The CAMKKβ Inhibitor STO-609 Causes Artefacts in CA2+ Imaging and Selectively Inhibits BKCa in Mouse Carotid Body Type I Cells

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THE CAMK KB INHIBITOR STO-609 CAUSES ARTEFACTS IN Ca^{2+} IMAGING AND SELECTIVELY INHIBITS BK_{Ca} IN MOUSE CAROTID BODY TYPE I CELLS

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

By

JENNIFER GRACE JURCSISN
B.S., Wright State University, 2011

2014
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY JENNIFER GRACE JURCSISN ENTITLED THE CAMKKB INHIBITOR STO-609 CAUSES ARTEFACTS IN Ca²⁺ IMAGING AND SELECTIVELY INHIBITS BKCa IN MOUSE CAROTID BODY TYPE I CELLS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT

Jurcsisn, Jennifer Grace. M.S., Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2014. The CamKKβ inhibitor STO-609 causes artefacts in Ca\(^{2+}\) imaging and selectively inhibits BK\(_{\text{Ca}}\) in mouse carotid body type I cells.

It has previously been reported that AMP-activated protein kinase (AMPK) may be critical for hypoxic chemotransduction in carotid body type I cells (Wyatt et al, 2007). This study sought to determine the importance of the regulatory upstream kinase of AMPK, CamKKβ, in the acute response to hypoxia in isolated mouse type I cells.

Initial studies indicated several previously unreported artefacts associated with using the CamKKβ inhibitor STO-609 and calcium imaging techniques. Strong STO-609 autofluorescence meant that Fura-2 could not be used and X-Rhod-1 imaging revealed that STO-609 quenched fluorescence even in the absence of intracellular Ca\(^{2+}\). The whole-cell configuration of the perforated-patch clamp technique revealed for the first time that STO-609 (100µM) rapidly (seconds) inhibited outward macroscopic currents at +10 mV by 38.0% ± 7.4% (n = 5, P<0.03, mean ± SEM) and that this inhibition was abolished in the presence of the selective BK\(_{\text{Ca}}\) inhibitor paxilline (1 µM).

Taken together these data suggest that STO-609 should be used with caution during Ca\(^{2+}\) imaging studies as it can directly interact with dyes. The rapid inhibitory effect of STO-609 on BK\(_{\text{Ca}}\) was unexpected as the majority of studies using this compound required an incubation of approximately 10 minutes to inhibit the kinase. Furthermore, as AMPK activation inhibits BK\(_{\text{Ca}}\), inhibiting AMPK’s upstream kinases would, if anything, be predicted to have the opposite effect on BK\(_{\text{Ca}}\). Future work will determine if the inhibition of BK\(_{\text{Ca}}\) is via CamKKβ or via an off target action of STO-609 on the channel itself.
This work has appeared in abstract form at the Ohio Physiological Society meeting 2012, Experimental Biology 2013 and will be presented in its final form at the 19th meeting of the International Society for Arterial Chemoreception, Leeds, UK, July 2014.
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Thanks to Dr. Wyatt for being a true mentor.
I: INTRODUCTION

HISTORY

In the 1600’s, Danish anatomist Jacques-Bénigne Winslow was the first to describe the Carotid Body (CB) (Kellogg, 1981). After Winslow, Carl Samuel Andersch went on to explain the CB as a type of “ganglion” in the 1750’s (Kellogg, 1981). Later, Hubert Luschka improved the anatomical description in 1862, saying the CB was a “gland” due to the “glandular-looking cells” and the “very fine nerve fibers” associated with them (McDonald, 1981). However, in 1926, some of the most important histological discoveries were made by Santiago Ramón y Cajal and Fernando de Castro. De Castro stated the CB was able to detect substances in the blood and had an endocrine-like function; thus, responded by a release of a secretion (de Castro, 1926). In 1928, he rejected the neurosecretory hypothesis and embraced a new theory of the CB cells as a sensory mechanism capable of detecting changes in the blood (de Castro, 1928).

In 1930, Corneille Heymans strove to perfect cross-circulation techniques via parabiosis. With these experiments, Heymans proved that the chemoreceptors in the CB elicited a response as a result of the change in the arterial blood composition (Fitzgerald and Lahiri, 1996). The parabiosis was demonstrated by connecting the arteries of canine A, to the head of canine B (see figure 1). Upon exposing canine A to hypoxia, it was canine B that responded by hyperventilating (Heymans et al, 1930). Heymans determined that it was the carotid bodies that were mediating the hyperventilatory response. For this pioneering work, Heymans won the Nobel Prize in 1938 (Fitzgerald and Lahiri, 1996).
Heymans’ experiment in which two living canines were attached via parabiosis. The thoracic vessels of Canine A were connected to the cervical vessels of Canine B. Canine A was subjected to hypoxic conditions resulting in a hyperventilatory response by Canine B.
ANATOMY

The Carotid Bodies (CB) are small oxygen sensing organs that can be found at the bifurcation of the common carotid arteries. These organs function as chemoreceptors by detecting arterial partial pressure of oxygen (PO$_2$), partial pressure of carbon dioxide (PCO$_2$) and pH levels. They largely contribute to the hyperventilatory response seen during hypoxia, hypercapnia and metabolic acidosis (Gonzalez et al, 1994).

Ganglioglomerular nerves, branches of the superior cervical ganglion (SCG), provide sympathetic innervation to the CB, and are also the main efferent component in regulating blood flow to the bifurcation (Gonzalez et al, 1994). The carotid sinus nerve (CSN), a branch of the glossopharyngeal nerve, joins with filaments of the vagus nerve to provide both sympathetic and parasympathetic influence over the CB (Gonzalez et al, 1994). The CSN exits the CB and its cell bodies lie in the Petrosal ganglion. Projections of the CSN terminate ipsilaterally and bilaterally in the nucleus of the solitary tract via the Glossopharyngeal nerve, acting as the main source of afferent input (Finley and Katz, 1992). The action potential is then propagated to communicate with the central pattern generator to supply motor neurons innervating the diaphragm via the phrenic nerve. The frequency of generated action potentials is regulated by the release of neurotransmitters from the CB, which ultimately controls the hyperventilatory response to hypoxia.

The ultrastructure of the parenchyma in the CB is composed of two types of cells: Type I cells (glomus) and Type II cells (sustentacular) (Lahiri et al, 2006) (see figure 2). The Type I cells populate a majority of the CB, as they are the primary source of sensory receptors accountable for the detection of inappropriate arterial PO$_2$, PCO$_2$ and pH levels (Stea and Nurse, 1991, Heymans et al, 1930). The Type I cells are responsible for the release
of the neurotransmitters mentioned previously. Upon the falling of PO$_2$ and pH levels, or the rising of PCO$_2$ levels, Type I cells respond to this detection by discharging neurotransmitters and initiating an action potential down the CSN. There is also some evidence that cellular communication occurs between Type I cells and nerve endings via gap junctions (McDonald, 1981). The function of Type II cells, on the other hand, is thought to be comparable to that of a glial cell in the nervous system.

**TYPE I CELL SIGNAL TRANSDUCTION**

Carotid Body Type I cells’ hypoxic-excitation reaction is correlated with O$_2$ sensitive ion channels, specifically K$^+$ channels (Shimoda and Polak, 2010). Similar to the rat, two types of chemosensitive K$^+$ channels exist in the mouse: the high-conductance Ca$^{2+}$-dependent K$^+$ channels (BK$_{Ca}$) and the TASK-like background K$^+$ channels (Ortega-Sáenz, 2010, Yamaguchi, 2004). Inhibition of these K$^+$ channels, as a result of exposure to hypoxic conditions, leads to a membrane depolarization. When the outward-moving potassium currents decrease, the cell membrane depolarizes and voltage-gated Ca$^{2+}$ channels open as a result, causing an influx of Ca$^{2+}$ (Buckler and Vaughan-Jones, 1994). The rise in intracellular [Ca$^{2+}$] subsequently induces exocytosis and the release of neurotransmitters from the Type I Cells (Gonzalez et al, 1994). This stimulates excitatory post-synaptic potentials (EPSPs) in the carotid sinus nerve (CNS), which communicates with the respiratory centers in the brain to adjust breathing patterns and ultimately return blood oxygen levels to homeostasis, completing the negative feedback loop. (See Figures 2 and 3). Currently, there are two
existing hypotheses that attempt to explain the underlying cause of the K\(^+\) channels’ inhibition: the membrane hypothesis and the mitochondrial hypothesis.
Figure 2

A schematic demonstrating the anatomical location of the carotid body (CB).  

A. The CB shown at the bifurcation of the common carotid artery (CCA), lying on the internal carotid artery (ICA) across from the external carotid artery (ECA).  

B. Type I cells enveloped by type II cells in the CB. Type I cells are innervated by the carotid sinus nerve (CSN).
MEMBRANE HYPOTHESIS

During hypoxia, plasma membrane K\(^+\) channels are inhibited (Lopez-Barneo et al., 1988). This inhibition leads to Type I cell depolarization and an increase in intracellular Ca\(^{2+}\) via the opening of voltage-gated Ca\(^{2+}\) channels. There are many classes of K\(^+\) channels that exist; however, the specific K\(^+\) channels that contribute to the depolarization of the Type I cells vary from species to species (Prabhakar, 2000). For the sake of brevity only the two types of K\(^+\) channels, which are chemosensitive in the rat, will be discussed. While it is known that the mouse shares these two chemosensitive K\(^+\) channels (Ortega-Sáenz, 2010, Yamaguchi, 2004), they vary from strain to strain and have not been fully characterized. As such, the following research focuses on the rat.

It has been suggested that the first type of channel, high-conductance Ca\(^{2+}\) - dependent K\(^+\) (BK\(_{\text{Ca}}\)) channels, plays an important role in regulating the hypoxic response of Type I cells (Peers, 1990). Some BK\(_{\text{Ca}}\) channels can be found in the open state at resting membrane potential, thus implying a possible function in propagation of action potentials (Lahiri et al., 2006, Wyatt & Peers, 1995). Williams et al (2004) describe an enzyme that is responsible for oxygen sensing known as hemeoxgenase-2 (HO-2), which has a primary purpose of creating carbon monoxide (CO). BK\(_{\text{Ca}}\) channels are activated by this CO resulting from the work of the HO-2 (Williams et al, 2004, Kemp, 2005). In a hypoxic situation, however, CO levels become so low it is impossible to sustain activation of BK\(_{\text{Ca}}\) channels in Type I cells (Kemp 2005). Interestingly enough, Ortega-Saenz et al (2006) used HO-2 null mice to show unchanged CB output in response to hypoxia, thus proposing that HO-2 may not be the sole O\(_2\) sensor, or involved at all.
A second type of O₂-sensing K⁺ channel was discovered to be open at resting membrane potential in Type I cells known as Twik-related acid sensing K⁺ (TASK-like) channels (Lahiri et al, 2006). Although these channels were first thought to be voltage-insensitive background K⁺ channels (Buckler, 1997), it was later discovered these channels bared a similar resemblance to TASK channels, hence the name “TASK-like” (Buckler et al, 2000). There are a variety of TASK-like channels expressed in Type I cells including TASK-1, TASK-3 and a TASK-1/TASK-3 heteromer (Kim et al, 2009). The heteromer is accountable for a majority of the oxygen-sensing background K⁺ current according to Kim et al (2009). Hypoxia leads to an inhibition of TASK channels resulting in depolarization of Type I cells and ultimately causing an influx of [Ca²⁺]₀ (Buckler et al, 2000, Buckler, 1997). In addition to hypoxic conditions, mitochondrial uncouplers and inhibitors are also known to inhibit TASK channel activity. Moreover, these channels are sensitive to mitochondrial metabolism, providing the implication that they are capable of detecting O₂ levels in blood by way of a mitochondrial mechanism rather than a membrane-delimited one. This demonstrates the potential necessity of mitochondria for hypoxic inhibition of the chemosensitive TASK-like channels in Type I cells of the CB (Buckler, 2006).

There are some pharmacological and patch-clamp studies that indicate the membrane alone is not solely responsible for Type I cell depolarization. Using various patch-clamp techniques, it has been demonstrated that hypoxia affects BKCa channels differently. Riesco-Fagundo et al (2001) demonstrated that BKCa channels of Type I cells are sensitive to hypoxia with the whole-cell and inside-out configuration. However, Wyatt and Peers (1995) showed when using the outside-out patch-clamp configuration, the same channels were no longer sensitive to hypoxia. These different results illustrate that an important factor is lost
from the contents of the cytoplasm and suggests another component is necessary for depolarization.

MITOCHONDRIAL HYPOTHESIS

Mitochondria are able to produce ATP via the process of oxidative phosphorylation. This is possible given that oxygen serves as the final electron acceptor in cellular respiration. When oxygen levels are low, cells cannot rely solely on oxidative phosphorylation and must fall back on other mechanisms for energy production. Demonstrating the vital role oxygen plays in this process led to the development of the mitochondrial hypothesis of Type I cell chemotransduction, (Lahiri et al., 2006). Mills and Jobsis (1972) showed further support for this hypothesis by suggesting that specific complexes of mitochondria in Type I cells must have a low affinity for oxygen in order for oxygen sensing to occur. Depolarization of the mitochondrial membrane in Type I cells occurs during hypoxia at 60 mm Hg O₂; however, in non-oxygen-sensing chromaffin cells of the adrenal gland and sensory neurons of the dorsal root ganglion, depolarization of the mitochondrial membrane does not occur until an almost anoxic state is reached at 5 mm Hg O₂ (Duchen and Biscoe, 1992a, Duchen and Biscoe, 1992b). These studies demonstrate that the response to hypoxia of mitochondria in Type I cells is specialized.

The effects of hypoxia cause an increase in [Ca^{2+}]; in Type I cells which ultimately leads to a release in neurotransmitters acting post-synaptically on the CSN. Some compounds have been used to pharmacologically mimic these effects of hypoxia, such as mitochondrial electron transport chain inhibitors and uncouplers, as well as ATP synthase inhibitors. This
was first demonstrated in 1931 when Heymans et al used cyanide, a recognized mitochondrial IV complex inhibitor in order to increase CB activity. An ATP synthase inhibitor, Oligomycin, was used to inhibit ATP mitochondrial production, ultimately uncovering a connection between the function of mitochondria and increased activity of the CSN due to hypoxia (Wyatt and Buckler, 2004). Specific mitochondrial inhibitors also cause an increase in $[\text{Ca}^{2+}]_i$ in addition to inhibition of background $\text{K}^+$ channels (Wyatt and Buckler, 2004). These mitochondrial inhibitors also prevent Type I cells from responding to hypoxia suggesting a similar mechanism mediating both of these processes (Mulligan et al, 1981).

It is not well understood how the mitochondria of Type I cells couple to the $\text{K}^+$ channels of the plasma membrane, however, one theory suggests ATP regulates TASK-like $\text{K}^+$ channels such that when there is a decrease in ATP levels, $\text{K}^+$ channels become inhibited (Varas, Wyatt and Buckler, 2007). As a result of decreased ATP levels caused by hypoxia, there is an increase in cellular AMP/ATP ratio, ultimately leading to the activation of adenosine monophosphate-activated protein kinase (AMPK). This activation of AMPK causes inhibition of $\text{K}^+$ channels which then depolarizes the cell and opens voltage-gated calcium channels. The resulting calcium influx induces exocytosis, releasing neurotransmitters from the CB Type I cells (Evans, 2006, Wyatt et al, 2007). (See Figure 3.)
Figure 3

A schematic illustrating hypoxic chemotransduction within a CB Type I cell. Low blood oxygen levels caused by hypoxia results in reduced mitochondrial ATP production and subsequent activation of AMPK. AMPK inhibits K\(^+\) channels, depolarizing the cell and opening voltage-gated Ca\(^{2+}\) channels. The influx of Ca\(^{2+}\) induces exocytosis and neurosecretion which allows the CB to communicate with the respiratory centers in the brain via the CSN, mediating breathing and returning blood oxygen to homeostasis.
AMPK

Adenosine monophosphate-activated protein kinase (AMPK) has been identified as a master regulator of energy balance throughout the body (Hardie, 2008). Recent evidence suggests that AMPK can also influence neuronal excitability (Ross, 2011) by regulating a multitude of ion channels, including TASK3 and BK$_{Ca}$ (Dallas, 2009; Ross, 2011). Moreover, there is increasing evidence that AMPK is critical in the transduction cascade whereby hypoxic inhibition of K$^+$ channels causes depolarization and subsequent neurotransmitter release from CB Type I cells (Evans, 2006, Wyatt et al, 2007). Activation of AMPK is dependent on one of its three known upstream kinases: LKB1 (Hawley, 2003, Woods, 2003), CamKKβ (Hawley, 2005, Woods, 2005), and TAK1 (Momcilovic, 2006) (See Fig 4). These upstream kinases phosphorylate AMPK at the threonine-172 (Thr-172) location on the catalytic alpha subunit (Carling, 2008, Fogarty, 2010). Once activated, AMPK is then free to phosphorylate different targets, including the aforementioned background, TASK-like K$^+$ channels and BK$_{Ca}$ channels. This suggests that inhibition of the upstream kinases of AMPK may attenuate the hypoxic response in CB Type I cells.

The compound STO-609 has been widely used as a selective pharmacological inhibitor of CamKKβ in a variety of preparations (Hawley, 2005, Tamas, 2005, Tokumitsu, 2002). The crystallized structure of the STO-609-CamKKβ complex shows that STO-609 binds near the ATP-binding site of the kinase, inducing structural changes that result in a closed conformation state (Kukimoto-Niino et al, 2011). This specific inhibitor has been used in conjunction with calcium imaging experiments (Monteiro, 2008, Wayman, 2004) as well as electrophysiological recordings (Tamas, 2006, Schmitt, 2005). Therefore, STO-609 seemed suitable for examining the potential role of CamKKβ as a regulator in the hypoxic
chemotransduction of Type I cells. If CamKKβ is inhibited by STO-609, the AMPK activation cascade will be disrupted, allowing the chemosensitive K⁺ channels that would normally close under hypoxic circumstances to stay open, inhibiting depolarization of the cell membrane and subsequent influx of Ca²⁺. The hypoxic response would then ultimately be attenuated.
Figure 4

A schematic showing the activation of AMPK by upstream kinases via phosphorylation of the Thr-172 on the alpha-subunit. AMPK is then able to phosphorylate K⁺ channels, inhibiting them and causing membrane depolarization and Ca^{2+} influx through voltage-gated Ca^{2+} channels.
HYPOTHESIS

It has been shown that AMPK may play a role in the chemotransduction of hypoxia in the carotid bodies. AMPK is activated by upstream kinases such as LKB1 and TAK1. It can also be activated by a Ca\(^{2+}\) mediated pathway involving CamKK\(\beta\) through phosphorylation of Thr-172 in an AMP-dependent manner. It is anticipated that if CamKK\(\beta\) is required for a full response to hypoxia, then its inhibition via the selective CamKK\(\beta\) inhibitor STO-609 will attenuate this response.

SUMMARY

The Type I cells of the carotid body, located at the bifurcation of the common carotid artery, are chemosensors, possessing the ability to detect changes in the levels of pH, CO\(_2\) and O\(_2\) in arterial blood composition. Upon hypoxic exposure, the Type I cells depolarize potentially through an AMPK-regulated mechanism, resulting in the release of neurotransmitters which communicate with the brain to correct breathing patterns and restore homeostasis. This cellular cascade involving AMPK may also be governed by the upstream kinases of AMPK, including CamKK\(\beta\). Inhibiting this AMPKK with the compound STO-609 may cause an attenuation of the hypoxic response.
II: 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 MOUSE CAROTID BODY TYPE I CELLS

On days of experimentation, two to five adult mice (25 – 35 g) were individually placed into a chamber and quickly euthanized with a rising concentration of CO₂. Using a dissection microscope (Omâna, Japan) under low magnification, the legs of the mouse were taped down and secured. Two incisions were made between the shoulders and along the sternum to expose the subcutaneous fascia. After removal of the local skin, the salivary glands and lateral tracheal muscles were cut and removed using fine forceps and scissors (Moria, Fine Science Tools, USA) to reveal the common carotid arterial bifurcation. The overlying vagus nerve was snapped. The entire carotid bifurcation containing the carotid body tissue was then carefully removed and placed directly into ice-cold, Dulbecco’s phosphate buffered saline (DPBS) without Ca²⁺ or Mg²⁺ (Sigma). The carotid bifurcations were then dissected further, separating the arterial and connective tissues from the carotid body tissue using fine forceps underneath the Omâna dissecting microscope. After taking tail snips of each mouse for PCR, the carcasses were disposed of according to the Lab Animal Research specifications.
The cleaned carotid bodies were placed in a digestive enzyme solution (0.4mg ml\(^{-1}\) collagenase type I, 220u mg\(^{-1}\) (Worthington Biochemical Corporation), 0.2mg ml\(^{-1}\) trypsin type I, 8550 BAEE u mg\(^{-1}\) (Sigma)) in DPBS with low CaCl\(_2\) (86 μM) and MgCl\(_2\) (350 μM) for 20 minutes at 37°C to digest the connective tissue holding the carotid body together. The carotid bodies were teased apart and incubated again at 37°C for 7 minutes. After this final digestion, the carotid body tissue was removed from the Petri dish using a fire-polished, silanized (Sigmacote, Sigma) Pasteur pipette and placed into a test tube. The tissue was triturated and centrifuged at 770 rpm (200 x g) for 5 minutes. The cells were then re-suspended in tissue culture medium (Ham’s F12 (Sigma) supplemented with 10% heat inactivated fetal bovine serum (Biowest)), centrifuged again for 5 minutes at 770 rpm (200 x g), re-suspended in tissue culture medium, and plated onto 12 mm diameter, matrigel (BD Biosciences) coated glass coverslips. The growth factor reduced, phenol-red free matrigel prep was used. 57 μL matrigel aliquots were thawed and diluted with 220 μL ice cold F-12 Ham’s nutrient medium (Sigma) before plating 30-50 μL onto the center of the coverslips. Slips were maintained at 37°C in a humidified, 5% CO\(_2\)/air incubator. Cells were allowed to adhere for two hours, and all cells were used for experimentation within eight hours.

CULTURE METHOD OF N2A CELLS

Mouse neuroblastoma cells (N2A) lines were maintained, split (1:20 ratio), and grown the week prior to usage. On the day of experimentation, the 75 mm\(^2\) Tissue Culture flask (Corning) containing the cells was removed from the 37°C, humidified, 5% CO\(_2\)/air incubator. The remaining growth media in the flask taken from a stock comprised of 250 ml DMEM with high glucose (Gibco Life Technologies), 250 ml Opti-MEM + GlutaMAX.
(Gibco Life Technologies), 25 ml 5% Fetal Bovine Serum (BioWest) and 5 ml penicillin/streptomycin solution (10,000 units/ml penicillin 10,000 μg/ml streptomycin) (Gibco Life Technologies) was removed and the cells were rinsed with 5 ml Phosphate Buffered Saline (Gibco Life Technologies). 2 ml of 0.25% Trypsin-EDTA (Gibco Life Technologies) was added and allowed to incubate at 37°C for 2 minutes to loosen the cells from the flask. 5 ml of the growth media was added to stop digestion and the cells were drawn up and placed in a 50 ml tube. The cells were centrifuged at 750 rpm for 5 minutes. After removing the supernatant, the pellet was resuspended in 10 ml of the growth media and plated in 50 μl increments onto 35 mm diameter Fluoro dishes with 10 mm diameter cover glass bottoms (World Precision Instruments) coated with poly-d-lysine (0.01% w/v). All procedures were performed within a fume hood under sterile conditions.

CALCIUM IMAGING EXPERIMENTS – FURA-2

Isolated Type I cells were loaded with the Ca^{2+}-sensitive fluorescent dye FURA-2-AM (5 μM, Invitrogen) in an extracellular solution of composition (in mM): NaCl, 140; KCl, 4.5; CaCl₂, 2.5; MgCl₂, 1; glucose, 11; HEPES, 11; pH 7.4 with NaOH. After removing the remaining media from the cells, the coverslips were added to the dishes containing the prepared dye solution. The cells were left for 30 minutes to allow the dye to permeate the cells. The slips were then rinsed in another dish containing 3 ml HEPES solution and transferred to a third dish of 3 ml HEPES solution in which they rested for another 10-15 minutes to allow the cells time to cleave the –AM groups from the dye. For each rest period, the cells were kept in a drawer to minimize light exposure.
Coverslips were mounted in a chamber (0.4 ml, RC-25F, Warner Instruments) and perfused at 8 ml/minute with extracellular solution of composition (in mM): NaCl, 140; KCl, 4.5; CaCl₂, 2.5; MgCl₂, 1; glucose, 11; HEPES, 11; pH 7.57 with NaOH. Cells were visualized with a Nikon TE 2000U inverted microscope with a CFI super fluor x40 oil immersion objective. The Fura-2 loaded cells were excited with 50 msec exposures to 340 nm and 380 nm light at 0.2 – 0.5 Hz using a Lambda 10-3 filter wheel (Sutter). The filtered light was reflected up to the cells by way of a 400 DCLP dichroic mirror (Chroma). The emitted fluorescence measured at 510 nm was then allowed to pass through the Fura-2 Set filter (Chroma) and be captured using a CoolSNAP HQ2 CCD camera (see Figure 6). Neutral density filters of 0.7 optical density (Chroma) were placed in the excitatory light path to prevent any photodamage to cells. Data acquisition and analysis was controlled using Metafluor 7.1.2 imaging software (Molecular Devices) (see Figure 5). Fluorescence levels were measured before, during, and after perfusing STO-609 (100 μM, Tocris) over the cells for approximately ten minutes.
Figure 5

Schematic illustrating the lab setup for the calcium imaging experiments.
Figure 6

Figure illustrating the Fura-2 dichroic filter transmission. Fura-2 emission is high when excited with 340 light when Ca$^{2+}$ is bound, conversely, its emission is high when excited with 380 light when Ca$^{2+}$ is unbound. It emits fluorescence in the range of 510 nm. The grey line represents the dichroic mirror, reflecting the 340 and 380 nm light, and letting pass the 510 nm light.
CALCIUM IMAGING EXPERIMENTS – X-RHOD-1

Isolated Type I cells were loaded with the Ca\(^{2+}\)-sensitive fluorescent dye X-Rhod-1-AM (5 \(\mu\)M, Invitrogen) using the same procedure as Fura-2.

The X-Rhod-1 loaded cells were excited with 50 msec exposures to 580 nm light at 0.2 Hz reflected up to the cells by way of a Texas Red dichroic mirror (Chroma). The emitted fluorescence measured at 602 nm was then allowed to pass through the dichroic mirror and be captured using a CoolSNAP HQ2 CCD camera (see Figure 7). Neutral density filters were reduced to 0.2 optical density (Chroma) to improve the signal. Fluorescence levels were measured before, during, and after perfusing STO-609 (100 \(\mu\)M, Tocris) over the cells for approximately ten minutes. For these experiments, a Type I cell excited by a high K\(^+\) extracellular solution of composition (in mM): NaCl, 64; KCl, 80; CaCl\(_2\), 2.5; MgCl\(_2\), 1; glucose, 11; HEPES, 11; pH 7.57 at 24\(^\circ\) C with KOH were used, as well as unexcited N2A cells. All other imaging and recording parameters were identical to those used for the Fura-2 experiments.
Figure 7

Figure illustrating the X-Rhod-1 dichroic filter transmission. X-Rhod-1 emission is high when excited with 580 light when Ca\textsuperscript{2+} is bound. It emits fluorescence in the range of 602 nm. The grey line represents the dichroic mirror, reflecting the 580 nm light, and allowing to pass the 602 nm light.
CALCIUM IMAGING EXPERIMENTS – IONOMYCIN

Experiments were also conducted both in cells loaded with Fura-2 and X-Rhod-1 at zero [Ca$^{2+}$]$\textsc{i}$ by perfusing cells with the extracellular solution described previously, but with Ca$^{2+}$ removed and 10 mM EGTA and 2 μM of the calcium ionophore ionomycin added. After applying the solution containing ionomycin, Ca$^{2+}$ levels were allowed to reach a zero baseline before applying STO-609 (100 μM, Tocris) in conjunction with the modified extracellular solution. Fluorescence emission levels were measured throughout this process, as well as upon removal of the drug.

IMAGING DATA ANALYSIS

The dissociation procedure utilized here predominantly yields individual Type I cells. Occasionally clusters of cells were observed, but these were not recorded from as locally released neurotransmitters could affect the nearby cells and this could confuse any results obtained. Regions of interest (ROI) were drawn over the isolated cells using Metafluor software and the fluorescence signal was taken as the average within the ROI. Data were background subtracted. Application of STO-609 was only performed once per coverslip to avoid any possible sensitization or desensitization effects.
ELECTROPHYSIOLOGY EXPERIMENTS

Isolated Type I cells were mounted in a chamber (0.4 ml, RC-25F, Warner Instruments) and perfused at 8 ml/minute with extracellular solution of composition (in mM): NaCl, 117; KCl, 4.6; CaCl₂, 2.5; MgCl₂, 1; glucose, 11; NaHCO₃, 23; pH 7.57 via bubbling with CO₂. Cells were visualized with a Nikon TE 2000U inverted microscope. The traditional whole-cell configuration of the patch clamp technique was used (see Figure 8). Data were acquired using an Axopatch 200B amplifier (Molecular Devices). Currents were digitized at 5 kHz and filtered at 1 kHz. Experiments were performed at 35-37°C. Results were not leak subtracted, seal resistance was typically >5 GΩ, access resistance was typically 20-30 MΩ and was not compensated, and holding currents were typically less than 20 pA throughout the recordings. All data were recorded using Clampex v10.0 software and analyzed using Clampfit v10.0 software (Molecular Devices). All experiments were carried out on an anti-vibration microscopy table (TMC).

Micropipettes were made from 1.5 mm O.D. x 0.86 mm I.D. borosilicate glass capillaries (Harvard Apparatus) inserted into a Narishige PC-10 Puller. The tips were polished using a Narishige MF-900 Microforge and the ends were fire-polished using a methanol lamp. Only micropipettes with tip resistances between 5 and 12 MΩ were used. After carefully dipping the tips in an intracellular solution of composition (in MM): NaCl, 10; KCl, 130; CaCl₂, 1; MgSO₄, 2; EGTA, 11; HEPES, 11; ATP, 2; pH 7.2 with KOH, micropipettes were back-filled with the same solution. The Ag/AgCl electrode wire was threaded into the micropipette and mounted onto a micromanipulator (Sutter). After gradually lowering the tip to make contact with the cell membrane, gentle suction was
applied to form a gigaseal and further suction to quickly break the membrane and allow electrical access to the cell interior.

Once a stable patch was achieved, recordings were taken approximately every minute following. STO-609 (100 µM, Tocris) was applied after achieving several baseline recordings. In later experiments, the BK$_{Ca}$ channel blocker paxilline (1 µM, Sigma) was applied prior to, and in conjunction with STO-609. Procedural methods were otherwise identical.
Figure 8

Schematic illustrating the lab setup for the electrophysiology experiments.
STATISTICS

Data are presented as mean ± S.E.M. Differences between individual means were determined by a paired or unpaired Student’s t-test as appropriate. A value of P < 0.05 was taken to indicate statistical significance.
III: RESULTS

CALCIUM IMAGING CONTROL EXPERIMENTS

Initial experiments were to see if intracellular Ca\(^{2+}\) increase during hypoxic exposure (10 Torr) could be altered by the CamKK\(\beta\) inhibitor STO-609 (100 \(\mu\)M). Intracellular calcium concentration is an indicator of cellular excitation. In hypoxia-depolarized Type I cells, intracellular calcium levels rise to induce neurosecretion. Therefore, calcium imaging was the technique chosen to examine this topic. It was expected that STO-609 would attenuate the hypoxic response in mouse Type I cells by inhibiting the CamKK\(\beta\)/AMPK cascade thereby prevent a rise in intracellular Ca\(^{2+}\) concentrations (see Figure 9). However, as STO-609 is not a commonly used or well-researched drug, control experiments were first performed on resting, normoxic Type I cells to discern whether or not STO-609 had any unforeseen extraneous or artefactual effects.
Figure 9

Plot illustrating anticipated calcium imaging results (using Fura-2) from application of STO-609 on a hypoxia-depolarized Type I cell. STO-609 reduces intracellular $[\text{Ca}^{2+}]$ over the course of several minutes and the cell recovers upon removal of the drug.
CALCIUM IMAGING EXPERIMENTS – FURA-2

The green calcium-fluorescent dye Fura-2 was used in these experiments due to its common laboratory use and ratiometric properties.

Isolated, resting Type I cells loaded with Fura-2 (5 µM) were perfused with normoxic Tyrode’s solution. Application of STO-609 (100 µM) caused an increase in 340 and 380 nm fluorescence in both the presence and absence (blank area of coverslip) of cells and decreased upon drug removal (see Figure 10). Both the 340 and 380 nm signals increased upon drug application instead of opposing one another. The 380 nm fluorescence increased faster than 340 nm fluorescence in both instances, resulting in an overall decrease in signal ratio. The changes in fluorescence were observed within seconds of STO-609 application, which is uncharacteristically rapid (Hawley, 2005).

The changing background fluorescence indicated that STO-609, which is green in solution, likely autofluoresces in the green spectrum (510 nm). To avoid this signal artefact, a calcium dye that fluoresces in the red spectrum was used in subsequent experiments.
Figure 10

CALCIUM IMAGING EXPERIMENTS – X-RHOD-1

Isolated, resting Type I cells loaded with the red calcium-fluorescent dye X-Rhod-1 (5 µM) were perfused with normoxic Tyrode’s solution. A high K\(^+\) challenge was used to depolarize the cell. Though it did not recover, STO-609 (100 µM) was applied regardless. The drug caused a rapid decrease in X-Rhod-1 emission fluorescence and reversal upon removal (Figure 11). Because a high K\(^+\) depolarization does not operate via the CamKKβ pathway, it was not expected that STO-609 would cause a decrease in fluorescence levels. Background fluorescence (no cell) levels did not change over the course of the experiment, indicating that the artefactual effect of the drug’s green color had been successfully eliminated.

At this point, it became clear that the effect seen may be due, at least in part, to interactions between STO-609 and the dyes independent of the cell. Since hypoxic depolarization is not required to examine this effect, the remaining calcium imaging experiments were carried out in mouse neuroblastoma cells (N2A), which are easy to divide and culture, instead of mouse Type I cells, which must be dissected out, are fragile, and have low yields.

Experiments were repeated in resting N2A cells. Application of STO-609 (100 µM) caused a rapid decrease in emission fluorescence and reversal upon drug removal. Resting cells characteristically have low intracellular Ca\(^{2+}\) levels, thus such a dramatic decrease in fluorescence seen here was not expected. Once again, background fluorescence (no cell) levels showed no change.
To discern if any portion of these effects are also artefactual and not caused by STO-609 acting via the CamKKβ pathway, the following experiment was designed to eliminate Ca$^{2+}$ itself as a variable.

If a decrease in fluorescence levels is seen in the presence of 0 mM Ca$^{2+}$, then the effect must be artefactual, as there is no Ca$^{2+}$ to bind and unbind to the dyes and alter their fluorescence emission.
Figure 11

A.i., B.i. No Cell. High K⁺ perfusion (A.ii.) and application of the drug (A.ii., B.ii.) result in no fluorescence change. A.ii. Type I cell. The cell is depolarized with a high K⁺ perfusion, but fails to recover. Application of STO-609 caused a decrease in X-Rhod-1 fluorescence and an increase upon removal. B.ii. N2A Cell. X-Rhod-1 fluorescence decreased with STO-609 application and increased upon removal.
A.i. High K⁺ STO-609

A.ii.

B.i. STO-609

B.ii.
CALCIUM IMAGING EXPERIMENTS – IONOMYCIN

Changes in calcium-fluorescent dye emissions correlate directly with changes in intracellular \([\text{Ca}^{2+}]\). If \([\text{Ca}^{2+}]\) is eliminated as an experimental variable, then any fluorescence changes seen after application of STO-609 must be artefactual – possibly due to direct drug-dye interaction. To do this, a calcium ionophore Ionomycin (2 µM) was used to perforate the cell membrane, allowing free passage of \([\text{Ca}^{2+}]\). This, in conjunction with a 0 mM \([\text{Ca}^{2+}]\) HEPES solution with added EGTA (5 mM) was perfused over N2A cells loaded with Fura-2 and repeated in N2A cells loaded with X-Rhod-1. After a 0 \([\text{Ca}^{2+}]\) baseline was reached, STO-609 (100 µM) was applied. Fluorescence emission signals immediately decreased upon drug application and subsequently recovered upon drug removal for both experiments (see Figure 12). The Fura-2 data were background subtracted to account for the STO-609 autofluorescence in the 510 nm range.

These changes in fluorescence levels without any \([\text{Ca}^{2+}]\) present suggest that STO-609 may be directly interacting with the dyes and causing artefacts. Continuing to test different calcium fluorescent dyes may well result in a repeat of the pattern of artefacts, thus a different experimental approach was ultimately recommended.
Figure 12

A. N2A Cell. Background subtracted data of N2A cells loaded with Fura-2. Application of STO-609 decreased emission signal in the presence of 0mM Ca$^{2+}$, indicating artefact. B. N2A Cell. Background subtracted data of N2A cells loaded with X-Rhod-1. Application of STO-609 decreased emission signal in the presence of 0mM Ca$^{2+}$, indicating artefact. 0mM Ca$^{2+}$ solution containing 5mM EGTA and 2 μM ionomycin was applied to cells loaded with each dye causing the large, initial spike as the bath calcium temporarily mixed with the intracellular solution before washing out of the cell.
ELECTROPHYSIOLOGY CONTROL EXPERIMENTS

Instead of indirectly measuring cellular activity via intracellular calcium levels, cellular activity can be *directly* measured via electrophysiology. As was done with the calcium imaging experiments, control electrophysiology experiments were first performed on resting, normoxic Type I cells prior to excited, hypoxia-depolarized Type I cells to account for any unforeseen interactions and/or effects.

Type I cells exhibit small outward potassium currents at rest. These potassium channels close as the cell is exposed to hypoxia, leading to depolarization of the cell membrane, opening of voltage-gated calcium channels, an influx of intracellular calcium, and neurosecretion (see Figure 3). Furthermore, upon depolarization large conductance voltage-activated $K^+$ currents are activated to repolarize the cell. However, in the presence of hypoxia a component of these (the $BK_{Ca}$ currents) are inhibited. As such, hypoxia-exposed cells should display substantially reduced outward $K^+$ currents. Therefore, if CamKKβ is involved in the upstream control of the AMPK cascade that aids in the closing of these $K^+$ channels, then application of the CamKKβ inhibitor STO-609 during hypoxic exposure may result in an attenuated effect of hypoxia – currents somewhere between the resting and hypoxic levels (see Figure 13). Applied to an unstimulated cell, STO-609 is not expected to have any effect (unless AMPK has tonic activity), but it may inhibit any stimulated closing of these $K^+$ channels by way of AMPK, and thus increase outward-moving currents.
Figure 13
Current-voltage (I-V) plot illustrating expected electrophysiology results from application of STO-609 on a hypoxia-depolarized Type I cell. Example corresponding raw current traces at +20 mV shown inset. Normoxic outward currents are substantially reduced upon hypoxia exposure. Applying STO-609 during this exposure might be expected to attenuate the hypoxic inhibition.
ELECTROPHYSIOLOGY EXPERIMENTS – STO-609

Traditional whole-cell configuration of the voltage-clamp technique was used. A programmed voltage step protocol from -80 mV to +60 mV in 10 mV steps at 200 ms duration per step was used. Protocols were executed at one minute intervals. Once a gigasealed patch was achieved and several stable baseline recordings were taken, STO-609 was applied to the cells for several minutes. If the cell did not detach during this period, STO-609 was removed to observe any reversal effects.

Application of STO-609 (100 µM) caused a rapid decrease in evoked outward current (see Figure 14). Measured at +10 mV, currents were reduced by an average of 51.78 pA ±19.73, (n=5). This decrease was statistically significant (P = 0.029). The recordings did not last long enough to observe any effects of drug removal. This effect is the reverse of what might be expected of STO-609. Notably, the raw traces observed after STO-609 application appeared smoother and more level. This could indicate that STO-609 may be inhibiting large-conductance Ca\(^{2+}\)-activated potassium channels (BK\(_{Ca}\)), the opening and closing of which is visible on raw traces, making them appear rugged. If STO-609 is selectively inhibiting these channels, then applying a BK\(_{Ca}\) blocker prior to and in conjunction with STO-609 should prevent any further channel inhibition and current reduction by the drug.
**Figure 14**

Example I-V plot from an isolated Type I cell. Application of STO-609 caused a decrease in outward current. Corresponding raw current traces at +20 mV shown inset.
ELECTROPHYSIOLOGY EXPERIMENTS – STO-609 + PAXILLINE

Paxilline is blocker of large-conductance calcium-activated potassium channels (BK\textsubscript{Ca}). Application of this inhibitor will reduce outward currents by blocking these channels. If STO-609 is responsible for any inhibition of BK\textsubscript{Ca} channels, then application of it after and in conjunction with paxilline should result in no further reduction in current.

Application of paxilline (1 µM) caused a decrease in outward current relative to baseline recordings over the course of several minutes (see Figure 14). Measured at +20 mV, currents were reduced by an average of 75.10 pA ±18.22 (n=3). This decrease was statistically significant (P = 0.027). Once no further reduction was observed, STO-609 (100 µM) was applied in conjunction with the paxilline (1 µM). Currents either decreased slightly or not at all (see Figure 15). Measured at +20 mV, currents were reduced by an average of 35.77 pA ±17.94, (n=3), but this mean reduction was not statistically significant (P = 0.092).
**Figure 15**

Example I-V plot from an isolated Type I cell. Application of the $\text{BK}_{\text{Ca}}$ channel inhibitor Paxilline caused a decrease in current. Application of Paxilline and STO-609 caused no further decrease in current. Corresponding raw traces at +20 mV shown inset.
IV: DISCUSSION

The results of this thesis illustrate two major findings: STO-609 directly interacts and alters the fluorescent properties of the Ca$^{2+}$-fluorescent dyes Fura-2 and X-Rhod-1, and that STO-609 selectively inhibits BK$_{Ca}$ channels. This is the first time that either of these artefactual effects has been observed. Each will be discussed in kind.

The calcium imaging data shows that the application of STO-609 caused artefactual changes in the fluorescent emissions of both Fura-2 and X-Rhod-1. The rapid pace at which the drug showed effects, the decrease in intracellular calcium in a non-hypoxically excited cell, and the large effect seen on resting intracellular calcium levels were all signs that indicated the data may be artefactual. The ionomycin experiments, which eliminated Ca$^{2+}$ as a variable, confirmed that the drug must be directly interacting with the dyes in both cases. While it is possible that STO-609 may be compatible with other non-green spectrum emission dyes, it is likely that it could interact directly with those other dyes in a similar fashion due to the common mechanism by which they all bind calcium.

The electrophysiology results show that STO-609 selectively interacts with and inhibits BK$_{Ca}$ channels. Not only was it predicted that STO-609 would have the opposite effect on K$^+$ currents when applied during normoxic conditions (an increase, if anything – see Fig 13), but the lack of any further significant decrease in currents after the application of paxilline, a specific BK$_{Ca}$ blocker, indicated that STO-609 was without effect on other channels contributing to the macroscopic current.

This pattern of direct interaction and inhibition suggests that STO-609 may be acting via the Ca$^{2+}$-binding domains on these kinases, dyes, and channels. The drug may be binding competitively at the active site or binding near the active site and altering the shape and
functionality of the molecules, as it does with CamKKβ (Kukimoto-Niino et al, 2011). It is therefore recommended that STO-609 not be used in conjunction with calcium imaging techniques, and caution should be taken when using STO-609 in the presence of other molecules that bind Ca\(^{2+}\), as it may interfere with their function, although this remains to be tested. If it is used in the presence of Ca\(^{2+}\)-binding elements, then good control experiments must be performed to ensure that any effects seen from the application of STO-609 is not artefactual or are via targets other than CamKKβ. Otherwise, alternate techniques or the use of an alternate specific inhibitor of CamKKβ is recommended.

FUTURE EXPERIMENTS

To bolster the findings that STO-609 inhibits BK\(_{\text{Ca}}\) channels, similar electrophysiology experiments could be performed on cells that have known BK\(_{\text{Ca}}\) channel expression but not CamKKβ expression. If application of the drug causes a decrease in K\(^{+}\) currents, then this must be due to direct channel inhibition and not due to indirect influence from the CamKKβ/AMPK cascade.

Instead of pharmacological inhibition, other experiments using siRNA or shRNA to specifically knock down CamKKβ could be performed. However, in addition to the potential off-target effects, transfection and incubation of siRNA and shRNA typically takes 24+ hours, while our experiments using Type I cells must be performed the same day they are extracted.

It is worth noting that while more experiments could be designed to parse out exactly with what channel or by which mechanism STO-609 is creating artefactual effects, the end result of these experiments will not change the ultimate conclusion. STO-609 cannot be used
to test the original question posed in this thesis – whether or not inhibition of upstream kinases that activate AMPK can attenuate the hypoxic response in Type I cells. To continue investigating this topic, a new specific inhibitor of CamKKβ must be used, or the focus must be shifted to one of the other upstream kinases, LKB1 or TAK1. There is evidence that another pharmacological inhibitor of CamKKβ exists, KN-93 (Jensen, 2007), however it is also a potassium channel blocker and therefore would be incompatible with these studies (Rezazadeh, 2005).

CONCLUSION

STO-609 directly interacts with Fura-2 and X-Rhod-1, altering their fluorescent properties even in the absence of Ca$^{2+}$. STO-609 also inhibits outward currents of mouse Type I cells, likely via large-conductance calcium-activated K$^+$ channels ($\text{BK}_{\text{Ca}}$). Due to these novel findings, this drug should not be used in conjunction with calcium imaging techniques and caution should be taken when using it in the presence of other compounds that bind Ca$^{2+}$.

At present, STO-609 is used as a selective CamKKβ inhibitor in a variety of preparations in many different cell types due to its ease of use as drug. However, these new data indicate that in addition to care moving forward regarding STO-609’s unanticipated effects, previous work in which the drug was used in the presence of calcium imaging dyes or $\text{BK}_{\text{Ca}}$ channels should be carefully scrutinized and potentially reevaluated. For example, Monteiro, et al showed that “using spectrofluorometry and the Ca$^{2+}$-sensitive probe Fura-2-AM, STO-609 was demonstrated to elicit a rapid increase of [Ca$^{2+}$]i in MCF-7 cells” (2008). However, control experiments were not performed to demonstrate the effects of STO-609
alone on these cells, and given the strong evidence presented here that STO-609
autofluoresces in the green spectrum, it is possible that their results and the conclusions they
drew from them may be erroneous.
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