EFFECTS OF LACTIC ACID ON BRONCHOMOTOR TONE IN THE NEWBORN

by

Michael A. Nault

A thesis submitted to the Department of Physiology in conformity with the requirements for the degree of

Master of Science

Queen’s University
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Abstract

Stimulation of the pulmonary C-fibre vanilloid receptor (VR1) with capsaicin (CAPS) evokes reflex bronchoconstriction in both the adult and newborn dog. Subsequent studies have suggested that lactic acid (LA) acts as an endogenous ligand of C-fibre afferents (Lee, Morton and Lundberg, 1996), although preliminary studies suggested LA was unable to evoke bronchoconstriction in the newborn (Ducros and Trippenbach, 1991). We tested the hypotheses that LA behaves as an endogenous C-fibre stimulant in the newborn to cause bronchoconstriction and that LA causes reflex bronchoconstriction via the same pulmonary C-fibre/VR1 receptor mechanism as CAPS. Right heart injection of LA (0.4 mmol·kg⁻¹) caused a significant increase (49 ± 11%) in lung resistance (R_L) that was atropine-sensitive (reduced by 80 ± 11%; P < 0.05). Right heart injections of CAPS (25 µg·kg⁻¹) caused a significant increase (41 ± 8.5%) in R_L that was reduced by 83 ± 5%, (P < 0.01) during infusion of the competitive CAPS-antagonist capsazepine (500 µg·kg⁻¹·min). The CAPS response returned toward the baseline response (71 ± 22%) 15 min post capsazepine infusion. In contrast to CAPS, LA-evoked increases in R_L were unaffected by CAZP (reduced by 16 ± 12%, P > 0.05). We conclude that: 1) C-fibre stimulation with LA causes reflex bronchoconstriction in the newborn dog, 2) CAZP reversibly antagonizes reflex bronchoconstriction elicited by right heart injection of CAPS in the newborn dog, presumably by acting at the C-fibre “capsaicin” receptor and 3) LA-induced reflex bronchoconstriction is not attenuated by CAZP, suggesting that CAPS and LA evoke reflex bronchoconstriction by stimulating
different C-fibre receptor signal transduction mechanisms, or by stimulating different C-fibre subpopulations.
Statement of Co-Authorship

Under the guidance of Dr. J. T. Fisher, Michael A. Nault designed, performed and analyzed the experiments described in this thesis. Original drafts of all text were written by Michael Nault. The finished product includes editorial suggestions by Dr. Fisher.
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<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AP</td>
<td>area postrema</td>
</tr>
<tr>
<td>ASIC</td>
<td>acid-sensing ionic channel</td>
</tr>
<tr>
<td>ASM</td>
<td>airway smooth muscle</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>BPD</td>
<td>bronchopulmonary dysplasia</td>
</tr>
<tr>
<td>CLdyn</td>
<td>dynamic lung compliance</td>
</tr>
<tr>
<td>Ca^2+</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CAPS</td>
<td>capsaicin</td>
</tr>
<tr>
<td>CAZP</td>
<td>capsazepine</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DmnX</td>
<td>dorsal motor nucleus of the vagus</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>ECG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>ELdyn</td>
<td>dynamic lung elastance</td>
</tr>
<tr>
<td>FRC</td>
<td>functional residual capacity</td>
</tr>
<tr>
<td>H⁺</td>
<td>hydrogen ion</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LA</td>
<td>lactic acid</td>
</tr>
<tr>
<td>MX</td>
<td>muscarinic receptor subtype (X = 1, 2, 3, 4, or 5)</td>
</tr>
<tr>
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</tr>
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<td>sodium ion</td>
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<tr>
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<td>sodium chloride</td>
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<td>NANC</td>
<td>non-adrenergic non-cholinergic</td>
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<tr>
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<td>neurokinin receptor subtype (x = 1 or 2)</td>
</tr>
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<tr>
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<td>NTS</td>
<td>nucleus tractus solitarius</td>
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<tr>
<td>P&lt;sub&gt;TP&lt;/sub&gt;</td>
<td>transpulmonary pressure</td>
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<td>arterial partial pressure of oxygen</td>
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<td>phenylbiguanide</td>
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<td>prostaglandins</td>
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<td>prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
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<td>prostaglandin F&lt;sub&gt;2α&lt;/sub&gt;</td>
</tr>
<tr>
<td>R&lt;sub&gt;L&lt;/sub&gt;</td>
<td>inspiratory lung resistance</td>
</tr>
<tr>
<td>RAR</td>
<td>rapidly adapting receptor</td>
</tr>
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SAR  slowly adapting receptor
SOC  store-operated channel
SP   substance P
T_c  core temperature
T_i  inspiratory time
TRP transient receptor potential
TxA_2 thromboxane A_2
\dot{V}  ventilation
VIP vasoactive intestinal peptide
VR1 vanilloid receptor subtype 1
V_T tidal volume

**Units and Symbols**

bpm beats per minute
cmH_2O centimeter of water
hr hour
g gram
kg kilogram
ml milliliter
mmHg millimeter of mercury
mmol millimole
mOsm milliosmole
s  second
°C  degree Celsius
µg  microgram
CHAPTER 1

Introduction
Introduction

The rich innervation to the lungs and its functional significance have long been appreciated. Bartholinus (1663) described nerve branches to human bronchi which were ascribed a role in coordinating the distribution of ventilation by Sir Thomas Willis (1679). Contemporary thought holds that bronchomotor reflexes are of physiological relevance in minimizing dead space, limiting exposure of the sensitive respiratory membranes to noxious stimuli and matching the distribution of ventilation to lung perfusion (Widdicombe, 1963; as reviewed in Coleridge & Coleridge, 1994). Bronchomotor reflexes have not only long been appreciated for their contribution to regulatory and protective reflexes, but were also recognized for their potential role in the pathophysiology of asthma as long ago as 1698 when Floyer suggested that an asthma attack was caused when “nerves are filled with Windy Spirits” (as cited in Barnes, 1986).

At the turn of the century, Brodie (1900) implicated fine bronchopulmonary afferents in the reflex control of airway smooth muscle (as reviewed in Coleridge & Coleridge, 1984). Perhaps the most important class of afferents that affect airway smooth muscle is the C-fibres, which outnumber other receptors 10 to 1 (Coleridge et al., 1989).

Appreciation that the behaviour of at least certain pulmonary afferents differs between the adult and newborn is more recent (see Fisher et al., 1991 for review). Continued development of numerous organs and organ systems extends well into postnatal life, conferring a variable ability of many neonatal physiological systems to mount responses similar to those of the mature system. As such, our understanding of the neural control of airway smooth muscle and the potential therapeutic implications for the
management of respiratory diseases in the adult may not directly address the unique physiology of the newborn.

One potentially important difference between the adult and neonate is the possible discrepancy in the reflex effects of bronchopulmonary C-fibres on airway smooth muscle tone. In the adult dog, activation of pulmonary C-fibres reflexly increases airway resistance, highlighting both the known regulatory, protective and defensive roles of C-fibres, as well as their suspected pathophysiological role in inflammatory lung disease (Barnes, 1986; Coleridge et al., 1989). Identification of C-fibre-mediated reflexes in the newborn has been more controversial and initially raised questions regarding the adaptive benefits of mature C-fibre-mediated reflexes in the neonate (Kalia, 1976; Ducros & Trippenbach, 1991; Haxhiu-Poskurica et al., 1991).

The pungent extract of hot peppers, capsaicin, has proven to be a powerful tool for investigating C-fibre-induced reflexes and receptor recordings. Nevertheless, as capsaicin is not a physiological C-fibre stimulant, our understanding of C-fibre biology continues to progress even as researchers endeavor to identify the endogenous C-fibre "capsaicin receptor" ligand. Among the many candidates for the in vivo agonist, lactic acid is known for its ability to stimulate pulmonary C-fibres and elicit respiratory reflexes in the adult rat (Lee et al., 1996; Hong et al., 1997). Additionally, lactic acid is produced in pathological conditions characterized by increased airway reactivity in both the adult (Canny et al., 1993) and newborn (Abman & Groothius, 1994). The potential contribution of lactic acid to the etiology and/or pathophysiology of neonatal airway disease is accentuated by the metabolic acidosis characteristic of parturition and immediate post-natal life (Koch &
Wendel, 1968; Koch, 1968). Although early studies led to the conclusion that lactic acid was not capable of reflexly increasing airway smooth muscle tone in the newborn, methodological as opposed to physiological limitations may explain this observation. In this thesis, measurements of lung mechanics were used to assess the ability of lactic acid to reflexly alter airway caliber in the newborn and to address the role of lactic acid as an endogenous C-fibre “capsaicin” receptor ligand.

Anatomy and Development

The Tracheobronchial Tree

The primary function of the airways is to provide a pathway between the atmosphere and the gas exchange surfaces of the lung. The airways are organized in a dichotomously branching, tree-like structure with the trunk represented by the trachea; 23-27 branching generations of smaller airways ultimately distribute gas to the gas-exchanging alveoli (Weibel, 1963). In humans, the trachea is characterized by 16-20 C-shaped cartilaginous rings which, although incomplete dorsally, are joined by connective tissue and a band of smooth muscle (Jeffrey, 1998, see below). The first bifurcation of the tracheobronchial tree occurs intrathoracically at the carina, giving rise to two primary or “mainstem” bronchi. The next 3-7 branchings are termed “bronchi” and are morphologically characterized by cartilaginous, highly elastic walls containing little airway smooth muscle (ASM) (Jeffrey, 1998). The lower bronchi give rise to approximately 14 generations of bronchioles, distinguishable from the bronchi on the basis of relatively greater smooth muscle content and the absence of cartilaginous structures. The first 10
generations of bronchioles, in combination with the trachea and bronchi, are termed the “conducting airways” and do not display gas exchange properties. In contrast, the last 4-5 bronchiolar generations are characterized by increasingly frequent alveolar outpouchings which participate in gas exchange and are thus termed “respiratory bronchioles” (Staub & Albertine, 1988). The final generation of bronchioles enters the alveolar ducts, whose walls consist almost exclusively of alveolar outpouchings. The alveolar ducts variably branch an additional 1-2 generations before terminating in alveolar sacs, the principle sites of gas exchange (Staub & Albertine, 1988).

Basic tracheobronchial morphology is complete at birth in most species (human, canine) and no further division of the conducting airways occurs post-natally (Jeffrey, 1998). Canine airways continue to increase uniformly in diameter at a rate proportional to the cube root of body weight until adulthood (Horsfield, 1977). In humans, post-natal respiratory system development includes further alveolarization, which continues into the eighth year of post-natal life (Staub & Albertine, 1988), as well as an increase in anatomical dead space volume which parallels somatic development (Polgar & Weng, 1979).

All airways contained within the confines of the thoracic cavity are termed “intrathoracic airways”. These airways are subject to mechanical deformation as pleural pressure varies during respiration, expiratory manoeuvres, or during defensive airway reflexes (see below). The highly compliant airways of the newborn (Bhutani et al., 1981; Panitch et al., 1989) are especially susceptible to the collapsing forces accompanying forced expiratory manoeuvres (Penn et al., 1988) as well as the distending forces
accompanying positive pressure ventilation, the latter of which are instrumental in the etiology of bronchopulmonary dysplasia (BPD) (Bhutani et al., 1981; Abman & Groothius, 1994).

**Airway Smooth Muscle**

Airway smooth muscle (ASM) distribution varies throughout the lung, generally contributing more to airway wall thickness along the length of the tracheobronchial tree (Jeffrey, 1998). ASM is present in the trachea and large airways as a band of transversely oriented muscle fibres, *trachealis*, which runs along the posterior airway wall, completing the tips of the C-shaped cartilaginous rings (Stephens & Kroeger, 1980). In addition to the typical transverse fibres, human newborn *trachealis* is characterized by intermittent bundles of longitudinally oriented fibres beneath the transverse layer of smooth muscle in an arrangement that is similar to the organization of smooth muscle in the gastrointestinal tract (Hakansson et al., 1976). Despite their presence in the newborn, the functional role of these longitudinal fibres remains unknown.

ASM connects the edges of the cartilaginous plates in the smaller bronchi, forming a distinct layer under the mucosa in the remaining bronchi and bronchioles (Stephens & Kroeger, 1980). This layer is organized in two helices which wind around the airways in both directions (Miller, 1947; Stephens & Kroeger, 1980; Bates & Martin, 1990). ASM is continuous into the smallest bronchioles where characteristic sphincter-like rings of muscle surround the openings to alveolar ducts (von Heyek, 1960). Human neonatal ASM distribution is thought to be comparable to that of the adult in the large airways but
contributes relatively less to the walls in more peripheral airways (Matsuba & Thurlbeck, 1972; Jeffrey, 1998). Furthermore, ASM is differentially distributed between the large intra- and extrapulmonary airways (Ma et al., 1997), with relatively lower distribution in intrapulmonary bronchi possibly conferring protection to these airways by limiting constriction in vivo.

ASM contraction confers stability to the airways (Olsen et al., 1967; Bhutani et al., 1986) and appears to participate in the subtle coordination of ventilation-perfusion matching (Forkert & Fisher, 1992) as well as in playing a supportive role in airway reflexes (see below). In studies using cholinergic agonists to assess airway mechanics, contraction of ASM decreased lung compliance in both the adult and newborn (Bhutani et al., 1986; Penn et al., 1988), thereby conferring stability in the face of supra-atmospheric, collapsing forces. However, the smaller contribution of ASM to the bronchioles of neonates (Jeffrey, 1998) prevents newborn airways from achieving comparable stability to the adult during bronchoconstriction (Matsuba & Thurlbeck, 1972; Jeffrey, 1998). This may account for the increased susceptibility of newborn airway smooth muscle to barotrauma-induced hyperplasia (Bhutani et al., 1981; Panitch et al., 1992).
Innervation

Contemporary experimental techniques have substantially advanced our understanding of sensory innervation and motor control of the airways. Current respiratory biology recognizes bronchopulmonary plasticity and the dynamic role the airways play in an array of regulatory, protective and defensive reflexes (for review see Paintal, 1973; Sant'Ambrogio, 1982; Barnes, 1986; Coleridge et al., 1989; Coleridge & Coleridge, 1994).

Airway innervation arises from both the sympathetic and parasympathetic subdivisions of the autonomic nervous system (ANS). In the lung, nerves subserving the ANS converge to form two distinct neural plexuses: the peribronchial plexus and the periarterial plexus (Laitinen, 1985). Functional characterization of airway innervation identifies efferent and afferent subdivisions.

Efferent Innervation

Traditional concepts of the efferent control of ASM describe contributions from the sympathetic and parasympathetic subdivisions of the ANS. Excitatory innervation to the airways is primarily parasympathetic, within the vagus nerve (X), while adrenergic inhibitory innervation to the airways arises from the cervical sympathetic trunk (Russell, 1980; Barnes, 1986). Additionally, a vagal non-adrenergic non-cholinergic (NANC) system of inhibitory efferent innervation has been described in several species (human, baboon, cat, guinea pig) while vagal cholinergic excitatory innervation is complemented by an additional adrenergic source of excitatory innervation in some species (Russell, 1980;
Figure 1. Innervation of the airways

Schematic representation of airway innervation. Afferent vagal innervation is composed of myelinated axons consisting of rapidly adapting and slowly adapting receptors (RAR’s and SAR’s, respectively) and unmyelinated bronchopulmonary C-fibres. Efferent innervation consists of vagal cholinergic and non-adrenergic, non-cholinergic (NANC) fibers and extra-vagal sympathetic adrenergic fibers. C-fibres ramify widely and are associated with glands, epithelium and vasculature. ACh, acetylcholine; NO, nitric oxide; NE, norepinephrine; VIP, vasoactive intestinal peptide.
VAGAL AFFERENTS

SYMPATHETIC

VAGAL EFFERENTS

Epithelium

Smooth muscle

RAR

C-FIBRE

ACHE
NO
VIP

NE
Excitatory efferent airway innervation consists of preganglionic efferent fibres originating from the nucleus ambiguus (NA) and the dorsal motor nucleus of the vagus (DmnX) which project to ganglia in the airway walls (Barnes, 1986). From these ganglia, short post-ganglionic fibres project to ASM (Barnes, 1986). Acetylcholine (ACh) released from presynaptic and prejunctional fibres mediates its airway effects through pharmacomechanical coupling with muscarinic receptors. Acetylcholinesterase (AChE) staining indicates that cholinergic innervation extends to the level of the terminal bronchioles in dogs (Richardson, 1979) and humans (Laitinen, 1985) with AChE-positive fibres most numerous in the upper bronchi of humans. This characteristic distribution of cholinergic fibres is similar to that in the dog, in which the distribution of AChE-positive motor ganglia decreases with airway size (Laitinen, 1985; Richardson, 1979). To date, 5 muscarinic receptor subtypes have been identified, of which 3 (M1-3) possess bronchopulmonary actions (Fisher et al., 1998).

Activation of post-ganglionic fibres by nicotinic and perhaps also M1 receptor agonists stimulates the release of ACh from pre-junctional terminals (Barnes, 1986; Fisher et al., 1998). Pharmacomechanical coupling of ACh to post-junctional ASM muscarinic subtype 3 (M3) receptors stimulates a cascade of intracellular events which ultimately leads to ASM contraction (Barnes, 1986; Fisher et al., 1998). The remaining airway muscarinic receptor subtype (M2) is pre-junctional and autoinhibitory to ACh release (Barnes, 1986; Maclagan & Barnes, 1989). M2 receptors have also been localized to ASM and have been tentatively identified as inhibitory to contraction (Fisher et al., 1998).
Receptor binding studies suggest that, in neonatal porcine airways, M2 receptor subtype differentiation may be lacking (Haxhiu-Poskurica et al., 1993). This observation is supported by research demonstrating reduced effects of selective blockade of airway M2 receptors in the newborn dog (Fisher et al., 1995). A lack of M2 receptor function may predispose the neonate to airway narrowing (Fisher et al., 1995).

While the primary source of excitatory innervation is parasympathetic, inhibitory airway innervation arises primarily from cervical divisions of the sympathetic nervous system (SNS) (Suzuki et al., 1976; Russell, 1980). Typically, post-ganglionic efferents arising from the stellate ganglia innervate parasympathetic airway ganglia where β-adrenergic sympathetics appear to inhibit parasympathetic transmission (Baker et al., 1983). Although functional sympathetic innervation in human airways is thought to be weak or absent (Widdicombe, 1985; Laitinen, 1989), direct adrenergic innervation of ASM is present in the dog and represents the major source of inhibitory airway innervation (Russell, 1980). In the presence of vagal tone, stimulation of the stellate ganglia produces bronchodilation and reduces the degree of bronchoconstriction during concurrent vagal stimulation (Cabezas et al., 1971). In dogs, pretreatment with β-adrenergic blockers such as propranolol or isoproterenol abolishes the bronchodilatory effects of selective β-adrenergic stimulation (Barnes, 1986). In contrast, β-adrenergic blockers do not increase resting airway resistance in healthy adult humans (Nadel & Barnes, 1984) suggesting resting sympathetic efferent tone is not present. In contrast, β-blockade in asthmatics increases airway resistance, unmasking the presence of significant constrictor tone (Barnes, 1986).
While predominantly thought of as inhibitory, the role of adrenergic innervation to the airways has also received attention for the potential role that α-adrenoreceptors may play in the airway hyperreactivity of asthma (Barnes, 1986). In the adult, contractile effects of α-adrenergic stimulation on smooth muscle can be demonstrated only under conditions of aggressive β-blockade (Barnes, 1986; Waldron & Fisher, 1991). In contrast, epinephrine or norepinephrine (mixed α and β receptor agonists) evoke tracheal smooth muscle contraction in the neonate, highlighting what appears to be a greater α-adrenergic receptor density in neonatal ASM (Waldron & Fisher, 1991). To date, this contraction has only been demonstrated directly in newborn canine airways although it has been suggested to be present in human newborns (Koslo et al., 1986).

In addition to inhibitory β-adrenergic control, the airways are innervated by non-adrenergic non-cholinergic (NANC) inhibitory nerves. Evidence for NANC innervation was initially based on the observation that preconstricted human airway strips are relaxed by a neural mechanism that is insensitive to β-blockade with propranolol (Richardson & Beland, 1976). NANC airway innervation is now recognized to be present across several species including guinea pig (Coburn & Tomita, 1973), cat (Irvin et al., 1980) and baboon (Middendorf & Russell, 1980) and has been suggested to be the only direct functional neural bronchodilator pathway in humans. The principle NANC neurotransmitter is nitric oxide (NO) (Barnes, 1995) although vasoactive intestinal peptide (VIP) is also a NANC neurotransmitter and appears to be colocalized with ACh (Waldron & Fisher, 1991). NANC innervation is functional in the neonatal gut (summarized in Waldron & Fisher, 1991) and has also been demonstrated in newborn cat airways (Waldron et al., 1989).
Although reflex recruitment of NANC has been demonstrated in the human adult, evidence for reflex activation of NANC in the newborn rests solely on studies in the guinea pig (Waldron & Fisher, 1991).

Afferent Innervation

The lungs are richly innervated with sensory afferents which provide the CNS with information regarding respiratory system status and initiate a number of bronchopulmonary reflexes. Both myelinated and unmyelinated lung afferents run in the vagus nerve, relaying information from airways and parenchymal tissue to the nucleus tractus solitarius (NTS) (Kubin & Davies, 1995). Bronchopulmonary afferents are classified on the basis of three different receptor types: slowly adapting or pulmonary stretch receptors (SAR), rapidly adapting irritant receptors (RAR) and unmyelinated bronchopulmonary C-fibres.

SAR are large myelinated vagal afferents (conduction velocities ranging from 14-59 m·s⁻¹; Sant'Ambrogio, 1982) and comprise the majority of myelinated pulmonary afferents. Terminal SAR arborizations are associated with ASM (as reviewed in Coleridge et al., 1989) where they appear to be bound to connective tissue elements. Stimulation of SAR afferents mediates the Heuring-Breuer inflation inhibitory reflex which plays a major role in the resting breathing patterns of anesthetized animals and, to a lesser extent, adult humans (Coleridge et al., 1989). SAR inhibit inspiration in response to circumferential airway tension while their reflex effect on airway smooth muscle is bronchodilation (Coleridge et al., 1989). SAR are insensitive to most forms of chemical stimulation. In
particular the chemicals used extensively to investigate C-fibre neurobiology (bradykinin, prostaglandin, capsaicin, phenylbiguanide; see below) have virtually no direct effect on SAR (Coleridge et al., 1989). In contrast to the adult, SAR display little or no activity at FRC in newborns (Fisher et al., 1983; Sant'Ambrogio, 1987). Despite this, human neonates possess a Heuring-Breuer reflex similar to that in anesthetized animals (Cross et al., 1976).

Like their larger counterparts, RAR are small myelinated vagal axons with average conduction velocities of 16-37 m·s⁻¹ (Sant'Ambrogio, 1987). RAR terminal arborizations are located between columnar cells in the airway epithelium, where they respond primarily to changes in transpulmonary pressure and are responsible for evoking augmented inspiratory breaths (sighs) (Coleridge et al., 1989). RAR, so named for their rapid adaptation to maintained lung inflation, also respond to decreased lung compliance, pulmonary edema and histamine release (Coleridge et al., 1989). RAR are stimulated by a variety of chemicals with bronchoconstrictor actions (histamine, serotonin, prostaglandins E₂ and F₂α and muscarinic agonists) (Mills et al., 1969; Coleridge et al., 1989). Furthermore, they play a significant role in defensive airway reflexes (see below), mediating cough and reflex bronchoconstriction. In the newborn, RAR contribute less to the overall afferent receptor population than in the adult (Fisher et al., 1983; Sant'Ambrogio, 1987).

Despite the significant role played by myelinated vagal afferents in airway reflexes, unmyelinated bronchopulmonary vagal fibres outnumber their myelinated counterparts by as much as 10 to 1 in some species (Waldron & Fisher, 1991). These sensory afferents
contribute to the C-wave of the compound action potential and are thus termed C-fibres (average conduction velocities ranging from 0.9-2.3 m·s⁻¹; Coleridge et al., 1989). C-fibre afferents innervate all levels of the lower respiratory tract and ramify extensively in airway epithelium and subepithelial structures (Coleridge et al., 1989). Lung C-fibres were initially thought to be juxta-alveolar capillary in location due to their stimulation by pulmonary edema (Paintal, 1973). Termed “J-receptors”, Paintal initially proposed that these afferents served as interstitial stretch receptors (Paintal, 1973); however, lung C-fibres were subsequently classified as either pulmonary or bronchial on the basis of vascular accessibility and response to injected stimuli (Coleridge et al., 1989). Pulmonary C-fibres are preferentially stimulated by injection of noxious stimuli (capsaicin, phenylbiguanide) into the right atrium or pulmonary artery with latencies of 1-2 s. Bronchial C-fibres are activated by injection of capsaicin into the right heart with latencies of 6-9 s and with latencies of 3-5 s by left atrial injections of capsaicin. Pulmonary and bronchial C-fibre populations differ subtly in their response to numerous stimuli, including autocoids, hyperinflation and certain chemicals (Coleridge et al., 1989).

Pulmonary C-fibres are stimulated by hyperinflation (2-3 times tidal volume) and do not respond to most autocoids, displaying only weak responses to high doses of prostaglandins (specifically PGE₂). In contrast, bronchial C-fibres are strongly stimulated by prostaglandin, bradykinin and histamine but display weak discharge to hyperinflation up to 3-4 times V̇̇̇R.

The C-fibre terminals of certain species, most notably the rodents, exhibit high immunoreactivity to substance P (SP), neurokinin A (NKA) and calcitonin gene related
peptide (CGRP) (Lundberg & Saria, 1987; Lundberg, 1995; Spina & Page, 1996). High performance liquid chromatography has localized SP to human airway tissue while CGRP and NKA, as well as other neuropeptides (neuropeptide Y), are co-localized in human airway sensory nerves (Spina & Page, 1996). These sensory neuropeptides presumably act as trophic factors or neuromodulators (Lundberg, 1995) but are also potent effectors of neurogenic inflammation, a condition characterized by vascular leak, edema, infiltration of inflammatory cells and, in the airways, bronchoconstriction (Barnes, 1986; Lundberg & Saria, 1987). The tachykinins (SP, NKA) effect their responses by activation of neurokinin (NK) receptors. SP is preferential for neurokinin subtype 1 receptors (NK₁) and is primarily responsible for vascular leak, while activation of neurokinin subtype 2 (NK₂) receptors with NKA mediates bronchoconstriction (Lundberg & Saria, 1987). The role of tachykinins in neonatal ASM reflex control differs from those of the adult; SP-induced modulation of ACh release increases with post natal age in rabbits (Grunstein et al., 1984). Furthermore, Murphy et al. (1994) demonstrated that dry gas hyperpnea-induced bronchoconstriction was markedly diminished in newborn guinea pigs despite adequate airway contractility and sensorimotor function (Murphy et al., 1994).

In the past 100 years, a host of stimuli have been described which activate bronchopulmonary C-fibres. While early descriptions tied the activation of bronchopulmonary C-fibres only to pulmonary edema (Paintal, 1973), contemporary understanding of bronchopulmonary C-fibres couples their stimulation to a much broader category of stimuli; those evoking protective and defensive airway reflexes.
Airway Reflexes

Protective and Defensive Reflexes

Comroe (1954) classified bronchopulmonary reflexes as either regulatory or protective/defensive (as reviewed in Coleridge & Coleridge, 1994). He defined regulatory reflexes as those subject to “continuous neural input from low threshold afferents which mediate events tightly centered around a control setpoint.”. These reflexes typically exert continuous control over respiratory and cardiovascular homeostasis. In contrast, reflexes characterized as protective and defensive are evoked by stimuli that threaten lung function. Such reflexes are involved both in limiting exposure of the sensitive respiratory membranes to noxious stimuli (protective reflexes) and in the active expulsion of particulate irritants from the airway lumen (defensive reflexes) (as reviewed in Coleridge & Coleridge, 1994).

Cough, a forced expiratory maneuver serving to dislodge mucus and particulate irritants entrapped within with high flow, is the principle defensive airway reflex (Barnes, 1986; Karlsson et al., 1988; Coleridge & Coleridge, 1994). Reflex bronchoconstriction invariably accompanies the cough reflex, playing a key role in enhancing its effectiveness by establishing turbulent flow in lower airways and increasing laminar airflow velocity in the larger conducting airways (Coleridge et al., 1989). Turbulent flow favours the deposition of particulate pollutants in the lower airway mucus lining while high flow rates increase impaction of particulate irritants in the airway mucus lining at sites of upper airway bifurcation (Karlsson et al., 1988). In addition to its complementary role in enhancing the expulsive effectiveness of cough, reflex bronchoconstriction also serves to
stabilize the airways and maintain airway patency during the explosive, collapsing forces accompanying cough (Karlsson et al., 1988).

Where cough serves to remove inhaled irritants from the respiratory mucosa, apnea, laryngeal narrowing and bronchoconstriction are reflexes which limit entry of particulate irritants into the airway lumen. In this way, reflex bronchoconstriction can act independently of cough to manifest as a protective airway reflex (Karlsson et al., 1988; Coleridge & Coleridge, 1994). Despite the autonomous and supportive roles reflex bronchoconstriction plays in airway reflexes, reflex bronchoconstriction is best appreciated for its role in the pulmonary chemoreflex. Initially described by Brodie (1900), and later named by Dawes and Comroe (1954), the pulmonary chemoreflex consists of an initial triad of apnea, bradycardia and hypotension that is accompanied by tachypnea and reflex bronchoconstriction (as reviewed in Coleridge et al., 1989; Coleridge & Coleridge, 1994). Pulmonary extravasation and tracheal mucous gland secretion have also been variably described in this reflex (Coleridge & Coleridge, 1994). The pulmonary chemoreflex is not only triggered by inhalation of exogenous irritants such as ozone, cigarette smoke or sulphur dioxide (Coleridge et al., 1989; Lee & Lundberg, 1994), but also by locally released autacoids or introduction of various chemical stimulants into the pulmonary circulation, including phenylbiguanide (PBG) and capsaicin (Coleridge et al., 1989; Coleridge & Coleridge, 1994).

Capsaicin, the pungent extract of hot peppers, is a specific stimulant of pulmonary C-fibres (Holzer, 1991) and has been used extensively to investigate the role of pulmonary C-fibres in the reflex control of ASM. Capsaicin evokes the characteristic
cardiorespiratory depression typical of the pulmonary chemoreflex in guinea pigs (Lundberg & Saria, 1982; Biggs & Goel, 1985), dogs (Coleridge et al., 1989; Coleridge & Coleridge, 1994) and monkeys (Ravi & Singh, 1996). Capsaicin mediates its effects on bronchomotor tone via two independent mechanisms: a central cholinergic reflex and a peripheral "axon" reflex (Coleridge et al., 1989). The relative importance of each mechanism varies remarkably between species.

C-Fibre-Mediated Bronchomotor Reflexes

In dogs, inhalation or vascular delivery of capsaicin stimulates pulmonary C-fibres (Coleridge et al., 1989; Coleridge & Coleridge, 1994) and evokes an atropine-sensitive reflex bronchoconstriction (Coleridge et al., 1989) that is abolished by bilateral vagotomy or vagal cooling to block C-fibres (Coleridge et al., 1989). Anterograde degeneration studies, amino acid autoradiography and horseradish peroxidase (HRP) histochemistry have identified the primary vagal afferent termination sites as the nucleus tractus solitarius (NTS) and adjacent area postrema (AP) (Kubin & Davies, 1995). The projections of second order neurons ultimately synapse at the DmnX and NA (Kubin & Davies, 1995) where they stimulate vagal cholinergic efferents to release ACh at airway ganglia. While this central cholinergic mechanism describes the reflex activation of ASM in the dog, C-fibre stimulation in rodents evokes reflex bronchoconstriction through a different mechanism.

In the guinea pig, activation of pulmonary C-fibres stimulates the release of the bronchoactive tachykinins NKA and SP as well as the sensory neuropeptide CGRP from
C-fibre axon collaterals (Fox et al., 1993; Lundberg, 1995; Coleridge et al., 1989). Release of these neuropeptides initiates a cascade of responses including vascular leak, mucus hypersecretion and, in the airways, bronchoconstriction which are collectively termed neurogenic inflammation (Barnes, 1986; Coleridge et al., 1989; Lundberg, 1995). NKA, SP and CGRP are potent activators of ASM (Lundberg & Saria, 1982; Barnes, 1986; Biggs & Goel, 1985; Karlsson et al., 1988). Although other rodents, such as rats, display tachykinin innervation, the magnitude of its effects on airway reflexes is much weaker than in guinea pigs.

Several experimental observations support a potential role for axon reflex-mediated increases in ASM tone in the pathophysiology of human inflammatory lung disease. The relative inability of cholinergic antagonists to reduce non-specific airway hyperreactivity (Barnes, 1986), the localization of CGRP, SP and NKA in pulmonary C-fibre afferent terminals (Lundberg & Saria, 1987; Coleridge & Coleridge, 1984), and the effectiveness of SP and CGRP to excite human bronchial smooth muscle in vitro (Saria et al., 1985; Lundberg et al., 1984), support a role for axon reflex regulation of ASM in humans. Furthermore, increased CGRP-like immunoreactivity in the epithelial layer of infants suffering from BPD (Johnson et al., 1988; Johnson & Wobken, 1987) implies a role for the axon reflex in human inflammatory lung disease (Ghatei et al., 1987; Lundberg et al., 1984; Barnes, 1986). However, apart from identification of SP involvement in neurogenic inflammation in the rat (Baluk et al., 1992), responses of the axon reflex type have not been identified in animals other than rodents, nor do SP or CGRP appear to evoke reflex bronchoconstriction in humans in vivo (Lundberg & Saria, 1987). Thus the
relative contribution of this mechanism to human bronchomotor reflexes remains unclear. In contrast, others have challenged the emphasis on the role of tachykinins in reflex bronchoconstriction. Coleridge *et al.* (1989) argue that cholinergic reflex control of airway smooth muscle remains an important contributor in larger species including humans and indeed may be predominant.

Despite elucidation of C-fibre-mediated reflexes in the adult, early attempts to identify the existence of mature C-fibre-mediated respiratory chemoreflexes in the newborn were inconclusive. Kalia (1976) reported that the characteristic ventilatory response evoked by C-fibre stimulation with right atrial injection of PBG was weak or absent in 1-6 d old kittens. Additionally, initial attempts to quantify bronchomotor competence in newborns of other species (pig, rabbit) suggested that bronchoconstrictor reflexes characteristic of the adult were not present in the neonate (Ducros & Trippenbach, 1991; Haxhiu-Poskurica *et al.*, 1991). Using measurements of isometric tracheal tension *in situ* from segments of extrathoracic trachea, Haxhiu-Poskurica *et al.* (1991) were unable to elicit a bronchomotor response to presumptive C-fibre stimulation with capsaicin in newborn piglets. This apparent absence of mature bronchoconstrictor reflexes in the newborn was initially interpreted as beneficial since the highly compliant airways of the newborn would be at an increased risk of collapse (Bhutani *et al.*, 1986). On the other hand, it was argued that the absence of bronchoconstrictor reflexes in the newborn could be detrimental if reflex bronchoconstriction was designed to stabilize the airways (Olsen *et al.*, 1967) or reduce dead space (Widdicombe, 1963) during rapid shallow breathing and forced expiratory maneuvers.
Radiographic studies have demonstrated that the magnitude of airway narrowing varies with respect to airway caliber (Cabezas et al., 1971) such that, in the adult dog and pig, small airways (1-1.5 mm diameter) exhibit relatively greater narrowing than do larger airway such as the main stem bronchi and trachea (Cabezas et al., 1971; Murphy et al., 1991). This suggests that the inability of Haxhiu-Poskurica et al. (1991) to identify a bronchomotor response of the trachea to presumptive C-fibre stimulation in newborn pigs does not preclude the existence of the mature reflex. Indeed, others have since shown that the newborn does mount a competent bronchomotor response to presumptive C-fibre stimulation. Using the method of LaFramboise et al. (1993) to measure pulmonary resistance, Anderson and Fisher (1993) demonstrated that capsaicin causes a robust, atropine-sensitive increase in lung resistance in the newborn dog and pig comparable to that evoked by capsaicin in the adult (Coleridge et al., 1982). Thus recent data indicate that presumptive C-fibre stimulation with capsaicin evokes reflex bronchoconstriction in the newborn.

The Capsaicin Receptor

Recent developments in our understanding of the molecular mechanisms activating C-fibre afferents and the development of capsaicin antagonists have significantly enhanced our ability to study the physiologic mechanisms activating pulmonary C-fibres. The selectivity of capsaicin for thin sensory nerves, combined with the similarity of the reflex effects evoked by structural capsaicin analogues (resiniferatoxin, mustard oil), suggests a specific capsaicin recognition site on C-fibre endings. Using ion flux and patch clamp
studies, this putative "capsaicin receptor" was initially identified as a relatively non-selective cation conductance permeable to both mono- and divalent cations (Marsh et al., 1987, for review see Holzer, 1991). Due to the high specificity of the capsaicin receptor for vanilloid compounds the putative capsaicin receptor was termed "vanilloid receptor" (Szallasi et al., 1995). The vanilloid receptor has recently been cloned from DRG neurons and identified as a non-specific cation conductance preferential for Ca\(^{2+}\) (Caterina et al., 1997). Dubbed vanilloid receptor subtype 1 (VR1), it is structurally related to the *Drosophila* retinal TRP gene, a putative store-operated channel (SOC) that appears to mediate extracellular Ca\(^{2+}\) entry in response to depletion of intracellular Ca\(^{2+}\) stores (Clapham, 1996).

Despite molecular, pharmacological and biophysical characterization of VR1, its endogenous ligand has not been identified. Capsaicin stimulates neurogenic inflammation (see above) and evokes C-fibre-mediated reflex bronchoconstriction, both conditions characterizing inflammatory lung disease (Barnes, 1986; Canny et al., 1993; Abman & Groothuis, 1994). As such, the metabolic byproducts of inflammation (H\(^+\), lactic acid) as well as inflammatory mediators themselves (histamine, serotonin, prostanoids) have been suggested as potential endogenous VR1 ligands (Bevan & Geppetti, 1994; Lee et al., 1996; Hong et al., 1997; Stahl & Longhurst, 1992).

Early evidence favouring H\(^+\) as an endogenous VR1 ligand was based on the finding that ischemically sensitive afferents and small, unmyelinated DRG neurons are activated by acidic solutions (Stahl & Longhurst, 1992; Fox et al., 1995; Lou & Lundberg, 1992; Bevan & Geppetti, 1994). Furthermore, extracellular acidification at
pathophysiological concentrations excites cutaneous nociceptors in both rats (Steen et al., 1996; Steen et al., 1995) and humans (Steen et al., 1996; Steen et al., 1995) and lactic acid can establish comparable acidification at sites of tissue ischemia and inflammation in vivo (Hong et al., 1997). An endogenous source of H⁺, lactic acid evokes the pulmonary chemoreflex (Lee et al., 1996) and activates bronchopulmonary C-fibres (Trenchard, 1986a; Trenchard, 1986b; Lee et al., 1996; Hong et al., 1997). Furthermore, while H⁺ is a potent activator of C-fibres and mediator of the pulmonary chemoreflex, the reflex effects and C-fibre activation evoked by H⁺ are potentiated in the presence of the lactate anion (Lee et al., 1996; Stahl & Longhurst, 1992). However, the lactate anion itself does not appear to increase C-fibre activity, nor evoke the characteristic cardiorespiratory depression that accompanies pulmonary C-fibre activation (Trenchard, 1986b; Hong et al., 1997). Acid-evoked reflex responses are abolished by perineural capsaicin treatment to selectively block C-fibres (Lou & Lundberg, 1992; Lee et al., 1996) and blockade of the capsaicin receptor inhibits the acid-induced pulmonary chemoreflex in vivo (Lee & Lundberg, 1994) and reduces acid-evoked C-fibre activity in vitro (Urban & Dray, 1991; Fox et al., 1995; see below). Combined with the finding that H⁺ inhibits the binding of the capsaicin analogue resiniferatoxin to vanilloid receptors in vitro (Szallasi et al., 1995) the above mentioned studies imply that H⁺ and capsaicin activate common C-fibre populations, possibly through a common receptor transduction mechanism.

Despite identification of LA's role as a C-fibre stimulant and mediator of the pulmonary chemoreflex in the adult, there is no direct evidence that LA-induced C-fibre stimulation evokes reflex bronchoconstriction in either the adult or the newborn.
Although newborns display a capsaicin-induced reflex bronchoconstriction attributed to stimulation of pulmonary C-fibre afferents (Anderson & Fisher, 1993), using dynamic lung compliance (C_{dyn}) as an index of bronchoconstriction, others have shown that lactic acid does not evoke bronchomotor responses in newborn rabbits (Ducros & Trippenbach, 1991). However, lung compliance is an indirect index of bronchoconstriction and may be insensitive to small changes in smooth muscle tone or resistance (Widdicombe & Nadel, 1963). Given these considerations, the evidence for the ability of lactic acid to evoke reflex bronchoconstriction in the newborn remains controversial.

Development of the competitive capsaicin antagonist capsazepine\(^1\) provided an experimental means with which to investigate capsaicin-evoked, C-fibre-mediated reflexes as well as those attributed to other substances, including lactic acid (Dickenson & Dray, 1991; Bevan \textit{et al.}, 1992; Belvisi \textit{et al.}, 1992; Urban & Dray, 1991; Lou & Lundberg, 1992). Capsazepine reversibly attenuates or abolishes the pattern of breathing response and C-fibre activity evoked by capsaicin in the adult rat (Lee & Lundberg, 1994) and guinea pig (Fox \textit{et al.}, 1995). Capsazepine also antagonizes the reflex contraction of smooth muscle, inhibiting the contractile response evoked by capsaicin in isolated rat vas deferens (Maggi \textit{et al.}, 1993). Biophysical studies demonstrate that capsazepine reversibly inhibits capsaicin- and resiniferatoxin-induced Ca\(^{2+}\) uptake in rat DRG and reduces or abolishes the inward current response to capsaicin of voltage-clamped rat DRG neurons (Bevan \textit{et al.}, 1992). In addition, capsazepine reversibly antagonizes the inward cation current evoked by capsaicin in \textit{Xenopus} oocytes transfected with VR1 (Caterina \textit{et al.}... \footnote{\(2\)-[2-(4-chlorophenyl)ethylaminothiocarbonyl]-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-2-benzazepine)}
al., 1997). In addition to capsaizepine, which is a competitive capsaicin-antagonist, the inorganic dye ruthenium red antagonizes the reflex effects of capsaicin in a complex, non-competitive manner (Maggi et al., 1993).

Studies of capsaicin and lactic acid-induced apnea, bradycardia and hypotension, as well as C-fibre recordings (Lee & Lundberg, 1994; Lee et al., 1996), suggest that capsaizepine abolishes both the capsaicin- and possible lactic acid-evoked bronchomotor responses, indicating a common capsaicin and lactic acid mechanism of C-fibre activation. In contrast, biophysical studies suggest that H⁺ per se is not a strong agonist of the VR1 receptor (Caterina et al., 1997) and favour the hypothesis that lactic acid activates an alternate C-fibre receptor or stimulates the release of an intermediary endogenous C-fibre ligand to mediate its potential bronchomotor effects (Karla et al., 1992; Shams et al., 1988; Vyklicky et al., 1998). Discovery of a proton-gated Na⁺ channel localized to capsaicin-sensitive frog and rat DRG neurons (Akaike et al., 1990; Bevan & Yeats, 1991) supports the hypothesis that lactic acid and/or H⁺ mediate C-fibre reflex effects by activating a receptor signal transduction mechanism independent of VR1. Additional evidence supporting separate pulmonary C-fibre receptor mechanisms involved in capsaicin and lactic acid sensing comes from the recent cloning of an acid-sensing ionic channel (ASIC) from Xenopus DRG (Waldmann et al., 1997; Waldmann et al., 1997).

Unlike direct C-fibre stimulation with H⁺, acid-induced release of an intermediary endogenous C-fibre/VR1 receptor ligand, likely an inflammatory mediator (Steen et al., 1995), is an alternate hypothesis. Slow infusion of HCl stimulates platelets to release thromboxane A₂ (TxA₂), an inflammatory mediator produced in the cyclooxygenase
pathway of arachadonic acid metabolism (Campbell, 1990). TxA₂ is a potent C-fibre stimulant (Shams et al., 1988), and additional inflammatory mediators produced in the cyclooxygenase pathway (prostaglandins E₂ (PGE₂) and F₂α (PGF₂α)) also stimulate unmyelinated sensory afferents and/or potentiate the effects of other C-fibre stimulants (Coleridge et al., 1978; Lee & Morton, 1995; Steen et al., 1996; Steen et al., 1995). Recent evidence that a cocktail of inflammatory mediators (PGE₂, bradykinin and 5-HT) potentiates the inward cation current evoked by low pH in cultured newborn rat sensory neurons (Steen et al., 1996; Steen et al., 1995) supports this hypothesis. However, application of these inflammatory mediators alone at physiological pH does not induce an inward cation current in these cells. The potentiating effects of these inflammatory mediators on the current response evoked by extracellular acidosis is blocked by capsazepine (Vyklicky et al., 1998) and the potentiating effect of acidic pH on the inflammatory mediator-evoked current in sensory neurons is similar to that evoked by acid pH during submaximal capsaicin application (Caterina et al., 1997). Based on these observations, inflammatory mediators such as PGE₂ may represent endogenous ligands for the capsaicin or VR1 receptor (Vyklicky et al., 1998).
Purpose

The lack of consensus regarding the influence of lactic acidosis on the reflex control of ASM tone in the newborn is apparent. The potential role of lactic acid and H⁺ is particularly relevant in the newborn since metabolic acidosis is a normal component of parturition (Koch & Wendel, 1968; Koch, 1968) as well as an important constituent of neonatal inflammatory lung disease (Penn et al., 1988; Abman & Groothius, 1994). In addition to questions concerning the actions of lactic acid on ASM, biophysical studies and in vivo electrophysiological investigations raise questions regarding the mechanism of action of lactic acid on pulmonary C-fibre receptors.

We tested two hypotheses regarding the actions of lactic acid on bronchomotor tone in the newborn: 1) lactic acid behaves as an endogenous C-fibre stimulant which evokes atropine-sensitive reflex bronchoconstriction; and 2) lactic acid acts via the same capsaicin/VR1 C-fibre receptor mechanism to cause reflex bronchoconstriction.
CHAPTER 2

Methods
Preparation

Surgery

All experimental procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee. Experiments were performed on 38 newborn dogs ranging in age from 1 to 13 d (mean 5.1 ± 0.4 d) and ranging in weight from 256 to 1,063 g (mean 569 ± 29.6 g). Animals were anesthetized with an initial i.p. injection of chloralose (75-100 mg·kg⁻¹) and urethane (0.75-1.0 g·kg⁻¹) and supplemental anesthesia was administered intravenously at approximately 1 h intervals to maintain abolition of a response to a painful stimulus or acute changes in blood pressure and heart rate. The electrocardiogram (ECG) was monitored via needle electrodes connected to a preamplifier, oscilloscope and audio speaker. Animals were tracheostomized and ventilated with 40% O₂/balance N₂ at a ventilator frequency of ~40-45 bpm and a tidal volume ~8-10 ml·kg⁻¹ adjusted to provide normal pH (blood gases: pH = 7.41 ± 0.01, PaCO₂ = 32.3 ± 1.2 mmHg, PaO₂ = 191.0 ± 12.6 mmHg). A mid-line thoracotomy was performed and an end-expiratory load of ~2.3 cmH₂O was established to provide a normal functional residual capacity (Fisher & Mortola, 1981). Transpulmonary pressure (P_{TP}) was measured via a differential pressure transducer (Validyne MP45, ± 2 cmH₂O) connected to the side port of the tracheal cannula. A femoral arterial line was inserted in most animals for blood gas sampling and arterial blood pressure (BP) measurement. A femoral venous line was established for administration of supplemental anesthesia and infusion of capsazepine (CAZP) (Harvard Apparatus Syringe Infusion Pump, model 22). The right jugular vein was cannulated and
Figure 2. Experimental set-up

Animals were placed in a flow plethysmograph and mechanically ventilated with 40% oxygen/balance nitrogen through a tracheostomy tube. A positive end-expiratory pressure (PEEP) of ~2.3 cmH$_2$O was established to maintain normal FRC. Flow ($V$) and transpulmonary pressure ($P_{TP}$) were acquired on-line for calculation of inspiratory lung resistance ($R_L$) and dynamic lung compliance ($C_{L,dyn}$). The electrocardiogram (ECG) was monitored with needle electrodes. Animals were instrumented with a right heart catheter for capsaicin and lactic acid administration (CAPS/LA), a femoral venous line for capsaquine infusion (CAZP) and a femoral arterial line for arterial blood pressure (BP). Core temperature ($T_c$) was monitored with a rectal temperature probe.
a catheter (dead space ~0.3-0.4 ml) advanced into the right heart (location confirmed post mortem) for capsaicin (CAPS) and lactic acid (LA) injections. Inspiratory efforts were abolished by bilateral phrenic nerve section or paralysis with doxacurium chloride (0.025 mg·kg⁻¹ i.v.), a non-depolarizing muscle relaxant with no muscarinic side effects (Okanlami et al., 1996). During paralysis, additional anesthesia was administered based on the schedule used prior to paralysis (approximately every hour). Core temperature (Tc) was regulated with a heat lamp and a servo-controlled heating blanket (Harvard Apparatus Animal Blanket Control Unit, model # 50-6956). Animals were placed in a body plethysmograph and respiratory flow (VT) was measured with a pneumotachograph (Hans Rudolph, model 8300) connected to a differential pressure transducer (Validyne MP45, ±2 cmH₂O). Flow, ECG, BP and PRTP were acquired with a computerized chart recorder data acquisition package (CODAS, DATAQ Instruments).

Analysis of Pulmonary Mechanics

Transpulmonary pressure and respiratory flow were acquired on-line by an additional computer at a sampling frequency of 100 samples·s⁻¹ per channel for breath-by-breath calculation of inspiratory lung resistance (RL, cmH₂O·ml⁻¹·s⁻¹) and dynamic lung compliance (CLdyn, ml·cmH₂O⁻¹). RL was calculated based on the method of Hildebrandt, as described by LaFramboise et al. (1983). This method calculates RL by dividing the average pressure required to overcome the flow resistive properties of the lung by the mean inspiratory flow (Waldron & Fisher, 1988). CLdyn was calculated by dividing the
Figure 3. Pulmonary mechanics analysis

Schematic illustrating the calculation of mean inspiratory lung resistance ($R_L$) and dynamic lung compliance ($C_{L_{dyn}}$). The computer-acquired signals of respiratory flow ($\dot{V}$), transpulmonary pressure ($P_{TP}$) and tidal volume ($V_T$) were used to calculate pulmonary mechanics. $C_{L_{dyn}}$ is calculated by dividing $V_T$ by the $P_{TP}$ measured at end-inspiration (elastic pressure). The pressure above that required to overcome the elastic properties of the lung describes the pulmonary resistance to flow and is termed the "resistive area". Mean "resistive pressure" is calculated by dividing the resistive area by the inspiratory time ($T_i$) and $R_L$ is then calculated by dividing the resistive pressure by the mean inspiratory flow ($V_T/T_i$). Dynamic lung elastance ($E_{L_{dyn}}$) was calculated as the reciprocal of $C_{L_{dyn}}$. 
\[
R_L = \frac{\text{Resistive Pressure}}{V_T/T_i}
\]

\[
C_{L\text{dyn}} = \frac{V_T}{\text{Elastic Pressure}}
\]
tidal volume by the transpulmonary pressure swing between points of zero flow during inspiration. Dynamic lung elastance ($E_{L\text{dyn}}$) was calculated from the reciprocal of $C_{L\text{dyn}}$.

Protocols

Effect of Lactic Acid on Bronchomotor Tone

To determine the effects of LA on neonatal bronchomotor tone, we recorded the breath-by-breath pulmonary mechanics response to right heart injection of LA. In preliminary experiments, we assessed the bronchomotor response to right heart injections of various doses of lactic acid ranging from 0.02-2.0 mmol·kg$^{-1}$ (Fig. 4). On the basis of these results we chose to use 0.4 mmol·kg$^{-1}$ for the present study since it was twice the threshold dose of 0.2 mmol·kg$^{-1}$, and it elicited a robust bronchomotor response on which we could test the effects of pharmacological antagonism.

In order to assess the reflex nature of the responses to LA, $R_L$ and $E_{L\text{dyn}}$ responses to right heart injection of LA were measured before and after muscarinic receptor blockade with atropine (2 mg·kg$^{-1}$) in 17 newborn dogs ($4.9 \pm 0.6$ d). The protocol consisted of 3 acquisition periods separated by 15 min. Each acquisition period was preceded by flushing the jugular catheter with 37°C saline and inflating the lungs to $\geq 20$ cmH$_2$O ($4 \times V_T$) to establish a constant volume history. Two min after the inflation a 5-8 minute acquisition period began. The first trial consisted of saline (0.2 or 0.4 ml) and ACh (10 µg) control injections at 1 and 3 min, respectively, to assess ASM responsiveness. In the second trial, we measured the bronchoconstrictor response to right heart injection of LA (0.4 mmol·kg$^{-1}$). In 11 animals, LA was delivered in 0.2 ml. Due to the elevated
Figure 4. Effect of Dosage on the peak resistance response to lactic acid

Average peak $R_L$ response (% change from baseline) to lactic acid at 4 doses: 0.2, 0.4, 0.5, and 0.75 mmol·kg$^{-1}$. Lactic acid at 1.0 and 2.0 mmol·kg$^{-1}$ evoked severe cardiac dysrhythmias that proved terminal. Injections at 0.02 mmol·kg$^{-1}$ did not cause any significant change in $R_L$. 
osmolality of this stimulus (~600-2300 mOsm·l⁻¹, mean: 1,464 ± 166 mOsm·l⁻¹), an additional 6 animals were studied in which LA was dissolved in 0.4 ml which reduced osmolality to ~285-950 mOsm·l⁻¹ (mean: 510 ± 57 mOsm·l⁻¹). Solution osmolalities were measured using the freezing point depression method (Osmette A Automatic Analyzer, model # 5002, Precision Instruments Inc.). The pH of 0.2 ml and 0.4 ml volume LA injections was 2.04 ± 0.03 and 2.22 ± 0.02, respectively (Accumet model 815MP, Fisher Scientific). A second LA challenge was delivered after atropine to assess the reflex contribution of vagal cholinergic efferents to the bronchomotor response.

**Effect of Osmolality on Bronchomotor Tone**

To investigate the possible effects of solution osmolality on pulmonary mechanics we measured the breath-by-breath pulmonary mechanics response to injection of hypertonic saline (500, 1000, 1500, 2000, 2500 and 5000 mOsm·l⁻¹) in 3 newborn dogs (13.0 d). The protocol consisted of a saline and ACh control trial (as described for the effects of lactic acid on bronchomotor tone) and 2-3 hypertonic saline trials in which animals were randomly challenged with right heart bolus injections (0.4 ml) of hypertonic saline (500-5000 mOsm·l⁻¹) separated by 5 min.

**Capsazepine Delivery Method**

To determine the most effective method of delivering CAZP for VR1 receptor antagonism, we compared the effects of bolus injections (3.5 or 5.0 mg·kg⁻¹ i.v.) versus infusions (500 μg·kg⁻¹·min⁻¹ i.v.) of CAZP on the bronchoconstrictor response to 25
μg·kg⁻¹ capsaicin in 5 newborn dogs (mean age 3.8 ± 0.5 d). These doses were chosen based on the findings of Lee and Lundberg (1994) in which either bolus (2.0-3.5 mg·kg⁻¹) or infusion (250-350 μg·kg⁻¹·min⁻¹) of CAZP abolished the reflex apnea, bradycardia and hypotension elicited by injection of 1-2 μg·kg⁻¹ capsaicin in the adult rat (Lee & Lundberg, 1994). Our protocol consisted of 4 (bolus study) or 5 (infusion study) acquisition periods, of which the first 2 were common to both and consisted of an initial trial of saline and ACh to establish baseline bronchomotor responsiveness, and bronchoconstrictor response to a control injection of CAPS (25 μg·kg⁻¹), respectively. For the CAZP bolus study, the third and fourth trials consisted of a CAZP block trial, in which we measured the pulmonary mechanics response to CAPS 2 min after bolus injection of CAZP (3.5 or 5.0 mg·kg⁻¹ i.v.), and a recovery trial, in which we tested the airway response to CAPS 15 min following CAZP. For the CAZP infusion study the third acquisition period consisted of a CAZP trial in which we measured R_L and E_L_syn during the onset of the CAZP infusion (500 μg·kg⁻¹·min⁻¹). The fourth or "blockade" trial measured the pulmonary mechanics response to CAPS at the 10 min mark of CAZP infusion. In the "recovery" trial, we measured the pulmonary mechanics response to CAPS and ACh at 15 and 18 min following CAZP, respectively.

Bolus CAZP injection reduced the CAPS-evoked R_L responses by 25% (3.5 mg·kg⁻¹) and by 59% (5.0 mg·kg⁻¹). Although the reductions in R_L were significant (one way ANOVA), it was difficult to determine whether the animals were in a steady-state or how critical the timing of the CAPS response was following the CAZP bolus. For this reason we pursued the CAZP-blockade studies using the CAZP infusion technique.
Effect of Capsazepine Infusion on Capsaicin- and Lactic Acid-Induced Reflex Bronchoconstriction in the Newborn

In order to test the hypothesis that LA and CAPS activate the same C-fibre receptor mechanisms, we first had to determine whether CAZP was capable of abolishing capsaicin-induced reflex bronchoconstriction. Experiments were performed on 11 newborn dogs (mean age 5.4 ± 0.9 d) in which the bronchomotor responses to right heart injections of CAPS were measured before, during and after CAZP infusion (Fig. 5). In a second set of experiments (n=8, mean age 4.9 ± 0.4 d) we tested the actions of CAZP on LA-induced bronchoconstriction. Protocols for both the CAZP/CAPS and CAZP/LA experiments consisted of five acquisition periods separated by 15 min (Fig. 5). Prior to each trial, the jugular catheter was flushed with 37°C saline and the animal inflated to ≥ 20 cmH₂O (4 x V₇) to establish a constant volume history. The first trial (saline and ACh) was the same as that described above for the muscarinic blockade study. The second trial measured the response to right heart injection of capsaicin (25 μg·kg⁻¹) or LA (0.4 mmol·kg⁻¹ in 0.4 ml). The third trial measured Rₐ and Eₐdyn prior to the onset and during the capsazepine infusion (500 μg·kg⁻¹·min) into the femoral vein. The fourth trial assessed the lung mechanics response to CAPS and LA 10 min after initiation of the CAZP infusion. The fifth trial was performed 15 to 20 min post CAZP infusion to assess the reversibility of the CAZP blockade.
Figure 5. Experimental protocol

Baseline and control trials were designed to assess the bronchomotor responses to saline and ACh (BASELINE) and CAPS or LA (CONTROL). The BLOCKADE trial consisted of challenge with CAPS or LA at 10 min into CAZP infusion. A RECOVERY trial was performed at the end of the experiment to assess the reversibility of the blockade on the response to CAPS or LA. Trials were separated by at least 15 min. Injections, denoted by arrows, occurred at approximately 1 and 4 minutes into the 8 min trial. ACh injections were 10 µg and CAPS and LA injections were 25 µg·kg⁻¹ and 0.4 mmol·kg⁻¹, respectively. Break in BLOCKADE trial represents ~9 min.
BASELINE

saline  ACh 1
(10 μg)

CONTROL

CAPS (25 μg·kg⁻¹)
or LA (0.4 mmol·kg⁻¹)

BLOCKADE

CAPS or LA  ACh 2

CAZP 500 μg·kg⁻¹·min
or Atropine 2 mg·kg⁻¹

RECOVERY

CAPS or LA  ACh 3

8 min
Drugs

The following drugs were used: Chloralose (ICN Biochemicals) and urethane (Sigma) dissolved as a mixture (0.25 g chloralose, 2.5 g urethane) in 10 ml heated saline, administered at doses of 2-2.5 ml·kg⁻¹ with supplemental injections of 0.5 ml·kg⁻¹ to induce and maintain anesthesia, respectively. A stock solution of capsaicin (Sigma) was prepared by dissolving 10 mg capsaicin in 1 ml ethanol, 2 drops Tween 80 and 9 ml saline to arrive at a final concentration of 1 mg·ml⁻¹. The stock was diluted further with saline to the required concentration for injection. A stock solution of 3.3 M lactic acid (Sigma, L(+)lactic acid) was diluted with distilled H₂O to the required concentration for injection. Acetylcholine chloride (Sigma) was dissolved in saline to a concentration of 50 μg·ml⁻¹. Atropine (Sigma) was prepared as a 10 mg·ml⁻¹ stock solution and diluted with saline to the required concentration for injection. Capsazepine (RBI) was prepared as a 2.5 mg·ml⁻¹ stock solution by dissolving 25 mg in dimethyl sulfoxide and diluting it in a solution of 1 ml Tween 80, 1 ml ethanol and 8 ml saline. Injection volumes for capsaicin and ACh were 0.2 ml. Injection volumes for saline were 0.2 ml or 0.4 ml. Injection volumes for LA were 0.2 ml or 0.4 ml in the LA/atropine study but 0.4 ml in the CAZP protocols. Saline, ACh, capsaicin and lactic acid were injected into the jugular catheter dead space then rapidly flushed with saline. Chloralose/urethane and capsazepine were administered via the femoral venous catheter and flushed with saline. All solutions were warmed to 37°C.
Statistical Comparisons

Baseline $R_L$ and $E_{L\text{dyn}}$ were calculated on the average of the 10 breaths prior to injection. The peak lung mechanics response for each animal was defined as the average resistance and elastance calculated for the two largest consecutive values following challenge. $R_L$ response latency was defined as the time from jugular line injection to the onset of a maintained or continued increase in $R_L$ above baseline. The time to peak was calculated as the time from jugular line injection to the first breath of the maximal response. HR baselines were calculated as the average values over the 10 seconds preceding injection. Bradycardia was calculated as the lowest instantaneous HR following injection. Statistical comparisons between responses were based on one way ANOVA, Mann-Whitney rank sum or paired $t$-test. Tukey post hoc analyses were performed if the ANOVA was significant. A significant difference was defined as $P < 0.05$. All results are expressed as mean ± SEM unless otherwise indicated.
CHAPTER 3

Results
**Lung Mechanics Response to Lactic Acid**

The average breath-by-breath response of $R_L$ to 0.4 mmol·kg$^{-1}$ LA is shown in Fig. 6 (filled symbols). The 0.2 ml LA injectate resulted in a peak increase in $R_L$ of $52 \pm 6.7\%$ ($P < 0.01$, Fig. 7) and the 0.4 ml LA injectate caused a peak increase in $R_L$ of $49 \pm 10.9\%$ (Fig. 7). Peak LA-evoked $R_L$ increases were not significantly different for 0.2 and 0.4 ml injectate volumes ($P > 0.05$). Latency and time to peak values for the response of $R_L$ to LA are presented in Table 2.

The average breath-by-breath response of $R_L$ to LA during muscarinic blockade with atropine is shown in Fig. 6 (open symbols). The maximal response of $R_L$ to the 0.2 ml LA injectate was relatively atropine-resistant (Fig. 7), displaying a reduction of only 33 • 13.5% from the control value ($P < 0.05$). In contrast, the maximal responses associated with the 0.4 ml LA injectate (Fig. 7) were atropine-sensitive and were reduced by 80 ± 10.9% compared to control ($P < 0.01$). The effect of atropine antagonism on the response of $R_L$ was significantly different ($P < 0.01$) for the 0.2 and 0.4 ml LA injectates.

**Effect of Osmolality on Bronchomotor Tone**

Inspiratory lung resistance was unaffected by right heart injection of solutions of 500 to 2500 mOsm·l$^{-1}$. However, $R_L$ displayed a small (8.4%) decrease to 5000 mOsm·l$^{-1}$. Although 0.4 mmol·kg$^{-1}$ LA delivered in a 0.4 ml injectate evoked atropine-sensitive reflex bronchoconstriction, the atropine-resistance of the bronchoconstriction evoked by the 0.2 ml LA injectate suggests a non-cholinergic component. Since hypertonic saline solutions (2400 to 4800 mmol·l$^{-1}$) injected into the pulmonary circulation of adult
Figure 6. Breath-by-breath mechanics response to lactic acid pre and post atropine

Average (± SEM) breath-by-breath values of percent change in lung resistance (R_L, upper panels) and elastance (E_{L,dy}, lower panels) in response to right heart injections of lactic acid (0.4 mmol·kg⁻¹) in 0.2 ml (high osmolality; left panels) or 0.4 ml (low osmolality; right panels) injectate before (closed symbols) and after (open symbols) atropine (2 mg·kg⁻¹). Note that atropine abolished the response to the 0.4 ml injectate whereas the 0.2 ml injectate was atropine-resistant. Arrows indicate injection of lactic acid. Mean ventilator frequency was approximately 45 breaths per minute.
Figure 7. Average maximal mechanics response to lactic acid in 0.2 and 0.4 ml

Mean peak pulmonary resistance ($R_L$, top panels) and dynamic lung elastance ($E_{L,dyn}$, lower panels) responses to saline and lactic acid (0.4 mmol·kg$^{-1}$) in 0.2 ml (left) or 0.4 ml (right) injectate volume before (INTACT) and after atropine (ATROPINE). Peak values are slightly greater than maximal values for breath-by-breath averages in Figure 6 due to minor variations between animals for the ventilatory cycle at which maximal response occurred. Note: atropine reduced the $R_L$ response to the 0.4 ml injectate such that it was significantly different from the intact response but was not different from saline. +, significantly different from INTACT lactic acid response; *, significantly different from SALINE control. $P < 0.05$ (ANOVA and Tukey post hoc).
dogs stimulate pulmonary C-fibres and cause reflex bronchoconstriction via cholinergic mechanisms (Pisarri et al., 1991), the atropine-resistant bronchoconstrictor effects of the high osmolality LA injectate in our studies were not likely due of C-fibre stimulation. Furthermore, injection of 5000 mOsm·l⁻¹ saline (twice our highest LA osmolality) had no bronchoconstrictor action in 12-14 d newborn dogs. Therefore, high osmolality per se likely did not affect ASM tone, but evoked an as yet undetermined non-muscarinic mechanism of increasing airway tone.

**Lung Mechanics Responses During Capsazepine**

CAZP infusion alone was associated with an increase in $R_L$ of $23 ± 6.7\%$ at 8 min ($n = 19$) (Fig. 8). Hyperinflation ($4 \times V_T$) did not significantly reduce the CAZP infusion-evoked elevation in $R_L$, which was $18 ± 0.6\% \ (P < 0.05)$ 2 min after inflation. Eight min of tidal breathing following hyperinflation without any other intervention was associated with an increase in $R_L$ of $\sim 11\%$ relative to control. In preliminary studies, infusion of CAZP vehicle alone did not alter the $R_L$ response to LA, which was $68.2\%$ and $60.5\%$ prior to and following CAZP vehicle, respectively.

The average breath-by-breath response of $R_L$ to right heart injection of capsaicin for 11 animals is shown in Fig. 10. Capsaicin caused a maximal increase of $41 ± 8.5\%$ in $R_L$ (Fig. 11, top panel). During CAZP infusion, the maximal response of $R_L$ to capsaicin was inhibited by $83 ± 4.7\% \ (P < 0.01)$, indicating that competitive antagonism of the capsaicin/VR1 receptor effectively blocks reflex bronchoconstriction. Fifteen min following CAZP infusion the response of $R_L$ to capsaicin had recovered to $71 ± 21.9\%$ of
the control trial (Fig. 11, top panel).

The average breath-by-breath response of $R_L$ and $E_{L_{\text{dyn}}}$ to 0.4 ml LA injectate (low osmolality) during CAZP is shown in Fig. 13. LA caused a maximal increase of $50 \pm 7.0\%$ in $R_L$ (Fig. 14, upper panel) but, in contrast to CAPS, the LA-induced increase in $R_L$ was unaffected by CAZP infusion ($39 \pm 5.7\%; P > 0.05$). At 15 min following CAZP infusion the response of $R_L$ to LA was $95 \pm 13.7\%$ of the control response. Figure 14 illustrates the average of the group peak $R_L$ (upper panel) and $E_{L_{\text{dyn}}}$ (lower panel) responses during control, attempted CAZP blockade and recovery LA injections. $R_L$ response latencies and times-to-peak for CAPS and LA are summarized in Table 3. Heart rate responses are summarized in Table 4.
Figure 8. Acute breath-by-breath response of mean lung resistance to capsazepine infusion

Average lung resistance response to capsazepine (500 μg·kg⁻¹·min i.v.) in 19 newborn dogs. Infusion of capsazepine (~ 6:45 min) caused an increase in inspiratory lung resistance (R_L) that was not significantly reduced by hyperinflation.
Figure 9. Cardiopulmonary response to capsaicin pre and post capsazepine

Cardiopulmonary response illustrating the antagonistic effect of capsazepine (500 µg·kg⁻¹·min i.v.) on reflex responses to right heart injection of capsaicin (CAPS, 25 µg·kg⁻¹) in a newborn dog. Transpulmonary pressure (P_{TP}), flow (V), tidal volume (Vₜ) and arterial blood pressure (BP) before (upper panel) and during (lower panel) capsazepine infusion. The bronchoconstrictor response is indicated by the increase in P_{TP} and is accompanied by hypotension and bradycardia. During capsazepine the P_{TP} and cardiovascular response were abolished. Arrows represent capsaicin inject and flush, respectively; - indicates expiratory flow.
CAPSAZEPINE

INTACT

FLUSH
Figure 10. Breath-by-breath response of pulmonary mechanics to capsaicin

Average (± SEM) breath-by-breath values of percent change in inspiratory lung resistance ($R_L$) and dynamic lung elastance ($E_{Ldyn}$) in response to right heart injections of capsaicin (arrow) in 11 newborn dogs for three separate conditions: INTACT (left panels) - Right heart injection of capsaicin caused a brisk bronchoconstrictor response. CAPSAZEPINE (middle panels) - Capsaicin response was blocked by capsazepine infusion. RECOVERY (right panels) - Partial recovery of the capsaicin response 15-20 min after CAZP.
Figure 11. Average maximal changes in the response of pulmonary mechanics to capsaicin

Mean peak response (± SEM) of inspiratory lung resistance (R_L, top panel) and dynamic lung elastance (E_Ldyn, bottom panel) plotted as % change from baseline for saline and three different capsaicin injections (INTACT, BLOCK and RECOVERY). +, significantly different from INTACT capsaicin response; * significantly different from SALINE control. P < 0.05 (ANOVA with Tukey post hoc).
SALINE INTACT BLOCK RECOVER

% CHANGE FROM BASELINE

RL

E_Ldyn

SALINE INTACT BLOCK RECOVER

* indicates significant difference from baseline
+ indicates trend toward significance
Figure 12. Cardiopulmonary response to lactic acid pre- and post- capsazepine

Cardiopulmonary response illustrating the effect of capsazepine on reflex responses to right heart injection of lactic acid (0.4 mmol·kg\(^{-1}\)) in a single newborn dog. Note that capsazepine did not abolish the increase in \(P_{TP}\) or hypotension response to lactic acid. Abbreviations as in Figure 9.
Figure 13. Breath-by-breath mechanics responses to lactic acid

Mean responses of lung mechanics to lactic acid (0.4 mmol·kg⁻¹) in 8 newborn dogs. Average (± SEM) breath-by-breath values of percent change in inspiratory lung resistance (R_l, upper panels) and dynamic lung elastance (E_Ldyn, lower panels) in response to right heart injections of lactic acid (arrows) for three separate conditions. Right heart injection of lactic acid caused a robust bronchoconstrictor response (INTACT, left panels) that was maintained during capsazepine infusion (500 µg·kg⁻¹·min; CAPSAZEPINE, middle panels) and post capsazepine (RECOVERY, right panels).
Figure 14. Average maximal responses of pulmonary mechanics to lactic acid

Mean peak response (± SEM) of inspiratory lung resistance ($R_L$, top panel) and dynamic lung elastance ($E_{L, dyn}$, bottom panel) plotted as % change from baseline to saline and three different lactic acid responses (INTACT, BLOCK and RECOVERY). Lactic acid responses were all significantly different from saline ($P < 0.05$) but were not different from each other ($P > 0.05$). *, significantly different from SALINE control.
<table>
<thead>
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<th>Lactate Acid</th>
<th>Capsaicin</th>
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<tbody>
<tr>
<td>1.076 ± 0.134</td>
<td>0.986 ± 0.004</td>
</tr>
<tr>
<td>1.075 ± 0.134</td>
<td>0.986 ± 0.004</td>
</tr>
<tr>
<td>1.086 ± 0.135</td>
<td>0.976 ± 0.004</td>
</tr>
<tr>
<td>0.960 ± 0.030</td>
<td>0.906 ± 0.030</td>
</tr>
<tr>
<td>0.918 ± 0.133</td>
<td>0.905 ± 0.030</td>
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Baseline values of lung mechanics (mean ± SEM)

**TABLE 1.**
TABLE 2.
Latency of lung resistance response to lactic acid (mean ±SEM)

<table>
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<tr>
<th>Lactic Acid</th>
<th>INTACT</th>
<th>ATROPINE</th>
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<tr>
<td>Latency (s)</td>
<td>Time to peak (s)</td>
<td>Latency (s)</td>
</tr>
<tr>
<td>0.2 ml injectate</td>
<td>6.8 ± 1.2</td>
<td>18.7 ± 2.7</td>
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<tr>
<td>0.4 ml injectate</td>
<td>8.4 ± 2.1</td>
<td>26.3 ± 6.0 *</td>
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* different from 0.2 ml injectate time to peak (P > 0.05); n/a not applicable.
<table>
<thead>
<tr>
<th>Protocol</th>
<th>INTACT</th>
<th>CAPSAZEPINE</th>
<th>RECOVERY</th>
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<tbody>
<tr>
<td></td>
<td>Latency</td>
<td>Time to peak</td>
<td>Latency</td>
</tr>
<tr>
<td></td>
<td>(s)</td>
<td>(s)</td>
<td>(s)</td>
</tr>
<tr>
<td>CAPS</td>
<td>2.3 ± 0.4</td>
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<tr>
<td>ACh</td>
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<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>LA</td>
<td>5.2 ± 1.7</td>
<td>17.8 ± 3.1</td>
<td>8.2 ± 1.8</td>
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</table>

n/a not applicable.
<table>
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<th>RECOVERY</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (bpm)</td>
<td>% Decrease (s)</td>
<td>Baseline (bpm)</td>
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<tr>
<td>saline</td>
<td>185 ± 4.4</td>
<td>4 ± 1.1*</td>
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</tr>
<tr>
<td>CAPS</td>
<td>206 ± 6.3</td>
<td>8 ± 2.9*†</td>
<td>208 ± 5.8</td>
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<tr>
<td>LA</td>
<td>181 ± 5.6</td>
<td>31 ± 7.8*†</td>
<td>207 ± 5.5</td>
</tr>
</tbody>
</table>

* different from baseline value \( (P < 0.05) \); † different from capsaicin \( (P < 0.05) \); ‡ different from saline \( (P < 0.05) \); n/a, not applicable.
CHAPTER 4

Discussion
Discussion

Our results show that LA evokes an atropine-sensitive increase in bronchomotor tone that is consistent with that described for other stimulants of C-fibre afferents (Coleridge et al., 1989). Our study also found that capsazepine, the competitive capsaicin/VR1 receptor antagonist, significantly attenuates the bronchomotor reflex response to capsaicin, but not the response to LA. Taken together, these results indicate that LA is not an endogenous VR1 ligand but suggest that LA and CAPS stimulate reflex bronchoconstriction via different receptor signal transduction mechanisms.

In both the adult and neonate, activation of pulmonary C-fibres by the vanilloid excitotoxin capsaicin evokes reflex bronchoconstriction (Coleridge et al., 1989; Fisher et al., 1998), which is variably accompanied by mucus hypersecretion, vascular exudation and airway edema (Coleridge et al., 1989; Lundberg & Saria, 1982). Physiological stimulation of pulmonary C-fibres initiates airway reflexes aimed at limiting exposure of the airways to noxious stimuli (as in Coleridge & Coleridge, 1994; Paintal, 1973; Karlsson et al., 1988); however, activation of pulmonary C-fibres has also been implicated in the airway hyperreactivity accompanying inflammatory lung diseases such as asthma or, in the newborn, BPD (Barnes, 1986; Barnes, 1995; Spina, 1996). The magnitude of the bronchoconstriction we observed to both CAPS and LA is submaximal (Forkert & Fisher, 1992) and may reflect a mechanism to enhance gas exchange by reducing dead space volume (Widdicombe, 1963) or to protect the highly compliant airways of the newborn from dynamic compression (Olsen et al., 1967).

Despite characterization of the in vivo reflex effects of CAPS, the effects of
capsaicin on pulmonary C-fibres and elucidation of the molecular mechanism of action of the putative C-fibre “capsaicin” receptor (i.e. VR1), the endogenous VR1 ligand remains to be identified. Likely activation of pulmonary C-fibres during inflammatory conditions has led to the proposal that biological compounds released in the inflammatory process may represent endogenous agonists for the capsaicin receptor (Bevan & Geppetti, 1994; Franco-Cereceda et al., 1994; Longhurst et al., 1991). Among these compounds, lactic acid (LA) has received considerable attention. Acidic pH has been shown to evoke a capsazepine-sensitive bronchoconstriction in the isolated perfused guinea pig lung, presumably by acting directly on C-fibre endings to cause release of tachykinins (Lou & Lundberg, 1992). The ability of LA to act as an endogenous stimulant of reflex bronchoconstriction in vivo however, has not been tested in the adult despite its excitatory actions on C-fibres. The newborn is particularly interesting with respect to the actions of H⁺ on C-fibres since metabolic and respiratory acidosis are normal components of parturition as well as in certain pathological complications of birth (Koch & Wendel, 1968; Koch, 1968; Penn et al., 1988; Abman & Groothius, 1994). While C-fibre recordings have not been made in the newborn, C-fibre type reflexes have been examined (Kalia, 1976; Ducros & Trippenbach, 1991; Haxhiu-Poskurica et al., 1991; Anderson & Fisher, 1993).

Initial studies in the newborn led to the conclusion that LA (0.25 mmol·kg⁻¹) does not evoke a bronchoconstrictor response in 1-6 d old rabbit pups (Ducros & Trippenbach, 1991). In this study, we directly measured total pulmonary resistance following presumptive C-fibre stimulation with comparable LA doses and found that LA caused a
robust, cholinergic, reflex bronchoconstriction in the newborn dog. While the discrepancy between Ducros and Trippenbach (1991) and our findings could be attributable to species differences, other factors are equally as likely. Ducros and Trippenbach used the ratio of tidal volume to oesophageal pressure swing (i.e. $C_{L_{dyn}}$) to monitor changes in bronchomotor tone. However, $C_{L_{dyn}}$ is an indirect index of bronchoconstriction and may be insensitive to small changes in ASM tone or resistance (Widdicombe, 1963). Furthermore, $C_{L_{dyn}}$ is constrained in its relative response since it can decrease only in the range of 100 to 0% of the control value, whereas $R_L$ can increase several fold above control. Indeed, this is why we evaluated $E_{L_{dyn}}$ in the present study. In preliminary experiments we found that right heart injection of 0.2 mmol·kg$^{-1}$ LA evoked modest but quantifiable bronchoconstrictor responses in the newborn dog (8.3% increase in $R_L$ versus a 5% decrease in $C_{L_{dyn}}$). The small $C_{L_{dyn}}$ response associated with the response of $R_L$ to 0.25 mmol·kg$^{-1}$ LA may make bronchoconstriction more difficult to detect with dynamic compliance measurements. Alternatively, 0.2-0.25 mmol·kg$^{-1}$ may be inadequate to sufficiently stimulate C-fibres in the newborn rabbit, which is more immature at birth than the dog.

Development of the competitive C-fibre receptor antagonist CAZP allowed us to specifically investigate the putative mechanism of C-fibre activation with LA. *In vivo*, CAZP attenuates the cardiorespiratory depressor effects evoked by CAPS in the adult rat (Lee & Lundberg, 1994) and both *in vivo* (rat) and *in vitro* (guinea pig) recordings of C-fibre responses to CAPS support this observation (Lee et al., 1996; Fox et al., 1995). Furthermore, CAZP abolishes the CAPS-evoked inward cation current in neonatal rat
DRG cells (Bevan et al., 1992). Nevertheless, to our knowledge the effect of CAZP on CAPS-evoked reflex bronchoconstriction had not been investigated prior to our study. We found that CAZP effectively antagonized CAPS-evoked bronchoconstriction *in vivo* (Fig. 10), which supports the notion that therapeutic targeting of sensory afferents in the newborn may be a beneficial strategy for reducing both the cholinergic reflex component as well as potential tachykinin components of the bronchomotor response associated with C-fibre activation in inflammatory lung disease (Spina, 1996; Spina & Page, 1996; Barnes, 1986; Barnes, 1995; Szallasi & Blumberg, 1996).

In contrast to CAPS-evoked bronchoconstriction, LA-induced bronchomotor reflexes were essentially unaffected by CAZP (Fig. 13). The CAZP-resistance of the LA-evoked bronchomotor response indicates that LA and CAPS stimulate reflex bronchoconstriction either via different receptor signal transduction mechanisms within the same pulmonary C-fibre afferent, or via different pulmonary C-fibre subpopulations. The former explanation is the most likely, as single fibre recordings from adult rat have shown that the same C-fibres are stimulated by CAPS and LA (Lee et al., 1996; Hong et al., 1997). Descriptions of a proton-evoked cation current in frog and rat DRG neurons (Akaike et al., 1990; Bevan & Yeats, 1991), the latter localized in fibres sensitive to CAPS, combined with the recent cloning of an acid-sensing ionic channel (ASIC) from *Xenopus* DRG (Waldmann et al., 1997; Waldmann et al., 1997) provide additional evidence in support of separate pulmonary C-fibre receptor mechanisms mediating CAPS- and LA-evoked reflex bronchoconstriction. Alternatively, the finding that acidic extracellular pH below the threshold required to activate C-fibres facilitates capsaicin-
induced cation current (Caterina et al., 1997) is consistent with Vycklicky et al.'s (1998) later suggestion that the facilitatory actions of protons on the capsaicin-evoked cation current may be mediated by protonation of VR1. While this latter speculation addresses a possible mechanism by which protons potentiate the activation of VR1 with capsaicin, its mechanistic focus does not offer an alternative endogenous VR1 ligand candidate. In order for the facilitatory actions of protons to explain our results, H+ would have to unmask a reflex, C-fibre-mediated response to a circulating endogenous ligand.

Others have alluded to the potential role of additional inflammatory compounds as endogenous stimulants of VR1 (Karla et al., 1992; Shams et al., 1988; Vycklicky et al., 1998). The CAZP-insensitivity of the LA response, combined with the longer latency and time-to-peak of the bronchomotor response to LA (Tables 2 and 3) compared to CAPS, could reflect a LA-induced metabolic intermediary that activates pulmonary C-fibre receptors. Based on our data, it is not possible to state whether the time course of the RL response supports either a direct proton/LA-mediated mechanism of C-fibre activation or the generation of an intermediary endogenous C-fibre stimulant released by LA. Slow infusion of hydrochloric acid stimulates platelets to release thromboxane A2 (TxA2), an arachadonic acid cyclooxygenase metabolite and C-fibre stimulant (Shams et al., 1988). Other products of the arachadonic acid cyclooxygenase pathway, such as PGE2 and PGF2α, also stimulate unmyelinated sensory afferents and/or potentiate their activation and reflex effects (Coleridge et al., 1978; Lee & Morton, 1995; Steen et al., 1995; 1996). While application of PGE2, bradykinin and 5-HT together does not evoke the inward cation current characteristic of VR1 activation in cultured newborn rat sensory neurons,
the acidic pH-evoked cation current is greatly potentiated in the presence of inflammatory mediators (Steen et al., 1995; 1996). Furthermore, capsazepine appears to block the potentiating effect of acid pH on inflammatory mediator-evoked current but does not reduce the underlying acid-evoked current itself (Vylicky et al., 1998). The potentiating effect of acidic pH on the inflammatory mediator-evoked current in sensory neurons is similar to that evoked by acid pH during submaximal capsaicin application (Caterina et al., 1997) and has prompted the hypothesis that inflammatory mediators, specifically PGE₂, may represent endogenous ligands for the “capsaicin” or VR₁ receptor (Vylicky et al., 1998). Whether these mechanisms play a role in the newborn dog’s response to LA that we observed is unclear, although they appear unlikely. Lee and colleagues found that cyclooxygenase intermediaries were not involved in the LA-induced pulmonary chemoreflex in the rat in which reflex apnea, bradycardia and hypotension evoked by LA were unaffected by pretreatment with the cyclooxygenase enzyme antagonist indomethacin (Lee & Morton, 1995; Lee et al., 1996). In a single animal, we assessed the effect of indomethacin (15 mg·kg⁻¹); it did not attenuate the bronchomotor response to LA, supporting Lee and colleague’s conclusion that an LA-evoked cyclooxygenase intermediary mechanism of C-fibre activation is unlikely.
Potential Mechanisms of Action

Based on the available data in the literature and our study, it appears that C-fibres are stimulated by at least 2 parallel receptor transduction mechanisms (Fig. 15). The first, VR1, is a non-specific cation conductance which specifically binds vanilloid compounds (i.e. capsaicin, resiniferatoxin) and displays a secondary putative recognition site for H⁻ that facilitates or amplifies the response to CAPS. While H⁻ alone appears to be only a weak stimulant of this receptor (Caterina et al., 1997), it potentiates the inward cation current evoked by both vanilloids and inflammatory mediators (specifically PGE₂), the latter of which may represent an endogenous VR1 agonist (Vyklicky et al., 1998). The second receptor transduction mechanism (ASIC) is a H⁻-gated Na⁺ conductance. Since the lactate anion potentiates the acid-evoked pulmonary chemoreflex, ASIC might also display a secondary recognition site for the lactate anion (Fig. 15). Our findings show a clear separation of the mechanisms with respect to in vivo bronchomotor reflexes. C-fibre recordings in the newborn are required to further clarify the hypothesized parallel C-fibre receptor transduction mechanisms by quantifying activation latencies and afferent discharge frequencies. Biophysical studies of the vanilloid receptor-induced cation current and its pH sensitivity will also be critical in elucidating the mechanisms activating C-fibre afferents.
Hypotheses for the activation of pulmonary C-fibres with capsaicin and lactic acid based on the present study and the literature. Capsazepine abolished the “capsaicin”/VR1 receptor-mediated response to capsaicin but not the LA-evoked response. The present study indicates that CAPS and LA act via different sensory mechanisms, consistent with the 2 parallel activation mechanisms shown: VR1, a non-specific cation conductance with a primary activation site which binds capsaicin and a secondary recognition site for H+ that enhances the response to CAPS (Caterina et al., 1997); and ASIC (Acid-Sensing Ionic Channel), a H+-gated conductance (Waldmann et al., 1997) with a secondary recognition site for the lactate anion that reflects the reported ability of lactate anion to enhance the H+ response (Hong et al., 1997).
Summary

In summary, we conclude that LA behaves as an endogenous C-fibre stimulant to cause reflex bronchoconstriction. Further, we conclude that capsazepine reversibly antagonizes the reflex bronchoconstriction elicited by right heart injections of capsaicin, presumably by acting at the C-fibre “capsaicin”/VR1 receptor. Finally, the CAZP-resistance of the LA-induced airway response indicates that LA acts via an independent mechanism to that of CAPS to evoke reflex bronchoconstriction, possibly by activation of an acid-sensing ionic channel-like C-fibre receptor transduction mechanism.
References


