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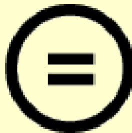

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Modulation of Transient Receptor Potential Vanilloid Subtype 1 by Dexmedetomidine

덱스메데토미딘에 의한 Transient Receptor Potential Vanilloid Subtype 1 조절

2017년 10월

서울대학교 치의학대학원

치의학과

장 윤 선

Modulation of Transient Receptor Potential Vanilloid Subtype 1 by Dexmedetomidine

지도교수 정 지 훈

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Abstract

1. Background

Dexmedetomidine, an α2 adrenergic receptor agonist and novel sedative drug with minimal respiratory suppression, shows anti-nociceptive activity in various pain models by poorly understood mechanisms. Because α2 adrenergic receptor is up-regulated and co-localized with Transient Receptor Potential Vanilloid Subtype 1 (TRPV1) polymodal nociceptive receptor in neuropathic pain animal models, the analgesic activity might be mediated through inhibition of TRPV1. The purpose of the study is to confirm whether analgesic effects of dexmedetomidine is mediated by TRPV1.

2. Method

Dorsal root ganglion (DRG) neurons from adult C57BL/6 male mice were prepared. To test inhibition of TRPV1 activity by dexmedetomidine, we measured the capsaicin-induced increase of intracellular calcium concentration with and without dexmedetomidine pre-treatment in mice DRG neurons. We also tested whether the effect of dexmedetomidine on capsaicin-induced calcium response is in dose-dependent manner. Lastly, expression of specific receptors, α2 adrenergic receptor and TRPV1, was confirmed by using RT-PCR.

3. Result and Conclusion

Increased intracellular calcium concentration induced by 400nM capsaicin decreased after treating dexmedetomidine with 10-minute interval. Also, dexmedetomidine (2, 10, 50 μM) significantly reduced capsaicin responses ($P<0.01$) in dose-dependent manner. In addition, RT-PCR analysis revealed expression of TRPV1 and all three subtypes of α2 adrenergic receptor in mice DRG neurons. In summary, these results suggested that the inhibition of TRPV1 by dexmedetomidine

might be a plausible mechanism that contributes to the anti-nociceptive action of the drug.
Keywords: DRG neuron, analgesic effect, adrenergic receptor, dexmedetomidine (DEX), Transient Receptor Potential Vanilloid Subtype 1 (TRPV1), capsaicin response, calcium
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I. Introduction

Dexmedetomidine (DEX) is a novel sedative drug that became increasingly popular due to its minimal respiratory suppression (Hsu et al. 2004). In addition to its sedative effect, several lines of studies reported potential anti-nociceptive function of DEX in various pain models (Smith and Elliott 2001). DEX reduced nociceptive signals in the rat spinal cord neurons (Kendig et al. 1991) and dorsal horn neurons (Sullivan et al.1992). Intraperitoneal injection of DEX reduced nocifensive responses in chemotherapy-induced neuropathic pain (Park et al. 2012), chronic nerve injury-induced neuropathic pain models in rats (Guneli et al. 2007; Lee et al. 2013), and acute inflammatory pain models, in which intraplantar injection of 1% carrageenan was done into hindpaws of rats (Honda et al. 2015). Intrathecal DEX administration reduced hyperalgesia and allodynia in chronic nerve injury models (Kimura et al. 2012; Yaksh et al.1995). In addition, local injection of DEX in human subjects prolonged the effect of co-injected local anesthetics (Yamane et al. 2015). Despite many evidences, pharmacology of DEX other than its selective and specific activation of the α2 adrenergic receptor remained poorly understood, and the mechanism underlying the anti-nociceptive effect of DEX is mostly unknown.

TRPV1 is a polymodal receptor protein that responds to noxious high temperature, low pH, and inflammatory mediators (Montell et al. 2002). TRPV1 was implicated in the development and maintenance of neuropathic and inflammatory pain (Kanai et al. 2005; Palazzo et al. 2010), and its functional modulation was considered as a potential pharmacologic target for treatment of chronic pain. Interestingly, alpha 2 adrenergic receptors are up-regulated and co-localized with TRPV1 in spinal dorsal root ganglion

cells in neuropathic pain model (Ma et al. 2005). Also, it has been reported that alpha 2 adrenergic agonist modulates intracellular signaling pathways critical in the function of TRPV1 receptor (Wu et al. 1988).

These reports strongly suggest that subsidization of neuropathic or inflammatory chronic pain might involve modulation of TRPV1, yet direct evidence supporting such molecular pathway has not been investigated and remained unclear. This study aims to investigate whether DEX would inhibit TRPV1 activity to elucidate the mechanism underlying analgesic action of DEX.

II. Materials and Methods

1. Preparation of dorsal root ganglion neurons

All procedures for animal use were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University prior to the experiments. Animal treatments were performed according to the guidelines of the International Association for the Study of Pain (Zimmermann 1983). Adult C57BL/6 male mice were purchased from Orientbio (Sungnam, Korea) and bred in an in-campus facility. Animals were habituated for at least one week prior to experiments in a conventional facility with a 12:12 hr light cycle (lights on 8.00am) and had ad libitum access to water and food.

Dorsal root ganglion neurons from adult C57BL/6 male mice were prepared as previously described (Park et al. 2009; Yeon et al. 2011). Briefly, dorsal root ganglia prepared in 4 °C Hank's Balanced Salt Solution (HBSS; Welgene, Daegu, Korea) were incubated in 2 mL HBSS containing 0.167% trypsin (Invitrogen, Carlsbad, CA, USA) at 37 °C for 40 mins. The cells were washed, triturated with a flame-polished Pasteur pipette, and placed on 0.5 mg/mL poly-L-ornithine (Sigma, St. Louis, MO, USA)-coated glass coverslips. The cells were maintained at 37 °C in a 5% CO2 incubator and were used for recording within 12hours after being plated.

2. Intracellular Ca^{2+} *Imaging*

Fura-2AM-based Ca^{2+} imaging experiments were performed as previously described (Park et al. 2006). Briefly, dorsal ganglion neurons were loaded with fura-2AM (2μM; Molecular Probes, Eugene, OR, USA) for 40 min at 37°C in HBSS, and then rinsed and incubated in HBSS for

additional 30 mins. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured at room temperature with an intensified CCD camera (Cascade, Roper Scientific, Trenton, NJ, USA)coupled to an inverted microscope (IX70, Olympus, Tokyo, Japan) and a computer with imaging software (MetaFluor, Universal Imaing Corp., West Chester, PA, USA). Cells were illuminated with a 175W xenon arc lamp, and excitation wavelengths (340/380nm) were selected by a Lambda DG-4 monochromatic wavelength changer (Sutter Instrument, Novato, CA, USA). The imaging chamber was continuously perfused with extracellular solution containing (in mM) NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10, adjusted to pH 7.3 with NaOH.

3. RT-PCR

To assess all three subtypes of α2 adrenergic receptors (α2AR), α2A, α2B, α2C, and TRPV1 channels in mice DRG neurons, total RNA was isolated by using the RNeasy mini kit (Qiagen). According to manufacturer's protocols, complementary DNA was synthesized from 1 μg of total RNA by M-MLV reverse Transcriptase (Invitrogen). The following PCR primers were used (Table 1). PCR amplification was done by ® HotStart PCR PreMix (Bioneer). The PCR products were then displayed on the Safe-Pinky (GenDEPOT) stained 1.5% agarose gel and photographed using a UV digital camera.

4. Drugs

Capsaicin (Sigma, St. Louis, MO, USA), fura-2AM were dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution and were stored at −20 °C before starting the experiment. The final concentration of DMSO was restricted to be less than 0.1% (v/v), which had a negligible effect on

intracellular calcium concentration. Dexmedetomidine was purchased as 100 μg/ml solution from Precedex (Hospira, IL, USA). All drugs were diluted to their final concentration with the extracellular solution.

5. Statistical analysis

Data are expressed as mean ± SEM and were compared by Mann-Whitney-Wilcoxon test and Kruskal-Wallis test after normality test by D'Agostino-Pearson omnibus test using Prism 6 (GraphPad Software, Inc. La Jolla, CA, USA). *P* values less than 0.05 were considered statistically significant.

III. Results

Calcium responses induced by capsaicin (TRPV1 agonist) application of DRG neurons were tested prior to investigating influence of DEX to TRPV1 activity. Capsaicin (400 nM) produced calcium influx mostly in small sized dorsal root ganglion neurons (data not shown). Three consecutive application of capsaicin, with interval of 10 minutes, (Fig. 1A, black horizontal bar) induced TRPV1 responses which slightly decreased each time. However, the difference of each peak amplitudes was within statistically non-significant range (Fig 1C, $p=0.08$), suggesting that desensitization of TRPV1 was minimal in our experimental condition. Pre-treatment of 10 μM DEX for 10 minutes before application of the second capsaicin reduced the peak amplitude (60.7 %, $p<0.0001$, $n=34$, Fig. 1B). The capsaicin-induced calcium peak recovered after wash-out of DEX for 10 minutes, but not to the peak amplitudes before DEX application (68.2 %, $p<0.0001$, $n=34$). Normalized peak amplitudes of calcium responses were summarized in Fig 1C.

We next tested whether modulation of TRPV1 by DEX was dose-dependent. While calcium responses were elicited by capsaicin application twice in each cell, DEX (0, 1, 10 and 50 μM) was treated for 10 minutes before the second capsaicin application. The first (black) and second (red) calcium traces were superimposed in Fig. 2A-D (A: DEX-free control, B: 1 μM, C: 10 μM, D: 50 μM DEX). The second calcium transients after DEX application was always smaller than the first ones before DEX application, and the relative peak amplitudes of the second calcium responses compared to the first suggested stronger inhibition of TRPV1 by higher concentration of DEX (Fig. 2E; 0 μM: 87.97±2.30 %,

n=41; 1 μM: 81.15±1.48 %, $p=0.004$, n=148; 10 μM: 64.29±7.39 %, $p<0.0001$, n=30; 50 μM: 54.89±8.58 %, $p=0.0002$, n=15).

RT-PCR analysis indicated expression of all three subtypes of α2 adrenergic receptors (α2AR) and TRPV1 channels in mice DRG neurons. Electrophoresis of DRG from 3 mice revealed PCR products positive for 538, 199, 156 and 233 bp amplicons, as expected for adrenergic receptor α2A, α2B, α2C and TRPV1 respectively (Fig. 3). PCR performed with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and without any primer served as a positive and a negative controls.

IV. Discussion

In current study, we found that dexmedetomidine inhibits TRPV1 polymodal nociceptive receptor activity. To the best of our knowledge, this study is the first to investigate the effect of TRPV1 as a potential mechanism underlying analgesic action of dexmedetomidine.

The analgesic effects of dexmedetomidine have been investigated by many studies. For example, intraoperative dexmedetomidine reduces postoperative opioid consumption and improves pain management in postanesthesia care unit (Bellon et al. 2016). The addition of 10 μg of intrathecal dexemedetomidine decreases the required doses of postoperative analgesics in patients undergoing lower abdominal and lower limb surgeries without any increase in side effects (Gupta et al. 2016).

TRPV1 is a polymodal receptor activated by noxious heat (>43 °C), low pH, inflammatory mediators, and irritable chemicals, and such TRPV1 receptors are widely expressed in peripheral endings of nociceptive C- and Aδ-fibers (Guo et al. 1999). The upregulation of TRPV1 in inflammation or nerve injury and its crucial roles in the development of inflammatory pain and neuropathic pain conditions are supported by numerous studies (Palazzo et al. 2010) (Kanai et al. 2005) (Hudson et al. 2001). Consistently, intrathecal administration of TRPV1 antisense oligonucleotides significantly reduced tactile hypersensitivity in spinal nerve ligation neuropathic pain model (Christoph et al. 2007). TRPV1 has been extensively investigated as the potential pharmacological target of novel analgesics (Szallasi et al. 2007). Therefore drugs such as cinnamides, carboxamides, andimidazole derivatives have been discovered and developed. Interestingly, recent studies have shown that α2 adrenergic receptors are co-localized with TRPV1 in

spinal DRG. Also, α2 adrenergic agonist modulates intracellular signaling pathways critical in the function of TRPV1 receptor (Wu et al. 1988). In this study, assuming that DEX may modulate the activity of TRPV1 receptor expressed in DRG cells , we measured its effect by comparing the intracellular calcium response induced by capsaicin to investigate whether dexmedetomidine has analgesic effect by directly modulating nociceptive DRG cells.

In order to see whether dexmedetomidine modulate TRPV1 activity, we analyzed the calcium responses elicited by capsaicin, a well-known TRPV1 agonist, in dose-dependent manner after applying the drug. Also, through RT-PCR analysis, we confirmed that adult mice dorsal root ganglion neurons expressed all three α2 adrenergic receptor (α2-AR) subtypes and TRPV1. These results confirmed the modulating effect of dexmedetomidine on TRPV1 receptors. The influx of calcium is prerequisite to intracellular signal pathways as well as neurotransmitter release. Therefore, changes of intracellular calcium concentration are essential to maintain cell functions, and inhibition of calcium influx reflects an integrated depressed response on cell excitability. In other words, neurotransmitter release at peripheral terminals is critical in nociception, which requires the increase in intracellular calcium concentrations. Therefore, the decrease of intracellular calcium after DEX application may be associated with DEX-induced antinociception. A real time calcium imaging videomicroscopy used in this study allows us to define small to medium sized capsaicin-sensitive DRG cells, which are known as nociceptive neurons, in phenotypic and functional way. It is also possible to measure the intracellular calcium responses in more than 100 cells simultaneously at a magnification of 20. In addition, the overall actions of DRG cells after DEX application can be easily observed with the use of real time calcium imaging videomicroscopy.

In this study, we found that capsaicin-induced TRPV1 activation was attenuated in the presence of DEX. Although the exact mechanisms of DEX induced alterations of TRPV1 activity remains unclear, it may be speculated that DEX acts on the signaling pathways of TRPV1 regulating its functional activity. It has been shown that TRPV1 sensitivity is increased by activating phosphokinase A (PKA) activation through stimulation of inflammation associated receptors (Amadesi et al. 2006; Tominaga et al. 2001; Vellani et al. 2004; Zhang et al. 2005) The increase in cAMP levels by activating adenyl cyclase (AC) is prerequisite for PKA activation. Interestingly, the sympatholytic actions of alpha 2 adrenergic agonist is associated with inhibiting cAMP generation to beta receptor activation.(Wu et al. 1988) Therefore, it is likely that DEX may decrease TRPV1 activity by modulating intracellular signaling pathway. DEX is also reported to modulate voltage gated calcium and sodium channel. The activation of voltage gated ion channel could induce a cell depolarization contributing to the conformational changes of TRPV1 channel essential in channel activation. The sensitization of TRPV1 can occur by the release of proinflammatory factors (Chuang et al. 2001; Tominaga et al. 2001; Zhang et al. 2007). Interestingly, DEX is reported to have an anti-inflammatory activity by inhibiting the release of the inflammatory mediators: Activation of TRPV1 is associated with anti-inflammatory mediators such as interleukin-6 (Sappington and Clakins 2008).

The anti-inflammatory activity of DEX may lead to inhibit the sensitization of TRPV1 by capsaicin application, although a further study should be conducted to clarify the relationship between DEX anti-inflammatory actions and TRPV1 functions. Interestingly, the upregulation of alpha 2 adrenergic receptors at the DRG is related to the pathogenesis of neuropathic pain. Also, alpha 2 adrenergic agonist possess

the ability to modulate the critical pathway of activate TRPV1 function (Wu et al. 1988). It can be inferred that DEX, acting at alpha adrenergic receptors may affect TRPV1 activity morphologically and functionally, which can be one of explanation of attenuated TRPV1 activity in the presence of DEX as shown in this study.

Taken together, DEX may modulate TRPV1 activity which may contribute to analgesic actions of DEX in neuropathic pain. The present study demonstrated reduction of intracellular calcium increase after capsaicin administration by DEX, suggesting reduced activity of TRPV1 polymodal nociceptive receptor might be a potential mechanism underlying the analgesic effect of DEX.

V. Limitations & Future Perspective

In summary, we demonstrate that DEX significantly attenuated the activity of TRPV1, even though the exact mechanism of DEX induced could not be determined by this study. In fact, the present study had another limit in that the neuron sample was not from neuropathic pain model animal but from normal dorsal root ganglion neurons from healthy adult C57BL/6 male mice. Also, whether DEX directly inhibits TRPV1 or indirectly inhibit TRPV1 via alpha 2 adrenergic receptor is still unclear in this study. Therefore, We propose that further investigation might develop a currently used sedative, DEX, as an effective analgesic drug targeting TRPV1-mediated neuropathic or inflammatory hyperalgesia.

VI. Acknowledgement

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Tables and Figures

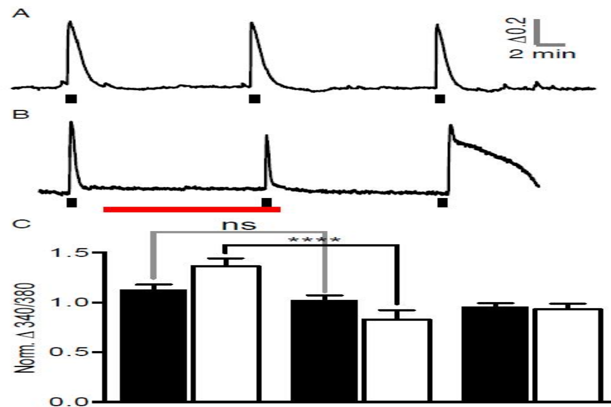

Figure 1.

(A) Representative calcium transients of dorsal root ganglion neurons under three repeated applications (black horizontal bar) of capsaicin (400 nM). (B) Representative calcium traces of dorsal root ganglion neurons under capsaicin (black horizontal bars) and dexmedetomidine (red horizontal bar). (C) Normalized peak amplitudes of three consecutive calcium transients from panel A (black) and panel B (white). Data shown are means ± SEMs from # neurons; statistical significance was assessed using Student t-test (**** $p<0.0001$; NS not-significant)

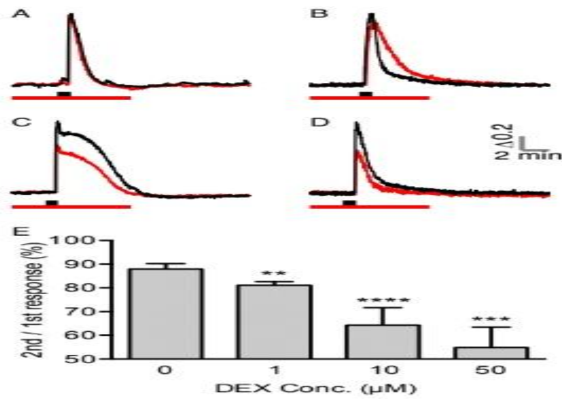

Figure 2.

(A-D) Representative calcium transients of dorsal root ganglion neurons before (black) and after (red) application of dexmedetomidine. Concentration of dexmedetomidine are as follows: A, 0 μM (control); B, 1 μM; C, 10 μM; D, 50 μM. (E) Relative peak amplitudes of capsaicin responses with and without dexmedetomidine treatment. Data shown are means ± SEMs from # neurons; statistical significance was assessed using Student t-test ((** p<0.01; (*** p<0.001; **** p<0.0001; NS not-significant)

Figure 3.

RT-PCR analysis of α2 adrenergic receptor (α2-AR) subtypes and TRPV1 in adult mice dorsal root ganglion neurons. Representative gel

images show expression of α2A-AR(α2A),α2B-AR(α2B),α2C-AR(α2C), TRPV1, and control GAPDH.

Target Gene (Product Size)	Forward Primer	Reverse Primer	Gen Bank Ref.
Adra2a (538 bp)	CTCGCTGAACCCTGTTATCTAC	GACCGCCCTGAATGATCTTTAT	NM_007417
Adra2b (199 bp)	CCCTGCCTCATCATGATTCT	GTCCATTAGCCTCTCCGACA	NM_009633
Adra2c (156 bp)	TCATCGTTTTCACCGTGGTA	GCTCATTGGCCAGAGAAAAG	NM_007418
Trpv1 (233 bp)	AGCGAGTTCAAAGACCCAGA	TTCTCCACCAAGAGGGTCAC	NM_001001445
Gapdh (230 bp)	ACTCCCACTCTTCCACCTTC	TGAGGGAGATGCTCAGTGTT	GU214026

Table 1.

List of primers used.

국문초록

1. 목 적

진정제 덱스메데토미딘 (Dexmedetomidine)은 적은 호흡 억제 부작용과 부가적인 진통 효과로 다양한 활용도를 보인다. 덱스메데토미딘의 진정 효과는 alpha 2 adrenergic receptor의 효현제로 작용하여 나타나는 것으로 알려져 있으나, 진통효과의 분자적 기전에 대해서는 아직 알려진 바가 많지 않다. 본 연구에서는 덱스메데토미딘의 진통 효과가 통각 유발 수용체 Transient Receptor Potential Vanilloid Subtype 1 (TRPV1)를 매개로 일어나는지 확인해보고자 한다.

2. 방 법

실험용 C57BL/6 쥐의 척수후근신경절 (DRG)에서 신경세포를 분리하여 캡사이신 약물에 의한 세포내 칼슘 농도의 증가가 덱스메데토미딘 전처리에 의해 줄어드는지를 Fura-2 기반 calcium ratiometry로 알아보았다. 캡사이신(400nM)을 연속해서 적용하여 탈감작이 최소로 일어나는 조건을 찾은 후, 두 번째 캡사이신을 적용하기 전 덱스메데토미딘을 10분간 전처리 하였으며, 첫 번째와 두 번째 캡사이신 적용에 의해 증가한 칼슘 농도의 비율을 비교하였다. 척수후근신경절에서 $\alpha 2$ adrenergic receptor의 3가지 subtype, α_{2A}-, α_{2B}-, 그리고 α_{2C}-adrenergic receptor와 TRPV1의 발현을 확인하였다.

3. 결 과

덱스메데토미딘이 TRPV1 활동에 끼치는 영향을 알아보기에 앞서 척수후근신경절 뉴런에 캡사이신 (TRPV1 작용제)를 처리해줌으로써 유도된 칼슘 반응을 살펴보았다. 그 결과, 400 nM의 캡사이신이 주로 작은 크기의 척수후근신경절 뉴런에 칼슘 유입을

유도한 걸 발견할 수 있었다. 10분 간격으로 캡사이신을 3번 연속적으로 처리하자 TRPV1 반응은 점점 줄어들었으나, 최대 TRPV1 반응치 수준의 차이는 통계적으로 유의하지 않은 범위 내에 있었다 (p=0.08). 이는 실험 조건에서 TRPV1의 탈감작화가 적었다는 것을 의미한다.

캡사이신에 의한 척수후근신경절 뉴런의 칼슘 반응은 덱스메데토미딘에 의해 유의하게 감소하였고, (60.7%, p<0.0001, n=34) 이는 10분간의 인공세포외액 처리에 의해서 일부 회복되었다 (68.2%, p<0.0001, n=34). 이러한 덱스메데토미딘에 의한 캡사이신 반응의 억제는 덱스메데토미딘의 농도 증가에 따라 더 크게 나타났다. 이로써 덱스메데토미딘에 의한 반응이 농도에 의존한다는 것 또한 알 수 있었다.

척수후근신경절 신경의 RT-PCR 결과 alpha-2 adrenergic receptor 세 가지 아형(α2A, α2B, α2C) 모두의 발현을 RT-PCR을 통해 확인 할 수 있었으며, 이는 덱스메데토미딘의 캡사이신 반응 억제가 alpha-2 adrenergic receptor의 활성화를 통해서 일어났을 가능성을 시사한다.
주요어 : 덱스메데토미딘 (dexmedetomidine), 척수후근신경절 신경, 진통 효과, Adrenergic 수용체, Transient Receptor Potential Vanilloid Subtype 1 (TRPV1), 캡사이신 (capsaicin), 칼슘 (calcium)

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