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Epinephrine enhances the sensitivity of rat vagal chemosensitive neurons: role of β_3 -adrenoceptor

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Abstract

This study was carried out to determine whether epinephrine alters the sensitivity of rat vagal sensory neurons. In anesthetized rats, inhalation of epinephrine aerosol (1 and 5 mg/ml, 3 min) induced an elevated baseline activity of pulmonary C-fibers and enhanced their responses to lung inflation (20 cmH₂O, 10 s) and right atrial injection of capsaicin (0.5 μ g/kg). In isolated rat nodose and jugular ganglion neurons, perfusion of epinephrine (3 μ M, 5 min) alone did not produce any detectable change of the intracellular Ca²⁺ concentration. However, immediately after the pretreatment with epinephrine, the Ca²⁺ transients evoked by chemical stimulants (capsaicin, KCl and ATP) were markedly potentiated; for example, capsaicin (50 nM, 15 s) - evoked Ca²⁺ transient was increased by 106% after epinephrine ($P < 0.05$; $n = 11$). The effect of epinephrine was mimicked by either BRL 37344 (5 μ M, 5 min) or ICI 215,001 (5 μ M, 5 min), two selective β_3 -adrenoceptor agonists, and blocked by SR 59230A (5 μ M, 10 min), a selective β_3 -adrenoceptor antagonist; whereas pretreatment with phenylephrine (α_1 -adrenoceptor agonist), guanabenz (α_2 -adrenoceptor agonist), dobutamine (β_1 -adrenoceptor agonist) or salbutamol (β_2 -adrenoceptor agonist) had no significant effect on capsaicin-evoked Ca²⁺ transient. Furthermore, pretreatment with SQ 22536 (100 300 μ M, 15 min), an adenylate cyclase inhibitor, and H89 (3 μ M, 15 min), a PKA inhibitor, completely abolished the potentiating effect of epinephrine. Our results suggest that epinephrine enhances the excitability of rat vagal chemosensitive neurons. This sensitizing effect of epinephrine is likely mediated through the activation of β_3 -adrenoceptor and intracellular cAMP-PKA signaling cascade.

Keywords

Stress; vagal afferents; fura-2; adenylate cyclase; PKA

INTRODUCTION

Epinephrine is the primary catecholamine secreted by the adrenal medulla in response to various physiological stresses (41). The physiological functions of epinephrine are mediated through the activation of cell membrane α - and β -adrenoceptors, both of which belong to the superfamily of seven-transmembrane G-protein-coupled receptors (13). Vagus nerves provide the primary afferent innervation of a broad spectrum of visceral organs, and play an important role in the initiation of visceral/viscerosomatic reflexes and the regulation of vegetative functions (24,29). Numerous studies have identified the presence of β -adrenoceptors on the vagus nerve in both rats and humans (23,28). Although a sensitizing effect of epinephrine on

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nociceptors was recently reported (21), whether epinephrine can modulate the excitability of vagal sensory neurons is largely unknown.

The aims of the present study were: 1) to determine whether epinephrine alters the excitability of pulmonary vagal afferents in-vivo and the chemosensitivity of isolated vagal sensory neurons invitro; 2) if so, to identify the specific subtype of adrenoceptors subserving the effect of epinephrine; and 3) to investigate the involvement of cAMP-PKA intracellular transduction cascade in mediating this effect. Since intracellular Ca^{2+} is known to play a critical role in regulating a diverse range of cellular processes such as neuronal membrane excitability, neurotransmitter release, synaptic plasticity, cell proliferation and gene transcription (3), fura-2-based ratiometric Ca^{2+} imaging was employed to determine the sensitivity of these vagal sensory (nodose and jugular ganglion) neurons isolated from adult Sprague-Dawley rats. Three chemical agents (capsaicin, KCl and ATP) known to stimulate vagal sensory neurons (12) by activating different ion channels were used as the chemical stimuli in the Ca^{2+} imaging study.

METHODS

The procedures described below were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

In-Vivo Study:

Recording of Single-fiber Activity of Pulmonary Afferents—Sprague-Dawley rats (280–455 g; $n = 31$) were initially anesthetized with an intraperitoneal injection of α -chloralose (100 mg/kg) and urethane (500 mg/kg) dissolved in a borax solution (2%); supplemental doses of these anesthetics were injected intravenously to maintain abolition of pain reflexes. A short tracheal cannula was inserted after a tracheotomy, and tracheal pressure (P_t) was measured (Validyne MP 45-28, Northridge, CA) via a side-port of the tracheal cannula. The expiratory outlet of the respirator was placed under 3 cmH₂O pressure to maintain a near-normal functional residual capacity. Tidal volume (V_T) and frequency (f) were set at 8–10 ml/kg and 50 breaths/min, respectively. One femoral artery was cannulated for recording the arterial blood pressure (ABP). For right-atrial injection of capsaicin, the left jugular vein was cannulated and a catheter was advanced until its tip was positioned just above the right atrium. The volume of each bolus injection was 0.15 ml, which was first injected into the catheter (dead space, ~0.2 ml) and then flushed into the circulation by an injection of 0.4 ml saline. Body temperature was maintained at ~36°C by means of a heating pad placed under the animal. At the end of the experiment, the animal was killed by an i.v. injection of KCl.

Vagal pulmonary afferents can be broadly classified into three major types, based upon the criteria established by previous investigators (33): slowly adapting receptors (SARs), rapidly adapting receptors (RARs) and pulmonary C-fibers. These pulmonary afferents were identified using the procedures described previously (14), and the fiber activity (FA) measured by the conventional method of single-fiber recording. Briefly, right cervical vagus nerve was sectioned as far rostrally as possible, and the caudal end of the cut vagus was placed on a small dissecting platform and immersed in a pool of mineral oil. A thin filament was teased away from the desheathed nerve trunk and placed on a miniature platinum-iridium electrode. Action potentials were amplified, monitored by an audio monitor, and displayed on an oscilloscope. The thin filament was further split until the action potentials arising from a single unit was electrically isolated. FA, P_t , ABP and heart rate were analyzed with an on-line data acquisition system (Biocybernetics TS-100, Taipei, Taiwan).

Experimental Protocols—To study the effect of epinephrine on pulmonary C-fiber afferents, the single-unit responses to lung inflation (20 cmH₂O, 10 s) and to capsaicin (0.5 µg/kg) were investigated before, 2 min, 10 min and 20 min after the administration of epinephrine. To minimize systemic effects, epinephrine was delivered into the airways by aerosol; during the delivery of the epinephrine, the ultrasonic nebulizer (Lumiscopes 6610; Lumiscopes Co., East Brunswick, NJ) that generated the aerosol was connected between the outlet of the respirator and the tracheal tube to prevent contamination of the breathing circuit by the residual epinephrine. The mass mean diameter of aerosol and the total volume of solution delivered over 3 min under our experimental setting were estimated to be 3–4 µm and 0.05–0.06 ml, respectively. To determine whether the effect of epinephrine was dose-dependent, three doses of epinephrine were used in different groups of rats: 0 mg/ml (vehicle: 5 mg/ml ascorbic acid in saline), 1 mg/ml (low concentration) and 5 mg/ml (high concentration). No more than two doses of epinephrine were administered in each animal. Because of the long-lasting effect of the high-concentration of epinephrine, responses were also measured at 60 min after the aerosol challenge. Identical protocols were followed to study the effect of epinephrine on the responses of SARs and RARs to lung inflation (20 cmH₂O, 10 s) in a separate group of rats.

In-Vitro Study:

Isolation and Culture of Nodose and Jugular Ganglion Neurons—Because subtypes of adrenoceptors are known to be expressed in different cell types in the airways and their individual involvements cannot be differentiated in the in-vivo preparation, the following experiments were performed in isolated vagal sensory neurons to identify the receptor subtypes subserving the effect of epinephrine. Young adult male Sprague-Dawley rats (150–220 g; *n* = 35) which were anesthetized with 4% halothane and decapitated. The head was immediately immersed in ice-cold Hank's balanced salt solution. Nodose and jugular ganglia were extracted under a dissecting microscope and placed in ice-cold Dulbecco's minimal essential medium/F-12 (DMEM/F12) solution. Each ganglion was desheathed, cut into ~10 pieces, placed in 0.125% type IV collagenase, and incubated for 1 h in 5% CO₂ in air at 37°C. The ganglion suspension was centrifuged (150 × *g*, 5 min) and supernatant-aspirated. The cell pellet was resuspended in 0.05% trypsin in Hanks' balanced salt solution for 5 min and centrifuged (150 × *g*, 5 min); the pellet was then resuspended in a modified DMEM/F12 solution (DMEM/F12 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 100 µM MEM nonessential amino acids) and gently triturated with a small bore fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged (500 × *g*, 8 min) through a layer of 15% bovine serum albumin to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM/F12 solution supplemented with 50 ng/ml 2.5S nerve growth factor, plated onto poly-L-lysine-coated glass coverslips, and then incubated overnight (5% CO₂ in air at 37°C).

In a subset of experiments, sensory neurons innervating the lungs and airways were identified by retrograde labeling from the lungs (22) with the fluorescent neuronal tracer, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). Briefly, rats (140–180 g) were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg) and intubated with a polyethylene catheter (PE-150) with its tip positioned in the trachea above the thoracic inlet. DiI was initially sonicated and dissolved in ethanol, diluted in saline (1% ethanol, vol/vol), and then instilled into the lungs (0.2 mg/ml; 0.2 ml × 2) with animal's head tilted upwards at ~30°C. The animal was allowed to recover for 7–10 days to permit DiI to be transported back to the cell soma of pulmonary vagal sensory neurons.

Intracellular Ca²⁺ Measurement—Intracellular Ca²⁺ was monitored using the fluorescent Ca²⁺ indicator fura-2 AM. Cells were loaded with 5 µM fura-2 AM for 30 min at 37°C, then

rinsed ($\times 3$) with extracellular solution, and allowed to de-esterify for at least 30 min before use. Ratiometric Ca^{2+} imaging was performed using a fluorescence inverted microscope (Axiovert 100; Carl Zeiss, Thornwood, NY) equipped with a variable filter wheel (Lambda 10-2; Sutter Instrument, Novato, CA) and a digital CCD camera (Princeton Instruments, Trenton, NJ). Dual images (340 and 380 nm excitation, 510 nm emission) were collected and pseudocolor ratiometric images monitored during the experiments by using the software Axon Imaging Workbench (Axon Instruments, Union City, CA). The imaging system was standardized with a two-point calibration, using a Ca^{2+} -free standard (–) and a Ca^{2+} -saturated standard (+). Both standards contained 11 μM fura-2 [44 μl of 10 mM fura-2 Penta K^+ salt, 8 ml of 20 mM HEPES-Na (pH 7.4), 32 ml H_2O] and were prepared as follows: (– standard) 18 ml fura-2, 1.98 ml of 10 mM EGTA-Na (pH 7.6); (+ standard) 18 ml fura-2, 1.98 ml of 10 mM CaCl_2 . The parameters used for the two-point calibration include: the dissociation constant of fura-2 (K_d ; 225), the ratio values for the (–) and (+) concentration standards ($R_{\min}$ and $R_{\max}$), and the denominator wavelength intensities for the (–) and (+) concentration standards ($\text{Den}_{\min}$ and $\text{Den}_{\max}$). The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in nM was estimated according to the following equation described by Grynkiewicz *et al.* (11): $[\text{Ca}^{2+}]_i = K_d (R - R_{\min}) / (R_{\max} - R) (\text{Den}_{\min} / \text{Den}_{\max})$. Typical $R_{\min}$ and $R_{\max}$ values were 0.586 and 2.54, respectively.

Experimental Protocols—Following the incubation period with fura-2 AM, the coverslip containing vagal sensory neurons was mounted onto a chamber (0.2 ml) placed on the stage of the microscope. During the experiments, the entire chamber was continuously perfused with the standard extracellular solution (containing in mM: 5.4 KCl, 136 NaCl, 1 MgCl_2 , 1.8 CaCl_2 , 0.33 NaH_2PO_4 , 10 glucose, 10 HEPES; pH 7.4) or with standard extracellular solution containing various pharmacological and chemical agents by a gravity-fed valve-control system (VC-66CS; Warner Instruments, Hamden, CT). At the onset of each test, the perfusing solution was switched from one to another (e.g., from extracellular solution to epinephrine or from epinephrine to capsaicin) and delivered via the common outlet of a manifold operated by the valve-control system. The perfusion was kept at a constant rate of ~ 2 ml/min; a complete change of bath solution occurred in 6 s. KCl solution (100 mM) was perfused at the end of each experimental run to test for cell viability. All experiments were performed at room temperature (20–23°C).

Three study series were performed to determine: 1) the effect of epinephrine on the Ca^{2+} transients evoked by low doses of chemical stimulants including capsaicin, KCl and ATP; 2) the adrenoceptor subtype(s) potentially involved in the effect of epinephrine; 3) the possibility of the involvement of cAMP-PKA transduction cascade.

Chemicals—DMEM/F12, trypsin and 2.5S-nerve growth factor were obtained from Invitrogen (Calsbad, CA). Fura-2 AM and DiI were purchased from Molecular Probes (Eugene, OR). Urethane, α -chloralose, borax, collagenase, capsaicin, KCl, ATP, epinephrine, ascorbic acid, phenylephrine (α_1 -adrenoceptor agonist), guanabenz (α_2 -adrenoceptor agonist), dobutamine (β_1 -adrenoceptor agonist), salbutamol (β_2 -adrenoceptor agonist), BRL 37344 (β_3 -adrenoceptor agonist), ICI 215,001 (β_3 -adrenoceptor agonist), SR 59230A (β_3 -adrenoceptor antagonist), SQ 22536 (adenylate cyclase inhibitor) and H89 (PKA inhibitor) were obtained from Sigma (St. Louis, MO).

In the Ca^{2+} imaging study, stock solution of capsaicin (1 mM) was prepared in a vehicle of 10% Tween 80, 10% ethanol, and 80% extracellular solution; H89 (10 mM) was dissolved in dimethyl sulphoxide; epinephrine (20 mM) was dissolved in a mixture of extracellular solution with equivalent amount of ascorbic acid before each experiment and was kept on ice in subdued lighting conditions. These stock solutions were then diluted with the extracellular solution to yield the appropriate concentration prior to application. All other chemicals were prepared

daily in extracellular solution before applications. The concentrations of BRL 37344 and SR 59230A used have been reported to effectively and selectively activate and antagonize, respectively, the rat β_3 -adrenoceptors (18). The concentration of ICI 215,001 was selected based on a concentration-response relationship demonstrating that this concentration produces ~75% of β_3 -adrenoceptor-mediated maximal relaxing effect in guinea pig ileum strips whereas showing no significant agonist activities on guinea pig atrium β_1 - and trachea β_2 - adrenoceptors (39). The concentrations of SQ 22536 and H89 used have been shown to be effective in selectively inhibiting adenylate cyclase and PKA, respectively, in various cell types (12,34).

In the single-fiber recording study, desired concentrations of the pharmacological agents were prepared in a similar manner, except that isotonic saline, instead of extracellular solution, was used as vehicle.

Statistic Analysis—A one-way or two-way repeated-measures ANOVA was used for the statistical analysis. When results of the ANOVA showed a significant interaction, pair-wise comparisons were made with a *post hoc* analysis (Newman-Keuls test). Data are reported as means $\pm$ SE. A *P* value < 0.05 was considered significant difference.

Results

In-Vivo Study:

Effect of Inhalation of Epinephrine Aerosol on Rat Vagal Pulmonary Afferents

—A total of 25 pulmonary C-fiber afferents were studied in 17 anesthetized, open-chest rats. The distribution of locations of these receptors was as follows: 5 in the upper lobe, 11 in the middle lobe, 8 in the lower lobe, and 1 in the accessory lobe of the right lung.

Inhalation of epinephrine aerosol at both low and high concentrations markedly enhanced the pulmonary C-fiber response to lung inflation (Figs. 1 and 2). This potentiating effect sustained a substantially longer duration after the high concentration of epinephrine was administered. In comparison, a significant potentiating effect on C-fiber response to capsaicin was only found after the challenge with high concentration of epinephrine (Fig. 2, lower panel). In addition, inhalation of epinephrine aerosol increased the baseline activity of the pulmonary C-fibers in a dose-dependent manner (Fig. 2, upper panel). This stimulatory effect of epinephrine had a rapid (< 2 min) onset and returned slowly to control in 10 min and 60 min after deliveries of the low and high concentrations of epinephrine, respectively. Inhalation of low concentration of epinephrine aerosol did not cause any significant change in either ABP or heart rate; whereas high-concentration of epinephrine induced an intense hypertension (Δ ABP = 37.5 mmHg; $n = 8$) during aerosol delivery, but this hypertensive response was short-lasting (compared to the effect of epinephrine on C-fiber sensitivity), and completely returned to control in < 10 min after termination of aerosol delivery.

In contrast, we did not find any detectable effect of epinephrine aerosol on the activity of either SARs or RARs. For example, the average FA of SARs in response to lung inflation did not change significantly after termination of aerosolized epinephrine treatment (pre-aerosol control: 91.0 ± 29.1 imp/s; 2 min after epinephrine: 127.3 ± 34.1 imp/s; $P = 0.15$; $n = 8$). Furthermore, a similar pattern of response was found after the delivery of vehicle (5 mg/ml ascorbic acid in saline) in the same group of receptors (pre-aerosol control: 95.4 ± 32.3 imp/s; 2 min after saline: 102.3 ± 29.7 imp/s; $P = 0.55$; $n = 8$). Similarly, epinephrine did not cause any detectable effect on the average FA response of RARs to lung inflation ($n = 5$).

In-Vitro Study:

Epinephrine Potentiated the Chemical Stimulation-evoked Ca^{2+} Transients in Rat Vagal Sensory Neurons

—Consistent with what we have demonstrated in a previous

study (12), application of capsaicin (50 nM, 15 s) evoked a reversible Ca^{2+} transient in small- and medium-size (diameter < 35 μm) nodose and jugular ganglion neurons. As shown in Fig. 3A, epinephrine pretreatment (3 μM , 5 min) alone did not have any significant effect on the basal $[\text{Ca}^{2+}]_i$, but dramatically increased the peak Ca^{2+} transient evoked by capsaicin. The group data shown in Fig. 3C illustrated that the capsaicin-induced Ca^{2+} transient was significantly increased after the epinephrine pretreatment by 106% (control: 55.8 ± 12.6 nM; after epinephrine: 115.1 ± 24.7 nM; washout: 75.2 ± 15.7 ; $P < 0.05$; $n = 11$). Pretreatment with the vehicle of epinephrine had no detectable effect on the capsaicin-induced Ca^{2+} transient ($P = 0.81$; $n = 8$) (Fig. 3, B and C).

The potentiating effect of epinephrine was not limited only to response to capsaicin, the Ca^{2+} transient evoked by KCl (15 mM, 15 s) was also significantly potentiated after pretreatment with epinephrine (3 μM , 5 min) (Fig. 4). The KCl-evoked Ca^{2+} transient was elevated after epinephrine pretreatment in both capsaicin-sensitive (control: 83.3 ± 9.7 nM; after epinephrine: 130.5 ± 20.1 nM; washout: 96.3 ± 18.2 ; $P < 0.05$; $n = 6$) and capsaicin-insensitive neurons (control: 86.4 ± 8.3 nM; after epinephrine: 126.5 ± 16.5 nM; washout: 92.6 ± 14.9 ; $P < 0.05$; $n = 9$) (Fig. 4D). In addition, pretreatment with same dose of epinephrine potentiated the ATP (0.3 μM , 15 s) -evoked Ca^{2+} transient from a control level of 102.1 ± 11.6 nM to 143.5 ± 9.8 nM after the treatment ($P < 0.05$; $n = 8$). We should point out that the responses to these chemical stimuli and also the potentiating effect of epinephrine varied considerably among different cells (e.g., Figs. 3 and 4).

The potentiating effect of epinephrine on pulmonary vagal sensory neurons identified by DiI labeling was investigated in separate groups of neurons. Our results showed no significant difference in the capsaicin (50 nM, 15 s) and KCl (15 mM, 15 s) -evoked Ca^{2+} transients between labeled neurons and non-labeled ones matched in size from the same cultures, either during control or after epinephrine pretreatment (Fig. 5).

Selective Activation of α_1 -, α_2 -, β_1 -, or β_2 -adrenoceptor Did Not Alter the Sensitivity of Rat Vagal Sensory Neurons—Because epinephrine has affinity for both α - and β -adrenoceptors, our first approach was to determine whether activation of either or both of these major types of adrenoceptor could mimic the potentiating effect of epinephrine. As shown in Fig. 6, pretreatment with neither phenylephrine (5 μM , 5 min; $P = 0.74$; $n = 12$), a α_1 -adrenoceptor agonist, nor guanabenz (5 μM , 5 min; $P = 0.35$; $n = 11$), a α_2 -adrenoceptor agonist, altered the vagal sensory neurons responses to capsaicin (50 nM, 15 s) (Fig. 6, A and B). Similarly, pretreatment with either β_1 -adrenoceptor agonist dobutamine (5 μM , 5 min; $P = 0.23$; $n = 10$) or β_2 -adrenoceptor agonist salbutamol (5 μM , 5 min; $P = 0.16$; $n = 10$) had no significant effect on capsaicin (50 nM, 15 s) -evoked Ca^{2+} transient (Fig. 6, C and D).

The Effect of Epinephrine Was Mediated through β_3 -adrenoceptor in Rat Vagal Sensory Neurons—As illustrated in Fig. 7A, pretreatment with BRL 37344 (5 μM , 5 min), a selective β_3 -adrenoceptor agonist, produced a similar potentiating effect as epinephrine. The group data showed that capsaicin (50 nM, 15 s) -evoked Ca^{2+} transient was significantly increased from the control level of 97.5 ± 13.0 nM to 204.4 ± 39.9 nM after BRL 37344 ($P < 0.05$; $n = 17$) (Fig. 7B). Pretreatment with another selective β_3 -adrenoceptor agonist ICI 215,001 (5 μM , 5 min) also significantly potentiated the capsaicin-evoked Ca^{2+} transient but to a less extent (control: 86.3 ± 15.6 nM; after ICI 215,001: 127.7 ± 19.9 nM; $P < 0.05$; $n = 13$) (Fig. 7, C and D). Furthermore, pretreatment with SR 59230A (5 μM , 10 min), a selective β_3 -adrenoceptor antagonist, completely abolished the potentiating effect of epinephrine on the Ca^{2+} transients evoked by both capsaicin ($P = 0.23$; $n = 14$) and KCl ($P = 0.24$; $n = 15$) (Fig. 8), whereas pretreatment with SR 59230A alone did not affect the sensory neurons responses to these two chemical stimulants ($P = 0.88$; $n = 12$).

Adenylate Cyclase Inhibitor Abolished the Effect of Epinephrine in Rat Vagal Sensory Neurons—To investigate whether the activation of adenylate cyclase and subsequent increase of intracellular cAMP is critical to the potentiating effect of epinephrine, we tested the effect of SQ 22536, a membrane-permeable adenylate cyclase inhibitor. As shown in Fig. 6, pretreatment with SQ 22536 (100 μ M, 15 min) completely prevented the potentiating effect of epinephrine on capsaicin-evoked Ca^{2+} transient ($P = 0.32$; $n = 8$) (Fig. 9, A and B). In addition, pretreatment with SQ 22536 also prevented the effect of epinephrine on the Ca^{2+} transient induced by KCl ($P = 0.44$; $n = 16$) (Fig. 9, C and D).

Inhibition of PKA Prevented the Epinephrine-induced Potentiation in Rat Vagal Sensory Neurons—To determine whether the potentiating effect of epinephrine was due to the cAMP activation of PKA, we examined the effect of H89, a membrane-permeable PKA inhibitor, on the epinephrine-mediated enhancement of chemical stimulation-induced Ca^{2+} transient. Pretreatment with H89 (3 μ M, 15 min) completely abolished the potentiating effect of epinephrine on the Ca^{2+} transients evoked by both capsaicin ($P = 0.87$; $n = 10$) and KCl ($P = 0.51$; $n = 9$) (Fig. 10), whereas the vehicle of H89 had no significant effect.

Discussion

β -adrenoceptors are among the most extensively characterized members of the G-protein-coupled receptors. Three different subtypes of β -adrenoceptor (β_1 , β_2 and β_3) have been characterized on the basis of biochemical, functional and receptor-cloning studies. Although the fourth adrenoceptor (β_4) has been reported in heart muscle (17), it is now recognized that this newly proposed β -adrenoceptor most likely represents only a particular state of the β_1 -adrenoceptor (25). Functional assignment of β -adrenoceptor subtypes suggests that β_1 -adrenoceptor is the predominant subtype regulating heart rate and contractility, although all three β -adrenoceptors appear to be involved in the cardiac stimulation (17). β_2 -adrenoceptor is the predominant subtype mediating the vascular smooth muscle relaxant properties (32). β_3 -adrenoceptor was initially identified in adipose tissue, and its presence has been recently demonstrated in a variety of tissues including blood vessel (32), gastrointestinal tract (9), urinary bladder smooth muscle (43) and retinal endothelial cell (37). In the present study, our results have demonstrated that in anesthetized, open-chest and artificially ventilated rats, inhalation of epinephrine aerosol substantially enhanced pulmonary C-fiber responses to lung inflation and capsaicin injection (Figs. 1 and 2). Our data have also shown that pretreatment with epinephrine significantly potentiated chemical stimulation-evoked Ca^{2+} transient in isolated rat vagal sensory neurons including those specifically innervating the lungs (Figs. 3-5). In addition, the potentiating effect of epinephrine was mimicked by both BRL 37344 and ICI 215,001, two selective β_3 -adrenoceptor agonists (Fig. 7), and abolished by SR 59230A, a selective β_3 -adrenoceptor antagonist (Fig. 8), whereas pretreatment with none of the α_1 , α_2 , β_1 , and β_2 -adrenoceptor agonists had any significant effect on capsaicin-evoked Ca^{2+} transient (Fig. 6). Taken together, these results strongly suggest that activation of β_3 -adrenoceptor mediates the sensitizing actions of epinephrine on these vagal sensory neurons, although additional evidence obtained from immunohistochemistry and/or molecular cloning will be required to further confirm the expression of this specific β -adrenoceptor in these neurons.

All three β -adrenoceptor subtypes are known to be coupled to the G_s protein leading to the activation of adenylate cyclase and accumulation of the second messenger cAMP, which has been demonstrated both in native tissues and in reconstitution systems (13,38). It has been proposed that epinephrine, acting on nociceptors, produces mechanical hyperalgesia through three different signaling pathways involving: 1) cAMP-dependent PKA; 2) epsilon isozyme of PKC; and 3) extracellular signal-regulated kinases 1 and 2 (1,20). It has been demonstrated that the latter two pathways may also be initiated by the accumulation of cAMP (8,35). However, recent pharmacological and electrophysiological studies have reported the presence

of β -adrenoceptor-mediated cAMP-independent signaling pathways (38); for example, the β_3 -adrenoceptor-induced relaxation of gastrointestinal smooth muscle has been proposed to be mediated through the activation of a delayed rectified K^+ channel (15). In the present study, our results suggest that intracellular cAMP-PKA transduction cascade is substantially involved in, if not solely responsible for, the sensitizing effect of epinephrine in rat vagal sensory neurons, since pretreatment with either adenylate cyclase inhibitor SQ 22536 (Fig. 9) or PKA inhibitor H89 (Fig. 10) completely prevented the potentiating effect of epinephrine in these neurons.

We have recently demonstrated that capsaicin evoked a Ca^{2+} transient in small- to medium-size rat vagal sensory neurons; the response was mediated through transient receptor potential vanilloid receptor type 1 (TRPV1), and was dependent on the extracellular Ca^{2+} (12). TRPV1, a member of the transient receptor potential ion channel superfamily (27), is a polymodal nonspecific cation channel expressed predominantly in unmyelinated (C-) fibers, and is activated not only by capsaicin but also by noxious heat, protons, anandamide and lipoxygenase metabolites (4,16). Therefore, TRPV1 may act as a thermal and chemical transducer and contribute to neurogenic inflammation (4). It has been demonstrated that cAMP-dependent PKA activation can enhance capsaicin-induced inward current and Ca^{2+} transient in dorsal root ganglion neurons (36) as well as in vagal sensory neurons (12,22). Indeed, recent studies have successfully delineated several candidate PKA-phosphorylation sites in TRPV1, through which PKA may mediate the sensitization of this channel as well as prevent its desensitization (2).

ATP, another chemical stimulant used in this study, is known to activate the ligand-gated $P2X_3$ purinoceptor that is coupled to nonselective cation channel and widely expressed in both the central and peripheral nervous systems (31). PKA-dependent sensitization of TRPV1 and $P2X_3$ may increase the permeability of these channels to cations such as Ca^{2+} and Na^+ , which can lead to cell membrane depolarization and subsequent activation of voltage-dependent Ca^{2+} channels (VDCCs). On the other hand, VDCCs are also the potential phosphorylation targets of PKA activation, which may increase the channel availability (19) or modulate the channel properties (10). This assumption is supported by our observation that epinephrine also potentiates the KCl-evoked Ca^{2+} transient (Fig. 4), a response resulting from the cell membrane depolarization and subsequent activation of VDCCs. We can not compare the extent of the potentiation of epinephrine on these three chemical stimulants (capsaicin, ATP and KCl) because a full range of the dose responses of these activators were not established in this study. Our in-vitro study was carried out in cultured neurons, and the responses were recorded from the neuronal soma. Hence, we could not determine the specific types of afferent terminals of these individual isolated neurons, although we have purposely selected the small- to medium-size neurons for this study. In fact, in the experiments testing the responses to ATP and KCl, we did not exclude capsaicin-insensitive neurons because our previous studies indicated that ~40% of small- to medium-size (diameter < 35 μm) nodose and jugular ganglion neurons prepared under similar conditions do not exhibit any sensitivity to capsaicin (6,42). More importantly, our in-vivo single-fiber recording data clearly indicate that the sensitizing effect of epinephrine is found exclusively in C-fiber afferents, and not in SARs or RARs in the rat lungs. In addition, activation of TRPV1, $P2X_3$ or VDCCs may initiate Ca^{2+} release from intracellular stores via a process of Ca^{2+} -induced Ca^{2+} release (CICR) (40). Indeed, previous studies have provided direct evidence that CICR can be triggered solely by Ca^{2+} influx in various sensory neurons including dorsal root (36) and nodose (7) ganglion neurons. On the basis of our results, we can not rule out the possibility that increased CICR may have also contributed to the enhanced chemical stimulation-evoked Ca^{2+} transients after the epinephrine pretreatment.

The present study was carried out using two different experimental approaches. We used the in-vivo single-fiber recording to determine the effect of epinephrine in intact animals, and the in-vitro Ca^{2+} imaging to identify the subtypes of adrenoceptor mediating this effect in isolated vagal sensory neurons. Whether there are differences in the receptor concentrations and the sensitivities of these receptors between the sensory terminal and the soma of these neurons remains to be determined. Nevertheless, our study have shown that inhalation of epinephrine aerosol markedly enhanced pulmonary C-fiber sensitivity in anesthetized rats, and that epinephrine pretreatment significantly potentiated chemical stimulation-evoked Ca^{2+} transient in isolated rat vagal sensory neurons; these results are in general agreement with the previous findings from other investigators (1,5,21,28). Under the conditions of acute stress, the systemic plasma concentration of epinephrine increases dramatically in various species including humans (41). Intradermal administration of epinephrine has been shown to produce mechanical and thermal hyperalgesia (5,21), which may be explained by a direct sensitizing effect of epinephrine on primary nociceptive afferents (1,21). It has been recently reported that neural discharge in vagal afferent fibers is significantly increased by elevations of peripheral concentrations of epinephrine in rats (28).

The primary afferents innervating various visceral organs are mainly conducted through vagus nerves. It is known that the chemosensitive vagal afferent endings can be stimulated by various endogenous substances such as H^+ , adenosine, and serotonin (24,29); and by various inhaled irritants such as cigarette smoke, SO_2 , ozone, and also by lung expansion (24). Under normal physiological conditions, these stimuli (endogenous chemicals, inhaled irritants and lung expansion) may not generate a significant stimulatory effect on the vagal afferent terminals. However, when the excitability of these afferents is up-regulated by epinephrine as shown in the present study, their stimulation thresholds will be lowered (e.g., Fig. 1B), which may then lead to afferent activation. In addition, some of these endogenous activators (e.g., H^+) are known to be secreted at high concentrations under acute stressful conditions (e.g., strenuous exercise), during which the circulating level of epinephrine is also expected to increase substantially (30). Therefore, the consequent augmented visceral/viscerosomatic reflexes may, presumably, contribute to the adaptive responses of these visceral organs to the acute stresses and play a role in maintenance of homeostasis under these conditions.

In summary, our results show that inhalation of epinephrine aerosol induced a pronounced increase in the sensitivity of pulmonary C-fibers to lung inflation and right atrial injection of capsaicin. Furthermore, pretreatment with epinephrine markedly and consistently potentiated the chemical stimulation-evoked Ca^{2+} transients in isolated rat vagal sensory neurons. The effect of epinephrine was mimicked by either BRL 37344 or ICI 215,001, two specific β_3 -adrenoceptor agonists, and blocked by SR 59230A, a selective β_3 -adrenoceptor antagonist. In contrast, pretreatment with none of α_1 , α_2 , β_1 , or β_2 -adrenoceptor agonists had any significant effect. Furthermore, pretreatment with either adenylate cyclase inhibitor, SQ 22536, or PKA inhibitor, H89, completely blocked the potentiating effect of epinephrine. Taken together, these results suggest that epinephrine up-regulates the sensitivity of rat vagal chemosensitive neurons; this sensitizing effect is probably mediated through the β_3 -adrenoceptor and the subsequent activation of intracellular cAMP-PKA transduction cascade.

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Fig. 1.

Experimental records illustrating the effect of aerosolized epinephrine on pulmonary C-fiber responses to lung inflation (20 cmH₂O, 10 s) in three anesthetized, open-chest rats. *A*: treatment with vehicle aerosol (5 mg/ml ascorbic acid in saline); receptor location: middle lobe of the right lung; body weight: 410 g. *B*: treatment with low concentration of epinephrine aerosol (1 mg/ml); receptor location: right lower lobe; body weight: 350 g. *C*: treatment with high concentration of epinephrine aerosol (5 mg/ml); receptor location: right lower lobe; body weight: 380 g. Panels from top to bottom: before, 2 min, 10 min and 20 min after aerosol inhalation (duration = 3 min). Epi, epinephrine; conc., concentration; AP, action potential; P_t , tracheal pressure; ABP, arterial blood pressure.

Fig. 2.

Time course of the effect of aerosolized epinephrine on afferent activity of pulmonary C-fibers in anesthetized, open-chest rats. *A*, *B* and *C*: 3-min treatments with aerosol of vehicle (5 mg/ml ascorbic acid in saline; $n = 12$), low concentration of epinephrine (1 mg/ml; $n = 18$) and high concentration of epinephrine (5 mg/ml; $n = 10$), respectively. Upper panel: baseline fiber activities (FA). Middle panel: responses to lung inflation (20 cmH₂O for 10 s). Lower panel: responses to right-atrial injections of capsaicin (0.5 µg/kg). FA at each time point was averaged over a 20-s interval for each fiber. ΔFA was measured as the difference in fiber activity between the 10-s average during lung inflation or 2-s average after capsaicin injection, and the 10-s average at the baseline in each fiber. No more than two doses of epinephrine were administered in each animal. Epi, epinephrine; conc., concentration; * $P < 0.05$ significantly different from the corresponding control. Data are means ± SE.

Fig. 3.

Epinephrine potentiates capsaicin-evoked Ca^{2+} transient in isolated rat vagal sensory neurons. **A:** experimental record illustrating that the Ca^{2+} transient evoked by capsaicin (50 nM, 15 s) was potentiated after pretreatment with epinephrine (3 μ M, 5 min). **B:** pretreatment with vehicle of epinephrine had no effect on capsaicin (50 nM, 15 s)-evoked Ca^{2+} transient. **C:** group data showing the effects of epinephrine ($n = 11$) and its vehicle ($n = 8$) on capsaicin-evoked Ca^{2+} transient. * $P < 0.05$ significantly different from the corresponding control. † $P < 0.05$ significant difference between the corresponding data obtained from pretreatments with epinephrine and its vehicle. Data are means $\pm$ SE. Cap, capsaicin; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+} in nM; $\Delta[Ca^{2+}]_i$, the peak amplitude of Ca^{2+} transient measured as the difference between the 4-s average at peak and the 30-s average at baseline.

Fig. 4.

Epinephrine potentiates KCl-evoked Ca^{2+} transient in isolated rat vagal sensory neurons. **A:** experimental record illustrating that the Ca^{2+} transient evoked by KCl (15 mM, 15 s) was potentiated after pretreatment with epinephrine (3 μM , 5 min). **B:** pretreatment with vehicle of epinephrine had no effect on KCl (15 mM, 15 s) -evoked Ca^{2+} transient. **C:** group data showing the effects of epinephrine (epi, $n = 8$) and its vehicle (veh, $n = 7$) on KCl-evoked Ca^{2+} transient. * $P < 0.05$ significantly different from the corresponding control (Con). † $P < 0.05$ significant difference between the corresponding data obtained from pretreatments with epinephrine and its vehicle. **D:** group data showing the effect of epinephrine on KCl-evoked Ca^{2+} transient in capsaicin-sensitive (Cap+, $n = 6$) and capsaicin-insensitive (Cap-, $n = 9$) neurons. * $P < 0.05$ significantly different from the corresponding control. Data are means $\pm$ SE.

Fig. 5.

Comparison of the potentiating effect of epinephrine between pulmonary (DiI labeled) and non-pulmonary (unlabeled) rat vagal sensory neurons matched in size from the same cultures. *A*: effect of epinephrine (3 μ M; 5 min) on the capsaicin (50 nM; 15 s) -evoked Ca^{2+} transient (DiI labeled: $n = 10$; unlabeled: $n = 11$). *B*: effect of epinephrine (3 μ M; 5 min) on the KCl (15 mM; 15 s) -evoked Ca^{2+} transient (DiI labeled: $n = 8$; unlabeled: $n = 9$). Data are means $\pm$ SE. * $P < 0.05$ significantly different from the corresponding control.

Fig. 6.

Effects of α_1 -, α_2 -, β_1 - and β_2 -adrenoceptor agonists on the capsaicin-evoked Ca^{2+} transient in rat vagal sensory neurons. A–D: effects of pretreatments with phenylephrine (α_1 -adrenoceptor agonist; 5 μ M, 5 min; $n = 12$), guanabenz (α_2 -adrenoceptor agonist; 5 μ M, 5 min; $n = 11$), dobutamine (β_1 -adrenoceptor agonist; 5 μ M, 5 min; $n = 10$) and salbutamol (β_2 -adrenoceptor agonist; 5 μ M, 5 min; $n = 10$), respectively, on the Ca^{2+} transient evoked by capsaicin (50 nM, 15 s). Data are means $\pm$ SE.

Fig. 7.

Effects of β_3 -adrenoceptor agonists on the capsaicin-evoked Ca^{2+} transient in rat vagal sensory neurons. *A* and *C*: experimental records illustrating the effects of pretreatments with BRL 37344 (β_3 -adrenoceptor agonist; 5 μM , 5 min) and ICI 215,001 (β_3 -adrenoceptor agonist; 5 μM , 5 min), respectively, on the Ca^{2+} transient evoked by capsaicin (Cap; 50 nM, 15 s). *B* and *D*: group data showing the effects of pretreatments with BRL 37344 ($n = 17$) and ICI 215,001 ($n = 13$), respectively, on the capsaicin-evoked Ca^{2+} transient. Data are means $\pm$ SE. * $P < 0.05$ significantly different from the control response.

Fig. 8. Effect of SR 59230A on the potentiation of capsaicin- and KCl-evoked Ca^{2+} transients by epinephrine in rat vagal sensory neurons. *A* and *C*: experimental records illustrating the effect of pretreatment with SR 59230A (5 μM , 10 min), a β_3 -adrenoceptor antagonist, on the potentiation of capsaicin (Cap; 50 nM, 15 s) and KCl (15 mM, 15 s) -evoked Ca^{2+} transients by epinephrine (3 μM , 5 min), respectively. *B* and *D*: group data showing the effect of SR 59230A (SR) on the potentiating effect of epinephrine (Epi); $n = 14$ and 15 in *B* and *D*, respectively. Data are means $\pm$ SE.

Fig. 9.

Effect of SQ 22536 on the potentiating effect of epinephrine in rat vagal sensory neurons. *A* and *C*: experimental records illustrating the effect of pretreatment with SQ 22536 (100 μ M in *A*, 300 μ M in *C*; 15 min), an adenylate cyclase inhibitor, on the potentiation of capsaicin (Cap; 50 nM; 15 s) and KCl (15 mM, 15 s) -evoked Ca^{2+} transients by epinephrine (3 μ M, 5 min). *B* and *D*: group data showing that the effect of SQ 22536 (SQ) on the potentiating effect of epinephrine (Epi); $n = 8$ and 16 in *B* and *D*, respectively. Data are means $\pm$ SE.

Fig. 10.

Effect of H89 on the potentiation of capsaicin- and KCl-evoked Ca^{2+} transients by epinephrine in rat vagal sensory neurons. *A* and *B*: experimental records illustrating the effects of pretreatments with H89 (3 μM , 15 min), a membrane permeable PKA inhibitor, and its vehicle, respectively, on the potentiation of capsaicin (Cap; 50 nM, 15 s)-evoked Ca^{2+} transient by epinephrine (3 μM , 5 min). *C*: group data showing the effects of H89 ($n = 10$) and its vehicle ($n = 8$) on the potentiating effect of epinephrine (Epi) on the Ca^{2+} transient evoked by capsaicin (50 nM, 15 s). *D*: group data showing the effects of H89 ($n = 9$) and its vehicle ($n = 9$) on the potentiating effect of epinephrine (Epi) on the Ca^{2+} transient evoked by KCl (15 mM, 15 s). * $P < 0.05$ significantly different from the corresponding response to capsaicin or KCl alone. † $P < 0.05$ significant difference between the corresponding data obtained from pretreatments with H89 and its vehicle. Data are means $\pm$ SE.