European Journal of Pharmacology 609 (2009) 105–112

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular Pharmacology

Telmisartan, an angiotensin II type 1 receptor antagonist, attenuates T-type Ca^{2+} channel expression in neonatal rat cardiomyocytes

Masaki Morishima [a,*,1], Yan Wang [a,1], Yuko Akiyoshi [a,b], Shinji Miyamoto [b], Katsushige Ono [a]

[a] Department of Pathophysiology, Oita University School of Medicine, Oita 879-5593, Japan
[b] Department of Cardiovascular Surgery, Oita University School of Medicine, Oita 879-5593, Japan

ARTICLE INFO

Article history:
Received 28 November 2008
Received in revised form 25 February 2009
Accepted 3 March 2009
Available online 16 March 2009

Keywords:
T-type Ca^{2+} channel
Angiotensin II
p38MAPK

ABSTRACT

Recently, it has been revealed that angiotensin II type 1 receptor (AT_1) antagonists act as antiarrhythmic agents and that the T-type Ca^{2+} channel plays an important role in arrhythmia. However, it remains unclear how the T-type Ca^{2+} channel expression system is involved in angiotensin II-mediated arrhythmogenesis in cardiomyocytes. In this study, we investigated the effect of telmisartan, an AT_1 receptor antagonist, on transcriptional regulation of T-type Ca^{2+} channel isoform ($Ca_v3.1$ and $Ca_v3.2$) expression and cardiac contractility using rat neonatal cardiomyocytes. Cultured cardiomyocytes were stimulated with telmisartan and/or angiotensin II for 24 h. T-type Ca^{2+} currents ($I_{Ca.T}$) were then measured with the patch clamp technique, while $Ca_v3.1$ and $Ca_v3.2$ mRNA expression were assessed by real-time PCR. Expression of $Ca_v3.1$ and $Ca_v3.2$ mRNA as well as $I_{Ca.T}$ current density in cardiomyocytes increased significantly after long-term application of angiotensin II (24 h), which was accompanied by extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) phosphorylation. In contrast, telmisartan decreased $Ca_v3.1$ and $Ca_v3.2$ mRNA expression as well as $I_{Ca.T}$ in a dose-dependent manner in the absence of angiotensin II. In addition, the basal phosphorylation level of p38MAPK but not ERK1/2 was decreased by telmisartan in the absence of angiotensin II. Valsartan, an AT_1 receptor antagonist, did not mimic the action of telmisartan, while the action of telmisartan was completely blocked by valsartan. These results indicate that telmisartan attenuates T-type Ca^{2+} channel expression likely through p38MAPK activity in an agonist-independent manner, which suggests a novel pharmacological action of telmisartan.

1. Introduction

Activation of the renin–angiotensin system is well known to target vascular smooth muscle cells and elevate blood pressure leading to hypertension. The renin–angiotensin system has also recently been implicated in cellular hypertrophy as well as lethal arrhythmias because inhibitors of this pathway reduce the incidence of sudden death in patients with heart failure (Paradis et al., 2000; Kacimi and Gerdes, 2003; Metha and Griendling, 2007). The primary effective molecule of this system, angiotensin II, has emerged as a critical hormone that affects the function of many cell types and organs, including the heart, kidney, and vasculature (Geisterfer et al., 1988; Bkaily et al., 2005). In addition, angiotensin II is also involved in various aspects of cardiac remodeling, which plays an important role in the development of arrhythmias (Nakashima et al., 2000; Ferron et al., 2003; Kumagai et al., 2003). Recently, Healey et al. observed that renin–angiotensin system inhibition by an AT_1 receptor antagonist not only reduced blood pressure but also prevented new-onset atrial fibrillation (Healey et al., 2005; Saygili et al., 2007). AT_1 receptor antagonists also have been shown to promote regression of left ventricular hypertrophy and to decrease cardiovascular morbidity (Zou et al., 2004). Although AT_1 receptor antagonists have both direct and indirect effects on the cardiovascular system, AT_1 receptor antagonists have a direct effect to prevent structural as well as electrical remodeling in cardiomyocytes, resulting in reversal of cardiac hypertrophy (Zou et al., 2004; Saygili et al., 2007), reduction of tissue fibrosis (Bueno and Molkentin, 2002; Kumagai et al., 2003), and prevention of atrial and ventricular arrhythmias (Nakashima et al., 2000).

Cardiac electrical remodeling is often associated with cellular Ca^{2+} overload, resulting in abnormal up-regulation or down-regulation of several ion channels in cardiomyocytes (Ferron et al., 2003; Takebayashi et al., 2006; Vassort et al., 2006). In the heart, two types of voltage-dependent Ca^{2+} channels, the L-type and the T-type Ca^{2+} channel, act to bring Ca^{2+} into the myocytes. It is suggested that T-type Ca^{2+} channels play a physiological role in cardiac automaticity and cell growth (Zhang et al., 2003; Vassort et al., 2006). Under pathological conditions such as pressure-overload cardiac hypertrophy, myocardial infraction, and heart failure, T-type Ca^{2+} currents (I_{CaT}) are functionally re-expressed in ventricular myocytes (Fareh et al., 1999; Ferron et al., 2003; Takebayashi

* Corresponding author. Department of Pathophysiology, Oita University School of Medicine, 1-1 Idaigaoka, Hasama, Yufu, Oita 879-5593, Japan. Tel.: +81 97 586 5652; fax: +81 97 586 6646.
E-mail address: mmoris@med.oita-u.ac.jp (M. Morishima).
[1] These authors equally contributed to this study.

0014-2999/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2009.03.024

et al., 2006), which are reportedly associated with increased renin–angiotensin system activation (Nakashima et al., 2000; Ferron et al., 2003). Indeed, changes of electrical properties in hearts prone to arrhythmias are related to underlying changes in expression of several ion channel genes, including T-type Ca^{2+} channels (Borlak and Thum, 2003; Ferron et al., 2003). Alternatively, suppression or reverse electrical remodeling of the myocyte may reduce the elevated risk of arrhythmias risks that are caused by angiotensin II-mediated ion channel transcriptional alterations (Li et al., 2001; Shang et al., 2007). Moreover, it is recently recognized that voltage-gated Ca^{2+} channels including the T-type Ca^{2+} channel contribute to pulmonary vein pacemaker activity that are responsible to initiate paroxysmal atrial fibrillation (Chen et al., 2004). Although the antiarrhythmic actions of AT_1 receptor antagonists have been recently demonstrated (Kumagai et al., 2003; Zou et al., 2004), it is largely unknown whether and how the T-type Ca^{2+} channel expression system is involved in angiotensin II-mediated arrhythmogenesis in cardiomyocytes.

Telmisartan, one of the clinically available AT_1 receptor antagonists, is highly selective for the AT_1 receptor and has unique pharmacological properties characterized by a long duration of action and high lipophilicity (Derosa et al., 2004). Recently, the molecular mechanism underlying inverse agonist activity of AT_1 receptor antagonists binding to AT_1 receptors has been demonstrated (Miura et al., 2006). Moreover, receptor-independent intracellular radical scavenging action of telmisartan has been proposed (Shao et al., 2007). Nevertheless, there is no therapeutic role for AT_1 receptor antagonists as an agonist-independent action to modulate arrhythmogenic substrates, which are related to the T-type Ca^{2+} channel remodeling in the heart.

In this study, we investigated the action of telmisartan, a member of the AT_1 receptor antagonist family supposedly possessing an agonist-independent action, focusing on the long-term effects on T-type Ca^{2+} channel expression in neonatal cardiac myocytes.

2. Materials and methods

2.1. Preparation and culture of neonatal cardiomyocytes

The experimental protocol was approved in advance by the Ethics Review Committee for Animal Experimentation of Oita University School of Medicine. Neonatal cardiomyocytes were prepared from 3- to 5-day-old Wistar rats as described previously (Wang et al., 2007). Twenty-four hours after plating, myocytes were incubated for 24 h with telmisartan (10^{-10} M to 10^{-6} M) or telmisartan with other signal inhibitors (see Inhibitor experiments section) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. The spontaneously beating myocytes were subjected to electrophysiological experiments 24 h after isolation.

2.2. Electrophysiological measurements

Whole-cell current clamp and voltage clamp experiments were performed as described previously (Wang et al., 2007). Action potentials were recorded at the sample rate of 1 kHz using EPC-8 software (HEKA Electronik, Lambrecht, Germany) and 400-series Power Lab with Chart v4 software (ADInstruments, Castle Hill, Australia). The L-type Ca^{2+} channel current ($I_{Ca.L}$) was recorded from a holding potential (V_{HP}) of −50 mV followed by various test potentials. The T-type Ca^{2+} channel current ($I_{Ca.T}$) was recorded by subtracting the current obtained at a V_{HP} of −50 mV from the current obtained at a V_{HP} of −100 mV in the same patch. All experiments were conducted at 37 °C.

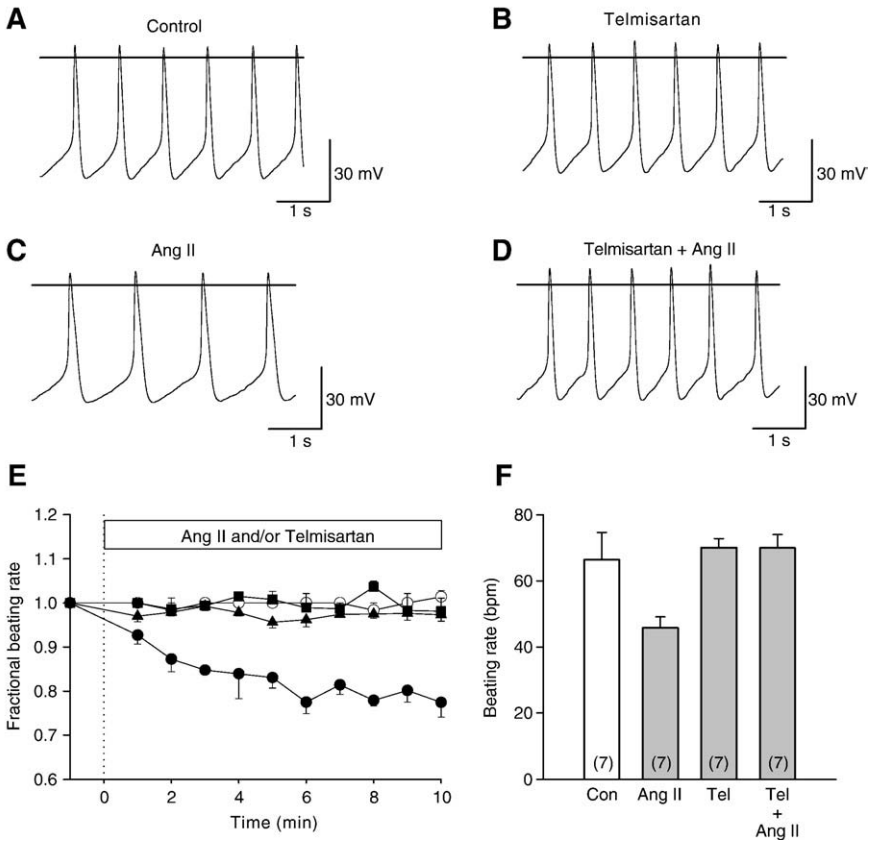

Fig. 1. Short-term effects of angiotensin (Ang) II and/or telmisartan on action potentials of neonatal rat cardiomyocytes. A–D, Representative recordings of action potentials are shown for the control myocytes (A), myocytes treated with 20 μM telmisartan (B), 0.1 μM angiotensin II (C), or telmisartan (20 μM) plus angiotensin II (0.1 μM) for 10 min (D). E, The time course of spontaneous beating rate in control myocytes (○, $n=7$), during application of 20 μM telmisartan (▲, $n=7$), 0.1 μM angiotensin II (●, $n=7$), or telmisartan (20 μM) plus angiotensin II (0.1 μM) (■, $n=7$). The beating rate was normalized to the initial rate (assigned a value of 1.0) in each group. F, Mean spontaneous beating rate (not normalized) of cardiomyocytes 10 min after application of vehicle, angiotensin II, telmisartan, or telmisartan plus angiotensin II. Data are expressed as the means ± S.E.M. *$P<0.05$ compared with the control group.

2.3. Solutions

For measuring membrane potentials, the bath solution (Tyrode's solution) contained (mM): NaCl 140, $MgCl_2$ 1, KCl 5.4, HEPES 10, $CaCl_2$ 1, and glucose 10 (pH of 7.4 adjusted with 1 M NaOH), and the pipette solution contained (mM): KCl 140, $MgCl_2$ 2, creatine phosphate 5, HEPES 10, EGTA 0.05, and Mg-ATP 5 (pH of 7.2 adjusted with 1 M KOH). For measuring $I_{Ca.L}$ and $I_{Ca.T}$, the bath solution was Na^+–K^+ free solution and contained (mM): TEA-Cl 120, CsCl 6, 4-AP 5, $MgCl_2$ 0.5, DIDS 0.1, HEPES 10, $CaCl_2$ 1.8, and glucose 10 (pH of 7.4 adjusted with TEA-OH). The pipette solution contained (mM): CsCl 130, Mg-ATP 2, EGTA 5, and HEPES 10 (pH of 7.2 adjusted with 1 M CsOH).

2.4. Chemicals

Angiotensin II, PD98059 [2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one], SB203580 [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole], and SP600125 [1,9-Pyrazoloanthrone] were purchased from Calbiochem (San Diego, CA). Valsartan, actinomycin D, and other all chemicals were from Wako Pure Chemical Industries (Osaka, Japan). Angiotensin II and actinomycin D were dissolved in distilled water, while PD98059, SB203580, and SP600125 were dissolved in dimethyl sulfoxide (DMSO) as stock solutions (5–20 mM), and then diluted to final concentrations in cell culture solutions. Telmisartan, a gift from Boehringer Ingelheim Co, was dissolved in DMSO to give to a stock solution of 20 mM. The final concentration of DMSO in the bathing solution was 0.01% or less. Prior to electrophysiological and real-time PCR analyses, these concentrations of DMSO were confirmed not to significantly influence $I_{Ca.T}$ or Ca_v3 mRNA expression.

2.5. Inhibitor experiments

Several inhibitors were used to identify the role of intracellular signaling pathways in the action of telmisartan. The concentration of these inhibitors used in this study was optimized based on the results of previous studies (Ferron et al., 2003; Tsai et al., 2007): PD98059 [a extracellular signal-regulated kinase (ERK)1/2,5 inhibitor] 10 μM, SB203580 [a p38 mitogen-activated protein kinase inhibitor] 1 μM, SP600125 [a c-Jun N-terminal kinase (JNK) inhibitor] 10 μM, and actinomycin D [nucleolus transcription mediated by RNA polymerase inhibitor] 5 μM. Each inhibitor was added alone or simultaneously with telmisartan (1 μM) or angiotensin II (0.1 μM).

2.6. Quantitative real-time PCR

Total RNA was extracted from rat neonatal myocytes using TRIzol (Invitrogen, Carlsbad, CA) 24 h after the treatment with agents described above. The single-stranded cDNA was synthesized from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular System Inc., Alameda, CA). Real-time PCR was performed on Light Cycler

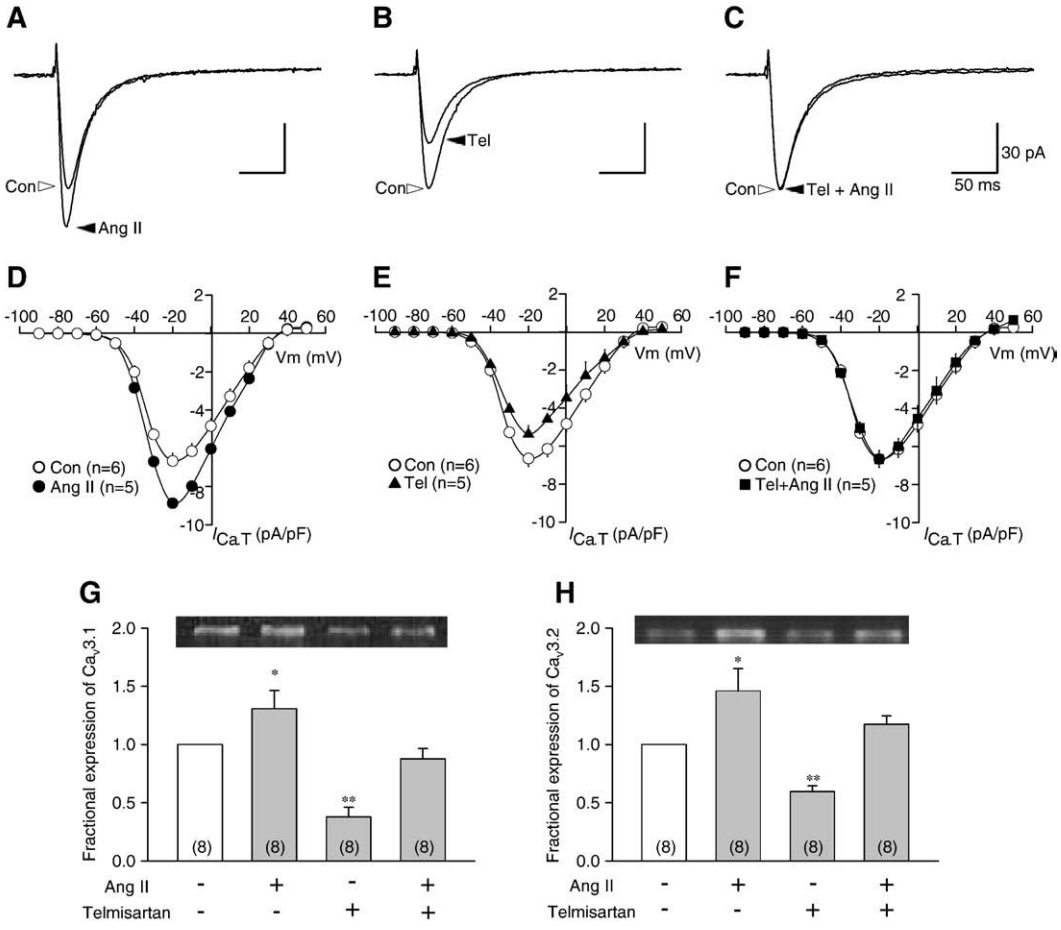

Fig. 2. Long-term effects of angiotensin (Ang) II and/or telmisartan on $I_{Ca.T}$ and the expression of T-type Ca^{2+} channel isoforms ($Ca_v3.1$, $Ca_v3.2$) mRNA in cardiomyocytes. A–C, Typical current traces of $I_{Ca.T}$ recorded from myocytes cultured in vehicle (Con), 0.1 μM angiotensin II (A), 1 μM telmisartan (Tel) (B), or telmisartan (1 μM) plus angiotensin II (0.1 μM) for 24 h (C) by subtracting the currents obtained at a V_{HP} of −40 mV from the ones obtained at a V_{HP} of −100 mV in the same patch. D–F, The current (I)–voltage (V) relationship of $I_{Ca.T}$ from myocytes under control conditions (○, $n=6$), with 0.1 μM angiotensin II (●, $n=5$), 1 μM telmisartan (▲, $n=5$), or telmisartan (1 μM) plus angiotensin II (0.1 μM) (■, $n=5$) for 24 h. Numbers of cells are indicated in parentheses. G, H, Fractional expression of $Ca_v3.1$ (G) and $Ca_v3.2$ (H) mRNA and their modulation by angiotensin II and/or telmisartan. Cardiomyocytes were preincubated with 1 μM telmisartan for 1 h and then stimulated with 0.1 μM angiotensin II for 24 h. Data were normalized to GAPDH (defined as 1.0 fold), and expressed as the mean ± S.E.M. ($n=8$). *$P<0.05$, **$P<0.01$ compared with the control group.

(Roche) using the FastStart DNA Master SYBR Green I (Roche) as a detection reagent. The primers for rat T-type Ca^{2+} channel α1G ($Ca_v3.1$) and α1H ($Ca_v3.2$) subunits were designed according to the published gene sequences [CACNA1G (GenBank accession no. AF027984; 5′-TCTCTAGGGCTATAGGCG-3′ and 5′-GGAGATTTTGCAGGAGCTAT-3′) and CACNA1H (GenBank accession no. AF290213; 5′-GGCGAAGA-AGGCAAAGCTGA-3′ and 5′-GCGTGACACTGGGCATGTT-3′), respectively] (Wang et al., 2007). Rat Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. M17701; 5′-GCCATCAACGACCCCTTCAT-3′ and 5′-TTCACACCCATCACAAACAT-3′) mRNA was used as an internal control. Data were calculated by $2^{-\Delta\Delta CT}$ and presented as fold change of transcripts for $Ca_v3.1$ and $Ca_v3.2$ genes in myocytes and normalized to GAPDH (defined as 1.0 fold). The size of the PCR products was confirmed by 2% agarose gel electrophoresis.

2.7. Assay of ERK1/2 and p38MAPK activity

ERK1/2 and p38MAPK activities were measured using the Cellular Activation of Signaling ELISA kit following the manufacture's protocol (Superarray Bioscience Corporation, Frederick, MD). Briefly, primary neonatal cardiomyocytes were seeded onto 96-well plates. After the treatment with telmisartan (1 μM) and/or angiotensin II (0.1 μM) for 24 h, the cells were fixed to preserve any activation-specific protein modification, such as phosphorylation. We used two primary antibodies in the kit: one antibody recognizes only the phosphorylated form (phospho-ERK1/2 and phosphor-p38MAPK antibodies) of the specific target protein, while the other recognizes the specific target protein (total-ERK1/2 and total-p38MAPK antibodies) regardless of the activation state. Following incubation with primary and secondary antibodies, the amount of bound antibody in each well was determined using a developing solution and detected at 450 nm on a spectrophotometer (TFCAN, Wako, Osaka, Japan). The absorbance readings were then normalized to relative cell number as system determined by a cell staining solution. The amount of phosphorylated protein, once normalized to the amount of total protein, was then directly related to the extent of downstream pathway activation.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. Between group and among group comparisons were conducted with one-way ANOVA followed by a Scheffe test. A P value <0.05 was considered significant.

3. Results

3.1. Short-term effects of telmisartan on action potential configuration in cardiomyocytes

Acute effects of angiotensin II and the AT_1 receptor antagonist, telmisartan, on action potential configuration were examined in rat neonatal cardiomyocytes (Fig. 1). Angiotensin II at a concentration of 0.1 μM significantly reduced the spontaneous beating rate of myocytes (Fig. 1C, F), and the effect was prevented by telmisartan (Fig. 1D, F). In contrast, telmisartan did not cause any significant changes in action potentials during the 10-min observation period of 10 min, indicating that this drug has no acute effect on pacemaker ion channels.

3.2. Changes in the Ca^{2+} channel expression in cultured myocytes

To investigate the long-term effect of telmisartan on cellular excitability, we measured $I_{Ca.L}$ and $I_{Ca.T}$ in primary cultured cardiomyocytes treated with telmisartan, angiotensin II, and telmisartan plus angiotensin

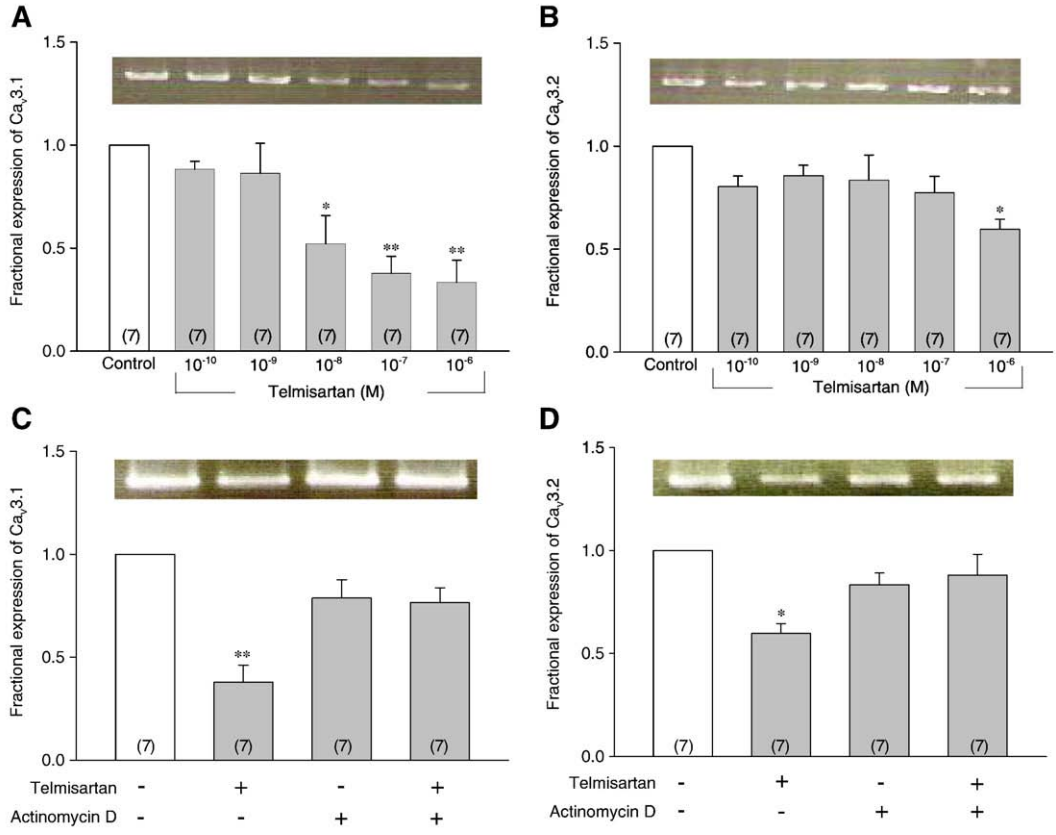

Fig. 3. Effects of telmisartan on the level of T-type Ca^{2+} channel isoforms ($Ca_v3.1$, $Ca_v3.2$) mRNA in neonatal rat cardiomyocytes. A, B, Dose-dependent changes of $Ca_v3.1$ (A) and $Ca_v3.2$ (B) mRNA by telmisartan treatment (10^{-10} M to10^{-6} M) for 24 h. C, D, Effects of transcriptional inhibition by 5 μM actinomycin D on $Ca_v3.1$ (C) and $Ca_v3.2$ (D) mRNA expression. Cardiomyocytes were preincubated with actinomycin D for 1 h and then stimulated with 1 μM telmisartan for 24 h. Data were normalized to GAPDH, and then expressed relative to vehicle-treated cardiomyocytes (defined as 1.0 fold). The results are shown as the mean ± S.E.M. ($n=7$). *$P<0.05$, **$P<0.01$ compared with the control group.

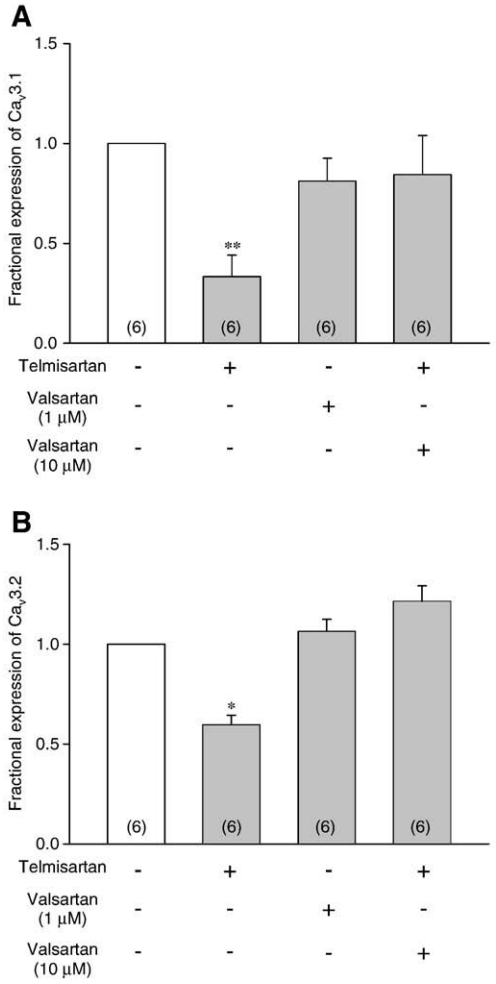

Fig. 4. Fractional expression of $Ca_v3.1$ (A) and $Ca_v3.2$ (B) mRNA and their modification by telmisartan and valsartan. A, B, Cardiomyocytes were treated with 1 μM telmisartan for 24 h, 1 μM valsartan for 24 h, or 10 μM valsartan for 1 h and then stimulated with 1 μM telmisartan for 24 h. Data were normalized to GAPDH (defined as 1.0 fold), and expressed as mean ± S.E.M. ($n = 6$). *$P<0.05$, **$P<0.01$ compared with the control group.

II (Fig. 2). As shown in Fig. 2, a long-term treatment of cardiomyocytes with angiotensin II (0.1 μM, 24 h) augmented the maximum $I_{Ca.T}$ (133.2 ± 6.8%, at −20 mV) without a shift of the current–voltage (*I–V*) relationship. In contrast, the addition of telmisartan (1 μM, 24 h) significantly decreased the I_{CaT} density (−19.3 ± 6.8%, at −20 mV). However, changes of $I_{Ca.T}$ by angiotensin II (0.1 μM) were not observed when myocytes were pretreated and concomitant application of telmisartan (1 μM) (Fig. 2C, F). Meanwhile $I_{Ca.L}$ was not modified by angiotensin II (0.1 μM), telmisartan (1 μM), or telmisartan (1 μM) plus angiotensin II (0.1 μM) for 24 h (data not shown).

3.3. Long-term effects of angiotensin II and telmisartan on the expression of T-type calcium channel subunits in cardiomyocytes

We thereafter investigated the effect of angiotensin II and telmisartan on the expression of T-type Ca^{2+} channel isoforms, $Ca_v3.1$ and $Ca_v3.2$, in cardiomyocytes by quantifying transcript amount (Fig. 3). Treatment of myocytes with angiotensin II (0.1 μM) significantly increased the level of $Ca_v3.1$ (130 ± 16%, $n = 8$, $P<0.05$) and $Ca_v3.2$ mRNA (146 ± 19%, $n = 8$, $P<0.05$) (Fig. 2G, H). The increase of $Ca_v3.1$ and $Ca_v3.2$ mRNA levels was consistent with the increase of $I_{Ca.T}$ density (Fig. 2D–F). Actinomycin D (5 μM), an inhibitor of nucleolus transcription mediated by RNA polymerase, suppressed augmentation of $Ca_v3.1$ and $Ca_v3.2$ mRNA by angiotensin II (data not shown). In contrast, pretreatment of myocytes with telmisartan completely blocked the action of angiotensin II. Importantly, telmisartan itself strongly suppressed the level of both $Ca_v3.1$ and $Ca_v3.2$ mRNA expression (Fig. 2G, H). We then examined whether a clinical concentration of telmisartan has an effect on T-type Ca^{2+} channel expression. Fig. 3A and B shows the dose–response relationship of the long-term action of telmisartan on $Ca_v3.1$ and $Ca_v3.2$ mRNA expression. A therapeutic concentration of telmisartan (10^{-9}–10^{-8} M) significantly depressed $Ca_v3.1$ but not $Ca_v3.2$ mRNA expression in cardiomyocytes (Fig. 3A, B), whereas no changes of the L-type Ca^{2+} channel isoforms ($Ca_v1.2$ and $Ca_v1.3$ mRNA) were observed (data not shown). The up-regulation of $Ca_v3.1$ and $Ca_v3.2$ mRNA by angiotensin II and the down-regulation of $Ca_v3.1$ and $Ca_v3.2$ mRNA by telmisartan combined with actinomycin D indicate that a transcriptional mechanism is indeed involved in this modulation (Fig. 3C, D). Furthermore, to investigate whether a similar effect was produced by other AT_1 receptor antagonists and whether the effect of telmisartan to reduce $Ca_v3.1$ and $Ca_v3.2$ was mediated by the AT_1 receptor, we examined the action of valsartan, which is an AT_1 receptor antagonist with a pharmacological profile similar to telmisartan. Valsartan (1–10 μM) did not modify expression of $Ca_v3.1$ and $Ca_v3.2$ mRNA expression, and importantly, the action of telmisartan was completely masked by the concomitant treatment with valsartan (Fig. 4). Thus, we speculate that the T-type Ca^{2+} channel reduction by telmisartan in cardiomyocytes is caused by a unique action of this drug that is unrelated to AT_1 receptor blockade.

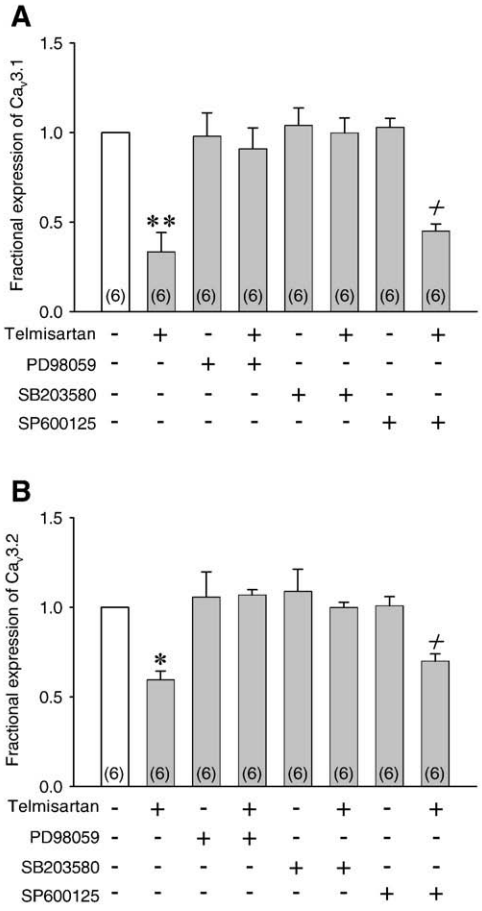

Fig. 5. Effects of an ERK1/2,5 inhibitor (PD98059, 10 μM), a p38MAPK inhibitor (SB203580, 1 μM), and a JNK inhibitor (SP600125, 10 μM) on the action of telmisartan in $Ca_v3.1$ (A) and $Ca_v3.2$ (B) mRNA. Cardiomyocytes were treated by these agents for 1 h and then stimulated with 1 μM telmisartan for 24 h. Data were normalized to GAPDH (defined as 1.0 fold), and expressed as the mean ± S.E.M. ($n = 6$). *$P<0.05$, **$P<0.01$ compared with the control group. +$P<0.05$ compared with SP600125 alone group.

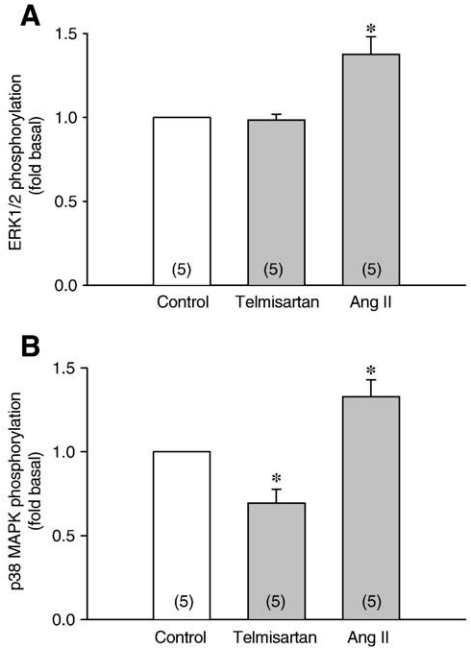

Fig. 6. Phosphorylation modulation of ERK1/2 and p38MAPK by telmisartan and angiotensin II. A, B, Amount of phosphorylated ERK1/2 (A) and p38MAPK (B) as assessed by ELISA in cardiomyocytes incubated with 1 μM telmisartan or 0.1 μM angiotensin II for 24 h. Data were expressed as the mean ± S.E.M. ($n = 5$). Data in A and B are representative of three independent experiments. *$P < 0.05$ compared with the control group.

3.4. Telmisartan attenuates the expression of the T-type Ca^{2+} channel in neonatal rat cardiomyocytes via the p38MAPK pathway

Extracellular signal-regulated kinase (ERK), p38MAPK, and c-Jun N-terminal kinase (JNK), belong to the mitogen-activated protein kinase (MAPK) family, and are generally activated as myocardial remodeling-related molecules. Furthermore, their activities are enhanced in cardiomyocytes by angiotensin II (Bueno et al., 2000). If telmisartan-induced reverse electrical remodeling is physiologically relevant, then blocking this pathway should affect T-type Ca^{2+} channel expression in the heart, and this is what was observed as shown in Fig. 5. Inhibitors of ERK1/2,5 (PD98059), p38MAPK (SB203580), and JNK (SP600125) were added to the culture medium of cardiomyocytes 1 h prior to 1 μM telmisartan treatment and left in the culture medium for 24 h until the cells were harvested. As shown in Fig. 5A and B, PD98059 and SB203580 but not SP600125 inhibited the action of telmisartan on $Ca_v3.1$ and $Ca_v3.2$ mRNA expression. These observations indicate that the transcriptional modulation of $Ca_v3.1$ and $Ca_v3.2$ by telmisartan is mediated either by the ERK 1/2 or p38MAPK pathway. In order to clarify this, we used ELISA to investigate the amount of phosphorylation of ERK1/2 and p38MAPK in cardiomyocytes treated with telmisartan (Fig. 6). Stimulation of cardiomyocytes with telmisartan (1 μM, 24 h) reduced the phosphorylation of p38MAPK but not ERK1/2 activity, whereas both ERK1/2 and p38MAPK phosphorylation levels were elevated by treatment with angiotensin II (0.1 μM, 24 h). Given these results, the p38MAPK pathway is postulated to play a crucial role in modulation of $Ca_v3.1$ and $Ca_v3.2$ T-type Ca^{2+} channel expression by long-term treatment of cardiomyocytes with telmisartan.

4. Discussion

The most striking observation in this study was that an AT_1 receptor antagonist, telmisartan, blunted the expression of low-voltage-activated T-type Ca^{2+} channels in an agonist-independent manner through the p38MAPK-dependent pathway in cardiomyocytes. We also demonstrated that telmisartan decelerated cardiac cellular automaticity by reducing $I_{Ca.T}$ distinctly suppressing the $Ca_v3.1$ isoform at a therapeutic concentration. Our results provide novel information that the AT_1 receptor could modulate the T-type Ca^{2+} channel, which may account for the electrical remodeling of cardiomyocytes in pathological conditions of the heart.

Many basic and clinical studies have demonstrated that the renin–angiotensin system plays a crucial role in the development of various cardiovascular diseases (Griendling et al., 1996; Kumagai et al., 2003; Zou et al., 2004). Angiotensin II is a well-known molecule of the renin–angiotensin system and plays an important role in cardiovascular homeostasis, including constriction of vascular smooth muscle cells, modulation of blood pressure, and cardiac hypertrophy and remodeling (Geisterfer et al., 1988; Mehta and Griendling, 2007). In cardiomyocytes, angiotensin II induces cardiac myocyte apoptosis and hypertrophy that contributes to heart failure possibly through enhanced oxidative stress (Ferron et al., 2003; Shang et al., 2007), all of which are hallmarks of myocardial fibrosis and remodeling (Geisterfer et al., 1988, Kumagai et al., 2003; Grobe et al., 2007). Members of the MAPK cascade such as ERK, JNK, and p38MAPK can all be activated by angiotensin II and are implicated as important regulators of pathological stress in the heart (Kacimi and Gerdes, 2003). Once activated in response to pathological stress, these kinases phosphorylate a wide array of intracellular targets that include numerous transcription factors resulting in the reprogramming of cardiac gene expression as part of the hypertrophic program in the heart (Grobe et al., 2007; Mehta and Griendling, 2007). In agreement with these studies, we found that the expression levels of T-type Ca^{2+} channels, $Ca_v3.1$ and $Ca_v3.2$ mRNA, in cardiomyocytes significantly increased after long-term administration of angiotensin II (24 h) accompanied by ERK1/2 and p38MAPK phosphorylation. Taken together, angiotensin II-induced augmentation of the T-type Ca^{2+} channels and its inhibition by telmisartan can be attributed, at least in part, to the effect of transcriptional regulation related to ERK 1/2 and p38MAPK phosphorylation.

In our study, there was a significant decrease of p38MAPK activity by telmisartan in the absence of angiotensin II in myocyte, which led to induce a reduction of $Ca_v3.1$ and $Ca_v3.2$ T-type Ca^{2+} channel expression. Meanwhile, SB203580, a p38MAPK inhibitor, blocked the inhibitory effect of telmisartan on expression of $Ca_v3.1$ as well as $Ca_v3.2$ mRNA but failed to decrease them by itself (Fig. 5 and 6). Taken together, it is postulated that a reduction of p38 MAPK activity is necessary but not sufficient to depress expression of the T-type Ca^{2+} channels. These observations lead us to consider that telmisartan not only exerts a reduction of p38MAPK phophorylation but also may affect other factor(s) to decrease expression of $Ca_v3.1$ and $Ca_v3.2$ channels probably in a synergistic manner. In the previous studies, it has been reported that p38MAPK signaling promotes multiple intracellular signal molecules including transcription factors, such as NF-κB (Raingeaud et al., 1996), and phosphorylation of several proteins, such as heat shock protein 27 (Chevalier and Allen, 2000) in the heart. We therefore assume that some other factor(s) are linked to p38MAPK activation in telmisartan-induced down-regulation of $Ca_v3.1$ and $Ca_v3.2$ in cardiomyocytes. Intriguingly, both p38MAPK and ERK1/2 activities were needed for GATA4 activation in the intact isolated heart in response to acute mechanical stretch (Tenhunen et al., 2004). Because GATA4 is known as a transcription factor responsible for diverse pathological stimuli including angiotensin II (Hautala et al., 2002), and because agonist-independent activity of AT_1 receptor in cardiomyocytes was associated with mechanical stretch, angiotensin II-induced augmentation of the T-type Ca^{2+} channels and its inhibition by telmisartan without any agonist activity can be attributed, at least in part, to the effect of transcriptional regulation related to ERK1/2 and p38MAPK phosphorylation.

Because telmisartan is known as a partial agonist of PPARγ, it is conceivable that telmisartan controls cardiac gene transcription by

direct interaction with the ligand binding domain of PPARγ in the nucleus (Imayama et al., 2006). Interestingly, Xing et al. reported that the PPARγ activation by pioglitazone inhibited p38MAPK phosphorylation, while the addition of pioglitazone did not change basal p38MAPK phosphorylation levels in microglia-enriched cultured cells (Xing et al., 2008). In our study, however, telmisartan down-regulated basal p38MAPK phosphorylation levels in the absence of angiotensin II in myocytes (Fig. 6). These findings lead us to conclude that inhibition of $Ca_v3.1$ and $Ca_v3.2$ by telmisartan may be independent of PPARγ receptor antagonism. Moreover, the lack of an effect of valsartan, another AT_1 receptor antagonist, on Ca_v3 expression and a complete block of telmisartan's effect by this agent firmly supports our conclusion that the action of telmisartan is independent of PPARγ activation in nucleus.

As far as we know so far, agonist-independent activity of AT_1 receptor in cardiomyocytes is associated solely with mechanical stretch. In this study, however, we demonstrate that agonist-independent activity may be present in the absence of exogenously applied stress, which suggests that according to the cellular effect considered the agonist-independent activated state of AT_1 receptor may be different. This hypothesis is supported by the observation that valsartan, one of those AT_1 receptor antagonists, which were shown to have inverse agonist properties (Miura et al., 2008) behaves as a neutral antagonist (Fig. 4). Since we only evaluated changes of T-type Ca^{2+} channel expression as an agonist-independent activity of AT_1 receptor without mechanical stretch, further study is obviously needed to reconfirm this mechanism.

Atrial fibrillation is the most common cardiac rhythm disorder found in clinical practice. Importantly, several experimental studies have shown that the T-type Ca^{2+} channel has a pivotal role in atrial and/or pulmonary vein myocytes for the generation and maintenance of atrial fibrillation (Fareh et al., 1999; Fareh et al., 2001; Ohashi et al., 2004; Chen et al., 2004). Moreover, recent experimental and clinical studies revealed that angiotensin-converting enzyme (ACE) inhibitors and AT_1 receptor blockers improved electrical dysfunction of the myocardium after successful cardioversion of atrial fibrillation to sinus rhythm pharmacological therapy by means of AT_1 receptor blockers prevented the shortening of effective refractory period and shortened the duration of induced atrial fibrillation episodes (Nakashima et al., 2000; Kumagai et al., 2003). Nevertheless, experimental data on the molecular mechanism of AT_1 receptor antagonists for the regulation of T-type Ca^{2+} channel expression with regard to atrial fibrillation prevention are still lacking. There is some limitation of this study that we used cardiomyocytes from neonatal heart but not from adult heart. It has been reported that T-type Ca^{2+} channels are developmentally regulated and that they are much less expressed in normal adult myocytes (Nuss and Houser, 1993): it is important to use neonatal cardiomyocytes, because of an abundantly expressed T-type Ca^{2+} channels during embryonic development in ventricular myocytes. The objective of this study was to determine if the expression of T-type Ca^{2+} channel is altered with a treatment of AT_1 receptor antagonist. In this context, we have successfully demonstrated up-regulation of the $Ca_v3.1$- and $Ca_v3.2$-T-type Ca^{2+} channel by angiotensin II and down-regulation of the $Ca_v3.1$-T-type Ca^{2+} channel by telmisartan (Fig. 2G, H). Our findings, particularly in combination with some previous studies that identified up-regulation of the T-type Ca^{2+} channels by angiotensin II, profile a possible mechanism for the treatment of atrial fibrillation by AT_1 receptor antagonists (Izumi et al., 2003; Ferron et al., 2003). The crucial question that needs to be answered is the evidence for the T-type Ca^{2+} channel in human atrial fibrillation. Despite clear evidence for the presence of mRNA, physiological and pathophysiological roles of $I_{Ca.T}$ are still controversial in the human myocardium (Cribbs, 1998). The direct clinical application of our findings will therefore have to await evidence supporting a role for $I_{Ca.T}$ in human atrial fibrillation.

In conclusion, our observations indicate a novel pharmacological action of an AT_1 receptor antagonist, telmisartan, in an agonist-independent manner on T-type Ca^{2+} channel expression. These results support the proposition that telmisartan could be an attractive drug for the treatment or prevention of atrial fibrillation by blunting p38MAPK signaling, which is related to the modulation of arrhythmogenic substrates such as the T-type Ca^{2+} channel in the heart.

Acknowledgements

This study was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (KAKEN, Nos. 15590759, 17590775) to K.O. M.M. was supported in part by a Japan Heart Foundation Young Investigator's Research Grant.

References

Bkaily, G.B., Sculptoreanu, A., Wang, S., Nader, M., Hazzouri, K.M., Jacques, D., Regoli, D., D'Orleans-Juste, P., Avedanian, L., 2005. Angiotensin II-induced increase of T-type Ca^{2+} current and decrease of L-type Ca^{2+} current in heart cells. Peptides 26, 1410–1417.

Borlak, J., Thum, T., 2003. Hallmarks of ion channel gene expression in end-stage heart failure. FASEB J. 17, 1592–1608.

Bueno, O.F., Molkentin, J.D., 2002. Involvement of extracellular signal-regulated kinase 1/2 in cardiac hypertrophy and cell death. Circ. Res. 91, 776–781.

Bueno, O.F., Windt, L.J.D., Tymitz, K.M., Witt, S.A., Kimball, T.R., Klevitsky, R., Hewett, T.E., Jones, S.P., Lefer, D.J., Peng, C.F., Kitsis, R.N., Molkentin, J.D., 2000. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO J. 19, 6341–6350.

Chen, Y.C., Chen, S.A., Chen, Y.J., Tai, C.T., Chan, P., Lin, C.I., 2004. T-type calcium current in electrical activity of cardiomyocytes isolated from rabbit pulmonary vein. J. Cardiovasc. Electrophysiol. 15, 567–571.

Chevalier, D., Allen, B.G., 2000. Two distinct forms of MAPKAP kinase-2 in adult cardiac ventricular myocytes. Biochemistry 39, 6145–6156.

Cribbs, L.L., 1998. T-type Ca^{2+} channels in vascular smooth muscle: multiple functions. Cell Calcium 40, 221–230.

Derosa, G., Ragonesi, P.D., Mugellini, A., Ciccarelli, L., Fogari, R., 2004. Effects of telmisartan compared with eprosartan on blood pressure control, glucose metabolism and lipid profile in hypertensive, type 2 diabetic patients: a randomized, double-blind, placebo-controlled 12-month study. Hypertens. Res. 27, 457–464.

Fareh, S., Bénardeau, A., Thibault, B., Nattel, S., 1999. The T-type Ca^{2+} channel blocker mibefradil prevents the development of a substrate for atrial fibrillation by tachycardia-induced atrial remodeling in dogs. Circulation 100, 2191–2197.

Fareh, S., Bénardeau, A., Nattel, S., 2001. Differential efficacy of L- and T-type calcium channel blockers in preventing tachycardia-induced atrial remodeling in dogs. Cardiovasc. Res. 49, 762–770.

Ferron, L., Capuano, V., Ruchon, Y., Deroubaixe, Coulombe, A., Renaud, J.F., 2003. Angiotensin II signaling pathways mediate expression of cardiac T-type calcium channels. Circ. Res. 93, 1241–1248.

Geisterfer, A.A.T., Peach, M.J., Owens, G.K., 1988. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circ. Res. 62, 749–756.

Griendling, K.K., Lassègue, B., Alexander, R.W., 1996. Angiotensin receptors and their therapeutic implications. Annu. Rev. Pharmacol. Toxico. 36, 281–306.

Grobe, J.L., Mecca, A.P., Lingis, M., Shenoy, V., Bolton, T.A., Machado, J.M., Speth, R.C., Raizada, M.K., Katovich, M.J., 2007. Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1–7). Am. J. Physiol. Heart Circ. Physiol. 292, H736–H742.

Hautala, N., Tenhunen, O., Szokodi, I., Ruskoaho, H., 2002. Direct left ventricular wall stretch activates GATA4 binding in perfused rat heart: involvement of autocrine/paracrine pathways. Pflugers Arch. 443, 362–369.

Healey, J.S., Baranchuk, A., Crystal, E., Morillo, C.A., Garfinkle, M., Yusuf, S., Connolly, S.J., 2005. Prevention of atrial fibrillation with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers: a meta-analysis. J. Am. Coll. Cardiol. 45, 1832–1839.

Imayama, I., Ichiki, T., Inanaga, K., Ohtsubo, H., Fukuyama, K., Ono, H., Hashiguchi, Y., Sunagawa, K., 2006. Telmisartan downregulates angiotensin II type 1 receptor through activation of peroxisome proliferators-activated receptor γ. Cardiovasc. Res. 72, 184–190.

Izumi, T., Kihara, Y., Sarai, N., Yoneda, T., Iwanaga, Y., Inagaki, K., Onozawa, Y., Takenaka, H., Kita, T., Noma, A., 2003. Reinduction of T-type calcium channels by endothelin-1 in failing hearts in vivo and in adult rat ventricular myocytes in vitro. Circulation 108, 2530–2535.

Kacimi, R., Gerdes, A.M., 2003. Alterations in G protein and MAP kinase signaling pathways during cardiac remodeling in hypertension and heart failure. Hypertension 41, 968–977.

Kumagai, K., Nakashima, H., Urata, H., Gondo, N., Arakawa, K., Saku, K., 2003. Effects of angiotensin II type I receptor antagonist on electrical and structural remodeling in atrial fibrillation. J. Am. Coll. Cardiol. 41, 2197–2204.

Li, M., Shinagawa, K., Li, P., Leung, T.K., Cardin, S., Wang, Z., Nattel, S., 2001. Effects of angiotensin-converting enzyme inhibition on the development of the atrial fibrillation substrate in dogs with ventricular tachypacing-induced congestive heart failure. Circulation 104, 2608–2614.

Mehta, P.K., Griendling, K.K., 2007. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am. J. Physiol. Cell. Physiol. 292, C82–C97.

Miura, S., Fujino, M., Hanzawa, H., Kiya, Y., Imaizumi, S., Matsuo, Y., Tomita, S., Uehara, Y., Karnik, S.S., Yanagisawa, H., Koike, H., Komuro, I., Saku, K., 2006. Molecular

mechanism underlying inverse agonist of angiotensin II type 1 receptor. J. Biol. Chem. 281, 19288–19295.
Miura, S., Kiya, Y., Kanazawa, T., Imaizumi, S., Fujino, M., Matsuo, Y., Karnik, S.S., Saku, K., 2008. Differential bonding interactions of inverse agonists of angiotensin II type 1 receptor in stabilizing the inactive state. Mol. Endocrinol. 22, 139–146.
Nakashima, H., Kumagai, K., Urata, H., Gondo, N., Ideishi, M., Arakawa, K., 2000. Angiotensin II antagonist prevents electrical remodeling in atrial fibrillation. Circulation 101, 2612–2617.
Nuss, H.B., Houser, S.R., 1993. T-type Ca^{2+} current is expressed in hypertrophied adult feline left ventricular myocytes. Circ. Res. 73, 777–782.
Ohashi, N., Mitamura, H., Tanimoto, K., Fukuda, Y., Kinebuchi, O., Kurita, Y., Shiroshita-Takeshita, A., Miyoshi, S., Hara, M., Takatsuki, S., Ogawa, S., 2004. A comparison between calcium channel blocking drugs with different potencies for T- and L-type channels in preventing atrial electrical remodeling. J. Cardiovasc. Pharmacol. 44, 386–392.
Paradis, P., Dali-Youcef, N., Paradis, F.W., Thibault, G., Nemer, M., 2000. Overexpression of angiotensin II type 1 receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. Proc. Natl. Acad. Sci. USA 95, 10140–10145.
Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B., Davis, R.J., 1996. MKK3- and MKK6-regulated gene expression is mediated by p38 mitogen-activated protein kinase signal transduction pathway. Mol. Cell. Biol. 16, 1247–1255.
Saygili, E., Rana, O.R., Saygili, E., Reuter, H., Frank, K., Schwinger, R.H.G., Muller-Ehmsen, J., Zobel, C., 2007. Losartan prevents stretch-induced electrical remodeling in cultured atrial neonatal myocytes. Am. J. Heart Circ. Physiol. 292, H2898–H2905.
Shang, L.L., Sanyal, S., Pfahnl, A.E., Jiao, Z., Allen, J., Liu, H., Dudley Jr., S.C., 2007. NF-κB-dependent transcriptional regulation of the cardiac scn5a sodium channel by angiotensin II. Am. J. Physiol. Cell. Physiol. 294, C372–C379.
Shao, J., Nangaku, M., Inagi, R., Kato, H., Miyata, T., Matsusaka, T., Noiri, E., Fujita, T., 2007. Receptor-independent intracellular radical scavenging activity of an angiotensin II receptor blocker. J. Hypertens. 25, 1643–1649.
Takebayashi, S., Li, Y., Kaku, T., Inagaki, S., Hashimoto, Y., Kimura, K., Miyamoto, S., Hadama, T., Ono, K., 2006. Remodeling excitation–contraction coupling of hypertrophied ventricular myocytes is dependent on T-type calcium channels expression. Biochem. Biophys. Res. Commun. 345, 766–773.
Tenhunen, O., Sarman, B., Kerkela, R., Szokodi, I., Papp, L., Toth, M., Ruskoaho, H., 2004. Mitogen-activated protein kinases p38 and ERK 1/2 mediate the wall stress-induced activation of GATA4 binding in adult heart. J. Biol. Chem. 279, 24852–24860.
Tsai, C.T., Wang, D.L., Chen, W.P., Hwang, J.J., Hsieh, C.S., Hsu, K.L., Tseng, C.D., Lai, L.P., Tseng, Y.Z., Chiang, F.T., Lin, J.L., 2007. Angiotensin II increases expression of α1C subunit of L-type calcium channel through a reactive oxygen species and cAMP response element-binding protein-dependent pathway in HL-1 myocytes. Circ. Res. 100, 1476–1485.
Vassort, G., Talavera, K., Alvarez, J.L., 2006. Role of T-type Ca^{2+} channels in the heart. Cell Calcium 40, 205–220.
Wang, Y., Morishima, M., Zheng, M., Uchino, T., Mannen, K., Takahashi, A., Nakaya, Y., Komuro, I., Ono, K., 2007. Transcription factors Csx/Nkx2.5 and GATA4 distinctly regulate expression of Ca^{2+} channels in neonatal rat heart. J. Mol. Cell. Cardiol. 42, 1045–1053.
Xing, B., Xin, T., Hunter, R.L., Bing, G., 2008. Pioglitazone inhibition of lipopolysaccharide-induced nitric oxide synthase is associated with altered activity of p38 MAP kinase and PI3K/Akt. J. Nueroinflammation 5, 1–11.
Zhang, Y.M., Shang, L., Hartzell, C., Narlow, M., Cribbs, L., Dudley Jr., S.C., 2003. Characterization and regulation of T-type Ca^{2+} channels in embryonic stem cell-derived cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol. 285, H2770–H2779.
Zou, Y., Akazawa, H., Qin, Y., Sano, M., Takano, H., Minamino, T., Makita, N., Iwanaga, K., Zhu, W., Kudoh, S., Toko, H., Tamura, K., Kihara, M., Nagai, T., Fukamizu, A., Umemura, S., Iiri, T., Fujita, T., Komuro, I., 2004. Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. Nat. Cell Biol. 6, 499–506.