

Olmesartan (OLM)/azelnidipine (AZ) and candesartan (CAN)/amlodipine (AM) combinations are often used as fixed-dose ARB/CCB combination therapies in Japan and are reported to have the powerful antihypertensive action (5,6). In general, ARBs have been shown to decrease sympathetic activity, although not equal in each ARB (7–9). In contrast, a long-acting CCB, AM is suggested to be neutral or elicit a slight increase in sympathetic activity probably due to reflex-mediated activation (10,11). Another long-acting CCB, AZ is suggested to have a sympathoinhibitory effect (12,13). In addition, accumulating evidence indicates that oxidative stress has an important role in hypertension (14). In particular, oxidative stress in the brain contributes to the activation of the sympathetic nervous system (8,9,15–18). It has been demonstrated that the reduction of oxidative stress in the brain decreases BP and heart rate (HR) through sympathoinhibition in hypertensive rats (7–9,17,19). However, it remains unknown whether OLM/AZ and CAN/AM combination therapies still have the antioxidant effect in the brain and the sympathoinhibitory effect without eliciting reflex sympathoexcitation due to the powerful antihypertensive action. Therefore, in this study, it was investigated whether there are differences in the antioxidant effect in the brain and the subsequent sympathoinhibitory effect between combination therapies using OLM/AZ and CAN/AM.

METHODS

Animals and General Procedures

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical and Pharmaceutical Sciences, and performed in accordance with the Guidelines for Animal Experiments at Kyushu University. Male stroke-prone spontaneously hypertensive rats (SHRSP) (11 weeks old; SLC Japan, Hamamatsu, Japan) were housed in a room with controlled lighting, temperature, and humidity. After a 1-week acclimatization period, SHRSP were assigned to one of four groups: (i) treatment with a combination of olmesartan medoxomil (OLM)/AZ (OLM/AZ group, OLM: 10 mg kg⁻¹ day⁻¹; AZ: 8 mg kg⁻¹ day⁻¹); (ii) treatment with a combination of candesartan cilexetil (CAN)/amlodipine besylate (AM) (CAN/AM group, CAN: 4 mg kg⁻¹ day⁻¹; AM: 2.5 mg kg⁻¹ day⁻¹); (iii) treatment with vehicle (untreated group, vehicle: 0.5% of methyl cellulose); and (iv) no treatment (before treatment group). All drugs were dissolved in 0.5% of methyl cellulose and administered once a day by oral gavage, and treatment continued for 30 days. OLM, AZ, and CAN were supplied by Daiichi-Sankyo Company, Ltd., Tokyo, Japan, and AM was supplied by LKT Laboratories, Inc., Tokyo, Japan.

Measurements of BP, HR, and Urinary Norepinephrine Excretion

Systolic blood pressure (SBP) and HR were measured using the tail-cuff method for each group (BP-98A; Softron, Tokyo, Japan). Urine was collected from each rat for 24 hours in a metabolic cage. Urinary norepinephrine concentration was measured using high-performance liquid chromatography to calculate urinary norepinephrine (UNE) excretion as a marker of sympathetic activity (7,12,17,19,20).

In Vivo Electron Spin Resonance Spectroscopy

Oxidative stress in the brain was evaluated using in vivo electron spin resonance (ESR) spectroscopy as previously described (7). [3-(Methoxycarbonyl)-2,2,5,5-tetramethylpyrrolizinoxy]radical (MC-PROXYL), a nitroxyl radical species, was used as the blood-brain barrier permeable spin probe because of its lipophilicity (21). MC-PROXYL undergoes an in vivo biological reaction to nonradical derivatives such as hydroxylamine (22). Additionally, increased reactive oxygen species (ROS) precipitate in the reaction nonenzymatically (23). This provides a basic mechanism for the in vivo ESR method; increased ROS production increases the in vivo ESR signal decay rate in comparison with an appropriate control value.

An anesthetized rat (sodium pentobarbital, 50 mg kg⁻¹, intraperitoneally) was placed on a handmade rat carrier. MC-PROXYL solution (150 mmol L⁻¹ in saline, 0.33 mmol kg⁻¹ body weight) was injected into the tail vein of the rat, and the carrier was slid into the resonator cavity such that the head of the rat, the area between interorbital and interaural lines, was located in the ESR detecting area (coaxial discoid space; 6 cm in diameter, 1.5 cm in depth). Measurement was started immediately using 300 MHz ESR spectroscopy (JEOL, Tokyo, Japan) under the following conditions: radiowave frequency, 300 MHz; radiowave power, 2.5 mW; field modulation width, 0.1 mT; field modulation frequency, 100 kHz; scan rate, 0.083 mT second⁻¹; scan width, 1.0 mT; time constant, 0.1 second; and accumulation number, 5.

Signal intensity, defined as the height of the center line of a spectrum, was obtained using an averaged spectrum processed using five consecutively accumulated spectra within a 60-second interval. Measurement data were collected for 5 minutes. Natural logarithmic values of the data were plotted as a function of time, and the signal decay rate was determined using the negative slope of the linear regression of the plots, which showed good linearity.

Measurement of Thiobarbituric Acid Reactive Substances

To obtain brain tissues, rats were deeply anesthetized using sodium pentobarbital (100 mg kg⁻¹ intraperitoneally) and perfused transcardially with

phosphate-buffered saline (150 mol L⁻¹ NaCl, 3 mmol L⁻¹ KCl, and 5 nmol L⁻¹ phosphate; pH 7.4, 4°C). The brain was quickly removed and divided into four parts: the cerebral cortex, cerebellum, hypothalamus, and brainstem. All tissues were homogenized in 1.15% KCl (pH 7.4), 0.4% sodium dodecyl sulfate, and 7.5% acetic acid adjusted to pH 3.5 with NaOH. Thiobarbituric acid (0.3%) was added to the homogenate. The mixture was maintained at 5°C for 60 minutes, followed by heating to 100°C for 60 minutes. After cooling, the mixture was extracted using distilled water and *n*-butanolpyridine (15:1) and centrifuged at 1600 × *g* for 10 minutes. The absorbance of the organic phase was measured at 532 nm, and the amount of thiobarbituric acid reactive substances (TBARS) was determined by absorbance (17).

Statistical Analysis

All values are expressed as the mean ± SEM. Two-way repeated measures analysis of variance with Bonferroni post hoc tests was used to compare SBP and HR between groups. One-way analysis of variance with Bonferroni post hoc tests was used to compare UNE, ESR signal decay rate, and TBARS levels between groups. Differences were considered to be statistically significant for *P* < .05.

RESULTS

BP, HR, and UNE Levels

SBP was markedly decreased in OLM/AZ and CAN/AM groups after treatment for 30 days (Figure 1). Although the decreases in BP after 30 days of treatment were not different between the OLM/AZ and CAN/AM groups, OLM/AZ decreased BP more slowly than CAN/AM. In both treated groups, HR was significantly lower compared with the untreated group (Figure 1); however, the HR in OLM/AZ-treated SHRSP was significantly lower than that in the CAN/AM-treated SHRSP. UNE excretion was significantly lower in the OLM/AZ group than in the untreated group (*n* = 8 for each, *P* < .05) or before treatment group (*n* = 8 for each, *P* < .05), but was not significantly different between the CAN/AM and untreated groups (Figure 2). In addition, when changes in UNE from baseline between OLM/AZ and CAN/AM groups were compared, the changes were found to be greater in the OLM/AZ group than in the CAN/AM group (-0.32 ± 0.05 vs. -0.11 ± 0.04 μg 24 h⁻¹, *n* = 4 for each, *P* < .05).

Oxidative Stress in the Whole Brain as Measured Using In Vivo ESR

The signal decay rates in the whole brain were significantly decreased in both the OLM/AZ and CAN/AM groups compared with the untreated or before treatment groups (Figure 3). Decreases in the signal decay

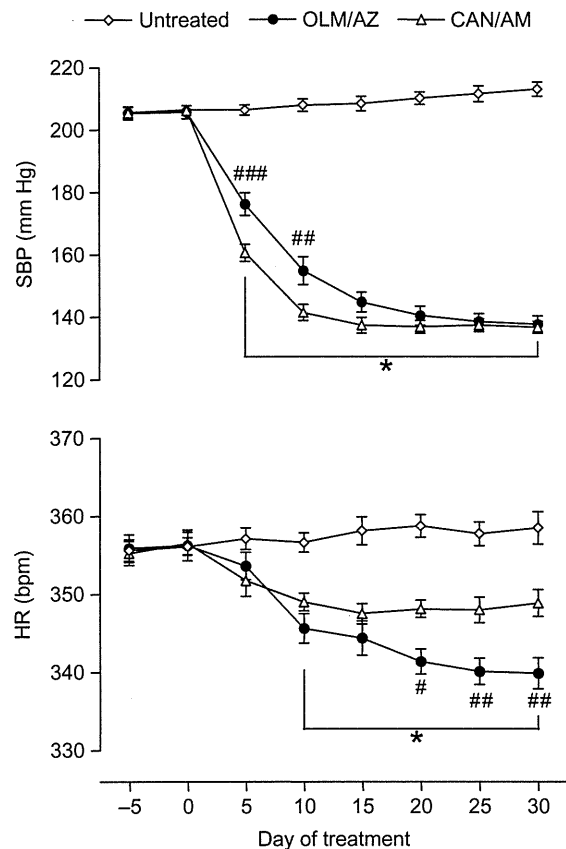


Figure 1. The effect of combination therapies of olmesartan/azelnidipine (OLM/AZ) and candesartan/amlodipine (CAN/AM) on systolic blood pressure (SBP) and heart rate (HR). Time courses of SBP and HR in the groups during the study period. **P* < .001 versus untreated group for both OLM/AZ and CAN/AM groups; #*P* < .05, ##*P* < .01, ###*P* < .001 versus CAN/AM group.

rate did not differ between the OLM/AZ and CAN/AM groups.

TBARS Levels in the Brain

Figure 4 shows TBARS levels in each region of the brain. In the cerebral cortex, cerebellum, and hypothalamus, TBARS levels were significantly lower in both the OLM/AZ and CAN/AM groups compared with the untreated or before treatment groups. However, in the brainstem, TBARS levels were significantly lower only in the OLM/AZ group relative to the untreated group (*n* = 8 for each, *P* < .05) or before treatment group (*n* = 8 for each, *P* < .05) and were not significantly altered in the CAN/AM group compared with the untreated group or before treatment group.

DISCUSSION

The effects of oral treatment using an OLM/AZ combination and CAN/AM combination for 30 days on the BP, HR, sympathetic activity, and brain oxidative stress in SHRSP were investigated. Major findings of

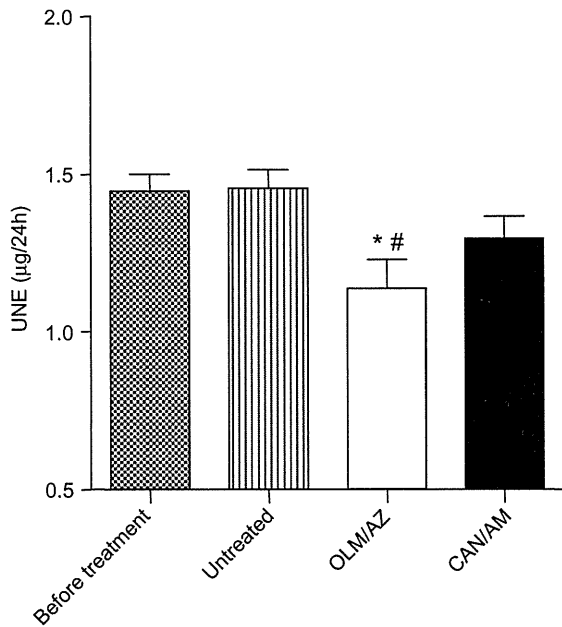


Figure 2. The effect of combination therapies of olmesartan/azelnidipine (OLM/AZ) and candesartan/amlodipine (CAN/AM) on urinary norepinephrine (UNE) excretion for 24 hours. UNE excretion as an indicator of sympathetic activity was significantly lower only in the OLM/AZ group compared with the untreated or before treatment groups. * $P < .05$ versus before treatment group; # $P < .05$ versus untreated group.

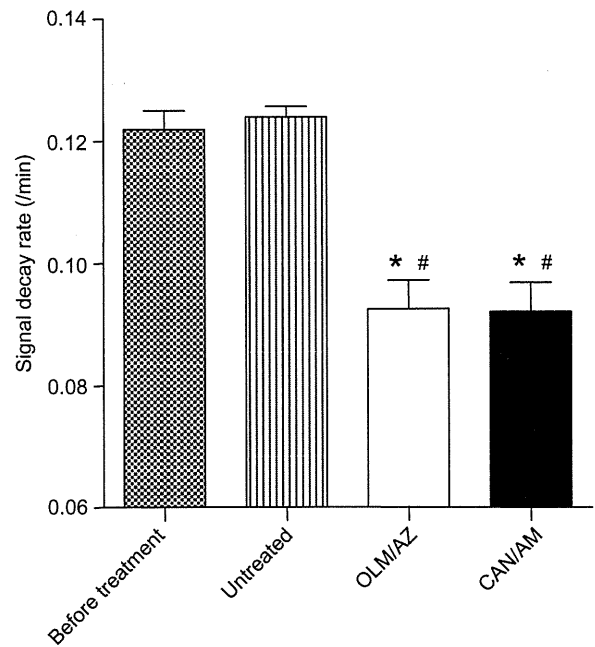


Figure 3. The effect of combination therapies of olmesartan/azelnidipine (OLM/AZ) and candesartan/amlodipine (CAN/AM) on in vivo electron spin resonance (ESR) signal decay rates. In vivo ESR signal decay rate was significantly lower in both the OLM/AZ and CAN/AM groups than in untreated or before treatment groups. * $P < .001$ versus before treatment group; # $P < .001$ versus untreated group.

this study were as follows: (i) combination therapy of either OLM/AZ or CAN/AM markedly decreased BP and did not elicit a baroreflex-mediated increase in HR and sympathetic activity; (ii) OLM/AZ and CAN/AM reduced oxidative stress in the brain; and (iii)

OLM/AZ, not CAN/AM, significantly decreased sympathetic activity and oxidative stress in the brainstem. These results suggest that both OLM/AZ and CAN/AM combination therapies have a powerful antihypertensive effect and do not elicit a baroreflex-mediated increase in

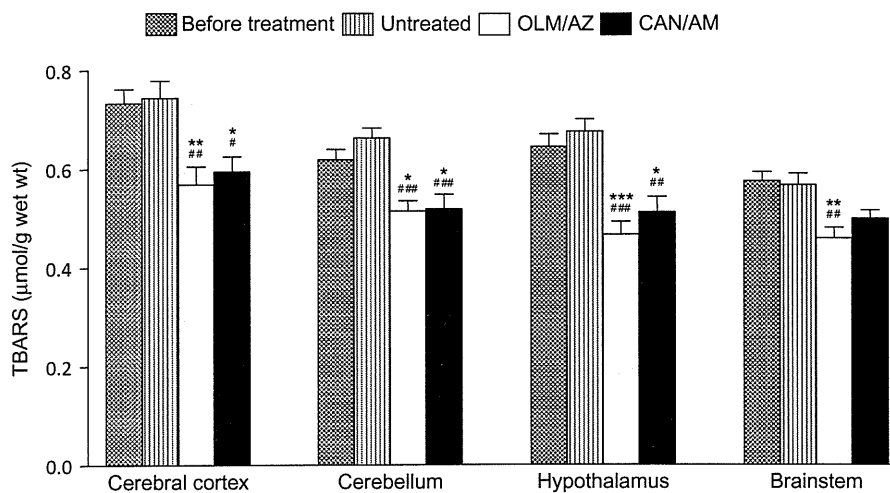


Figure 4. The effect of combination therapies of olmesartan/azelnidipine (OLM/AZ) and candesartan/amlodipine (CAN/AM) on the levels of thiobarbituric acid reactive substances (TBARS) in the brain (cerebral cortex, cerebellum, hypothalamus, and brainstem). In the cerebral cortex, cerebellum, and hypothalamus, TBARS levels were significantly lower in both the OLM/AZ and CAN/AM groups compared with the untreated or before treatment groups. In the brainstem, however, TBARS levels were significantly lower only in the OLM/AZ group compared with the untreated or before treatment groups. * $P < .05$, ** $P < .01$, *** $P < .001$ versus before treatment group; # $P < .05$, ## $P < .01$, ### $P < .001$ versus untreated group.

HR or activate the sympathetic nervous system associated with an antioxidant action in the brain. Particularly, combination therapy using OLM/AZ inhibited the sympathetic activity, probably due to a greater antioxidant effect in the brainstem.

In this study, combination therapy using either OLM/AZ or CAN/AM in SHRSP markedly decreased BP considering the antihypertensive effect of each drug alone. For example, previous studies have shown that each of these combination therapies showed a stronger antihypertensive effect compared with monotherapy (24,25). In this study, despite significant reductions in BP, OLM/AZ and CAN/AM did not elicit a baroreflex-mediated increase in HR and sympathetic activity; in fact, these treatments decreased HR. The authors' group as well as other groups have previously showed that treatment using OLM, AZ, and AM did not increase the HR and/or sympathetic activity in SHRSP or spontaneously hypertensive rats (SHR) (7,12,20,26,27). Additionally, CAN did not elicit reflex tachycardia and sympathoexcitation in diabetic hypertensive rats (28). Activation of the sympathetic nervous system has an important role in hypertension pathogenesis (4); therefore, both OLM/AZ and CAN/AM are beneficial for treating hypertension because of their significant BP-lowering effect without causing sympathoexcitation.

OLM/AZ and CAN/AM reduced oxidative stress in the brain of SHRSP in this study. By measuring *in vivo* ESR signal decay rates in the brain and TBARS levels in several brain regions, it was confirmed that OLM/AZ and CAN/AM decreased oxidative stress in the brain. It has been previously demonstrated that the *in vivo* ESR method is useful for noninvasively evaluating the effect of antihypertensive drugs on brain oxidative stress in a hypertensive rat model (7). Several reports have supported the authors' finding that ARBs and CCBs exhibit antioxidant action in the brain. First, activation of angiotensin type 1 (AT₁) receptors by angiotensin II (Ang II) results in ROS production. Ang II binds AT₁ receptors, activating the nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase/Rac1 pathway, which produces superoxides in the brain (15,19). Chronic oral treatment with OLM and CAN, respectively, abolished the pressor effect of Ang II in the rostral ventrolateral medulla (RVLM) of the brainstem, where the vasomotor center is located (29), in SHR (27,30). In other studies, chronic oral treatment with OLM or CAN decreased oxidative stress in the brain in SHRSP and in salt-loaded SHRSP (7,31), respectively. Second, regarding the involvement of calcium channels in ROS generation, L-type CCBs, such as AZ and AM, can scavenge free radicals and donate protons to stabilize free electrons (32). Calcium overload, particularly in the mitochondria, generates free radicals (33). A recent study suggested that in the brain, AZ and AM reduced calcium overload and consequent oxidative stress in the neurons (34). It has been previously showed that

chronic oral treatment with AZ and AM reduces oxidative stress in the brains of SHRSP (12,20) and that AZ activates copper/zinc-dependent superoxide dismutase and manganese-dependent superoxide dismutase in the RVLM of SHRSP (12). Oxidative stress in the brain, particularly in the autonomic nuclei, such as the paraventricular nucleus of the hypothalamus, nucleus tractus solitarius, and RVLM of the brainstem, contributes to the neural mechanism of hypertension (8,9,15–18). The findings of this study suggest that OLM/AZ and CAN/AM may affect the brain, thereby inhibiting the sympathetic activity, at least in part, by reducing oxidative stress in the brain, including the autonomic nuclei of the brain (8,9,15–18).

The differences in the sympathoinhibitory effect between combinations of OLM/AZ and CAN/AM were shown in this study. OLM/AZ, but not CAN/AM, significantly decreased UNE, a marker of sympathetic activity. This might have resulted from the differences in the antioxidant effect in the brainstem between OLM/AZ and CAN/AM combinations. Compared with the untreated or before treatment groups, TBARS levels in the cerebral cortex, cerebellum, and hypothalamus were significantly lower in both the OLM/AZ and CAN/AM groups; however, TBARS levels in the brainstem were significantly lower only in the OLM/AZ group. It was also demonstrated that reducing oxidative stress in the brainstem and/or hypothalamus of hypertensive rats decreased sympathetic activity, thereby reducing BP (8,9,15–18). Thus, a greater antioxidant effect of OLM/AZ on the brain, including the brainstem, compared with CAN/AM, might inhibit the sympathetic nervous system.

The extent of the actions of the ARBs and CCBs within the brain is partially dependent on the lipophilicity and pharmacokinetics of these drugs (26,35,36). Although OLM, AZ, and AM exhibit lipophilic properties (32,37), CAN exhibits a hydrophilic property. Additionally, AZ is more lipophilic than AM (32). Thus, OLM/AZ may be more beneficial for acting on the brainstem than CAN/AM because of its higher blood-brain barrier permeability. Furthermore, other mechanisms, such as active transport of the drugs through the blood-brain barrier, should be considered (38) because CAN, a hydrophilic ARB, also acts within the brain (38,39). Additionally, the disruption of the blood-brain barrier of SHRSP may affect the effect of the drugs on the brainstem (40). Further study is necessary to determine each drug's affinity in the brainstem region.

Another different effect between OLM/AZ and CAN/AM was the speed of BP lowering. Rapid BP reduction causes reflex tachycardia through the activation of the sympathetic nervous system (3,41). A previous study comparing the effects of AZ and AM showed that AZ decreased HR partly due to the slow BP-lowering effect, whereas AM increased HR, despite a similar extent of reduction in BP (41). In this study,

OLM/AZ decreased BP more slowly than CAN/AM, despite a similar powerful extent of reduction in BP after 30 days of treatment, which is a similar BP level of Wistar–Kyoto rats. Thus, the slower BP-lowering effect of OLM/AZ might also contribute to sympathoinhibition observed as a reduction of UNE.

There are some limitations in this study. The effects of combination therapies were not compared with those of each monotherapy, because the aim of this study was to investigate whether there are differences in the antioxidant effect in the brain and the sympathoinhibitory effect between combination therapies using OLM/AZ and CAN/AM in SHRSP with a powerful BP reduction. Further studies are required to evaluate the effects of monotherapies at the same dose as administered in this study to understand the differences in sympathoinhibitory and antioxidant effects, as additive effects, between OLM/AZ and CAN/AM. In this study, a dose of ARB/CCB that is proportional to the dose used in clinical combination therapies was selected because it was considered that the results obtained with these doses would more accurately reflect the effects of the clinical fixed-dose combination therapies. The doses of each drug that are in the range of doses commonly used for the experimental study using hypertensive animals (7,20,42,43) were selected for this study. Finally, this study did not determine whether the combination therapy indeed inhibited sympathetic activity by reducing oxidative stress in the brain. However, it was considered that this mechanism plays an important role in sympathetic control of BP in hypertensive rats as reported in a series of previous studies by the authors themselves and others (8,9,15–18).

In conclusion, the results of this study suggest that chronic oral treatment with OLM/AZ combination and CAN/AM combination, respectively, has a powerful antihypertensive effect and does not elicit baroreflex-mediated sympathetic activation associated with an antioxidant action on the brain in SHRSP. The antioxidant effect on the brain, which is probably due to the action on the brainstem, and the sympathoinhibitory effect of OLM/AZ combination therapy may be greater than those caused using CAN/AM combination therapy.

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5 **Calcium influx through a possible coupling of cation channels impacts**
6 **skeletal muscle satellite cell activation in response to mechanical stretch**

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34

35 **ABSTRACT**

36 When skeletal muscle is stretched or injured, satellite cells, resident myogenic stem
37 cells positioned beneath the basal lamina of mature muscle fibers, are activated to enter the
38 cell cycle. This signaling pathway is a cascade of events including calcium-calmodulin
39 formation, nitric oxide (NO) radical production by NO synthase, matrix metalloproteinase
40 activation, release of hepatocyte growth factor (HGF) from the extracellular matrix, and
41 presentation of HGF to the receptor c-met, as demonstrated by assays of primary cultures
42 and in vivo experiments. Here, we add evidence that two ion channels, the
43 mechano-sensitive cation channel (MS-channel) and the long-lasting-type voltage-gated
44 calcium-ion channel (L-VGC-channel), mediate the influx of extracellular calcium ions in
45 response to cyclic stretch in satellite-cell cultures. When applied to 1-hr stretch cultures
46 with individual inhibitors for MS- and L-VGC-channels (GsMTx-4 and nifedipine,
47 respectively) or with a less specific inhibitor (gadolinium chloride, Gd), satellite cell
48 activation and upstream HGF release were abolished, as revealed by
49 bromodeoxyuridine-incorporation assays and western blotting of conditioned media,
50 respectively. The inhibition was dose-dependent with a maximum at 0.1 μ M (GsMTx-4),
51 10 μ M (nifedipine) or 100 μ M (Gd) and cancelled by addition of HGF to the culture media;
52 a potent inhibitor for transient-type VGC-channels (NNC55-0396, 100 μ M) did not show
53 any significant inhibitory effect. The stretch response was also abolished when
54 calcium-chelator EGTA (1.8 mM) was added to the medium, indicating the significance of
55 extracellular free calcium ions in our present activation model. Finally, cation/calcium
56 channel dependencies were further documented by calcium-imaging analyses on stretched
57 cells; results clearly demonstrated that calcium ion influx was abolished by GsMTx-4,
58 nifedipine and EGTA. Therefore, these results provide an additional insight that calcium
59 ions may flow in through L-VGC-channels by possible coupling with adjacent MS-channel
60 gating that promotes the local depolarization of cell membranes to initiate the satellite cell
61 activation cascade.

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64 **Key Words:** Calcium ion influx; Mechano-sensitive channel; Muscle regeneration; Satellite
65 cells; Stretch-activation; Voltage-gated channel

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68 INTRODUCTION

69 Satellite cells, a population of resident myogenic stem cells positioned between the
70 basal lamina and the sarcolemma of post-natal skeletal muscle fibers, are normally found in
71 a mitotically and metabolically quiescent (or near-dormant) protracted G₁ state (also
72 referred to as G₀) in adult muscles. When muscle is injured, overused, or mechanically
73 stretched, these cells are activated to enter the cell cycle, proliferate to produce large
74 numbers of myoblast progeny, differentiate, and fuse with existing muscle fibers or form
75 new fibers. Through this process, many myonuclei are accumulated in the tissue, and are
76 responsible for increasing protein synthesis and providing the potential to enhance muscle
77 growth (hypertrophy and hyperplasia) and regeneration. Consequently, satellite cell
78 activation is an initial and crucial step in the process of post-natal myogenesis (reviewed in
79 Refs. 12, 28, 45, 50, 66) and may be triggered by mechanical perturbation of satellite cells
80 and muscle fibers. However the detailed mechanism is not fully elucidated.

81 Of all growth factors studied thus far, including fibroblast growth factors (FGFs),
82 insulin-like growth factor-1 (IGF-1), platelet-derived growth factor BB (PDGF-BB),
83 transforming growth factor- β s (TGF- β 1 and 2) and epidermal growth factor (EGF),
84 hepatocyte growth factor (HGF) is the only mitogen with an established ability to stimulate
85 quiescent satellite cells to enter the cell cycle early in primary culture assay and in vivo (1,
86 58; reviewed in Refs. 15, 38, 72), even though Nagata et al. (40) first demonstrated that
87 sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite, induces satellite cells
88 to cycle and very recently Sassoli et al. (48) showed that S1P promotes satellite cell renewal
89 and differentiation in damaged muscle. HGF is a heparin-binding protein localized in the
90 extracellular domain of un-injured skeletal muscle fibers by possible association with
91 glycosaminoglycan chains of proteoglycans, and its predominant form is the active
92 disulfide-linked heterodimer of a 60-kDa α -chain and a 30-kDa β -chain (62). The
93 intracellular signaling receptor for HGF is the c-met proto-oncogene; its message and
94 protein have been found in quiescent and activated satellite cells (1, 19, 58). Thus, release
95 of HGF from its sequestration in the matrix and subsequent presentation to the receptor
96 c-met may be a critical aspect of the activation of quiescent satellite cells. Recently,
97 Wozniak and Anderson (73) provided a novel insight that HGF released from the matrix
98 may induce c-met RNA expression as an immediate-early gene within 30 min in response to
99 muscle fiber stretch and thus enhance HGF-c-met signaling in the satellite-cell activation
100 process.

101 In a series of previous reports, we employed a FlexerCell system (Flexcell
102 International, Hillsborough, NC) to apply cyclic stretch to isolated rat satellite cells and
103 found that mechanical stretch triggers satellite cell activation by rapidly releasing HGF from
104 its tethering in the extracellular matrix and its subsequent presentation to c-met (59, 60, 63,
105 65; reviewed in Ref. 66). This phenomenon is relevant to satellite cells in living muscle as
106 revealed by an in vivo muscle-stretch model (64). The most important observation from
107 these previous experiments was that the release of HGF was completely dependent on nitric
108 oxide (NO) radical synthesis by NO synthases (NOS) from L-arginine of the substrate (60,
109 61). Anderson (4) first pointed to production of NO as a key signal responsible for
110 satellite cell hypertrophy and detachment from the adjacent fiber following crush injury;
111 those results were later verified in in vitro cultures of isolated muscle fibers and their
112 associated satellite cells (5, 6, 71-73; reviewed in Refs. 7, 8). In our subsequent studies,
113 Yamada et al. (75, 76) demonstrated that matrix metalloproteinases (MMPs), a large family
114 of zinc-dependent endopeptidases that collectively degrade one or more extracellular matrix
115 constituents, specifically MMP2, mediate HGF release, possibly by shedding
116 proteoglycan-core proteins in response to the NO radical (reviewed in Refs. 66, 68).

117 In an effort to understand the events upstream from NO-radical production, recent
118 studies showed that NO-radical/MMP-mediated HGF release and the resulting activation of
119 satellite cells are blocked if calmodulin activity is abolished by the specific inhibitors,
120 calmidazolium, W-13 and W-12; the same report showed that the activation cascade can be
121 turned on when calcium ionophores A23187 or ionomycin are simply added to un-stretched
122 control cultures (67). Therefore, the influx of calcium ions and their binding to calmodulin
123 are thought to be involved in the segment of the activation pathway between sensing the
124 mechanical stimulus and synthesis of the NO radicals, consistent with the observation that
125 the calcium-calmodulin complex associates with constitutive NOS (cNOS: i.e., neuronal
126 and endothelial NOS proteins) to activate NOS enzyme activity, as shown in neuronal and
127 endothelial cells (43, 69). However, it is still unclear how quiescent satellite cells sense
128 mechanical stimuli for transduction into an elevation of the intracellular calcium-ion
129 concentration at the most upstream step of the satellite-cell activation cascade.

130 Experiments in this study were designed to test the hypothesis that mechano-sensitive
131 cation channels (MS-channels) are involved in the mechano-transduction system.
132 MS-channels are mechanosensing machinery proteins and are widely distributed from
133 single-celled bacteria to animal and plant cells. A variety of experimental approaches

134 revealed that MS-channels respond to a variety of mechanical stimuli, including shear stress,
135 gravity, osmotic pressure and stretch, by opening nanoscale protein pores that are selectively
136 permeable to cations (mainly calcium and sodium) entering into the cytosol down their steep
137 electrochemical gradients (22, 42; reviewed in Refs. 3, 9, 27, 31, 33). Therefore,
138 MS-channel gating may transduce physical stimuli into electrical signals by generating
139 localized depolarization of the cell membranes (through changes in membrane voltage).
140 This depolarization potentially enables the activation of voltage-gated calcium channels
141 (VGC-channels). Indeed, calcium signaling has a central role in regulating activity of many
142 cell types including skeletal muscle fibers in which the long-lasting-type of VGC-channels
143 (L-VGC-channels) in transverse tubules act as a voltage sensor in excitation-contraction
144 coupling (44, 56, 57, 70; reviewed by Refs. 14, 21).

145 In the present paper, we examined the effect of a calcium chelator, EGTA, and
146 selective inhibitors for MS-channels (GsMTx-4) and VGC-channels (nifedipine and
147 NNC55-0396) with and without cyclic stretch of satellite-cell primary cultures on HGF
148 release from the matrix and satellite-cell activation. We provide evidence that extracellular
149 calcium-ion influx through a possible coupling of MS- and L-VGC-channels, may be
150 responsible for the mechano-sensing machinery of quiescent satellite cells.

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154 **MATERIALS AND METHODS**

155 **Materials.** Dulbecco's modified Eagle's medium (DMEM, low glucose type, 31600-034),
156 normal horse serum (HS, 16050-122), antibiotic-antimycotic (15240-062) and gentamicin
157 (15710-064) were purchased from Invitrogen (Grand Island, NY). Poly-L-lysine (P9155),
158 bovine plasma fibronectin (F1141), protease type XIV (P5147) and
159 5-bromo-2'-deoxyuridine (BrdU, B5002) were obtained from Sigma (St. Louis, MO).
160 Recombinant mouse HGF (2207-HG) was purchased from R&D Systems (Minneapolis,
161 MN). Cation-channel inhibitors, nifedipine (141-05783, 1,4-dihydro-2,6-dimethyl-4-
162 (2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester), gadolinium chloride
163 (078-02661) and NNC55-0396 (2268, (1*S*,2*S*)-2-[2-[[3-(1*H*-Benzimidazol-2-yl)propyl]
164 methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-(1-methylethyl)-2-naphthalenyl
165 cyclopropanecarboxylate dihydrochloride) were obtained from Wako Pure Chemical
166 Industries (Osaka, Japan), and GsMTx-4 (4393-s, synthetic peptide of the spider venom
167 toxin) was from Peptide Institute (Osaka, Japan).

168 Fluo3-AM (F026, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)
169 phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid,
170 pentaacetoxymethyl ester) was obtained from Dojindo Laboratories (Kumamoto, Japan).
171 EGTA (15214, *O,O'*-Bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid) was from
172 Nacalai Tesque (Kyoto, Japan). Calcium ionophore A23187 (21186) and nonionic
173 copolymer surfactant Pluronic F-127 (P2443) were purchased from Sigma.

174 The following materials were obtained for protein expression analysis: D3 mouse
175 monoclonal anti-desmin and G3G4 mouse monoclonal anti-BrdU antibodies from the
176 Developmental Studies Hybridoma Bank (Iowa City, IA); goat polyclonal anti-human HGF
177 antibody (AB-294-NA) and goat polyclonal anti-c-met antibodies (AF527) from R&D
178 Systems; AC-15 mouse monoclonal anti- β -actin antibody (ab6276) from abcom
179 (Cambridge, MA); horseradish peroxidase (HRPO)-conjugated AffiniPure donkey Anti-goat
180 IgG (705-036-147) from Jackson ImmunoResearch Laboratories (West Grove, PA);
181 affinity-purified biotinylated horse anti-mouse IgG (BA-2000) and the HRPO-labeled
182 avidin kit (PK-6100) from Vector Laboratories (Burlingame, CA); affinity-purified
183 HRPO-conjugated goat anti-mouse IgG (A-4416) and 3,3'-diaminobenzidine (DAB,
184 D5637) from Sigma; HRPO-labeled rabbit anti-goat IgG (414331, Histofine Simple Stain
185 Rat MAX-PO (G)) and normal rabbit serum (426051) from Nichirei BioScience (Tokyo,
186 Japan); Amersham enhanced chemiluminescence (ECL) detection kit (PRN2106),

187 nitrocellulose membrane (Hybond ECL, RPN2020D), and Hyperfilm ECL (28-9068) from
188 GE healthcare (Little Chalfont, UK); and MagicMark XP molecular weight standards
189 (LC5602) from Invitrogen; CanGetSignal immunoreaction solutions (NKB-101) from
190 Toyobo (Osaka, Japan). For the mRNA expression analysis, an RNeasy Micro kit (74004)
191 and QIAshredder homogenizer spin column (79654) from Qiagen (Hilden, Germany),
192 SuperScript III reverse transcriptase (18080-044) from Invitrogen, Oligo-dT primer
193 (H09876) from Roche (Mannheim, Germany), ExTaq DNA polymerase (RR001A) from
194 Takara Bio (Otsu, Japan), agarose-LE (01157) from Nacalai Tesque (Kyoto, Japan), and Gel
195 Red (41000) from Biotium (Hayward, CA) were additionally used.

196

197 ***Animal care and use.*** All experiments involving animals were conducted according to
198 institutional guidelines and with the approval of Kyushu University Institutional Review
199 Board, in collaboration with investigators at the University of Arizona and the University of
200 Manitoba.

201

202 ***Satellite cell isolation and primary culture.*** Satellite cells were isolated from muscle in
203 the hind limb and back of 9-month-old male Sprague-Dawley rats according to Allen et al.
204 (2) with a slight modification (65). Briefly, muscle from the upper hind limb and back
205 were excised, trimmed of fat and connective tissue, minced with scissors, and digested for 1
206 hr at 37°C with 1.25 mg/ml protease type XIV. Cells were separated from muscle-fiber
207 fragments and tissue debris by differential centrifugation and filtration through nylon cell
208 strainers (100 µm and 40 µm mesh size) prior to a final centrifugation step at 1500 x g for 3
209 min, and then plated on poly-L-lysine and fibronectin-coated dishes in DMEM containing
210 10% horse serum (HS), 1% antibiotic-antimycotic mixture, and 0.5% gentamicin
211 (DMEM-10% HS, pH 7.2). Cultures were maintained in a humidified atmosphere of 5%
212 CO₂ at 37°C. In addition, companion satellite-cell cultures, prepared at the same time,
213 were immunostained for the presence of desmin at 30 hr after plating, using a D3
214 monoclonal anti-desmin antibody, biotinylated anti-mouse IgG antibody and HRPO-labeled
215 avidin, in order to determine the percentage of myogenic cells present; cultures with less
216 than 95% DAB-positive cells were not used for experiments.

217 Satellite-cell cultures on Bioflex-Amino silicone-bottom plates (BF-3001A, Flexcell
218 International) were treated with one of the cation-channel inhibitors (GsMTx-4, nifedipine,
219 gadolinium chloride, or NNC55-0396) or a highly selective calcium chelator EGTA (1.8

220 mM) for 30 min in DMEM-10% HS. GsMTx-4, nifedipine and NNC55-0396 are highly
221 selective peptide/chemical-inhibitors, and are therefore intensely useful for the study of
222 cellular excitability and intracellular calcium-ion signaling and for characterizing effects of
223 therapeutically beneficial drugs. Although detailed inhibitory mechanisms of these
224 compounds still remain unclear, GsMTx-4 selectively inhibits MS-channel activity when
225 applied to the extracellular face of the cell membrane. It does this by increasing the
226 membrane tension required for activation, suggesting that GsMTx-4 acts as a gating
227 modifier (54). By comparison, nifedipine binds directly to inactive L-VGC-channels,
228 which stabilizes their inactive conformation. NNC55-0396 may bind to transmembrane or
229 intracellular domains of the channels to exert their inhibitory effects specifically on
230 transient-type VGC-channels (T-VGC-channels) (29; reviewed in Ref. 53). Cells were
231 then subjected to a cyclic-stretch environment [optimized previously at 25% stretch at 12
232 sec intervals (59); see Fig. 1 panel *A* for more detail] in a vacuum-operated cyclic
233 strain-providing instrument FlexerCell FX-2000 System (Flexcell International) for 1 hr
234 starting at 24-hr post-plating, as described in the result section with each experiment. In
235 the present study, the stretch periods were all set to 1 hr [half the duration from our original
236 report by Tatsumi et al. (59)] in order to fit calcium-imaging experiments that compare the
237 calcium-influx activity between stretch cultures (in the presence or absence of
238 cation-channel inhibitors or EGTA) and un-stretched control cultures in the same set of
239 experiments within a limited time-period. The stretch-activation activity of satellite cells
240 after 1 hr was not significantly different from that in 2-hr-stretch cultures, as revealed by the
241 BrdU-incorporation assay described below (see Fig. 1 panel *B*, compare *black bars b* and *c*)
242

243 **Conditioned medium.** In experiments in which samples of conditioned media from stretch
244 cultures were assayed by western blotting for the presence of HGF protein released from the
245 cells, cultures were washed with serum-free DMEM at 24-hr post-plating and treatments
246 were imposed for as short as 1 hr in the presence of serum-free DMEM (pH 7.2). The
247 culture medium was supplemented with one of the following for additional treatments: a
248 cation-channel inhibitor GsMTx-4 (0.1 μ M), nifedipine (10 μ M), or EGTA (1.8 mM) 30
249 min prior to the addition of cyclic stretch. Conditioned medium from each treatment was
250 collected, centrifuged for 4 min at 1300 x g, treated with SDS-sample buffer (2% SDS, 5%
251 β -mercaptoethanol, 10% glycerol, 0.01% bromophenol blue and 62.5 mM Tris-HCl buffer,
252 pH 6.8), and stored at -80°C until use.

253

254 ***In vitro activation assay.*** Cultures were pulse-labeled with 10 μ M BrdU in DMEM-10%
255 HS for the final 2 hr from 46 to 48 hr post-plating, followed by immunocytochemistry for
256 detection of BrdU using a G3G4 anti-BrdU monoclonal antibody [1:100 dilution in 0.1%
257 bovine serum albumin (BSA) in phosphate-buffered saline (PBS)] and a HRPO-conjugated
258 anti-mouse IgG antibody (1:500 dilution) according to Tatsumi et al. (58). The percentage
259 of BrdU-labeled cells was used as an indicator of activation and entry into the cell cycle.

260

261 ***Immunoblotting and ECL.*** Mouse recombinant HGF and conditioned media from 1-hr
262 stretch cultures with or without a cation-channel inhibitor or EGTA were applied to 10%
263 polyacrylamide gels for electrophoresis under reducing conditions (35) and transferred to
264 nitrocellulose membranes (58). The blots were blocked with 10% powdered milk in 0.1%
265 polyethylene sorbitan monolaurate (Tween 20)-Tris buffered saline (TTBS) prior to
266 incubation with goat polyclonal anti-HGF antibody (1:500 dilution in 1% powdered
267 milk-TTBS additionally containing 0.05% sodium azide) overnight, and subsequently
268 treated with HRPO-labeled donkey anti-goat IgG antibody at 1:10,000 dilution for 1 hr,
269 followed by ECL detection on Amersham Hyperfilm ECL X-ray film. Internal β -actin in
270 cell lysates was visualized with mouse monoclonal anti- β -actin antibody (1:1,000 dilution
271 in CanGetSignal solution 1, overnight) and with biotinylated horse anti-mouse IgG (1:5,000
272 dilution in CanGetSignal solution 2, for 1 hr) and HRPO-labeled avidin (1:500 dilution in
273 TTBS, for 30 min).

274

275 ***Calcium imaging.*** Satellite-cell cultures on a silicon membrane (11-004-006, Flexcell
276 International) were loaded with 5 μ M Fluo3-AM in 0.2% Pluronic F-127 for 1 hr in the dark
277 at 24-hr post-plating, and then rinsed with PBS and left to equilibrate in DMEM-10% HS
278 (pH 7.2) for 60 min at room temperature. Cultures were then exposed to 2-cycles of
279 stretch in the presence of 1.8 mM EGTA or a cation-channel inhibitor (0.1 μ M GsMTx-4 or
280 10 μ M nifedipine, each added another 30 min prior to the stretch stimulation) under the
281 StageFlexer Jr. system equipped with a 18.5-mm cylindrical loading post; positive-control
282 cells received a calcium ionophore A23187 (3 μ M) in the media, according to Tatsumi et al.
283 (67). Calcium images were monitored at intervals of 3 sec under a Nikon ECLIPSE 80i
284 fluorescence microscope system (Tokyo, Japan) with an excitation wavelength of 488 nm
285 and a maximum emission wavelength of 526 nm. Fluorescence intensity was assigned

286 from blue to red in color-coded images by the installed Nikon computer software. After
 287 imaging, cells were fixed for 10 min in cold methanol containing 0.1% H₂O₂ and blocked
 288 with 5% normal rabbit serum containing 0.6% H₂O₂ in PBS for 20 min. Blocking was
 289 followed by immunostaining for the presence of c-met using R&D Systems goat polyclonal
 290 primary antibody (1:100 dilution in PBS containing 5% normal rabbit serum) and Histofine
 291 Simple Stain HRPO-labeled anti-goat IgG secondary antibody in order to determine if the
 292 calcium-imaged cells were myogenic.

293

294 **Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was purified
 295 from cultured satellite cells using an RNeasy Micro kit according to the manufacturer's
 296 recommendation. cDNA was synthesized from total RNA by a reverse-transcriptase
 297 SuperScript III using oligo-dT primer. PCR was performed using ExTaq DNA polymerase
 298 on the mRNA expression of α_{1S} , α_{1C} , α_{1D} and α_{1F} subunits of L-VGC-channels (accession
 299 no. NM_053873, NM_012517, NM_017298, and NM_053701, respectively) and transient
 300 receptor potential canonical channel (TRPC) 1 and 6 (accession no. NM_053558 and
 301 NM_053559, respectively). The intron-spanning primer sets designed by Primer3
 302 software were as follows: for rat α_{1S} subunit, forward
 303 5'-ACATCTTTGTGGGCTTCGTC-3', reverse 5'-TCCGACTGGTTGTAATGCTG-3',
 304 annealing temperature 62°C, amplicon 259 nucleotides (nt); for rat α_{1C} subunit, forward
 305 5'-CTCGAAGTTGGGAGAACAGC-3', reverse 5'-GACGAAACCCACGAAGATGT-3',
 306 annealing temperature 60°C, amplicon 239 nt; for rat α_{1D} subunit, forward
 307 5'-GCAGCACTATGAGCAATCCA-3', reverse 5'-CGGAAAAGACGGAAAAAGGT-3',
 308 annealing temperature 60°C, amplicon 249 nt; for rat α_{1F} subunit, forward
 309 5'-GCTGCTCAGTAAGGGTGAGG-3', reverse 5'-GTCCTGAAGAGCCACTTTGC-3',
 310 annealing temperature 62°C, amplicon 154 nt; for rat TRPC1, forward
 311 5'-CGGCAGAATCATTACACAC-3', reverse 5'-CCAACCCTTCATACCACAGC-3',
 312 annealing temperature 57°C, amplicon 215 nt; for rat TRPC6, forward
 313 5'-TGAAATTCCTCGTGGTCCTC-3', reverse 5'-GAGCTTGGTGCCTTCAAATC-3',
 314 annealing temperature 57°C, amplicon 197 nt. The annealing temperatures and cycle
 315 numbers were optimized so that amplification reactions were within the linear range. The
 316 PCR products were visualized by 1.5% agarose gel electrophoresis with 0.01% Gel Red
 317 reagent.

318

319 ***Statistical analysis.*** Analysis of variance procedures were employed to analyze
320 experimental results using the general linear model procedures of SRISTAT2 for Windows
321 software (Social Survey Research Information, Tokyo, Japan). Least-squares means for
322 each treatment were separated, based on least significant differences. Data were
323 represented as mean and standard error for four cultures per treatment, and statistically
324 significant differences from the mean of control cultures at $p < 0.05$ and $p < 0.01$ were
325 indicated by (*) and (**), respectively. Each experiment was repeated two or three times
326 to verify the reproducibility of results and, in most cases, one rat was used for each
327 experiment.

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330

331 **RESULTS**

332 It is now clear that, in attempting to construct a cascade pathway that translates
333 mechanical perturbation of quiescent satellite cells or muscle fibers into an activation signal,
334 calcium-calmodulin formation is required for NOS activation that produces the NO radical
335 from L-arginine and which subsequently releases HGF from extracellular tethering (for
336 review see Ref. 66). Therefore, the purpose of this study was to clarify a mechanism that
337 responds to mechanical perturbation of stretch by increasing intracellular calcium-ion
338 concentrations in satellite-cell cultures.

339 The first experiment was designed to examine the significance of extracellular
340 calcium-ion influx in our stretch-activation model of satellite cells in vitro (Fig. 1). Satellite
341 cells were prepared from adult rat skeletal muscles and applied to cyclic-stretch cultures (25%
342 stretch at 12-sec intervals as shown in panel *A*) for 1 hr beginning at 24-hr post-plating in
343 DMEM-10% HS (pH 7.2) with or without 1.8 mM EGTA (calcium chelator that does not
344 enter into the cytosol), followed by the BrdU-incorporation assay at 48-hr post-plating (panel
345 *B*). This concentration of EGTA decreases free calcium ion concentrations in the culture
346 media (1.8 mM) to about 10 μ M and without diminishing cell viability, even through the last
347 24-hr period, as monitored by effects on cell density and BrdU-incorporation at the 48-hr
348 time-point of the assay (*black bar e* and *open bar i*); therefore EGTA was applied to the pilot
349 experiments to determine the dependence of activation on the level of extracellular calcium.
350 Results clearly showed that EGTA treatment abolished stretch-activation (*black bar d*) down
351 to a level equivalent to that in un-stretched control cultures without (*open bar a*) and with 1.8
352 mM EGTA (*open bar h*). The 1-hr cyclic-stretch cultures (*black bar c*) showed activating
353 activity comparable to cultures receiving 2.5 ng/ml HGF for 24 hr from 24-hr post-plating in
354 the absence or presence of 1.8 mM EGTA (*gray bars f* and *g*, respectively), thereby serving as
355 important controls for other treatment groups in this assay. Together these results provide
356 evidence that extracellular calcium ions are essential in stretch-induced activation of satellite
357 cells in primary culture.

358 This issue was further examined by studying the effect of cation-channel inhibitors on
359 stretch-activation of satellite cells (Fig. 2 panel *A*). In this experiment, stretch was applied to
360 satellite-cell cultures for 1 hr beginning 24-hr post-plating in the presence of an increasing
361 concentration of one of the selective inhibitors for MS-channels, L-VGC-channels and T-type
362 VGC-channels (0.01-1 μ M GsMTx-4, 0.1-10 μ M nifedipine and 1-100 μ M NNC55-0396,
363 respectively) or a less specific classic-inhibitor for MS-channels (0.1-100 μ M gadolinium

364 chloride), and then subsequently assayed for satellite-cell activation at 48-hr post-plating as
365 described previously. The T-VGC-channel inhibitor NNC55-0396 (*open square*) did not
366 show a significant effect on activation in the range examined here or in another report (29, 55).
367 Exposures to one of the other three cation-channel inhibitors all abolished stretch-stimulated
368 activation in a dose-dependent manner. GsMTx-4 (*closed circle*) was the most powerful of
369 the three inhibitors, with blocking at an optimal concentration of 0.1 μM versus 10 μM for
370 nifedipine (*closed square*) and 100 μM for Gd (*open circle*). None of these cation-channel
371 inhibitors diminished cell viability, again as monitored at 48-hr by cell density and in
372 comparison to the BrdU-labeling of control cultures receiving cation-channel inhibitors (*open*
373 *bars h-j*), and by results showing that addition of HGF (2.5 ng/ml) to individual-inhibitor
374 cultures (*gray bars d-g*) stimulated satellite cell activation to a level equivalent to HGF (*gray*
375 *bar c*) and stretch (*black bar b*). These observations, therefore, indicated that both MS- and
376 L-VGC-channels are essential in the stretch activation of satellite cells and may be mediating
377 the influx of extracellular calcium ions in response to mechanical stretch. Also, as shown in
378 Fig. 2 panel *B*, the implication of MS- and L-VGC-channels in the activation cascade was
379 further emphasized by western blot detection of the 60 kDa α -chain of HGF in conditioned
380 media (serum-free DMEM, pH 7.2); media were collected from the 1-hr stretch cultures that
381 had received the optimal dose of GsMTx-4 (0.1 μM), nifedipine (10 μM), or EGTA (1.8 mM)
382 and assayed for HGF. Results qualitatively demonstrated that the cyclic stretch culture
383 stimulates HGF release (consistent with our original results) (*lane m*), and even in the
384 presence of stretch, GsMTx-4 (*lane n*), nifedipine (*lane o*) and EGTA (*lane p*) diminish
385 release of HGF with an apparently similar potency. This indicates that HGF release from
386 extracellular matrix depends on the up-stream MS- and L-VGC-channel activities. In fact,
387 the messages of α_{1S} and α_{1C} subunits of L-VGC-channels were detected by RT-PCR analysis
388 for satellite cells at 24-hr post-plating, which is the time at which the stretch treatment was
389 applied in the present and our previous experiments (65, 75-77) (Fig. 3 panel *A*). Because
390 L-VGC-channel proteins are now classified into $\text{Ca}_{v1.1}$, $\text{Ca}_{v1.2}$, $\text{Ca}_{v1.3}$ and $\text{Ca}_{v1.4}$ according to
391 their corresponding α_1 -subunits, α_{1S} , α_{1C} , α_{1D} and α_{1F} (34, 36), our PCR results indicate that
392 $\text{Ca}_{v1.1}$ and $\text{Ca}_{v1.2}$ may exist in quiescent satellite cells and be responsible for calcium ion influx
393 by coupling with MS-channel gating that senses mechanical perturbation of satellite cells.

394 Finally in a series of extracellular calcium ion dependency experiments, calcium ion
395 influx was documented by analysis of calcium imaging studies for two-cycle stretched
396 satellite cells at 24-hr post-plating in the presence or absence of cation-channel inhibitors (Fig.

397 4). Calcium ionophore treatment (3 μ M A23187, without stretch or treatment with channel
398 inhibitors) stimulated increase in intracellular calcium ion concentrations, consistent with our
399 previous result (67) and therefore served as a positive control within this assay (*row A*).
400 Cyclic stretch also affects calcium ion influx as shown in *row B*, in which the fluorescence
401 intensity reached a maximum at 30-60 sec post-stretch and the calcium signal maintained a
402 plateau within at least 210 sec (far-right image) and typically 300 sec of the maximum
403 recording time-point of the present analysis (image not shown), and that response was almost
404 abolished to reach a level comparable to 1.8 mM EGTA-treated cultures when stretch cultures
405 received 0.1 μ M GsMTx-4 or 10 μ M nifedipine (*rows C and D*, respectively). These
406 observations provide direct evidence that both MS- and L-VGC-channels are essential in
407 mediating the extracellular calcium ion influx in response to mechanical stretch in skeletal
408 muscle satellite cells in culture.
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