Contractile Responses and Myosin Phosphorylation in Reconstituted Fibers of Smooth Muscle Cells From the Rat Cerebral Artery

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ABSTRACT—String-shaped reconstituted smooth muscle fibers were prepared in rectangular wells by thermal gelation of a mixed solution of collagen and cultured smooth muscle cells derived from the rat cerebral artery. The fibers contracted in response to KCl, 5-hydroxytryptamine (5-HT), noradrenaline, endothelin-1, endothelin-2, angiotensin II, prostaglandin F₂α and prostaglandin E₂. 5-HT-induced contraction was partially inhibited by the L-type voltage-dependent Ca²⁺ channel inhibitor nifedipine, putative non-selective cationic channel inhibitor SKF96365 and intracellular Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM), and completely abolished by the myosin light chain kinase inhibitor ML-9. The fibers pre-contracted by 5-HT were completely relaxed by the Rho kinase inhibitor Y-27632, serine/threonine kinase inhibitor staurosporine, 8-bromo cyclic GMP and papaverine, and partially relaxed by dibutyryl cyclic AMP. Moreover, 5-HT as well as endothelin-1 and KCl enhanced 20-kDa myosin light chain phosphorylation in the fibers. These results suggested that the characteristics of contraction of the fibers reflect typical contractilities of vascular smooth muscle tissues. This technique will allow us to directly address questions relating to heterogeneity of receptor mechanisms and intracellular pathways of vascular smooth muscle contraction as a function of vessel type.

Keywords: Cultured smooth muscle cell, Middle cerebral artery, Collagen gel fiber, Isometric force, Contractility

Studies of the stimulus-contraction coupling of smooth muscle have largely been performed in intact tissues. Although there is a considerable body of information about the functions of these tissues, results obtained from intact tissue often result from the complex organization of smooth muscle cells into a multicellular unit rather than the properties of the contractile cells themselves. By isolating single smooth muscle cells from the smooth muscle layer of a blood vessel, it is possible to study signal transduction mechanisms in these cells without the need to consider indirect effects resulting from actions on other cell types. Isolated cells also have the advantage of being more amenable to experimental techniques that can directly address questions relating to receptor mechanisms and intracellular pathways.

Several investigators have investigated the physiology of smooth muscle utilizing isolated smooth muscle cells and obtained new insights into smooth muscle contraction. The major disadvantage of using isolated smooth muscle cells is that it is almost never possible to provide a constant source of the relatively large amounts of biological material that is often needed for biochemical or molecular studies. Smooth muscle cells in primary culture are good experimental models for the study of stimulus-contraction coupling at the cellular level. It has been shown that smooth muscle cells derived from a variety of blood vessels and species can be grown under conditions in which they continue to express smooth muscle contractile proteins (1–3). In most of these cases, smooth muscle cell contraction was assessed on cells grown on deformable substrates rather than directly on non-deformable plastic dishes, thus permitting cell shortening. The magnitude of cellular force in these experiments was inferred from the degree of wrinkling of the substrates. Using cultured smooth muscle cells from the guinea pig stomach, we have recently developed a method of reconstituting hybrid smooth muscle fibers that retain responsiveness to typical contractile agonists to produce isometric contraction (6). In our system, force can be measured
directly and quantitatively. This technique enables us to make direct observations of the reactivity of cultured smooth muscle cells from various cerebrovascular vessels. The majority of in vitro pharmacological studies have been carried out with large vessels in a viable form. The techniques for isolating and recording small arteries from the cerebrovascular bed are still somewhat limited and restricted to pial arteries. The available evidence indicates variable smooth muscle characteristics in different arterial branches (7, 8), suggesting that the pharmacology of specific, large arteries such as the basilar artery cannot be extrapolated to the cerebral circulation in general. The present study was, therefore, performed to explore the possible application of our technique for reconstituting smooth muscle fibers to investigation of heterogeneity of cerebrovascular responses.

MATERIALS AND METHODS

Cell isolation and culture

All procedures using animals were performed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science and were approved by the Institutional Animal Use and Care Committee at Meiji Pharmaceutical University. Male Wistar-Kyoto rats (200 – 300 g) were anesthetized with pentobarbital (35 mg/kg, i.v.) and exsanguinated. Middle cerebral arteries with diameters in the range of 200 – 300 μm were surgically isolated from the lateral cerebral hemispheric surface of the rat brain under a dissection microscope as described by Hill et al. (7). Smooth muscle cells were isolated by the method described previously for guinea pig stomach (9). The viability of isolated single cells, measured by trypan blue exclusion, was more than 90%. Isolated smooth muscle cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, high glucose) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 10% fetal bovine serum (FBS) on plastic culture dishes. Spindle-shaped smooth muscle cells attached and spread within the first 3 days after seeding and began to proliferate. When cultures reached confluence, cells were subcultured using 0.05% trypsin for dissociation. One of the clones, RcSM-924B, was obtained after the cloning of smooth muscle cells using cloning rings. RcSM-924B cells were passaged twice a week, and cultures up to 30 passages were used in this study. The smooth muscle cells in culture extended laterally and showed a highly flattened configuration. They proliferated with a doubling time of approximately 3.1 days through at least 30 passages. Based on the results of immunocytochemical analyses of RcSM-924B cells passaged 10 times, more than 95% of cells in culture expressed differentiated smooth muscle cell marker proteins including SM-1 and SM-2 myosin heavy chains, α-smooth muscle actin and smooth muscle calponin (data not shown). RT-PCR experiments also indicated the presence of mRNAs for differentiated smooth muscle cell proteins such as SM-22 and α-smooth muscle actin. They also expressed SMemb and nonmuscle myosin heavy chains, which are marker proteins for developing (immature) smooth muscle cells (2, 10, 11). These results indicated that RcSM-924B cells were smooth muscle cells. There were no significant differences in the expression of these marker proteins in cell cultures after 10 or 30 passages (data not shown). These results suggested that RcSM-924B cells continue to express some differentiated smooth muscle cell marker proteins after multiple passages in cell culture.

Antibodies

The anti-α-smooth muscle actin antibody was a monoclonal antibody raised against a synthetic NH2 terminus decapetide of the smooth muscle α-isofrom of actin (Progen Biotechnik GmbH, Heidelberg, Germany). The anticalponin antibody was an IgG1 monoclonal antibody (clone hCP) specific for smooth muscle calponin (Sigma Chemical Co., St. Louis, MO, USA). Anti-SM-1 (clone 1C10), SM-2 (clone 2B8) and SMemb (clone 3H2) were monoclonal antibodies that specifically recognized the isoforms of SM-1, SM-2 and SMemb smooth muscle myosin heavy chains, respectively (Yamasa Co., Tokyo). The anti-MLC20 was a monoclonal antibody (clone MY-21) specific for smooth muscle 20-kDa myosin light chains (Sigma Chemical Co.). Biotinylated anti-mouse IgG secondary antibody raised in a horse was obtained from Vector Labs., Inc. (Barlingame, CA, USA).

Immunocytochemistry

 Cultures grown on glass coverslips were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4·7H2O and 1.4 mM KH2PO4) for 3 min, fixed in 4% paraformaldehyde for 15 min, and then incubated in PBS containing 0.5% Triton X-100 for 15 min at room temperature (RT). After fixation, cells were stained with anti-α-smooth muscle actin (1:10), anti-SMemb (1:3,000), anti-SM-1 (1:3,000), anti-SM-2 (1:400) or anti-calponin antibody diluted in PBS containing 2% horse serum for 60 min at RT. The cells were then incubated for 60 min at RT with biotinylated horse anti-mouse IgG secondary antibody diluted 1:500 in PBS followed by a second 60 min incubation in an avidin-biotin solution (Vectastain ABC Elite kit, Vector Labs., Inc.). Both incubations were preceded by thorough rinses with PBS. The reaction products were visualized with 3’-3’ diaminobenzidine and hydrogen peroxide (DAB kit, Sigma Chemical Co.). Cells were then washed three times with PBS, and the slides were mounted in Permount. Cells were viewed and photographed on photomicroscope (Olympus, Tokyo).
Preparation of reconstituted smooth muscle fibers

String-shaped reconstituted smooth muscle fibers were prepared in rectangular wells as described previously (6). Briefly, RcSM-924B cells (cultures from 10 to 30 passages were used unless otherwise indicated) were suspended in ice-cold collagen solution containing 3 × 10⁶ cells/ml cultured cells, 2.2 mg/ml collagen type I-A and 0.24 mg/ml collagen type IV in DMEM. Aliquots (2 ml) of the collagen-cell suspension were poured into rectangular wells (0.8 × 5.0 × 0.5 cm deep) with two poles placed 4 cm apart on the bottom of each well, and placed in a CO₂ incubator (humidified 5% CO₂ / 95% air atmosphere) at 37°C. The collagen gel suspension gelled within 30 min. After 30 min, 15 ml of DMEM was added to each Petri dish. The preparations were incubated until the cells shrank the gel and formed a string-shaped fiber.

Measurement of isometric force

Smooth muscle fibers prepared as described above were cut into two pieces (20 mm each length) and mounted vertically in a 10-ml organ bath containing normal HEPES-buffered Tyrode’s solution, pH 7.4, at 37°C. The fibers were equilibrated in the same medium for 1 h at a resting tension of 5 mN. The tension development was recorded isometrically with a force displacement transducer (TB-612T; Nihon Kohden, Tokyo). Contractile studies were performed by adding various chemical agents to the final desired concentration or by replacing the medium with potassium-rich solution. In some experiments to observe Ca²⁺-dependence of contraction, Ca²⁺-free HEPES-buffered Tyrode’s solution was also used as bathing solution. The normal solution had the following composition: 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5.6 mM glucose and 4.2 mM HEPES (pH 7.4 at 37°C). The Ca²⁺-free solution had the same composition as normal solution except that CaCl₂ was omitted. BAPTA-AM (40 μM) and BAPTA (1 mM) were added to the Ca²⁺-free solution for chelation of cytosolic and extracellular Ca²⁺, respectively. To prepare potassium-rich solutions, equimolar amounts of NaCl were replaced with KCl.

Myosin light chain phosphorylation assay

The 20-kDa myosin light chain (MLC20) phosphorylation assay using urea-glycerol PAGE coupled with Western blotting was performed essentially as described previously (12, 13). Briefly, after stimulation with 10 nM endothelin-1 (ET-1), 1 μM 5-hydroxytryptamine (5-HT) or 80 mM KCl for 2 min, the fibers were flash-frozen with dry ice-cold acetone containing 10% TCA and 10 mM DTT; and then they were allowed to warm to RT in the same solution. TCA was washed out with acetone-DTT and the fibers were dried. The dried fiber was mixed with urea-sample buffer (20 mM Tris, 22 mM glycine, 10 mM DTT, 8 M deionized urea and 0.1% bromphenol blue) and allowed to stand for 1 h at RT. The samples were filtered through a 0.45-μM membrane filter (Millipore Corp., Bedford, MA, USA), and the filtrates were subjected to urea-glycerol PAGE. Proteins were transferred onto nitrocellulose membranes and Western blotting was performed with a monoclonal anti-MLC20 antibody at 1:200 dilution. Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody at 1:4000 dilution and the immunoreactive proteins were visualized on x-ray film with an enhanced chemiluminescence detection system (ECL Western Blotting Detection System; Amersham Pharmacia Biotech, Buckinghamshire, UK). Quantitative analysis of MLC20 phosphorylation was carried out by densitometry of Western blots using macros for the NIH Image program (version 1.62; NIH, Bethesda, MD, USA).

Immunocytochemistry on sectioned fibers

The gel matrix including cultured smooth muscle cells was fixed with freshly prepared 4% paraformaldehyde in PBS for at least 3 h at 4°C followed by cryoprotection in 30% sucrose in PBS at 4°C for 24–48 h. The gels were immersed in Tissue-Tek O.C.T. (Miles Inc., Elkhart, IN, USA) and frozen in liquid nitrogen. Longitudinal sections (10 μm) were cut using a cryostat. The sections were then subjected to immunostaining.

RT-PCR analysis

Total RNA was isolated from the reconstituted fibers (approx. 15 mm in total length) using an Isogen RNA purification kit (Nippon Gene, Tokyo) according to the method supplied by the manufacturer. Aliquots of 1 μg of total RNA were reverse transcribed using the Superscript premultiplication system (Life Tech., Inc., Gaithersburg, MD, USA) with oligo (dT)₁₂ – ₁₈ primers (20 μg/ml) in 25-μl reaction mixtures according to the method supplied by the manufacturer. PCR reactions were performed in 25-μl reaction mixtures using a Perkin-Elmer Thermocycler (Model 9600). An aliquot of the cDNA derived from 50 ng total RNA was subjected to PCR amplification with primer sets specific to the gene of interest. Thirty-five PCR cycles were performed to achieve amplification of the mRNA of interest with typical cycle parameters of 30 s at 94°C, 30 s at an appropriate annealing temperature, and 60 s at 72°C with 7 min of 94°C treatment before starting thermal cycles. PCR amplification was followed by a final extension step of 7 min at 72°C. The reaction mixtures contained cDNA template, 0.4 μM each of 5’- and 3’-primers, 200 μM each of the four dNTPs and 1 U of Taq polymerase (Takara Co., Kyoto), in a buffer containing 10 mM Tris-HCl, 50 mM KCl and 1.5 mM MgCl₂. To ensure that the PCR signals detected were not caused by amplification of genomic DNA, control RT-PCR experiments were performed in
which cDNA was synthesized without reverse transcriptase. Primers for \( \beta \)-actin were used as an internal control to confirm that the amount of input RNA was the same for each sample. The oligonucleotide primers used are listed in Table 1. PCR products were resolved in 2% agarose gels stained with 0.5 \( \mu \)g/ml ethidium bromide. The PCR products were visualized by ultraviolet transillumination and photographed using 667 Polaroid film.

| Table 1. PCR primers |
|----------------------|
| **Sequence (5'-3')** | **Product size (bp)** | **Annealing temperature (°C)** | **Reference** |
| \( \beta \)-Actin sense | AAGCTCAACACCCCAGCCATGTACG | 703 | 54 | 29 |
| \( \beta \)-Actin antisense | CTGATGACTCACCTGCTGGGAAAGTGG | | | |
| \( \alpha \)-Smooth muscle actin sense | GATCACCATCGGGAATGAACGC | 389 | 57.5 | 30 |
| \( \alpha \)-Smooth muscle actin antisense | CTTAGAAGCATTTGCGGTGGAC | | | |
| SM-22 sense | TGTGCGAGCTTTGAGGATC | 369 | 54.9 | 31 |
| SM-22 antisense | GTGATACCTCAAAGACTGTC | | | |
| L-type Ca\(^{2+}\) channel sense | CAGAGTTGCTACATACATCGT | 437 | 57 | 32 |
| L-type Ca\(^{2+}\) channel antisense | ATTCCAGGCTTTCCAAAATCTGC | | | |
| N-type Ca\(^{2+}\) channel sense | AAGAAAGTGTTGACACATCTG | 671 | 57 | 32 |
| N-type Ca\(^{2+}\) channel antisense | CAGCATCAGCTGTACCTGAAG | | | |
| Endothelin ET\(_{x}\) receptor sense | TTCGTCATGTTACCATCGA | 546 | 54 | 33 |
| Endothelin ET\(_{x}\) receptor antisense | GATACTCGTTCCATACATGG | | | |
| Histamine H\(_{1}\) receptor sense | AAAAGGCCATCAAGAGACCC | 350 | 55.6 | 34 |
| Histamine H\(_{1}\) receptor antisense | CTTTCAGATTGACTCTGC | | | |
| Serotonin 5-HT\(_{1A}\) receptor sense | CCAAAGAGCACCTCCATCTCG | 388 | 54.8 | 35 |
| Serotonin 5-HT\(_{1B}\) receptor sense | CCAAAGAGCACCTCCATCTCA | 203 | 53.6 | 36 |
| Serotonin 5-HT\(_{1D}\) receptor sense | GCGATTCCATGAGGACCT | 367 | 56.4 | 36 |
| Serotonin 5-HT\(_{1E}\) receptor sense | AACAGAGGATAAAGTTGCTC | 198 | 56 | 37 |
| Serotonin 5-HT\(_{1F}\) receptor sense | AGCCGGCTTCAACTCGAG | 410 | 55.6 | 38 |
| Serotonin 5-HT\(_{2A}\) receptor sense | AGCCGGCTTCAACTCGAG | 410 | 55.6 | 38 |
| Serotonin 5-HT\(_{2B}\) receptor sense | ATAGCCATCAAAAAGCCAA | 318 | 53.3 | 39 |
| Serotonin 5-HT\(_{2C}\) receptor sense | GTGATGCTGCTTCAACTCGAG | 170 | 51.6 | 40 |
| Serotonin 5-HT\(_{2D}\) receptor sense | GTGATGCTGCTTCAACTCGAG | 249 | 53.9 | 41 |
| Serotonin 5-HT\(_{3}\) receptor sense | CTGATGCTGCTTCAACTCGAG | 285 | 51.2 | 42 |
| Serotonin 5-HT\(_{4}\) receptor sense | GCAGAAGGGATAAAGTTGCTC | 115 | 50.2 | 43 |
| Serotonin 5-HT\(_{5A}\) receptor sense | GCAGAAGGGATAAAGTTGCTC | 115 | 50.2 | 43 |
| Serotonin 5-HT\(_{5B}\) receptor sense | GCAGAAGGGATAAAGTTGCTC | 115 | 50.2 | 43 |
| Serotonin 5-HT\(_{6}\) receptor sense | GCAGAAGGGATAAAGTTGCTC | 115 | 50.2 | 43 |
| Serotonin 5-HT\(_{7}\) receptor sense | GCAGAAGGGATAAAGTTGCTC | 115 | 50.2 | 43 |
**Drugs used**

Collagen type I-A and IV were purchased from Nitta Gelatin Co. (Osaka); noradrenaline (NA), 5-HT, histamine, dopamine, BAPTA-AM, nifedipine and papaverine hydrochloride were from Wako Pure Chemicals (Osaka); ML-9, staurosporine, prostaglandin F_{2α}, prostaglandin E_{1}, prostaglandin E_{2}, dibutyryl cyclic AMP (db-cAMP) and 8-bromo cyclic GMP (8-Br-cGMP) were from Sigma Chemical Co.; DMEM was from Gibco BRL (Gaithersburg, MD, USA). Y-27632 was a generous gift from Yoshitomi Pharmaceutical Industries Ltd. (Osaka). All other chemicals were of reagent grade.

**Data analyses**

All data are presented as means ± S.E.M. Standard analysis of variance and unpaired *t*-tests were used to assess differences.

**RESULTS**

**Reconstitution of smooth muscle fiber**

A collagen gel containing $3 \times 10^6$ cells/ml RcsM-924B cells was prepared. The collagen-cell suspension gelled within 30 min after the inoculated collagen-cell suspension was placed in a CO_{2} incubator. By 1 day after casting, smooth muscle cells began to contract the gel from an initial thickness of 5.0 mm to about 1.4 mm in diameter and maintained this string shape for over 2 weeks. We examined the expression of smooth muscle-specific proteins in the fibers by immunocytochemistry and RT-PCR. Immunocytochemical analyses revealed that RcsSM-924B cells in 7-days incubated fibers were immunoreactive for smooth muscle-specific marker proteins such as α-smooth muscle actin, SM-1, SM-2 and SMemb myosin heavy chains, and smooth muscle calponin (Fig. 1). RT-PCR also indicated the presence of mRNAs for smooth muscle-specific marker proteins such as SM-22 and α-smooth muscle actin (Fig. 2). Immunostaining of a fiber section for α-smooth muscle actin revealed that the smooth muscle cells in the fiber exhibited elongated bipolar spindle shapes and were oriented parallel to the direction of the isometric axis (Fig. 1).

**Contractile responses to KCl-induced depolarization**

After 7 days of incubation in a CO_{2} incubator at 37°C, isometric contractions of the fibers were studied. Figure 3A shows a representative contractile response to 80 mM KCl-induced depolarization. KCl (80 mM) caused a sustained increase in tension (Fig. 3A) with the force value of $1.74 \pm 0.21$ mN (n = 4). KCl-induced contraction was observed at a concentration of 20 mM, and increasing the KCl concentration up to 120 mM increased the tension (Fig. 3B). The maximal force observed at 120 mM KCl was $1.86 \pm 0.22$ mN (n = 4), which was 2.1-fold higher than that for the smooth muscle cells of guinea pig fundus (140 mM KCl) reported previously by our group (6). The KCl-induced contraction is considered to result from depolarization of smooth muscle membranes and an increase in transmembrane influx of Ca^{2+} via voltage-dependent Ca^{2+} channels. The L-type voltage-dependent Ca^{2+} channel inhibitor nifedipine (2 μM) significantly inhibited the contraction (Fig. 3B). RT-PCR analysis of total RNA isolated from the smooth muscle fibers using sequence-specific oligonucleotide primers designed with reference to the published sequence of the rat voltage-dependent Ca^{2+} channel α-subunit gene revealed the presence of L-type voltage-dependent Ca^{2+} channel mRNA in the fibers (Fig. 2). These results strongly suggested that Ca^{2+} entry from extracellular sources, primarily through nifedipine-sensitive L-type voltage-dependent Ca^{2+} channels causes the observed increase in contractile force.

**Agonist-stimulated contraction**

Figure 4A shows representative contractile responses of the fiber to 5-HT and ET-1. 5-HT at a concentration of 1 μM caused a sustained increase in tension followed by a gradual decrease. Cumulative dose-response curves indicated that the maximal tension induced by 5-HT (100 μM) was $1.82 \pm 0.14$ mN (n = 24) (Fig. 4Ba). ET-1 (10 nM) induced a contraction of similar magnitude ($1.86 \pm 0.16$ mN, n = 8) (Fig. 4Bb). Endothelin-2 (ET-2, 10 nM) also caused contraction with a force value of $1.72 \pm 0.47$ mN (n = 4). Prostaglandin F_{2α} at a concentration of 1 μM caused contraction with a force value of $3.65 \pm 0.18$ mN (n = 4). Prostaglandin E_{2} was less effective than prostaglandin F_{2α}. Prostaglandin E_{1} had little effect. Angiotensin II also caused contraction with a maximal force value of $0.92 \pm 0.06$ mN at a concentration of 1 μM. Noradrenaline at a concentration of 100 μM caused contraction with a maximal force value of $1.11 \pm 0.12$ mN (n = 14). Histamine and dopamine showed virtually no effect.

**Expression of endothelin receptor and 5-HT receptor subtypes**

To clarify which endothelin receptor was expressed in the fibers, RT-PCR was performed for endothelin ET_{A} and ET_{B} receptor subtypes using total RNA samples from 7-day-incubated smooth muscle fibers. As shown in Fig. 5, a PCR product amplified with the ET_{A} subtype-specific primers with the predicted size of 546 bp was observed. A minor product amplified with the ET_{B} subtype primers with the predicted size of 475 bp was also observed. Next, RT-PCR for 5-HT receptor subtypes was performed to clarify which 5-HT receptor was expressed in the fibers. PCR products amplified with 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{3A} and 5-HT_{7} subtype primers
Fig. 1. Immunostaining of longitudinal sections of smooth muscle fibers for smooth muscle-specific proteins. Expression of several smooth muscle-specific proteins in 7-day incubated fibers was examined by immunostaining of longitudinal sections of the fibers with monoclonal antibodies against $\alpha$-smooth muscle actin (A), SM-1 (B), SM-2 (C), SMemb (D) and calponin (E). Scale bars represent 100 $\mu$m.
were observed. No predicted bands were observed with 5-HT<sub>2B</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3</sub>, 5-HT<sub>4</sub>, 5-HT<sub>5B</sub> or 5-HT<sub>6</sub> subtype primers.

**Contractile properties of agonist-induced contraction**

To clarify whether the characteristics of contraction of these fibers reflect typical contractilities of natural smooth muscle tissues, we studied the effects of a myosin light chain kinase (MLCK) inhibitor, Rho kinase inhibitor, serine/threonine kinase inhibitor, db-cAMP, 8-Br-cGMP and papaverine on 5-HT-induced contraction. 5-HT-induced contraction was completely inhibited by the pretreatment of the MLCK inhibitor ML-9 (30 μM), suggesting the involvement of MLCK (Fig. 6A). The addition of the Rho kinase inhibitor Y-27632 (1 μM) (14) or the serine/threonine kinase inhibitor staurosporine (300 nM) caused complete relaxation of the fibers pre-contracted by 5-HT (Fig. 6B and C). The fibers pre-contracted by 5-HT were also completely relaxed by addition of 10 μM papaverine (Fig. 7A) or 100 μM 8-Br-cGMP (Fig. 7C), and they were partially relaxed by 100 μM db-cAMP (Fig. 7B), indicating that the cells in the fibers exhibit relaxation responses to papaverine, cGMP and cAMP.

We next examined the Ca<sup>2+</sup>-dependence of agonist-induced contractions (Fig. 8A). These experiments were performed in Ca<sup>2+</sup>-free HEPES-buffered Tyrode’s solution. When the external solution was changed to Ca<sup>2+</sup>-free solution (with 1 mM BAPTA) and 5-HT was cumulatively added, the maximal force induced by 10 μM 5-HT was decreased from 1.64 ± 0.20 to 1.22 ± 0.11 mN (n = 6) (Fig. 8Aa). 5-HT-induced contraction in the presence of Ca<sup>2+</sup> was inhibited by the voltage-dependent Ca<sup>2+</sup> channel inhibitor 2 μM nifedipine (Fig. 8Ba) and 30 μM SKF96365 (Fig. 8Bb). These results suggested that contraction was accompanied by Ca<sup>2+</sup> influx. This was supported by the

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**Fig. 2.** Expression of several smooth muscle-specific proteins in the smooth muscle fibers was examined by RT-PCR. Total RNAs extracted from the smooth muscle fibers were subjected to RT-PCR analysis using primers specific for α-smooth muscle actin (aAC), SM-22 (SM22), L-type (LC) and N-type (NC) Ca<sup>2+</sup> channels and β-actin (bAC). The PCR products were resolved by electrophoresis on 2% agarose gels prestained with ethidium bromide. No signals were detected when samples had not been reverse-transcribed. The PCR primers used are shown in Table 1. The number of PCR cycles was ×35. LM: Molecular weight size markers (100 bp DNA ladder). RT-PCR analysis indicated that the fibers expressed SM-22, α-smooth muscle actin and L-type Ca<sup>2+</sup> channels.

**Fig. 3.** Contractile responses of smooth muscle fibers to KCl. A: Representative tracing showing contractile responses of smooth muscle fibers to 80 mM KCl. Tension development was recorded isometrically with a force displacement transducer. Contractions were induced by replacing the medium with potassium-rich solution (80 mM KCl). W, wash. B: Effects of nifedipine on KCl-induced contraction. The fiber was incubated for 20 min in normal solution (hatched column) or absence (open column) of nifedipine (2 μM) and contractions were induced by replacing the medium with potassium-rich solution (20–140 mM KCl). Data points and error bars are means ± S.E.M. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.005, compared with controls.
observation that the calcium ionophore A23187 (5 \mu M) evoked a small but significant increase in tension (0.65 \pm 0.10 mN, n = 4). Similar results were also obtained with ET-1 as an agonist (Fig. 8Ab). When the fiber was pretreated with 40 \mu M BAPTA-AM for 15 min in Ca\textsuperscript{2+}-free solution, contractions induced by 5-HT or ET-1 were decreased further (Fig. 8A: a, b), indicating that the intracellular Ca\textsuperscript{2+} chelator BAPTA-AM is able to partially inhibit those parts of the contraction that are independent of extracellular Ca\textsuperscript{2+}.

**MLC phosphorylation**

To examine whether the contraction of the fibers is accompanied by phosphorylation of MLC20, we performed Western blotting analysis for MLC20 phosphorylation (Fig. 9). The basal phosphorylation as expressed by (monophosphorylated MLC20 + diphosphorylated MLC20) / total MLC20 in the absence of agonist was 19.3 \pm 2.4\% (n = 6) (Fig. 9A, lane 1). Stimulation of fibers with ET-1 (10 \text{nM}), 5-HT (1 \mu M) and KCl (80 mM) for 2 min enhanced the extent of MLC20 phosphorylation to 57.9 \pm 5.1 (n = 3), 47.3 \pm 2.5 (n = 4) and 40.8 \pm 2.5\% (n = 4), respectively (Fig. 9A: lanes 2–4). As shown in Fig. 9, A and B, time course experiments of KCl-induced MLC20 phosphorylation revealed that the extent of MLC20 phosphorylation was maximal at 2 min after stimulation and declined to the basal phosphorylation level at 10 min. It is noteworthy that the phosphorylated MLC20 was almost extensively monophosphorylated. Pretreatment of the fibers with 30 \mu M ML-9 inhibited 5-HT-induced MLC20 phosphorylation to 42.5\% of the control level (Fig. 8C). Pretreatment of the fibers with 2 \mu M nifedipine inhibited KCl-induced MLC20 phosphorylation to 24.7\% of the control level (Fig. 8C).

**DISCUSSION**

The present results are the first quantitative measurements of contractions produced by cultured cerebrovas-
Fig. 5. Expression of receptor subtypes for endothelin, 5-HT and histamine in the smooth muscle fibers was examined by RT-PCR. Total RNAs extracted from the smooth muscle fibers were subjected to RT-PCR analysis using primers specific for endothelin receptor subtypes ET-A and ET-B, 5-HT receptor subtypes (type 1A – F, 2A – C, 3, 4, 5A, 5B, 6 and 7) and histamine receptor subtypes H₁ and H₂. The PCR primers used are shown in Table 1. The number of PCR cycles was ×35. LM, 100 bp DNA ladder; EM, λ-EcoT14 I/Bgl II digest.
Contraction of Cerebrovascular Fiber

The results of the present study suggested that the characteristics of contractions reflect typical contractilities of vascular smooth muscle.

The first important observation in the present study was that the fibers responded to typical cerebral vasoconstrictors. 5-HT, ET-1, prostaglandin F$_2\alpha$, angiotensin II and noradrenaline have been demonstrated to induce vasoconstriction of cerebral arteries (15, 16). Isometric force measurements revealed contraction of the fibers in response to these cerebrovasoconstrictors. 5-HT has been implicated in various disorders including the pathogenesis of migraine. Consistent with this, we found that 5-HT (10 nM – 100 µM) elicited contraction of the smooth muscle fibers in a dose-dependent manner. Previous studies have demonstrated that 5-HT$_1$ and 5-HT$_2$ receptor subtypes are involved in the 5-HT-induced vasoconstriction in rat and

Fig. 6. Inhibition of 5-HT-induced contraction by ML-9 and relaxation by Y-27632 and staurosporine. A: The first contraction was induced by adding 1 µM 5-HT (a). The fiber was then washed for 60 min and treated with 30 µM ML-9 (b) for 20 min. The second contraction was induced by adding 1 µM 5-HT (b). The fiber was then washed for 60 min. The third contraction was then induced by adding 5-HT (c). Representative traces of repeated experiments (n = 4). B. Representative tracing showing relaxation of the fibers pre-contracted by 5-HT by Y-27632. The contraction was induced by adding 1 µM 5-HT, and then 1 µM Y-27632 was applied 20 min later. Representative traces of repeated experiments (n = 4). C. Representative tracing showing relaxation of the fibers pre-contracted by 5-HT by staurosporine. The contraction was induced by adding 1 µM 5-HT, and then 300 nM staurosporine was applied 20 min later. Representative traces of repeated experiments (n = 4). W, wash.

Fig. 7. Representative tracing showing relaxation of the fibers pre-contracted by 5-HT by papaverine (A), db-cAMP (B) and 8-Br-cGMP (C). The contraction was induced by adding 1 µM 5-HT, and then 10 µM papaverine, 100 µM db-cAMP or 100 µM 8-Br-cGMP was applied 10 – 30 min later. Representative traces of repeated experiments (n = 4). W, wash.
bovine cerebral arteries (17, 18). The present study demonstrated the presence of mRNAs for both 5-HT₁ and 5-HT₂A receptor subtypes in the fibers. ET-1 may play roles in maintenance of cerebral vascular tone as well as in the pathogenesis of cerebral vasospasm (19). The endothelin receptor subtype ETₐ is involved in the ET-1-induced vasoconstriction. A recent finding indicated that ETₐ receptors also modulate constriction. RT-PCR experiments indicated the presence of mRNAs for both endothelin receptor subtypes ETₐ and ETₖ in the fibers. These observations were consistent with these earlier studies by functional analysis and RT-PCR (20, 21).

The extent of noradrenaline-induced vasoconstriction in the cerebral arteries was markedly less than that seen in extracerebral arteries (22, 23). Consistent with this, the present findings demonstrated that noradrenaline caused little contractile response as compared with 5-HT, ET-1 and prostaglandin F₂α.
However, there was virtually no contractile response to histamine. In contrast, isolated cerebral arteries from various species contract in response to histamine via histamine $H_1$ receptors (16). RT-PCR analysis of total RNA isolated from the smooth muscle fibers, using sequence-specific oligonucleotide primers designed with reference to the published sequence of rat histamine $H_1$ and $H_2$ subtype genes, revealed that mRNA for $H_1$ subtype was present in smooth muscle fibers. The reason for the lack of response to histamine is currently unknown.

The second major finding of the present study was that the fibers responded to typical cerebral vasodilators such as db-cAMP and 8-Br-cGMP. Cerebral vascular smooth muscle relaxation is thought to be mediated by intracellular cAMP and cGMP (24); increases in cAMP and cGMP result in inhibition of contraction. Moreover, the contraction was completely abolished by the smooth muscle relaxant papaverine. These findings strongly suggested that typical vasodilation mechanisms are maintained in the reconstituted fibers.

The contractions were dependent on Ca$^{2+}$. In general, contraction of vascular smooth muscle is regulated by increases in intracellular calcium levels as a result of rapid Ca$^{2+}$ release from intracellular stores, mainly the sarcoplasmic reticulum, and from Ca$^{2+}$ influx via plasma membrane Ca$^{2+}$ channels (25). An increase in intracellular Ca$^{2+}$ causes smooth muscle cell contraction by activation of the Ca$^{2+}$/calmodulin-dependent MLCK, which phosphorylates myosin light chain and activates contractile myosin adenosine triphosphatase (ATPase). A decrease in intra-

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**Fig. 9.** Myosin light chain phosphorylation of the smooth muscle fibers. A: The fibers were preincubated for 10 min. Vehicle (lane 1), 10 nM ET-1 (lane 2), 1 μM 5-HT (lane 3) or 80 mM KCl (lanes 4 – 6) was then added and incubation was continued for 2 min (lanes 1 – 4), 5 min (lane 5) and 10 min (lane 6). The samples from the fibers were subjected to ureaglycerol PAGE and electrotransferred onto nitrocellulose membranes. Unphosphorylated MLC20 (MLC), monophosphorylated MLC20 (MLC-P) and diphosphorylated MLC20 (MLC-2P) were simultaneously detected with anti-MLC20 antibody. B: Time-dependent changes in the phosphorylation of monophosphorylated (open circles) and diphosphorylated (solid circles) MLC20s after stimulation with 80 mM KCl. Data represent means ± S.E.M. (n = 4). C: a, Effects of ML-9 on 5-HT-induced MLC20 phosphorylation. b, Effects of nifedipine on KCl-induced MLC20 phosphorylation. The fibers were pretreated with vehicle, 30 μM ML-9 or 2 μM nifedipine for 20 min. Vehicle, 1 μM 5-HT or 80 mM KCl was then added and incubation was continued for 2 min. Unstimulated phosphorylation of MLC20 in the presence and absence of ML-9 or nifedipine was subtracted from the percentages of myosin light chain phosphorylation stimulated with agonists in the presence and absence of ML-9 or nifedipine, respectively. Data represent means ± S.E.M. (n = 4). ***p < 0.005, compared with the control (with agonists in the absence of inhibitors).
cellular calcium causes inactivation of MLCK, accompanied by dephosphorylation of myosin light chain by the myosin light chain phosphatase, PP1M (26, 27). Cerebro-vascular contractions induced by 5-HT as well as ET-1 are considered to be driven by increased influx of Ca\textsuperscript{2+} and increased release of Ca\textsuperscript{2+} from intracellular storage sites (16, 28). The removal of extracellular Ca\textsuperscript{2+} resulted in a decrease in the force induced by 5-HT. A similar decrease in the force was also observed when the fiber was pretreated with the L-type voltage-dependent Ca\textsuperscript{2+} channel inhibitor nifedipine and then stimulated with 5-HT in Ca\textsuperscript{2+}-containing medium. RT-PCR analysis of total RNA isolated from the smooth muscle fibers, using sequence-specific oligonucleotide primers designed with reference to the published sequences of rat L-type and N-type voltage-sensitive Ca\textsuperscript{2+} channels, revealed that mRNA for L-type, but not N-type, voltage-dependent Ca\textsuperscript{2+} channel was present in smooth muscle fibers. There are six subtypes of voltage-dependent Ca\textsuperscript{2+} channels. The main type of voltage-dependent Ca\textsuperscript{2+} channel in the vascular smooth muscle is the L-type. These results strongly suggested that Ca\textsuperscript{2+} entry from extracellular sources, primarily through nifedipine-sensitive L-type voltage-dependent Ca\textsuperscript{2+} channels causes an increase in contractile force induced by 5-HT. This was supported by the findings that KCl-induced depolarization of smooth muscle cell membrane caused significant force development and that pretreatment of the fiber with nifedipine inhibited the contraction. Taken together, these results suggested that Ca\textsuperscript{2+} entry through nifedipine-sensitive voltage-dependent Ca\textsuperscript{2+} channels causes an increase in contractile force. 5-HT-induced contraction was also partially inhibited by the non-selective cationic channel inhibitor SKF96365. In vascular smooth muscles, the main pathway of Ca\textsuperscript{2+} entry into smooth muscles is via L-type voltage-dependent Ca\textsuperscript{2+} channels. The putative non-selective cationic channels are also partially involved in agonist-stimulated Ca\textsuperscript{2+} influx. It was, therefore, suggested that Ca\textsuperscript{2+} entry through non-selective cationic channels is also involved in the increase in contractile force.

The contractions were dependent on MLC20 phosphorylation and Rho-kinase. 5-HT as well as ET-1 and KCl induced marked contraction of the fibers accompanied by a pronounced increase in MLC20 phosphorylation. ML-9 inhibited both MLC20 phosphorylation and contraction evoked by 5-HT. These results strongly suggested the importance of MLC20 phosphorylation catalyzed by MLCK. Furthermore, the fibers pre-contracted by 5-HT were completely relaxed by the Rho kinase inhibitor Y-27632. These findings suggested that the contraction is regulated by MLCK-dependent and Rho-kinase-dependent mechanisms. However, at present, we cannot eliminate an alternative possibility that the effect of Y-27632 is due to the inhibition of other protein kinases such as protein kinase C.

In conclusion, string-shaped reconstituted smooth muscle fibers were prepared in rectangular wells by thermal gelation of a mixed solution of collagen and cultured smooth muscle cells derived from rat cerebral arteries. The fibers contracted in response to KCl, 5-HT, noradrenaline, ET-1, ET-2, angiotensin II, prostaglandin F\textsubscript{2}\alpha, and prostaglandin E\textsubscript{2}. 5-HT-induced contraction was partially inhibited by nifedipine, SKF96365 and the intracellular Ca\textsuperscript{2+} chelator BAPTA-AM, and it was abolished by the MLCK inhibitor ML-9. The fibers pre-contracted by 5-HT were completely relaxed by the Rho kinase inhibitor Y-27632, serine/threonine kinase inhibitor staurosporine, 8-Br-cGMP and papaverine, and partially relaxed by db-cAMP. Moreover, 5-HT as well as ET-1 and KCl enhanced MLC20 phosphorylation in the fibers. These results suggested that the characteristics of contraction of these fibers reflect typical contractilities of vascular smooth muscle tissues. Molecular analyses of the mechanisms of vasoconstrictor actions in cerebrovascular smooth muscle cells are frequently difficult because the quantity of materials available for studies is quite limited. This technique will allow us to directly address questions relating to heterogeneity of receptor mechanisms and intracellular pathways of vascular smooth muscle contraction as a function of vessel type.

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