative cells express only L-type, while the cells in the proliferative state frequently express T-VOC in addition to L-VOC [Akaike et al., 1989; Kuga et al., 1990; Hirakawa et al., 1995]. We also demonstrated the cell cycle-dependent difference in the expression of VOCs [Kuga et al., 1996]. The L-VOC was expressed in all cell cycle phases, while T-VOC was expressed in G_1 and S phases. The cells in G_0 and M phase expressed only L-VOC. Collectively, we concluded that the expression of T-VOC is associated with the proliferative state, while L-VOC is expressed in the cells in the non-proliferative state and down-regulated in the proliferative state [Akaike et al., 1989; Kuga et al., 1996]. Gollasch et al. [1998] demonstrated that the expression of L-VOC was down-regulated in VSMCs of a synthetic phenotype, and thus suggested that L-VOC serves as a differentiation marker for VSMCs. However, the mechanism of down-regulation of the expression of L-VOCs in the proliferating smooth muscle cells still remains to be determined. Such a mechanism may be related to the loss of differentiated phenotype of VSMCs and development of vascular diseases.

In the present study, we focused on the mechanism of down-regulation of the functional expression of L-VOC in the proliferative state. We first elucidated the correlation between the functional expression of L-VOC and a proliferative state of rat aortic VSMCs in primary culture, and then investigated the contribution of mitogenic signals such as mitogen-activated protein kinase (MAPK) cascade and phosphatidylinositol 3-kinase (PI3-K) in the down-regulation of L-VOC. The functional expression of L-VOC was evaluated by the responsiveness to high K^+ depolarization. It is well established that the elevation of the extracellular K^+ concentration depolarizes the membrane potential and causes $[Ca^{2+}]_i$ elevation due to the Ca^{2+} influx through L-VOC [Moreno Davila, 1999]. L-VOC is composed of α_1, α_2/δ, β, and γ subunits, and α_1 subunit is the essential subunit that forms the pore of the channel, dihydropiridine binding sites, and a voltage sensor [Moreno Davila, 1999]. Therefore, its expression were evaluated by immunofluorescence staining and reverse transcription-polymerase chain reaction (RT-PCR). On the other hand, the inhibition of the Ca^{2+}-ATPase and the Ca^{2+} uptake into the intracellular stores by cyclopiazonic acids (CPA) [Seidler et al., 1989] was shown to activate capacitative Ca^{2+} influx and induce $[Ca^{2+}]_i$ elevation [Schilling et al., 1992]. The response to CPA was used to evaluate the functional expression of channels other than VOCs as a control.

MATERIALS AND METHODS

Primary Cell Culture

The study protocol was approved by the Animal Care and Committee of Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University. VSMCs were enzymatically dispersed from the aortic media of male Wistar rats, seeded on 35 mm culture dishes (Nunc, Copenhagen, Denmark) and cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Sanko Junyaku, Tokyo, Japan), 100 U/ml penicillin, 100 μg/ml streptomycin. The growth medium was renewed every 2 days.

Cell Count

VSMCs were harvested by treatment with 2.5 mg/ml trypsin, 0.5 mM EDTA in phosphate-buffered saline (PBS). The cell number was determined on a hemocytometer.

Analysis of DNA Synthesis of the Rat VSMCs

The DNA synthesis of VSMCs was analyzed by incorporation of BrdU in nuclear DNA as previously described [Hirano et al., 2000]. The cells were labeled with 10 μM BrdU in growth media for 24 h. The BrdU incorporation was detected by immunofluorescence staining with monoclonal anti-BrdU antibody and FITC-labeled secondary antibody (Boehringer-Mannheim, Tokyo, Japan). The fraction of BrdU-incorporated nuclei was determined by counting more than 1,000 nuclei on photographs.

Fura-2 Fluorometry in the Rat VSMCs

The change in $[Ca^{2+}]_i$ in VSMCs on 35 mm dish was monitored with a front-surface fluorometer as previously described for the intact vascular strips [Kanaide, 1999; Ihara et al., 2000]. The cells cultured on 35 mm plastic dishes were loaded with Ca^{2+} indicator dye, fura-2, by incubating them in Dulbecco's modified Eagle medium containing 5 μM fura-2

acetoxymethyl ester for 1 h at 37°C [Hirano et al., 1993]. After being loaded with fura-2, the cells were washed and equilibrated in HEPES-buffered saline (HBS) for at least 30 min before starting the measurements. The change in fura-2 fluorescence was monitored with a front-surface fluorometer, CAM-OF3 (JASCO, Tokyo, Japan) [Ihara et al., 2000]. The fluorescence intensities (500 nm) at 340 nm (F340) and 380 nm (F380) excitation and their ratio (Ratio = F340/F380) were continuously monitored. All measurements were performed at 25°C to prevent any leakage or sequestration of the dye [Kobayashi et al., 1986]. At the end of the experimental protocol, the cells were exposed to 25 μM ionomycin in HBS (containing 1 mM Ca^{2+}) to obtain the maximal level of the fluorescence ratio. The fluorescence ratio data were expressed as a percentage, assigning the values at rest and at the maximal response obtained with 25 μM ionomycin to be 0 and 100%, respectively. Ionomycin is a Ca^{2+} ionophore and causes the $[Ca^{2+}]_i$ elevation up to submillimolar level and a resultant saturation of fura-2 signal [Hirano et al., 1990]. Therefore, the ionomycin-induced $[Ca^{2+}]_i$ elevation is considered to be the same among different culture days and treatments. All data were collected using a computerized data acquisition system (Mac Lab, Analog Digital Instruments, Castle Hill, NSW, Australia; Macintosh, Apple Computer, USA.

Immunofluorescence Staining of α_{1C} and α_{1D} Subunits of L-VOC

The cells cultured on 25 mm square glass cover slips (Matsunami, Tokyo, Japan) in 35 mm dishes were fixed in 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then incubated in PBS containing 5% bovine serum albumin at room temperature for 20 min. The α_{1C} and α_{1D} subunits were detected by rabbit polyclonal antibodies (Alomone Labs, Jerusalem, Israel), and a Texas Red-conjugated secondary antibody (ICN, Aurora, OH). The fluorescence images were observed under a fluorescence microscope (Axioskop, Zeiss, Germany) equipped with an objective lens Plan-Apochrmat × 63 (Zeiss), and photographed on Kodak Ektachrome 400 reversal film. The images on films were scanned with a film scanner LS-2000 (Nikon, Tokyo, Japan), and then were processed using the Adobe Photoshop program (Adobe system, San Jose, CA).

RT-PCR Analysis of the Expression of α_1 Subunit mRNA

Total RNA was isolated from cultured VSMCs as previously described [Chomczynski and Sacchi, 1987], and was treated with RNase-free DNase to remove any contaminating genomic DNA. The RT-PCR analysis of the expression of α_{1C} and α_{1D} subunit and β-actin mRNA was performed as previously described [Hirano et al., 2000]. One microgram total RNA was used for 20 μl RT reaction, and 1 μl RT product was subjected to 10 μl PCR. The thermal cycle profile used for amplification of α_{1C} and α_{1D} subunit mRNA was the same as that used for the RT-PCR detection of α-tropomyosin mRNA [Hirano et al., 2000], except for that PCR amplification was performed with 30 cycle. Based on the sequences deposited to the Genbank database with the accession numbers M76516 (rat α_{1C} subunit) and D38101 (rat α_{1D} subunit), we designed the primers within the region of epitopes of anti-α_{1C} and anti-α_{1D} subunit antibodies, respectively, to detect α_{1C} and α_{1D} subunit specifically. The primer used in the RT reaction of α_{1C} subunit was 5′-GTC ATT GAC AAT GCG GTG GCA-3′ (residues 3431–3451), and the primers used in PCR were 5′-ACT GCC AGC CCA GAA AAG AAA-3′ (the sense primer: 3095–3115) and 5′-CAG GCG GAA CCT GTT GTT TGG-3′ (the antisense primer: 3407–3427). The primer used in the RT reaction for α_{1D} subunit was 5′-GTT GAT GAG TTT GTG GCA ACC-3′ (3163–3183), and the primers for PCR were 5′-AAA GAA AGC CTA GAA AAC AAA-3′ (the sense primer: 2868–2878) and 5′-CAC ACG GAT CGG GTT GGT CTT-3′ (the antisense primer: 3142–3162). The primers and conditions for the RT-PCR analysis of β-actin mRNA were all as previously described [Nishimura et al., 1992]. The expected size of the PCR product for α_{1C} subunit, α_{1D} subunit, and β-actin was 333, 294, and 227 bp, respectively. The PCR product was separated on 3% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide. The densities of bands were determined with Gel Plotting Macros of the NIH image ver. 1.61 after obtaining the fluorescence images with a CCD camera (Atto, Tokyo, Japan).

Drugs and Solutions

The composition of PBS was (in mM): 136.9 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.47 KH_2PO_4, pH 7.4. The composition of normal HBS was

(in mM): 135 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 5.5 glucose, and 10 HEPES, pH 7.4 at 25°C. HBS containing high K^+ was prepared by replacing NaCl with equimolar KCl. Fura-2 acetoxymethyl ester was purchased from Dojindo Laboratories (Kumamoto, Japan). Dulbecco's modified Eagle medium was purchased from Life Technologies (Grand Island, NY). CPA, ionomycin, and diltiazem were from Sigma (St. Louis, MO). SKF96365 (1-{β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl}-^{1}H-imidazolehydrochloride) was purchased from Calbiochem (La Jolla, CA). PD98059, 2′-amino-3′-methoxyflavone, was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The oligonucleotides for primers were synthesized by Hokkaido System Sciences (Sapporo, Japan).

Statistical Analysis

Data are expressed as the mean ± SE mean. Student's *t*-test was used to determine statistical significance between the two groups. *P* values of less than 0.05 were considered to be significant.

RESULTS

Changes in the Responsiveness to 100 mM K^+-Depolarization and CPA in Relation to the Culture Days of the Rat Aortic VSMCs in Primary Culture

When VSMCs of the rat aorta were cultured, almost 100% of the cells incorporated BrdU during the 24 h labeling period, and the cell number increased exponentially on days 3–5 (Fig. 1). Thereafter, the cells reached the confluence on day 7 and the BrdU incorporation decreased gradually with the culture day. There was a further decrease in the BrdU incorporation after the cell density reached the confluence ($8.1 \pm 0.6 \times 10^5$ cells/9.6 cm^2), and 8.2 ± 1.2% of the cells incorporated BrdU within 24 h in the normal growth media containing 10% serum on day 11.

The VSMCs in the proliferative state on day 4 scarcely responded to 100 mM K^+ depolarization in any measurement. In contrast, 30 μM CPA caused a large sustained elevation of $[Ca^{2+}]_i$ in the cells on day 4 (Fig. 2a). The $[Ca^{2+}]_i$ reached its peak (38.4 ± 6.9%, n = 4) at 5 min after the application of CPA, and then gradually declined (27.7 ± 1.7% at 20 min and 19.2 ± 1.5% at 40 min, n = 4) (Fig. 2c). On the other hand,

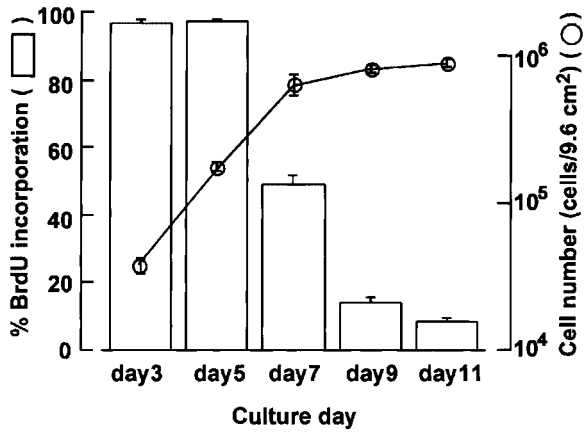

Fig. 1. Changes in the cell number and DNA synthesis in relation to the culture day of the rat aortic smooth muscle cells in primary culture. The cells were seeded on 35 mm culture dish (9.6 cm^2) and cultured in the 10% serum-containing media. Most of the cells on days 3–5 incorporated BrdU, and the culture reached confluence at days 7–8. Data are the mean ± SE mean (n = 4).

the cells on days 8 did respond to 100 mM K^+ depolarization in all measurements (Fig. 2b), and a sustained $[Ca^{2+}]_i$ elevation was observed. The level of $[Ca^{2+}]_i$ (3.9 ± 0.5% of the ionomycin-induced maximal elevation, n = 4) was significantly ($P < 0.05$) higher than the resting level and the level obtained with the cells on day 4 (Fig. 2c). In the cells on day 8, CPA also induced a $[Ca^{2+}]_i$ elevation, however, the levels of $[Ca^{2+}]_i$ elevation were much lower ($P < 0.05$) than those obtained on day 4 (Fig. 2c). In the post-confluent cells on day 12, the responsiveness to 100 mM K^+ and CPA did not significantly ($P > 0.05$) differ from that observed with the cells on day 8 (Fig. 2c). On the other hand, the BrdU incorporation of the cells on day 12 was significantly lower ($P < 0.05$) than that seen with the cells on day 8 (Fig. 1).

The expression of α_1 subunit, a major subunit of L-VOC, was examined by immunofluorescence staining. The cells on day 4 did not show any specific staining with anti-α_{1c} subunit antibody (Fig. 2d). On the other hand, the cells on days 8 and 12 showed the positive staining at the cell periphery (Fig. 2d). We also examined the expression of α_{1D} subunit. However, no specific staining was observed in the rat aortic VSMCs either on day 4 or day 8 (data not shown). The expression of the α_{1c} subunit on the cell membrane thus closely correlated with the responsiveness to 100 mM K^+ depolarization.

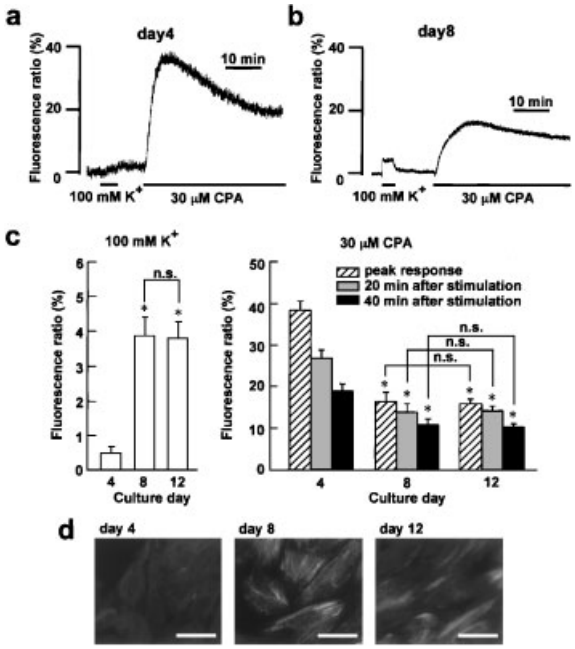

Fig. 2. The responses to K^+ depolarization and cyclopiazonic acid, and the expression of α_{1C} subunit in the rat aortic smooth muscle cells in the proliferative state and at confluence. **a**,**b**: The representative recordings showing changes in $[Ca^{2+}]_i$ induced by 100 mM K^+ and 30 μM cyclopiazonic acid (CPA) in smooth muscle cells on day 4 (a) and day 8 (b). The cells were exposed to 25 μM ionomycin at the end of the measurement (data not shown). The levels of $[Ca^{2+}]_i$ (fluorescence ratio) at rest and at the maximal response obtained with 25 μM ionomycin were designated as 0 and 100%, respectively. **c**: Summary of the responses to 100 mM K^+ and CPA. Data are the mean ± SE mean (n = 4). *, Significantly different from the value obtained with the cells on days 4 ($P < 0.05$); n.s., not significantly different ($P > 0.05$). **d**: Immunofluorescence detection of α_{1C} subunit protein in smooth muscle cells on days 4, 8, and 12. Bar = 50 μm.

Differential Effect of Diltiazem on the CPA-Induced $[Ca^{2+}]_i$ Elevation Based on the Culture Days

We compared the effects of diltiazem, a blocker of VOCs, on the CPA-induced $[Ca^{2+}]_i$ elevation between day 4 and day 8, and determined the involvement of L-VOC in the CPA-induced $[Ca^{2+}]_i$ elevation. Diltiazem (10 μM) was applied 20 min after the stimulation with 30 μM CPA, and its effect was evaluated at 40 min (20 min after the application of diltiazem) (Fig. 3). Diltiazem had no effect on the $[Ca^{2+}]_i$ elevation induced by CPA on day 4 (20.2 ± 1.5% with diltiazem vs. 22.3 ± 1.2% without diltiazem, n = 4; Fig. 3a,c). On the other hand, diltiazem decreased the CPA-induced $[Ca^{2+}]_i$ elevation on days 8 (Fig. 3b,c). The levels of $[Ca^{2+}]_i$ obtained with diltiazem (7.5 ± 0.2%,

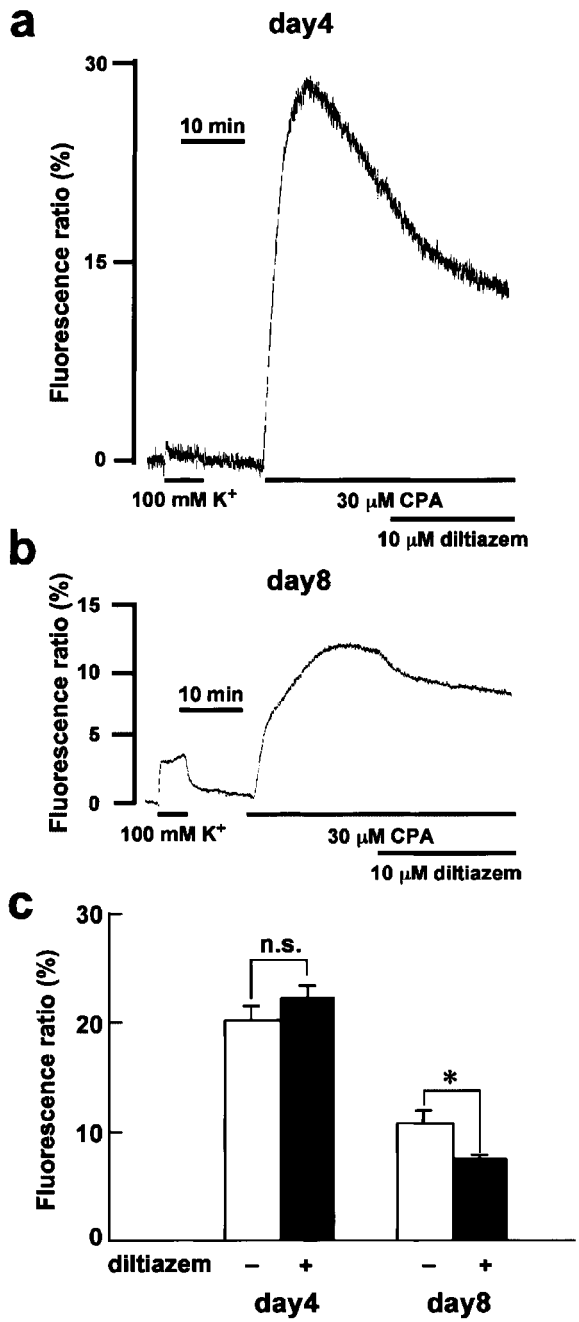

Fig. 3. Effects of diltiazem on the cyclopiazonic acid-induced $[Ca^{2+}]_i$ elevation in the rat aortic smooth muscle cells in the proliferative state and at confluence. **a**,**b**: Representative recordings showing changes in $[Ca^{2+}]_i$ induced by 30 μM cyclopiazonic acid (CPA) and 10 μM diltiazem in the cells on day 4 (a) and day 8 (b). Diltiazem was applied 20 min after the stimulation of CPA. The levels of $[Ca^{2+}]_i$ (fluorescence ratio) at rest and at the maximal response obtained with 25 μM ionomycin were designated as 0 and 100%, respectively. **c**: Summary of the effects of 10 μM diltiazem on the CPA-induced $[Ca^{2+}]_i$ elevation. The $[Ca^{2+}]_i$ level obtained 40 min after stimulation with CPA was compared between the presence and absence of diltiazem. The data are the mean ± SE mean (n = 4). *, Significantly different ($P < 0.05$); n.s., not significantly different ($P > 0.05$).

n = 4) were significantly ($P < 0.05$) lower than that obtained without it ($10.8 \pm 1.3\%$, n = 4). The addition of SKF96365, a Ca^{2+} entry blocker, to diltiazem completely inhibited the CPA-induced $[Ca^{2+}]_i$ elevations both on day 4 and day 8 (data not shown). The pre-treatment of the cells on days 8 with diltiazem for 10 min completely inhibited the $[Ca^{2+}]_i$ elevation induced by the subsequent stimulation with 100 mM K^+ (data not shown).

Up-Regulation of Functional L-VOC by Serum Starvation and PD98059

We examined the effects of serum starvation, PD98059 (an inhibitor of MAPK kinase [Reiners et al., 1998]) and wortmannin (a PI-3K inhibitor [Arcaro and Wymann, 1993]) on the responsiveness to 100 mM K^+ depolarization and the expression of the α_{1C} subunit. The cells on day 3 were cultured in the serum-free media or treated with 10 μM PD98059 or 10 μM wortmannin in the normal growth media for 2 days, and the responsiveness to 100 mM K^+ was evaluated on day 5. When cultured in the normal growth media, 100 mM K^+ did not induce any significant elevation of $[Ca^{2+}]_i$, and no immunoreactive α_{1C} subunit was detected on day 5 (Fig. 4a). On the contrary, the serum-deprived cells demonstrated a significant elevation of $[Ca^{2+}]_i$ in response to 100 mM K^+, and the expression of α_{1C} subunit at the cell periphery on day 5 (Fig. 4b). Similarly, the treatment with 10 μM PD98059 rendered the cells responsive to 100 mM K^+, and induced the expression of α_{1C} subunit. However, the extent of $[Ca^{2+}]_i$ elevation ($10.7 \pm 2.9\%$, n = 4) observed in the PD98059-treated cells was smaller than that observed in the serum-starved cells on day 5 ($18.7 \pm 2.9\%$, n = 4). On the other hand, the 2-day treatment with 10 μM wortmannin did not induce any responsiveness to 100 mM K^+ or expression of α_{1C} subunit on day 5 (Fig. 4d). None of the treatment had effect on the $[Ca^{2+}]_i$ elevation induced by CPA as a whole (Fig. 4e). In the normal growth media, the cell number increased from $3.7 \pm 0.4 \times 10^4$ cells/9.6 cm^2 on day 3 to $14.5 \pm 1.7 \times 10^4$ cells/9.6 cm^2 on day 5 (Table I). The serum starvation completely inhibited an increase in the cell number, while 10 μM PD98059 and 10 μM wortmannin had no effect on cell growth (Table I). In line with these effects on cell growth, the serum starvation markedly inhibited the BrdU incorporation, while PD98059 and wortmannin had no effect

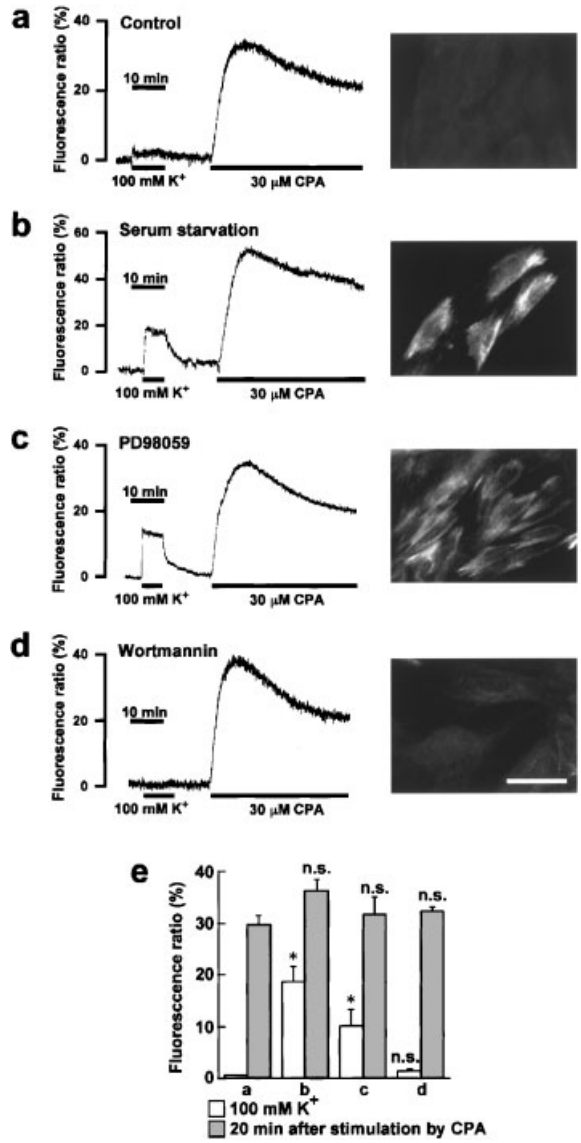

Fig. 4. Effect of serum starvation, PD98059, and wortmannin on the responses to 100 mM K^+ and cyclopiazonic acid and the expression of α_{1C} subunit in the rat aortic smooth muscle cells in primary culture. **a–d**: The representative recordings showing the changes in $[Ca^{2+}]_i$ induced by 100 mM K^+ and 30 μM cyclopiazonic acid, and the representative immunofluorescence staining of α_{1C} subunit in the cells on day 5. The cells were treated with serum-free (b), 10 μM PD98059 (c), and 10 μM wortmannin (d) for 2 days from day 3 through day 5. The control responses to 100 mM K^+ and cyclopiazonic acid and the control immunofluorescence staining was shown in panel a. In photos, bar = 50 μm. The levels of $[Ca^{2+}]_i$ (fluorescence ratio) at rest and at the maximal response obtained with 25 μM ionomycin were designated as 0 and 100%, respectively. **e**: Summary of the $[Ca^{2+}]_i$ elevation induced by 100 mM K^+ and 30 μM cyclopiazonic acid in protocol a, b, c, and d. The responses to 100 mM K^+ and 30 μM cyclopiazonic acid were evaluated at the sustained level and 20 min after the stimulation, respectively. The data are the mean ± SE mean (n = 4). *, Significantly different from the control value obtained with protocol a ($P < 0.05$); n.s., not significantly different from the control value ($P > 0.05$).

TABLE I. Effects of Serum Starvation, PD98059, and Wortmannin on the Proliferative State of the Rat Aortic VSMCs in Primary Culture

	Control	Serum free	PD98059	Wortmannin
Cell number ($\times 10^4$ cells/9.6 cm^2)	14.5 ± 1.7	3.4 ± 0.10^a	15.5 ± 2.9	17.4 ± 3.8
BrdU incorporation (%)	97.2 ± 0.36	19.1 ± 2.5^a	95.8 ± 1.2	91.5 ± 1.4

The cells on day 3 ($3.7 \pm 0.4 \times 10^4$ cells/9.6 cm^2) were treated with either serum-free media or the normal growth media containing 10 μM PD98059 and 10 μM wortmannin for 2 days. The cell number and BrdU incorporation were evaluated on day 5. Data are the mean ± SE mean (n = 4).
[a]Significantly different from control ($P < 0.05$).

on the BrdU incorporation (Table I). As a result, the responsiveness to 100 mM K^+ and the expression of the α_{1C} subunit were associated with the decrease in cell growth in the serum-starved cells, but not in the PD98059-treated cells.

Expression of α_{1C} Subunit mRNA in the Rat VSMCs in Primary Culture

We next examined the effects of serum starvation and PD98059 on the expression of α_{1C} subunit mRNA (Fig. 5). The α_{1C} subunit mRNA was detected by PCR even on day 3, when the cells did not respond to 100 mM K^+ or express α_{1C} subunit protein. The level of α_{1C} subunit mRNA increased with the culture day, resulting in approximately 1.5 fold increase on day 8 (Fig. 5). When the cells on day 3 were incubated for 2 days in the serum-free media, the level of α_{1C} subunit mRNA increased twofold on day 5 (Fig. 5). However, treatment with 10 μM PD98059 for 2 days had no significant effect on the level of the α_{1C} subunit mRNA (Fig. 5).

DISCUSSION

In the present study, we demonstrated that the functional expression of L-VOC was substantially suppressed in the proliferative state of the VSMCs. The density-dependent subsidence of proliferation induced the up-regulation of α_{1C} subunit mRNA and protein, and also caused the responsiveness to high K^+ depolarization. The cessation of the proliferation by serum starvation mimicked the density-dependent growth inhibition. On the other hand, a disruption of the MAPK cascade in the presence of mitogenic stimulations induced the up-regulation of α_{1C} subunit protein and rendered the cells responsive to high K^+, however, it had no effect on the expression of α_{1C} subunit mRNA and proliferative state. These findings suggest that the expression of functional L-VOC

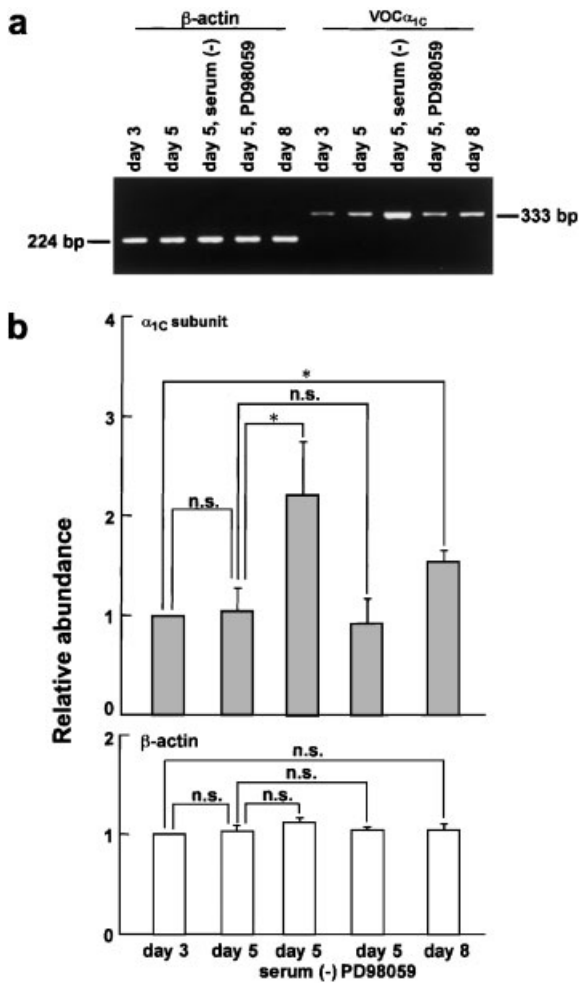

Fig. 5. The effects of culture day, serum starvation, and PD98059 on the expression of α_{1C} subunit mRNA in the rat aortic smooth muscle cells in primary culture. **a**: Representative photos of agarose gel electrophoresis showing the levels of α_{1C} subunit and β-actin mRNA. The expression of α_{1C} subunit and β-actin mRNA was examined on days 3, 5, and 8 in the cells cultured in the 10% serum-containing normal growth media, and on day 5 in the cells treated with serum-free media or 10 μM PD98059 for 2 days from day 3 through day 5. **b**: Summary of the expression levels of α_{1C} subunit and β-actin mRNA under the indicated conditions. The data are the mean ± SE mean (n = 4). *, Significantly different ($P < 0.05$); n.s., not significantly different ($P > 0.05$).

was suppressed at transcriptional and post-transcriptional levels in the proliferative smooth muscle cells. MAPK cascade was involved in the suppression at post-transcriptional level, because the α_{1C} mRNA level was not altered by PD98059 treatment.

The mitogens activate divergent intracellular signals, including Ca^{2+} signal, protein kinase C, MAPK, PI-3K, and src in smooth muscle cells [Williams, 1989; Hershenson et al., 1997]. The intracellular signaling pathways other than MAPK cascade were considered to be involved in the transcriptional suppression. Furthermore, these signaling pathways were suggested to be closely linked to the DNA synthesis and cell proliferation. It is thus conceivable that PD98059 specifically inhibited the MAPK cascade and released the post-transcriptional suppression of α_{1C} subunit, while the serum starvation removed all intracellular mitogenic signaling cascades and thereby induced an up-regulation of the expression of L-VOC at both the mRNA and protein levels. The dual effects of the serum starvation may be linked to the higher responsiveness to high K^+ depolarization than that seen in the PD98059-treated cells.

The present study demonstrated that the MAPK cascade regulates the expression of α_{1C} subunit at the post-transcriptional level. There are many reports showing the involvement of the MAPK cascade in the transcriptional regulation of the gene expression, most of which depend on the serum response element [Hershenson et al., 1997]. In fact, the major downstream effectors of the MAPK cascade include many transcription factors such as Ets, AP-1, Elk-1, and CREB [Whitmarsh and Davis, 1996; Wasylyk et al., 1998; Cobb, 1999]. However, the molecular mechanism of post-transcriptional regulation of the gene by the MAPK cascade is yet to be determined. It is possible that MAPK cascade is involved in the translational suppression of α_{1C} subunit protein [Mordret, 1993; Knauf et al., 2001]. The ribosomal S6 kinases $p90^{RSK}$ is a downstream target of the MAPK cascade [Ahn, 1993], and $p70^{RSK}$ is activated through PI-3K in response to mitogenic stimulation [Thomas, 1992; Peterson and Schreiber, 1998]. However, the phosphorylation of ribosomal protein S6 by the mitogen is linked to up-regulation but not suppression of translation in the mitogen-activated cells [Peterson and Schreiber, 1998; Volarevic and Thomas, 2000]. Recently, MAPK-interacting kinases 1 and 2 have been shown to phosphorylate the initiation factor eIF4E and inhibit cap-dependent translation [Knauf et al., 2001]. Our observation is thus consistent with this report. The expression of the α_1 subunit and the channel activity has been reported to depend on β subunit, although the α_1 subunit is the channel forming subunit and some isoforms are sufficient to form a functional channel by itself [Mori et al., 1991; Beguin et al., 2001]. A recent report demonstrated that the small G protein kir/Gem binds to β subunit and inhibits the translocation of the α_1 subunit to the cell membrane and the functional expression of L-VOC [Beguin et al., 2001]. There is a possibility that the MAPK-dependent suppression of α_{1C} subunit in the proliferating cells is due to the inhibition of translocation. However, no immunofluorescence staining of α_{1C} subunit was observed in any part of the proliferating cells, thus suggesting that the inhibition of the expression of α_{1C} protein on the cell membrane was due to the suppression of synthesis of α_{1C} protein, but not just due to translocation failure.

In the present study, we found that diltiazem inhibited the CPA-induced $[Ca^{2+}]_i$ elevation in the cells on day 8 that were responsive to high K^+ depolarization, but not in the cells on day 4 that were unresponsive to high K^+ depolarization. As a result, the L-VOC-mediated Ca^{2+} influx was suggested to contribute to the $[Ca^{2+}]_i$ elevation induced by CPA when the cells expressed the functional L-VOC. CPA is considered to cause a depletion of the intracellular Ca^{2+} stores by inhibiting Ca^{2+} pump, which in turn activates the capacitative Ca^{2+} entry [Thastrup et al., 1990; Higuchi et al., 1996]. Although the mechanism of activation of the capacitative Ca^{2+} entry pathways by the store depletion remain to be clarified, it is clear from the present study that L-VOC can be activated following the store depletion and contribute in part to the capacitative Ca^{2+} entry in the cells expressing L-VOC.

In conclusion, the functional expression of VOC was found to be substantially suppressed in the proliferative state of the VSMCs. The inhibition of MAPK cascade up-regulated the level of α_{1C} subunit protein and the functional expression of L-VOC. The total removal of mitogenic signals caused up-regulation of α_{1C} subunit at both protein and mRNA level, and the functional expression of L-VOC. These findings suggest that MAPK cascade down-regulates

L-VOC at the post-transcriptional level, and that other signaling pathways down-regulates L-VOC at the transcriptional level. The signals involved in the transcriptional down-regulation of α_{1C} subunit are therefore considered to be closely linked to those involved in the cell growth.

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