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Effects of acute exposure of alpha 1- and alpha 2-adrenoreceptor agonist or antagonist on capsaicin-evoked substance P release from dorsal root ganglion neurons *in vitro*

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Peripheral alpha-adrenoceptors are involved in mediating neurogenic inflammation. To characterize the effects of acute administration of selective alpha adrenoreceptor agonists or antagonists on capsaicin-evoked substance P (SP) release from dorsal root ganglion (DRG) neurons, dissociated cultured DRG neurons were preincubated with selective alpha 1-adrenoreceptor agonist phenylephrine (10^{-5} mol/L), alpha 1-adrenoreceptor antagonist prazosin (10^{-6} mol/L), alpha 2-adrenoreceptor agonist clonidine (10^{-5} mol/L), alpha 2-adrenoreceptor antagonist yohimbine (10^{-5} mol/L) for 10 min, followed by the addition of capsaicin (10^{-7} nmol/L) for additional 10 min. Radioimmunoassay (RIA) was employed to determine if the capsaicin-evoked enhancement of neuropeptide release was subject to adrenergic modulation. Expression of SP mRNA was determined by RT-PCR. Acute exposure of selective alpha 1-adrenoreceptor agonist phenylephrine could increase capsaicin-evoked SP release from primary cultured DRG neurons. Expression of SP mRNA was not affected by acute stimulation with these adrenoreceptor agonists or antagonists. The data provided in the present study suggest that the excitatory effect of alpha 1-adrenoreceptor agonist on capsaicin-evoked release of neuropeptide from primary cultured DRG neurons is likely to be mediated by activation of VR1 to influence capsaicin sensitivity but not by promotion of SP synthesis under acute stimulative states.

1. Introduction

The sympathetic system (SNS) is considered to be a major component of the neurogenic contribution to inflammation and hyperalgesia (Safieh-Garabedian et al. 2002). The noradrenergic system is subject to various plastic changes that influence its antinociceptive efficacy after injury or inflammation (Pertovaara 2006). Sympathetic post-ganglionic neurons may be involved in the generation of pain, hyperalgesia and inflammation under pathophysiological conditions (Jänig et al. 1996). Dorsal root reflexes-mediated neurogenic inflammation depends in part on intact sympathetic efferents acting on peripheral alpha 1-adrenoceptors, but not alpha 2-adrenoceptors, which augment the sensitization of primary afferent nociceptors induced by capsaicin injection, helping trigger dorsal root reflexes that produce vasodilation (Wang et al. 2004). Interestingly, functional alpha 1-adrenoceptors are expressed in primary sensory neurons and regulate neurogenic inflammation and nociceptive responses (Xie et al. 2001; Nicholson et al. 2005; Trevisani et al. 2007). Alpha 1-adrenoceptors mediate depolarization in cultured rat dorsal root ganglion (DRG) neurons (Pluteanu

et al. 2002). It has been demonstrated that alpha 2- adrenoreceptors are also expressed in DRG neurons (Gold et al. 1997; Shi et al. 2000; Ma et al. 2005). Alpha-adrenoreceptors have a key role in mediating pain regulatory effects of norepinephrine (NE) (Pertovaara 2006; Yang et al. 2009). These functionally active adrenoreceptors may vary with the presence of nerve injury, inflammation or other physiological and pathophysiological conditions (Gold et al. 1997; Birder and Perl 1999; Ma et al. 2005).

Capsaicin, the pungent component of hot peppers, elicits a sensation of burning pain, via activation of vanilloid receptor 1 (VR1, capsaicin receptor) expressed in primary sensory neurons (Winston et al. 2001; Tominaga et al. 2005). Capsaicin depolarizes DRG neurons (Oh et al. 1996; Lee et al. 2005) and evokes release of neuropeptide substance P (SP) (Hingtgen et al. 1995; Winston et al. 2001; Xing et al. 2006; Yang et al. 2008). SP, an 11-amino-acid peptide, is a member of the tachykinin family of peptide neurotransmitters that are derived from preprotachykinin gene by alternative splicing. SP is found in sensory nerves innervating peripheral tissues (Ribeiro-da-Silva and Hökfelt 2000). Release of SP from peripheral endings causes

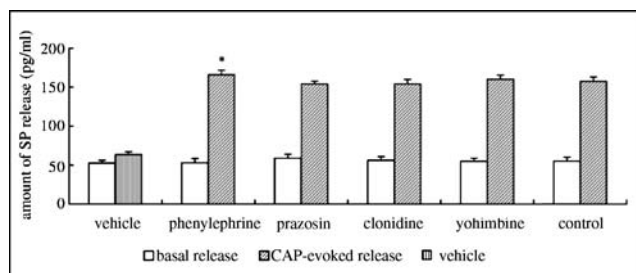


Fig. 1: Effects of alpha 1- and alpha 2-adrenoreceptor agonist or antagonist on SP release from DRG neurons. The basal SP release was not changed at different stimulative conditions ($P > 0.05$). Administration of alpha 1-adrenoreceptor agonist phenylephrine for 10 minutes could increase capsaicin-evoked SP release ($P < 0.05$). Exposure of prazosin, clonidine or yohimbine did not have effect on capsaicin-evoked SP release ($P > 0.05$). Bar graphs with error bars represent mean $\pm$ SD ($n = 6$). * $P < 0.05$ vs. control

a series of local inflammatory responses referred to as neurogenic inflammation (Trevisani et al. 2007).

Therefore, to provide the knowledge of the effects of acute administration of selective alpha adrenoreceptor agonist or antagonist on capsaicin-evoked neuropeptide release from dissociated cultured DRG neurons is important for understanding the adrenergic modulation on neurogenic inflammation and hyperalgesia. Here we have investigated preincubation with the alpha 1-adrenoreceptor agonist phenylephrine, the alpha 1-adrenoreceptor antagonist prazosin, the alpha 2-adrenoreceptor agonist clonidine, or the alpha 2-adrenoreceptor antagonist yohimbine on capsaicin-evoked SP release from dissociated cultured DRG neurons. Radioimmunoassay (RIA) was employed to determine if the capsaicin-evoked enhancement of neuropeptide release was subject to adrenergic modulation. In addition, expression of SP mRNA was determined by RT-PCR for analyzing the relevance of neuropeptide release to SP mRNA expression.

2. Investigations and results

2.1. Effects of exposure of phenylephrine or prazosin on DRG neurons

Exposure of alpha 1-adrenoreceptor agonist phenylephrine (10^{-5} mol/L) or alpha 1-adrenoreceptor antagonist prazosin (10^{-6} mol/L) for 10 minutes, followed by the addition of capsaicin (10^{-7} nmol/L) for additional 10 minutes, capsaicin-evoked SP release was promoted by phenylephrine, but not prazosin. The expression of SP mRNA was not changed with phenylephrine or prazosin treatment as compared with that in control group (Figures 1, 2).

2.2. Effects of exposure of clonidine or yohimbine on DRG neurons

Exposure of alpha 2-adrenoreceptor agonist clonidine (10^{-5} mol/L) or alpha 2-adrenoreceptor antagonist yohimbine (10^{-5} mol/L) for 10 minutes, the expression of SP mRNA and capsaicin-evoked SP release were not changed as compared with that in control group (Figures 1, 2).

3. Discussion

The present study demonstrates that selective activation of alpha 1-adrenoreceptors could influence capsaicin-evoked neuropeptide release from primary cultured DRG neurons. Acute exposure of these adrenoreceptor agonists or antagonists did not affect expression of SP mRNA. These results implicated that the

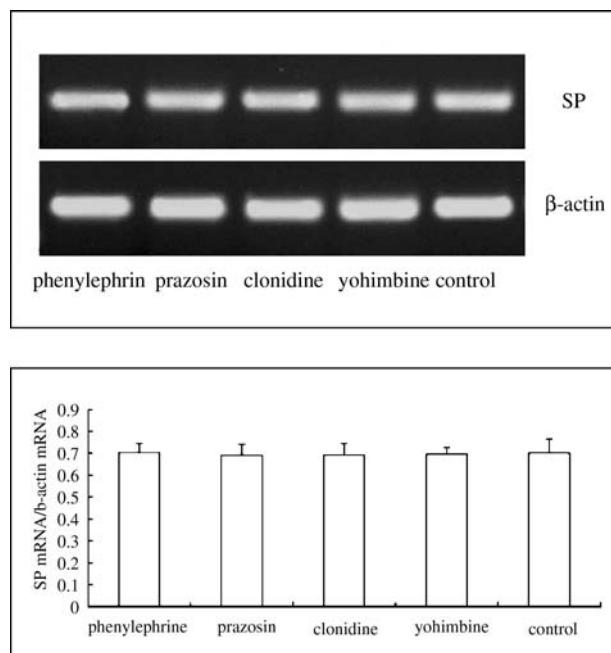


Fig. 2: Effects of alpha 1- and alpha 2-adrenoreceptor agonist or antagonist on SP mRNA expression in primary cultured DRG neurons. SP and β -actin mRNA were analyzed by RT-PCR. The quantitative analysis of the results are as follows: DRG cells were treated with 10^{-5} mol/L phenylephrine (SP mRNA/ β -actin mRNA = 0.7028 ± 0.0411). DRG cells were treated with 10^{-6} mol/L prazosin (SP mRNA/ β -actin mRNA = 0.6914 ± 0.0501). DRG cells were treated with 10^{-5} mol/L clonidine (SP mRNA/ β -actin mRNA = 0.6933 ± 0.0510). DRG cells were treated with 10^{-5} mol/L yohimbine (SP mRNA/ β -actin mRNA = 0.6955 ± 0.0291). DRG cells were cultured continuously in growth media as control (SP mRNA/ β -actin mRNA = 0.7008 ± 0.0654). Administration of alpha 1- and alpha 2-adrenoreceptor agonist or antagonist did not have effect on SP mRNA expression. Bar graphs with error bars represent mean $\pm$ SD ($n = 5$)

excitatory effect of the alpha 1-adrenoreceptor agonist is likely to be mediated by activation of VR1 to influence capsaicin sensitivity rather than by promoting neuropeptide synthesis since the exposure time is too short to initiate transcriptional or translational machinery of the neuropeptide. The present study provides important new evidence that adrenergic modulation on capsaicin-evoked neuropeptide release under acute stimulative states is by activation of alpha 1-adrenoreceptors in primary DRG neuronal cultures.

Alpha 1-adrenoreceptor agonists and antagonists have been reported to increase and reduce, respectively, neurogenic inflammatory responses mediated by capsaicin-sensitive sensory neurons (Andersson 2000, Wang et al. 2004; Milani et al. 2005; Trevisani et al. 2007). Alpha 1-adrenoceptors are functionally expressed by capsaicin-sensitive, nociceptive, primary sensory neurons, and their activation may contribute to signal irritative and nociceptive responses. Whereas the beneficial effect of alpha 1-adrenoceptor antagonists derives from their inhibitory effect on neurogenic inflammatory responses (Trevisani et al. 2007). As known, the initiation and development of neurogenic inflammation in rats are mainly mediated by dorsal root reflexes. The enhancement of dorsal root reflexes evoked by capsaicin was prevented by sympathectomy. Interestingly, the enhancement of dorsal root reflexes evoked by capsaicin could be restored by peripheral activation of alpha 1-adrenoceptors, but not alpha 2-adrenoceptors, after sympathectomy (Wang et al. 2004). The results observed in the present study are consistent with the previously observed effects of activation of alpha 1-adrenoceptors. However, it is still a matter of debate, which subtype of adrenoceptor in the periphery contributes to aggravation of pain and which one to suppression of pain, since different adrenoceptor types have at least partly different functions and their func-

tional effects vary with the pathophysiological condition. Both facilitatory and inhibitory neuronal mechanisms activated by noradrenergic compounds have been shown to exist in the DRG cells (Pertovaara 2006). Both pain facilitatory role (Banik et al. 2001) and inhibitory role (Lavand'homme et al. 2002; Dogrul and Uzbay 2004) of peripheral alpha-2-adrenoceptors were found in previous studies. The norepinephrine-induced hypersensitivity was attenuated by an alpha-2-adrenoceptor antagonist (Banik et al. 2001) suggesting a pain facilitatory role. Whereas peripheral administration of an alpha-2-adrenoceptor agonist attenuated nociceptive responses in control animals (Dogrul and Uzbay 2004) and hypersensitivity in inflammatory and neuropathic conditions (Lavand'homme et al. 2002) suggesting a pain inhibitory role. Even co-expression of alpha 2-adrenoceptors and VR1 was reported in injured DRG neurons (Ma et al. 2005), no effect on capsaicin-evoked neuropeptide release was observed in the present study in acute treatment experiment with alpha 2-adrenoceptor agonist or antagonist. One reason of this result may be that the exposure time is too short to initiate VR1 function. Thus, more chronic stimulative states of these adrenoceptor agonists or antagonists on DRG neurons should be further clarified.

Sensory neuropeptides are released from central and peripheral endings of the primary sensory neurons. Release in the spinal cord has been associated with nociceptive transmission, whereas release from peripheral endings causes a series of local inflammatory responses referred to as neurogenic inflammation (Winston et al. 2001; Trevisani et al. 2007). Primary cultured DRG neurons are the suitable model for these studies, since they are expressed alpha-adrenoceptors, VR1 and sensory neuropeptides (Kress and Fickenschner 2001; Winston et al. 2001; Schmidt et al. 2003). And these neuropeptides are released from cultured DRG neurons upon appropriate stimulation (Tang et al. 2006). Stimulus-evoked neuropeptide release is a key measure of sensory neuron function (Winston et al. 2001). Phenylephrine enhancement of capsaicin-evoked SP release from cultured DRG neurons observed in the present study may reflect that the excitatory effect of alpha 1-adrenoceptor agonist at the neurogenic inflammatory status.

In summary, the findings in the present study suggest that acute exposure of selective alpha 1-adrenoceptor agonists, but not alpha 1-adrenoceptor antagonists or alpha 2-adrenoceptor agonists or antagonists affects capsaicin-evoked neuropeptide release from primary cultured DRG neurons. The excitatory effect of alpha 1-adrenoceptor agonist is likely to be mediated by activation of VR1 to influence capsaicin sensitivity. Evaluation of the role of these adrenoceptor agonists or antagonists under more chronic stimulative states is warranted.

4. Experimental

4.1. Cell cultures

Dorsal root ganglia were dissected from embryonic 15-day-old Wistar rats obtained from the Experimental Animal Center of Shandong University of China. Dorsal root ganglia prior to establishment in culture were digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37 °C for 10 min, centrifuged, and triturated in growth media supplemented with 2.5% fetal bovine serum (Gibco). Dissociated DRG cells were then cultured in 24-well clusters (Costar, Corning, NY, USA) for monitoring SP levels using RIA or flasks (Costar, Corning, NY, USA) for detecting for SP mRNA by RT-PCR. The clusters and flasks were precoated with poly-L-lysine prior to plating DRG cells. DRG cells were plated at 1×10^5 cells/well in clusters and at a density of 5×10^5 cells/ml in flasks. Then DRG cells were cultured in culture media at 37 °C with 5% CO₂ for 24 h and then maintained in culture media containing cytarabine (ara-C) (5 µg/ml) for another 24 h to inhibit growth of non-neuronal cells, and then cultured in culture media for another 4 days with media change every 2 days. The composition of the culture media is D-MEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), insulin (0.25 µg/ml, Sigma), L-glutamine (0.1 mg/ml, Sigma), penicillin (100 U/ml), and streptomycin (100 µg/ml).

4.2. Exposure of selective alpha adrenoceptor agonists or antagonists and capsaicin treatment

DRG cell cultures were prepared as described above and allowed to grow processes for 6 days. Then the DRG neurons were preincubated with alpha 1-adrenoceptor agonist phenylephrine (10^{-5} mol/L), alpha 1-adrenoceptor antagonist prazosin (10^{-6} mol/L), alpha 2-adrenoceptor agonist clonidine (10^{-5} mol/L), alpha 2-adrenoceptor antagonist yohimbine (10^{-5} mol/L) for 10 min, followed by the addition of capsaicin (10^{-7} nmol/L) for additional 10 min before being examined by RIA.

4.3. RT-PCR analysis for mRNAs for SP

The mRNA levels of SP were analyzed by RT-PCR at 6 days of culture age with administration of alpha 1-adrenoceptor agonist phenylephrine (10^{-5} mol/L), alpha 1-adrenoceptor antagonist prazosin (10^{-6} mol/L), alpha 2-adrenoceptor agonist clonidine (10^{-5} mol/L), and alpha 2-adrenoceptor antagonist yohimbine (10^{-5} mol/L) for 10 min. The expression of β -actin was also determined as an internal control. Total DRG cell RNA of each flask was isolated by TRIzol (Gibco). cDNA synthesis was performed with M-MLV reverse transcriptase. The gene-specific primers were synthesized by use of the published cDNA sequences for SP and β -actin. The synthetic oligonucleotide primer sequences for SP and β -actin were as follows:

SP 5'-GCC CTT TGA GCA TCT TCT TC-3' (upper primer) and 5'-GTC TGA GGA GGT CAC CAC AT-3' (lower primer).

β -actin 5'-ATC ATG TTT GAG ACC TTC AAC-3' (upper primer) and 5'-CAT CTC TTG CTC GAA GTC CA-3' (lower primer).

The predicted size of the amplified SP and β -actin DNA products were 450 bp and 317 bp, respectively.

PCR amplification was performed for 35 cycles. The cycle profile included denaturation for 45 s at 94 °C, annealing for 60 s at 58 °C, and extension for 45 s at 72 °C. PCR was performed within the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products.

The amplified products were analyzed by standard agarose gel electrophoresis and stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned and the electrophoresis gel images were analyzed quantitatively by using an ImageJ analysis software. The levels of SP mRNA were expressed as the ratio of the gene to β -actin.

4.4. RIA analysis for SP release from DRG neurons

After 10 min incubation under different treatment conditions, DRG neuron cultures were washed with release buffer (Hank's balanced salt solution supplemented with 10.9 mmol/L HEPES, 4.2 mmol/L sodium bicarbonate, 10 mmol/L dextrose and 0.1% bovine serum albumin, pH 7.4) and incubated for 10 min at 37 °C in release buffer to measure basal SP release. Fresh release buffer containing capsaicin (10^{-7} mol/L) was added for an additional 10 min to measure capsaicin-evoked SP release. After each incubation, the culture media were removed and measured by RIA for SP release from DRG neurons.

The RIA technique for the measurement of SP was as follows. The samples were reconstituted in PBS. Standards of synthetic SP (rat amino acid sequence) ranging from 2.5 to 1280 pg/assay tube dissolved in a volume of 0.2 ml PBS. The dissolved SP was then incubated at 4 °C with 0.1 ml of anti-SP antibody (anti-rat SP antibody) for 24 h. The mixture was then incubated for an additional 24 h at 4 °C with 0.1 ml of ¹²⁵I-labeled SP (20,000 counts/min/tube) in PBS. Free and bound neuropeptide were separated by adding 0.5 ml separating agent 45 min. The RIA test tubes were centrifuged (4000 rpm at 4 °C, 20 min). After removal of the supernatant fraction, the RIA test tubes were counted for iodine-125 remaining in the tubes.

4.5. Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical analysis was evaluated with SPSS software by one-way ANOVA followed by the Student-Newman-Keuls test for significance to compare the differences among various groups. Significance was accepted at $P < 0.05$.

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