THERMAL SENSITIVITY OF VAGAL PULMONARY SENSORY NEURONS: ROLE OF TRANSIENT RECEPTOR POTENTIAL VANILLOID CHANNELS

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ABSTRACT OF DISSERTATION

Dan Ni

The Graduate School
University of Kentucky
2008
THERMAL SENSITIVITY OF VAGAL PULMONARY SENSORY NEURONS: ROLE OF TRANSIENT RECEPTOR POTENTIAL VANILLOID CHANNELS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Dan Ni
Lexington, Kentucky

Director: Dr. Lu-Yuan Lee, Professor of Physiology
Lexington, Kentucky
2008
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Hyperthermia can occur in lungs and airways during both physiological and pathophysiological conditions. A previous study carried out in our laboratory showed that hyperthermia activates and sensitizes vagal bronchopulmonary C-fiber afferents, whether this effect is through a direct action of hyperthermia on sensory nerves is not known. This dissertation study was aimed to investigate the thermal-sensitivity of pulmonary sensory neurons, and the roles of thermal-sensitive transient receptor potential vanilloid (TRPV) channels. Whole-cell patch-clamp recordings of neurons isolated from nodose/jugular ganglia were applied in the study. Results of this study showed that hyperthermia directly activates pulmonary sensory neurons, and this effect is partially mediated through the TRPV subtype 1 (TRPV1) channel as well as other thermal-sensitive TRPV (2–4) channels. In addition, hyperthermia exerts potentiating effects on responses of pulmonary sensory neurons to TRPV1 activators, but not to non-TRPV1 activators. Furthermore, results obtained in the study of TRPV1-null mice revealed that TRPV1 plays a dominant role in mediating the potentiating effect of hyperthermia on pulmonary sensory neurons, but is only partially involved in the direct activation of these sensory neurons by increasing temperature. These results suggested that the thermal-sensitivity of pulmonary sensory neurons is dependent upon the function of the TRPV1 channel, and TRPV1-mediated sensitization of these sensory neurons may contribute to airway hyperreactivity and augmented reflex responses under hyperthermic conditions.

KEYWORDS: Hyperthermia, TRPV, C-fibers, Exercise, Airways
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For my parents, Wang Fei and Ni Wenxue
I would like to acknowledge many people for helping me during my doctoral work. I would like to express my deepest gratitude to my advisor, Dr. Lu-Yuan Lee, for his excellent guidance, encouragement and patience. I could never have reached the heights or explored the depths without his support.

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Chapter One: Background

1.1 Bronchopulmonary C-fiber afferents and airway hyperreactivity.

The afferent activities that arise from sensory terminals located in lungs and airways are conducted primarily by branches of vagus nerves (Widdicombe, 1982; Lee and Undem, 2005). These bronchopulmonary vagal afferent fibers are known to play an important role in the regulation of respiratory functions and airway defense reflexes, such as controlling airway caliber, pattern of breathing and airway secretion (Coleridge and Coleridge, 1986). Among these sensory nerves, the majority (~75%) are unmyelinated (C-fiber) afferents (Agostoni et al., 1957) that function as a primary sensor for detecting various inhaled irritants and certain endogenous inflammatory mediators. Their sensitivity can be significantly elevated under various pathophysiological conditions in airways and lungs (Coleridge and Coleridge, 1984; Ho et al., 2001).

Cell bodies of sensory neurons innervating lungs and airways are located in the jugular and nodose ganglia (Figure 1.1), which relay the information from lungs and airways to the medullary respiratory centers, where the sensory information is integrated and processed to generate appropriate reflexive responses.

Figure 1.1 Structures of nodose and jugular ganglia. These two ganglia are located right within (jugular) and outside (nodose) the jugular foramen. Modified from (McDonald et al., 1988).
Nerve endings of bronchopulmonary C-fibers are located between airway epithelia, in the submucosa, near airway smooth muscles, and around pulmonary capillaries and mucous glands (Coleridge and Coleridge, 1984; Lee and Undem, 2005) (Fig. 1.2). When these nerve endings are stimulated either by inhaled irritants or by circulated autacoids, pulmonary chemoreflexes such as bronchoconstriction, bradycardia and hypotension, and increased mucus secretion will be elicited via the cholinergic pathway (Coleridge and Coleridge, 1984). In addition, another neural pathway called “axon reflex” also plays an important role in eliciting reflex responses upon the activation of these C-fibers. Several neuropeptides including tachykinins and calcitonin gene-related peptide (CGRP) are released upon activation of these sensory terminals, which then produce additional local effects such as bronchoconstriction, plasma extravasation, airway mucosal edema, and inflammatory cell chemotaxis (Lee and Undem, 2005).

Figure 1.2 Schematic illustration of the distribution pattern of vagal C-fiber afferent endings and their potential interaction with other cell types in airway mucosa. E, erythrocyte, L, leukocyte, TKs, tachykinins; N, neuroepithelial body. Adapted from (Lee and Undem, 2005).

The defense reflexes elicited both via the cholinergic and axon reflex pathways are protective under normal conditions. An example of the reflex regulation of
bronchomotor tone by these afferent nerves is illustrated schematically in Fig 1.3. However, under pathophysiologic conditions, sensitization of these C-fiber afferents and sensory neurons could elicit exaggerated reflex responses, such as uncontrollable cough, bronchospasm and mucous hypersecretion (Ho et al., 2001; Widdicombe and Lee, 2001), which are common pathophysiologic features of airway hyperresponsiveness.

Figure 1.3 Schematic illustration of reflex regulation of bronchomotor tone by vagal bronchopulmonary C-fibers.

1.2 Hyperthermia in the airway.
Hyperthermia is defined as an elevation of body or local temperature above the normal level. It occurs when metabolic rate or environmental heat load exceeds normal heat loss capacity of the body, or when there is an impaired ability for heat dissipation. It can occur under both physiological and pathophysiological conditions. For example, heavy exercise could elevate body temperature significantly. It has been reported that during strenuous exercise body core temperature can increase up to 41.9°C in human (Maron et al., 1977) and 43.4°C in rats (Brooks et al., 1971). Pathophysiological conditions, such as inflammation or infection, could lead to the induction of severe fever (Saper and Breder, 1994). In addition, a recent study reported a significant increase in the exhaled air temperature (Δ = 2.7°C) in patients with airway inflammation (Piacentini et al., 2007). The changes in airway function during hyperthermia may include
hyperventilation, pulmonary edema, bronchoconstriction and so on (Javorka et al., 1996). Many investigations have shown that as respiratory heat exchange rises in asthmatic individuals, the intensity of the airway obstruction follows suit (Bar-Or et al., 1977; Anderson et al., 1982). Furthermore, studies also have shown that inhalation of warm air could cause marked bronchoconstriction in asthmatic patients (Aitken and Marini, 1985; McFadden et al., 1999). Indeed, a previous study in our lab has demonstrated that elevation of intrathoracic temperature evokes both stimulatory and sensitizing effect on bronchopulmonary C-fibers (Ruan et al., 2005), as shown in Figure 1.4.
Figure 1.4 Effects of an increase in intrathoracic temperature ($T_{it}$) on the baseline activity and responses to lung inflation and capsaicin injection of pulmonary C-fibre afferents. Left panels, fibre activity (FA) was analysed by computer in 0.5 s intervals. Upper, average baseline FA ($n= 53$; data of only 30 s are shown to avoid clustering); middle, average response to lung inflation ($P_{t}= 30 \text{ cmH}_2\text{O}$ for 10 s; $n= 19$), depicted by the horizontal bar; lower, average response to right-atrial injection of capsaicin (arrow, $0.5 \mu \text{g kg}^{-1}$ in 0.1 ml; $n= 11$). Responses were tested at three different levels of $T_{it}$ in each fibre: control ($\sim 36^\circ \text{C}$), medium ($-38.5^\circ \text{C}$) and high ($-41^\circ \text{C}$). Each level of $T_{it}$ was maintained for 3 min, and at least 30 min elapsed between tests in each fibre; the average $T_{it}$ is shown in each panel. Right panels, peak responses at three different levels of $T_{it}$. $\Delta$FA represents the difference between the peak FA (average over 10 s and 2 s intervals for lung inflation and capsaicin, respectively) and the baseline FA (average over 60 s intervals) in each fibre. Responses during recovery were not tested in every fibre due to time limitation; recovery data shown in upper, middle and lower panels were collected from 36, 13 and 10 fibres, respectively. *† Significantly different ($P < 0.05$) from the corresponding data at control and medium $T_{it}$, respectively. Data are means ±S.E.M. Adapted from (Ruan et al., 2005).

1.3 Thermal-sensitive ion channels.

The transient receptor potential vanilloid (TRPV) channel is a non-specific cation channel receptor family with six identified members (Benham et al., 2003; Montell, 2005). TRPV channels are important for perception of pain, temperature sensing, osmotic regulation, and maintenance of calcium homeostasis (Montell, 2005). These TRPV channels have 6 transmembrane domains, and both terminals are intracellular. To date, four subtypes of the TRPV channels have been cloned and shown to be activated by thermal stimuli in the warm to hot range when expressed heterologously (Figure 1.5). In addition to their sensitivity to increase in temperature, the TRPV channels can also be activated by a variety of physiological and pharmacological stimuli, including acid, capsaicin, anandamide and certain lipooxygenase products for TRPV1, hypotonicity/low osmolarity and phorbol esters for TRPV4 (Alessandri-Haber et al., 2004), and 2-aminoethoxydiphenyl borate (2APB) for TRPV1–3 (Chung et al., 2004; Hu et al., 2004). The temperature activation threshold, activators and tissue distributions for these TRPV channels are well documented in the recent literature and summarized in Table1.1.
Figure 1.5 Structures of thermal-sensitive TRPV channels. Modified from (Jordt et al., 2003).

Table 1.1 Properties of four thermal-sensitive TRPV family members

<table>
<thead>
<tr>
<th>Temperature range (°C)</th>
<th>Agonist</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV1 &gt;43</td>
<td>Capsaicin, anandamide, OEA, PKA, leukotriene B4, decreased PI(4,5)P2, protons, resiniferatoxin, 2-APB, H+</td>
<td>DRG, TG, bladder, skin, epithelial cells,</td>
</tr>
<tr>
<td>TRPV2 &gt;52</td>
<td>2-APB, mechanical stimulation (stretch, swelling), growth factors, IGF-1, HA</td>
<td>DRG, TG, brain, spleen, intestine</td>
</tr>
<tr>
<td>TRPV3 &gt;34 or 39</td>
<td>2-APB, camphor</td>
<td>DRG, TG, keratinocytes</td>
</tr>
<tr>
<td>TRPV4 &gt;27</td>
<td>Hypo-osmolarity, shear stress, PKC, anandamide, 5’6’ EET, 4αPDD and other phorbols</td>
<td>TG, kidney, trachea, keratinocytes, spleen, hypothalamus, hair cells, merkel cells</td>
</tr>
</tbody>
</table>

Modified from Jordt et al., 2003 and Pederson et al., 2005

2-APB – 2-aminoethoxydiphenyl borate
DRG – dorsal root ganglion
5’,6’ EET – epoxyeicosatrienoic acid
HA – neuropeptide head activator
4αPDD – 4 α-phorbol 12,13-didecanoate
OEA – oleoylethanolamide
PKA – protein kinase A
PKC – protein kinase C
TG – trigeminal ganglion

Since TRP channel family is known to be intimately involved in the regulation of calcium homeostasis, upon channel activation, influx of Ca$^{2+}$ could elicit extensive downstream effects, such as alteration of neuron excitability, change of gene expression, activation of other receptors, and so on (Carafoli, 2002; Hardie, 2003). The TRPV1 channel, in particular, has been shown to be extensively involved in sensory transduction and pain sensation under both physiological and pathophysiological conditions. Heat-evoked responses mediated through the
TRPV1 channel have been demonstrated in humans and animals as well as in primary afferent nerve fibers and cultured sensory neurons (Petersen and LaMotte, 1993; Cesare and McNaughton, 1996; Vyklicky et al., 1999). In addition to the direct activation by physical/chemical activators of the TRPV1 channel, many studies have clearly demonstrated an important role of the TRPV1 channel in the development of hyperalgesia both by using TRPV1-null mice model and by applying TRPV1 antagonists (Caterina et al., 2000; Nagy et al., 2004; Nilius et al., 2007). More importantly, recent reviews have further revealed that TRPV1 is critically involved in the manifestation of various symptoms and pathophysiological responses in inflammatory airway diseases (Lee and Undem, 2005; Geppetti et al., 2006; Jia and Lee, 2007).

Figure 1.6 Cationic influx through VR1 can be regulated by the convergent actions of multiple pain-producing stimuli. Membrane-permeant second messengers, such as lipid metabolites, may modulate nociceptor activity in an autocrine fashion (as diagrammed here), or in a paracrine manner if produced by neighboring neural or nonneural cell types. Abbreviations: AA, arachidonic acid; G, heterotrimeric G protein; GPCR, G protein-coupled receptor; PL, phospholipase. Capsaicin and noxious heat are represented by pepper and flame, respectively. Adapted from (Caterina and Julius, 2001).

1.4 Specific aims of the dissertation project
In light of the existing information reviewed above, the purpose of this dissertation study was to evaluate the thermal-sensitivity of pulmonary sensory
neurons and the potential role of thermal-sensitive TRPV channels. The specific aims were 1) to determine whether hyperthermia has a direct effect on pulmonary sensory neurons, and if so, whether this effect is mediated through TRPV channels; 2) to study the modulatory effect of hyperthermia on responses of pulmonary sensory neurons to chemical stimulations, and the potential involvement of TRPV channels in this effect; 3) to determine the specific role of the TRPV1 channel in regulating the thermal sensitivity of pulmonary sensory neurons.

This studies reported in this dissertation were performed in accordance with 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.
Chapter Two: Thermal sensitivity of isolated vagal pulmonary sensory neurons: role of transient receptor potential vanilloid receptors

2.1 Introduction

A previous study carried out in our lab in anesthetized rats surgically prepared with isolated perfused thoracic chamber showed that hyperthermia activates and sensitizes vagal pulmonary C-fibers (Ruan et al., 2005). However, whether these effects are caused by a direct action of hyperthermia on sensory nerves or by indirect effects via local release of inflammatory mediators (Ruan et al., 2005) and/or cytokines (Jiang et al., 1999) is not known, because some of these endogenous mediators (e.g., prostaglandin E₂, hydrogen ion, etc.) are known to activate C-fiber endings (Coleridge and Coleridge, 1984; Ho et al., 2001; Lee and Undem, 2005).

One of the characteristic features of bronchopulmonary C-fiber afferents is their exquisite sensitivity to capsaicin and the expression of the transient receptor potential vanilloid type 1 (TRPV1) channel (Ho et al., 2001). A recent report from our laboratory has demonstrated that 2-aminoethoxydiphenyl borate (2-APB), a common activator of TRPV1–3 receptors (Chung et al., 2004; Hu et al., 2004), exerted a direct stimulatory effect on isolated vagal bronchopulmonary sensory neurons (Gu et al., 2005). The 2APB-induced stimulation was only partially blocked by a TRPV1 antagonist, suggesting the possible presence of TRPV1–3 channels in these neurons (Gu et al., 2005). However, whether and to what extent these channels are involved in the expression of thermal sensitivity of these neurons under normal physiological conditions is yet unknown.

In view of the background information described above and the existing unanswered questions, this study was carried out to investigate whether isolated vagal pulmonary sensory neurons could be activated by increase in temperature within the physiological range, and if so, whether the response was mediated through TRPV channels.
2.2 Materials and methods

2.2.1. Labeling vagal pulmonary sensory neurons with Dil.

Sensory neurons innervating lungs and airways were identified by retrograde labeling from lungs by using the fluorescent tracer Dil as described previously (Kwong and Lee, 2002). Briefly, young adult Sprague-Dawley rats (~160 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and intubated with a polyethylene catheter (PE 150) with its tip positioned in the trachea above the thoracic inlet. Dil was initially sonicated and dissolved in ethanol, diluted in saline (1% ethanol v/v), and then instilled into the lungs (0.2 mg/ml; 0.2 ml × 2) with the animal’s head tilted up at ~30º.

2.2.2. Isolation of nodose and jugular ganglion neurons.

After 7–10 days, an interval previously determined to be sufficient for Dil to diffuse to the cell body, the rats were anesthetized with halothane inhalation 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 separately in ice-cold Dulbecco’s minimal essential medium/F12 (DMEM/F-12) 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 37ºC. 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/F-12 solution (DMEM/F-12 supplemented with 10% (v/v) 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/F-12 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 37ºC).

2.2.3. Electrophysiology.

Patch-clamp recordings were performed in a small-volume (0.2 ml) perfusion chamber that was continuously perfused by gravity-feed (VC-6, Warner Instruments, Hamden, CT) with extracellular solution (ECS) at 1 ml/min. Recordings were made in the whole-cell perforated patch configuration (50 µg/ml gramicidin) using Axopatch 200B/pCLAMP 8.2 (Axon Instruments, Union City, CA). Borosilicate glass electrodes had tip resistance of 2–4 MΩ. The series resistance was usually in the range of 4–8 MΩ and was not compensated. The ECS contained (in mM): 136 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 0.33 NaH₂PO₄, 10 glucose, 10 Hepes, pH at 7.4. The intracellular solution contained (in mM): 92...
potassium gluconate, 40 KCl, 8 NaCl, 1 CaCl₂, 0.5 MgCl₂, 10 EGTA, 10 Hepes, pH at 7.2. Chemical and temperature stimulations were applied by using a three-channel fast-stepping perfusion system (SF-77B, Warner), with its tip positioned to ensure that the cell was fully within the stream of the perfusate (width: ~700 µM). The temperature of the solution perfusing the neurons was raised progressively (TC-344B and SHM-6, Warner) from the baseline, either 23°C (room temperature) or 35°C (body temperature), to 41°C (hyperthermic temperature) in a “ramp” pattern in ~20 s. The actual temperature was measured by a micro-thermal probe (time constant = 0.005 s) (Harvard Apparatus, Holliston, MA) placed within 100 µm downstream from the cell being recorded. The data were filtered at 5 kHz and digitized at 10 kHz. The resting membrane potential was held at −70 mV.

The temperature coefficient over a 10°C temperature range (Q₁₀) and the activation energy Eₐ were used to characterize the temperature dependency of the membrane current (Vyklicky et al., 1999). Arrhenius plot was obtained by plotting the common logarithm of the current against the reciprocal of the absolute temperature. In the temperature range where the Arrhenius plot was linear (correlation coefficient r > 0.95), Eₐ was expressed using the slope of the regression line: 

\[-E_a = 2.303 R \log_{10}(I_2/I_1)/(1/(T_2) - (1/T_1))\]

where I₁ and I₂ are the values of normalized currents at the lower and higher absolute temperatures T₁ and T₂, respectively, and R is the gas constant (8.314 J K⁻¹ mol⁻¹). Q₁₀ was determined using the formula: 

\[Q_{10} = \exp (10E_a/(RT_1T_2))\]

2.2.4. Chemicals.

All chemicals were obtained from Sigma Chemical (St. Louis, MO), except for AMG 9810 [(E)-3-(4-t-Butylphenyl)-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylamide], which was obtained from Tocris (Ellisville, MO). A stock solution of capsaicin (1 mM) was prepared in 1% Tween 80, 1% ethanol and 98% saline. Stock solutions of capsazepine, AMG 9810 and ruthenium red were prepared in dimethyl sulfoxide (DMSO) at the concentration of 15, 50 and 10 mM, respectively. Solutions of these chemical agents at desired concentrations were then prepared daily by dilution with ECS. No detectable effect of the vehicles of these chemicals was found in our preliminary experiments.

2.2.5. Statistical analysis.

Data were analyzed with a one-way ANOVA, unless mentioned otherwise. When the ANOVA showed a significant interaction, pair-wise comparisons were made with a post hoc analysis (Fisher's least significant difference). A P value < 0.05 was considered significant. Data are means ± SE.
2.3 Results

2.3.1. Responses of isolated rat vagal pulmonary sensory neurons to increase in temperature.

Sensory neurons innervating lungs and airways were identified by the fluorescence intensity of Dil. When the temperature of the extracellular solution surrounding the neuron was elevated from room temperature (~23ºC) to 41ºC in ~20 s in a ramp pattern, a whole-cell inward current was consistently evoked in 78% of the neurons (87 out of 112) tested in voltage-clamp mode at the holding potential of −70 mV (e.g., Fig. 2.1B). The increase in temperature also induced membrane depolarization in 6 of the 9 neurons tested, and action potentials in 3 of them in current-clamp mode; an example is shown in Fig. 2.1A.

The inward current induced by increasing temperature in vagal pulmonary sensory neurons became apparent when temperature exceeded a certain threshold and increased sharply in amplitude as the temperature was further increased; this is clearly illustrated when the temperature-current relationship of the response shown in Fig. 2.1B is plotted in Fig. 2.2A. The “threshold” is defined in this dissertation as the temperature at which neurons begin to exhibit detectable and consistent responses (e.g., Fig. 2.1B), but not as the temperature that triggers the opening of individual ion channels. The temperature threshold for neuron activation was defined as $T_{120\%}$, which is calculated as the temperature when the current reached 20% of the peak current amplitude generated at 41ºC (e.g., Fig. 2.2A). The average $T_{120\%}$ was 34.4 ± 0.4 ºC ($n = 87$). In majority of the neurons, the current-temperature relationship obtained from the ascending and the subsequent descending phases of the temperature ramp (between 23ºC and 41ºC, as in Fig. 2.3A) exhibited a counterclockwise hysteresis (Fig. 2.3B). To quantitatively express the neuron sensitivity to temperature increase, the temperature coefficient, $Q_{10}$, was measured; $Q_{10}$ was derived from the linear fits of the semilog Arrhenius plot, in which the evoked current was plotted against $1/T$ ($T$, absolute temperature) (e.g. Fig. 2.3C). In general, a $Q_{10}$ value of > 5 indicates the presence of temperature-sensitive channels (Hille, 2001). In this study, the $Q_{10}$ over the higher temperature range (35–41ºC) was 29.5 ± 6.4 ($n = 22$), which was distinctly higher than that over the lower temperature range (23–30ºC, $Q_{10} = 2.84 ± 0.56$; $P < 0.01$, $n = 22$) (Fig. 2.3D).

2.3.2. Correlation between sensitivities to capsaicin and increasing temperature.

Since capsaicin is known to be a selective activator of TRPV1 channel (Caterina et al., 1997), we examined the correlation between the responses of whole-cell inward currents evoked by capsaicin and increasing temperature in the same neurons. Indeed, inward currents were evoked by both capsaicin (0.3, 0.5 or 1.0 µM; 2–6 s) and hyperthermia (41ºC) in the same neuron in 58% (65 out of 112) of the cells tested (e.g., Fig. 2.1B). However, no significant correlation was found
between the peak amplitude of the currents evoked by capsaicin and increasing temperature \((r^2 = 0.01, n = 75; \text{Fig. } 2.2B)\).

2.3.3. Correlation between the neuron size and the response to increasing temperature.

We further investigated whether the neuron sensitivity to hyperthermia is related to the cell size. However, no correlation was found either between TI\(_{20\%}\) (33.4 ± 0.7ºC, \(n = 34\)) and cell diameter (32.5 ± 1.0 μm) \((r^2 = 0.01; \text{Fig. } 2.2C)\) or between the peak current density at 41ºC (6.37 ± 1.54 pA/pF, \(n = 34\)) and cell diameter \((r^2 = 0.0004; \text{Fig. } 2.2D)\). Furthermore, there was no significant difference in the peak inward current evoked by increasing temperature (41ºC) between the neurons isolated from nodose and jugular ganglia (nodose: 3.22 ± 0.90 pA/pF, \(n = 23\); jugular: 5.07 ± 0.84 pA/pF, \(n = 11\); \(P > 0.1\); un-paired \(t\)-test). In the 34 neurons tested, 24 responded to capsaicin and 10 did not; in comparison, both the peak current density and TI\(_{20\%}\) were significantly higher in the capsaicin-sensitive neurons (7.47 ± 2.13 pA/pF and 34.7 ± 0.7ºC, respectively) than that in the capsaicin-insensitive neurons (3.70 ± 0.54 pA/pF, \(P < 0.01\); 30.2 ± 0.8ºC, \(P < 0.01\); un-paired \(t\)-test).

2.3.4. Pulmonary neuron response to increasing temperature within the physiological range.

Normal body temperature of the rat during sleep is around 35–36ºC (Refinetti and Menaker, 1992). To determine whether the neuron response to increasing temperature in the range tested in this study (23–41ºC; room temperature: ∼23ºC) is different from that in the normal physiological temperature range (35–41ºC), we compared the current response to increase in temperature over these two different temperature ranges in the same neurons; the temperature-current relationship obtained from the two temperature ranges overlapped almost completely in individual cells (e.g., Fig 2.4A), clearly indicating that the neuron response to hyperthermia was mainly generated in the temperature range of 35–41ºC (Fig. 2.4B). A similar pattern of responses was also found in several other pulmonary sensory neurons tested in this study series following the same experimental protocols as that described above.

2.3.5. Role of TRPV channels in the response of pulmonary sensory neurons to increasing temperature.

To investigate the relative contributions of different subtypes of TRPV channels to the effect of hyperthermia on pulmonary sensory neurons, currently available channel blockers were used in the subsequent experiments; both capsazepine (Bevan et al., 1992) and AMG 9810 (Gavva et al., 2005) have been shown to be selective antagonists of TRPV1 channel, whereas ruthenium red is used frequently as a non-selective antagonist for TRPV1–4 channels (Benham et al., 2003). The whole-cell inward current evoked by increasing temperature (41ºC)
was only partially blocked by pretreatment with capsazepine (10 µM, 5 min) or AMG 9810 (0.3 µM, 4 min): before and after capsazepine, 389.3 ± 117.4 pA and 186.4 ± 38.5 pA ($P < 0.05$, $n = 11$), respectively; before and after AMG 9810, 367.1 ± 177.8 pA and 84.6 ± 17.4 pA ($P < 0.05$, $n = 8$), respectively (Fig. 2.5). Furthermore, $T_l_{20\%}$ was significantly reduced from 34.9 ± 0.7°C at control to 31.0 ± 0.7°C after capsazepine ($P < 0.05$, $n = 11$; paired t-test), and from 33.7 ± 1.5°C at control to 30.9 ± 1.0°C after AMG 9810 ($P < 0.05$, $n = 8$; paired t-test). In sharp contrast, the response was almost completely abolished by pretreatment with ruthenium red (3 µM, 5 min) alone (15.0 ± 6.8 pA; $P < 0.05$, $n = 9$) (Fig. 2.5). The inward current evoked by increasing temperature returned to the control level after ~60 min washout of ruthenium red (235.0 ± 84.3 pA; $P > 0.1$, $n = 9$) (Fig. 2.5C). In contrast, the vehicle (DMSO) at the same concentration (10–15 µM) as that of capsazepine and ruthenium red applied in the same manner did not produce any detectable effect ($n = 3$). The blocking effect of capsazepine was completely reversed after 30-min washout, whereas that of AMG 9810 lasted for > 60 min (data not shown).
2.4 Discussion

Results of this study clearly demonstrated that isolated vagal pulmonary sensory neurons can be directly activated by an increase in temperature within the normal physiological range, as demonstrated by the evoked inward currents (voltage-clamp mode) and membrane depolarization/action potentials (current-clamp mode) (Fig. 2.1). The inward current induced by hyperthermia was temperature dependent; the distinctly higher $Q_{10}$ value (29.5 ± 6.4) over the range of 35–41ºC suggests that the increase in temperature probably leads to the opening of temperature-sensitive ion channels. The inward current induced by increasing temperature was partially blocked by capsazepine and AMG 9810, selective TRPV1 antagonists, but almost completely abolished by ruthenium red, an effective blocker of TRPV1–4 channels, indicating the involvement of TRPV1 as well as other subtypes of thermal-sensitive TRPV channels. Taken together, these observations suggest that TRPV1–4 channels play a primary role in regulating the responses of these pulmonary sensory neurons to hyperthermia.

Increasing evidence from recent studies have collectively suggested that TRPV1 may also play an important role in the manifestation of various symptoms of airway hypersensitivity (cough, reflex bronchoconstriction, etc) associated with airway inflammatory reactions (Gunthorpe et al., 2002; Hwang and Oh, 2002; Lee and Undem, 2005). However, the potential roles of other TRPV channels (TRPV2–4) in the neural regulation of airway functions are yet unknown.

The lack of any correlation between peak current amplitude evoked by increasing temperature (41ºC) and capsaicin, a selective agonist of TRPV1 channel, in these neurons (Fig. 2.2B) suggests a possibility that the hyperthermia-induced response is not solely mediated through TRPV1 channel. Considering the possible limitation of capsazepine to block the heat-induced current mediated through the TRPV1 channel (Benham et al., 2003), we tested in a subsequent experiment the effect of a newly developed, selective TRPV1 antagonist, AMG 9810, which has been shown to effectively block capsaicin and heat-induced activation of TRPV1 channel in both in vivo and in vitro preparations (Gavva et al., 2005). The current induced by increase in temperature was not totally blocked by either capsazepine or AMG 9810 (Fig. 2.5). Although the experiments for obtaining more definitive evidence were limited by the unavailability of specific agonists/antagonists for other subtypes of TRPV channels, the combination of the partial blocking effect of either capsazepine or AMG 9810 and the almost complete abolition by ruthenium red (Fig. 2.5) indicated the possible involvement of other thermal-sensitive TRPV channels, in addition to TRPV1, in the response of these neurons to increase in temperature. Future studies in the knockout mice of specific TRPV channels should allow us to further delineate the relative contributions of different subtypes of TRPV channels to the thermal sensitivity of pulmonary sensory neurons.
Another interesting observation in this study is that the peak current density evoked by the same heat stimulation (41°C) was significantly smaller in the capsaicin-insensitive pulmonary neurons than the capsaicin-sensitive neurons (3.70 vs 7.47 pA/pF). Presumably, the capsaicin-insensitive neurons express either no or a low density of TRPV1 channel, and the whole-cell current recorded during increasing temperature represents a summation of all the currents conducted through open channels of that neuron. Furthermore, the temperature threshold for activation, $T_{I20\%}$, was also markedly higher (mean $\Delta = 4.5^\circ C$) in the capsaicin-sensitive neurons. These differences may be related to the fact that the temperature threshold for activating TRPV1 channel is much higher (~43°C) than that of TRPV3 and V4 (Tominaga and Caterina, 2004).

The average temperature threshold for activation of the pulmonary sensory neurons measured by the evoked current in the present study was ~34°C (Figs. 2.2A and 2.3A), which is considerably lower than that previously reported in the primary sensory neurons innervating other organ systems (e.g., dorsal root ganglion neurons, ~43°C)(Benham et al., 2003; Patapoutian et al., 2003). This difference is probably related to the different native environments and temperature ranges to which these sensory endings are normally exposed. We also believe that this threshold may reflect the relative contributions of different subtypes of thermal-sensitive TRPV channels to the response of these neurons to hyperthermia for the following reasons. Firstly, the activation threshold varied between cells in a wide range of 30−39°C. Secondly, the proportion of the high temperature-induced peak current that was blocked by capsazepine also varied substantially between cells. Lastly, the threshold temperature observed in our study does not match that of any existing subtypes of the thermal-sensitive TRPV channels, namely TRPV1–4. The functional diversity of TRPV channels, especially in primary sensory neurons, has not been fully explored. However, it has been suggested that TRPV channels can co-assemble into heteromeric pore complexes in native cells, and can possibly exhibit distinct channel gating functions from the individual homomeric channels. Indeed, a recent study (Liapi and Wood, 2005) has demonstrated the heteromultimer formation between TRPV1 and TRPV2 in adult rat cerebral cortex. In a TRPV1 and TRPV3 heterologously co-expressed system, a positive co-immunoprecipitation and an increased sensitivity to capsaicin and proton (compared with the transfection with TRPV1 alone) has been reported (Smith et al., 2002). It seems possible that one of the functional alterations of these heteromultimers could be a change in the activation threshold in response to hyperthermic stimulus. In the present study, the temperature threshold for activation is lower than that of TRPV1 or TRPV2, but higher than that of TRPV3 or TRPV4 (Fig. 2.2, A and C). Whether this difference is related to the possible heteromultimer formation between different TRPV subunits as well as a difference in the subunit composition remains to be determined.

Previous studies of other types of primary sensory neurons have reported that different subtypes of TRPV channels are expressed in sensory neuron
populations of different cell sizes. For example, TRPV1 is expressed exclusively in small- and medium-diameter neurons (Caterina et al., 1997; Caterina and Julius, 2001), whereas TRPV2 is preferentially expressed in medium- and large-diameter DRG neurons (Caterina et al., 1999; Ahluwalia et al., 2002). In contrast, our results do not indicate any correlation between the cell response to hyperthermia, expressed as the peak current density at 41°C, and the cell diameter (Fig. 2.2D). Furthermore, there is no clear correlation between the temperature threshold for activation and the cell size, regardless of the sensitivity to capsaicin (Fig. 2.2C). These results do not seem to indicate a clear pattern of size-dependent distribution of specific subtypes of TRPV channels in these pulmonary sensory neurons. Sensory neurons derived from different ganglionic origin are known to express distinct physiologic and pharmacologic properties (Ricco et al., 1996). However, we did not find any significant difference in the response to increase in temperature between nodose and jugular pulmonary neurons in this study.

It is well documented that vagal bronchopulmonary afferents play an important role in regulating various cardiopulmonary functions (Coleridge and Coleridge, 1986; Ho et al., 2001). However, the existing knowledge about the thermal sensitivity of these afferent nerves is extremely limited. It is, therefore, of particular significance to know that increasing temperature within the physiological range has a direct effect on isolated pulmonary sensory neurons as shown in the present study (Fig. 2.1). The fact that a high percentage of these neurons (78%) displayed pronounced temperature sensitivity suggests that this is a general property of the pulmonary sensory neurons. It also lends support to our previous observation of a stimulatory effect of hyperthermia on pulmonary C-fibers in anesthetized rats (Ruan et al., 2005). However, we should also point out that there are noticeable differences in the results between the present study and our previous in vivo study. The average temperature thresholds of activation (for detailed definition of “temperature threshold”, please see page 12, second paragraph) for bronchopulmonary C-fiber endings and the isolated sensory neurons are 39.2°C and 34.4°C, respectively. Several factors may account for this difference. In the in vivo study, the temperature threshold was determined from the action potential signal generated from the sensory terminal and recorded from the axon (Ruan et al., 2005). In contrast, the temperature threshold measured in this study was determined from the inward current signal, which reflected the increase in cation influx resulted from opening of ion channels. However, sub-threshold depolarization does not necessarily lead to the generation of action potentials unless it reaches the firing threshold. This is illustrated in the example shown in Fig. 2.1A, in which membrane depolarization and action potential occurred at the temperatures of ~35°C and ~40°C, respectively. In addition, the differences in membrane properties, the expression of channels/receptors and the signal transduction mechanism between the neuronal soma and the sensory terminal may have also contributed to this difference in the temperature threshold. Moreover, the cellular environment and milieu that surround and interact with the sensory terminals in living tissue are
very different from that used in the cultured neurons, which may also influence the thermal sensitivity of these afferents.

In our previous *in vivo* study, the stimulatory effect of hyperthermia was only observed in the capsaicin-sensitive afferents in the lungs (Ruan et al., 2005), whereas the thermal-sensitivity was found in both capsaicin sensitive and insensitive pulmonary neurons in the present study. Although this discrepancy cannot be adequately explained in this study, the various factors discussed above may have also contributed to the difference. More importantly, it is well documented that when capsaicin-sensitive bronchopulmonary afferents are stimulated (e.g., by hyperthermia), they can elicit centrally mediated reflex responses such as cough, bronchoconstriction and hypersecretion of mucus (Coleridge and Coleridge, 1984; Ho et al., 2001), and evoke the local “axon reflex” via the release of tachykinins (TKs) and calcitonin gene-related peptides (CGRPs) (Solway and Leff, 1991; Ho et al., 2001). In fact, it has been recently reported that tissue hyperthermia triggers the local release of TKs and CGRPs (Kessler et al., 1999; Zimmermann et al., 2005). In the airways and lungs, these neuropeptides are known to act on a number of effector cells (e.g., airway smooth muscles, cholinergic ganglia, inflammatory cells, mucous glands) and generate potent local effects such as bronchoconstriction, extravasation of macromolecules, and chemotactic responses (Lundberg and Saria, 1987; Solway and Leff, 1991).

In summary, this study has established the first evidence that isolated vagal pulmonary sensory neurons can be activated by an increase in temperature within the physiological range. Our results also suggest that the thermal sensitivity of these neurons is mediated through the activation of TRPV1 and other TRPV channels. This conclusion is further supported by the evidence that thermal-sensitive TRPV1–4 channels are expressed in these sensory neurons (Ni et al., 2006). However, the relative contributions of these different subtypes of TRPV channels to the thermal sensitivity of these neurons remain to be determined. Although this finding has provided the definitive evidence of a direct stimulatory effect of hyperthermia on vagal bronchopulmonary sensory nerves, the influence of the activation of these neurons by hyperthermia on the overall regulation of airway function under normal (e.g., strenuous exercise) or pathophysiological conditions (e.g., fever, airway inflammation, heat stroke) requires further investigations.
2.5 Figures

Figure 2.1 Representative experimental records illustrating responses of isolated rat vagal pulmonary sensory neurons to increase in temperature. **A**: in current-clamp mode, increasing the temperature in a ramp pattern evoked depolarization and action potentials in a jugular neuron (21.0 pF). V, membrane potential; notice that the membrane began to depolarize as the temperature reached ~35°C, but action potentials were not evoked until ~40°C. Insets display the action potential signals at an expanded time scale. **B**: in voltage-clamp mode (holding potential = −70 mV), capsaicin (0.5 µM, 2-s duration) and heat were applied to a jugular neuron (28.6 pF). In both A and B, ~2 min elapsed between the two hyperthermia challenges.
Figure 2.2 Thermal sensitive properties of isolated rat vagal pulmonary sensory neurons. A: the temperature-current relationships of the two hyperthermia challenges in the same neuron (data are identical to that in Fig. 2.1B). TI20% was determined by locating the temperature point where the amplitude of the current reached 20% of the peak current generated at 41°C. B: the relationship between the peak current induced by heat (I41°C) and that by capsaicin (I_cap) of either low dose (0.3 or 0.5 µM, closed circles, n = 60) or high dose (1.0 µM, open circles, n = 15). C: the relationship between cell diameter and TI20% (n = 34). D: correlation between cell diameter and current density; the later was calculated by dividing the peak current to increasing temperature by the capacitance of each individual neuron (n = 34). In both C and D: closed circles, capsaicin sensitive (Cap +); open circles, capsaicin insensitive (Cap -).
Figure 2.3 Whole cell inward current induced by increasing temperature and its temperature dependency in isolated rat vagal pulmonary sensory neurons. A: representative experimental record illustrating that an inward current was evoked in a jugular neuron (37.4 pF) when a temperature ramp of 23–41°C was applied. B: the temperature-current relationship of the same response in A exhibits a counterclockwise hysteresis; arrows indicate the direction of temperature change. C: Arrhenius plot of the data in A illustrating two distinct phases of the response to increase in temperature. $Q_{10}$ values were derived from linear fits of the data in low and high temperature ranges. D: group data for $Q_{10}$ values over the two temperature ranges. Data are means ± SE ($n = 22$). *, $P < 0.01$, significant difference between the $Q_{10}$ values in these two ranges with paired t-test.
Figure 2.4 Representative experimental records illustrating that the responses of rat vagal pulmonary sensory neurons to increase in temperature are generated mainly within the physiological temperature range. A: inward currents evoked by increasing temperature from a baseline of room (25ºC; left panel) or body temperature (35ºC; right panel) to high temperature (41ºC) in a jugular neuron (21.0 pF). B: temperature-current relationships plotted from the two current traces in A; closed circles, from the baseline of 25ºC; open circles, from 35ºC.
Figure 2.5 Blocking effects of capsazepine, AMG 9810 and ruthenium red on the hyperthermia-induced current in rat vagal pulmonary sensory neurons. A: representative traces illustrating the inward currents evoked by increasing temperature at control, after pretreatments with capsazepine (CPZ, 10 µM) and ruthenium red (RR, 3 µM), and after washout in a jugular neuron (28.6 pF). At least 10 min was allowed for the cell to recover between tests. B: the temperature-current relationships of the four current traces shown in A. C: group data showing the effects of CPZ and RR treatments on cell response to increasing temperature (n = 11; RR was studied in only 9 of these cells). D: representative traces illustrating the inward currents evoked by capsaicin (Cap, 1 µM, 2-s duration) and increasing temperature, before (control) and after pretreatment with AMG 9810 (AMG, 0.3 µM) in a jugular neuron (22.3 pF). E:
group data showing the effect of AMG treatment on cell response to capsaicin and increasing temperature \((n = 8)\); because of slow recovery after the AMG treatment, data after washout were not collected. Data are means ± SE. *, significantly different \((P < 0.05)\) from the corresponding control response; †, significant difference \((P < 0.05)\) between the responses after CPZ and RR treatments.
Chapter Three: Effect of increasing temperature on TRPV1-mediated responses in isolated rat pulmonary sensory neurons

3.1 Introduction

In the study described in Chapter 2, we showed that hyperthermia has a direct stimulatory effect on pulmonary sensory neurons, and this effect is partially mediated through the TRPV1 channel as well as other subtypes of thermal-sensitive TRPV (2–4) channels. Whether hyperthermia has any effect on the excitability of pulmonary sensory neurons and whether the responses of these neurons to chemical activators can be modulated by hyperthermia is not known. In addition, previous work in our laboratory has shown that the responses of bronchopulmonary C-fiber afferents to both lung inflation and capsaicin injection were markedly potentiated when the intrathoracic temperature was increased in anesthetized rats (Ruan et al., 2005). However, whether the stimulation was generated by a direct action of hyperthermia on these sensory nerves could not be determined in that study because in addition to their expression on pulmonary sensory neurons (Ni et al., 2006), these TRPV channels are also expressed on other cell types in lungs (e.g., epithelial cells, endothelial cells, airway smooth muscles, etc) (Jia and Lee, 2007), which upon activation can generate a secondary effect on these afferent endings. The present study was, therefore, designed to investigate the direct effect of increasing temperature within the physiological range on the excitability of isolated pulmonary sensory neurons, and to determine the involvement of TRPV receptors in these responses.

3.2 Materials and methods

3.2.1. Labeling and Isolation of nodose and jugular ganglion neurons.

Labeling and isolation procedures were identical as that described in Chapter 2, sections 2.2.1 & 2.2.2.

3.2.2. Electrophysiology.

Patch-clamp recordings were performed the same way as described in Chapter 2, section 2.2.3, except that the temperature of the ECS solution perfusing the neurons was maintained (TC-344B and SHM-6, Warner) at a constant level of either resting body temperature (BT; ~36°C) or hyperthermic temperature (HT; ~40.6°C) in rats for > 60 s before applying the chemical solution at the same temperature.

3.2.3. Chemicals.

Capsaicin, 2-APB, acid (pH 5.5, 6.0 and 6.5), acetylcholine (ACh) and adenosine 5'-triphosphate (ATP) are activators of pulmonary sensory neurons, and they were chosen in this study for the following reasons: capsaicin is a known selective TRPV1 agonist (Caterina et al., 1997); 2-APB is a common activator of
TRPV1, V2 and V3 (Hu et al., 2004); acid activates both TRPV1 and acid-sensing ion channels (ASICs) (Caterina et al., 1997; Waldmann et al., 1997); in contrast, ACh and ATP are not known to directly activate the TRPV1 channel.

All chemicals were obtained from Sigma Chemical (St. Louis, MO), except for 2-APB (Tocris, Ellisville, MO). Capsaicin solution was prepared the same way as described in Chapter 2. Stock solutions of 2-APB and amiloride were prepared in dimethyl sulfoxide (DMSO) at the concentration of 0.1 and 1 M, respectively. Solutions of these chemical agents at desired concentrations were then prepared daily by dilution with ECS. The responses of neurons to the vehicles of these chemicals were tested in our preliminary experiments, and no detectable effect was found.

3.2.4. Statistical analysis.

Data were analyzed with a one-way ANOVA analysis, followed by a post hoc Newman-Keuls test, unless mentioned otherwise. A $P$ value < 0.05 was considered significant. Data are mean ± S.E.M.
3.3 Results

Sensory neurons innervating lungs and airways were identified by the fluorescent intensity of DiI; those with spherical shape and smooth membrane were chosen for the study. Experimental protocols were completed in a total of 90 pulmonary sensory neurons isolated from nodose and jugular ganglia. The whole cell capacitances of these neurons were in the range of 12.3–40.8 pF (25.5 ± 0.8 pF; \( n = 90 \)); the majority of them (77 out of 90) were small in size (capacitance \( \leq 30 \) pF); 41.1% (\( n = 37 \)) of the cells were nodose neurons, and 58.9% (\( n = 53 \)) were jugular neurons. Although neurons were not selected based upon their sensitivity to capsaicin (except in the study of cell response to capsaicin), 81.1% (73 out of 90) of the cells were activated by a low concentration of capsaicin (0.3 or 1.0 µM; 1–6 s).

3.3.1. Effect of increasing temperature on responses of vagal pulmonary sensory neurons to capsaicin and 2-APB.

In current-clamp mode, when the temperature of the ECS surrounding the neuron was elevated from normal body temperature (BT; 35.9 ± 0.06ºC) to hyperthermic temperature (HT; 40.6 ± 0.07ºC) in rats, baseline membrane potential (Vm) was significantly elevated (Vm = -69.5 ± 3.08 mV at BT, Vm = -64.8 ± 4.3 mV at HT; \( n = 7 \), \( P < 0.05 \)) (Fig. 3.1B), and membrane depolarization evoked by capsaicin challenge (0.3 or 1.0 µM; 1–8 s) were also potentiated (\( \Delta V_m^{(Cap)} = 29.3 ± 7.0 \) mV at BT, \( \Delta V_m^{(Cap)} = 42.1 ± 8.3 \) mV at HT; \( n = 7 \), \( P < 0.01 \)); in 4 of the 7 cells, capsaicin evoked firing of action potentials, and the number of action potentials in response to the same capsaicin challenge was also clearly increased during HT (3 ± 3 at BT, 15 ± 7 at HT; \( n = 4 \), \( P < 0.05 \)) (Fig. 3.1C). To minimize a possible involvement of the voltage-sensitive currents generated by changes in membrane potentials, the rest of our experiments were conducted in voltage-clamp mode. Similarly, capsaicin (0.3 µM; 2–4 s)-evoked current (\( \Delta I \)) was greatly potentiated by increasing the temperature from ~36°C to ~40.6°C (\( \Delta I = 775 ± 140 \) pA at BT, \( \Delta I = 2242 ± 597 \) pA at HT; \( n = 7 \), \( P < 0.05 \)) (Fig. 3.1, D and E).

2-APB (0.3 mM; 2–8 s)-evoked current was also potentiated at the hyperthermic temperature (\( \Delta I = 484 ± 99 \) pA at BT, \( \Delta I = 1019 ± 209 \) pA at HT; \( n = 19 \), \( P < 0.01 \)) (Fig. 3.2A and B). To further investigate the contribution of the TRPV1 channel, we studied the effect of capsazepine (CPZ), a selective TRPV1 channel antagonist (Bevan et al., 1992). Increasing the temperature from 36.0 ± 0.10°C to 40.6 ± 0.08°C markedly enhanced the 2-APB (0.3 mM; 2–8 s)-evoked inward current both before (\( \Delta I = 435 ± 123 \) pA at BT; \( \Delta I = 1061 ± 282 \) pA at HT; \( n = 8 \), \( P < 0.01 \)) and after (\( \Delta I = 116 ± 25 \) pA at BT; \( \Delta I = 305 ± 85 \) pA at HT; \( n = 8 \), \( P < 0.05 \)) the pre-treatment with CPZ (10 µM; 2 min); CPZ at this concentration has been shown to completely block the effect of capsaicin on the TRPV1 channel in this model system (Ni et al., 2006). However, this increase in the current...
response to 2-APB resulting from the same temperature elevation (between BT and HT) was significantly attenuated by the CPZ pre-treatment (Fig. 3.2, C and D; before CPZ, $\Delta I = 625 \pm 177$ pA; after CPZ, $\Delta I = 189 \pm 65$ pA; $n = 8$, $P < 0.05$; analyzed by the linear mixed model two-factor ANOVA). In addition, another selective and more potent TRPV1 antagonist AMG 9810 (AMG), which has been shown to effectively block both capsaicin and heat-induced activation of the TRPV1 channel (Gavva et al., 2005), was also tested. Similarly, pretreatment of AMG (1 $\mu$M; 5 min) significantly attenuated the increase in the current response to 2-APB resulting from the temperature elevation (before AMG, $\Delta I = 689 \pm 192$ pA; after AMG, $\Delta I = 218 \pm 47$ pA; $n = 5$, $P < 0.05$; the linear mixed model two-factor ANOVA) (Fig. 3.2E and F). Pretreatment with the vehicle of CPZ (0.7 mM DMSO; 2 min) and AMG (10 $\mu$M DMSO; 5 min) had no effect. These results indicate that the stimulatory effect of 2-APB on these neurons consisted of two components: one mediated through TRPV1, and the other through non-TRPV1; both these components were potentiated by increasing the temperature from BT to HT.

In our preliminary studies, we found no difference between nodose and jugular pulmonary sensory neurons in the potentiating effect of increasing temperature on the cell responses to capsaicin or 2-APB; for example, the hyperthermia-induced increases in response to the same challenge of 2-APB (0.3 mM, 2–8 s) were 102 ± 17 % ($n = 10$) and 138 ± 24 % ($n = 9$) in nodose and jugular neurons, respectively ($P > 0.05$). Therefore, the data obtained from the neurons of these two different ganglion origins were pooled for group analysis in this study.

3.3.2. Effect of increasing temperature on the response of pulmonary sensory neurons to acid.

Acid-induced currents in pulmonary sensory neurons have been shown to be mainly mediated through the activation of both ASICs and the TRPV1 channel (Ni et al., 2006). Figure 3.3A illustrates the typical response of these neurons evoked by acid (pH 5.5; 2 s) at room temperature, consisting of a rapidly activated and inactivated current and a slow, sustained current, defined as the transient and sustained components, respectively. When the temperature was raised to ~36°C, the sustained current was potentiated in comparison to that at room temperature. Interestingly, however, the transient current induced by acid was inhibited by the increase in temperature. When the temperature was further increased to 40.2°C, the sustained current response induced by the same degree of acidity became larger and the transient current disappeared completely. Both the transient and sustained responses almost completely returned when the temperature was returned to body and room temperatures (Fig. 3.3A). Similar responses were also seen in 5 other cells. To further identify the types of channels involved in the effect of hyperthermia, amiloride, a known blocker of ASICs (Waldmann et al., 1997), and capsazepine were applied separately in this experiment. Figure 3.3A showed that the transient but not the sustained component was completely blocked by amiloride (1 mM; 1 min). In contrast, capsazepine (10 $\mu$M; 3 min) significantly attenuated the sustained but not the
transient component. These results indicate that the transient and sustained components of the acid-evoked current seen at room temperature are mediated mainly through ASICs and the TRPV1 channel, respectively, which is consistent with those reported previously in pulmonary sensory neurons (Ni et al., 2006).

As shown in the previous study (Ni et al., 2006), the acid-evoked responses in different pulmonary sensory neurons exhibited varying degrees of expression of these two different phenotypes of inward currents. In the following experiments, we selectively chose the neurons exhibiting a specific type of current response to acid to further investigate the effect of increasing temperature on these two different types of acid-induced current components. In the neurons that exhibited only the transient current component in response to acid stimulation (e.g. Fig. 3.3B), the inward current evoked by low pH (6.0 or 6.5, 2–6 s) at room temperature ($\Delta I = 593 \pm 116$ pA) was almost completely inhibited at $36.0 \pm 0.07^\circ C$ ($\Delta I = 28 \pm 18$ pA), and completely disappeared at $40.7 \pm 0.05^\circ C$ ($\Delta I = 0$ pA) (Fig. 3.3B and C) ($n = 7$, $P < 0.01$). The response was fully recovered upon returning to room temperature (Fig. 3.3C). In a sharp contrast, in the neurons that only exhibited sustained current in response to acid stimulation (e.g. Fig. 3.3D), the sustained inward current evoked by low pH (5.5 or 6.0; 2–6 s) was markedly potentiated by an increase in temperature (Fig. 3.3, D and E; $\Delta I = 558 \pm 208$ pA at BT, $\Delta I = 1074 \pm 353$ pA at HT; $n = 9$, $P < 0.05$).

3.3.3. Effect of increasing temperature on pulmonary neuron response to non-TRPV1 activators.

The results described above clearly indicate the potentiating effects of hyperthermia on the responses of pulmonary sensory neurons to TRPV1 channel activators. To further determine whether these potentiating effects are specific to TRPV1 channel activators, cell responses to other chemical stimulants that do not directly activate TRPV1, such as ACh and ATP, were studied.

ACh is known to activate both nicotinic and muscarinic ACh receptors, but its possible effects on pulmonary sensory neurons have not been well characterized. In our experiments, ACh in the range of 50–100 µM evoked an inward current in 22 of the 68 pulmonary sensory neurons tested, with a current magnitude comparable to that evoked by capsaicin (0.3 µM) (e.g. Fig. 3.4B). Furthermore, our results showed that hexamethonium (0.1 mM; 5 min), a specific antagonist of nicotinic ACh receptor, almost completely abrogated the ACh (100 µM; 2–6 s)-evoked current ($\Delta = 90.1 \pm 5.0\%$; $n = 6$) (e.g., Fig. 3.4, A and B), indicating that the response was mostly mediated through nicotinic ACh receptors.

ATP is a known activator of both P2X and P2Y receptors (Burnstock and Williams, 2000). ATP (0.3 µM; 1–6 s) reproducibly evoked an inward current in 34 of the 39 pulmonary sensory neurons (460 ± 135 pA), but the pattern of the current response varied between different cells (e.g. Fig. 3.5, A and C). Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS; 30 or 50 µM; 2 min), a non-selective P2X receptor antagonist (Khakh et al., 2001), completely
abolished the ATP-evoked current (Δ = 99.1 ± 0.6%; n = 7) (e.g., Fig. 3.5, A and B), indicating that the response was mostly mediated through P2X receptors.

Our results clearly showed that neither ACh (50 or 100 µM; 2–6 s)- nor ATP (0.3 µM; 1–6 s)- evoked current was potentiated by hyperthermia (Fig. 3.4, C and D, Fig. 3.5, C and D). ACh-induced current responses were 602 ± 397 pA and 640 ± 415 pA at 35.9 ± 0.08°C and 40.7 ± 0.08°C, respectively (n = 8, P = 0.27). Similarly, ATP-induced current responses were 460 ± 136 pA and 415 ± 118 pA at 35.9 ± 0.08°C and 40.7 ± 0.07°C, respectively (n = 13, P = 0.19).

3.3.4. Capsaicin-evoked responses at different temperatures.

To determine the temperature threshold of the potentiating effect of hyperthermia on capsaicin-evoked responses, in a subsequent series of experiments we successively increased the temperature in 1.5°C increments between 36.0 and 40.5°C, each step held in a steady state for 3 min with 15 min recovery between two steps, and tested the response to capsaicin (0.1 or 0.3 µM; 1–3 s) in each neuron. Although capsaicin-evoked current increased progressively when the temperature was raised (Δ I = 416 ± 68 pA at BT, Δ I = 601 ± 83 pA at 37.5°C, Δ I = 1092 ± 157 pA at 39.0°C, Δ I = 1336 ± 248 pA at 40.5°C), significant potentiation was found only after the temperature reached 39.0°C (n = 10, P < 0.05) (Fig. 3.6).
3.4 Discussion

The present study demonstrated that increasing temperature within the normal physiological range (36–40.6°C) resulted in sensitization of isolated pulmonary sensory neurons to chemical activators of the TRPV1 channel; this was clearly indicated by the observation that both membrane depolarization and the number of action potentials evoked by capsaicin were markedly potentiated by hyperthermia. When the membrane potential was held constant in voltage-clamp mode, both capsaicin- and 2-APB-induced currents were significantly augmented when temperature was increased. This potentiating effect was temperature-dependent, and became evident when the temperature reached ~39°C. The potentiating effect of hyperthermia on 2-APB-evoked current was attenuated by either capsazepine or AMG 9810, selective antagonists of the TRPV1 receptor. In contrast, the responses to ATP and ACh (neither is known to activate the TRPV1 receptor) remained unchanged with increasing temperature in these neurons. More interestingly, increasing temperature exerted a paradoxical effect on the acid-evoked current: the rapid, transient component mediated by ASICs was consistently inhibited, and the slow, sustained component mediated by the TRPV1 receptor was markedly potentiated.

The present study has provided the first evidence that hyperthermia exerts a direct potentiating effect on the sensitivity of pulmonary sensory neurons to chemical activators of the TRPV1 receptor. Several possible physiological implications of these findings should be considered. The temperature change applied in this study was well within the normal physiological range. The body temperature in rats was measured at 35.5–36.0°C during sleep (Refinetti and Menaker, 1992), whereas the hyperthermic temperature (~40.5°C) employed in this study can occur under both normal and pathophysiological conditions. Most importantly, the potentiating effect observed in our study was clearly present even at the moderate level of hyperthermia (39.0°C; Fig. 3.6). In healthy subjects, the most common cause of hyperthermia is an increase in metabolic rate (e.g., during vigorous exercise). As discussed earlier, body core temperature exceeding 41°C has been reported during exertional exercise in healthy men (Maron et al., 1977) and in animals (Brooks et al., 1971). Hyperthermia also occurs frequently under pathophysiological conditions caused by endogenous pyrogens or infection, such as in patients suffering from severe fever. Moreover, tissue inflammation is known to lead to local hyperemia and an increase in temperature in the inflamed area (Gourine et al., 2001).

Depolarization of baseline membrane potential at hyperthermic temperature shown in the current study (Fig. 3.1, A and B) is consistent with the previous results reported recently by Ni et al. (Ni et al., 2006). The results of that study indicate that TRPV1 and possibly other thermal sensitive TRPV channels (TRPV2–4) are involved in the expression of thermal sensitivity of these cells. However, we can not rule out the possible involvement of other ion channels.
exhibiting thermal sensitivity. Recent studies have demonstrated that the activities (e.g. single channel conductance, frequency of opening, etc.) of certain potassium channels, such as TREK-1, TREK-2 and TRAAK, were elevated with increasing temperature (Kang et al., 2005; Alloui et al., 2006), which is expected to induce membrane hyperpolarization. Hence, it is unlikely that these potassium channels play a part in inducing the membrane depolarization formed in pulmonary sensory neurons during hyperthermia in this study.

In addition, the small and sustained elevation of temperature by ~4.6ºC (from 36 to 40.6ºC) generated only a small inward current (average ~40 pA) in these neurons, which is considerably smaller than the average current response (~135 pA) evoked by a larger and transient temperature elevation (from 23 to 41ºC in a ramp pattern) found in our previous study (Ni et al., 2006). However, despite the relatively mild increase in baseline current during hyperthermia, the inward currents evoked by various chemical activators of the TRPV1 receptor (e.g., capsaicin, proton, 2-APB) in these neurons were drastically and consistently augmented not only in voltage clamp mode (Figs. 3.1, 3.2 and 3.3D) but also in current clamp mode (capsaicin; Fig. 3.1, A–C), clearly indicating a synergistic, not additive, effect of hyperthermia on the responses to these chemical activators. Although the specific site(s) and mechanism of the sensitizing effect can not be determined in this study, cytoplasmic C-terminal tail of the TRPV1 receptor may play an important part in mediating the positive interaction between hyperthermia and chemical activators of the channel since this region has been shown to be involved in the conformational changes leading to channel activation (Vlachova et al., 2003). Furthermore, the evidence that capsazepine or AMG pretreatment markedly attenuated the potentiating effect of hyperthermia on the neuron response to 2-APB suggests a primary role of the TRPV1 receptor (Fig. 3.2, C–F). However, because the potentiated response to 2-APB was not completely abolished by capsazepine or AMG (Fig. 3.2, C–F), the involvements of TRPV2 and V3 receptors, though to a lesser extent, can not be dismissed.

Our results clearly demonstrated that incremental increases in temperature induced a progressive increase in the capsaicin-evoked response in pulmonary sensory neurons (Fig. 3.6). Because of the long recovery time required after each capsaicin challenge and the limited time for maintaining a stable recording of these neurons, we were not able to identify more precisely the threshold temperature of the potentiating effect of hyperthermia. Nevertheless, our data indicated that the response to capsaicin was pronouncedly potentiated after the temperature exceeded 39ºC, which is at a level of moderate hyperthermia. This temperature is substantially lower than the threshold temperature (43ºC) for activating the heterologously expressed TRPV1 channel reported in the literature (Caterina et al., 1997; Clapham, 2003; Patapoutian et al., 2003), but is comparable to that found in pulmonary C-fibers recorded in anesthetized rats (39.7 ºC; (Ruan et al., 2005). Whether this discrepancy is due to a different activation threshold of the TRPV1 expressed in pulmonary sensory neurons (Ni et al., 2006) or a different transduction mechanism involved in the sensitizing
effect of hyperthermia on the response to TRPV1 activators remains to be determined.

Lactic acid is produced by anaerobic metabolism such as during strenuous exercise (Erickson et al., 1991), and local tissue acidosis frequently occurs in airway inflammatory and ischemic conditions (Kostikas et al., 2002). It is known that protons are capable of modulating the activity of a number of ion channels expressed on primary afferent sensory nerves, including ASIC channels (Waldmann et al., 1997) and the TRPV1 receptor (Caterina et al., 1997). Analyses of the stimulatory effect of acid on native and cloned TRPV1 receptors suggest that the TRPV1 channel is involved in the sustained response to acid challenge in vivo (Bevan and Geppetti, 1994; Caterina and Julius, 1999). This is further supported by the profoundly reduced responses to acid in cultured DRG neurons after a targeted disruption of the TRPV1 gene (Caterina et al., 2000). Similarly, our recent study (Ni et al., 2006) showed that the acid-evoked current in the majority of pulmonary sensory neurons consists of two components; the transient component is mainly mediated through activation of ASICs, whereas the slow, sustained component is mostly mediated through the TRPV1 channel. Our results in this study further demonstrated that hyperthermia exerted opposite effects on these two components of the acid-induced current in pulmonary sensory neurons (Fig. 3.3A). The inhibitory effect of hyperthermia on the ASICs in pulmonary sensory neurons is in general agreement with observations reported previously (Askwith et al., 2001; Asai et al., 2005), which showed that the proton-gated channel activation was inhibited by increasing temperature in both transfected cells and DRG neurons. Hyperthermia appears to exert an effect on the same site as proton activation on the ASIC channel, because mutation of a conserved residue that determines the channel gating abolished the desensitizing effect of increasing temperature on proton-induced current (Askwith et al., 2001). On the other hand, the potentiating effect of hyperthermia on the TRPV1-mediated response to low pH is consistent with the finding of a positive interaction at the TRPV1 channel between proton and heat (Vyklicky et al., 1999). In addition, more rapid activation and inactivation of the TRPV1-mediated current were noticeable during hyperthermia compared with that at room temperature (e.g., Fig. 3.3A), which was probably due to the faster opening and closing of the TRPV1 channel at a higher temperature; indeed, a similar increase in rate of gating caused by increasing temperature is known to occur in voltage-gated channels (Hille, 2001).

These results clearly indicated the distinct functional roles of these two types of channels in sensing acidity under different temperatures. TRPV1 played a dominant role, whereas ASICs exhibited very little or no response to acid in the physiological range of body temperature in pulmonary sensory neurons. Whether this finding is applicable to sensory neurons (e.g. DRG neurons) innervating peripheral tissue, where the temperature is generally lower than that of the viscera, remains to be determined. Nevertheless, the complete inhibition of ASICs after exceeding 40°C shown in this study may explain, at least in part, the
lack of a significant increase in pulmonary C-fiber sensitivity to lactic acid with increasing temperature reported in anesthetized rats (Ruan et al., 2005).

Our conclusion on the primary role of TRPV1 in the synergistic effect of hyperthermia on the response to TRPV1 chemical activators is further supported by the observation that the same temperature elevation failed to potentiate the response to either ACh or ATP in pulmonary sensory neurons (Fig. 3.4, C and D; Fig. 3.5, C and D). ACh, a primary neurotransmitter of the autonomic nervous system, evoked an inward current in pulmonary sensory neurons via activation of nicotinic ACh receptors (Fig. 3.4, A and B). ATP, in addition to its key role in cellular metabolism, also functions as an active extracellular messenger, producing its effects via the activation of both P2X receptor, a ligand-gated cation channel, and P2Y receptor, a G protein-coupled receptor (Burnstock and Williams, 2000). Our results showed that ATP stimulated pulmonary sensory neurons mainly via activation of P2X receptors (Fig. 3.5, A and B). Neither ACh nor ATP is known to activate TRPV1 directly, and their stimulatory effects on pulmonary sensory neurons were not affected by pre-treatment with capsazepine (Ni and Lee, unpublished data).

The potential of the TRPV1 channel as a signal integrator has attracted much attention in recent years; for example, a potentiating effect of chemical and physical stimuli on the gating of the TRPV1 channel has been suggested to play an important role in certain diseases (Nilius et al., 2007). Furthermore, a number of endogenous inflammatory mediators (e.g. prostaglandin E₂ (PGE₂), bradykinin, acid, etc.) can sensitize TRPV1 during tissue inflammation, which leads to nociceptor hypersensitivity and hyperalgesia (Carr et al., 2003). In pulmonary sensory neurons, the hyperthermic temperature activates TRPV1 and/or other subtypes of TRPV channels (Ni et al., 2006), which in turn can induce the influx of cations, mainly Ca²⁺ and Na⁺ (Montell, 2005). These cations could possibly trigger several cascading events. For example, the modulatory effects of cytosolic Ca²⁺, such as increasing fusion of receptor-containing vesicles to the plasma membrane (Clapham, 2003), may contribute to the hyperthermia-induced sensitizing effects on the response of pulmonary sensory neurons to capsaicin, 2-APB and acid (sustained component) by increasing the density of receptor expression on the cell membrane. In addition, the protein kinase C (PKC)- and protein kinase A (PKA)-mediated phosphorylation of the TRPV1 channel is believed to induce TRPV1 sensitization caused by endogenous inflammatory mediators such as PGE₂ and bradykinin (Premkumar and Ahern, 2000; Gu et al., 2003; Moriyama et al., 2005). Furthermore, a recent study has shown that high temperature (42°C) shifted the TRPV1 channel activation curve (open probability vs. voltage) from a non-physiological positive voltage range towards the negative potential (Nilius et al., 2005). This large shift of voltage-dependent activation curve to a physiologically relevant voltage range with a relatively small gating charge may contribute to not only the hyperthermia-induced hypersensitivity demonstrated in this study, but also the diverse functional properties of TRPV1 (Nilius et al., 2005).
In conclusion, this study showed that increasing temperature within the physiological range potentiated the responses of isolated pulmonary sensory neurons to TRPV1 activators. This potentiating effect was probably mediated through a positive interaction between hyperthermia and chemical activators primarily at the TRPV1 channel. Because tissue hyperthermia and endogenous release of certain chemical activators of the TRPV1 are known to occur concurrently during airway inflammatory reaction, selecting the TRPV1 channel as a potential therapeutic target for treating airway inflammatory diseases should merit further investigations.
3.5 Figures

Figure 3.1 Effect of increasing temperature on the response of vagal pulmonary sensory neurons to capsaicin (Cap). A: experimental records illustrating that both membrane depolarization and number of action potentials evoked by Cap (1 μM; 4 s) were increased in current-clamp mode when the temperature was increased from 36.0 to 40.6°C in a jugular neuron (22.9 pF); the response recovered when the temperature was returned. Vm, membrane potential; Temp., temperature. B: group data for the baseline membrane potential at the two different temperatures: BT, body temperature (35.7 ± 0.09°C); HT, hyperthermic temperature (40.5 ± 0.11°C). C: group data for capsaicin (0.3–1 μM; 1–8 s)-evoked membrane depolarization, ΔVm (Cap), at the two temperatures. D: experimental records illustrating that the Cap (0.3 μM; 2 s)-evoked current was increased when the temperature was increased from 35.8 to 40.6°C in a nodose neuron (23.8 pF) in voltage-clamp mode. E: group data for the Cap (0.3 μM; 2–4 s)-evoked current response (ΔI) at the two different temperatures. *P < 0.05 and **P < 0.01 compared with the corresponding response at BT.
Figure 3.2 Effect of increasing temperature on the response of vagal pulmonary sensory neurons to 2-aminoethoxydiphenyl borate (2-APB). A: experimental records illustrating that the 2-APB (0.3 mM; 3 s)-evoked current was clearly augmented when the temperature was increased from 36.4 to 40.4°C in a jugular neuron (17.0 pF), and the response recovered when the temperature was returned. B: group data for the 2-APB (0.3 mM; 2–8 s)-evoked current response at the two different temperatures: BT, body temperature (36.0 ± 0.10°C); HT, hyperthermic temperature (40.6 ± 0.08°C). C: experimental records illustrating the effect of capsazepine (CPZ; 10 µM; 2 min) pretreatment on the 2-APB (0.3 mM; 3 s)-evoked current at 36.2°C and 40.9°C in a nodose neuron (25.2 pF). D: group data illustrating the effect of CPZ pretreatment on 2-APB (0.3 mM; 2–8 s)-evoked currents at BT (36.1 ± 0.06°C) and HT (40.9 ± 0.07°C). E: experimental records illustrating the effect of AMG 9810 (AMG; 1 µM; 5 min) pretreatment on the 2-APB (0.3 mM; 2 s)-evoked current at 35.7°C and 40.4°C in a jugular neuron (21.4 pF). F: group data illustrating the effect of AMG pretreatment on 2-APB (0.3 mM; 2–6 s)-evoked currents at BT (35.8 ± 0.05°C) and HT (40.4 ± 0.08°C). *P < 0.05 and **P < 0.01 compared with the corresponding current response at BT.
Figure 3.3 Effect of increasing temperature on the response of vagal pulmonary sensory neurons to low pH. 

A: experimental records illustrating the typical acid-induced current exhibiting both rapid, transient and slow, sustained components in a jugular neuron (22.5 pF). Transient component of the acid (pH 5.5; 2 s)-induced current was almost completely inhibited whereas the sustained component was potentiated when the temperature was increased from 24.4 to 36.4°C, and then to 40.2°C; the responses recovered when the temperature was returned. Amiloride (1 mM; 1 min) pretreatment completely blocked the transient component, and the response was recovered after 2 min wash out. Pretreatment with capsazepine (CPZ; 10 µM; 3 min) almost completely blocked the sustained component.

B: experimental records illustrating that the acid (pH 6.5; 6 s)-evoked rapid transient current was completely inhibited when the temperature was increased from 24.1 to 36.0°C in a nodose neuron (27.9 pF).

C: group data for
the acid (pH 6 or 6.5; 1–6 s)-evoked transient current tested at the three temperatures in the order shown in A: RT, room temperature (22.8 ± 0.58°C); BT, body temperature (35.8 ± 0.10°C); HT, hyperthermic temperature (40.6 ± 0.12°C). **P < 0.01*, *P < 0.05 compared with the current response at RT. D: experimental records illustrating that the acid (pH 6.0; 4 s)-evoked slow, sustained current was increased when the temperature was increased from 35.7 to 40.3°C in a nodose neuron (24.6 pF). E: group data for the acid (pH 5.5 or 6.0; 4–6 s)-evoked sustained current response at the two temperatures: BT, 36.0 ± 0.07°C; HT, 40.5 ± 0.08°C. *P < 0.05 compared with the current response at BT.
Figure 3.4 Effect of increasing temperature on the response of vagal pulmonary sensory neurons to acetylcholine (ACh). A: experimental records illustrating that pretreatment with hexamethonium (Hex; 100 µM; 5 min) almost completely abolished the ACh (100 µM; 3 s)-evoked current in a nodose neuron (19.8 pF). B: group data illustrating the effect of Hex (100 µM; 5 min) on the ACh (50 or 100 µM; 2–6 s)-evoked current. Con, control; Rec, recovery. *P < 0.05 compared with control response. C: experimental records illustrating that the ACh (50 µM; 4 s)-evoked current was not changed when the temperature was increased from 36.0 to 40.3°C in a nodose neuron (27.9 pF), and the response remained unchanged when the temperature was returned. D: group data for the ACh (50 or 100 µM; 1–6 s)-evoked current response at the two different temperatures: BT, body temperature (35.9 ± 0.08°C); HT, hyperthermic temperature (40.7 ± 0.08°C). No significant difference (P = 0.27) of the current response was found between the two temperatures.
Figure 3.5 Effect of increasing temperature on the response of vagal pulmonary sensory neurons to adenosine 5'-triphosphate (ATP). A: experimental records illustrating that pretreatment with pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS; 50 µM; 2 min) completely abolished the ATP (0.3 µM; 2 s)-evoked current in a nodose neuron (21.9 pF). Con, control; Rec, recovery. B: group data illustrating the effect of PPADS (30 or 50 µM; 2 min) on ATP (0.3 µM; 2–6 s)-evoked current. *P < 0.01 compared with control response. C: experimental records illustrating that the ATP (0.3 µM; 6 s)-evoked current was not changed when the temperature was increased from 36.2 to 40.6°C in a jugular neuron (12.5 pF), and the response remained unchanged when the temperature was returned. D: group data for the ATP (0.3 µM; 1–6 s)-evoked current response at the two different temperatures: BT, body temperature (35.9 ± 0.08°C); HT, hyperthermic temperature (40.7 ± 0.07°C). No significant difference (P = 0.36) of the current response was found between the two temperatures.
Figure 3.6 Capsaicin-evoked response at body, moderate hyperthermic, and hyperthermic temperatures. A: experimental records illustrating that Cap (0.1 μM; 1 s)-evoked current was increased when temperature was elevated from 35.9 to 37.4, 39.0 and 40.4°C in a jugular neuron (29.9 pF). B: group data for the Cap (0.1 or 0.3 μM; 1–3 s)-evoked current response at the five different temperatures as indicated. *P < 0.05 compared with the current response at 36°C.
Chapter Four: A lack of potentiating effect of increasing temperature on the responses to chemical activators in vagal sensory neurons isolated from TRPV1-null mice

4.1 Introduction

It has been demonstrated that TRPV subtypes 1–4 (TRPV1–4) channels are expressed on the cell bodies of the nodose sensory neurons both in rats (Ni et al., 2006) and in mice (Zhang et al., 2004). The study in Chapter 2 showed that hyperthermia exerts a direct stimulatory effect on pulmonary sensory neurons, and the thermal-sensitive TRPV channels are involved in mediating such effect (Ni et al., 2006). The study in Chapter 3 further revealed a potential role of the TRPV1 channel in mediating the sensitizing effect of hyperthermia on the responses of pulmonary sensory neurons to chemical activators (Ni and Lee, 2008). All these previous studies seem to suggest that TRPV1 plays an important role in the effect of hyperthermia on the function and properties of vagal sensory neurons. However, these findings were based upon results obtained from experiments using the TRPV1 antagonists, of which the specificity have been repeatedly challenged and definitive evidence is still lacking. In view of the background information and unanswered questions, we used TRPV1-null mice as the animal model to assess the specific role of TRPV1 in mediating both the stimulatory and sensitizing effect of hyperthermia on vagal sensory neurons.

4.2 Materials and methods

4.2.1. Labeling vagal pulmonary sensory neurons with Dil

In the preliminary experiments, pulmonary sensory neurons were identified by retrograde labeling from the lungs by using the fluorescent tracer 3,3'-dioctadecylindocarbocyanine (DiI). Young adult C57/BL6 mice (~20 g, the Jackson Laboratory) were anesthetized with continuous inhalation of isoflurane administered by a nose cone connected to a vaporizing machine (AM Bickford Inc., NY, USA). A small (~0.5 cm) mid-line incision was made on the neck skin to expose the trachea with the mouse lying in the supine position and head tilted upwards at ~60°. A needle (30 guage) was inserted into the lumen of the trachea to instill DiI (0.2 mg/ml; 0.025 ml) into the lungs, and the incision was then closed. DiI was initially sonicated, dissolved in ethanol and diluted in saline (1% ethanol vol/vol). Animals were used for the study after 5–7 days to allow the time for the dye to reach the cell body located in the nodose and jugular ganglia. Results obtained from these preliminary experiments showed no detectable difference in the responses between Dil-labeled neurons and those isolated from unlabeled animals, the data collected from both groups were therefore pooled for analysis.
4.2.2. *Isolation of vagal sensory neurons.*

The procedures were almost identical to that described in Chapter 2, section 2.2.2, except the following: nodose/jugular ganglia were desheathed, cut into ~3 pieces, placed in 0.04% type IV collagenase and 0.04% dispase II mixture, and incubated for 1 h in 5% CO$_2$ in air at 37°C. Due to the small size of the mouse ganglion, nodose and jugular ganglia could not be clearly separated, thus sensory neurons from nodose and jugular ganglia were pooled to study.

4.2.3. *Electrophysiology.*

Patch-clamp recordings were performed the same way as described in Chapter 2, section 2.2.3.

4.2.4. *PCR.*

Four TRPV1-/- breeding pairs (purchased from the Jackson Laboratory, Bar Harbor, ME) and two pairs (female and male) of the offspring (one pair each from the two generations) were selected for genotyping. A small piece of tissue punched from the ear was collected from each mouse selected. Tissues were treated with 100 ml 50 mM NaOH solution at 100°C for 1 hour till homogenized. After brief spinning, 50 l of the supernatant was taken and 5 l 1 M Tris (pH 8.0) was added, and then followed by centrifugation at 10,000 rpm. PCR was performed using AccuPrime Taq DNA Polymerase System (Invitrogen) according to the manufacturer’s protocol. The amplification program consisted of an initial denaturation at 94°C for 3 min; 50 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 1 min, and elongation at 72°C for 1 min; and a final 2-min extension at 72°C. The amplification products were analyzed by electrophoresis in 1.5% agarose gels and were detected by ethidium bromide staining.

4.2.5. *Chemical applications.*

Chemical solutions were applied the same way as described in Chapter 2, section 2.2.3 and Chapter 3, section 3.2.2.

4.2.6. *Q$_{10}$ Calculations.*

The temperature coefficient $Q_{10}$ was calculated the same way as described in Chapter 2, section 2.2.3.

4.2.7. *Chemicals.*

All chemicals were obtained and prepared the same way as described in Chapter 2 & 3, sections 2.2.4 & 3.2.3, except for dispase II, which was purchased from Roche (Indianapolis, IN).
4.2.8. Data analysis.

Data were analyzed with a one-way ANOVA analysis, followed by a post hoc Newman-Keuls test. A $P$ value $< 0.05$ was considered significant. Data are presented as mean $\pm$ S.E.M.
4.3 Results

Vagal sensory neurons were chosen by using the same criteria described in Chapter 3, section 3.3. Experimental protocols were completed in a total of 155 and 98 nodose/jugular ganglion neurons of wild type (WT) and TRPV1-null (TRPV1-/-) mice, respectively. The whole cell capacitances of these neurons were in the range of 7.5–35.5 pF (19.2 ± 0.4 pF; \( n = 155 \)) for WT neurons, and 10.2–31.8 pF (18.7 ± 0.6 pF; \( n = 98 \)) for TRPV1-/- neurons.

4.3.1. Genotyping of TRPV1-/- mice colony.

To confirm that TRPV1 gene knock out was maintained through generations of offspring in our breeding colony, PCR experiment was performed. Our results confirmed the absence of the TRPV1 gene expression in the PCR reaction product obtained from the tissue in both breeding pairs and offspring of TRPV1-/- mice (Fig. 4.1). In contrast, the PCR reaction product from WT mice tissue contained the 984 bp fragment corresponding to the TRPV1 allele (Fig. 4.1).

4.3.2. Responses to increase in temperature of vagal sensory neuron isolated from WT and TRPV1-/- mice.

When the temperature of the ECS surrounding the neuron was elevated from RT (~ 23°C) to HT (~ 40.5°C) in ~ 20 s in a ramp pattern, whole cell inward current was evoked in both WT and TRPV1-/- neurons (Fig. 4.2A). The current densities of all vagal neurons tested were 4.26 ± 1.08 and 2.94 ± 0.5 pA/pF for WT (\( n = 66 \)) and TRPV1-/- neurons (\( n = 41 \)), respectively, and no significant difference was found between these two groups of neurons (\( P = 0.37 \)) (Fig. 4.2B).

In our preliminary experiments, only nodose and jugular neurons labeled with DiI (pulmonary sensory neurons) were studied in both WT and TRPV1-/- groups. There was no significant difference in the responses of current density to the same increase in temperature between pulmonary (DiI-labeled) neurons isolated from WT (4.35 ± 0.96 pA/pF) and TRPV1-/- (3.03 ± 0.44 pA/pF) mice (\( P = 0.15 \)) (Fig. 4.2B). Since there was no detectable difference in the results obtained from DiI-labeled neurons and those isolated from unlabeled mice, later experiments were carried out in unlabeled animals, and data collected from these two groups were pooled.

Within the WT group, hyperthermia-induced inward current density in capsaicin-sensitive (Cap+) neurons (5.75 ± 1.78; \( n = 33 \)) was not significantly different from the capsaicin-insensitive (Cap-) group (2.87 ± 0.48; \( n = 32 \)) (\( P = 0.13 \), Fig. 4.2B). \( Q_{10} \) was also measured in order to quantitatively determine the neuron sensitivity to temperature increase; a \( Q_{10} \) value > 3 is considered to be temperature-sensitive (Hille, 2001). There was no significant difference in \( Q_{10} \) in the temperature range of 30–40.5°C between the WT (\( n = 45 \)) and TRPV1-/- (\( n = 29 \)) neurons (\( Q_{10} = 4.52 ± 1.34 \) and 2.43 ± 0.35, respectively; \( P = 0.22 \); Fig. 4.2B).
The lack of difference in peak current response and $Q_{10}$ between WT and TRPV1-/- mice was probably due to the large variability within the WT group, because the $Q_{10}$ value of the hyperthermia-evoked inward current in WT Cap+ neurons ($n = 22$) was significantly higher than that in WT Cap- ($n = 23$) neurons ($Q_{10} = 7.16 \pm 2.59$ and $1.87 \pm 0.23$, respectively; $P < 0.05$; Fig. 4.2B).

4.3.3. Responses to TRPV1 activators of isolated vagal sensory neurons.

Inward current was evoked by capsaicin (0.3 or 1.0 µM; 1–6 s) application in nodose/jugular neurons isolated from WT ($52.2 \pm 9.7$ pA/pF; $n = 29$) mice, but not in those of TRPV1-/- mice ($n = 45$) (Fig. 4.3, A and B). Furthermore, 2-APB (0.3 mM; 6 s)-evoked inward current in WT neurons ($20.14 \pm 4.43$ pA/pF; $n = 49$) was significantly larger than that in TRPV1-/- neurons ($3.98 \pm 0.45$ pA/pF; $n = 45$) ($P < 0.01$; Fig. 4.3, C and D). Very similar responses to both capsaicin and 2-APB were recorded from DiI-labeled neurons in both WT and TRPV1-/- mice (Fig. 4.3, B and D).

Vagal sensory neurons from WT mice were concentration-dependently activated by low pH at different acidity; acid (6 s)-evoked response was significantly augmented when lowering the pH of the ECS: $I = 1.74 \pm 0.79, 4.16 \pm 1.08$ and $13.45 \pm 5.40$ pA/pF at pH = 6.0, 5.5 ($n = 13, P < 0.01$ compared with the response at pH 6.0) and 5.0 ($n = 13, P < 0.01$ compared with the response at pH 6.0), respectively (Fig. 4.4, A and B). However, acid (6 s)-evoked response in TRPV1-/- neurons was not significantly changed with decrease in ECS pH: $I = 0.71 \pm 0.18, 1.47 \pm 0.30$ and $2.61 \pm 0.51$ pA/pF at pH = 6.0 ($n = 24$), 5.5 ($n = 24, P = 0.28$ compared with the response at pH 6.0) and 5.0 ($n = 24, P = 0.16$ compared with the response at pH 6.0), respectively (Fig. 4.4, A and B). In addition, acid (6 s)-evoked response at pH 5.0 in WT neurons ($13.45 \pm 5.40$ pA/pF, $n = 13$) was significantly larger than that in the TRPV1-/- neurons ($2.61 \pm 0.51$ pA/pF, $n = 24$) ($P < 0.05$).

4.3.4. Responses to non-TRPV1 activators of isolated vagal sensory neurons.

To determine whether TRPV1 gene knock out affects cell response to non-TRPV1 activators, we investigated the inward current evoked by ATP and ACh, activators of the P2X/P2Y and nicotinic/muscarinic acetylcholine receptors, respectively. ATP (1 µM; 6 s)-evoked current density was $8.24 \pm 2.8$ ($n = 25$) and $8.24 \pm 2.1$ pA/pF ($n = 34$) in WT and TRPV1-/- mice, respectively, and no significant difference was found between them ($P = 0.72$) (Fig. 4.5, A and B). Similarly, there was no significant difference between the responses to ACh (100 µM; 6 s) in WT ($13.6 \pm 4.5$ pA/pF; $n = 17$) and TRPV1-/- ($10.3 \pm 5.4$ pA/pF; $n = 20$) neurons ($P = 0.69$) (Fig. 4.5, C and D).
4.3.5. Effect of increasing temperature on the responses of vagal sensory neurons to 2-APB.

Our previous study have shown that 2-APB-evoked response was potentiated by increasing in temperature in rat vagal pulmonary sensory neurons (Ni and Lee, 2008). In the present study, we compared the responses of vagal sensory neurons to 2-APB between WT and TRPV1-/- mice at three different temperatures in order to determine: 1) whether hyperthermia has the similar potentiating effect on 2-APB-evoked response in mouse vagal sensory neurons; 2) if so, whether the TRPV1 channel plays a dominant role in mediating such an effect. Our results showed that, in WT neurons, 2-APB (0.3 mM; 6 s)-evoked inward current was clearly augmented when temperature of the ECS surrounding the cells was elevated from RT (22.3 ± 0.11°C) to BT (35.9 ± 0.07°C), and then to HT (40.4 ± 0.05°C); the 2-APB-evoked current density was 9.0 ± 4.2 pA/pF at RT, 16.4 ± 6.1 pA/pF at BT and 50.6 ± 17.7 pA/pF at HT (P < 0.01 and 0.05, respectively, comparing the responses at BT and HT with that at RT), and it recovered when the temperature was returned to RT (Fig. 4.6, A and B). However, in TRPV1-/- mice, neuron response to 2-APB (0.3 mM; 6 s) was not changed when the temperature of the ECS was elevated: 2.7 ± 0.6 pA/pF at RT, 4.0 ± 1.3 pA/pF at BT and 5.0 ± 2.6 pA/pF at HT (P = 0.18 and 0.24, respectively, comparing with the responses at BT and HT with that at RT) (Fig. 4.6, C and D).

4.3.6. Effect of increasing temperature on the responses of vagal sensory neurons to acid.

In rat pulmonary sensory neurons, acid-evoked response has been shown to be mediated through the activation of both ASICs and the TRPV1 channel (Ni et al., 2006), and our previous study showed that hyperthermia exerts a potentiating effect on TRPV1-mediated acid-evoked currents in those neurons (Ni and Lee, 2008). This study series was carried out to determine: 1) whether the TRPV1 channel plays a role in mediating the acid-evoked response in mouse vagal sensory neurons; 2) whether a similar potentiating effect of increasing temperature on this TRPV1-mediated acid-evoked response also exists in mice. When the temperature of the ECS was increased, the acid (pH 5.5; 6 s)-evoked inward current was significantly augmented: 6.2 ± 2.9 pA/pF at RT, 24.0 ± 10.6 pA/pF at BT and 39.5 ± 17.3 pA/pF at HT (both P < 0.05 comparing the responses at BT and HT with that at RT) in WT neurons (Fig. 4.7, A and B), but was not significantly changed in TRPV1-/- neurons: 2.4 ± 0.6 pA/pF at RT, 2.5 ± 0.5 pA/pF at BT and 2.3 ± 0.5 pA/pF at HT (P = 0.45 and P = 0.37, respectively, comparing the responses at BT and HT with that at RT) (Fig. 4.7, C and D).
4.4 Discussion

Results of this study demonstrated that increasing temperature to 40.5°C evoked inward currents in isolated mouse vagal sensory neurons. The whole-cell current densities evoked by the temperature increase were not significantly different between neurons isolated from WT and TRPV1-/- mice, suggesting that the TRPV1 channel does not play an essential role in the response of vagal sensory neurons to hyperthermia. However, the cell response to capsaicin was completely absent, and those to other TRPV1 activators, 2-APB and acid, were distinctly attenuated in TRPV1-/- mice. In contrast, cell responses to non-TRPV1 activators, ATP and ACh, were not different between WT and TRPV1-/- mice. Furthermore, increasing temperature generated a pronounced potentiating effect on the responses of vagal sensory neuron to 2-APB and acid in WT mice, similar to that previously observed in the neurons isolated from rats (Ni and Lee, 2008); in sharp contrast, the same increase in temperature had no effect on the responses of these neurons to the same stimuli in TRPV1-null mice, indicating that the sensitizing effect of hyperthermia on these sensory neurons is almost exclusively mediated through the TRPV1 channel.

It has been reported previously that intrathoracic hyperthermia directly stimulates bronchopulmonary C-fiber sensory terminals (Ruan et al., 2005). A follow-up study on isolated sensory neurons innervating the airways and lungs reveals that the stimulatory effect of hyperthermia on these sensory afferents is mainly mediated through the direct activation, and not due to a secondary effect caused by hyperthermia-evoked release of inflammatory mediators or local changes of tissue mechanics (Ni et al., 2006). Application of a specific TRPV1 antagonist, either capsazepine (CPZ) or (E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylamide (AMG9810), attenuated the hyperthermia-evoked response by 52–77%, indicating that such response is primarily mediated through the TRPV1 channel (Ni et al., 2006). A more definitive conclusion could not be reached in that study because of the uncertainty about the selectivity of these TRPV1 antagonists on other TRPV channels and/or other receptors (McIntyre et al., 2001; Gavva et al., 2004; Roberts and Connor, 2006).

In the current study, we found no significant decrease in hyperthermia-evoked inward current density in vagal sensory neurons isolated from TRPV1-/- mice, as compared to that of the WT mice (Fig. 4.2, A and B). The lack of difference was also seen in Dil-labeled (pulmonary) sensory neurons between WT and TRPV1-/- mice (Fig. 4.2B). This finding was somewhat surprising, and suggested that TRPV1 may not play as a dominant role in mediating the direct stimulatory effect of hyperthermia on these sensory neurons as we previously suggested (Ni et al., 2006). Interestingly, recent studies have also reported that TRPV1 is not essential for the thermal sensitivity of dorsal root ganglion (DRG) sensory neurons; it has been shown that TRPV1 and TRPV2 are not required for nociception of heat (Woodbury et al., 2004). The lack of difference may be due to an extensive overlap of the temperature range among these different TRPVs.
Since temperature activation thresholds of heterologously expressed TRPV3 and TRPV4 are 34–39 and 25–34°C, respectively (Caterina, 2007), whereas the threshold temperature of the hyperthermia-evoked inward current in rat pulmonary sensory neurons is ~ 34.4°C (Ni et al., 2006). It seems also reasonable to speculate that changes in expression and/or sensitivity of other thermal-sensitive TRPV channels and other unidentified receptors in TRPV1-/- mice may have contributed to the lack of significant difference, although to our knowledge there is no existing evidence in support of this possibility. Whether an alteration (either downregulation or upregulation) in gene expression of other TRPV channels exists in vagal sensory neurons of TRPV1-/- mice remains to be determined. Nevertheless, results obtained in the present study clearly show that TRPV1 is not essential for triggering the direct stimulatory effect of hyperthermia on vagal sensory neurons. This finding seems to be in general agreement with a recent report that, although the TRPV1 antagonist induces hyperthermia, the signal essential for thermoregulation is not mediated by the TRPV1 channel (Steiner et al., 2007).

In isolated rat pulmonary sensory neurons, 2-APB-evoked response was attenuated by ~60% after application of CPZ (Gu et al., 2005). In comparison, the response to 2-APB in vagal sensory neurons isolated from TRPV1-/- mice was only <20% of that in WT mice (Fig. 4.3, C and D). This discrepancy in the relative contribution of TRPV1 to the response to 2-APB could be related partially to the fact that the previous study was carried out only in pulmonary sensory neurons, whereas all vagal sensory neurons were tested in this study. However, this possibility seems less likely because the 2-APB-evoked response in TRPV1-/- mice was not significantly different between Dil-labeled (pulmonary) neurons (17.0%) and all vagal neurons tested (19.8%) (Fig. 4.3D). It is also conceivable that CPZ may not be completely effective in blocking the TRPV1-mediated cell response to 2-APB, despite that it completely blocked the stimulatory effect of capsaicin on these neurons. Alternatively, the difference may be related to the species difference (mice vs. rats).

In a recent study (Ni and Lee, 2008), we have reported that the responses of pulmonary sensory neurons to chemical activators of the TRPV1 channel were potentiated by hyperthermia. Furthermore, treatment with CPZ or AMG9810 significantly attenuated but did not abolish this potentiating effect, suggesting an important role of TRPV1 in mediating the sensitizing effect of hyperthermia on pulmonary sensory neurons (Ni and Lee, 2008). In the present study, the potentiating effect of hyperthermia on the responses of these neurons to 2-APB (Fig. 4.6, C and D) and to acid (Fig. 4.7, C and D) were completely abolished in the absence of the TRPV1 channel, clearly indicating an essential role of TRPV1.

It has been suggested that TRPV1 is required for sensitization of nociceptive afferents, especially when an inflammatory reaction is involved. For example, thermal hyperalgesia was impaired in TRPV1-/- mice (Caterina et al., 2000; Nakagawa and Hiura, 2006), and TRPV1 expression is critical for maintenance of
inflammatory hyperalgesia (Ji et al., 2002; Amaya et al., 2003). The possible mechanisms underlying the tissue hyperthermia-induced potentiation of the TRPV1-mediated response are still not clear. However, several possible explanations have been proposed. First, it has been demonstrated that hyperthermia activates protein kinases (PK) C and PKA (Jones and Sorkin, 2005): phosphorylation of the TRPV1 channel by intracellular signaling pathways involving PKC and PKA may lead to the sensitization of the TRPV1 channel (Premkumar and Ahern, 2000; Lee et al., 2005). Further, TRPV channels are known to mediate the transmembrane influx of calcium (Clapham et al., 2001), which is a well-known intracellular second messenger that involves in many signaling pathways. Thus, it is reasonable to expect that influx of Ca^{2+} may trigger the activation of other receptors and/or pathways, which in turn may result in augmented cell response to activators during hyperthermia. In addition, increasing evidence has demonstrated a shift in voltage-dependent gating of TRPV1 channel with increasing temperature (Nilius et al., 2005; Matta and Ahern, 2007), and such shift may directly contribute to the hyperthermia-induced hypersensitivity of the pulmonary sensory neurons. Furthermore, tissue hyperthermia is known to induce the release of inflammatory mediators, such as tumour necrosis factor alpha, prostaglandin E_{2}, etc (Bouchama et al., 1991; Jiang et al., 1999; Roza and Reeh, 2001). The inflammatory mediators-mediated potentiating effect of hyperthermia could be due to an interaction between these mediators and the TRPV1 channel, which in turn sensitizes the TRPV1 channel, and ultimately elicits an augmented response of sensory neurons to TRPV1 activators. Finally, the augmented response under hyperthermic condition may be due to an increased functional expression of the TRPV1 protein on cell surface. Previous studies have reported an increase in TRPV1 expression on sensory neuron somata in rat DRG resulting from either nerve injuries or inflammation, which led to the development of hyperalgesia in those animals (Ma et al., 2005; Kasama et al., 2007; Xu et al., 2007).

In conclusion, results of this study indicate that the TRPV1 channel plays an essential role in the sensitizing effect of hyperthermia on isolated mouse vagal sensory neurons, which lends further support to the suggestion of the unique “integrator” function of the TRPV1 for various physiological stimuli (Nilius et al., 2005). This sensitizing effect may lead to augmented reflex responses to TRPV1 activation when hyperthermia develops under either normal physiological or pathophysiological conditions. However, deletion of TRPV1 does not seem to significantly alter the stimulatory effect of hyperthermia on vagal sensory neurons. Whether the lack of difference was due to an overlap of the temperature ranges between TRPV1 and other TRPV channels, or an overexpression of these other temperature-sensitive TRPVs in TRPV1-/- mice remains to be determined.
4.5 Figures

Figure 4.1 Genotyping of TRPV1 -/- mice breeding colony using PCR. TRPV1-/- mice tissue samples were taken from four breeding pairs as well as two pairs of the offspring (one pair each from the two generations). P, breeding pair; M, male; F, female; L, DNA ladder; WT, wild type; G, generation.
Figure 4.2 Responses to increase in temperature in vagal sensory neurons isolated from wild type (WT) and TRPV1-null (TRPV1−/−) mice. A. experimental record illustrating the inward current evoked by increasing temperature from 25 to 40.5°C evoked in both a WT (21.0 pF) and a TRPV1−/− (16.8 pF) neuron. B. group data for the current density evoked by increasing temperature as well as the derived Q_{10} values in the temperature range of 30–40.5°C for the hyperthermia-evoked inward current in all neurons (All) and pulmonary neurons (DiI-labeled) isolated from either WT or TRPV1−/− mice; the WT neurons were further divided into 2 groups based upon their responses to capsaicin (1 µM): capsaicin-sensitive (Cap+) and capsaicin-insensitive (Cap−) neurons. Number of neurons in each group is as indicated. * P < 0.05, compared between the corresponding groups. C. an example of Arrhenius plot illustrating different Q_{10} values were obtained in two distinct phases of the hyperthermia-evoked current.
Figure 4.3 Responses to Capsaicin and 2-APB in vagal sensory neurons isolated from WT and TRPV1 -/- mice. A. experimental record illustrating capsaicin (Cap; 1 µM; 4 s)-evoked inward current in a WT (25.1 pF), but not in a TRPV1-/- (20.6 pF) neuron. B. group data for the Cap (0.3 or 1 µM; 1–6 s)-evoked current density in WT and TRPV1-/- (-/-) neurons. C. experimental record illustrating 2-aminoethoxydiphenyl borate (2-APB; 0.3 mM; 6 s) evoked inward current in both a WT (18.5 pF) and a TRPV1-/- (20.1 pF) neuron. D. group data for the 2-APB (0.3 mM; 6 s)-evoked current density in all neurons (All) and pulmonary sensory neurons (Dil-labeled) isolated from WT and TRPV1-/- (-/-) mice. * P < 0.05 compared with the corresponding TRPV1-/- group.
Figure 4.4 Responses to non-TRPV1 activators in vagal sensory neurons isolated from WT and TRPV1-/- mice. A. experimental record illustrating inward currents evoked by adenosine 5'-triphosphate (ATP; 1 µM; 6 s) in a WT (21.4 pF) and a TRPV1-/- (22.8 pF) neuron. B. group data for the ATP (1 µM; 6 s)-evoked current density in WT and TRPV1-/- (-/-) neurons. No significant difference ($P = 0.72$) in the current response was found between the WT and TRPV1-/- neurons. C. experimental record illustrating inward currents evoked by acetylcholine (ACh; 100 µM; 6 s) in a WT (33.3 pF) and a TRPV1-/- (31.8 pF) neuron. D. group data for the ACh (100 µM; 6 s)-evoked current density in WT and TRPV1-/- (-/-) neurons. No significant difference ($P = 0.69$) in the current response was found between the WT and TRPV1-/- neurons.
Figure 4.5 Acid-evoked current in vagal sensory neurons isolated from WT and TRPV1 -/- mice. A. representative experimental record showing the responses of vagal sensory neurons to pH of 6.0, 5.5 and 5.0 isolated from WT and TRPV1-/- mice. B. group data for the current density responses of WT and TRPV1-/- neurons to increasing intensity of acidity. Open circle, TRPV1-/- (-/-); closed circle, WT. * P < 0.05 compared with the corresponding response to pH 6.0. † P < 0.05 compared with the response of TRPV1-/- neurons to the same pH challenge.
Figure 4.6 Effect of increasing temperature on response to 2-APB in vagal sensory neurons isolated from WT and TRPV1 -/- mice. A. experimental record illustrating that the 2-APB (0.3 mM; 2 s)-evoked current was augmented in a WT neuron (16.8 pF) when the temperature was increased from 22.8 to 36.0°C, and then to 40.3°C; the responses recovered when the temperature was returned to 36.1°C. B. group data for the 2-APB (0.3 mM; 2–6 s)-evoked current density tested at the temperatures in the order shown in A: RT, room temperature (23.6 ± 0.07°C); BT, body temperature (35.8 ± 0.07°C); HT, hyperthermic temperature (40.4 ± 0.09°C). * P < 0.05 compared with the response at RT. C. experimental record illustrating that the 2-APB (0.3 mM; 2 s)-evoked current was not changed in a TRPV1-/- neuron (19.4 pF) when the temperature was increased. D. group data for the 2-APB (0.3 mM; 2–6 s)-evoked current density tested at the temperatures in the order shown in C: RT (22.9 ± 0.09°C); BT (35.8 ± 0.07°C); HT (40.5 ± 0.06°C). No significant difference of the current response was found between either RT and BT, or BT and HT.
Figure 4.7 Effect of increasing temperature on response to acid in vagal sensory neurons isolated from WT and TRPV1 -/- mice. A. experimental record illustrating that the acid (pH 5.5; 2 s)-evoked current was augmented in a WT neuron (16.8 pF) when the temperature was increased from 22.9 to 35.9°C, and then to 40.5°C; the responses recovered when the temperature was returned. B. group data for the acid (pH 5.5; 2–6 s)-evoked current density tested at the temperatures in the order shown in A: RT (22.2 ± 0.07°C); BT (36.0 ± 0.05°C); HT (40.3 ± 0.08°C). * P < 0.05 compared with the current response at RT. † P < 0.05 compared with the current response at BT. C. experimental record illustrating that the acid (pH 5.5; 6 s)-evoked current was not changed in a TRPV1-/- neuron (23.6 pF) when the temperature was increased. D. group data for the acid (pH 5.5; 6 s)-evoked current density tested at the temperatures in the order shown in C: RT (21.7 ± 0.08°C); BT (35.9 ± 0.06°C); HT (40.4 ± 0.05°C). No significant difference of the current response was found between either RT and BT, or BT and HT.
Chapter Five: Summary of conclusions

Based upon the results obtained from these studies, three major conclusions can be drawn: 1) increasing temperature within the normal physiological range can exert a direct stimulatory effect on rat pulmonary sensory neurons, and this effect is mediated through the activation of TRPV1 as well as other subtypes of TRPV channels; 2) increasing temperature within the physiological range also exerts a potentiating effect on the response to TRPV1 activators in pulmonary sensory neurons, which is probably mediated through a positive interaction between hyperthermia and chemical activators of these neurons at the TRPV1 channel; 3) disruption of the TRPV1 channel does not significantly alter the thermal-sensitivity of nodose/jugular neurons, but eliminates the potentiating effect of increasing temperature on the response of these neurons to non-selective TRPV1 channel activators.

These results helped to improve our understanding of the mechanisms by which bronchopulmonary C-fibers are stimulated and sensitized by hyperthermia reported in the previous study carried out in our lab (Ruan et al., 2005). The increased activity and sensitivity of these C-fibers during hyperthermic stimulation are, at least in part, mediated through the direct action of hyperthermia on the afferent nerves.

We believe that the findings in this dissertation are physiologically relevant because some of the endogenous TRPV1 activators, such as hydrogen ion and certain lipoygenase metabolites of arachidonic acid (Jia and Lee, 2007), may be released in the airway tissue concurrently with an increase in tissue temperature during inflammatory reaction. In addition, the function of TRPV1 as a polymodal transducer for nociceptive stimuli in primary sensory neurons has been well recognized (Caterina and Julius, 2001), and recent studies have presented compelling evidence of an important role of TRPV1 in various symptoms of airway hypersensitivity associated with airway inflammation (Geppetti et al., 2006; Jia and Lee, 2007). For example, an elevated cough sensitivity to TRPV1 activators, such as capsaicin or citric acid aerosol, has been reported in patients with asthma or airway inflammation (O'Connell et al., 1996; Doherty et al., 2000). These studies may also have important pharmacological implications: thermal-sensitive ion channels, particularly the TRPV1 channel, may be an appropriate target for the development of antagonists capable of attenuating the effect of possible hyperthermia-related airway hyperresponsiveness.

There are still many important questions that can not be answered in the present study. Some of the examples are as follows.

1) How the absence of the TRPV1 channel may affect regulation of cardiopulmonary reflex responses under both physiological and pathophysiological conditions is not known. Several studies have suggested that TRPV1 may function as a signal integrator in sensory transductions. However, its
role in the neural regulation of cardiopulmonary function is poorly understood. Studies using the TRPV1-null mouse model can be conducted to answer this question. We have shown that TRPV1 activators such as capsaicin, 2-APB, acid and increasing temperature can activate sensory nerves innervating the lungs and airways in mice. Since the TRPV1 channel are also expressed in other cell types, such as smooth muscle cells, epithelial cells, endothelial cells and so on, how these responses are integrated could be investigated by performing the whole-animal cardiopulmonary reflex study. In addition, the relative contribution of the sensory innervations in the generation of the reflex responses could be further explored. Furthermore, if TRPV1 indeed plays an important role in the cardiopulmonary reflex responses as a signal integrator, reflex responses such as bradycardia, hypotension and apnea will be attenuated or abolished with the absence of the TRPV1 channel. It is expected that deletion of TRPV1 will abolish capsaicin-evoked reflex response and attenuate the hyperthermia-, acid- and 2-APB-evoked responses. This prediction is based upon the fact that capsaicin activates exclusively the TRPV1 receptor, whereas the rest of the three stimuli activate other known receptors in addition to TRPV1; for example, hyperthermia activates TRPV2-4, 2-APB activates TRPV2 and V3, and acid activates ASICs. Since we showed that TRPV1 does not seem to be a dominant player in mediating the hyperthermia-evoked response in pulmonary sensory neurons, so the hyperthermia-evoked reflex response is not expected to be significantly attenuated by the absence of the TRPV1 channel. However, 2-APB- and acid-evoked responses in whole-animals are expected to be significantly attenuated in TRPV1-/- animals, because we have shown in Chapter 4 that the majority of the responses evoked by these two activators were mediated through the TRPV1 channel in mouse pulmonary sensory neurons.

2) The relative contributions of different thermal-sensitive TRPV channels to the thermal sensitivity of pulmonary sensory neurons are also not known; particularly, that of the TRPV3 and TRPV4 channels. The activation temperature of these two channels (34–39°C and 25–34°C for TRPV3 and TRPV4, respectively) are well within the physiological temperature range of the lungs and airways. Studies using TRPV3- and TRPV4-null mouse models could help us to better define their roles. It is expected that deletion of either TRPV3 or TRPV4 may attenuate the hyperthermia-evoked response in pulmonary sensory neurons, because both their operating temperature ranges coincide with the normal body temperature.

3) The possible interactions between these TRPV channels, either through certain intracellular signaling molecules or protein-protein interactions, in the regulation of neuronal response to hyperthermia remain to be explored. Several studies have indicated the direct interactions between subtypes of TRPV channels. For example, TRPV1 and TRPV3 have been suggested to form heteromers: they were demonstrated to be co-expressed heterologously, showed positive co-immunoprecipitation as well as a synergistic functional response to capsaicin stimulation (Smith et al., 2003). Studies using cells co-transfected with
combination of any two different subtypes of TRPV1–4 channels could be used to study the interaction between the two channels. If there is a positive interaction between the two channels, upon hyperthermic stimulation, cells co-transfected with two channels are expected to have greater response than those transfected only with either of the individual channel.

4) The mechanisms by which responses of pulmonary sensory neurons to TRPV1-activators are potentiated by hyperthermia are not known, and the intracellular signaling pathways involving the TRPV1 channel and possibly other receptors remain unclear. It has been shown previously that hyperthermia activates PKC and PKA (Kasama et al., 2007), and several studies have demonstrated that the TRPV1 channel interacts extensively with several intracellular proteins, such as PKA, PKC and phosphatidylinositol-4,5-bisphosphate (PIP2) (Premkumar and Ahern, 2000; Prescott and Julius, 2003; Yajima et al., 2003). Whether such interactions also exist in pulmonary sensory neurons is not known, and whether these interactions play a role in the sensitizing effect of hyperthermia on these neurons merits further investigation. Since many studies have indicated that potentiation of the TRPV1 channel is closely associated with PKC activation, whether hyperthermia phosphorylates PKC in pulmonary sensory neurons should be further investigated. In addition, activators and inhibitors of PKC can be applied to study the involvement of PKC pathway. For example, if the potentiating effect of hyperthermia on TRPV1-mediated response can be attenuated by inhibitors of PKC, such as bisindolylmaleimide (BIM), and enhanced by activators of PKC, such as phorbol 12,13-dibutyrate (PDBu), it is likely that PKC plays an important role in mediating the sensitizing effect of hyperthermia on pulmonary sensory neurons. Furthermore, how PKC interacts with TRPV1 in pulmonary sensory neurons is not clear. It could be either through the direct phosphorylation of TRPV1 by activated PKC or through the indirect interaction with other molecules, such as the removal of PIP2 inhibition on TRPV1, or activation of other signaling molecules which may in turn activate TRPV1.

5) All the studies in this dissertation were performed on cell bodies of cultured neurons. Whether these findings are applicable to the responses of nerve terminals remains to be determined, although extensive and convincing evidence established in our previous studies indicate a close similarity between the in vivo (sensory terminals) and in vitro (neuron soma) responses to physiological and pharmacological stimuli of these pulmonary sensory nerves (Ho et al., 2000).
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PUBLICATIONS

*Journal Articles*


*Abstracts*

