**Purpose:** Malignant hyperthermia (MH) is a pharmacogenetic disorder arising from uncontrolled muscle calcium release due to an abnormality in the sarcoplasmic reticulum (SR) calcium-release mechanism triggered by halogenated inhalational anesthetics. However, the molecular mechanisms involved are still incomplete.

**Methods:** We aimed to identify transient receptor potential vanilloid 1 (TRPV1) variants within the entire coding sequence in patients who developed sensitivity to MH of unknown etiology. In vitro and in vivo functional studies were performed in heterologous expression system, trpv1−/− mice, and a murine model of human MH.

**Results:** We identified TRPV1 variants in two patients and their heterologous expression in muscles of trpv1−/− mice strongly enhanced calcium release from SR upon halogenated anesthetic stimulation, suggesting they could be responsible for the MH phenotype. We confirmed the in vivo significance by using mice with a knock-in mutation (Y324S) in the type I ryanodine receptor (Ryr1), a mutation analogous to the Y522S mutation associated with MH in humans. We showed that the TRPV1 antagonist capsazepine slows the heat-induced hypermetabolic response in this model.

**Conclusion:** We propose that TRPV1 contributes to MH and could represent an actionable therapeutic target for prevention of the pathology and also be responsible for MH sensitivity when mutated.

**Keywords:** TRP channel; TRPV1; Calcium; Hereditary disease; Malignant hyperthermia

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**INTRODUCTION**

The role of Ca$^{2+}$ as the main regulatory and signaling molecule in skeletal muscle contraction is well described, and pathogenic variants in several genes encoding Ca$^{2+}$ signaling and handling molecules are responsible for various myopathies, yet understanding of the molecular mechanisms involved is still incomplete.$^{1,3}$

Altered regulation of Ca$^{2+}$ release is a key contributor to the pathophysiology of core myopathies such as central core disease (CCD, OMIM 117000), as well as in the hypermetabolic response associated with anesthesia-induced malignant hyperthermia (MH, OMIM 145600), a triggered muscle disease.$^{4}$ These pathologies have so far been mainly linked to mutations in the RYR1 gene, which encodes the main intracellular Ca$^{2+}$-release channel of skeletal muscle.$^{5,6}$ Generally, these mutations lead to a hypersensitivity of the RyR1 channel to activation by a wide range of triggers, including caffeine, halothane, and Ca$^{2+}$, or to a decrease in voltage-induced activation.$^{6-8}$ The enhanced intracellular Ca$^{2+}$ results in abnormal skeletal muscle metabolism manifesting as activation of muscle contraction mediated by the binding of Ca$^{2+}$ on troponin, thereby allowing the movement of tropomyosin on actin filaments. This abnormal skeletal muscle metabolism is also characterized by increased oxygen consumption, adenosine triphosphate (ATP) hydrolysis, and heat production.$^{9,10}$ Although linkage to the RYR1

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ARTICLE

Gene is shown for more than 50% of all MH cases, mutations in two other genes were associated with MH: CACNA1S encoding the L-type plasma membrane channel regulating RyR1, and STAC3, a small adaptor protein interacting with both channels.

The search for a new candidate potentially involved in MH led us to the TRPV1 channel. To date, although playing a widely accepted role in nociception, this channel is not clearly linked to any hereditary disease. Efforts have been mainly focused on its implication in chronic pain syndromes, however, the latest available data suggest that TRPV1, as a highly $\text{Ca}^{2+}$-permeable channel, also plays an important role in the physiology of skeletal muscle. Indeed, after initial discovery as a neuronal sensory channel, TRPV1 was subsequently found in a few non-neuronal tissues, including skeletal muscle, and recent studies have clearly highlighted its functional role as a sarcoplasmic reticulum (SR) $\text{Ca}^{2+}$ channel. Interestingly, TRPV1 has recently been shown to be sensitive to volatile anesthetics in neurons. However, the potential implication of TRPV1 channel in the mechanism leading to altered regulation of $\text{Ca}^{2+}$ signaling in the pathophysiology of MH has not yet been investigated.

Here, using complementary approaches and models, we identify and functionally characterize human variants of the TRPV1 channel that confer muscle sensitivity to anesthetics exposure. Our results suggest that TRPV1 plays a critical role in the aberrant $\text{Ca}^{2+}$ homeostasis in MH.

MATERIAL AND METHODS

Patients

The 28 patients tested were referred to the laboratory for genetic screening of the RYR1 (OMIM 180901) gene in the context of malignant hyperthermia (MH, OMIM 145600). In all cases a familial history of MH during anesthesia was maintained in 5% $\text{CO}_2$, 95% air at 37 °C in a humidified incubator. Cells (50–60% confluency) were transiently transfected by either 1 µg (in 35-mm dishes) of plasmid (transfecting either TRPV1 wild-type or mutants plus pEGFP-N1 at a 5:1 ratio (pEGFP-N1 was used as a positive control of transfection)) using X-tremeGENE 9 DNA Transfection Reagent (Roche Diagnostics, France) as described by the manufacturer. Cells transfected with 1 µg of pEGFP-N1 alone were used as control (CTL) condition. Cells were mycoplasma-free.

Cloning

The coding sequence of human TRPV1 was amplified from the pCAGGS-M2-ires-GFP-TrpV1 vector (a gift from B. Nilius, Leuven, Belgium) and cloned into the pcDNAs5/FRT vector (Invitrogen, LifeTechnologies). TRPV1 mutants were obtained using in vitro mutagenesis (QuikChange Site-directed Mutagenesis kit, Agilent Technologies-Stratagene products) and the following primer pairs: T612M ccgtctgagtccatgtcgca-ccaggtg / ccacctgtgagtcagcttcagactcagccagtgc accctgagcttc/gaagctcagggtgcacttgacgccctcac, and V1394del caggtgg / ccacctgtgcgacatggactcagacgg, R722C gtgagggcgtcaagtgc acacctgacgct/gaagctcagggtgcacttgacgccctcac, and V1394del cactgctgagacttgctgcag / cctccagaccagcttccaggtc. TRPV1 wild-type and mutants were subcloned into the pmCherry-C1 vector as an EcoRI-KpnI fragment. All long polymerase chain reactions (PCRs) mentioned above were carried out with the High Fidelity Phusion DNA Polymerase (Finnzymes) and all constructs were verified by sequence analysis.

Ethics statement

All experiments were performed in accordance with the guidelines of the French Ministry of Agriculture (87/848) and of the European Community (86/609/EEC). They were approved by the local animal ethics committee of Rhône-Alpes, approval number 692660602.

Indirect calorimetry

Wild-type (WT) and YS (heterozygous RyR1$^{524S/WT}$ knock-in) male mice (on C57/B6 background, 6–10 weeks old) were weighed and injected with 5 mg/kg (IP) capsazepine dissolved in 2% DMSO and 10% Tween 20 in sterile saline (vehicle) or with vehicle alone. Ten minutes after injection, mice were placed individually into indirect calorimetry chambers (Oxymax System, Columbus Instruments) contained with a temperature controlled environment chamber set at 37 °C. Maximum oxygen consumption ($\text{VO}_2$, mL/kg/min) was monitored for 15 min before mice were removed from the chambers. All procedures were approved by the Animal Care Committee at Baylor College of Medicine.
Isolation of muscle fibers

Single skeletal muscle fibers were isolated from the flexor digitorum brevis muscles of 4 to 8-week-old wild-type (C57BL6) from Charles Rivers Laboratories or TRPV1+/− (from Jackson Laboratories) male mice. Mice were killed by cervical dislocation. Muscles were removed and treated with type 1 collagenase (45–60 min at 37 °C) in the presence of Tyrode as external solution. Single fibers were then obtained by triturating muscles within the experimental chamber. Cells were mounted into a glass bottom dish. Fibers were bathed in the presence of Fluo-4 AM (5 µM) during 30 min. Cells were then washed with Tyrode solution.

In vivo transfection

Expression of TRPV1 variants by electroporation was performed in the flexor digitorum brevis of 4 to 8-week-old trpv1+/− male mice using a previously described procedure. Mice were anesthetized by isoflurane inhalation (5 min, 3% in air, air flow at 300 ml.min⁻¹) using a commercial delivery system (Univentor 400 Anaesthesia Unit, Univentor, Zetjub, Malta). During anesthesia, 25 µl of a solution containing 2 mg/ml hyaluronidase dissolved in sterile saline was injected into the footpads of each hind paw. Mice recovered from anesthesia. Forty minutes later, mice were reanesthetized by isoflurane inhalation. First, 20 µl of plasmid DNAs (mcherry-hTRPV1, mcherry-T612M, or mcherry-N394del) were injected into the footpads of the animal (1.5 mg/ml in standard Tyrode solution). Then, 10 min after plasmid injection, two gold-plated stainless steel acupuncture needles connected to the electroporation apparatus were inserted under the skin, near the proximal and distal portion of the foot, respectively. Twenty pulses of 110 V/cm amplitude and 20 ms duration were delivered at a 2-Hz frequency by a BTX ECM 830 square wave pulse generator (Harvard Apparatus, Holliston, MA, USA). Mice recovered from anesthesia and experimental observations and measurements were carried out 8 days later.

Immunostaining

Fluorescence of immunostaining was measured on a Zeiss LSM 5 Exciter laser scanning confocal microscope.

Confocal Ca²⁺ imaging and image analysis

Unless otherwise specified, imaging was achieved on a Zeiss LSM 5 Exciter laser scanning confocal microscope. The microscope was equipped with a 63× oil immersion objective (numerical aperture (NA) = 1.4). Fluo-4 was excited with 488-nm argon laser. The emitted fluorescent light was measured at wavelengths >505 nm. Because Ca²⁺ responses to TRPV1 agonists revealed slow kinetics, images (512x512 pixels) were taken with a 5- or 15-second interval. Fluorescence of regions of interest was normalized to baseline fluorescence (F0). Experiments realized on HEK cells were performed using the membrane-permeable Ca²⁺-sensitive dye fura-2AM, as detailed previously. Each experiment was repeated three times (field of 35 to 45 cells) and representative experiments are presented (mean ± SE).

Reagents and preparation of anaesthetics

Capsaicin, capsazepine, and halothane were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France); isoflurane from Laboratoires Belamont (Neuilly Sur Seine, France). Other reagents were purchased from Sigma-Aldrich.

Biotinylation

Cells were transfected with 2 µg of each construct. Control experiments were performed by transfecting the empty vectors. Forty-eight hours after transfection, cells were subjected to cell-surface biotinylation and precipitated after lysis with neutravidin–agarose beads (Pierce Rockford, IL, USA) as described in. Anti-TRPV1 antibody (1/500, Santa Cruz) and anti-calnexin (1/2000, Millipore) were used.

Data analysis

Results were expressed as the means ± S.E.M. Normally distributed data are expressed as mean ± S.E.M and statistical comparison were made using Student’s t test. Data that were not normally distributed were made using the Mann–Whitney test or the Kruskal–Wallis tests with Dunn’s post hoc test. Differences were considered significant when p < 0.05 and Origin 5 software was used (Microcal Software, Inc.). Skeletal muscle fibers data were obtained from ≥6 cells from at least four different flexor digitorum brevis muscle from four mice. For animal studies, no randomization was used and no blinding was done. The investigators were not blinded to the group allocation during the experiment and/or when assessing the outcome. The variance is similar between the groups that are statistically compared.

RESULTS

TRPV1 expressed in HEK-293 cells is activated by volatile anesthetics

First, to assess the clinical relevance of studying TRPV1 in MH, we tested the effects of volatile anesthetics (VAs) on TRPV1 activity expressed in HEK-293 cells. Whole-cell patch-clamp experiments showed that isoflurane activated TRPV1 (Fig. 1a,b). No currents were observed in similar condition in untransfected HEK-293 cells as well as in cells transfected with pEGFP-N1 alone (CTL condition; see Materials and methods section). Next, we asked how VAs activate TRPV1. To address this, we hypothesized that VAs sensing could be tightly linked to voltage-dependent sensing as it has been demonstrated for temperature or the chemical agonist capsaicin. We investigated this possibility by performing the same patch-clamp protocol that has been used to demonstrate that the agonist capsaicin functions as a gating modifier shifting activation curves toward physiological membrane potentials. Comparison of the steady-state activation curves of the background membrane current carried through TRPV1 channels (iTRPV1) in HEK-293 cells with or without exposure to halothane showed that
anesthetic produces a depolarizing shift in the voltage dependence of the TRPV1 channel activation by about 100 mV (Fig. 1c,d), which may underlie its agonistic action mechanism.

TRPV1 is a SR Ca\(^{2+}\) channel activated by VAs in skeletal muscle cells

We have previously shown that Trpv1 is a functional SR Ca\(^{2+}\)-leak channel in adult mouse skeletal muscle cells.\(^{14}\) The
question remained as to whether endogenous Trpv1 could be involved in halothane-induced release of Ca^{2+} from internal stores in skeletal muscle. We tested this possibility on skeletal muscle cells isolated from C57BL/6j mice flexor digitorum brevis (FDB) muscle in an external Ca^{2+}-free solution. Fig. 1e shows that Fluo-4-loaded cells undergo massive increase of cytosolic [Ca^{2+}] after isoflurane perfusion (median of Δmax = 1.09; n = 29) (Fig. 1e,f). Interestingly a pretreatment with capsaizpine (CPZ), an inhibitor of Trpv1 significantly reduces isoflurane-induced Ca^{2+} release (median of Δmax = 0.3; n = 6), suggesting that muscle response to isoflurane is linked with Trpv1 activation. To confirm these results, we repeated those experiments in trpv1−/− mice muscle cells. As expected for these cells, the response to capsaicin (100 µM) was not observed (Fig. 1g). Next a dose-dependent response to isoflurane on SR Ca^{2+} release in WT and trpv1−/− muscle cells was tested. Isoflurane’s maximal effect was obtained at a concentration of 1 mM isoflurane in WT cells, but in the same conditions a clear reduction in trpv1−/− muscle cells’ Ca^{2+} release was recorded. This strongly suggests that SR Ca^{2+}-store release in differentiated skeletal muscle cells stimulated by isoflurane is mediated by the Trpv1 channel (Fig. 1h).

Identification of TRPV1 rare variants from patients who suffer from malignant hyperthermia

To date, TRPV1 has not been clearly linked to any hereditary disease, but we hypothesized that TRPV1 channel may be involved at a genetic level in MH, because it is well established that other MH-susceptible genes than RYR1 exist, and that TRPV1 can be activated by volatile anesthetics and mediate Ca^{2+} release in skeletal muscle. We sequenced the TRPV1 gene in a cohort of 28 MH patients who had been previously shown to have enhanced in vitro sensitivity to halothane in a presymptomatic test relying on the contraction developed by a skeletal muscle biopsy upon either caffeine or halothane stimulation (in vitro contracture test, IVCT), which is the gold standard for screening MH-susceptible patients. Because muscle biopsies from patients with RYR1 mutations trigger strong contractions in the presence of both caffeine and halothane, we focused our search on patients showing only hypersensitivity to halothane, and not caffeine, during IVCT. These patients are referred to as MHSsh. Two rare genetic variations in the TRPV1 gene were found in two independent patients (Fig. 2a, b). The first variant corresponded to a missense variation (c.1836C>T;p.Asn394del) corresponding to the inframe deletion of three nucleotides leading to the deletion of asparagine 394 (N394del) in an asymptomatic patient tested MHSsh during a family study. The RYR1 gene of the two patients was completely sequenced and only one displayed a significant change in the RyR1 sequence. The patient with the N394del variant was also carrier of a nonsense RYR1 mutation in the heterozygous state. This mutation leading to a premature stop codon in one allele of the gene could however not account for a MHS phenotype. Indeed, MH has a dominant mode of inheritance and a pathophysiology related to a hypersensitive RyR1 channel only caused by missense or inframe deletion variants. The T612M variation was found in the databases (rs199539626) with a minor allele frequency (MAF) of 0.0013 (ExAC browser), and classified as tolerated or benign by SIFT and Polyphen prediction software. The N394del was not reported, but the Asn in the 394 position was found to have mutated to Ser in only one allele in the ExAC database. Overall, the two TRPV1 variants were either unknown or below a low MAF threshold, as compatible with rare and triggered diseases such as MH.

Impact of the TRPV1 variants on Ca^{2+} homeostasis

We characterized the impact of the two TRPV1 variants on Ca^{2+} homeostasis by performing Ca^{2+} imaging experiments in HEK-293 cells. We found that both TRPV1 variants display a fast and transient Ca^{2+} response to isoflurane exposure compared with wild-type TRPV1 transfected cells (CTL) (Fig. 2c–h). Importantly, capsaicin does not alter [Ca^{2+}]i in cells transfected with the variant. We checked that the same level of TRPV1 proteins (wild-type or variants) were expressed at the plasma membrane as detected by cell-surface biotinylation assays (Fig. 2i). We also confirmed similar level of total fraction and ER enrichment of TRPV1WT, TRPV1 N394del, and TRPV1 T612M transiently transfected in HEK-293 (Fig. 2j). This suggests that the modification of the channel activity is not due to an altered trafficking of the mutated channels.

TRPV1 variants transfected in skeletal muscle cells are highly sensitive to VAs

We evaluated the consequences of the TRPV1 variants on SR Ca^{2+} leak in skeletal muscle. For this, we transfected trpv1−/− FDB muscles with plasmids coding for the wild-type or mutants human TRPV1 (T612M or N394del). Previously, we localized native mouse Trpv1 in the longitudinal part of SR in mouse muscle cells.14 Thus, human Trpv1 and its mutated forms seem to localize in the longitudinal part of SR (Fig. 2k, l). In accordance with our previous results, we did not notice any plasma membrane labeling.

To assess the role of TRPV1 mutants in the SR Ca^{2+} response to general anesthetics or capsaicin, we compared the sensitivity to isoflurane and capsaicin of WT mouse Trpv1 (C57BL6j) and human TRPV1 (hTRPV1), TRPV1, T612M, or TRPV1 N394del expressed in the FDB fibers of trpv1−/− mice. Nontransfected cells of trpv1−/− mice were used as a negative control. Cells were loaded with Fluo-4 AM to measure the increase in cytosolic Ca^{2+} level due to
SR Ca\(^{2+}\) release. As illustrated by Fig. 3a, b, except for the nontransfected FDB muscle fibers of \(trpv1^{-/-}\) mice, capsaicin (100 \(\mu\)M) induced an increase in cytosolic Ca\(^{2+}\) level. Capsaicin response was similar in endogenous mouse Trpv1 and hTRPV1 (median of max value = 1.74; \(n = 42\) and 2.81; \(n = 11\) respectively). Nevertheless, we noticed a significant decrease in amplitude of capsaicin response in cells expressing T612M variant (median of max value = 0.76; \(n = 12\)) and in cells expressing N394del variant (median of max value = 0.61; \(n = 10\)).

We further measured the amplitude of isoflurane response of these constructs (Fig. 3c, d) and found no differences between endogenous mouse Trpv1 and human TRPV1 (median of max value = 1.09; \(n = 29\) and median of max value = 0.98; \(n = 18\) respectively). Interestingly, the amplitude of isoflurane response in \(trpv1^{-/-}\) cells expressing T612M and N394del variants was significantly higher (2.80; \(n = 11\) and 3.65; \(n = 12\) respectively). The delay and time to peak of isoflurane responses were similar (data not shown). Altogether, these experiments strongly suggest that the T612M and N394del variants of TRPV1 have an altered channel function and are in particular much more sensitive to isoflurane that the wild-type channel.

On the basis of our previous experiments, we hypothesized that the TRPV1 channel could be an important part in the pathophysiology of MH triggering. We reasoned that TRPV1 activation could either directly result in massive Ca\(^{2+}\) release whenever mutations enhance its sensitivity to VAs, or that initial Ca\(^{2+}\) release by TRPV1 stimulated by VAs could trigger or enhance RYR1-mediated Ca\(^{2+}\) release. To explore this hypothesis, we investigated the role of Trpv1 in the phenotype of an MH mice model, the mice line with a knocked-in Y524S mutation in Ryr1 channel. These mice bear a mutation analogous to the Y522S mutation that is associated with MH in humans, and die after exposure to 37 °C for longer than 15 min. The Y524S mice display a dramatic heat-induced hypermetabolic response when exposed to 37 °C compared with WT mice that can be measured by VO\(_2\) consumption. We showed that treatment with the Trpv1 antagonist capsaizine significantly slows down the heat-induced hypermetabolic response in this mouse model (Fig. 3e). This suggested that Trpv1 may be contributing to the mechanism underlying the hyperthermia response of this Y524S Ryr1 model. These findings suggest that TRPV1 and related mutants could be a new therapeutic target for treating muscle diseases due to altered regulation of Ca\(^{2+}\) release.

### DISCUSSION

In the present study, we show that Trpv1 functions as a Ca\(^{2+}\)-release/Ca\(^{2+}\)-leak channel in adult skeletal muscle in response to VAs exposure. Trpv1 is a well-known polymodal cellular sensor for heat and other physiological stimuli. However, how this channel is activated by diverse physical and chemical stimuli remains largely unknown. Recently, we demonstrated that anesthetic produces a depolarizing shift in the voltage dependence of the TRPM8 channel activation, which might underlie its agonistic action mechanism. Our results suggest that Trpv1 shares a similar behavior, because VA exposure also produces a depolarizing shift in the voltage dependence of TRPV1.

Here, we also identified a new role of TRPV1 in a human pathology and we proposed it as a new therapeutic target. We have also identified, for the first time, two \(TPV1\) mutations in patients suffering from a human pathology and, to our knowledge, TRPV1 has never been involved before in any genetic disorders. Indeed, regarding our previous data and the fact that we identified VAs as potent activators of this channel in skeletal muscle cells, we hypothesized that TRPV1 could be part of malignant hyperthermia (MH) crisis. MH is a triggered muscle disease and is known to be a hereditary disease. Moreover, Ca\(^{2+}\) signaling is so far known to be linked to different muscle...
pathologies such as MH. For this reason, the regulation of intracellular Ca\(^{2+}\) signaling is an area of intense research to better understand muscle pathophysiology. Concerning MH, the pathogenic implications of different mutants of the main intracellular Ca\(^{2+}\) channel, RYR1, are well accepted in MH, where the release of Ca\(^{2+}\) through an abnormal RYR1 in the presence of volatile anesthetic activates an uncontrolled increase in Ca\(^{2+}\) release. This increase in cytosolic Ca\(^{2+}\) leads to the hypermetabolic response characteristic of MH.

Although central for MH susceptibility, RYR1 mutations do not constitute the exclusive genetic cause for this pathology. Accordingly, two TRPV1 variants were discovered in patients who tested MH sensitive. The first TRPV1 variant (T612M) was found in a patient with a postoperative hyperthermia after anesthesia. The second variant (N394del) was discovered in an asymptomatic patient. The two variations were either unknown or with very low prevalence in human genetic databases, as compatible with a triggered
pathology such as MH, but genetic evidence that these TRPV1 variations were responsible for MH was difficult to obtain for these cases. In the second family (N394del), RYR1 compound heterozygous mutations led to myopathy in the two children. Moreover, family members studied were limited, thus it is difficult to clearly establish a genotype to phenotype correlation.

We have also shown previously that Ca\(^{2+}\) release in skeletal muscle stimulated by the TRPV1 agonist capsaicin was a two-phase process consisting of a first step of Ca\(^{2+}\) release that is dantrolene resistant and a second step that is inhibited by dantrolene, which is a known inhibitor of RYR1 function.\(^{14}\) This suggested that Ca\(^{2+}\) released directly by TRPV1 from the SR could in turn activate a RYR1-mediated Ca\(^{2+}\) release. Therefore, TRPV1 could also directly be related to RYR1 function as an intracellular channel able to prime massive Ca\(^{2+}\) release by RYR1. To check this hypothesis, we performed in vivo experiments in transgenic mice expressing a ryr1 mutation responsible for MH.\(^{23}\) As already described, environmental heat induces increase in body temperature of these animals and a hypermetabolic response that can lead to death. A pretreatment of the mice with capsazepine, an inhibitor of Trpv1, significantly decreased the hypermetabolic crisis, suggesting that Trpv1-mediated Ca\(^{2+}\) activation of RyR1 underlies a causal mechanism of porcine malignant hyperthermia.\(^{54}\)

In conclusion, our study provides crucial understandings of pathological ryanodine receptors in mouse muscle fibres. J Physiol. 2006;174:535–544.

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**DISCLOSURE**

The authors declare no conflicts of interest.
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