Trp-His, a vasorelaxant di-peptide, can inhibit extracellular Ca^{2+} entry to rat vascular smooth muscle cells through blockade of dihydropyridine-like l-type Ca^{2+} channels

Wang, Zhengquan  
Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School of Kyushu University

Watanabe, Shimpei  
Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School of Kyushu University

Kobayashi, Yutaro  
Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School of Kyushu University

Tanaka, Mitsuru  
Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School of Kyushu University

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Trp-His, a vasorelaxant di-peptide, can inhibit extracellular Ca\(^{2+}\) entry to rat vascular smooth muscle cells through blockade of dihydropyridine-like L-type Ca\(^{2+}\) channels

Zhengquan Wang, Shimpei Watanabe, Yutaro Kobayashi, Mitsuru Tanaka, Toshiro Matsui*

*Corresponding author. Fax: +81-92-642-3012.

E-mail address: tmatsui@agr.kyushu-u.ac.jp (T. Matsui)
ABSTRACT

Our previous findings regarding the biological activities of small peptides revealed that a di-peptide, Trp-His (WH), could play a role in the prevention of vascular lesions, including cell proliferation and atherosclerosis. Its vasoprotective effects could be associated with suppression of the vasocontraction signaling cascade, but the underlying mechanism(s) remains obscure. In this study, we attempted to elucidate the vasoprotective mechanism of WH, in opposing the proliferation of rat vascular smooth muscle cells (VSMCs). In VSMCs from 8 week-old male Wistar rat thoracic aorta, WH evoked a significant dose-dependent anti-proliferation effect, without cytotoxicity. In mitogen-stimulated cell experiments, 300 μM WH inhibited cytosolic Ca\(^{2+}\) elevation in VSMCs induced by 10 μM angiotensin II (Ang II). Furthermore, WH suppressed extracellular Ca\(^{2+}\) entry into CaCl\(_2\)-stimulated VSMCs. The biological capacity of WH as an intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)_i]) suppressor was also proven when 50 μM Bay K8644 was used to enhance [Ca\(^{2+}\)_i] entry via a voltage-dependent L-type Ca\(^{2+}\) channel (VDCC) and 300 μM WH elicited a 23% reduction in [Ca\(^{2+}\)_i]. The absence of a reduction of the [Ca\(^{2+}\)_i] by the mixture of tryptophan and histidine revealed the importance of the peptide skeleton in the [Ca\(^{2+}\)_i] reduction effect. Furthermore, the WH-induced [Ca\(^{2+}\)_i] reduction was abolished by verapamil, but not by nifedipine, indicating that WH likely binds to an extracellular site of the VDCC at a site similar to that of the dihydropyridine type- Ca\(^{2+}\) channel blockers.

Keywords: Ca\(^{2+}\) channel blocker; small peptide; smooth muscle cells; anti-proliferation; anti-atherosclerosis
1. Introduction

Since the major cardiovascular diseases (CVDs), such as heart disease, cerebrovascular disease and hypertension, contribute to the increase of global mortality [1], there has been a growing focus on traditional drug therapy and alternative treatment by medicinal or functional foods in the prevention of CVDs [2]. A series of studies has provided insight into how small peptides, some of which are derived from nature, could play a role in preventing hypertension. A majority of the preventive effects of small peptides have focused on inhibition of the angiotensin I-converting enzyme or the suppression of the renin-angiotensin (RA) system [3]. Recent studies have provided us with a new insight on peptide functionalities regarding anti-hypertensive effects, in which some peptides, such as carnosine [4], Ile-Pro-Pro (IPP) [5] and Arg-Ala-Asp-His-Pro(-Phe) (RADHP(F)) [6,7] relaxed precontracted vessels and consequently enhanced blood flow.

A series of studies by our group have also revealed the possible involvement of small peptides in the regulation of vessel functions, since Val-Tyr (VY), which exhibited an anti-hypertensive effect in humans [8] via suppression of the RA systems in the aortae and kidney [9], and relaxation of precontracted rat aortae [10]. In our subsequent investigation, we demonstrated that Trp-His (WH) [11], as well as His-Arg-Trp (HRW) [12], caused a dose-dependent and endothelium-independent relaxation in pre-contracted rat aortic rings. To date, except for these three small peptides, no endothelium-independent vasoactive peptides have been reported, while other vasoactive peptides such as RADHP [6], RADHPF [7] and Met-Tyr (MY) [13] have been shown to evoke an endothelium-dependent relaxation. Vasoactive peptides that elicit endothelium-independent relaxation could be of benefit to prevent vessel disorders, such as atherosclerosis; the onset associated with hypertension is promoted by the proliferation and/or migration of VSMCs. Indeed, we firstly demonstrated that long-term administration of WH to apolipoprotein E-deficient mice led to a significant
reduction in atherosclerotic lesion formation [14]; although the anti-atherosclerotic mechanism induced by WH remains poorly understood.

The purpose of this study was, thus, to elucidate the underlying mechanism(s) of WH on the proliferation of VSMCs. Previous reports demonstrating that the endothelium-independent vasoactive peptides, VY and HRW, inhibited the elevation of cytosolic Ca\(^{2+}\) [12,15] allowed us to investigate a potential role of WH in the regulation of cytosolic Ca\(^{2+}\). We also examined a possible voltage-dependent L-type Ca\(^{2+}\) channel (VDCC) blocking mechanism of WH.

2. Materials and Methods

2.1. Materials

Fura-2/AM, Fluo-4/AM and WST-8 were purchased from Dojindo (Kumamoto, Japan). Angiotensin (Ang) II, S(-)-Bay K8644, (±)-verapamil, nimodipine and S(+)-PD 123177 were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-Aminoethoxydiphenylborate (2-APB) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Nifedipine was from Nacalai Tesque (Kyoto, Japan). WH was synthesized by an Fmoc-solid phase synthesis method according to the manufacturer’s instructions. All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. Cell cultures

VSMCs were isolated from rat thoracic aortae by enzymatic digestion, as described by Griendling et al. [16]. The thoracic aorta was removed aseptically from an 8 week-old male Wistar rat (Charles River Japan, Kanagawa, Japan) and was gently cleaned of fat in sterile physiological salt solution (PSS, pH 7.4, NaCl 145, KCl 5, Na\(_2\)HPO\(_4\) 1, CaCl\(_2\) 2.5, MgSO\(_4\) 0.5, glucose 10, and HEPES 5 mM). The endothelium was denuded by rubbing with sterile gauze in PSS buffer. The vessels were then incubated in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen,
Carlsbad, CA, USA) containing 315 units/ml collagenase and 10 units/ml elastase for 30 min at 37°C. The adventitia was then removed by sterile gauze in DMEM. The digestion was terminated by the addition of 10% fetal bovine serum (FBS, Invitrogen) containing DMEM, and the cells were then pelleted by centrifugation. VSMCs were plated at $1 \times 10^4$ cells/cm$^2$ in DMEM containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin (Meiji Seika, Tokyo, Japan) and 100 µg/ml streptomycin (Nacalai Tesque), and were gradually reduced to 10% FBS after two or three passages. Cells were maintained at 37°C in a humidified 5% CO$_2$ incubator. Passage numbers 5 to 7 were used in this study.

2.3. Cell proliferation assay

Cell numbers were determined by the Trypan blue (0.4% Trypan blue stain, Dojindo) dye exclusion method. VSMCs were seeded in 24-well tissue culture plates (500 cells/well) followed by a 24 h-attachment. After the cells were made quiescent by 24 h-serum starvation, the medium was replaced with 10% FBS/DMEM in the presence or absence of peptide. On day 5 of incubation the number of viable VSMCs was counted using a hemocytometer.

2.4. Cytotoxicity assay

The cytotoxicity of WH on VSMCs was determined using a cell counting WST-8. VSMCs were seeded on to 96-well tissue culture plates (500 cells/well). After 24 h-attachment and serum starvation, the medium was replaced with serum-free DMEM containing 1 mM WH and the cells were incubated for an additional 24 h at 37°C. The cells were then exposed to WST-8 reagent for 4 h at 37°C. Cell viability was determined by measuring the absorbance at 450 nm using a Wallac 1420 Multilabel counter (Perkin Elmer, Tokyo, Japan).

2.5. Measurement of intracellular Ca$^{2+}$ level ([Ca$^{2+}$]$_i$) in VSMCs

Measurement of [Ca$^{2+}$]$_i$ was performed using a Ca$^{2+}$-sensitive probe (Fura-2/AM)
as described previously [12], with one modification. Briefly, the modification involved cell deposition by centrifugation for 1 min at 1000 g; the detached cells were incubated with PSS buffer with 0.1% DMSO and 0.04% Cremophor EL (Nacalai Tesque) containing 1 µM Fura-2/AM for 30 min×2 and the probe-loaded cells were washed twice with PSS buffer. Ang II (10 µM) or Bay K8644 (50 µM) was then injected into the cuvette containing the probe-loaded cells (1×10^5 cells/ml) using a micro-syringe during real-time monitoring of fluorescence intensities with a fluorescent spectrometer (RF-5300PC, Shimadzu, Kyoto, Japan). The Ang II type2 (AT₂)-receptor antagonist, PD 123177 (1 µM), was added 10 min before the addition of Ang II to avoid activation of vasorelaxation-signaling pathways via the AT₂-receptor [17]. In order to focus on any [Ca^{2+}]_i increase from extracellular Ca^{2+} influx in the CaCl₂-stimulated VSMCs experiments, the probe-loaded cells were added into Ca^{2+}-free PSS buffer treated with 0.1 mM EGTA, and PD 123177 (1 µM) and 2-APB (200 µM) as an IP₃-receptor antagonist [18] were then added 10 min before the addition of Ang II (10 µM). After the addition of Ang II, 3 min pre-incubation with WH followed by stimulation with 2.5 mM CaCl₂ was performed. For the combination experiments of WH or a VDCC blocker (10 nM verapamil, 0.5 nM nifedipine or 0.5 nM nimodipine) with a VDCC blocker, Bay K8644 solution containing one of the blockers was added after a 10 min-pre-incubation with WH or the VDCC blocker. The [Ca^{2+}]_i was calculated according to a previous report by Grynkiewicz et al.[19] at the dual excitation wavelength of 340/380 nm and the emission wavelength of 500 nm.

2.6. Confocal microscopy

VSMCs (1×10^5 cells/dish) were cultured on a µ-Dish 35 mm (Ibidi GmbH, Martinsried, Germany) with 10% FBS/DMEM and the medium was changed to serum-free medium 24 h prior to the measurement. Thereafter, VSMCs were incubated in PSS buffer with 0.5% DMSO and 0.02% Cremophor EL containing 10 µM Fluo-4/AM at 37°C for 60 min and rinsed 3 times with PSS buffer followed by
incubation in the dark prior to the addition of 300 µM peptide (or its corresponding amino acids). After addition of the peptide, the cells were pre-incubated for 10 min. Bay K8644 (50 µM) was used to stimulate [Ca\textsuperscript{2+}]\textsubscript{i} in VSMCs maintained under the same experimental conditions described above. The stimulated VSMCs in the presence or absence of peptide were visualized using a Nikon confocal microscope (Nikon, Tokyo, Japan).

2.7. Statistical analyses

The results are expressed as the mean ± S.E.M. Statistical differences between the two groups were evaluated by unpaired Student’s t-test, and analyses between multiple groups were conducted using one-way analysis of variance (ANOVA) followed by post-hoc Tukey-Kramer’s t-test. A P value < 0.05 was considered statistically significant. All analyses were performed with Stat View J 5.0 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Inhibition of serum-stimulated proliferation of VSMCs by WH

Quiescent rat VSMCs were exposed to 10% FBS/DMEM with WH (0.01, 0.1, 0.5 and 1 mM) for 5 days to evaluate the anti-proliferative effect of WH. As shown in Fig. 1A, WH exhibited a dose-dependent anti-proliferative effect on VSMCs. Under our cell experiment conditions, a dose greater than 0.1 mM WH was required for this anti-proliferative effect; 0.5 mM WH exhibited a 43% decrease in proliferation of VSMCs (control: 4.7 ± 0.3×10\textsuperscript{4} cells/ml, 0.5 mM WH: 2.7 ± 0.4×10\textsuperscript{4} cells/ml, 1 mM WH: 1.8 ± 0.2×10\textsuperscript{4} cells/ml, n=4-8).

3.2. Cytotoxicity of WH in serum-free VSMCs

The cytotoxic effects of 1 mM WH were evaluated on serum-free VSMCs using the WST-8 incorporation assay. Although 1 mM WH exhibited a 61% decrease in
serum-stimulated proliferation of VSMCs (Fig. 1A), there were no significant changes in cell viability between the control and WH (1 mM)-groups (Fig. 1B), indicating that the anti-proliferative effects of WH on VSMCs occurred far below those required for its toxic or apoptotic effects.

3.3. WH inhibited the Ang II-stimulated \([Ca^{2+}]_i\) elevation in VSMCs

The \([Ca^{2+}]_i\) measurements in Ang II-stimulated VSMCs were performed using the \(Ca^{2+}\)-sensitive probe, Fura-2, to evaluate any WH-induced anti-proliferative effects. PD 123177 (1 µM), a selective AT\(_2\)-receptor antagonist [17], was used during the Ang II-stimulation cell experiments to prevent any relaxant signaling response via AT\(_2\)-receptors or due to any unexpected \([Ca^{2+}]_i\) decrease. As shown in Fig. 2A, WH markedly suppressed the magnitude of \([Ca^{2+}]_i\) elevation by Ang II under the present conditions (\(\Delta[Ca^{2+}]_i\): control; 620.0 ± 21.4 nM, 100 and 300 µM WH; 273.6 ± 55.7 and 53.3 ± 11.6 nM, respectively, \(P<0.01\) vs. control, \(n=3\)).

3.4. WH inhibited CaCl\(_2\)-stimulated \([Ca^{2+}]_i\) elevation in VSMCs

Prior to the CaCl\(_2\)-induced \([Ca^{2+}]_i\) measurements, \(Ca^{2+}\) release from the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) was completely abolished by 200 µM 2-APB, with which no increase in \([Ca^{2+}]_i\) from the ER/SR was confirmed by the addition of 10 µM Ang II in the presence of 1 µM PD 123177 (Fig. 2B). The subsequent addition of CaCl\(_2\) at a concentration of 2.5 mM to VSMCs led to a long-lasting \([Ca^{2+}]_i\) elevation. However, pre-incubation with 300 µM WH led to limited elevation of \([Ca^{2+}]_i\). A significant difference (\(P<0.05\)) was observed in the elevation of \([Ca^{2+}]_i\) by CaCl\(_2\) in the presence (\(\Delta[Ca^{2+}]_i\): 174.1 ± 12.0 nM, \(n=4\)) and absence of 300 µM WH (\(\Delta[Ca^{2+}]_i\): 254.9 ± 21.1 nM, \(n=6\)) (Fig. 2B).

3.5. WH inhibited Bay K8644-stimulated \([Ca^{2+}]_i\) elevation in VSMCs

As shown in Fig. 3, the addition of 50 µM Bay K8644 (a VDCC agonist) to VSMCs yielded a progressive increase in \([Ca^{2+}]_i\) (\(\Delta[Ca^{2+}]_i\): 83.7 ± 1.3 nM, \(n=4\)). By
contrast, 300 µM WH significantly ($P<0.01$) abolished the Bay K8644-induced $[\text{Ca}^{2+}]_i$ elevation ($\Delta[\text{Ca}^{2+}]_i$: 64.3 ± 3.0 nM, $n=5$), while a mixture of its corresponding amino acids (i.e., tryptophan and histidine, each 300 µM) did not. The fluorescence micrographs of Bay K8644-stimulated VSMCs also supported the finding that WH inhibited the Bay K8644-induced $\text{Ca}^{2+}$ entry via VDCC in VSMCs.

3.6. Inhibition of $[\text{Ca}^{2+}]_i$ increase by WH in combination with VDCC blockers

In order to elucidate the involvement of WH in $[\text{Ca}^{2+}]_i$ regulation via VDCC, the combination experiments of WH or one of the VDCC blockers with another blocker were performed in Bay K8644-stimulated VSMCs. The phenylalkylamine (PAA)-type blocker, verapamil (10 nM), and the dihydropyridine (DHP)-type blockers, nifedipine (0.5 nM) and nimodipine (0.5 nM), were used in this study. As shown in Fig. 4A, each VDCC blocker successfully inhibited the elevation of $\text{Ca}^{2+}$ entry through VDCC activation by Bay K8644. In Fig. 4B, a conflicting result was obtained regarding the combination of WH with either verapamil or nifedipine; the inhibitory potential of WH against $[\text{Ca}^{2+}]_i$ elevation was abolished in the presence of verapamil ($\Delta[\text{Ca}^{2+}]_i$: WH + verapamil, 85.9 ± 2.6 nM: WH, 64.3 ± 3.0 nM, $n=5-8$), while nifedipine did not interfere with the WH-potency ($\Delta[\text{Ca}^{2+}]_i$: WH + nifedipine, 43.3 ± 2.5 nM, $n=4$). A similar situation to the combination effect of WH with one of the VDCC blockers (Fig. 4B) was observed with the VDCC blockers as shown in Fig. 4A; namely, the combination of verapamil with nifedipine (or vice versa) resulted in the loss of their effectiveness, while nifedipine did not interfere with the effect of nimodipine.

4. Discussion

In our previous studies of the effects of peptides on blood vessels, WH exhibited a significant anti-atherosclerotic effect [14] along with an endothelium-independent vasorelaxation [11], while the underlying mechanisms remain unclear. At the beginning of the present study, we demonstrated the anti-proliferative effect of WH
on FBS-stimulated VSMCs in the absence of cytotoxic effects (Fig. 1). This finding implies that WH could have a great beneficial effect in preventing hypertension and related cardiovascular diseases, since the proliferation of VSMCs is crucial in the regulation of vascular function [20].

In order to elucidate the underlying mechanism(s) of WH on anti-proliferative effects or vascular function, changes of $[\text{Ca}^{2+}]_i$, which directly activate vasoconstriction-signaling pathways, were then evaluated in mitogen-stimulated VSMCs. As depicted in Fig. 2A and 2B, WH was found to inhibit the Ang II-induced elevation of $[\text{Ca}^{2+}]_i$ via stimulation of diverse pathways, such as AT$_1$R/PLC/IP$_3$ or VDCC [21,22]. Our subsequent $[\text{Ca}^{2+}]_i$ measurements in CaCl$_2$-stimulated VSMCs, in which WH inhibited the $\text{Ca}^{2+}$ influx into VSMCs (Fig. 2B), also allowed us to focus on the involvement of WH in the entry of extracellular $\text{Ca}^{2+}$ into VSMCs. In addition, the attenuation of the $[\text{Ca}^{2+}]_i$ elevation by WH in VDCC agonist (Bay K8644)-stimulated VSMCs (Fig. 3) demonstrated the direct effects of WH on VDCC.

It has been reported that some vasoactive small peptides, such as RADHP(F) and MY, were able to relax vessels, all of which were due to the activation of NO or CO/cGMP-dependent pathways in the endothelium [6,13]. However, to date no reports have revealed any involvement of small peptides in the regulation of $[\text{Ca}^{2+}]_i$ via VDCC blockade. While the data were not shown, HW also inhibited the elevation of $[\text{Ca}^{2+}]_i$ in Bay K8644-stimulated VSMCs to a similar degree as the mother WH ($\Delta[\text{Ca}^{2+}]_i$: control, 83.7 ± 1.3 nM; HW, 62.3 ± 5.0 nM; WH, 64.3 ± 3.0 nM), while WH in the D-configuration (D-W-D-H, D-W-L-H and L-W-D-H) failed to evoke any reduction in $[\text{Ca}^{2+}]_i$, indicating that VDCC may recognize the overall WH configuration. In VDCC drugs, photoaffinity-labeling studies [23,24] have clearly demonstrated that DHP-type blockers bind favorably to the extracellular side of VDCC proteins located at the loop connecting segments between S5-S6 on domain III and the end segment of S6 on domain IV in the $\alpha_1$-subunit [23]. In contrast, PAA-type blockers bind to an intracellular side of VDCC proteins located at the end of helix of S6 on domain IV in $\alpha_1$-subunit [24]. Thus, to elucidate the binding of WH to VDCC
protein or to predict a possible binding site(s), we conducted combination experiments of WH with DHP- or PAA-type blockers. As shown in Fig. 4B, the WH-induced $[\text{Ca}^{2+}]_i$ reduction in Bay K8644-stimulated VSMCs was significantly abolished by verapamil (PAA-type), while the presence of nifedipine (DHP-type) did not interfere with the $[\text{Ca}^{2+}]_i$ reduction power of WH. Our present study also revealed that the $[\text{Ca}^{2+}]_i$ reduction potential of nifedipine was abolished by verapamil, while nifedipine as a DHP-type blocker did not interfere with the nimodipine-induced $[\text{Ca}^{2+}]_i$ reduction. According to the report of Ruth et al. [25], the binding of the PAA-type blocker to intracellular VDCCs was apparently inhibited by DHP-type blockers via an allosteric mechanism, such that the DHP-type blocker did not bind to the extracellular site of VDCC, as supported by other researchers [26-29]. Indeed, Ehlert et al. [29] demonstrated a reduction of the binding potency of nitrendipine (DHP-type blocker) in the presence of D600 (PAA-type blocker), while nifedipine did not affect the potency of nitrendipine. Taken together, these findings strongly indicated that WH would play a role in the regulation of $[\text{Ca}^{2+}]_i$ in VSMCs by binding to the extracellular site of VDCC, similar to DHP-type blockers (Fig. 4C).

In conclusion, we demonstrated for the first time that the vasorelaxant di-peptide, WH, significantly inhibited the elevation of $[\text{Ca}^{2+}]_i$ in VSMCs. Our present study also revealed that WH acted as a VDCC blocker at a binding site similar to the DHP-type blockers. However, other possible roles of WH in the regulation of $\text{Ca}^{2+}$ signaling pathways including PLC/PKC, MAPKs (JNK/ERK/p38), $\text{Ca}^{2+}$-ATPase or NADP(H) oxidase [30] cannot be ruled out, since WH inhibited the $[\text{Ca}^{2+}]_i$ elevation by Ang II much stronger than that by Bay K8644, as shown in Figs. 2A and 3. The evidence indicating that VY activated the renal $\text{Ca}^{2+}$-ATPase system [31] also supported the possible role(s) of WH in diverse $\text{Ca}^{2+}$-signaling in VSMCs and further studies are currently in progress.
Acknowledgments

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Figure Legends

**Fig. 1.** Effects of WH on the proliferation of VSMCs. The anti-proliferative effect (A) of WH on serum-stimulated VSMCs was determined by the trypan blue dye exclusion method. After 24 h-serum starvation, the medium was replaced with 10% FBS/DMEM without (Cont) or with WH (0.01, 0.1, 0.5, 1 mM). The medium was changed every 48 h during the 5-day incubation period. The cytotoxic effect (B) of WH on VSMCs was determined by the WST-8 incorporation method. After 24 h-serum starvation, the medium was replaced by serum-free DMEM containing 1 mM WH, followed by a 5-day incubation. The medium was changed every 48 h during the incubation. Data are expressed as the mean ± S.E.M. (n=4-8).

**Fig. 2.** The effect of WH on [Ca\(^{2+}\)]\(_i\) in Ang II- or CaCl\(_2\)-stimulated VSMCs. The Ang II (10 μM)-induced [Ca\(^{2+}\)]\(_i\) elevation (A) in Fura-2/AM-loaded VSMCs was measured in the absence (Cont) or presence of WH (100 or 300 μM) using a fluorescence spectrophotometer at dual excitation wavelengths of 340/380 nm and an emission wavelength of 500 nm. The AT\(_2\)-receptor antagonist (PD 123177, 1 μM) was added 10 min before the addition of Ang II. For CaCl\(_2\)-induced [Ca\(^{2+}\)]\(_i\) elevation (B) in VSMCs, Ang II (10 μM) was added to VSMCs pre-incubated with Ca\(^{2+}\)-free buffer containing 0.1 mM EGTA, 1 μM PD 123177 and 200 μM 2-APB (IP\(_3\)-receptor antagonist) before the addition of 2.5 mM CaCl\(_2\) solution. Prior to CaCl\(_2\) stimulation, 300 μM WH was added to the VSMCs and incubated for 3 min. Data are expressed as the mean ± S.E.M. (n=3-6).

**Fig. 3.** Effect of WH on [Ca\(^{2+}\)]\(_i\) in Bay K8644-stimulated VSMCs. Fluorescent micrographs of Fluo-4/AM-loaded VSMCs in the absence (Cont) or presence of WH (300 μM) or its corresponding amino acids (tryptophan and histidine, each 300 μM) were obtained 3 min after Bay K8644-stimulation using a confocal microscope (A). The Bay K8644 (50 μM)-induced [Ca\(^{2+}\)]\(_i\) elevation in Fura-2/AM-loaded VSMCs was
measured under the same cell experimental conditions using a fluorescence spectrophotometer at dual excitation wavelengths of 340/380 nm and an emission wavelength of 500 nm (B). Data are expressed as the mean ± S.E.M. (n=3-5).

**Fig. 4.** The combination experiments of WH or a VDCC blocker with another VDCC blocker and the possible role of vasoactive WH in VSMCs. The combination [Ca^{2+}]_{i} experiments of DHP- and PAA-type VDCC blockers (A) were conducted in Bay K8644-stimulated VSMCs. VSMCs incubated with one of the VDCC blockers (shown below the abscissa) were stimulated with 50 µM Bay K8644 solution containing another VDCC blocker (shown above the abscissa). The combination [Ca^{2+}]_{i} experiments of WH with a VDCC blocker (B) were conducted in Bay K8644-stimulated VSMCs. VSMCs pre-incubated with 300 µM WH were stimulated with a 50 µM Bay K8644 solution containing one of the VDCC blockers (shown above the abscissa). Data are expressed as the mean ± S.E.M. (*P<0.05, **P<0.01 vs. control, *P<0.05, **P<0.01 vs. (-), n=3-8). (C) The possible role of vasoactive WH in VSMC. PLC; phospholipase C, DAG; diacylglycerol.
Fig. 1
Fig. 2
Fig. 3

(A) Graph showing changes in intracellular calcium ([Ca^{2+}]_i) over time with Bay K8644 and WH (300 μM) treatments. The graph indicates an increase in [Ca^{2+}]_i with each application of Bay K8644, followed by a decrease after WH treatment.

(B) Images showing fluorescence under different conditions: Cont, WH (300 μM), and W+H (300 μM). The images correspond to the changes in intracellular calcium levels observed in the graph.
**Fig. 4**

Panel A shows a bar graph comparing the change in calcium concentration (Δ[Ca\(^{2+}\)]\(_i\) in nM) under different conditions: Control (Cont), Verapamil, Nifedipine, and Nimodipine. The levels are depicted with error bars indicating standard deviation.

Panel B presents another bar graph with similar conditions: Control (Cont), Verapamil, and Nifedipine. The graph illustrates the effect on calcium levels with notable differences indicated by the bar heights and error bars.

Panel C illustrates a complex biochemical pathway involving various receptor and ion channel interactions, including receptors such as AT\(_2\)-Receptor and AT\(_1\)-Receptor, and calcium channels such as VDCC. The pathway highlights the effects of drugs like Nimodipine and Verapamil on calcium concentration and vasorelaxation vs. vasoconstriction.

The diagram includes chemical structures and annotations for specific compounds and mechanisms.