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The Presynaptic Regulation of Isolated Neonatal Rat Carotid Body Type I Cells by Histamine

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THE PRESYNAPTIC REGULATION OF ISOLATED NEONATAL RAT CAROTID BODY TYPE I CELLS BY HISTAMINE.

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Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Drew Burlon ENTITLED The Presynaptic Regulation of Neonatal Rat Carotid Body Type I Cells by Histamine BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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It has been previously shown that Carotid Body Type I cells have the ability to synthesize, package and release histamine in response to hypoxia, thereby contributing to the modulation of respiration within the rat. Here, isolated neonatal rat carotid body type I cells were used to identify the presynaptic effects of histamine and the specific receptor subtypes that modulate them. Although all four histamine receptor subtypes are expressed on the type I cells, and preliminary data showed promising results, further data proved that the activation of these receptors with histamine or selective agonists caused no rise in intracellular calcium ($[Ca^{2+}]_i$) and histamine did not augment calcium entry. Thus activation of histamine receptors on type I cells is unlikely to provide a presynaptic positive feedback mechanism during chemotransduction and any excitatory role attributed to the actions of histamine is likely to come from a postsynaptic effect on the carotid sinus nerve (CSN).

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONT'D)

TABLE OF CONTENTS (CONT'D)

LIST OF FIGURES

Page

LIST OF FIGURES (CONT'D)

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CHAPTER I

INTRODUCTION

Why do we need to breathe? Is it an important function? What controls our breathing?

Ever since oxygen became a freely accessible molecule on planet earth, organisms have been using it to fuel cellular respiration. Cellular respiration, or the process of using oxygen to produce ATP in mitochondria, is what allowed life to flourish and diversify on earth. Because of oxygen's role as the final electron acceptor in the electron transport chain, single as well as multi cellular organisms have the ability to increase their energy production. In animals, respiration is the process through which oxygen is exchanged in the lungs for carbon dioxide, a byproduct of cellular respiration. By inhaling air into our lungs, oxygen is delivered to the blood stream. The blood then transports oxygen to all the cells of the body, and allows for cellular respiration to take place. This fundamental process is pivotal for human life to exist as we know it.

For millennia man has known breathing, or respiration, was integral for survival. What was not known however, were the structures and processes involved with the control of our breathing. Toward the beginning of the 20^{th} century breathing was discovered to be influenced by specialized organs that sensed changes in the composition of the body fluids (PO_2, PCO_2, or $[H^+]$), which reflect O_2 demands and CO_2 production (Heymans, 1930; Gonzalez *et al.*, 1994). These organs were designated the peripheral arterial chemoreceptors. Later, during the middle of the century chemoreceptor sites within the brain also began to be identified (Leusen, 1950a, b). The work in this thesis is concerned with neurotransmission within the peripheral arterial chemoreceptors, in particular the carotid bodies of the rat and the role of histamine in their function.

CHAPTER II

LITERATURE REVIEW

General Anatomy

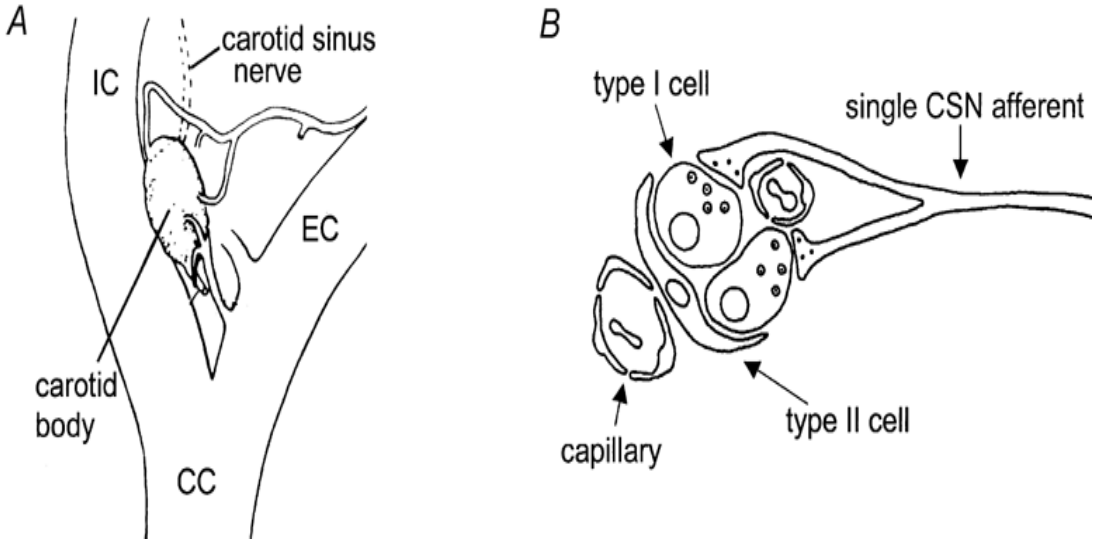
The carotid bodies are the primary peripheral arterial chemoreceptors for the control of respiration in the human body. They are sensitive to fluctuations in blood gas as well as pH. Any change in blood gas homeostasis, such as hypoxia, hypercapnia, or acidosis are sensed by the carotid bodies which then release neurotransmitters. This results in the increased activity of the carotid sinus nerve (CSN) which innervates the respiratory centers of the brain, causing increased breathing movements thereby returning arterial PO_2, PCO_2 and pH to normal physiological levels.

Located at the bifurcation of the common carotid artery, in humans carotid bodies are about the size of a grain of rice and about 1mg in weight. Although very small, the carotid body receives the highest perfusion of blood, per gram of tissue, of any organ in the body. Blood is delivered to the carotid bodies from multiple branches of both the internal and external carotid arteries, and perfused through the organ by an extensive network of capillaries. Venous drainage of the carotid bodies is completed through both the internal and external jugular veins (Fig 1A). Innervation of this particular organ is accomplished by two major nerves. The first is the afferent portion of the carotid sinus nerve (CSN), a branch of cranial nerve IX or glossopharyngeal nerve, and second is the efferent ganglioglomerular nerve from the superior cervical ganglion (SCG). The carotid bodies particular location not only allows the organ to sample blood that has recently passed through the lungs and heart, but allows it to relay what it senses directly to the brain allowing respiratory changes to be made extremely rapidly.

The carotid body contains two major types of cells (Fig 1B). The type I cells are responsible for sensing reductions in blood PO_2, pH and increases in PCO_2 (Buckler &

Vaughan-Jones, 1994b, a). Once changes are detected, the type I cell begins to release neurotransmitter. This release of neurotransmitter then evokes increased firing of the carotid sinus nerve which ultimately innervates cells of the commissural subnucleus of the solitary tract in the brainstem (Finley & Katz, 1992) and causes an increase in respiration, such that blood gases are returned to physiological levels (Gonzalez *et al.*, 1994). Until recently it was thought that the type II cell was glial in function and acted as 'physiological glue' to adhere the tissue together. However, recent research has demonstrated a more complex role for the type II cells. They have been found to have neuroprogenitor or stem cell-like properties. Under chronic hypoxia carotid bodies undergo hyperplasia and hypertrophy (Bee *et al.*, 1986). It is now known that the Type II cells are capable of division and formation of type I cells under chronic hypoxic conditions (Pardal *et al.*, 2007).

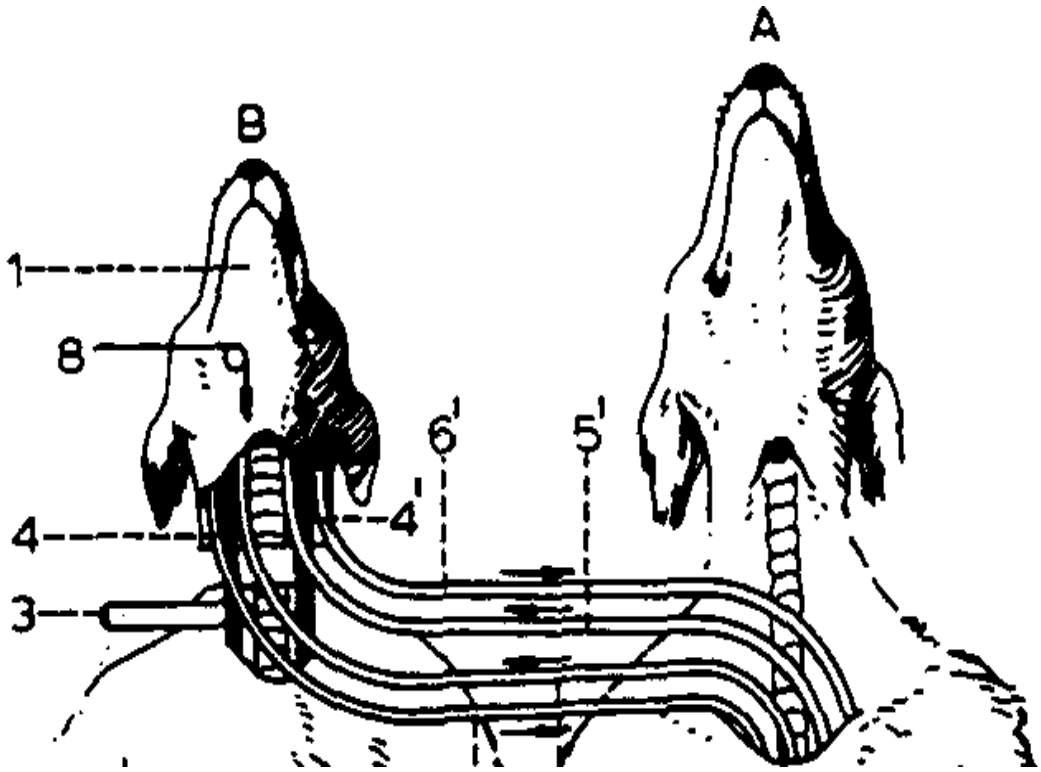
Fig 1. Structure and ultrastructure of the carotid body. A, Shows the local anatomy of the carotid body (CC, common carotid; EC, external carotid; IC, internal carotid). B. Shows the cellular ultrastucture of a glomoid (cluster of type I and II cells) within the carotid body (CSN, carotid sinus nerve). Reproduced from Pardal et al (2007).


A
carotid sinus
nerve
IC
EC
carotid
body
CC
B
type I cell
single CSN afferent
type II cell
capillary

History

The carotid bodies role as a chemosensory organ, and not a gland, was first identified by De Castro (De Castro, 1928). Proving that fibers from the carotid sinus nerve were sensory and not secretomotor, De Castro postulated that the carotid bodies function was to detect changes in the chemical composition of the blood and that special cells within the carotid body were the sensors (De Castro, 1928; Gonzalez *et al.*, 1994). This postulate was later confirmed by Corneille J. F. Heymans, who won the Nobel Prize in Physiology or Medicine in 1938 with his ingenious work on canines. By performing experiments on the isolated head of a canine (Fig 2) Heymans was able to show the carotid body's mediation of hyperventilation due to decreases in PO_2, pH, or increases in PCO_2 (Heymans, 1930). In Fig. 2, dog A was subjected to periods of hypoxia resulting in an increase in respiratory rate in dog B. This elegantly conducted research was pinnacle in defining the carotid body as the primary peripheral chemoreceptor.

Fig 2. This figure portrays the isolation of the canines head, connected with anastamoses to the isolated trunk of another animal. Arterial blood from dog A passes into the common carotid arteries of dog B below the level of the carotid bifurcation. Hypoxic gas given to dog A resulted in increased breathing movements in Dog B as hypoxaemic blood stimulated the carotid bodies of dog B.


A
B
1
8
6'
5'
4'
4
3

Chemotransduction by the carotid body

It is currently accepted that the basic mechanism of carotid body chemotransduction is that hypoxia, or a decrease in blood oxygen, and hypercapnia, an increase in blood CO_2, inhibit K^+ channels in the type I cell membranes leading to depolarization, voltage-gated Ca^{2+} entry and neurotransmitter release (Iturriaga & Alcayaga, 2004). However, type I cells release a wide variety of neurotransmitters in response to chemostimuli and the mechanisms by which these neurotransmitters regulate the output of the CSN are complex, exhibiting marked inter (and intra-) species differences (Shirahata *et al.*, 2007). Furthermore, both excitatory and inhibitory neurotransmitters are released in a tightly regulated manner by type I cells in response to chemostimuli with the overall response of the CSN being determined by the balance of excitation and inhibition. Indeed, modulation of the balance of excitatory to inhibitory neurotransmitter release has been proposed to underpin, in part, plasticity of the carotid body during chronic hypoxic conditions (Prabhakar, 2006).

Most recently it has been demonstrated that histamine plays a role in arterial chemoreception (Dutschmann *et al.*, 2003), being synthesized and released in response to hypoxia by carotid body type I cells (Koerner *et al.*, 2004). Moreover, application of histamine receptor 1 and 3 agonists to locally perfused carotid bodies mimicked the chemoreceptor reflex evoked by cyanide (a potent chemostimulant (Heymans *et al.*, 1931)) in a decerebrate rat preparation by increasing phrenic nerve output (Lazarov *et al.*, 2006). However, the precise mechanism by which histamine acted at the level of the type

I cells was not examined. An investigation of the role of histamine at the level of the type I cell is the focus of this thesis.

Role of the Type I cell as a Chemoreceptor

It is now widely accepted that the Type I, or glomus cells, are the main chemosensory elements within the carotid body (Verna *et al.*, 1975) and the work by Fricke Pietruschka on isolated Type I cells (Pietruschka & Acker, 1985) has allowed researchers to explore further into how these cells respond to changes in the partial pressure of oxygen.

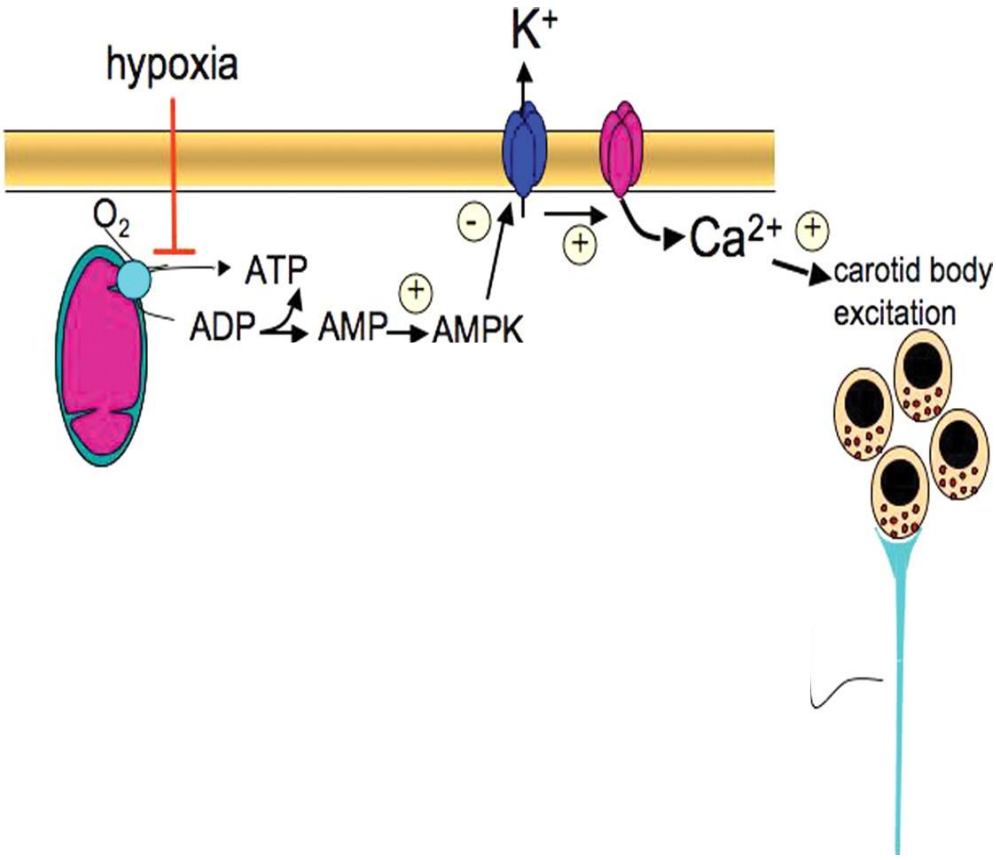
Throughout the past couple of decades research has shown that hypoxic inhibition of different K^+ channels within the Type I cells was crucial for chemotransduction to occur (Lopez-Barneo *et al.*, 1988; Stea & Nurse, 1991; Wyatt & Peers, 1995). Although the exact K^+ channel is different from species to species, the general mechanism of transduction was the same. A hypoxic event causes the closure, or inhibition, of K^+ channels leading to depolarization, calcium influx through voltage-gated calcium channels (Buckler & Vaughan-Jones, 1994b), and the eventual neurotransmitter release (Gonzalez *et al.*, 1994). Although this general mechanism is agreed upon, the actual process behind how these K^+ channels are inhibited continues to be a heated topic for debate. Carotid body researchers are constantly theorizing about what the exact mechanism is behind the hypoxic closure of these channels.

The Mitochondrial Hypothesis

Mitochondria are involved with the process of oxidative phosphorylation. When oxygen is delivered to the cell through the blood stream, mitochondria use it to produce

energy. It has been shown that application of inhibitors of the mitochondrial electron transport chain (ETC) or mitochondrial uncouplers to the carotid body increase the afferent activity of the carotid sinus nerve (Mills & Jobsis, 1972). Therefore, inhibiting oxidative phosphorylation will cause in increase in the amount of neurotransmitters released from the type I cell further stimulating the carotid sinus nerve. This proposal was supplemented by research finding that hypoxia and cyanide, an inhibitor of mitochondrial complex IV, lead to a rise in intracellular Ca^{2+} in dispersed, isolated, glomus cells (Duchen & Biscoe, 1992a, b). Further research conducted on the carotid body has shown that hypoxia may signal via a cascading chain of events. Decreasing the amount of oxygen a cell has available may ultimately decrease the amount of ATP that mitochondria can produce. In most cells oxygen has to be dropped to extremely low levels before oxidative phosphorylation is inhibited. However, this is not the case in the carotid body and physiologically relevant levels of hypoxia will cause inhibition of Type I cell mitochondrial oxidative phosphorylation (Duchen & Biscoe, 1992a, b). A decrease in ATP production could trigger a mechanism that might cascade down to membrane bound potassium channels, cause them to close, and thus depolarize the cell (Varas *et al.*, 2007). Another model of a mitochondrial cascade mechanism hypothesizes that by decreasing the amount of ATP production the ATP/AMP ratio will be reduced (Wyatt & Evans, 2007). This in turn would elicit a response from an enzyme, AMP-activated protein kinase (AMPK), which couples to the potassium channels resulting in their closure (see Fig. 3). Research testing this hypothesis is ongoing (Wyatt *et al.*, 2007).

Fig 3. Schematic showing the AMPK theory in detail. Hypoxia inhibits ATP production by mitochondria. Adenylate kinase converts rising levels of ADP to ATP and AMP causing AMP levels to rise. AMPK is activated and inhibits the 'O_2-sensitive' K^+ channels leading to Ca^{2+} influx and neurotransmitter release.


hypoxia
K+
O2
ATP
ADP
AMP
AMPK
Ca2+
carotid body
excitation

The Membrane Hypothesis:

During a hypoxic event the type I cell depolarizes, causing an influx of Ca^{2+} through voltage-gated Ca^{2+} channels leading to release of transmitters, and afferent nerve activation (Gonzalez *et al.*, 1994). This was believed to happen through the direct inhibition of K^+ channels within the membranes of type I cells (Lopez-Barneo *et al.*, 1988).

Although there is a huge variety of K^+ conductance channels within the membrane of the Type I cell (Patel & Honore, 2001) there seems to be several classes of more concern and importance than the others. One class is the large-conductance Ca^{2+}-activated K^+ channels also known as BK_{Ca} channels (Lahiri *et al.*, 2005). The research conducted on these channels has shown their importance in the hypoxic response of type I cells (Peers, 1990). The BK_{Ca} channels are thought to help control, or regulate, the excitatory hypoxic response of type I cells by closing during hypoxic membrane depolarization perhaps to sustain and prolong this depolarization (Lahiri *et al.*, 2005; Peers & Wyatt, 2007). Importantly, this occurs despite the rise in $[Ca^{2+}]_i$ that would activate the BK_{Ca} channels

To determine how hypoxia modulates BK_{Ca} channels the patch-clamp method was performed on individual type I cells. Many different types of experiments were performed on individual type I cells (Wyatt & Peers, 1995; Lewis *et al.*, 2002) and each showed that hypoxia alone could not directly inhibit the BK_{Ca} channels in isolated patches. Data showed that a cytoplasmic intermediary, one that is coupled to BK_{Ca} channels as well as chemically sensitive to drops in tissue O_2, was also involved in the excitatory hypoxic response within type I cells. However, there is also conflicting

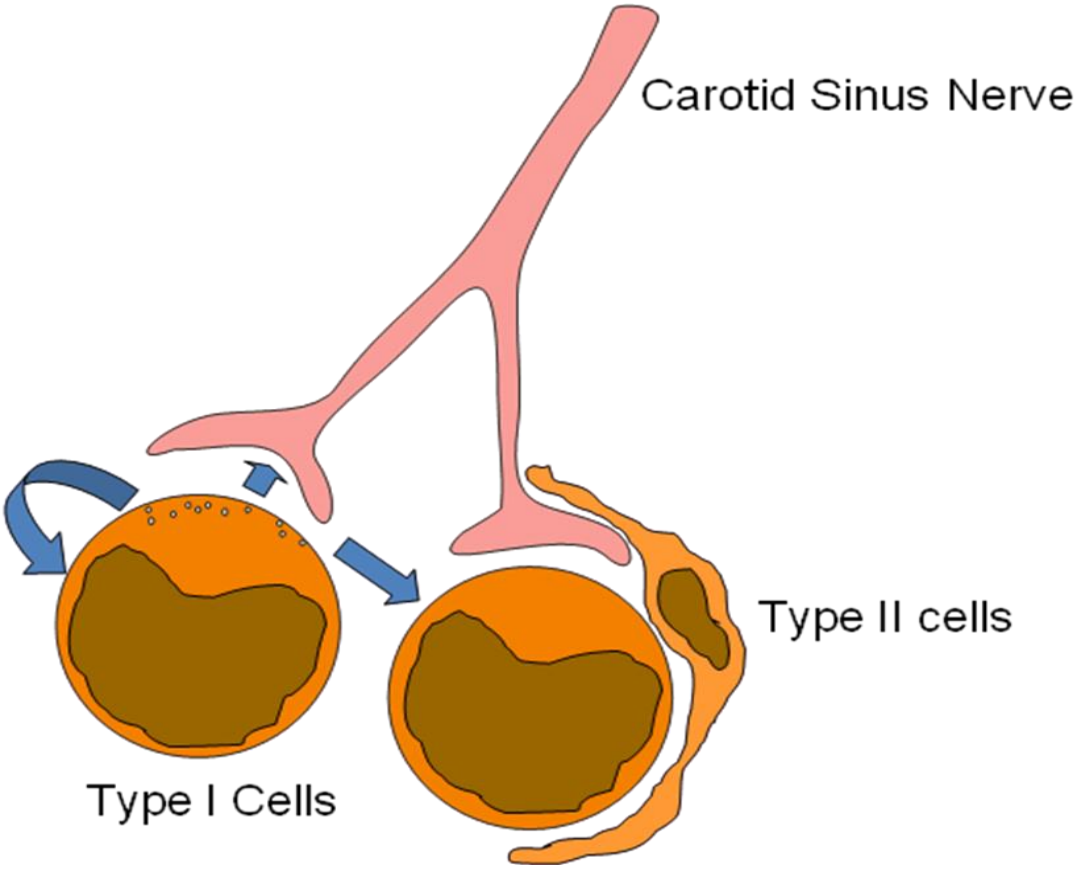
evidence indicating that hypoxia can inhibit BK_{Ca} in isolated patches (Riesco-Fagundo *et al.*, 2001). This issue remains unresolved.

A second class of oxygen-sensitive potassium channels in type I cells are background K^+ - channels, specifically those resembling TASK (TWIK-related Acid Sensing K^+) background channels. These channels show biophysical and pharmacological similarity to both TASK-1 and TASK-3 potassium channels and are consequently termed TASK-like background channels (Buckler *et al.*, 2006) as their molecular identity remains unknown. It has been shown that inhibition of these channels by hypoxia (Buckler, 1997; Buckler *et al.*, 2000) leads to membrane depolarization, calcium entry (Buckler & Vaughan-Jones, 1998; Wyatt & Buckler, 2004) and eventual neurotransmitter release (Ortega-Saenz *et al.*, 2003). Inhibition of oxidative phosphorylation in type I cells has also been shown to inhibit these leak conductances (Wyatt & Buckler, 2003, 2004). Applying known metabolic inhibitors to type I cells has been shown to mimic the response to hypoxia and in the presence of maximal concentrations of mitochondrial inhibitors hypoxia has no effect on the TASK-like current (Buckler *et al.*, 2006). This shows that the effects of mitochondrial inhibition and hypoxia on TASK-like background potassium channels are not additive, but mutually exclusive (Wyatt & Buckler, 2004; Buckler *et al.*, 2006), meaning, in order to generate the effects of hypoxia, mitochondrial metabolism must be active. Whether or not mitochondrial inhibition is directly coupled to background potassium channels is still a hot topic for debate, but what is known is that TASK- like background K^+ - channels play a central role in initiating the response of isolated type I glomus cells to a number of chemostimuli (Buckler *et al.*, 2006).

Neurotransmission within the rat carotid body

The carotid body is the key peripheral chemoreceptor for respiratory regulation within mammals. It accomplishes this task by communicating with other type I cells, as well as the CSN, through the use of neurotransmitters. The type I cell, for its size, has an enormously large nucleus capable of manufacturing a seemingly endless array of neurotransmitters, both excitatory and inhibitory. The excitatory neurotransmitters work both pre and post-synaptically (see Fig. 4), enhancing afferent nervous transmission to the brain and thereby increasing respiratory rate and tidal volume. Inhibitory neurotransmitters work pre-synaptically binding to receptors present on the membrane of the type I cells. These neurotransmitters work by decreasing the amount of excitatory neurotransmitter released, thus providing a negative feedback mechanism. Both types of neurotransmitters give the carotid body the ability to shape and refine its response to perturbations in blood gases. The next part of the introduction will discuss several of the major neurotransmitters released by type I cells and their functions relating to respiratory regulation.

Fig 4. Schematic showing neurotransmission within a glomoid in the carotid body. The blue arrows show how neurotransmitters may act postsynaptically on the carotid sinus nerve, presynaptically on the type I cell that has released the transmitter and also on other type I cells within the glomoid.


Carotid Sinus Nerve
Type II cells
Type I Cells

Excitatory Neurotransmitters: Post Synaptic

ACh and ATP – Acetylcholine and the co-release of ATP

Acetylcholine (ACh), already known to be an excitatory neurotransmitter released from the pre-synaptic terminals of motor neurons, has been linked to play an excitatory role in the modulation of breathing within the carotid body (Eyzaguirre & Zapata, 1968; Gonzalez *et al.*, 1994). ATP, a molecule used for energy, has also been shown to exhibit abilities as an extracellular signaling molecule within the central nervous system (Burnstock, 1997). Together, ACh and ATP are released from type I glomus cells of the carotid body in response to changes in blood chemistry (Zhang *et al.*, 2000). ATP then activates purinergic receptors of the afferent fibers of the carotid sinus nerve, increasing the rate of respiration during a hypoxic response.

Through the use of pharmacology as well as electrophysiology the effects of ACh and ATP on postsynaptic afferent neurons have been studied. When the carotid body is at rest, random spikes of depolarization occur in the CSN (Rong *et al.*, 2003), but the cause of these resting spikes of depolarization was unknown. Many neurotransmitter antagonists have been used to quiet these spikes, but nothing has provided conclusive results (Rong *et al.*, 2003). To determine the mechanism behind these spikes researchers in Colin Nurse's group investigated the role of ACh (a carotid body neurotransmitter that had fallen out of favor over the previous 15 years) and ATP in a novel type I cell-petrosal ganglion co-culture experiment (Zhang *et al.*, 2000).

Through the use of pharmacological reagents such as suramin (ATP receptor antagonist) and mecamylamine (neuronal nicotinic Ach receptor antagonist), it was proven that both ATP and ACh played a role in carotid bodies response to hypoxia

(Zhang *et al.*, 2000). In the normal control group recordings, random spiking in the petrosal neurons would occur throughout the testing period, but when hexamethonium or mecamylamine were applied (nicotinic ACh receptor blockers) the spontaneous activity was partially and reversibly repressed (Zhang *et al.*, 2000). As for ATP, when suramin was applied (P2 purinoceptor blocker) a partial suppression of spontaneous activity also occurred, even to those previously suppressed by mecamylamine (Zhang *et al.*, 2000). To demonstrate that the spontaneous synaptic events arose principally from co-release of ACh and ATP from Type I cells, both blockers were tested. The result was an almost complete abolishment of random spiking during the perfusion of suramin plus mecamylamine. As for when the cells were perfused with a hypoxic solution, the same results occurred. When each of the different blockers were applied there was a significant decrease in the firing in petrosal neurons (Zhang *et al.*, 2000).

ATP – Adenosine Tri-phosphate: Main excitatory neurotransmitter within the carotid body

Until recently, using antagonists to stop afferent impulses delivered from the CB, due to hypoxia, has produced conflicting results (Rong *et al.*, 2003). To determine the effects of ATP on purinergic P2X receptors subtypes within the carotid body (specifically $P2X_2$ and $P2X_3$), plethysmography as well as electrophysiology were used. Knock-out mice of $P2X_2^{-/-}$, $P2X_3^{-/-}$ and $P2X_2/P2X_3^{Dbl-/-}$ were created and experimented on using the above experimental techniques.

Plethysmography, or the use of pressure to determine changes in the respiration of an animal, was used first to determine the effects of hypoxia on knock-out mice as well

as their wild type littermates. Each group of knock-out mice as well as their wild type littermates were subjected to differing concentrations of O_2 starting at 21% O_2 and decreasing to 7.5% O_2. For the wildtype (WT) mice, a noticeable increase in respiratory rate as well as tidal volume were observed and recorded. Of the knockouts only the $P2X_2$ and double knockout showed significant results as compared to the WT using the student t-test. The results showed that removal of the $P2X_2$ receptor subtype resulted in an attenuation of the ventilatory response to hypoxia (Rong *et al.*, 2003).

In experiments involving electrophysiology, an intact carotid body – carotid sinus nerve preparation (Rong *et al.*, 2003) was used to study discharges from the afferent fibers of the carotid sinus nerve. Pharmacological studies were performed on this model using solutions of ATP as well as a stable analog α,βmetATP. In each of these experiments it was shown that ATP and its stable analog caused an immediate spike in discharge within the WT animals followed by a sustained increase for about 72-120 sec (Rong *et al.*, 2003). As for the knock-outs; the $P2X_2^{-/-}$ knockout showed a rapid increase to only ATP which lasted for only about 5 sec; $P2X_3^{-/-}$ did not show a rapid spike in discharge, but a sustained response after application of about 60sec; and the double knock-out showed no rapid increase and a very mild sustained response (Rong *et al.*, 2003).

These results show that ATP is acting on both receptors in different ways. The $P2X_3$ receptors are more easily desensitized and are what create the initial burst of afferent discharge, where $P2X_2$ receptors stay open longer allowing for discharges to continue over a longer period of time.

It is interesting to note that ATP can also activate purinergic P2Y receptors on the type I cells (Xu *et al.,* 2005). Activation of these receptors inhibited excitation in the type I cells and therefore served to slow or reduce the release of neurotransmitter. Consequently ATP can act as both an excitatory post synaptic transmitter and an inhibitory presynaptic inhibitor.

Excitatory Neurotransmitters: Pre- synaptic

5-HT: Seratonin

Paracrine and autocrine methods of cellular communication have been shown to help regulate the release of neurotransmitters within the carotid body (Zhang *et al.*, 2003). The monoamines (dopamine and serotonin) are a particular group of neurotransmitters released from the carotid body (Gonzalez *et al.*, 1994) that act pre-synaptically on type I glomus cells(Zhang *et al.*, 2003; Carroll *et al.*, 2005).

Serotonin, has been linked to the regulation of excitatory post-synaptic neurotransmitter secretion, ATP and ACh, from carotid body type I glomus cells (Zhang *et al.*, 2003). Serotonin works in a paracrine/autocrine method binding to 5-HT receptor sites present on type I cell membranes. During periods of normoxia, serotonin is released to create a stable resting membrane potential between adjacent cells. Working through a G-protein coupled receptor, 5-HT_{2a}, serotonin utilizes the second messenger, protein kinase C, to phosphorylate K^+ channels (TASK-like), closing them, resulting in a slight depolarization of the cell (Zhang *et al.*, 2003). It has also been shown that during a hypoxic event, serotonin is released creating a positive feedback mechanism. This results in a further depolarization of the cell and an enhanced

production, and release, of both excitatory post-synaptic neurotransmitters (Zhang *et al.*, 2003). Therefore, serotonin helps to both regulate, as well as help exacerbate the responses to chemostimuli perceived by the type I glomus cells.

Inhibitory Neurotransmitter: Pre-Synaptic

Dopamine

Of the two monoamines studied under carotid body physiology, dopamine has been studied in the greatest detail (Zhang *et al.*, 2003). This is because of the potential involvement of dopamine in neonatal fatality or sudden infant death syndrome. At birth the carotid body chemoreceptors have very low sensitivity to hypoxia and tend to become more sensitive after a couple of days, a process called resetting (Carroll *et al.*, 2005). Although the mechanisms of resetting are unknown, research has suggested the involvement of dopamine as a neuromodulator during this process (Carroll *et al.*, 2005). Similar to 5-HT, dopamine seems to be working through both paracrine and autocrine cellular communication methods. But, unlike 5-HT, dopamine acts as an inhibitory neurotransmitter, decreasing the amount of ATP and ACh released to the afferent fibers of the carotid sinus nerve (Carroll *et al.*, 2005).

Dopamine acting via D2 receptors in the carotid body of both mice and rats has been shown to inhibit the effects of hypoxia, resulting in a decrease in the ventilatory response (Carroll *et al.*, 2005; Prieto-Lloret, 2007). Dopamine receptor agonist/antagonists, as well as transgenic mice have been used to study this. In experiments using D2 receptor agonists (quinpirole) and antagonists (sulpiride), it was consistently shown that during periods of hypoxia, if dopamine is present, a decrease in

intracellular calcium concentration will occur (Carroll *et al.*, 2005). This decrease in intracellular calcium is correlated with a decrease in neurotransmitter release from the carotid body (Gonzalez *et al.*, 1994).

On the contrary, in D2 knockout mice there was not a significant decrease in ventilation as compared to their wildtype litter mates when both were subjected to periods of increasing hypoxia (Prieto-Lloret, 2007). These observations lead to the general conclusion that D_2 receptors contribute but are not essential to CB function or hypoxia transduction and play a role in modulating or shaping the secretory response to hypoxia (Prieto-Lloret, 2007).

Evidence for Histamine as excitatory neurotransmitter within the Carotid Body

Because biogenic amines have been found to contribute so greatly with respiratory modulation and control, researchers began wondering if histamine was also involved. It was recently found that Type I cells contain histamine and have the machinery to synthesize, store, and release it during periods of hypoxia (Koerner *et al.*, 2004) indicating a potentially significant role in arterial chemoreception.

In type I cells it was shown that histamine decarboxylase, the histamine biosynthesis enzyme necessary for the formation of histamine, was present (Koerner *et al.*, 2004). In this same study it was also shown that the packaging materials necessary for vesicular transport of histamine were also present. Histamine requires vesicular monoamine transporters VMAT1 and VMAT2 in order to be released from the cell. Both of these were found using antibodies directed against both transporters (Koerner *et al.*,

2004). This would suggest that type I cells have the capability to produce as well as release histamine if required.

Histamine is known to act via specific receptors within the mammalian body. These receptors H1, H2, H3, and H4 all act via G-protein coupled second messenger systems, but each elicit a different response when triggered. It has been shown that H1 and H3 are both present within rat carotid bodies (Koerner *et al.*, 2004; Lazarov *et al.*, 2006), but it has yet to be shown whether these receptors are present solely on the plasma membrane of Type I glomus cells.

By exposing the carotid body to histamine receptor 1 and 3 agonists, it was shown that phrenic nerve activity increased in a working heart-brainstem preparation (Lazarov *et al.*, 2006). This would suggest that histamine excites the carotid sinus nerve, increasing respiration during a period of hypoxia. What is not known is whether histamine is acting in a presynaptic (on the Type I cells) or postsynaptic fashion (directly on the sinus nerve) within the carotid body. Although not discussed in the Lazarov paper, some of the ways histamine could be acting in an excitatory manner are defined in the next few pages.

H1 receptor

H1 Receptor activation preferentially couples to Gαq leading to stimulation of phospholipase C (PLC) which catalyses the hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP2) to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Hill *et al.*, 1997). Release of IP3 may cause release of Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC). This PKC activation may lead to Ca^{2+} dependent cAMP accumulation, although $G_{\beta\gamma}$ subunits released from Gq may also contribute to a

rise in cAMP. More interestingly it has recently been demonstrated that Gαq may directly inhibit TWIK related acid-sensing potassium (TASK) channels by a mechanism that is independent of PLC-mediated PIP2 hydrolysis (Chen *et al.*, 2006). PKC activation has also been shown to inhibit TASK-1 (Besana *et al.*, 2004). Thus H1 activation alone could account for IP3-mediated release of Ca^{2+} from Type I cell stores and TASK channel inhibition (by direct G protein interaction and by PKC) leading to depolarization and voltage-gated Ca^{2+} entry. This potential ability to modulate TASK is interesting as inhibition of TASK-like K^+ channels has been shown to excite rat Type I cells (Buckler *et al.*, 2000). In addition PKC activation inhibits BK_{Ca} in rat Type I cells (Peers & Carpenter, 1998) and this would sustain any depolarization evoked by histamine.

H2 receptor

H2 receptor activation preferentially couples to the G_s family of G proteins which are positively coupled to adenyl cyclase, causing an increase in cAMP and thus activation of protein kinase A (PKA). A PKA dependent pathway has recently been shown to inhibit the TASK-like channels that contribute to setting the resting membrane potential in rat carotid body type I cells (Xu *et al.*, 2006; Xu *et al.*, 2007). Thus a H2 receptor mediated rise in cAMP could also lead to TASK channel inhibition, cell depolarization and voltage-gated Ca^{2+} entry.

H3 and H4 receptors

H3 and H4 receptor activation preferentially couples to the G_i family of G proteins which are negatively coupled to adenylate cyclase and would tend to decrease

cAMP. This would theoretically oppose the actions of histamine at H2 receptors via inhibition of adenylate cyclase and hence inhibition of PKA. Indeed, in general the H3 receptors are thought of as inhibitory auto receptors (Arrang *et al.*, 1983, 1985). However, there are limited reports suggesting that H3 receptor agonists can mobilize Ca^{2+} from intracellular stores (Bongers *et al.*, 2006).

The research conducted in this thesis project utilized isolated carotid body Type I cells and not the carotid body as a whole. This method was used to determine whether histamine acts presynaptically on the Type I cells to further excite or inhibit the cell during a period of hypoxia.

Summary

The carotid bodies lies at the bifurcation of the common carotid arteries, usually directly adhered to the internal carotid artery. They are composed of two different types of cells: The Type I glomus cell, which is chemosensory in nature, and the Type II cell, which is more glial-like. The Type I cell has the ability to react to changes in blood gases, as well as pH. During an adverse event, such as hypoxia or hypercapnia, the Type I cell releases neurotransmitters which act on afferent fibers of the carotid sinus nerve. These excitatory messages are sent to the medulla resulting in compensatory ventilatory adjustment.

Type I cells release many different neurotransmitters when activated by changes in blood chemistry. These neurotransmitters can either be excitatory (increasing respiratory rate) or inhibitory (decreasing respiratory rate). Furthermore, released

neurotransmitter may work in a presynaptic or postsynaptic fashion. The focus of the research performed in this thesis is on the neurotransmitter histamine. Histamine receptor agonists applied to intact rat carotid bodies have been shown to elicit increased firing in the rat phrenic nerve. Thus histamine receptor activation in the rat carotid body appears to be excitatory. The purpose of the work presented in this thesis was to determine if histamine could act presynaptically on the type I cells rather than directly on the carotid sinus nerve endings. Presynaptic Type I cell excitatory activity would amplify the hypoxic response possibly by augmenting the release of identified postsynaptic excitatory neurotransmitters such as ATP and Acetylcholine.

CHAPTER III

MATERIALS AND METHODS

All studies described in this paper were performed in accordance with protocols approved by the Wright State University Institutional Laboratory Animal Care and Use Committee (IACUC).These protocols are in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH publications No. 80-23) revised 1996.

Dissection and Dissociation of Neonatal Rat Carotid Body Type I Cells

On each day of experimentation two neonatal Sprague Dawley rats (aged 10-20 days) were placed into an induction chamber supplied with 4.5% isofluorane and oxygen to initially anesthetize and immobilize them. When unconscious, the rat was removed from the induction chamber and its head was carefully placed into a gas mask also supplied with the same mixture of anesthetic gas. Once deemed completely asleep (foot pinch withdrawal reflex was absent) the following procedure was performed: The rat was placed on its back with both forelegs and hind legs taped down, reducing any movement caused by the procedure. An initial incision was then made along the breast bone to expose the underlying subcutaneous fascia. In order to expose the common carotid artery, salivary glands as well as skeletal muscles laying lateral to the trachea were removed with very fine forceps (Moria, Fine Science Tools, USA), being careful not to cut or damage any of the surrounding arteries and veins.

The remaining dissection was done under low magnification using a dissection microscope (Omâno, Japan). Once the common carotid artery became visible, any bits of fat and fascia were removed to expose the bifurcation of the common carotid arteries external and internal branches. Removal of both the glossopharyngeal nerve and

occipital carotid artery revealed the small carotid body, usually adhered to the internal carotid artery (see Fig. 1 in the Introduction). The organ was then tweezed off very carefully, making sure to keep its integrity and placed directly into ice cold, oxygenated, Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} (to diminish neurotransmitter release) or Mg^{2+} (Sigma). The rats were then humanely killed by decapitation while still deeply anesthetized and disposed of accordingly to Lab Animal Research specifications. The organs were then transported to the lab and cleaned of any connective tissue or debris under increased magnification on the Omâno dissecting microscope.

Once cleaned the carotid bodies were then transferred to a digestive enzyme solution (0.4mg ml^{-1} collagenase type I, 220u mg^{-1} (Worthington Biochemical Corporation), 0.2 mg ml^{-1} trypsin type I, 10,100 BAEE u mg^{-1} (Sigma) in DPBS with low $CaCl_2$ (86 μM) and $MgCl_2$ (350 μM)) for 20 min at 37°C in order to degrade the connective tissue holding the organ together. The carotid bodies were then tweezed apart and placed back into the incubator for a further 7 min digestion. The tissue was then removed from the Petri dish and transferred to a test tube using a fire polished, silanized (Sigmacote, Sigma), Pasteur pipette; where it was triturated and then centrifuged at 110g for 5 min. Cells were resuspended in tissue culture medium (Ham's F12 (Sigma) supplemented with 10% heat inactivated fetal bovine serum (Biowest)), centrifuged again for 5 minutes at 110g, resuspended in tissue culture medium and plated onto 22 mm^2 poly-d-lysine coated coverslips for immunocytochemistry or 12 mm diameter poly-d-lysine (Sigma) coated glass coverslips for imaging. Coverslips were placed in 35mm diameter plastic Petri dishes and maintained at 37°C in a humidified, 5% CO_2 / air incubator. Cells were allowed to adhere to the coverslips for 2 hours and all cells were

used for experimentation within 8 hours of plating. Maintaining type I cells in tissue culture for longer periods of time results in changes to the physiological properties of the type I cells (Tse, 1996).

Immunocytochemistry

Coverslips with attached type 1 cells were fixed by immersion in methanol at -20˚C for 20min. Cells were then permeablized by 3 x 5min washes with 0.3% triton X-100 (Sigma) in phosphate buffered saline (PBS). Cells were given 3 x 5min washes with a blocking solution (1% bovine serum albumin, 1% donkey serum, 0.3% triton X-100 in PBS) to limit non-specific binding of the specific anti-histamine receptor antibodies. All anti-histamine receptor 1˚ antibodies were diluted 1:100 with blocking solution, added to the coverslips and incubated at 4˚C for 16 hours.

The specific selective anti-histamine receptor antibodies for receptors H1R, H2R and H4R were rabbit polyclonal antibodies (Santa Cruz Biotechnology: sc20633, sc-50315, sc-50313) and the selective anti-histamine antibody for receptor H3R was a goat polyclonal antibody (Santa Cruz Biotechnology: sc33977). Cells were also stained with a mouse anti-tyrosine hydroxyase antibody (1:2000, Sigma, T1299) to assist identification of type I cells. Following incubation with the 1˚ antibodies, coverslips were washed 4 x 5min with blocking solution and then incubated for 1 hour, at room temperature in the dark with 1:200 dilution of Rhodamine Red-X-conjugated affinipure donkey anti-rabbit IgG (Jackson Immunoresearch, for H1R, H2R and H4R), Rhodamine Red-X-conjugated affinipure donkey anti-goat IgG 2˚ antibody (Jackson Immunoresearch, for H3R) and FITC conjugated donkey anti-mouse IgG 2^{o} antibody (Jackson Immunoresearch, for

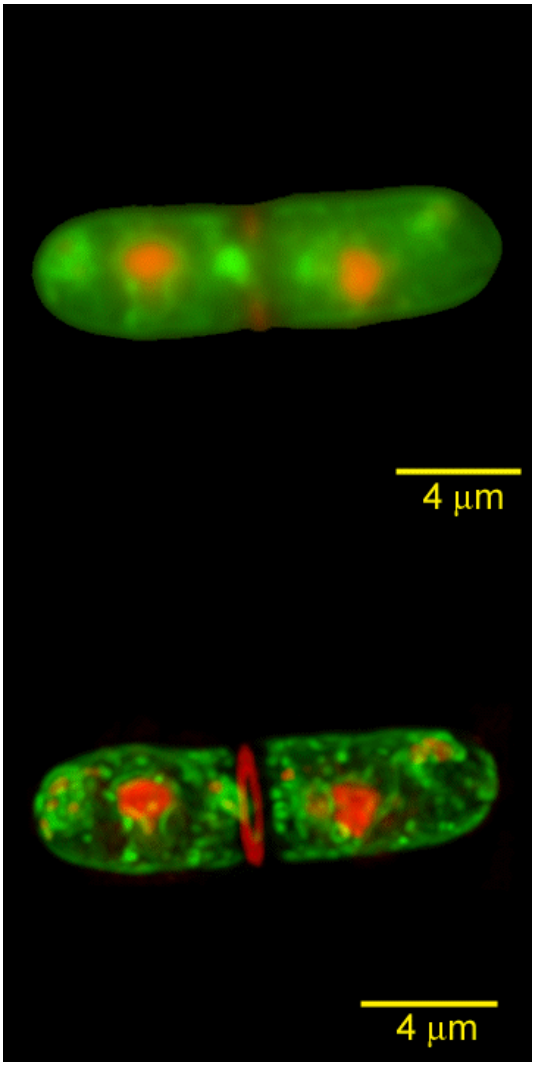
tyrosine hydroxyase). Coverslips were then washed for 5 x 5min with PBS before being inverted and attached to microscope slides with hard setting anti-fade mountant (2.4 g Mowiol 4-88, 6 g glycerol, 6 ml H_20, 12ml 0.2M Tris buffer pH 8.5, 2,5% diazobicyclo-octane) containing 4', 6-diamidino-2-2-phenylindole dihydrochloride (DAPI, 1mg ml^{-1}) for visualization of type I cell nuclei. For controls, 1˚ antibody was omitted during this procedure. As a further negative control histamine receptor staining was examined in undifferentiated NG108-15 cells that were processed in an identical manner to the carotid body cells.

Images were acquired using a DeltaVision microscope system (Applied Precision) on an inverted Olympus IX71 microscope with an oil immersion, x63 magnification, 1.4 n.a. objective and Coolsnap HQ CCD camera (Photometrics). Multiple z-sections (focal depth 0.28 μm, z-step 0.25 μm) were taken through individual cells. Images were deconvolved on-line via Softworx software (Applied Precision).

Deconvolution Microscopy

Deconvolution microscopy uses algorithms in order to redistribute out of focus light. When taking pictures of 3D images, such as cells, not all parts of the sample are in the same plane. This can cause the image to become blurred and out of focus. What deconvolution microscopy does is re-route the out of focus light, using specific algorithms in order to make all light shine from the same plane. This creates an image that appears to be much brighter, closer and in focus (Fig 5).

Fig 5. Image showing the effect of applying a Deconvolution algorithm to data acquired using a conventional light microscope. The top, blurry image is from raw data and the bottom is post-deconvolution. Image taken from http://micro.salk.edu/dv/dv.html


4 μm
4 μm

Fura-2AM

Fura-2AM is a molecular probe that is used to determine intracellular calcium concentrations through fluorescence. The acetoxymethyl ester portion of this probe allows for the molecule to diffuse easily through the plasma membrane of the cell, enabling researchers to avoid using more invasive techniques for loading. Once inside the cell, the AM portion is cleaved off by the cell's esterases and the cell-impermeant fluorescent indicator is left behind. Upon binding Ca^{2+}, Fura-2 exhibits an fluorescence shift that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at ~510 nm (invitrogen.com). In these experiments the Fura-2 was excited by exposing it to both 340nm and 380nm wavelengths of light while monitoring emissions at 510nm. Calculations made by looking at the ratios of emitted light evoked by 340nm/380nm excitation allowed for us to determine the intracellular concentration of calcium at specific instances in time (see below):

$$[Ca] = Kd\,(S_{f2}/S_{b2})(R_{exp} \times R_{min}/R_{max} - R_{exp})$$

$Kd = 224 \times 10^{-9} M$

S_{f2} = intensity 380nm Ca free
S_{b2} = intensity 380nm Ca saturated

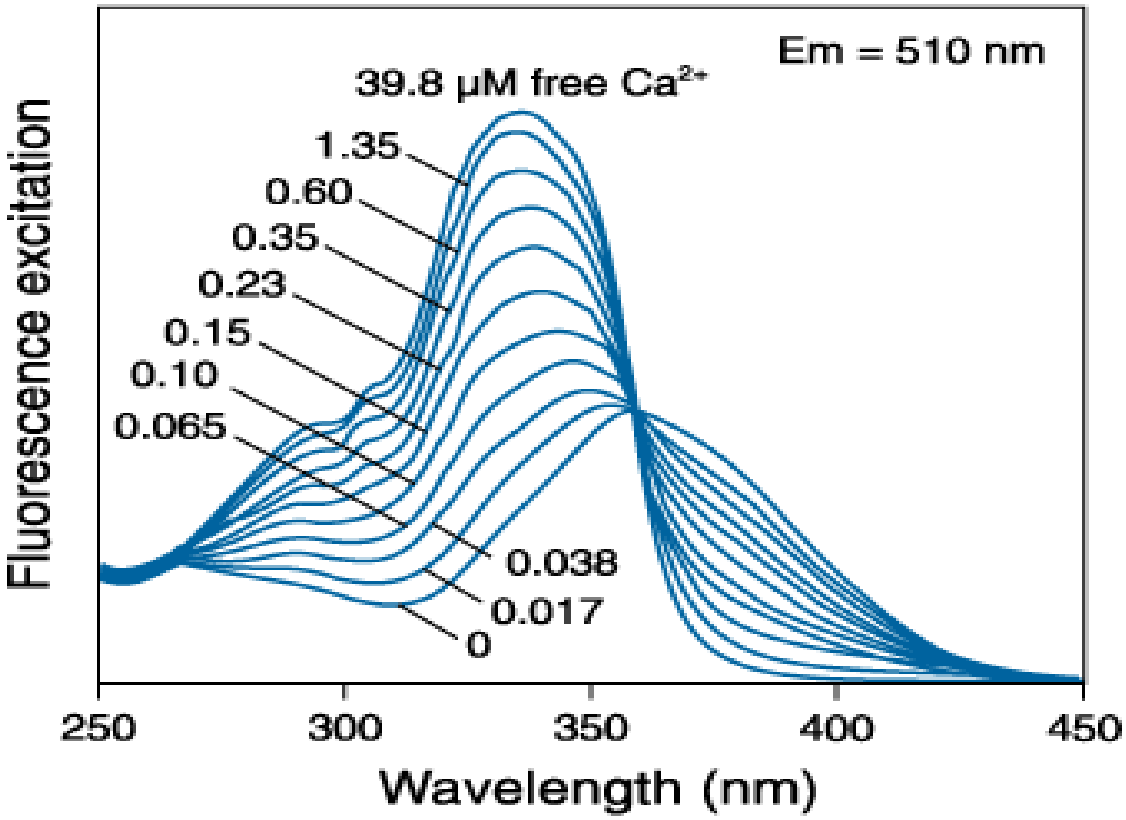
R_{exp} = measured experimental ratio
R_{min} = 0 Ca ratio
R_{max} = Ca saturated ratio

Thus the measured experimental ratio is directly proportional to the calcium concentration within the cell and can be calculated by calibrating the system using a Ca ionophore and a 0mM Ca^{2+} solution and a saturating Ca^{2+} solution (typically 5mM). Additionally, by using two different wavelengths of light to measure concentration, we

automatically canceled out variables such as dye bleaching and cell thickness that can result in unwanted artifact (Figure 6).

Fig 6: **Fluorescence emission of Fura-2 at differing wavelengths**

This graph shows the difference in fluorescence of Fura-2 due to excitation wavelength and intracellular free-calcium concentration. The dye was excited at both 340 nm and 380 nm wavelengths of light. At an intracellular Ca^{2+} concentration of zero, the fluorescence given off when excited with 340nm light is less than that at 380nm when viewed at 510nm. As intracellular calcium concentrations increase, the fluorescence at each wavelength changes such that more light is emitted when excited at 340nm. Image is taken from Molecular Probes catalogue and shows responses of Fura-2 to changes in Ca^{2+} in a cuvette.

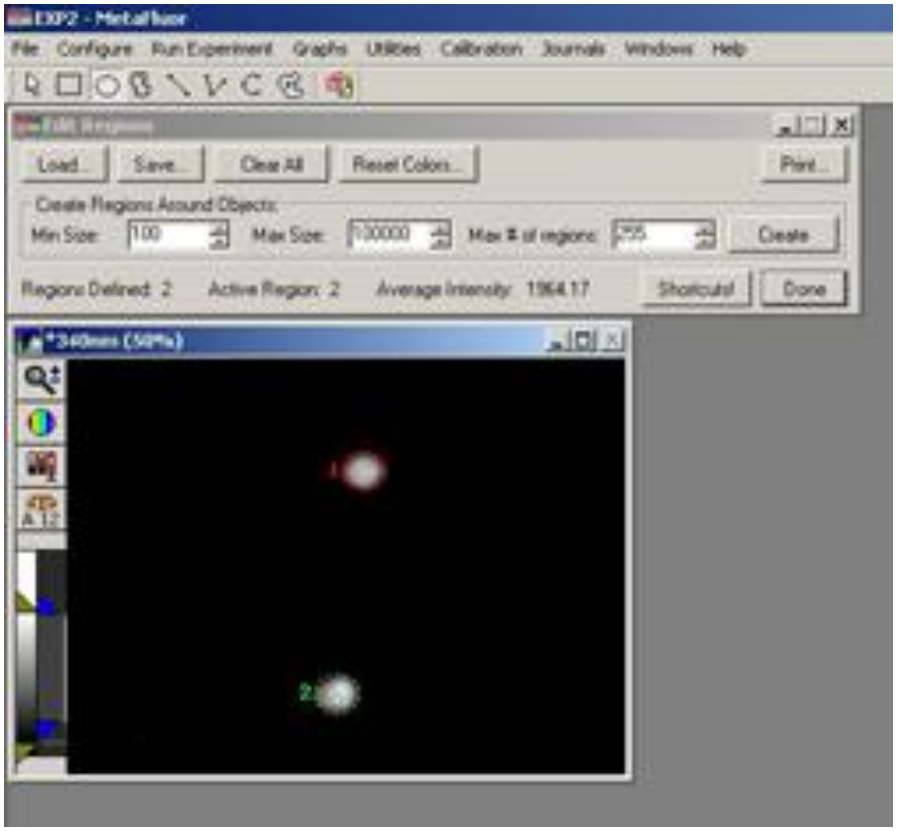

Em = 510 nm
39.8 µM free Ca2+
1.35
0.60
0.35
0.23
0.15
0.10
0.065
0.038
0.017
0
Fluorescence excitation
250
300
350
400
450
Wavelength (nm)

Ca^{2+} imaging

Cells were visualised with a Nikon TE2000U inverted microscope with a CFI super fluor x40 oil immersion objective. Fura-2 was excited with 50 msec exposures to 340 nm and 380nm light at 0.2 - 0.5 Hz using a Lambda 10- 3 filter wheel (Sutter) and emitted fluorescence measured at 510 nm using a CoolSNAP HQ2 CCD camera. Light was provided by a Lambda-LS xenon arc lamp (175 watt, Sutter). Because of the extremely high wattage of this lamp, the light was first passed through neutral density filters of 0.7 optical densities (Chroma, USA) before reaching the cellular specimens.

Neutral density filters are capable of decreasing the intensity of light from all wavelengths equally, therefore lowering the power of light from the source. Data acquisition and analysis was controlled using Metafluor 7.1.2 imaging software (Molecular Devices). 340/380 ratio images were generated on-line and time courses showing changes in the fluorescence ratio assessed by placing regions of interest over the type I cell images (see figure D). Only cells that responded to an 80mM K^+ challenge (KCl replaced NaCl in equimolar amounts, 80mM KCl was used to evoke maximal Ca^{2+} influx) with a rapid, robust and reversible increase in the fluorescence ratio were selected for study.

Fig 7: This figure is a screenshot from the Metafluor acquisition program. The program gives us the ability to select specific regions of interest for data acquisition as indicated by the dotted green and red lines. The two pale circles are type I cells excited at 340nm.


File Configure Run Experiment Graphs Utilities Calibration Journals Windows Help
Load... Save... Clear All Reset Colors... Print...
Create Regions Around Objects:
Min Size: 100 Max Size: 100000 Max # of regions: 255 Create
Regions Defined: 2 Active Region: 2 Average Intensity: 1964.17 Shortcuts! Done
1
2

Perfusion of Isolated Type I Cells

Isolated cells, plated on round coverslips, were placed into the perfusion chamber (0.16ml) and washed with HEPES buffered saline solution (34-36˚C) for approximately 5-7min. Temperatures were maintained at 34-36^{o}C by passing solutions through an in-line heater (SH-27F, Warner Instruments, USA) which was feedback controlled by an automatic temperature controller (TC-344B, Warner Instruments, USA).

After looking for cells through the Nikon TE2000U inverted microscope with a CFI super fluor x40 oil immersion objective, a group of cells were eventually chosen and data acquisition began using Metafluor 7.1.2 imaging software. Metafluor allows the researcher to select a specific area of interest within the field of view of the microscope. This program then plots the fluorescence intensity given off by the Fura-2 dye, at 510nm and converts it to a ratio graphically. Solution changes were accomplished by switching the solution inflow to a chamber containing the solution of choice; all solutions were perfused by gravity. Solution exchange in the chamber was usually complete within 5 seconds.

Statistical Analysis

Data are presented as means ± standard error of the mean. Where appropriate data were analyzed using students' paired t tests, a value of $P < 0.05$ was considered significant.

CHAPTER IV

RESULTS

Immunocytochemistry

We used anti-histamine receptor antibodies to determine the subtypes of histamine receptors expressed in isolated neonatal rat type I cells. All cells tested demonstrated strong staining for H1R, H2R, H3R and H4R and cytoplasmic staining for tyrosine hydroxylase (Figs. 8 and 9). Each receptor was stained on three different days with different cell isolates similar profiles were seen each time. Results are described qualitatively rather than quantitatively.

Staining Profiles for Histamine Receptors

H1 Receptor: Staining indicated that receptors were targeted to the cell membrane and also the cytoplasm (Fig. 8, H1). Little staining was seen in the nuclear space (see Fig 9, H1 where DAPI staining has been omitted).

H2 Receptor: Staining was present at the membrane and throughout the cytoplasm. The strongest staining was cytoplasmic (Figs. 8 & 9, H2) and some low level nuclear staining could also be observed (Fig. 9, H2).

H3 Receptor: H3 receptors were very clearly targeted to the type I cell plasma membranes (Figs. 8 & 9, H3) with little or no cytoplasmic staining. Nuclear staining could be observed in some cells (Fig. 9, H3) but this was variable and not every type I cell showed this pattern of distribution (see Fig. 8, H3 for an example where nuclear staining was not observed).

H4 Receptor: H4 receptors were also strongly targeted to the type I cell plasma membrane (Figs.8 & 9, H4). The H4 receptors also showed some cytoplasmic distribution but little or no nuclear staining was observed.

No staining was observed in control type I cells where 1˚ antibodies were omitted see Figs. 8 & 9, Con H1,2,3,4). Additionally the histamine receptor staining in undifferentiated NG108-15 cells was diffuse and of similar intensity to that seen when the 1˚ antibodies were omitted (data not shown) indicating that 1˚ antibodies were not non-specifically binding to cell membranes. Undifferentiated NG108-15 cells do not express histamine receptors (no citations found) and so any staining seen in these cells would be as a result of non-specific antibody interactions.

These results suggest, that in the neonatal rat carotid body, histamine released from type I cells may bind presynaptically to 4 histamine receptor subtypes. All four Histamine receptor subtypes showed staining at the plasma membranes. H3 and H4 receptors showed the clearest membrane targeting followed by H1 and H2 receptors. However, variation did occur and an attempt to quantify the staining data could be made to try and determine which receptor predominates at the membrane. This work was outside the scope of this thesis but is commented on in the discussion.

Activation of all 4 histamine receptors could engage a multitude of G-protein coupled second messenger systems ($G_{i/o}$, G_q, and G_s) which could excite or indeed inhibit type I cells. Furthermore, all 4 histamine receptors may signal via modulation of intracellular Ca^{2+} $[Ca^{2+}]_i$, see introduction. Therefore we used Fura-2-fluorescent imaging to assess the effect of histamine (10μM, 100μM and 300μM) on $[Ca^{2+}]_i$ in isolated neonatal rat type I cells.

Fig 8. Histamine receptor subtype immunofluorescence in isolated carotid body type I cells. Images show example z-sections taken through the center of type I cells. The fluorescent red color marks places where H1, H2, H3 and H4 receptors were found using immunocytochemistry. The fluorescent blue color represents the nucleus of Type I cells. Control images show absence of 2˚ antibody staining when 1˚ antibodies were absent.

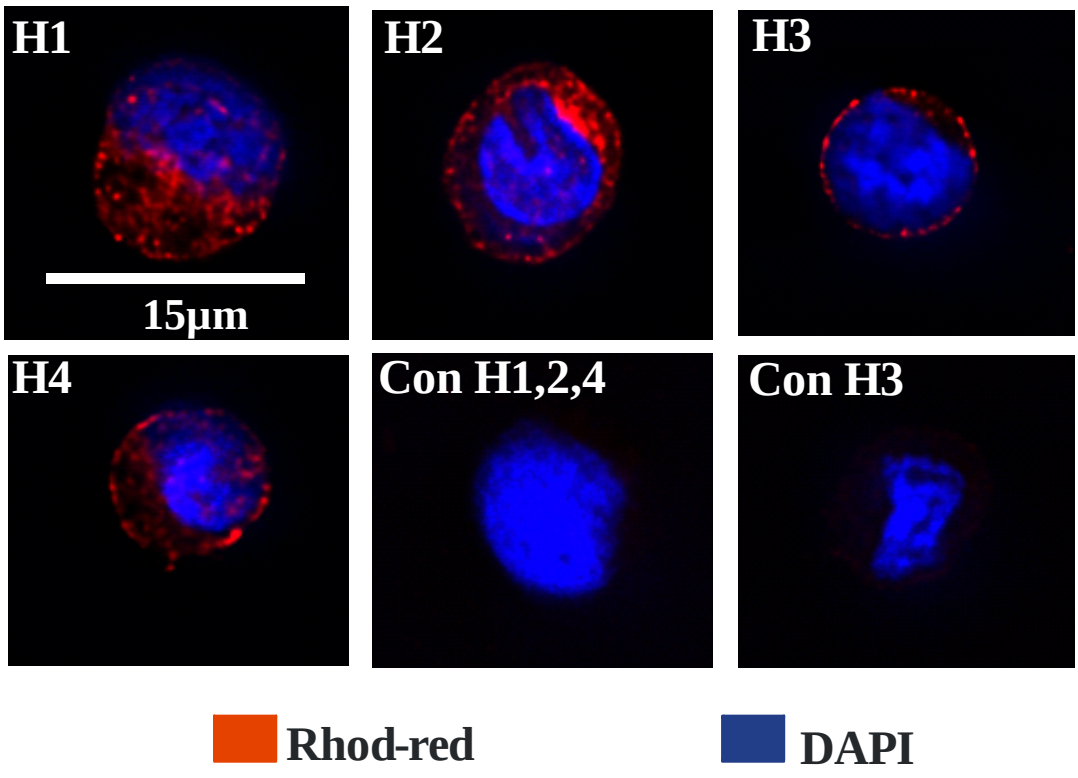
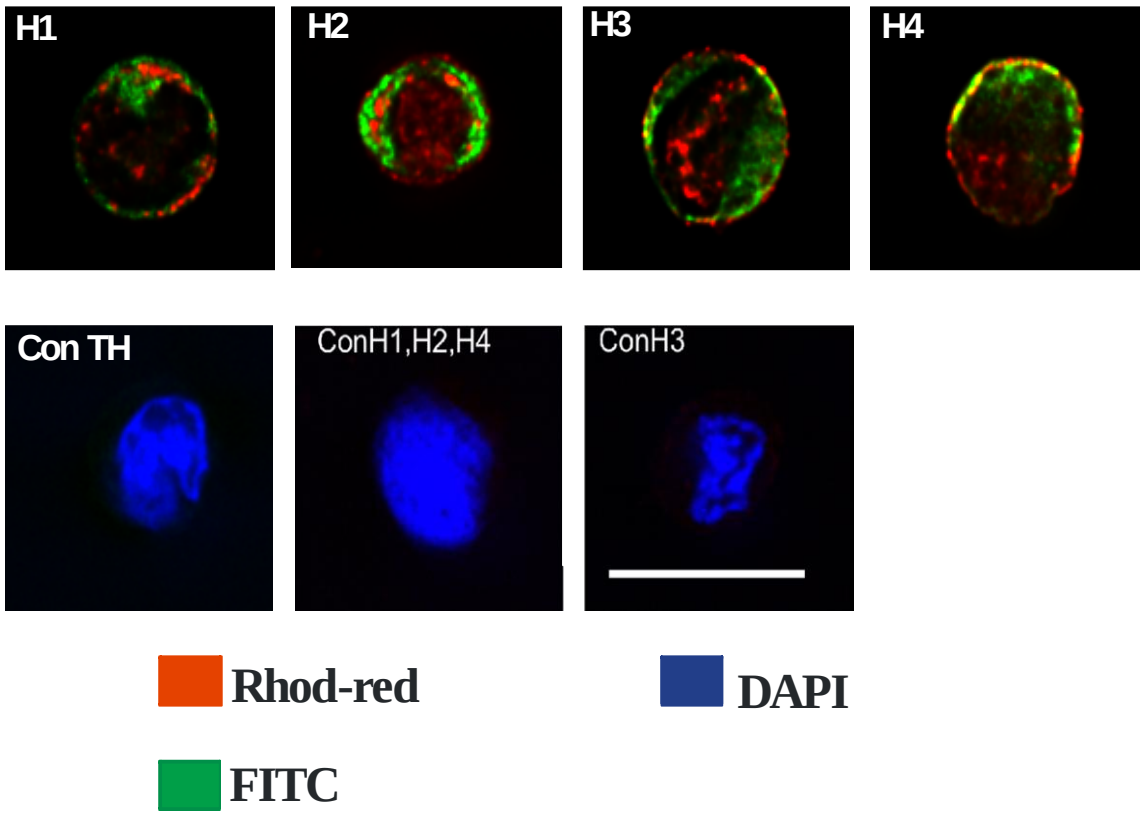

H1
15µm
H2
H3
H4
Con H1,2,4
Con H3
Rhod-red
DAPI

Fig 9. Type I cells are known to be dopaminergic, therefore containing the enzyme tyrosine hydroxylase (develops the precursor to dopamine, DOPA). The fluorescent green color represents the presence of tyrosine hydroxylase. This stain was used to assist in the identification of type I cells. Red staining represents histamine receptor localization. Scale bar is 10µm.

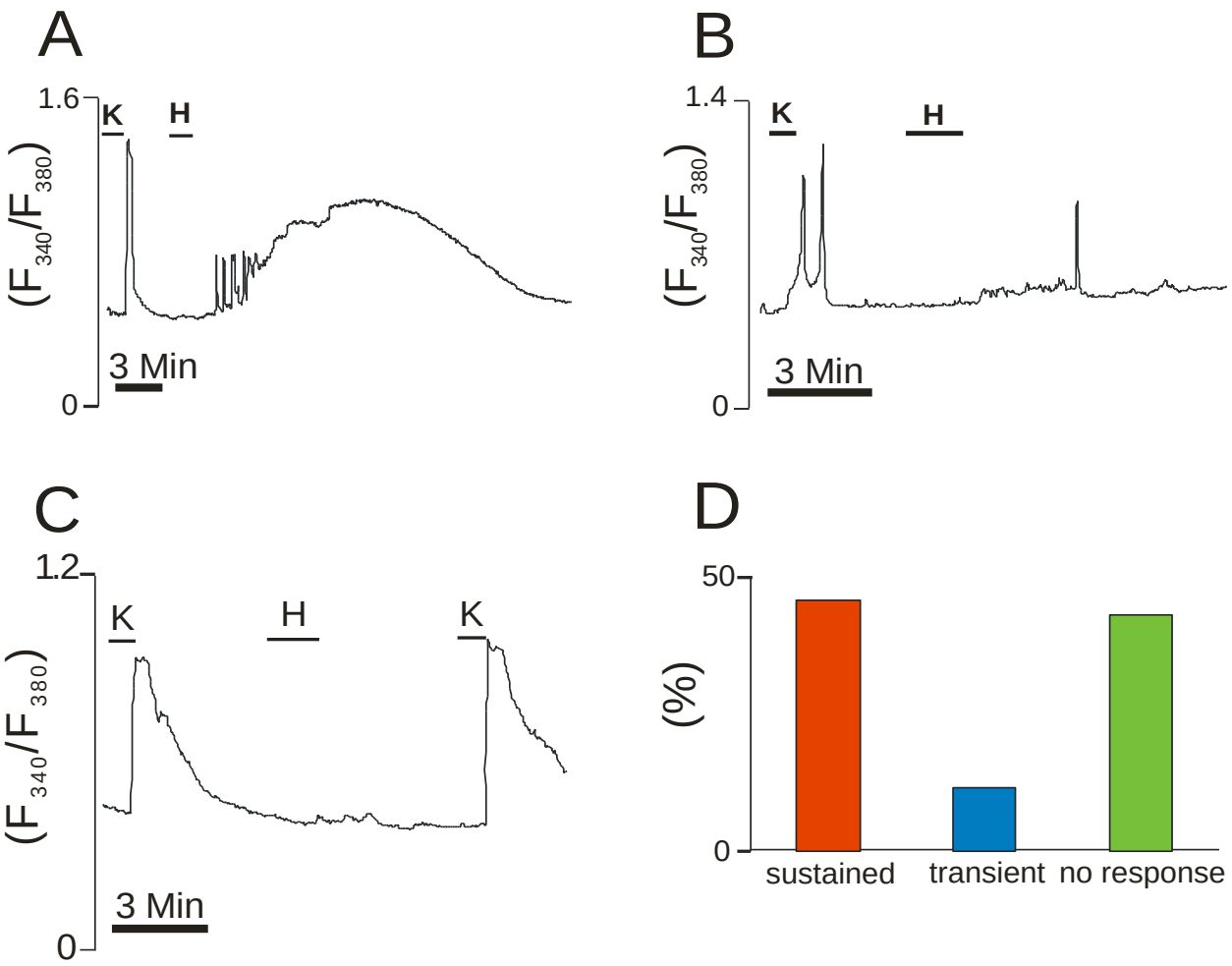

H1
H2
H3
H4
Con TH
ConH1,H2,H4
ConH3
Rhod-red
DAPI
FITC

Effect of Histamine on $[Ca^{2+}]_i$

Histamine (100μM) was bath applied to isolated neonatal rat type I cells. Histamine's activity at all 4 receptor sybtypes is similar with pD_2 values for histamine at H1R, 6.8; H2R, 6.0; H3R, 7.4; and H4R, 6.8. pD2 values represent the $-\log_{10}$ of the dissociation constant for an agonist at its receptor. The pD2 values therefore represent a measure of affinity of an agonist for its receptor. Here there is little difference between the histamine receptors affinity for histamine. Thus this concentration of histamine should not preferentially activate any one receptor and should evoke a robust response from all receptors present.

To make sure the cells we tested were living and viable, only cells that responded with rapid, robust and fully reversible responses to 80mM K^+ were selected for experimentation. 100μM histamine was the first concentration applied to the type I cells listed above. 100μM histamine was chosen based on the receptors pD2 values. At this concentration based on the pD2 values for this drug, all histamine receptor subtypes should be activated strongly. Of the 34 cells tested, histamine (100μM, 90 seconds) evoked a sustained rise in the Fura-2-fluorescence ration in 44.1% of cells or n=15 (Fig. 10A, 0.44 ± 0.08 ratio units, $P < 0.0001$) and the sustained response was usually preceded by rapid spiking activity. Rarely, brief single spike responses with no sustained component to histamine at this concentration were observed (11.7 % of cells, Fig. 10B) and the remainder of cells showed little or no change in Ca^{2+} in response to a histamine challenge (37.9% of cells, Fig. 10C).

Fig 10. Various effects of 100μM histamine on type I cell Fura-2 fluorescence ratio. K indicates the time interval 80mM K^+ solution was perfused over the cells; H is for histamine; all scale bars are 3 minutes. On each graph, histamine was perfused for 90 sec and the results were recorded using the program MetaFluor. A) Graph showing the sustained response when exposed to 100μM histamine. B) Example of transient response with 100μM histamine. C) Example of non responder with 100μM histamine. D) Bar graph showing the percentage of responders and non responders in 34 cells.


A
1.6
K
H
(F340/F380)
3 Min
0
B
1.4
K
H
(F340/F380)
3 Min
0
C
1.2
K
H
K
(F340/F380)
3 Min
0
D
50
(%)
0
sustained
transient
no response

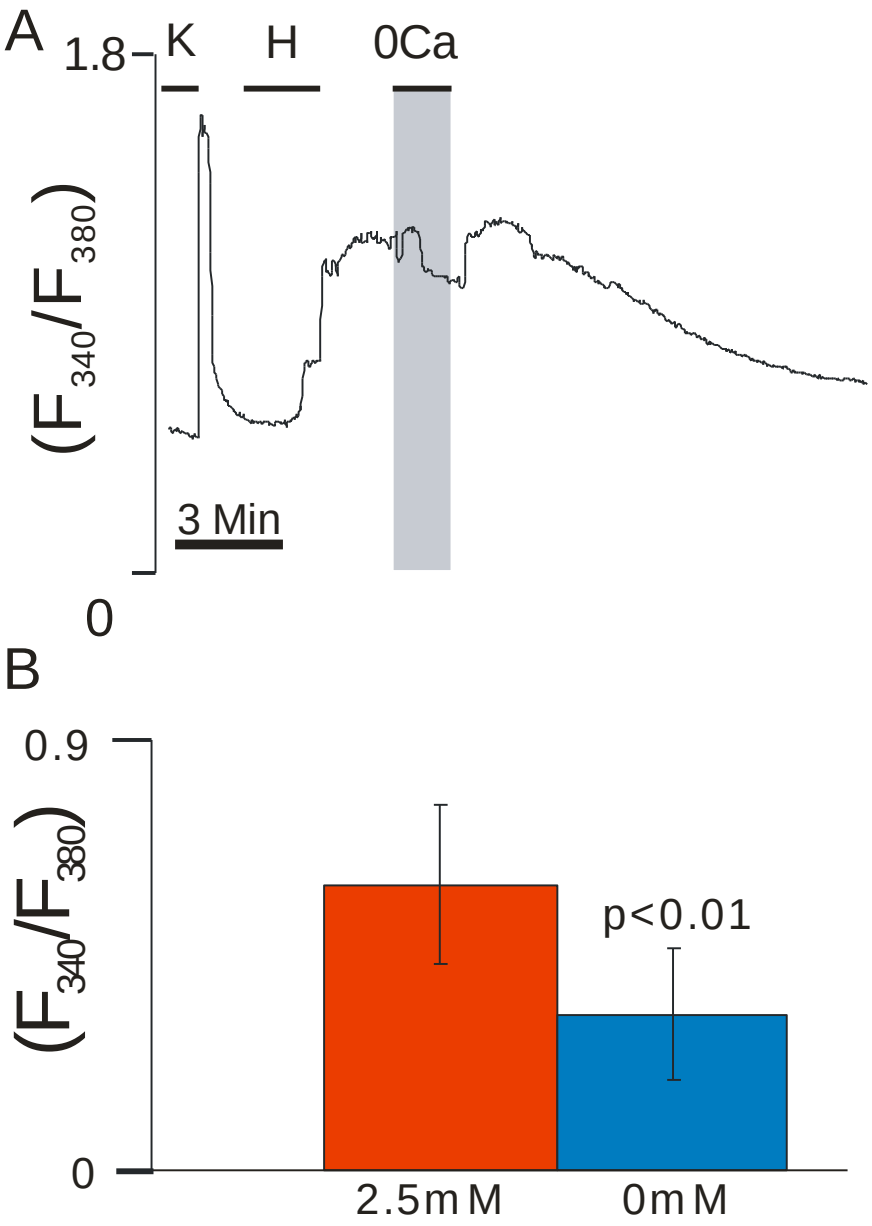
Dependence of Histamine evoked rise in $[Ca^{2+}]_i$ on extracellular Ca^{2+}

As mentioned in the introduction histamine could evoke a rise in $[Ca^{2+}]_i$ by many different signaling pathways. H1 and H2 receptor mediated pathways could induce depolarization and voltage-gated calcium entry as well as mediating Ca^{2+} release from intracellular stores. There are also limited reports that H3 receptors can couple to release of intracellular Ca^{2+}. Consequently, as the immunocytochemistry data indicated that all 4 histamine receptors are present on type I cells the source of the rise in $[Ca^{2+}]_i$ was examined.

To determine if histamine was evoking Ca^{2+} influx from the extracellular solution the $CaCl_2$ was removed from the solution and 1mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetra acetic acid (EGTA) was added to chelate any free Ca^{2+} in the extracellular solution. Histamine (100μM) was added to cells for 90 seconds and if a sustained rise in $[Ca^{2+}]_i$ was observed then the calcium free extracellular solution was perfused over the cells when the peak of the response had been reached.

Under these conditions removal of extracellular Ca^{2+} partially reversed the sustained rise in $[Ca^{2+}]_i$ to histamine (100 μM) by 50.9 ± 8.6% (n = 6, $P < 0.01$, Fig. 11A and B). Thus the sustained rise in $[Ca^{2+}]_i$ is caused by a combination of both Ca^{2+} influx and Ca^{2+} release from intracellular stores.

Fig 11. Effects of 100µM histamine on type I cell Fura-2 Fluorescence ratios. A) Example recording showing a sustained response to 100µM histamine followed by the removal of extracellular calcium. This was performed in order to tell whether Ca^{2+} was coming from extracellular on intracellular stores. B) The blue bar represents the amount Ca^{2+} dropped with 0mM Ca^{2+} as compared to the normal extracellular calcium concentration (red).


A
1.8
K
H
0Ca
(F340/F380)
3 Min
0
B
0.9
(F340/F380)
p<0.01
0
2.5mM
0mM

Effect of Histamine agonists on $[Ca^{2+}]_i$

In order to identify which histamine receptors were contributing to the rise in the Fura-2 ratios observed with histamine we investigated the effects of 4 selective histamine receptor agonists on Fura-2 fluorescence in isolated type I cells.

Bath application of the H1 receptor agonist histamine trifluoromethyl toluidide (HTMT dimaleate, 30μM, 90 seconds) failed to induce a sustained rise in the Fura-2 fluorescence ratio in all 17 cells tested. 2 cells showed a brief single Ca^{2+} spike however the majority of cells 98.2%) showed no response to HTMT dimaleate (Fig. 12). The selective H2 receptor agonist amthamine hydrobromide (30μM, 90 seconds) initiated spiking activity in the Fura-2 fluorescence ratio in isolated type I cells with 66.6% (10 cells from 15 tested) responding with a repeated spiking increase in the Fura-2 fluorescence ratio (Fig. 13A). This spiking activity developed into a sustained response in 40% of responding cells (Fig. 13B).

The H3 receptor agonist (R)-(-)-α-methylhistamine hydrobromide (30nM) consistently evoked sustained rises in the type I cell Fura-2 fluorescence ratio of 0.46 ± 0.06 ratio units ($P < 0.002$, $n = 5$, Fig. 14)

The H4 receptor agonist 4-methylhistamine dihydrochloride (1 μM) also evoked sustained rises in Fura-2 fluorescence ratio of 0.65 ± 0.09 ratio units ($P < 0.006$, $n=4$, Fig 15).

Fig 12. Pharmacological stimulation of the H1 histamine receptor subtype using the H1 selective histamine agonist, HTMT dimaleate, at a concentration of 30μM. Bath application of the agonist failed to raise intracellular Ca^{2+} concentrations in 17 different type I cells.

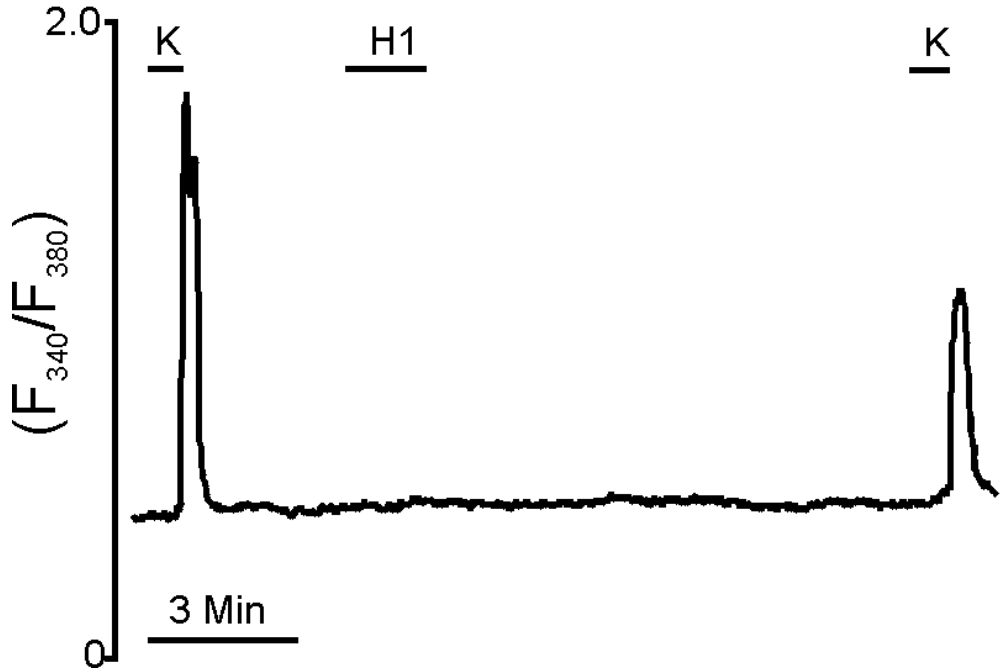

2.0
K
H1
K
(F_{340}/F_{380})
3 Min
0

Fig 13. Pharmacological stimulation of the H2 histamine receptor subtype using the H2 selective receptor agonist, Amthamine Hydrobromide, at a concentration of 30μM. Bath application of the H2 receptor subtype agonist elicited two different responses in the Fura-2 fluorescence ratio. Graphs showing the A) transient response in 10/15 cells and B) the transient to sustained response in 4/10 that responded. 5 of the cells did not respond at all.

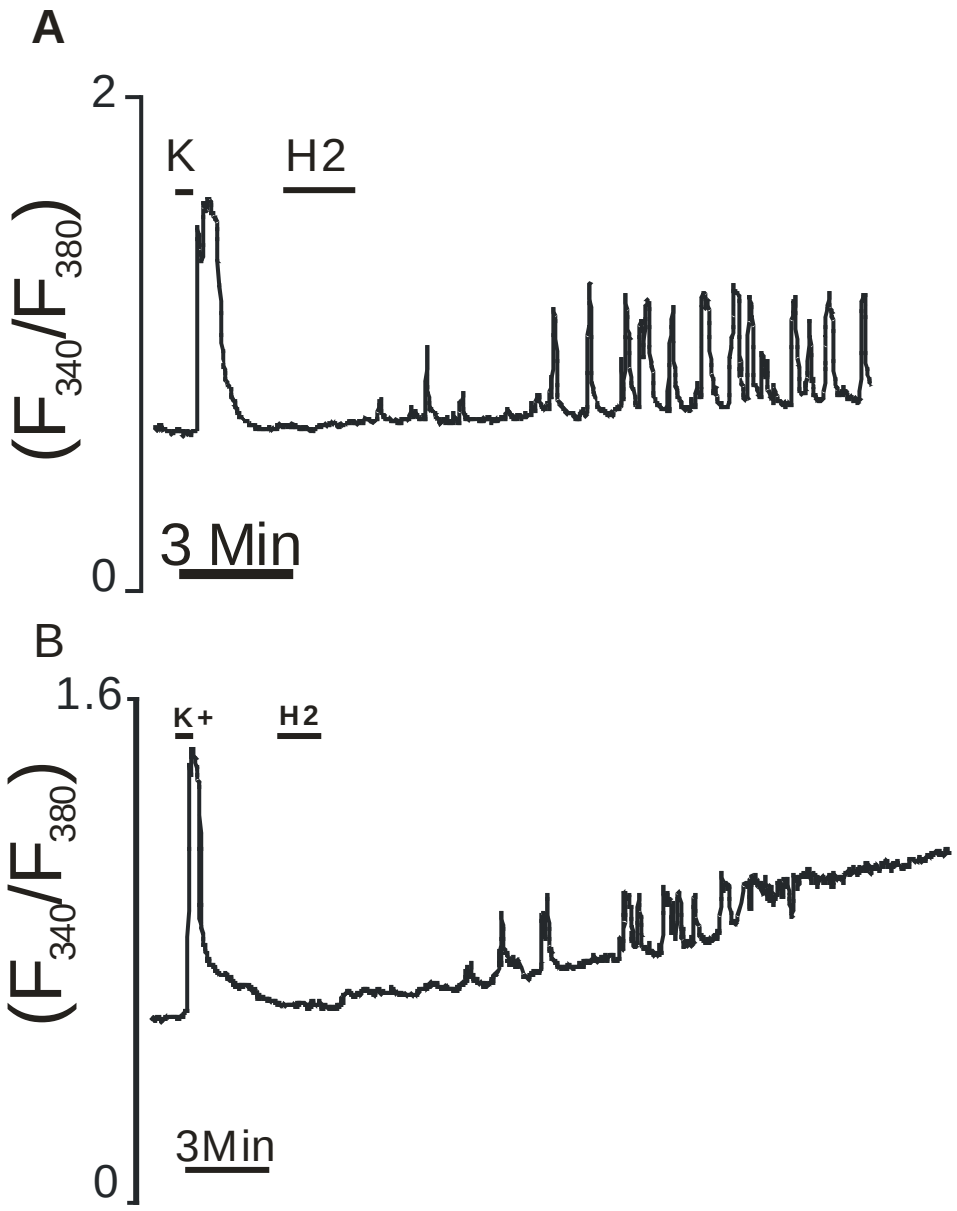

A
2
K
H2
(F340/F380)
0
3 Min
B
1.6
K+
H2
(F340/F380)
0
3Min

Fig 14. Pharmacological stimulation of the H3 histamine receptor subtype using the H3 selective receptor agonist, (R) - (-) – α – methylhistamine hydrobromide , at a concentration of 30nM. Bath application of the H3 receptor subtype agonist evoked sustained responses in 5 cells with a Fura-2 fluorescence ratio of 0.46 ± 0.06 ratio units.

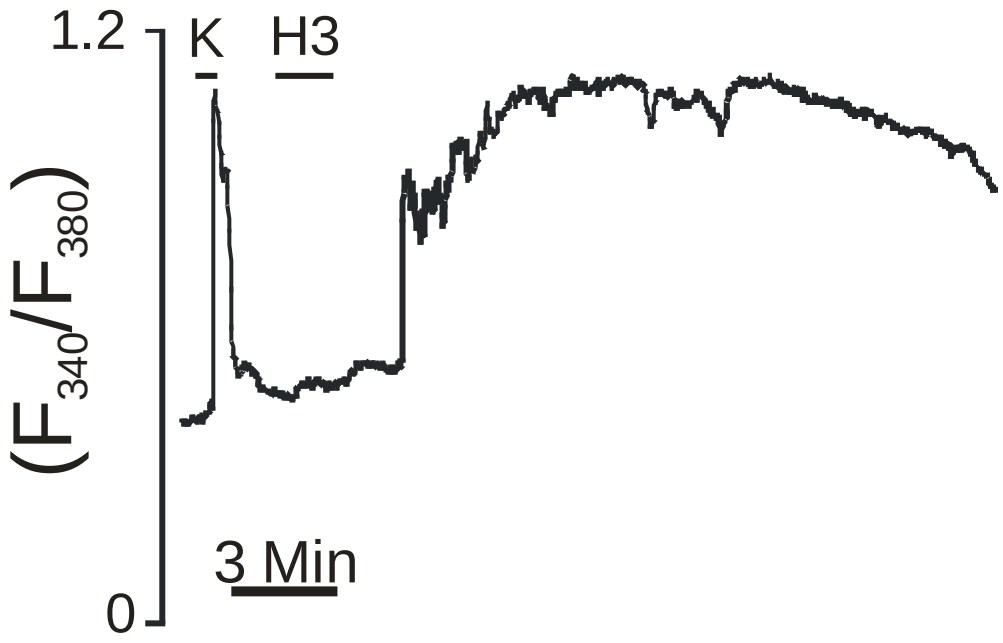
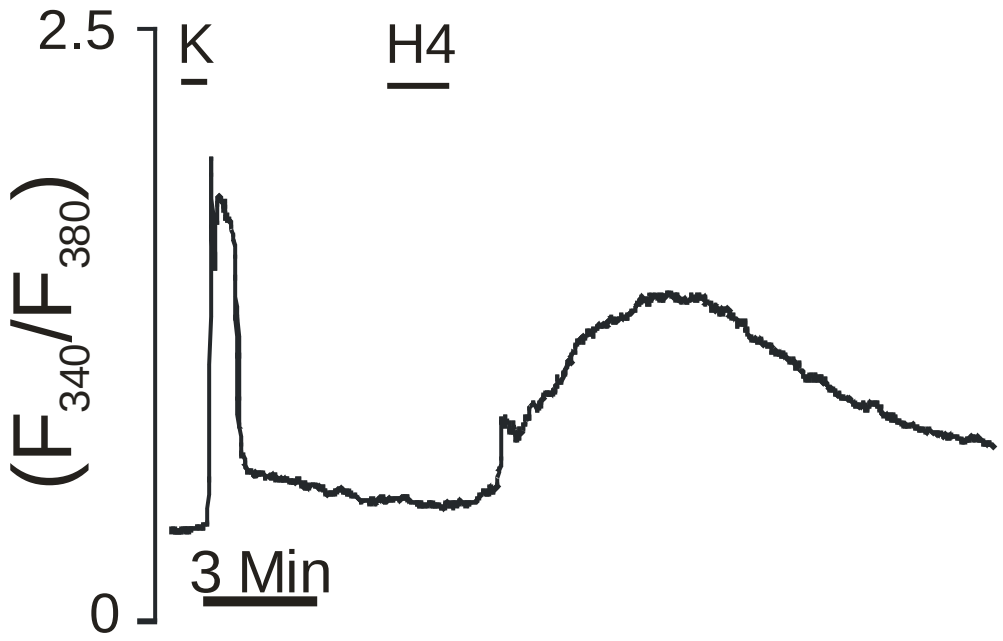

1.2
K
H3
(F340/F380)
3 Min
0

Fig 15. Pharmacological stimulation of the H4 histamine receptor subtype using the H4 selective receptor agonist, 4 – methylhistamine dihydrochloride, at a concentration of 1μM. Bath application of the H4 receptor subtype agonist evoked sustained rises in the Fura-2 fluorescence ratio of 0.65 ± 0.09 ratio units. ($P < 0.006$, $n = 4$).


2.5
K
H4
(F340/F380)
3 Min
0

Concentration Dependence of Histamine Response

After observing the Type I cells responses to the histamine (100μM), we began to wonder if the type I cell response was dose dependent. Approximately 40% of type I cells had not responded to histamine (100 μM). It appeared that type I cells did respond in a dose dependent manner to histamine with 100% of cells responding to histamine (300μM, 90 seconds) with a reversible sustained rise in the Fura-2-fluorescence ratio (Fig. 16A, 0.69 ± 0.05 ratio units, $P < 0,00001$, $n = 13$). In contrast, of 34 cells tested, histamine (100μM, 90 seconds) only evoked a sustained rise in the Fura-2 fluorescence ratio in 44.1% of cells (Fig. 16B, 0.44 ± 0.08 ratio uints, $P < 0.0001$, $n = 15$) and the sustained response was usually preceded by rapid spiking activity.

However, when the concentration of histamine was reduced it was noticed that at a concentration of 10μM and even 0μM histamine a large sustained response continued to occur (Fig. 17A, 0.53 ± 0.04 ratio units, $n = 5$ and Fig. 17B, 0.68 ± 0.09 ratio units, $n = 3$ respectively). Indeed the response to 10 μM was greater than that observed with 100 μM. The fact that similar responses were seen when control extracellular solution with no added histamine was perfused onto the cells suggested that the data being observed were artifactual.

Fig 16. Graphs representing dose response experiments performed on individual type I glomus cells with no neutral density filter. A) Bath application of 300μM histamine evoked a sustained rise in the Fura-2 fluorescence ratio of 0.69 ± 0.05 ratio units ($p < 0.00001$, $n = 13$) as compared to the application of B) 100μM histamine which only accounted for a rise of 0.44 ± 0.08 ratio units. ($p < 0.0001$, $n = 15$).

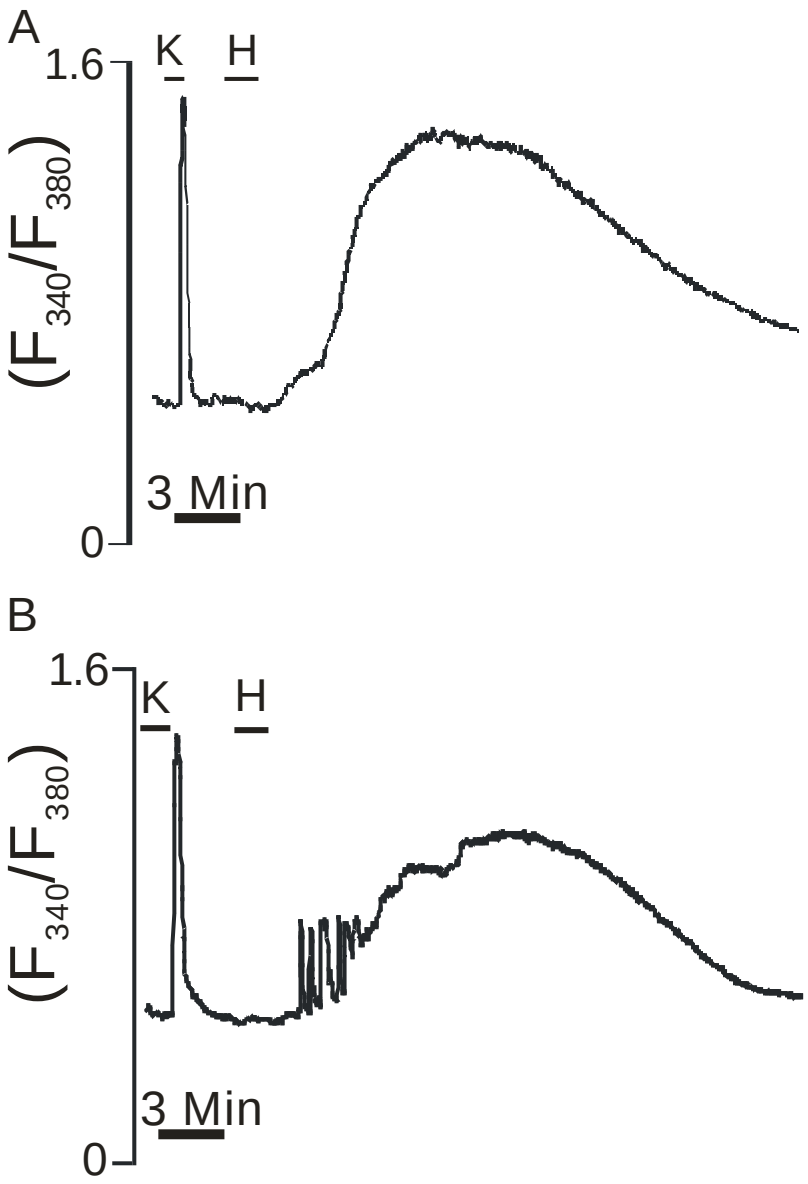
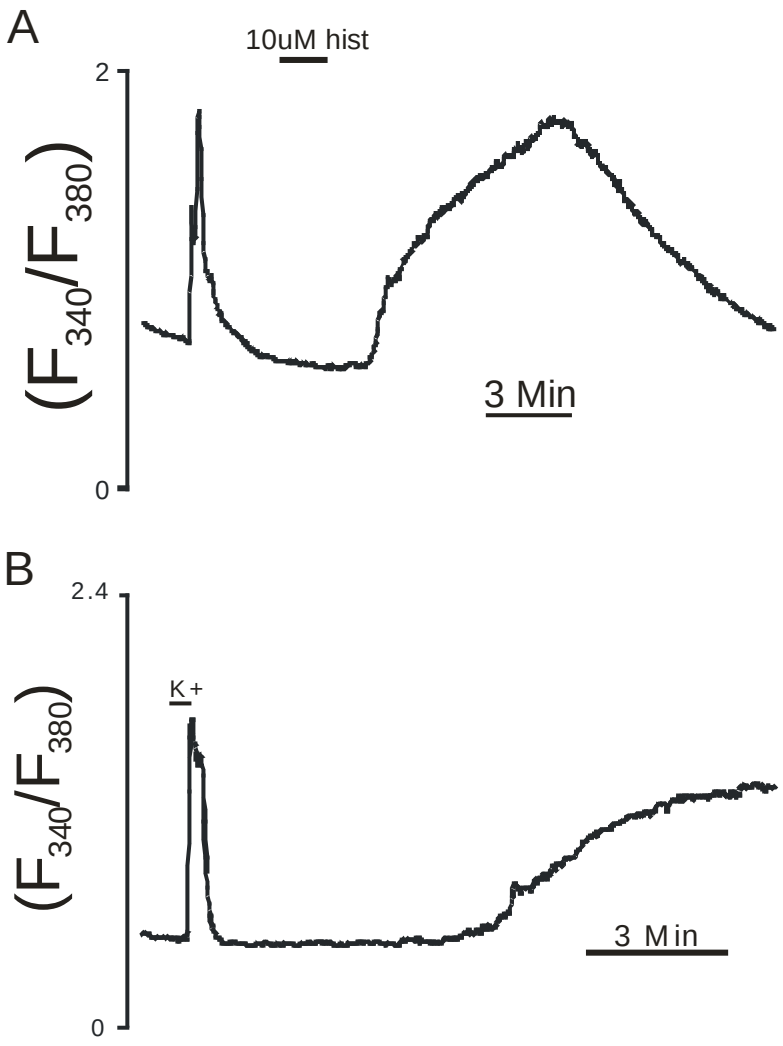

A
1.6
K
H
(F_{340}/F_{380})
3 Min
0
B
1.6
K
H
(F_{340}/F_{380})
3 Min
0

Fig 17. Graphs representing dose response experiments performed on individual type I glomus cells with no neutral density filter. A) Bath application of 10μM histamine evoked a sustained rise in the Fura-2 fluorescence ratio of 0.53 ± 0.04 ratio units (n = 13) as compared to the application of B) 0μM histamine which accounted for a rise of 0.68 ± 0.09 ratio units. (n = 3).


A
10uM hist
2
0
(F_{340}/F_{380})
3 Min
B
2.4
0
(F_{340}/F_{380})
K+
3 Min

Effects of Histamine and Agonists on $[Ca^{2+}]_i$ with the addition of a 0.7 Neutral Density Filter

It was decided to incorporate the use of a neutral density filter in order to dampen the suspected harmful effects of ultra violet light in the Ca^{2+} imaging system (see discussion). In order to determine what filter to use a range of filters from 0.6 – 0.9o.d (Chroma) were tested. With each filter only saline was bath applied and the effects were recorded from Fura-2 loaded cells. At the lowest range of 0.6, allowing only 25% light transmission, there continued to be noticeable spiking during the saline application similar to the results seen previously with H2 receptor agonist (Figs. 18A and B). A higher grade neutral density filter of 0.9 was then applied. This resulted in the eradication of random spiking. It was also found that a 0.7 (12.5% light transmission) neutral density filter eradicated random spiking of type I cells (Fig 18C).

After discovering that a neutral density filter was capable of decreasing the occurrence of random spiking within type I cells, new experiments were conducted testing its effects on the suspected artifact found during the initial rounds of experimentation. First a very high concentration of 300μM histamine was bath applied to the type I cells for a period of 90 seconds each after the initial 80mM K^+ stimulus. This resulted in 0 out of 20 cells responding to histamine at this concentration (Fig 19). Each histamine receptor agonist was then used again to determine their effects on type I cells together with the 0.7 neutral density filter. The first agonist used was the H1 receptor subtype agonist, HTMT dimaleate, at a concentration of 30μM. The agonist was bath applied for 90 seconds after the intial potassium stimulus. Out of 17 cells tested

0 responded to the application of the agonist (Fig. 20). Secondly, the H2 receptor subtype agonist, Amthamine Hydrobromide, was used at a concentration of 30μM. Again, out of the 6 cells tested 0 responded to the drug at this concentration (Fig. 21). Thirdly, the H3 receptor subtype agonist, (R) - (-) – α – methylhistamine hydrobromide, was used again at a concentration of 30nM. Similar results were found, out of the 6 cells tested 0 responded to the drug at this concentration (Fig. 22).

With the incoroporation of the neutral density filter, no cells responded with a sustained or transient response to histamine or the specific agonists. These data demonstrate that histamine as well as selective histamine receptor agonists do not have an effect on intracellular calcium in carotid body type I glomus cells.

Fig 18. Neutral density filter selection. A) and B) 0.6 ND filter was used allowing only a 25% transmission of light; Cells were only subjected to HEPES buffered saline solution; noticeable random spiking still occurred at this density. C) 0.7 ND filter (12.5% transmission) added, and resulted in the eradication of random spiking within the isolated cells.

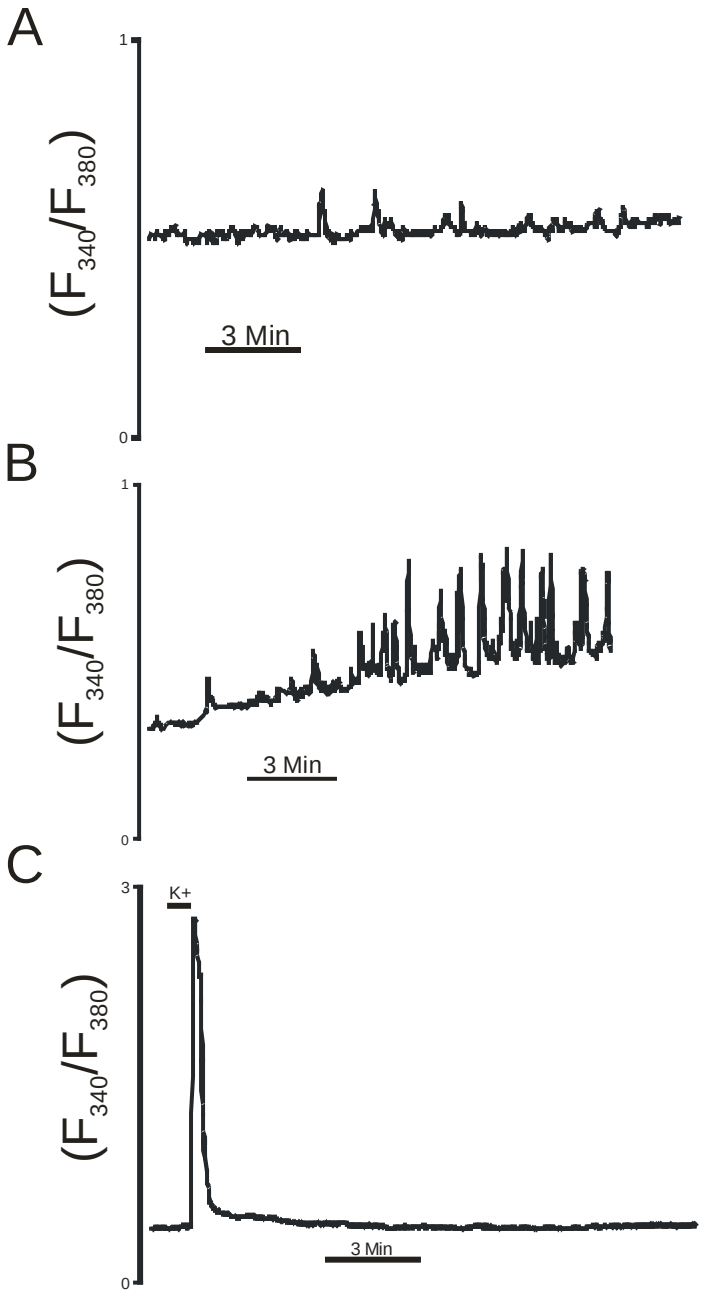

A
1
0
(F_{340}/F_{380})
3 Min
B
1
0
(F_{340}/F_{380})
3 Min
C
3
0
K+
(F_{340}/F_{380})
3 Min

Fig 19. Effects of 300μM histamine on type I cells with the incorporation of the 0.7 ND filter. Out of 20 different cells tested, not a single one responded to the bath application of 300μM Histamine. 80mM K^+ was initially perfused over the cells to determine whether or not the cells were viable to experimentation.

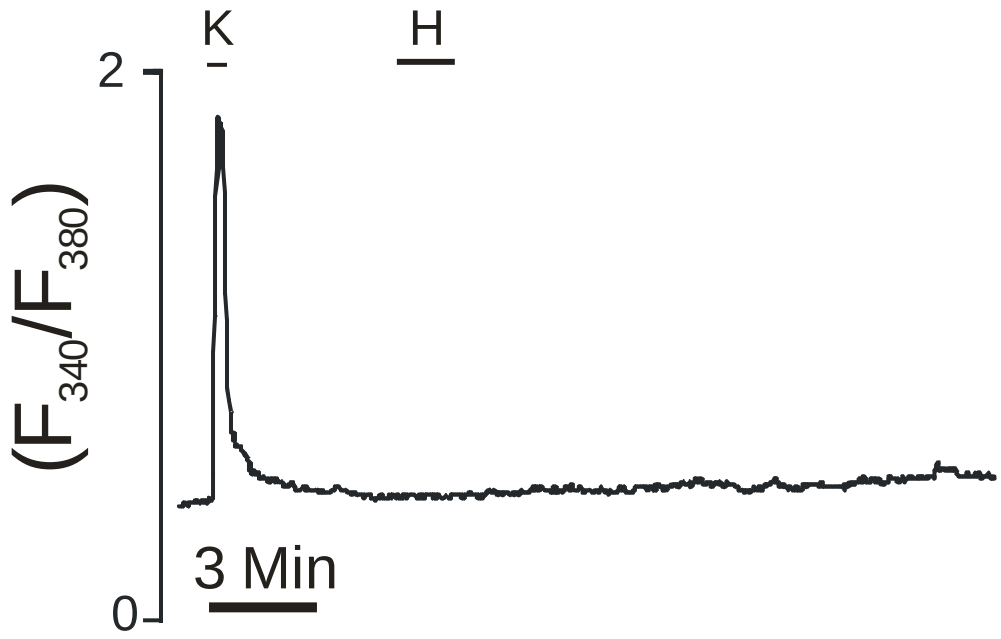

K
H
2
0
(F340/F380)
3 Min

Fig 20. Pharmacological stimulation of Type I cells with the H1 receptor subtype agonist, at a concentration of 30μM, with the incorporation of the 0.7 ND filter. Out of 17 cells tested, 0 showed a sustained response. 80mM K^+ was applied both before and after application of the agonist.

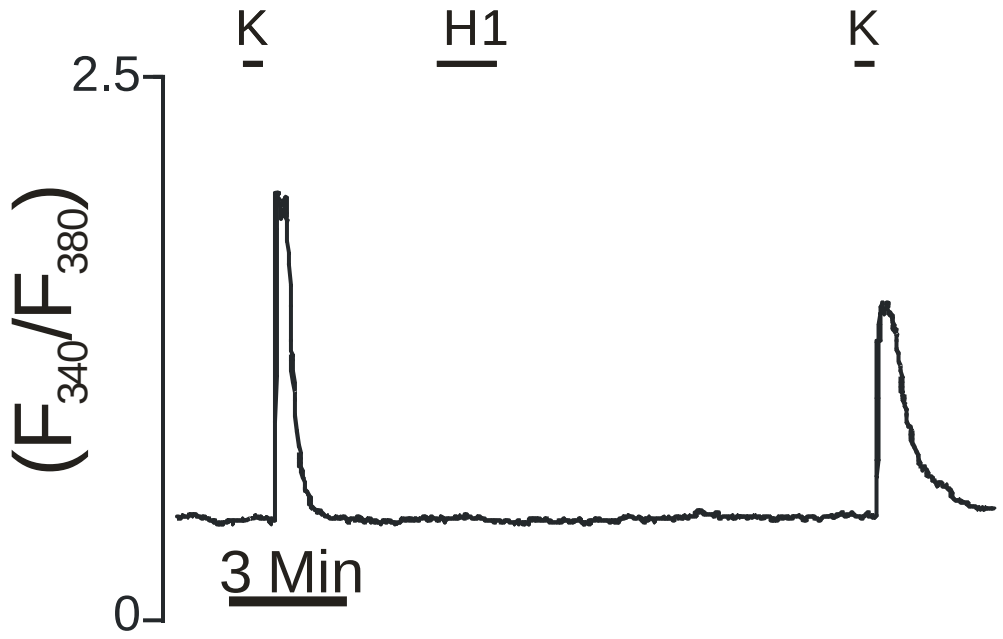

K
H1
K
2.5
0
(F340/F380)
3 Min

Fig 21. Pharmacological stimulation of Type I cells with the H2 receptor subtype agonist, at a concentration of 30μM, with the incorporation of the 0.7 ND filter. Out of 6 cells tested, 0 showed a sustained response. 80mM K^+ was applied both before and after application of the agonist.

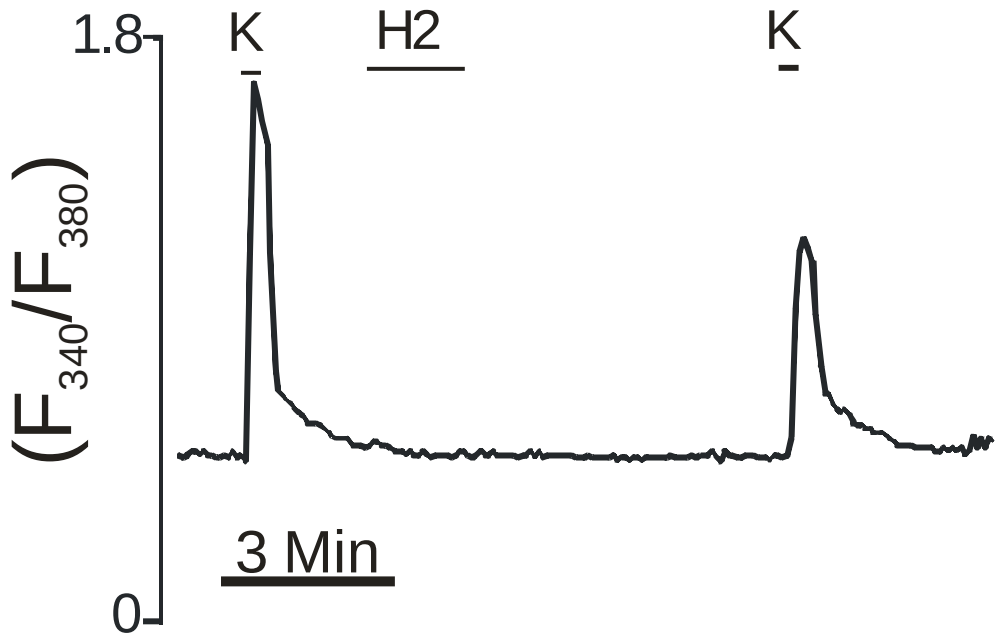
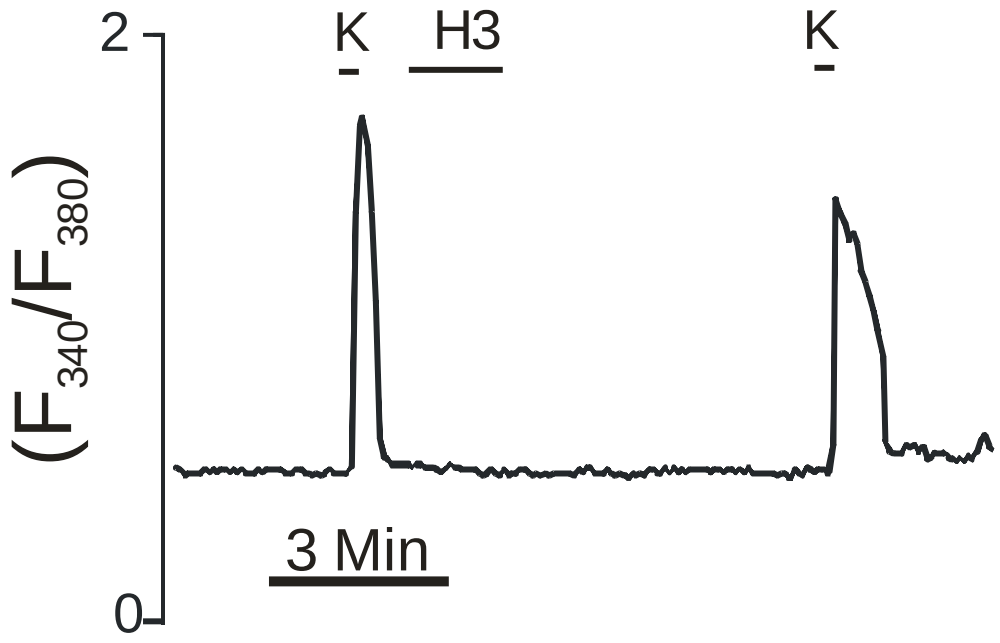

1.8
K
H2
K
(F340/F380)
3 Min
0

Fig 22. Pharmacological stimulation of Type I cells with the H3 receptor subtype agonist, at a concentration of 30nM, with the incorporation of the 0.7 ND filter. Out of 6 cells tested, 0 showed a sustained response. 80mM K^+ was applied both before and after application of the agonist.


2
K
H3
K
(F340/F380)
3 Min
0

CHAPTER V

DISCUSSION

Results Summary

Through the use of immunocytochemistry it was observed that all 4 histamine receptors were present on or around the plasma membrane of carotid body Type I cells. During the initial rounds of experimentation it was shown that histamine could play a role in the dose dependent excitation of Type I cells, but additional experimentation proved that the data gathered were a result of artifact instead. Following these findings further experiments were performed on isolated Type I cells and the data collected showed no excitatory effects of histamine on intracellular calcium in Type I cells isolated from neonatal rat carotid body.

Despite all the pathways described in the introduction histamine does not appear to couple to Ca^{2+} signaling in type I cells. Why might this be?

Are the Histamine Receptors Genuinely at the Membrane of Type I cells?

The limited staining performed in this study showed positive results for all 4 histamine receptor subtypes, but attempting to assess which receptors were most strongly targeted to the plasma membrane was not carried out. This procedure could have been done utilizing a specific program within the deltavision microscope. This program would have enabled the calculation of the percentage staining within a micron of the cell perimeter, and would have indicated which receptor subtype was the most strongly membrane targeted. However, comparing the staining intensity between antibodies is problematic because of different antibodies' affinities for their receptors. Although one antibody shows a more intense staining pattern around the membrane does not

necessarily mean it is more present. The higher signal could just be due to the stronger binding potential of that antibody to its specific receptor subtype.

Although staining for these receptors should be most visible at or around the plasma membrane, extensive staining within the cytoplasm as well as the nucleus was observed. One reason could be that the antibodies used for this particular set of experiments were of poorer quality than what was anticipated. An experiment using histamine antibodies from a different source and comparing staining patterns could rule out this finding. This notwithstanding it has been observed that the staining of ryanodine receptors, whose expression is usually restricted to the endoplasmic reticulum, were present in the nuclei of cells (Kinnear, 2008). Therefore the antibodies could be binding to premature histamine receptor subtypes before they are transported to the plasma membrane from the nucleus.

From the data collected (Fig. 8 & 9) the receptors showing strongest membrane staining appear to be the H3 and H4 histamine receptor subtypes. However, these subtypes are usually thought to be inhibitory when activated, not excitatory (Arrang *et al.*, 1983, 1985). However it should be noted that when Lazarov et al injected H3 agonist into the carotid body they observed excitation.

To absolutely confirm the expression of histamine receptor subtypes at the membrane of Type I cells antibodies that bind to extracellular epitopes of the receptors should be applied to non-permeablised living type I cells. The cells should then be washed and fixed. Any membrane staining observed should be due to the presence of histamine receptors in the type I cell plasma membrane.

Age and species differences

Age could have been an important factor in why different results were gathered. In Lazarov's experiments where histaminergic agonists stimulated respiration adult rats were used instead of the young rats used in ours (10-20 days). However, previous research has shown no significant difference between 10-20 day old animals and adults in the hypoxic response of the carotid body (Kholwadwala & Donnelly, 1992). Also, it has been shown that isolated Type I cells isolated from rats of the same age range as that used in our study have a mature hypoxic response (Pepper *et al.*, 1995), so age is unlikely to be a major influence on whether or not histamine is excitatory.

Interestingly it has been indirectly shown that histamine is excitatory in carotid body Type I cells in cats. Histamine does not appear to stimulate the post synaptic petrosal ganglion (Del Rio *et al.*, 2008) but does excite the CSN when injected into the carotid body. Unfortunately this experiment was performed using another species to the rat and there are numerous species differences between cat and rat carotid body. The parallel experiment using rats has not been performed. Furthermore the recordings in the cat were made on the cell bodies of the petrosal ganglion and not their dendrites within the carotid body. It is possible that receptors expressed at the cell bodies of petrosal ganglion neurons are radically different from those expressed at the postsynaptic terminals that type I cells communicate with. No attempt to characterize this has been made.

Were Cells Damaged by Enzyme Dissociation Process?

During the dissociation process harsh enzymes, such as trypsin, were used to break down the surrounding tissue in order to help free and isolate the carotid body Type I cells. During this process it is possible that the integrity of the proteins that compose histamine receptors were compromised resulting in the lack of response when exposed to histamine. If this was the case, then G-protein coupled receptor damage would be the cause for the lack of response to histamine application.

Experiments were performed to determine the integrity of G-protein coupled receptors using the muscarinic agonist acetyl-β-methylcholine (100μM). Previous work has shown that muscarinic agonists induce rises in $[Ca^{2+}]_i$ in neonatal rat type I cells (Dasso *et al.*, 1997). This agonist was bath applied to isolated Type I cells and the results were recorded using the MetaFluor program. The resulting data from this experiment showed that when the muscarinic agonist was bath applied the cells responded with spking rises in $[Ca^{2+}]_i$ (n = 6). Thus it appears that G-protein coupled receptors, or at least muscarinic receptors, were not enzymatically damaged during the dissociation process used during this thesis work (Fig. 23).

Does Histamine augment Calcium entry?

Another possibility for the lack of response to histamine during bath application is that it works in an additive fashion, effective only when the cell has already been depolarized. It could be that histamine is amplifying Ca^{2+} influx by regulating voltage gated calcium entry (see Introduction). If histamine did work in an additive fashion, then

applying it during a period of depolarization should further increase the effects causing a greater spike in the Fura-2-fluorescence ratio graph.

An experiment was performed to determine whether or not histamine further excited the cell during a depolarizing event. Type I cells were isolated as before and subjected to high levels of potassium. During these events, histamine (300 μM) was also applied and the results were recorded using the MetaFluor program. The resulting data showed that at no point during the application of both the high molarity potassium and histamine did the ratios increase, or even change (Fig. 24, n = 6). This is evidence for that histamine does not further amplify Ca^{2+} influx by regulating voltage gated calcium entry.

Fig 23. Graph demonstrating functional g-protein coupled muscarinic receptors in the plasma membrane of Type I cells. M represents the amount of time the muscarinic agonist was bath applied to the Type I cell.

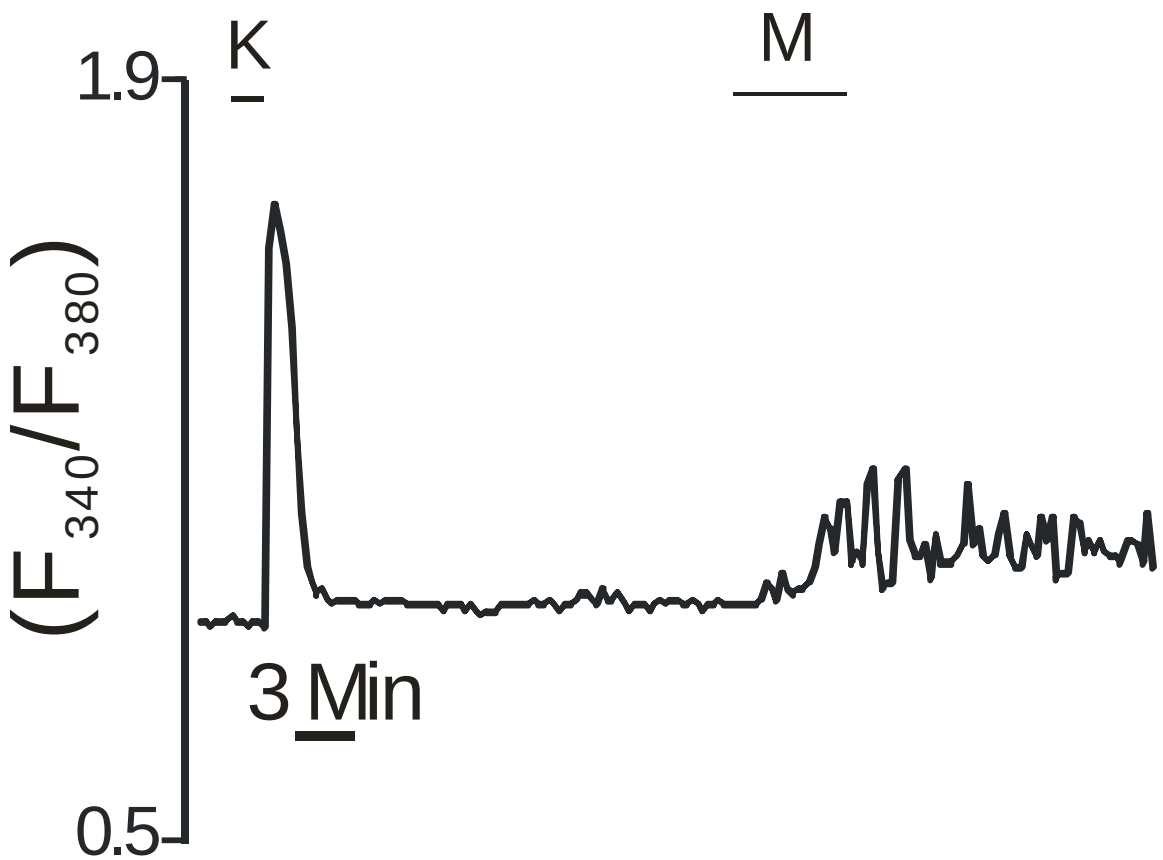
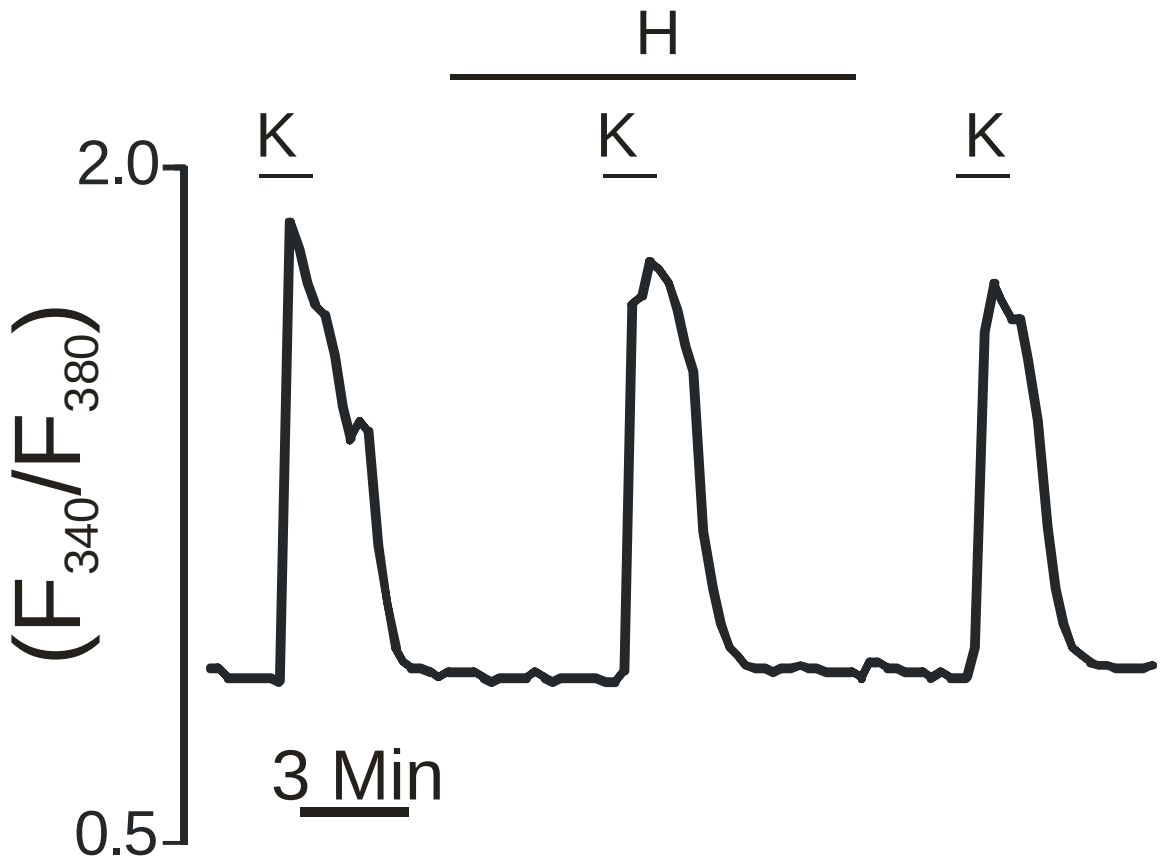

K
M
1.9
0.5
(F340/F380)
3 Min

Fig 24. Graph determining whether or not histamine amplifies Ca^{2+} influx by regulating voltage gated calcium channels. 80mM K^{+} solution was applied three times. After the first application of potassium, 300µM histamine was bath applied. During the middle of the histamine application high potassium was placed back on to see if there were any changes in the fura-2 fluorescence ratio. The graph clearly shows no significant changes to the spikes during or after the application of histamine.


H
K
K
K
2.0
0.5
(F340/F380)
3 Min

What Caused the Artifact?

Ultra Violet Light and Reactive Oxygen Species (ROS)

The artifact presented itself when our Type I cells were exposed to bright Ultraviolet light. Dimming this light by the use of neutral density filters enabled the artifact to be titrated out. The wavelengths of excitatory light used in all the preceding experiments were 340nm and 380nm of light. This puts them into the Ultraviolet A, or long wave UV light category. Although these particular wavelengths are not as harmful as shorter wavelength UVB light, when cells are exposed to them for extended periods of time cell damage can eventually occur (Orit Bossi, 2008).

Reactive oxygen species (ROS) are molecules known to participate in cellular damage, oncogenesis, mutagenesis and biological aging (Peter Koncz, 2006). The effects of ROS are determined by the rate of its formation and elimination (Peter Koncz, 2006). The major site of ROS production is within the mitochondria. It has been shown that UV light can induce the formation of ROS (Peter Koncz, 2006). This formation of ROS could explain the gradual, slow increase in intracellular Ca^{2+} as the experiment progressed, but could not explain why the cell was able to remove the excess calcium over a period of time and return to its original baseline Fura-2 Fluorescence ratio.

It is also possible that the overexposure to UV light was damaging the cellular plasma membrane, compromising its integrity and thereby allowing calcium to flow into the cell (Maglio, 2005).

CHAPTER VI

FUTURE DIRECTIONS

The data collected during this thesis prove there are still many questions left unanswered in determining whether or not histamine plays a role in the modulation of respiration by the carotid body. The direction that the lab is going after this thesis is looking at whether histamine acts to potentiate hypoxia within the carotid body, meaning, that during a hypoxic event, histamine will cause an even greater influx of calcium within the type I cell. In this hypothesis, histamine is not acting as a modulator as previously thought but as a potentiator, increasing the release of excitatory neurotransmitters. Furthermore, it would be interesting to investigate whether histamine can potentiate the effects of other excitatory presynaptic neurotransmitters. During a hypoxic episode multiple neurotransmitters will be released. It is conceivable that histamine alone is not excitatory but in combination with acetylcholine or serotonin causes enhanced calcium signaling. The muscarinic receptor subtypes in rat type I cells have not been characterized and histamine could, in theory, synergise with the G-protein coupled signaling cascades activated by these receptors.

It would also be interesting to see if histamine is acting in a negative fashion, acting as a sort of double negative. It could be possible that histamine is inhibiting an inhibitory neurotransmitter, thereby increasing the release of excitatory neurotransmitters to the post synaptic carotid sinus nerve. An experiment that could be run to test this is applying histamine with a known type I cell inhibitor, such as dopamine, and seeing whether or not there is any excitation or attenuated inhibition within the type I cell.

Finally, if histamine does not seem to have any excitatory mechanisms presynaptically its actions postsynaptically should be investigated. This could be done using an intact carotid body, carotid sinus nerve preparation. Selective potent antagonists

of the various histamine receptors should be able to determine if any of the postsynaptic CSN activity observed during hypoxia is attributable to histaminergic neurotransmission.

CHAPTER VII

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