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Regulation of the Chloride Store in the Retinal Amacrine Cells

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REGULATION OF THE CHLORIDE STORE IN RETINAL AMACRINE CELLS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biological Sciences

by

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B.S., University of Chennai, 2003
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August 2015
இதுவும் கடந்து பாகும்
(This Too Shall Pass)
Dedicated To My Mother
ACKNOWLEDGEMENTS

I remember sitting in the last bench of my classroom during my 6th grade and wondering what I would best become in life. I eventually figured out biology was one subject I can understand and internalize concepts thoroughly. I would never regret making biology as my career choice.

Everybody at some point in their life would benefit from a guru (mentor) to help guide them. I have not had that opportunity until I met Dr. Evanna Gleason. It was one of the most fortunate moments in my career when I received an email from Evanna that she was interested in taking me up as a graduate student in her lab. Evanna has been an excellent guide and has forged and tempered me over the years. She has provided me with great latitude in pursuing research ideas and placed her confidence in putting me in challenging research projects. I am thankful to her for preparing me for a career in neuroscience.

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ABSTRACT

Nitric Oxide (NO) is a key gaseous messenger that has been shown to be produced by multiple cell types in the vertebrate retina. Research in our lab is aimed at unlocking critical synaptic functions of NO. A major finding from our lab is that NO affects synaptic responses in amacrine cells by altering the plasma membrane gradient for Cl\(^-\). This is due to release of Cl\(^-\) from an internal store and this in turn dependent on a decrease in cytosolic pH.

Determining the factors regulating cytosolic Cl\(^-\) in neurons is fundamental to our understanding of the function of GABAergic and glycinergic synapses. This is because the Cl\(^-\) distribution across the postsynaptic plasma membrane determines the sign and strength of postsynaptic voltage responses. Here, my goals were to confirm the compartmental nature of the internal Cl\(^-\) store and to test the hypothesis that Cl\(^-\) is being released from acidic organelles such as the Golgi, synaptic vesicles, endosomes or lysosomes.

To accomplish this, I made whole cell voltage clamp recordings from cultured chick retinal amacrine cells and used GABA-gated currents to track changes in cytosolic Cl\(^-\). The compartmental pH was monitored using LysoSensor imaging. My results demonstrate that increasing compartmental pH with internal weak bases leads to release of Cl\(^-\) into the cytosol and subsequent addition of NO causes a reduction rather than the usual increase in cytosolic Cl\(^-\). In contrast, collapsing proton gradients and thus proton-dependent membrane potentials completely blocked the ability of NO to release compartmental Cl\(^-\).

These results indicate that maintenance of internal proton gradients is critical to the mechanisms of Cl\(^-\) release and that Cl\(^-\) is likely to come from acidic organelles. Additionally, I tested the hypothesis that the store can be emptied and refilled. I determined the conditions under which the store can be depleted and the dependence of refilling on extracellular Cl\(^-\). These results
demonstrate that the regulation of cytosolic Cl⁻ is closely linked to multiple regulatory processes and further our understanding of repertoire of NO signaling mechanisms.
CHAPTER 1
INTRODUCTION

Vertebrate retina

The vertebrate retina captures photons from the environment and processes that stimulus to form an image of visual space. The input side of the vertebrate retina consists of the photoreceptors where photons are transduced into an electrical signal. This signal passes through multiple synapses in the retina before being sent to the higher visual centers in the brain. The vertebrate retina is relatively accessible part of the central nervous system so it has long been studied as a model system for neural information processing.

The vertebrate retina consists of 6 layers: the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer, the ganglion cell layer (GCL) and the nerve fiber layer (Figure 1.1). The ONL consists of the photoreceptors, the INL contains horizontal cells, bipolar cells, and amacrine cells, and the GCL consists of ganglion cells and amacrine cells. The synaptic connections of photoreceptors, horizontal cells are contained within the OPL and the IPL consists of synapses among bipolar cell, amacrine cell and ganglion cell synapses.

After entering the retina, light travels through all the layers before reaching the photoreceptors. This signal then is carried back through the layers of the retina by vertical and horizontal interactions. The vertical pathway involves photoreceptors, bipolar cells, and ganglion cells. The horizontal pathway consists of photoreceptors, horizontal cells, and amacrine cells (Cook and McReynolds 1998).
Figure 1.1. A schematic illustration of retinal cell types and their organization in the retina. The retina is divided into three cellular layers: the outer nuclear layer (ONL), the inner nuclear layer (INL) and the ganglion cell layer (GCL). The outer nuclear layer (ONL) contains the rod and cone photoreceptors. The inner nuclear layer (INL) contains horizontal cells, bipolar cells, and amacrine cells. The ganglion cell layer (GCL) contains ganglion cells and amacrine cells. The synaptic layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL) contain the synapses between retinal cell types.
Retinal amacrine cells

Amacrine cells are a diverse class of interneurons that are predominantly found in the INL and GCL of the retina and project their processes to the IPL. Amacrine cells can be classified into about 40 subtypes based on their morphology, their interactions with bipolar and ganglion cells, and neurotransmitter content (Masland, 2012).

Two broad categories of amacrine cells are the wide-field amacrine cells that release GABA and the narrow-field amacrine cells that release glycine (Pourcho and Goebel, 1983, Menger et al., 1998). The majority of amacrine cells contain either glycine or GABA, which are both usually considered inhibitory neurotransmitters (Vaney, 1990, Marquardt et al., 2001). GABAergic amacrine cells have extensive dendritic branches and large receptive fields to fine tune signals from distant regions of the retina (de Vries et al., 2011). Amacrine cell synapses are found in the IPL and amacrine to amacrine cell synapses have been demonstrated to mediate contrast sensitivity (Jarsky et al., 2011), light adaptation (Herrmann et al., 2011) and directional selectivity (Euler et al., 2002, Wei et al., 2010).

Amacrine cells also make synapses with bipolar and ganglion cells (Figure 1.2). Bipolar cells are presynaptic to amacrine cells and provide excitatory glutamatergic input. Some GABAergic amacrine cells synapse back onto the bipolar cells forming reciprocal synapses (Dowling and Boycott, 1966). Amacrine cells also make extensive inhibitory synapses onto ganglion cells that shape their response properties. For example, directionally selective ganglion cells, fire when presented with a light stimulus moving in a particular direction, but are quiescent when the stimulus moves in the opposite direction. Directionally selective ganglion cell function depends mainly on inhibitory input from GABAergic starburst amacrine cells [reviewed in (Hausselt and Euler, 2009), (Mills and Massey, 1995, Demb, 2007)]. Thus the regulation of amacrine cell synaptic output plays a central role in retinal signal processing.
Figure 1.2 Synaptic interactions of amacrine cells, bipolar cells and ganglion cells. Amacrine cells receive synaptic input from bipolar cells and other amacrine cells and serve as presynaptic partners conveying information to bipolar cells and ganglion cells.

Nitric oxide in the retina

Nitric oxide (NO) is generated through an enzymatic reaction involving conversion of L-arginine to L-citrulline (Figure 1.3). This reaction is catalyzed by enzyme the nitric oxide synthase (NOS). Of the two Ca\(^{2+}\)-sensitive isoforms of NOS: neuronal (nNOS) and endothelial (eNOS), nNOS has been the major candidate for NO generation in the visual system (Park et al., 1996, Kaur et al., 2006). However, in the chicken retina, there is evidence for the expression and activity of eNOS as well (Tekmen-Clark and Gleason, 2013).
Figure 1.3. Production of NO. NO is generated from arginine by the enzyme nitric oxide synthase (NOS). NO signaling can occur through multiple mechanisms. The canonical mechanism involves the NO-dependent stimulation of soluble guanylate cyclase but NO can also directly alter target function by S-nitrosylation. The nNOS and eNOS enzymes are calcium dependent.

The production of NO in subsets of all classes of retinal cells (Dawson et al., 1991, Yamamoto et al., 1993, Cheon et al., 2002) suggests the importance of this molecule in the retina. Amacrine cells have been shown to be a strong producer of NO especially among the subtypes AI, AII and AIII in the turtle and mammalian retinas (Kim et al., 1999, Eldred and Blute, 2005). The canonical mechanism by which NO signals, is through the activation of soluble guanylate cyclase (sGC) leading to the synthesis of cyclic guanosine monophosphate (cGMP) (Arnold et al., 1977). The NO donor S-nitroso-N-acetylpenicillamine (SNAP) has been shown to increase cGMP levels in different retinal cell types including amacrine cells (Baldridge and Fischer, 2001). Continuous light stimulation of the rabbit retina evokes NO release from amacrine cells (Neal et al., 1998). NO has shown to control the light adaptation in the rabbit.
retina (Mills and Massey, 1995). NO has also been demonstrated to modulate GABA and glycine release from amacrine cells (Yu and Eldred, 2005) and endogenously produced NO has also been shown to modulate GABAergic transmission (Maggesissi et al., 2009). Together, these studies suggest an active role of NO in regulating signaling in the inner retina.

**Significance of nitric oxide in amacrine cells**

The expression of nitric oxide synthase and the production of NO has been found for subsets of each of the retinal cell types. The expression of nNOS and eNOS in amacrine cells and the evidence supporting the production of NO by amacrine cells led to our interest in studying how NO affects synaptic function in amacrine cells. The sign of the GABAergic and glycinergic synapses depends upon the distribution of Cl\(^-\) across the plasma membrane. Any change in concentration of cytosolic Cl\(^-\) can alter the function of GABAergic and glycinergic synapses. This is because GABA and glycine activate receptors that are Cl\(^-\) channels. An NO-induced Cl\(^-\) release from internal stores was shown by previous work from our lab (Hoffpauir et al., 2006). If cytosolic Cl\(^-\) is elevated, and E\(_{Cl^-}\) is moved positive, then activation of ionotropic GABA or glycine receptors can contribute to the depolarization of the postsynaptic neuron making the synapse excitatory. They demonstrated that after NO formerly inhibitory GABAergic synapses were converted to excitatory synapses as illustrated in Figure 1.4. Furthermore, we predict that increasing excitatory input to amacrine cells will lead to an increase in inhibitory output onto ganglion cells (Figure 1.4). This prediction is consistent with a study in the mouse retina where NO was shown to suppress ganglion cell activity by an increase in inhibitory amacrine cell synaptic output (Wang et al., 2003). My project involves studying the mechanisms of Cl\(^-\) release from internal stores by NO and will help to expand the current understanding of NO-mediated signaling mechanisms in amacrine cells.
Figure 1.4. Model for NO effects on amacrine cell signaling. An nNOS-expressing GABA- or glycinergic amacrine cell receiving both inhibitory and excitatory input generates a moderate inhibitory output (top). After NO (bottom), $E_{Cl^-}$ shifts positive, all inputs become excitatory, and inhibitory output from the amacrine cell is enhanced. The cells depicted are highly simplified in that amacrine cell synaptic inputs and outputs are not typically segregated.
Lysosomotropism

The term lysosomotropism was first introduced by (De Duve et al., 1974) to designate the uptake and selective accumulation of certain weak bases substances into lysosomes. Certain types of amines such as methylamine and chloroquine diffuse in a deprotonated form resulting in an increase in intralysosomal pH (Oda and Ikehara, 1985, Strous, 1985, Caplan et al., 1987). Some of the intracellular compartments such as the endosomes and lysosomes are acidic in nature (Poole, 1981). Lysosomotropic agents such as chloroquine and methylamine are weak bases that tend to accumulate in these compartments. Lysosomotropic agents get trapped in acidic compartments (AOs) through its association with H⁺.

Figure 1.5: Schematic model illustrating the trapping of lysosomal agents and maintenance of pH across acidic compartments. Lysosomal agents (LY) have been demonstrated to alkalinize compartments including lysosomes and endosomes, and synaptic vesicles. When applied via the patch pipet, these compounds will deprotonate in the cytosol then diffuse across organellar membranes. The deprotonated weak base will become trapped when they re-protonate in the acidic environment of the AOs. The dominant effect of these compounds is to increase the pH of acidic compartments.
The most suitable weak bases are those with a pKa around 8 (Ashfaq et al., 2011). These compounds ability to alkalinize acidic compartments allowed me to study the effects of selective manipulation of AO pH and its modulation of Cl\textsuperscript{−} release and NO-mediated Cl\textsuperscript{−} release

**Sources of internal Cl\textsuperscript{−}**

Cl\textsuperscript{−} was traditionally viewed as a passively distributed ion. Over the last couple of decades, however it has been clear that Cl\textsuperscript{−} is actively transported by a diverse collection of plasma membrane and internal membrane transporters. The concentration of Cl\textsuperscript{−} in organelles varies from 5-40 mM. Several intracellular organelles serve as excellent candidates for being the source of NO releasable Cl\textsuperscript{−}. The dominant members of focus here are the AOs because they contain 40-80 mM Cl\textsuperscript{−} (Faundez and Hartzell, 2004).

One determinant of organellar Cl\textsuperscript{−} concentration is organellar pH because Cl\textsuperscript{−} can be used as an electrical shunt for acidification. The acidification process is carried out by the V-type ATPase (Marshansky and Futai, 2008). Consistent with this mechanism, the Cl\textsuperscript{−} level is related to pH. [(Sonawane, 2003), Table 1.1].

The concentration of Cl\textsuperscript{−} in the lysosomes has been difficult to calculate as the currently available Cl\textsuperscript{−} detection dyes cannot accurately measure Cl\textsuperscript{−} concentration due to its inefficiency in the low pH environment of the lysosomes. Theoretical calculations however have estimated the luminal Cl\textsuperscript{−} concentration to be around 80 mM (Weinert et al., 2010). Because of the link between pH and Cl\textsuperscript{−} content, I tested the hypothesis that AOs are the source of NO releasable Cl\textsuperscript{−}. 

![Image](image-url)
Table 1.1: List of intracellular organelles with respective pH and Cl\(^-\) content (Sonawane, 2003)

<table>
<thead>
<tr>
<th>Intracellular Organelle</th>
<th>pH</th>
<th>Chloride Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golgi Complex</td>
<td>6.4</td>
<td>50 mM</td>
</tr>
<tr>
<td>Early Endosome</td>
<td>6.2</td>
<td>40 mM</td>
</tr>
<tr>
<td>Late Endosome</td>
<td>5.3</td>
<td>60 mM</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>4.4</td>
<td>80 mM</td>
</tr>
<tr>
<td>Synaptic Vesicles</td>
<td>5.5</td>
<td>50 mM</td>
</tr>
<tr>
<td>Mitochondrial Matrix</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Endoplasmic Reticulum</td>
<td>7.2</td>
<td></td>
</tr>
</tbody>
</table>

**Cl\(^-\) transport mechanisms**

Cl\(^-\) ions serve as an important constituent of both the intra and extracellular environment of multicellular organisms. The cytosolic Cl\(^-\) ion concentration in varies between 5–30 mM in neurons and depends on the activity of different transporters and ion channels. Volume changes in the cell are primarily determined by the movement of Cl\(^-\) ions across membranes. The acid base balance of the cell is also affected by Cl\(^-\) ions (Shcheynikov et al., 2015). Further, Cl\(^-\) fluxes are key in determining the sign of GABAergic and glycinergic synapses. The levels of intracellular Cl\(^-\) are strongly governed by the action of plasma membrane cation chloride co-transporters. These include the NKCC using its Na\(^+\) inward gradient to elevate intracellular Cl\(^-\)
and the KCC mediating Cl̄ efflux with the outward movement of K⁺ ions. Up or down regulation of these transporters or changes in intracellular Cl̄ due to dysfunctional GABAergic signaling has been implicated in a variety of diseases such as hypertension (Cho et al., 2012), epilepsy (Huberfeld et al., 2007, Hubner, 2014) and has been implicated in mechanisms of nociception (Price et al., 2009). In addition, NO mediated Cl̄ release from intracellular compartments results in an increase in cytosolic Cl̄. This can serve as a complementary model in understanding mechanisms involving alterations in Cl̄.

The regulation of cytosolic Cl̄ plays a key role in the inhibitory components of neuronal circuitry. The postsynaptic effect of the neurotransmitters GABA and glycine acting on their ionotrophic receptors is dependent upon the distribution of Cl̄ across the plasma membrane. Cl̄ transporters of the plasma membrane are categorized as either the Cl̄ accumulators like the anion exchangers (AE1-3), Na-K-Cl co-transporter (NKCC1) and the Na-Cl (NCC, SLC12A1-2) cotransporter or the Cl̄ extruders such as the K-Cl co-transporter (KCC1-4, SLC12A4-7) along with the Na dependent Cl-HCO₃ exchangers (NDCBE, SLC12A8-10). The expression and function of NKCC and KCC are well known to adjust cytosolic Cl̄ during development (Payne et al., 2003) and in some adult neurons (Nakanishi and Kukita, 2000). A contradictory result has also shown the non-involvement of NKCC in the accumulation of Cl̄ in immature retinal neurons (Zhang et al., 2007). The distribution of NKCC2 in tandem with KCC2 controls GABA-evoked responses on starburst amacrine cells and influences the directionally selectivity of retinal ganglion cells (Gavrikov et al., 2006).

Cl̄ is also transported across internal membranes. Four classes of Cl̄ transporters have been localized to internal membranes: these include anoctamins, bestrophins, chloride intracellular channels (CLICs) and chloride channels (CLCs) (For review see (Jentsch, 2008).
Some CLCs have turned out to be Cl⁻/H⁺ exchangers and have been firmly established to reside on endosomal membranes (Jentsch, 2008). The CLC’s participate in a diverse array of physiological functions within prokaryotic and eukaryotic organisms (Wellhauser et al., 2010). The CLC family comprises nine members in mammals. The subtypes CIC 3-7 localize to compartments along the endosomal-lysosomal pathway including synaptic vesicles (Novarino et al., 2010). These intracellular Cl⁻ transporters play an important role in regulating the pH of intracellular vesicles and, the intravesicular pH and [Cl⁻] in turn affects mechanisms of vesicular trafficking (Faundez and Hartzell, 2004). It has been shown that disruption of CIC Cl⁻ transporter function by knockout of CIC-3 or CIC-5 results in decreased vesicular acidification (Faundez and Hartzell, 2004). Together, these data generate a picture of CIC Cl⁻ transporters playing a key role in regulation of vesicular pH (Gunther et al., 1998). One mechanism by which Cl⁻ transporters can increase luminal pH is by Cl⁻ ions acting as counter ions to dissipate the voltage gradient produced by the V-type proton pump (Faundez and Hartzell, 2004) which in turn enhances the ability of the pump to transport H⁺. CIC-5 is an H-Cl exchanger that is expressed primarily in endosomes. CIC-5 can be more easily studied when overexpressed in a cell line because it becomes localized to the plasma membranes. In this configuration, the extracellular side of the transporter corresponds to the lumen of the endosome and the intracellular side corresponds to the cytosol. Currents mediated by this transporter show extreme outward rectification corresponding to the efflux of Cl⁻ from endosomes and into the cytosol in native systems (Smith and Lippiat, 2010a). This suggests that CIC-5 is also directly involved in endosomal acidification by exchanging endosomal Cl⁻ for cytosolic H⁺.

The CLICs or Cl⁻ intracellular channels are a novel class of Cl⁻ channel with six known members in vertebrates (Littler et al., 2010, Singh, 2010). It exists as both soluble and membrane
linked forms. The soluble form inserts into lipid bilayers in a pH-dependent way to form active Cl$^-$ channels (Warton et al., 2002). These channels regulate Cl$^-$ ion concentration, stabilization of cell membrane potential and trans-epithelial transport. A member of the CLIC group i.e. the CLIC-4 has been shown to be localized to the mitochondria. CLIC4 is thought to be a Cl$^-$ channel that is involved in maintaining the pH gradient across the mitochondrial membrane (Fernandez-Salas et al., 1999).

Calcium-activated calcium currents (CACCs) have long been observed physiologically. CACCs have been specifically characterized in neurons with sensory function like rods and cones (Bader et al., 1982) taste cells (Herness and Sun, 1999) and somatosensory neurons (Cho et al., 2012). The genes encoding this sort of channel, however, have only recently been identified. A new family of CACCs is the anoctamins (ANO). The ANOs consist of ANO1 (also known as TMEM16A) (Caputo et al., 2008); (Schroeder et al., 2008) and ANO2 (TMEM16B) (Rasche et al., 2010). The activation of ANO1 has been studied in cerebral arteries and its activation has been shown to be blocked by BAPTA, a Ca$^{2+}$ chelator, thereby showing a close association between ANO1 and Ca$^{2+}$ influx channels (Bulley et al., 2012). ANO2 has been identified as a subunit of CACC in olfactory neurons (Billig et al., 2011). The only accepted mechanism of ANO1/ANO2 activation is an increase in intracellular Ca$^{2+}$.

The bestrophins are a family of Ca$^{2+}$ regulated transporters that transport both Cl$^-$ and Ca$^{2+}$ (Qu and Hartzell, 2008, Bharill et al., 2014). The bestrophin-1 transporter has been shown to reside on the ER and compelling evidence that bestrophins can act as Ca$^{2+}$-activated Cl$^-$ channels (Hartzell et al., 2008), Bestrophin-1 has also been reported to facilitate the release of GABA by glia (Lee et al., 2010). Mutations in bestrophins have been implicated in juvenile macular dystrophy and retinopathies (Hartzell et al., 2008).
Summary of dissertation

In Chapter 2, I investigated the influences of the Cl\textsuperscript{-} store on cytosolic Cl\textsuperscript{-} and confirmed that the Cl\textsuperscript{-} store is sequestered. Furthermore, the role of compartmental pH in determining cytosolic Cl\textsuperscript{-} levels, both before and after NO stimulus was also studied. To achieve this I used whole cell voltage clamp recordings as well as Lysosensor imaging on cultured amacrine cells derived from the chicken retina. I have used the lysosomotropic compounds to alter pH inside AOs and subsequently evaluate the effects of NO. These studies demonstrate that the pH of acidic compartments has a substantial influence on cytosolic Cl\textsuperscript{-} and in the NO dependent release of Cl\textsuperscript{-}. This also suggests that acidic compartments such as the Golgi, endosomes synaptic vesicles or lysosomes likely play a critical role in the ability of NO to regulate cytosolic Cl\textsuperscript{-}.

Because of the importance of cytosolic Cl\textsuperscript{-} in neuronal function, it is essential to understand the physiological properties of the store and its regulation by NO. In Chapter 3, I tested the hypothesis that the Cl\textsuperscript{-} store can be successfully depleted by NO. I also tested conditions under which plasma membrane mechanisms restore store Cl\textsuperscript{-}.

References


CHAPTER 2
COMPARTMENTAL PH INFLUENCES CYTOSOLIC Cl⁻ AND ALTERS THE EFFECT OF NITRIC OXIDE

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Introduction

The regulation of cytosolic Cl⁻ plays a key role in determining the inhibitory strength within neuronal circuits. The postsynaptic effect of the neurotransmitters GABA and glycine acting on their ionotropic receptors is dependent upon the distribution of Cl⁻ across the plasma membrane. Thus, regulation of intracellular Cl⁻ levels influences the sign and synaptic strength of GABAergic and glycinergetic synapses by influencing the reversal potential of GABA- or glycine-gated synaptic currents. In the retina, GABAergic and glycinergetic amacrine cells are known to generate inhibitory output onto bipolar cells, ganglion cells and other amacrine cells. This signaling is known to be key in regulating the response properties of different classes of retinal ganglion cells (Zhou and Lee, 2008, Masland, 2012b, Venkataramani et al., 2014). Therefore, transient and possibly localized modifications in cytosolic Cl⁻ levels have the potential to alter synaptic signaling in the inner retina and ultimately, the output of the retina.

The expression and function of the Na-K-Cl (NKCC) and K-Cl co-transporters (KCC) are well known to adjust cytosolic Cl⁻ during development (Payne et al., 2003a) and in some adult neurons reviewed in (Kaila et al., 2014). More recently it has been reported that the level of internal and external organic anions may also be a determinant of the distribution of Cl⁻ across the neuronal plasma membrane (Glykys et al., 2014b). Almost completely neglected, however,
is a consideration of the role of subcellular compartments that harbor Cl\(^-\) and their ability to release Cl\(^-\) into the cytosol.

Previous work from our lab has demonstrated that nitric oxide (NO) can alter the signaling properties of neurons by releasing Cl\(^-\) from an internal compartment in cultured avian retinal amacrine cells and rat hippocampal neurons (Hoffpauir et al., 2006). All organelles are known to contain some Cl\(^-\) and express mechanisms to transport Cl\(^-\) (reviewed in (Edwards and Kahl, 2010). For example the endoplasmic reticulum and mitochondria contain Cl\(^-\) and express Cl\(^-\) transporters but have neutral and basic (matrix) pH, respectively.

![Figure 2.1 Model for NO-dependent release of internal Cl\(^-\). This model depicts our understanding of the NO-dependent release of Cl\(^-\) as well as the hypothesis tested in this paper. With NO, a cytosolic acidification occurs that is required for internal Cl\(^-\) release. The presence of Cl\(^-\) in the cytosol can be measured as an inward Cl\(^-\) current due to Cl\(^-\) leaving open GABA\(_A\)Rs.](image)

There is a strong correlation, however, between the level of acidity in organelles and their Cl\(^-\) content (Sonawane and Verkman, 2003), reviewed in (Stauber and Jentsch, 2013). Because
of this relationship, we hypothesize the NO releases Cl\(^-\) from Cl\(^-\)-rich acidic organelles (AOs) (Figure 2.1). These compartments include the Golgi, endosomes and lysosomes.

Here we test the hypothesis that AOs are the source of NO-releasable Cl\(^-\). To achieve this, we use whole cell voltage clamp recordings on cultured amacrine cells derived from the chicken retina. Chick retinal amacrine cells have been well-characterized and together with our previous related studies provide an ideal model system for further elucidating mechanisms underlying Cl\(^-\) homeostasis in central neurons. The studies on the details of synaptic physiology and signaling mechanisms in our lab, has been facilitated by the utilization of relatively simple cell culture system based on chick retinal neurons. It has been shown that that after six days in culture, amacrine cells express the same voltage and ligand gated ion channels expressed in the amacrine cells from the intact adult chicken retina (Mills and Massey, 1995).

The cultures contain GABAergic amacrine cells and it has been demonstrated that amacrine cells form functional GABAergic synapses with each other seven days after plating (Gleason and Wilson, 1989, Gleason et al., 1993a). Thus, these cells in culture provide us with a useful model system, to investigate the mechanisms and regulation of GABAergic amacrine cell signaling. Here, we further investigate the influences of the Cl\(^-\) store on cytosolic Cl\(^-\) and confirm that this store is compartmentalized.

Additionally, we demonstrate that internal proton gradients are required for the NO-dependent release of internal Cl\(^-\). Furthermore, we examine the role of compartmental pH and find that it is influential in determining cytosolic Cl\(^-\) levels, both before and after an NO stimulus. Overall, these results support the hypothesis that NO-dependent release of cytosolic Cl\(^-\) is coming from AOs and link compartmental pH to the function of GABAergic synapses.
Methods

Cell Culture

Our methods were determined to be exempt by the LSU Institutional Animal Care and Use Committee (IACUC). Briefly, retinas from eight day chick embryos were dissected and separated from the pigment epithelium. Retinal tissue was mechanically dissociated in calcium-free Hank’s solution (Life Technologies, Grand Island, NY). The tissue was centrifuged, re-suspended and treated with trypsin (0.125%) for 30 minutes. Following trituration with DNAase I (Sigma-Aldrich, St. Louis, MO) and centrifugation at 4000 rpm for 5 min, cells were re-suspended in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies) supplemented with 5% fetal calf serum (Hyclone, Logan UT) and 1,000 U of penicillin/mL, 100 µg/mL streptomycin and 2 mM glutamine (Life Technologies). Cells were plated on to poly-L-ornithine treated dishes at a density of 2.5 x 10^5 cells/35 mm dish. One day after plating, DMEM was replaced with Neurobasal, 1% B-27 nutrient medium (Life Technologies) and pen-strep glutamine (Sigma-Aldrich). Cells were fed every other day until the cultures were no longer viable for experimentation (10-12 days). Amacrine cells to be used for electrophysiology and imaging experiments were identified based on their morphology as previously described (Gleason et al., 1993b).

Electrophysiology

Amacrine cells were used for electrophysiological experiments after 6-12 days in culture. Culture dishes were mounted on the stage of an inverted Olympus IX-70 microscope. A reference Ag/AgCl pellet in 3 M KCL was connected to the dish via a 3M KCL and agarose-filled glass bridge. Patch electrodes with tip resistance values of (8-11 MΩ) were pulled from thick-walled borosilicate glass (Sutter instruments, Novato, CA) using a P-97 micropipette puller
Electrophysiology experiments were performed in the whole cell voltage clamp mode using Axopatch 1D-patch clamp (Molecular Devices, Sunnyvale, CA) and Clampex 9.2 software (Molecular Devices). For experiments involving voltage ramps, corrections were made for errors due to junction potential and series resistance.

**pH Imaging**

Compartmental pH changes over time were monitored using the LysoSensor™ Green DND-189 (Life Technologies). LysoSensor is a pH sensitive fluorescent probe that accumulates in acidic organelles, specifically. Cells were loaded by incubating in 1µM LysoSensor for 20 min at room temperature. Imaging was performed on an Olympus IX70 inverted microscope with continuous perfusion of normal external solution containing the dye to avoid washout of the dye. Images were captured at 0.33 or 0.2 Hz using a Sensicam QE CCD camera (Cooke Corporation, Romulus, MI). Fluorescence was quantified in regions of interest using Slidebook Imaging Software (3I Imaging Innovations, Inc. Denver, CO) and analyzed using Origin 8.0 software (Origin Lab, Northampton, MA).

**Solutions**

Unless otherwise specified, reagents were obtained from Sigma-Aldrich. Imaging experiments used normal external solution containing (in mM): NaCl 136.7, KCl 5.3, CaCl₂ 3.0, MgCl₂ 0.41, HEPES 10.0, and glucose 5.6. For electrophysiology recordings carried out in Cl⁻ free condition, the external solution contained (in mM) Na methanesulfonate 145.0, glucose 5.6 and HEPES 10.0. External solutions were adjusted to pH 7.4 with NaOH. The internal zero Cl⁻ solution consisted of Cs methanesulfonate 145.0 and HEPES 10.0. Voltage ramp experiments were performed using TEA-Cl⁻ external contained the following ingredients (in mM): NaCl 116.7, KCl 5.3, TEA-Cl 20.0, CaCl₂ 3.0, MgCl₂ 410 µM, HEPES 10.0, and glucose 5.6. The
TEA-Cl external solution was supplemented with 300 nM TTX (Alomone Labs, Jerusalem, Israel) and 50µM LaCl₃ to block voltage-gated sodium and calcium channels, respectively. The internal pipette solution for voltage ramp experiments contained in mM: cesium acetate 100.0, CsCl 10.0, CaCl₂ 0.1, MgCl₂ 2.0, HEPES 10.0 and EGTA 1.1 along with the ATP regeneration system. For experiments involving methylamine hydrochloride, methylamine replaced CsCl to maintain EₐCl⁻. The internal recording solutions were pH adjusted to 7.4 with CsOH. Both pipette solutions were supplemented with an ATP regeneration system containing: 50 U/ml creatine phosphokinase, 20 mM creatine phosphate, 1mM ATP disodium, 3mM ATP dipotassium and 2mM GTP disodium. Methylamine hydrochloride (MA, 10 mM), chloroquine (CQ, 100 µM) and Monensin (10 µM) were added to pipet solutions. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP 1 µM) and bafilomycin (1 µM) were added to the bath. External solutions were controlled by a ValveLink pinch valve gravity perfusion system (Automate Scientific, Berkeley, CA). For whole cell voltage clamp experiments, solution changes were achieved with a computer-controlled automated SF-77B perfusion fast stepper (Warner Instruments, Hamden, CT). As a control, cells were routinely exposed to NO-free, low pH solution (McMains and Gleason, 2011) and none of the results reported here were reproduced by this treatment. Estimations of cytosolic Cl⁻ content were made without considering the contribution of acetate which for these GABAₐ receptors (GABAₐRs) seems to be small because we observe that under normal Cl⁻ and control conditions, EₐCl⁻ and EGABA are within a few millivolts of each other.

**NO Solutions**

NO was prepared by bubbling Fisher ultra-distilled pure water for 15 minutes followed by bubbling with pure NO gas for 15 minutes. The NO was then sealed and protected from light
and stored at room temperature until use. NO (30-50 µL) was injected into the perfusion line and has been previously estimated to reach the recorded cell in ~3 seconds and to remain for ~3-5 sec (Hoffpauir et al., 2006). The NO donor 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC-5) was obtained from Dojindo Molecular Technologies (Rockville, Maryland) and stored at -20° C. NOC-5 solutions were prepared in zero Cl⁻ external solution just prior to use.

Data analysis

Decay indices (Figure 2.4) were calculated by the following formulae: DI=1-(amp P5/amp P1) or DI=1- (amp P2/amp P1) with “amp” indicating GABA-gated current amplitude. Data were analyzed using Origin 8.0 (OriginLab, Northampton, MA) analysis software and data are presented as means ± SD. Data were generally evaluated using the paired and unpaired student’s t-test as appropriate. Levels of significance are denoted by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

The Cl⁻ store is sequestered

Our previous work has supported the regulated communication between three separate Cl⁻ containing compartments: an intracellular compartment, the cytosolic compartment and the extracellular compartment. The intracellular compartment is envisioned to be a membrane bound compartment but an alternative hypothesis is that the NO-releasable Cl⁻ is not sequestered but is instead bound up to proteins in the cytosol (Fiedler et al., 2000, Hornberg et al., 2005, Zhou et al., 2010). To determine whether the store is actually contained within an intracellular membrane-bound compartment, we examined the GABA-gated currents in the absence of both external and recording pipet Cl⁻. GABA-gated current in cultured amacrine cells are known to
be entirely due to the activation of GABA$_A$ receptors (GABA$_AR$s) (Hoffpauir et al., 2006). The assumption in this experiment is that the Cl$^-$-free pipet solution can dialyze the cytosol and will eventually wash out both cytosolic and protein-bound Cl$^-$.  

Cells recorded in the whole cell recording configuration (ruptured patch) were held at -70 mV, and five, 400 msec pulses of GABA (20 µM) were delivered. Inward GABA-gated currents were often observed in response to the first few GABA applications, but when observed, these currents typically dissipated as Cl$^-$ washed out of the cytosol (see below). In all cells tested (n=14), after GABA-gated currents were no longer visible (see fifth GABA application before NO, Figure 2.2 A), injection of NO produced inward GABA-gated currents consistent with Cl$^-$ being released from an internal membrane bound compartment and exiting the cell via GABA$_AR$s (Figure 2.2 B&C, mean current amplitude pre NO 11.9 pA ±11.6 pA; post NO 51.8 pA ± 29.5 pA; n=14; p<0.0001). To rule out any involvement of a GABA transport current, muscimol was substituted for GABA. Muscimol activates GABA$_AR$s but does not activate the GABA transporter. With muscimol as the agonist, the NO-dependent appearance of inward current was still observed (mean current amplitude pre NO 3.0 pA ± 3.3 pA; post NO 66.3 pA ± 26.9 pA; n=6, not shown). To confirm the involvement of NO, this same experiment was done using the NO donor NOC-5 (500 µm, Figure 2.2 D&E). NOC-5 also increased the amplitude of the GABA-gated currents (mean current amplitude control 1.0 pA ± 2.2 pA; NOC-5 12.0 pA ± 4.2 pA; n=5; p=0.01) further supporting an NO-dependent release of Cl$^-$ from an internal store.  

Because both Cl$^-$ and bicarbonate (HCO$_3^-$) permeate GABA$_A$ receptors (Bormann et al., 1987), it is possible that an increase in intracellular HCO$_3^-$ could contribute to the NO-dependent increase in GABA-gated current amplitude. Although this is unlikely because these experiments
have been conducted in HEPES-buffered solutions, the cells are still producing metabolic CO₂ and it is possible that NO stimulates an endogenous carbonic anhydrase (CA) in amacrine cells.

Figure 2.2. The NO-dependent Cl⁻ store is sequestered. (A), Representative traces from an amacrine cell recorded in the whole-cell voltage clamp configuration with Cl⁻ free pipette and external solutions. The cell was held at -70 mV and pulses of GABA (20 µM, 400 msec) were applied. Injection of NO temporarily increased the amplitude of the GABA-gated currents indicating a release of Cl⁻ into the cytosol. Cartoons at the top depict the Cl⁻ distribution at the point in time being sampled directly below. The darker color represents more Cl⁻. (B), Current amplitude data are plotted for each cell recorded. Data were collected from the response to the first GABA pulse and from the response to the 6th GABA pulse delivered just after NO. (C), Mean current amplitude of the data shown in (B). (D &E), Data from an experiment like the one shown in (A) but here the NO donor NOC-5 (500 µM) is used rather than the NO-bubbled solution. The NO donor also causes a significant increase in the amplitude in the NO-dependent GABA-gated currents. (F), Mean NO-dependent current amplitude of GABA-gated currents recorded before and after the addition of acetazolamide (400 µm).
Expression of CA2 has been demonstrated for a subset of amacrine cells in the developing chick retina but is mostly confined to Müller glia in the adult chicken retina (Linser and Moscona, 1984). To rule out the unlikely participation of CA-generated HCO$_3^-$ in the NO-dependent increase in GABA-gated current amplitude, the CA inhibitor acetazolamide was used (Figure 2.2 F). The same protocol was followed as for the experiments shown in Figure 2.2 A & D. No effect of acetazolamide on the NO-dependent GABA-gated currents were observed (mean current amplitude control 26.0 pA ± 22.0 pA; acetazolamide 29.2 pA ± 33.5 pA; n=6; p=0.6) providing evidence that CA-dependent HCO$_3^-$ is not involved in the reappearance of GABA-gated current after NO.

**Influence of the patch pipette on cytosolic Cl$^-$**

As indicated above, there was often evidence for residual Cl$^-$ in the cytosol even with a Cl$^-$ free internal solution. To more fully define the influence of the patch pipet in these experiments, we recorded from a population of cells and looked at the fraction of cells that retained inward Cl$^-$ currents in recordings obtained immediately after patch rupture (Figure 2.3 A & B). We observed that about half the cells showing residual inward GABA-gated currents (0 pA 47.6%; <10 pA 23.7% >10 pA 23.7%, n=42). The time required to dialyze cytosolic Cl$^-$ with repeated delivery of the 5 GABA pulse protocol in those cells that demonstrated inward currents was also estimated and this time period ranged from about 10-60 sec (mean=18.2 sec, ± (14.6 sec). Although the time frame could vary, elimination of the GABA-gated current was always achieved. In these experiments, we are assuming that loss of GABA-gated current indicates the absence of cytosolic Cl$^-$. It is possible, however, that the loss of the current is due to reduced activity of the receptors themselves. To address this possibility, we recorded from amacrine cells in normal external Cl$^-$ but with zero Cl$^-$ pipet solution. Under these conditions,
loss of internal Cl\textsuperscript{-} should lead to an actual reversal of the GABA-gated current from inward to outward. With normal extracellular Cl\textsuperscript{-}, we observed that the time course of depletion was prolonged, presumably due to Cl\textsuperscript{-} import mechanisms operating at the plasma membrane opposing the influence of the zero Cl\textsuperscript{-} pipet. Nonetheless, over the time course of minutes (<10 pA mean 5.9 min ± 5.5 min; reversal mean 8.6 min ± 6.8 min, the GABA-gated current reversed (Figure 2.3 C & D) indicating that the GABA\textsubscript{A} receptors were still functional.

Figure 2.3 Cytosolic Cl\textsuperscript{-} is incompletely controlled by the patch pipet. (A), Representative recordings from three different amacrine cells made within 5 seconds of membrane rupture. Cells were recorded with zero Cl\textsuperscript{-} pipet and extracellular solutions and were held at -70 mV. GABA (20 µM) was applied in 400 msec pulses. (B), Forty-two cells recorded under the conditions in (A) are categorized by the initial amplitude of their GABA-gated currents. (C), Representative recordings from a single amacrine cell, 5 sec (top), 6 min (middle) and 14 min (bottom) after membrane rupture. Recording conditions are the same as in (A) except that the extracellular solution contains normal Cl\textsuperscript{-} concentration. (D), Data are plotted from cells recorded under the conditions in (C).
It is expected that the flux of Cl\textsuperscript{-} through the GABA\textsubscript{A} receptors themselves contributed to the cytosolic Cl\textsuperscript{-} depletion that we observe in Figure 2.3. To evaluate this, we examined the effect that varying the number of GABA pulses delivered had on the current amplitude (Figure 2.4). We compared the GABA-gated current amplitudes elicited during the first recording after patch rupture. Some cells were tested with the usual 5 GABA pulse protocol while others were tested with a 2 GABA pulse protocol (delivered over the same time frame). A decay index (DI) was calculated (see methods) for each recording. Recordings were made either in zero Cl\textsuperscript{-} solutions inside and out (Figure 2.4 A & C) or with normal internal Cl\textsuperscript{-} and normal external Cl\textsuperscript{-} solutions (Figure 2.4 D & E). In complete zero Cl\textsuperscript{-} solutions, the 5 pulse protocol showed a significantly larger DI indicating that, as expected, the increased opening of GABA\textsubscript{A}Rs contributes to the depletion of cytosolic Cl\textsuperscript{-} (Figure 2.4 A & B; mean 2 pulse DI -0.12 ± 0.42; n=9; mean 5 pulse DI 0.62 ± 0.26; n=8; p=0.007) Interestingly, there was no significant difference in the DI for the cells recorded in normal Cl\textsuperscript{-} (Fig. 2.4 D-F; mean 2 pulse DI 0.07 ± 0.09; n=6; mean 5 pulse DI 0.23 ± 0.2; n=7; p=0.1). This result suggests that Cl\textsuperscript{-} import mechanisms are able to minimize the effect of the loss of cytosolic Cl\textsuperscript{-} through GABA\textsubscript{A}Rs under these conditions. Together, these results demonstrate that although pipet Cl\textsuperscript{-} eventually dominates over cytosolic Cl\textsuperscript{-}, this effect is not immediate, is variable from cell to cell and is affected by extracellular Cl\textsuperscript{-}. Most importantly, the zero Cl\textsuperscript{-} experimental design allows us to isolate the internal store unequivocally.

**Disruption of compartmental proton gradient alters the NO-dependent Cl\textsuperscript{-} release**

AOs express the V-type proton pump and the activity of this active transporter generates a large proton gradient. If AOs harbor NO-releasable Cl\textsuperscript{-}, then it is possible that the proton gradient is linked to the NO-dependent export of Cl\textsuperscript{-}.
Figure 2.4 Cl⁻ escape via GABA<sub>A</sub> receptors can be offset by extracellular Cl⁻. (A&B), Whole cell recordings made from two different amacrine cells immediately after membrane rupture. In the first, two 400 msec pulses of GABA are applied and in the second, five are applied. Recordings were made under zero Cl⁻ conditions. (C), Decay indices were calculated as indicated in the Methods. These data indicate that the decay in the amplitude of GABA-gated currents is accelerated when the receptors are activated more frequently. (D&E), Data from two different amacrine cells recorded as in (A) but in the presence of normal intra and extracellular Cl⁻ concentrations. (F), Decay indices are plotted for cells recorded under the conditions in (C) and no significant difference in current amplitude is observed.

We have previously shown that pre-incubation with the V-type ATPase inhibitor bafilomycin produces a small but significant inhibition in the NO-dependent positive shift in the reversal potential (E<sub>rev</sub>) under normal Cl⁻ conditions (McMains and Gleason, 2011b). Here, we examine the effects of bafilomycin applied acutely under zero Cl⁻ conditions to test the hypothesis that there is a relationship between the proton gradient across AO membranes and the
level of NO-dependent Cl⁻ release. The NO-dependent increase in GABA-gated current amplitude was examined for amacrine cells held at -70 mV both before and after exposure to bafilomycin (1 µM, Figure 2.5 A). We observed that the NO-dependent GABA-gated currents were usually smaller than control (Fig. 2.5 B&C) and this was independent of the order of control and bafilomycin treatments. These results suggest that a reduction in the proton gradient across AO membranes impairs the ability of NO to release Cl⁻.

Figure 2.5 Reducing AO proton gradients inhibits the NO-dependent release of Cl⁻. (A), Data from a representative amacrine cell before and after addition of 1 mM bafilomycin under zero Cl⁻ conditions. On the left, GABA produces no current indicating that the cytosol is Cl⁻ free. After NO GABA elicits an inward current due to Cl⁻ moving from the cytosol out across the plasma membrane. After bafilomycin (grey trace), the NO-dependent GABA-gated current is smaller indicating a reduction in the release of internal Cl⁻. (B), NO-dependent GABA-gated current amplitude is plotted for each cell tested both before and after exposure to bafilomycin. (C), Mean GABA-gated current amplitude is significantly smaller in bafilomycin.
Bafilomycin blocks the V-type ATPase and upsets the balance between inward proton pumping and outward proton leak (Fuchs et al., 1989, Rybak et al., 1997, Wu et al., 2000, Grabe and Oster, 2001) The effect of brief (~30 sec) bafilomycin exposure as we used here, depends upon the amplitude of the proton leak.

An alternative approach is to induce a proton leak with an ionophore such as carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). FCCP is typically used to disrupt the mitochondrial proton gradient where its effect will be to allow protons to move down their electrochemical gradient across the inner mitochondrial membrane and into the mitochondrial matrix. FCCP will also disrupt the AO proton gradient (Forgac et al., 1983) and allow protons to move from AOs into the cytosol. The effects of FCCP were tested in Cl\(^-\) free GABA pulse experiments. Cells were first shown to have NO-dependent Cl\(^-\) release by demonstrating the appearance of GABA-gated currents after NO. After FCCP, no NO-dependent GABA-gated currents were detected (Figure 2.6 A & C). If FCCP had caused an emptying of AOs Cl\(^-\) content, we would expect to see evidence of cytosolic Cl\(^-\) as inward GABA-gated currents both before and after NO but this was never observed. However, to test this explicitly, some cells were first exposed to FCCP and tested with NO (Figure 2.6 B & D). In these experiments FCCP had the same inhibitory effect on the NO-dependent Cl\(^-\) release as in the experiment depicted in Figure 2.6A. FCCP was washed out then the experiment was immediately repeated under control conditions. In all cases, an NO-dependent release of Cl\(^-\) was observed indicating the Cl\(^-\) content was preserved during the brief period of the FCCP treatment (~1-2 min).
Figure 2.6 Internal proton gradients are required for the NO-dependent release of Cl⁻. (A), Whole cell recording of an amacrine cell held at -70 mV and exposed to 5 pulses of GABA under Cl⁻ free conditions (timing of pulses indicated by horizontal bars). Control record show some residual GABA-gated currents that diminish over the 5 GABA pulses. After NO, the GABA-gated current amplitude is enhanced indicating and increase in cytosolic Cl⁻. A (bottom) after FCCP GABA-gated currents are negligible and after NO, the increase in the GABA-gated current amplitude is not observed. (B), A recording from a different cell where FCCP is applied first. No GABA-gated currents are observed either before or after NO. (B) (bottom), A recording made from the same cell after washout of FCCP. NO elicits an increase in the GABA-gated current demonstrating a NO-releasable Cl⁻ store in this cell. (C&D), GABA-gated current amplitude data are plotted for each cell recorded. Cells are sorted according to the order of FCCP application (after control, (C); before control, (D)). Data plotted are the GABA-gated current amplitudes recorded just after NO application. (E), Combined mean current amplitude data are plotted to compare control to FCCP.
Overall, FCCP had a dramatic inhibitory effect on the ability of NO to release internal Cl\textsuperscript{−} (Figure 2.6 E, mean NO-dependent current amplitude control 116.9 pA ± 82.8; FCCP 5.1 pA ± 4.9, n=11, p=0.001). These results suggest that in the absence of proton gradient, the mechanism underlying the NO-dependent release of internal Cl\textsuperscript{−} is disabled.

**Increasing compartmental pH releases Cl\textsuperscript{−} into the cytosol**

Both bafilomycin and FCCP disrupt the normal AO proton gradient which disrupts both the AO membrane potential and increases luminal pH. In order to dissect the role of AO membrane potential and pH, we have tested the effects of lysomotropic weak bases methylamine (MA) and chloroquine (CQ). These compounds have been demonstrated to alkalinize compartments including lysosomes (Ohkuma and Poole, 1978, Poole and Ohkuma, 1981) and endosomes, including synaptic vesicles (Cousin and Nicholls, 1997, Abreu et al., 2008). When applied via the patch pipet, these compounds will deprotonate in the cytosol then diffuse across organellar membranes. The deprotonated weak base will become trapped when they re-protonate in the acidic environment of the AOs. The dominant effect of these compounds is to increase the pH of acidic compartments without affecting the AO membrane potential (Cousin and Nicholls, 1997). Deprotonation in the cytosol will likely acidify the cytosol to some degree but this effect will be at least partially offset by the activity of the plasma membrane Na/H exchanger (McMains and Gleason, 2011b) and the 10 mM HEPES in the pipet.

**Manipulating compartmental pH**

Compartments such as endosomes are known to contain millimolar concentrations of Cl\textsuperscript{−} (Sonawane and Verkman, 2003) and are therefore one candidate for harboring NO-releasable Cl\textsuperscript{−}. Endosomes also express the V-type proton pump and the activity of this active transporter generates low luminal pH. If endosomes AOs harbor NO-releasable Cl\textsuperscript{−}, then it is possible that
their low internal pH might be linked to the NO-dependent export of Cl\(^-\). Furthermore, we have previously established that NO produces a transient acidification of the cytosol (McMains and Gleason, 2011b) that might be due to release of protons from AOs. LysoSensor Green DND-189 localizes to acidic compartments and exhibits very low fluorescence outside of an acidic environment. Amacrine cells were loaded with LysoSensor and maintained in LysoSensor containing external solution during imaging to avoid dye depletion. The LysoSensor signal was most concentrated in cell bodies usually in a region eccentric to the nucleus (Figure 2.7 A, arrow). LysoSensor fluorescence was found throughout neuronal processes and was typically punctate consistent with the dye being partitioned into discrete compartments (Figure 2.7 A, small arrows).

In order to test the hypothesis that AOs pH affects the NO-dependent release of internal Cl\(^-\), the methods for manipulating AOs pH were established. The buffering action of MA and CQ will reduce the pH gradient across internal membranes but will not reduce the AOs membrane voltage generated by the V-type proton pump (Cousin and Nicholls, 1997). To test their effect on amacrine cell acidic compartments, the influence of these compounds on LysoSensor fluorescence was examined. Data were collected from both cell bodies and from bright regions out in the processes. Amacrine cell bodies and fluorescent regions in processes consistently responded to both MA and CQ with decreases in fluorescence, indicating that the pH of acidic compartments was increased by both of these structurally distinct weak bases (Figure 2.7 B & C; MA vs. Control p<0.0001; CQ vs. Control p<0.0001; n=37).
Figure 2.7 Methylamine and chloroquine reduce the LysoSensor signal in acidic compartments.

(A), An image of a LysoSensor-loaded cultured amacrine cell showing the typical distribution of dye. Strong fluorescence is typically seen in the cell body eccentric to the nucleus (long arrow). In processes, the dye is distributed in a punctate pattern that includes some especially distinct elements (short arrows). Scale bar is 10 µm. (B), Representative data collected from a cell body and one of the associated processes. Both methylamine (MA) and chloroquine (CQ) decrease the LysoSensor Fluorescence. These data were collected in normal external solution. (C&D), LysoSensor responses to MA and CQ are quantified for cells recorded under normal (C, n=37) and zero external Cl⁻ (D, n=63) conditions.

Because some of our experiments are conducted in normal external Cl⁻ (voltage ramp experiments) conditions and some are done in the absence of external Cl⁻ (GABA pulse experiments), we tested the effectiveness of MA and CQ under both conditions. The effectiveness of MA and CQ was not altered in a Cl⁻ free external environment (Figure 2.7 D;
MA vs. Control p<0.0001; CQ vs. Control p<0.0001; n=63). These LysoSensor imaging experiments do not exactly mimic our electrophysiology experiments because there, MA and CQ are added to the recording pipet. Nonetheless, they demonstrate that MA and CQ will lower compartmental pH and delivering these reagents via the patch pipet should bias their effects towards internal compartments over the cytosol.

Either 10 mM MA or 100 µm CQ was included in the pipette solution which was otherwise Cl⁻ free. The external solution was also zero Cl⁻. Recordings were begun about a minute after plasma membrane rupture to allow time for the reagents to gain access to the cell. Amacrine cells were held at -70 mV and exposed to GABA pulses as in previous experiments. In control conditions, the GABA-gated currents were small and often negligible by the fifth GABA pulse. With MA internal, the pre-NO GABA-gated currents were always substantial and did not wash out over the first five GABA pulses (Figure 2.8 A & B; mean current amplitude control 3.9 pA ± 6.1 pA; MA 79.8 pA ± 65.4 pA; n=17; p=0.0003). The larger currents could be entirely due to the 10 mM Cl⁻ in the MA pipet solution (methylamine hydrochloride) or could be due to methylamine somehow stimulating release of Cl⁻ from the internal store. If methylamine was causing release of Cl⁻ from stores because of its ability to buffer protons in AOs, then we would expect to see a similar enhancement of GABA-gated current amplitude with CQ in the pipet. Confirming this expectation, larger than control GABA-gated currents were consistently observed with CQ in the pipet (Figure 2.8, bottom trace). Although CQ had a smaller effect on the pre-NO GABA-gated current amplitude than MA, the difference from control was still strongly statistically significant (Figure 2.8 B; mean current amplitude CQ 26 pA ± 14.2 pA, n=8, p<0.0001).
Figure 2.8 Increasing compartmental pH releases Cl⁻ into the cytosol. (A), Representative traces from three different amacrine cells recorded under zero Cl⁻ conditions. Cells were held at -70 mV. MA and CQ labeled traces are from cells that were recorded with pipet solutions containing these compounds. Traces shown were the first collected after membrane rupture. Cartoons depict cytosolic Cl⁻ levels under the three conditions. (B), Mean amplitudes of the first response to GABA (20 µm, 400 msec) are plotted for each of the three conditions. Both MA and CQ significantly increased the GABA-gated current amplitude over control suggesting higher internal Cl⁻ concentration than control. (C), Leak-subtracted GABA-gated currents recorded from two different amacrine cells in 10 mM Cl⁻ internal and external solutions. Currents were elicited by ramping the voltage from −90 mV to +50 mV in the presence of GABA (20 µM). Recordings were made just after membrane rupture. The control cell has a GABA-gated current reversal potential near the predicted equilibrium potential for Cl⁻ of 0mV whereas the MA-containing cell’s GABA-gated current reverses at around +15 mV. (D), Cells recorded with MA had significantly more positive reversal potentials than control.

These data suggest that raising the pH of internal compartments releases Cl⁻ into the cytosol in the absence of an NO stimulus. To confirm this and to account for the 10mM internal Cl⁻ that comes along with the methylamine, the reversal potential of the GABA-gated current (E_GABA) was determined by applying voltage ramps during application of GABA.
These experiments were done in 10 mM external Cl\(^-\) and either 10 mM CsCl internal solution or 10 mM methylamine HCl internal solution with the predicted \(E_{\text{Cl}^-}\) being 0 mV for both combinations. The reversal potential of the GABA-gated current 1-2 min after plasma membrane rupture was determined and these measurements indicated that while control cell currents reversed near 0mV, the cells containing MA had significantly more positive reversal potentials even though both pairs of solutions had equivalent \(E_{\text{Cl}^-}\)s (Figure. 2.8 C & D; mean \(E_{\text{GABA}}\) control -1.7 mV ± 10.7 mV, n=6). The mean \(E_{\text{GABA}}\) in MA was about +12 mV (MA 12.4 mV ± 5.8 mV, n=9, p=0.006) which would correspond to an additional 6 mM cytosolic Cl\(^-\).

**Increasing AO pH reverses the effect of NO on cytosolic Cl\(^-\)**

If raising the pH in AOs causes Cl\(^-\) release, how does compartmental pH affect the ability of NO to release Cl\(^-\) from internal stores? GABA pulse experiments were conducted in zero Cl\(^-\) solutions to address this question. Control cells initially had typically small GABA-gated currents. After NO, control cells showed the expected increase in the GABA-gated current amplitude indicating an increase in cytosolic Cl\(^-\) (mean current amplitude control pre-NO 4.8 pA ± 7.6 pA; post-NO 23.9 pA ± 20.1 pA n=9, p=0.02). As expected, the amplitude of the pre-NO GABA-gated current was larger with MA and CQ internal. However, in cells with MA and CQ, no NO-dependent increase in the GABA-gated current amplitude was observed (Figure 2.9 A&B; MA pre-NO 79.7 pA ± 65.4 pA post-NO 38.2 pA ± 33.3 pA, n=17; CQ pre-NO 26.1 pA ± 14.2 pA; post-NO 14.4 pA ± 8.3 pA, n=8). In fact, the post-NO GABA-gated currents were significantly reduced rather than enhanced (MA p=0.001; CQ p=0.02) raising the possibility that under these conditions, NO reversed the movement of Cl\(^-\) between cytosol and AOs or generated export across the plasma membrane.
Figure 2.9 Increasing compartmental pH eliminates the NO-dependent release of Cl\(^-\) under zero Cl\(^-\) conditions. (A), Representative recordings from three different amacrine cells before and after addition of NO. The control cell starts out with a small inward GABA-gated current that diminishes over the 5 GABA pulses. After NO, the current is enhanced. In contrast, MA and CQ containing cells had larger GABA-gated currents before NO. After NO, the currents were not enhanced but rather significantly reduced in amplitude. (B), Mean current amplitudes are plotted before and after exposure to NO for control, MA and CQ.

To clarify whether the NO response was inhibited or actually reversed, reversal potentials for the GABA-gated currents were measured. These experiments were done in standard Cl\(^-\) containing solution with Cl\(^-\)\(_{out}\) 148.8 mM and Cl\(^-\)\(_{in}\) 14.2 mM (E\(_{Cl^-}\) = -59 mV) with MA and CQ.
added to the internal solution. As expected under control conditions, NO caused a positive shift in $E_{GABA}$ consistent with a release of Cl$^-$ into the cytosol (Figure 2.10 A; mean $E_{GABA}$ pre- NO -60.9 mV ± 8.0 mV; post NO -43.2 mV ± 2.9 mV, n=5, p=0.01). These mean values correspond to 14 mM Cl$^-$ before NO and 25 mM Cl$^-$ after NO (see methods).

In this control cell, the NO-dependent GABA-gated current enhancement that we have previously reported was also observed (Hoffpauir et al., 2006). With either MA or CQ internal solution, the $E_{GABA}$ started out significantly more positive than control (Figure 2.10 B-E, MA -43.2 mV ± 9.2 mV, n=5, p=0.01; CQ -46.1 mV ± 4.2 mV, n=4, p=0.04). These $E_{GABA}$ values would correspond to the following cytosolic Cl$^-$ values: MA 25 mM and CQ 23 mM. After NO, $E_{GABA}$ shifted in the negative direction indicating an NO-dependent depletion of cytosolic Cl$^-$ (MA -61.2 mV ± 8.3 mV; CQ -79.6 ± 15.3 mV). Post-NO values for $E_{GABA}$ were significantly more negative than pre NO values (MA p=0.002; CQ p=0.001).

Mean post-NO $E_{GABA}$ values lead to an estimation of 14 mM Cl$^-$ in MA and 6 mM Cl$^-$ in CQ for cytosolic Cl$^-$. We also consistently observed that in both MA and CQ, the GABA-gated ramp current was reduced in amplitude after NO. Presumably, this is a separate effect on the GABA$_A$ receptors that might be due to the altered pH environment when NO is co-applied with MA or CQ and could contribute to the post NO reduction in current amplitude we observe in the GABA pulse experiments. Nonetheless, the data in Figure 2.10 confirm that the reversal potential of the GABA-gated current shifts in the negative direction after NO indicating an NO-dependent reduction in cytosolic Cl$^-$. 

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Figure 2.10 In normal Cl\(^{-}\), increasing compartmental pH promotes NO-dependent Cl\(^{-}\) efflux from the cytosol. GABA-gated currents in response to voltage ramps were recorded in normal internal and external solutions. (A), A recording from a control cell is shown with about a 10 mV positive shift in reversal potential in response to NO. (B&C), GABA-gated currents in cells containing MA or CQ, respectively. In both cases the reversal potentials of the currents before NO are more positive than the calculated \(E_{\text{Cl}^{-}}\) , -59 mV. After NO, the reversal potential of the GABA-gated current shifts in the negative direction indicating a decrease in cytosolic Cl\(^{-}\). (D) NO-dependent shifts in \(E_{\text{GABA}}\) are shown for individual cells. (E), Data from experiments like A-C are quantified and show that GABA-gated current reversal potentials before NO are significantly more positive with MA and CQ than in control. NO elicits significant shifts in reversal potentials under all three conditions and the post-NO reversal potentials were significantly more negative than control with MA and CQ. Asterisks to the left (pre-NO) and right (post-NO) of the data points indicate significance of differences from control.
Discussion

These results demonstrate that cytosolic Cl$^-$ is tightly coupled to the physiological environment of internal stores. Here, we show that a compartmentalized Cl$^-$ store releases Cl$^-$ into the cytosol after exposure to NO. Reduction of AO proton gradients by acute inhibition of the V-type ATPase limited the release of internal Cl$^-$ and more severe disruption of the proton gradient with the protonophore FCCP completely blocked the NO-dependent release of internal Cl$^-$. Disruption of compartmental pH with lysomotropic bases demonstrates two things. First, an increase in compartmental pH, allows Cl$^-$ to escape into the cytosol. Second, under this same condition, the effect of NO is to promote Cl$^-$ uptake or removal rather than release.

Detecting Cl$^-$ changes in the ruptured-patch configuration

In order to track Cl$^-$ release from internal stores specifically, it was important to study the Cl$^-$ store in isolation and for this reason, we used ruptured patch in conjunction with zero Cl$^-$ solutions. Although our methods in this work were a compromise between isolation of the Cl$^-$ store and disruption of the cytosol, is important to recognize that we have demonstrated in previous work that the NO-dependent release of internal Cl$^-$ and its dependence on cytosolic pH are the same in both ruptured patch and gramicidin whole cell recordings (Hoffpauir et al., 2006, McMains and Gleason, 2011b).

Although the patch pipet is generally considered to rapidly dialyze the interior of the cell, standing Cl$^-$ gradients have been detected in ruptured patch recordings of Clomeleon-expressing hippocampal neurons (Kuner and Augustine, 2000b). Our data demonstrate that the washout of Cl$^-$ is highly variable from cell to cell, possibly due to differences in cellular architecture. Furthermore, we could readily detect alterations in cytosolic Cl$^-$ levels in response to elevated compartmental pH both before and after addition of NO. Although our recording configuration
may have minimized changes in cytosolic Cl\(^{-}\) or made them more transient, significant changes in Cl\(^{-}\) concentration were clearly detectable. 

**Interactions between Cl\(^{-}\) and pH**

AO Cl\(^{-}\) import has been thought to function primarily to provide counter ions to minimize the positive membrane potentials generated by the acidifying activity of the V-type proton pump. However, it has also become clear that endosomal Cl\(^{-}\) transport also plays a key role in endocytosis (Piwon et al., 2000, Wang et al., 2000) as well as other aspects of membrane trafficking. For review see (Stauber and Jentsch, 2013)). Cl\(^{-}\) and pH are also linked by the activity of Cl/ HCO\(_3\) anion exchangers (AE1-3) and the Na/Cl/HCO\(_3\) (NCBE) transporters at the plasma membrane. Interestingly, knockout of AE3 produces increased susceptibility to seizure activity (Hentschke et al., 2006) and knockout of NCBE exhibit decreased excitability and a higher threshold for seizure activity (Jacobs et al., 2008). Both of these defects are consistent with alterations in cytosolic Cl\(^{-}\) and/or HCO\(_3\) that could alter GABA\(_A\) receptor-mediated inhibition. In the retina, knockout of NCBE led to alterations in the normal response properties of the electro-retinogram (Hilgen et al., 2012). Additionally, a recent report demonstrates that Cl\(^{-}\) ions can bind and regulate the function of Na/HCO\(_3\) exchangers, suggesting that cytosolic Cl\(^{-}\) has previously unrecognized regulatory functions (Shcheynikov et al., 2015).

**Collapsing organellar membrane potentials**

We have previously shown that under normal Cl\(^{-}\) conditions, both bafilomycin and FCCP reduce the NO-dependent shift in \(E_{GABA}\) (McMains and Gleason, 2011) but do not eliminate it. With acute bafilomycin exposure in zero Cl\(^{-}\) conditions we see a reduction in the NO-dependent release of Cl\(^{-}\). The rapid nature of this effect implies that the AOs involved have a substantial
proton leak. Here, we also demonstrate that with the store isolated from all other sources of Cl\(^-\), FCCP completely inhibits the release of Cl\(^-\). This difference in the effect of FCCP in the presence and absence of external and cytosolic Cl\(^-\) may be a reflection of the normal influence that extracellular Cl\(^-\) and plasma membrane Cl\(^-\) transporters have on both cytosolic and internal Cl\(^-\). Because of the well documented effects of FCCP in collapsing the mitochondrial membrane potential (Buckler and Vaughan-Jones, 1998, Nicholls, 2006), the action of FCCP also raises the possibility that mitochondria contribute to the NO-dependent release of internal Cl\(^-\). (Garcia et al., 1997) provide evidence for a mitochondrial Cl\(^-\) store in pituitary lactotrophs that contributes to relatively high levels of cytosolic Cl\(^-\) found in those cells. Cl\(^-\) channel currents have also been detected directly in the inner mitochondrial membrane but they appear to have diverse properties and for the most part, their molecular identity is unknown (for review see (Tomaskova and Ondrias, 2010). However, MA and CQ should have no effect on mitochondria because the mitochondrial matrix is basic.

**Increasing AO pH**

We have previously shown that acidification of the cytosol by inhibition of the NHE shifts \(E_{GABA}\) positively and alkalization promotes a negative shift in \(E_{GABA}\) indicating an increase and decrease in cytosolic Cl\(^-\), respectively (McMains and Gleason, 2011). With internal MA and CQ, deprotonation before entry into AOs, will have some acidifying effect on the cytosol that will be mitigated by plasma membrane NHE activity and the 10 mM HEPES in the pipet. Thus any acidification of the cytosol is likely to be more transient than the increase in AO pH. Although the cytosolic acidification could contribute to the increase in cytosolic Cl\(^-\) observed in MA and CQ the contribution should be relatively small because inhibition of NHE with amiloride generated an \(E_{GABA}\) shift of only +5 mV whereas MA and CQ shifted \(E_{GABA}\) by +18
mV and +14 mV, respectively. It is also important to note that our results in normal internal and external Cl⁻ were consistent with GABA pulse experiments where the only source of Cl⁻ was compartmental. While there are multiple mechanisms available to move Cl⁻, these experiments reveal that compartmental Cl⁻ transport mechanisms respond to changes in pH within the compartment itself as well as pH changes in the cytosol.

The effects of cytosolic and AO pH changes on basal cytosolic Cl⁻ levels are, however, quite distinct from the effects of those factors on the NO-dependent release of Cl⁻. In our previous work, we discovered that alkalization of the cytosol suppressed the NO-dependent shift in $E_{\text{GABA}}$ while acidification had no significant effect on the NO-dependent shift (McMains and Gleason, 2011b). Here, we see that raising AO pH actually reverses the effect of NO such that $E_{\text{GABA}}$ is shifted in the negative direction consistent with removal of Cl⁻ from the cytosol. The mechanisms of this reversal are not yet understood. One of our working hypotheses is that internal CIC H/Cl exchangers mediate the NO-dependent release of compartmental Cl⁻ that we observe under control conditions. Indeed, we have RT-PCR and immunocytochemical evidence that CICs 3, 4, 5, 6 and 7 are expressed in amacrine cells (McMains et al., 2011). Outward movement of Cl⁻ and inward movement of H⁺ conform to the preferred direction of transport demonstrated by current measurements from CICs 3, 4 and 5 expressed in heterologous systems (Friedrich et al., 1999, Li et al., 2000, Scheel et al., 2005, Smith and Lippiat, 2010). The increase in cytosolic Cl⁻ we see upon elevation of compartmental pH with MA and CQ would be consistent with CIC involvement because the coupled import of H⁺ and export of Cl⁻ is favored under these conditions, at least for CIC5 (Smith and Lippiat, 2010). However, the resistance of the CIC exchangers to reversal makes it unlikely that these transporters could mediate NO-dependent Cl⁻ uptake. Instead, we are compelled to envision either a complex interaction
between compartmental transport mechanisms or activation of Cl⁻ export at the plasma membrane that is promoted by an increase in compartmental pH plus NO.

**Potential Sources of NO-releasable Cl⁻**

AOs include the Golgi, endosomes and lysosomes. The Golgi complex is typically located in neuronal cell bodies but Golgi outposts have been discovered that assemble in dendrites (Gardiol et al., 1999, Horton et al., 2005) and even in dendritic spines (Pierce et al., 2001). Thus in neurons, the distribution of Golgi can be nearly as widespread as endosomes and lysosomes. The Golgi is not as acidic as endosomes and lysosomes but all three contain Cl⁻ and Cl⁻ transport proteins including ClCs (Edwards and Kahl, 2010). We are actively pursuing the identification of the Cl⁻ transporter that mediates the NO-dependent release of internal Cl⁻. Once in hand, this information will allow us use co-localization studies with AO-specific markers to assess the relative contributions of these three classes of AO.

Because establishment of proton gradients can consume ATP (AOs) or are required to generate ATP (mitochondria), the movement of Cl⁻ is linked not only to protons but also to the energetic balance of the cell and its organelles. In cells with complex architectures such as neurons, cytosolic Cl⁻ levels also have the potential to be spatially diverse. Amacrine cells have complex dendritic morphology and local synaptic interactions dominate (Marc and Liu, 2000, Marc et al., 2014). Thus, it is important to understand how postsynaptic cytosolic Cl⁻ levels are regulated and ultimately, how highly localized regulation might occur. Our results thus far suggest that local cytosolic Cl⁻ levels might be influenced by regional cytosolic and organellar pH changes as well as synapse-specific generation of NO.
References


CHAPTER 3
PROPERTIES OF NO INDUCED Cl\textsuperscript{−} RELEASE FROM INTERNAL STORES:
DEPLETION AND REFILLING

Introduction

Previous experiments in our lab have shown that NO elicits internal Cl\textsuperscript{−} release after brief exposures to NO (Hoffpauir, 2006). We also have strong evidence that this release of Cl\textsuperscript{−} is from internal acidic compartments (Chapter 2). The maintenance of internal Cl\textsuperscript{−} gradients depends upon the nature of Cl\textsuperscript{−} release, depletion from internal stores and also refilling mechanisms associated with NO. Release of intracellular Cl\textsuperscript{−} has not been studied in detail before and studying the flux of Cl\textsuperscript{−} is important for a couple of reasons: 1) the intracellular Cl\textsuperscript{−} concentration determines the Cl\textsuperscript{−} equilibrium potential. 2) Cl\textsuperscript{−} concentration is tightly coupled to pH regulatory mechanisms. 3) the involvement of Cl\textsuperscript{−} in transport mechanisms in cell volume regulation. 4) Cl\textsuperscript{−} homeostasis is required for maintaining synaptic inhibition.

Under what conditions can the store become depleted? First, I tested the hypothesis that the Cl\textsuperscript{−} store can be depleted and defined the circumstances under which depletion can occur. If the store is resistant to depletion, it could respond to multiple NO signals with several Cl\textsuperscript{−} release events. After successful depletion of the store, I then defined more specifically the conditions under which the store can become depleted. To address this, different patterns of GABA applications and NO exposures were examined to establish the temporal properties of store depletion.

If store depletion is achieved then can the store be refilled? After depletion it was important to study then the involvement of an efficient store Cl\textsuperscript{−} recapture. This is because recovery of Cl\textsuperscript{−} after depletion would determine the duration of changes in the synaptic strength.
of GABAergic and glycinergic synapses. I also tested the hypothesis that NO-released Cl\(^{-}\) is exported across the plasma membrane. One exit pathway should be through the GABA\(_{A}\) receptors (GABA\(_{ARs}\)) and my results are consistent with this because increasing the exposure to GABA speeds the recovery back to control levels of cytosolic Cl\(^{-}\). With these experiments we gain important insight into the fidelity of the store and the conditions of NO generation under which the store can continue to regulate postsynaptic function. The nature of Ca\(^{2+}\) store and mechanisms of Ca\(^{2+}\) release and refilling has been extensively studied before (Clapham, 1995, Parekh and Penner, 1997) but the same has not been elucidated for Cl\(^{-}\). The importance of Cl\(^{-}\) regulation by NO and the availability of Cl\(^{-}\) compartments compelled me to study the physiological properties of this store.

**Methods**

Electrophysiology procedure and solutions were the same as described in Chapter 2. Data are reported as means ± SE and statistical significance was determined using the t-test.

**Results**

**Activation of GABA\(_{A}\) receptors contributes to the recovery of cytosolic Cl\(^{-}\) by NO**

When NO is applied, Cl\(^{-}\) is released into the cytosol within seconds and \(E_{Cl}^{-}\) becomes more positive. These changes are transient with normally low cytosolic Cl\(^{-}\) being restored within 60-90 seconds (Hoffpauir et al., 2006). What are the mechanisms involved in the recovery of cytosolic Cl\(^{-}\) to pre NO levels? In order to test for the influence of Cl\(^{-}\) exit via GABA receptors I designed two different protocols one with 5 GABA pulses and another with 2 GABA pulses, which has the same duration as the 5-pulse protocol, but with an increased inter-stimulus interval (Figure 3.1).
I performed whole cell voltage clamp recordings of retinal amacrine cells held at -70 mV. These experiments were carried out in Cl⁻ free external and internal solutions to isolate the Cl⁻ store. NO solution (50 µl) was injected through the perfusion line delivering it along with the 0 Cl⁻ external solutions. The GABA applications were initiated immediately after the appearance of the NO dependent inward current (Hoffpauir et al., 2006) which serves as a signpost for the arrival of NO. Calculation of the decay index (1-current amplitude of the 5ᵗʰ GABA pulse/current amplitude of the 1ˢᵗ GABA pulse) reflects the change in the GABA-gated current amplitude with repeated GABA applications and serves as an indicator of available cytosolic Cl⁻. GABA-gated currents were negligible prior to the application of NO (Figure 3.1 A & B).

NO application resulted in GABA-gated inward currents signifying Cl⁻ release from internal compartments into the cytosol in both the 5-pulse and 2-pulse protocols. Decay Indices were calculated from both the 5-pulse and 2-pulse protocols. It was seen that the reduction in current amplitude of the GABA gated current was significantly larger in the 5-pulse as opposed to the 2-pulse protocol (Figure 3.1 C; mean 2 pulse DI 0.35 ± 0.04 vs. 5 pulse DI 0.65 ± 0.01; n=15; p<0.0001) suggesting that the chloride lost through the open GABAₐ receptors contributes to the recovery of cytosolic Cl⁻ back to pre-NO Cl⁻ levels.

**The NO dependent Cl⁻ release from the internal store is initially resistant to depletion**

The activation of GABAₐ receptors can contribute to the recovery of Cl⁻, but processes involving Cl⁻ reuptake back into the store might also play a role. If reuptake plays a significant role then we would expect the store to be resistant to depletion. To explore this, GABA pulses and multiple NO injections were performed to establish the temporal properties of store
depletion. First we tested the effectiveness of four NO exposures. Multiple NO injections did show a decrease in the current amplitude over the course of NO injections; however it was not

Figure 3.1 A fraction of NO-dependent cytosolic Cl\(^-\) escapes through open GABA\(_A\) receptors. (A&B), Data from a representative amacrine cell is held at -70 mV in 0 Cl\(^-\) solutions. GABA (20\(\mu\)M, 400msec) is applied at times indicated by the dashed lines. Recordings begin just after an NO injection. The GABA-gated current amplitudes decay more rapidly when the GABAA receptors are opened more frequently. (C), Return to resting (low or no) cytosolic Cl\(^-\) occurs more rapidly with the 5-pulse protocol than with the 2-pulse protocol (p<0.0001, n=15). These data suggest that activation of the GABAA receptor hastens the removal of the NO-dependent cytosolic Cl\(^-\).
depleted (n=6) over the course of 4 NO injections (Figure 3.2A). The amplitude ratios for both the first (P1/P1) and fifth (P5/P5) GABA pulse amplitude for all the 4 NO injections were calculated in order to estimate the degree of store depletion. Comparison of mean amplitude ratios between the injections (mean P1/P1) and (mean P5/P5) did not show any significant trend towards Cl⁻ depletion. These data suggest that the Cl⁻ store is fairly resistant to depletion (Figure 3.2B).

Figure 3.2 The NO-sensitive Cl⁻ store is resistant to depletion. (A), Whole cell recordings from a representative amacrine cell were made in 0 internal and external Cl⁻ solutions. The cell is held at -70 mV and 400 msec pulses of GABA (20 µM) are delivered at the times indicated by the bars. Prior to NO injection, no GABA-gated current was observed (not shown). NO-bubbled solution (50 µl) is injected prior to the beginning of the record and causes release of Cl⁻ into the cytosol. Subsequent GABA pulses result in inward currents due to the cytosolic Cl⁻ originating from the store, leaving the cell. (B), Comparison of mean amplitude ratios of the first and fifth pulse with both showing decline in the DI initially but recovers by the 4 NO injection. Similar results were obtained in all 6 cells examined suggesting that the store is resistant to depletion.
Properties of NO-dependent Cl- depletion

If most of the NO-dependent Cl- leaves via GABAA receptors, then cells with larger NO-induced GABA-gated current should deplete faster. In order to address this, I tested to the relationship between NO-dependent GABA-gated current amplitude and the number of NO injections required to deplete the Cl- store. I performed voltage clamp recordings on amacrine cells held at -70 mV and GABA was applied for 400 msec. The cells were then subjected to multiple rounds of NO injections. Figure 3.3A serves as an example of NO applications on the same cell resulting in the elimination of GABA-gated currents due to the NO dependent release of internal Cl-.

I analyzed the relationship between current amplitude of the first NO response against the number of NO injections taken to deplete the store. Figure 3.3 B shows data from 15 cells where NO injections were repeated until GABA gated currents were abolished indicating store depletion. These data demonstrate that there is variability in the number of NO injections required to deplete the store of internal Cl-. This could be due to a number of reasons including variability in store content and variability in store size. Regression analysis in Figure 3.3 C does not identify a correlation between the initial GABA gated current amplitude and resistance of the store to depletion ($R^2=0.03$). This suggests that although Cl- escape through open GABAA receptors is a factor in store depletion, other unknown mechanisms play a significant role.

**Internal Cl- stores can be depleted and subsequently refilled**

The previous experiment demonstrated that the store can be depleted of its Cl- content (Figure 3.3). I next asked whether extracellular Cl- can be a source for refilling. In order to test for refilling mechanisms that involve extracellular Cl- refilling was tested with and without extracellular Cl-. Measuring the NO-dependent GABA-gated current allowed me to track the refilling of the store. This experiment was done with 0 Cl- internal solution and started in 0 Cl- external solution. NO was applied until the Cl- store was depleted (Figure 3.4 A). After depletion, the cells were incubated in either 0 Cl- external in Figure 3.4 B or normal external solution in
Figure 3.4 C for 3 minutes then NO was re-applied. NO did not increase the GABA-gated current amplitude in cells incubated in 0 Cl- external (Figure 3.4 D) but did increase the amplitude in external 0 Cl- solution (Figure 3.4 E n=9, p=0.0021) suggesting that a refilling mechanism(s) route Cl- from the extracellular environment to replenish internal stores with Cl-.

The primary mechanism that mediates Cl- uptake from the extracellular environment is the NKCC plasma membrane transporter (Haas and Forbush III, 1998).

Figure 3.3 Properties of store depletion. (A), Shows a whole cell voltage clamp recording from a representative cell held at -70 mV and subjected to multiple NO recordings (numbered top to bottom) showing lack of any NO GABA-gated currents indicating that NO has depleted the store of Cl-. (B), Current amplitude of the first NO response is plotted against the number of NO injections taken to completely deplete the store. Regression analysis does not identify a correlation between the two values. (C), Data plot representing the progressive current amplitudes of NO induced GABA-gated current from start of first NO injection to their respective NO injection that results in the total loss of the current.
NKCC serves to co-transport K⁺, Na⁺ and Cl⁻ into the cytoplasm. I hypothesized that NKCC is involved in the plasma membrane Cl⁻ refilling mechanism. I tested the involvement of NKCC by using a selective NKCC inhibitor bumetanide at a concentration of 300 µM. Figure 3.5B shows that post NO depletion the refilling response are not affected by NKCC inhibition, suggesting that NKCC is not involved in supplying Cl⁻ from the extracellular environment. Experiments with bumetanide are preliminary and will be repeated before reaching a conclusion about the involvement of NKCCs.

Figure 3.4 The NO-dependent Cl⁻ store can be depleted and subsequently refilled. (A-E), Representative data trace from a cell showing whole cell recordings held at -70 mV, in chloride free solutions and exposed to GABA (20 µM, 400 msec) at regular intervals. Multiple NO injections subsequently deplete Cl⁻ from intracellular stores as shown by NO-dependent GABA-gated currents decline in amplitude. Following depletion, cells were incubated in either (B), 0 Cl⁻ external or (C), Normal Cl⁻ external solution to experimentally confirm the possibility of refilling of internal stores after depletion. (D), Data from cells that have been exposed to 0 Cl⁻ external solution do not significantly increase (refill) GABA gated current amplitude (n=8). (E), Averaged data from cells showing significant increase in GABA gated current amplitude post refilling with normal external (n=9, p=0.0021). This shows that the store has been successfully refilled.
I have presented evidence that the Cl⁻ store can be effectively depleted by multiple applications of NO and subsequently can be refilled when incubated in normal Cl⁻ after depletion. Additional data is required to test the involvement of NKCC and other plasma membrane Cl⁻ accumulators. These experiments would require an (empty) Cl⁻ store. Using multiple NO applications to deplete the store has downsides as it is difficult to keep the cells healthy for multiple NO applications. Studies in literature have shown that pre-incubating cells in 0 Cl⁻ solutions along with a GABA agonist isoguvacine has been shown to deplete compartmental Cl⁻ (MacVicar et al., 1989). We tested the feasibility of this method and found that in cells preincubated with isoguvacine for 30 min no NO dependent Cl⁻ release was achieved (Figure 3.6B).

Figure 3.5 Bumetanide does not block NO mediated Cl⁻ release, depletion or refilling. (A) Schematic cellular representation depicting possible involvement of plasma membrane Cl⁻ co-transporter (NKCC1) in the refilling of internal stores post depletion. (B), Control data from individual cells showing successful refilling post depletion. (C), Blocking the activity of NKCC1 with bumetanide (300 µM) does not affect the refilling response as shown by its similar response to that of control.
Discussion

These results demonstrated the exit of Cl\(^-\) from the cytosol after NO is facilitated by an increased GABA\(_A\) receptor activation. My objective was also to study the temporal properties of store depletion and elucidate the mechanisms that influence refilling. I discovered that an NO-mediated Cl\(^-\) release is resistant to depletion. Additionally, under the same experimental parameters the number of NO injections required for depletion ranged from 4 to 10 injections. Finally, after depletion of Cl\(^-\) from internal stores, successful refilling was achieved only if extracellular Cl\(^-\) was available. Future experiments would involve testing the hypotheses that plasma membrane as well as store expressed Cl\(^-\) transport mechanisms contribute to restoration of normal cytosolic Cl\(^-\). Pilot experiments blocking the activity of a plasma membrane Cl\(^-\) transporter (NKCC) with bumetanide did not block the refilling mechanism. This suggests that NKCC is not involved in refilling. However a large number of cells need to be tested to reach a firm conclusion.

Figure 3.6 Pre-incubation with GABA\(_A\) agonist effectively depletes the Cl\(^-\) store. (A), Voltage clamp recordings from cells held at -70 mV in Cl\(^-\) free solution. Control cell showing GABA-gated NO induced Cl\(^-\) release. (B), A different cell pre-treated with GABA\(_A\) agonist Isoguvacine (1 mM) in Cl\(^-\) free external solution (30 min) blocked the NO-dependent release of internal Cl\(^-\).
(C). Comparison of GABA gated mean current amplitude between control (n=4) and isoguvacine treated cell (n=5).

Also, another key member of the Cl⁻ exit pathway is the ClC2 chloride channels. These have been shown to facilitate Cl⁻ exit from hippocampal neurons receiving GABAergic synaptic input, in order to maintain low cytosolic Cl⁻ (Földy et al., 2010, Rinke et al., 2010). The peptide GaTx2 (gating modifier of anion channels 2) is the only known inhibitor of ClC-2. Maintenance of low [Cl⁻]ᵢ in neurons has been attributed to the K-Cl cotransporter KCC2 (extrudes Cl⁻, reviewed in Payne et al., 2003, Blaesse et al., 2009). Blocking the activity of the Cl⁻ extruder KCC2 will improve our understanding of Cl⁻ recovery.

I also predict that there will be involvement of plasma membrane store-refilling mechanisms opposing our attempt to deplete the store. If such a Cl⁻ store-refilling mechanism is exists, then the store will be more resistant to depletion under normal Cl⁻ conditions. There are also multiple potential entry pathways for Cl⁻ across the plasma membrane that could be involved in refilling. The involvement of plasma membrane Cl⁻ accumulators like the anion exchangers (AE1 -3), NKCC1 and the Na- Cl⁻ cotransporter (NCC, SLC12A1-2) will be evaluated and consequently the mechanisms associated with refilling of Cl⁻ can be established. With these experiments we will gain important insight into the fidelity of the store and the conditions of NO generation under which the store can continue to regulate postsynaptic function.

Together, these data provides an outlook on studying the nature of the Cl⁻ store. This release of Cl⁻ has important implications in addressing the dynamic nature of cytosolic Cl⁻. The shift in intracellular Cl⁻ from high to low has been associated in the development of neurons. This is primarily due to the differential distribution of NKCC and KCC (Zhang et al., 2007). From imaging studies, it is known that transmembrane chloride gradients are dynamic, and
regionally different across the dimensions of individual neurons (Duebel et al., 2006). A decrease in the transmembrane Cl\(^-\) gradient has shown to eliminate the directional responses of DS ganglion cells and starburst amacrine cells. The response properties of retinal starburst amacrine cells depend on the relative activities of NKCC and KCC (Gavrikov et al., 2006).

In hippocampal neurons, brain-derived neurotrophic factor has been shown to induce a positive shift in the GABA-gated current reversal potential by altering transport of Cl\(^-\) across the plasma membrane (Woodin et al., 2003). The regulation of cytosolic Cl\(^-\) is also determined by internal Cl\(^-\) release as described in my research. Further studies would enable us to further establish the temporal properties of and hopefully the identity of the unknown Cl\(^-\) store.

**References**


CHAPTER 4
CONCLUSIONS

Nitric Oxide (NO) has been demonstrated to convert inhibitory synapses into excitatory synapses and this synaptic conversion is achieved by an increase in intracellular Cl\(^-\) concentration. It has further been established that this increase in cytosolic Cl\(^-\) is due to a NO-mediated release of Cl\(^-\) from cytosolic compartments and not from Cl\(^-\) entry across the plasma membrane. Voltage ramp recordings from cultured amacrine cells have shown that NO is able to induce a shift in the equilibrium potential for Cl\(^-\) (E\(_{Cl^-}\)) to more positive values (Hoffpauir, 2006). This Cl\(^-\) release is dependent upon a transient acidification of the cytosol (McMains and Gleason, 2011).

My work expands our understanding of mechanisms underlying the NO-dependent release of internal Cl\(^-\). Experiments in Chapter 2 provide evidence that Cl\(^-\) release mediated by NO is coming from acidic organelles (AOs). I have shown that altering the pH of these AOs affects both NO-dependent and independent Cl\(^-\) release. In Chapter 3 I further demonstrated that this store can be successfully depleted of its Cl\(^-\) content by repeated exposures to NO. Furthermore, the store can be refilled by extracellular Cl\(^-\). The experimental evidence presented in this dissertation advances our understanding of the mechanisms that underlie the ability of NO to convert inhibitory synapses into excitatory synapses.

The biological relevance of NO-bubbled solutions

NO electrode measurements from the inner surface of the retina have demonstrated that micromolar concentrations of NO can be produced by the retina (Donati et al., 1995, Heiduschka and Thanos, 1998, Groppe et al., 2003). NO imaging with 4-amin-4-methylamino-2',7' –
difluorofluorescein diacetate (DAF) has demonstrated that NO-producing amacrine cells have a strong DAF signal that remains within the boundaries of the cell indicating that NO is somehow prevented from freely diffusing out of the cell. In the ganglion cell layer, however, cell bodies produce a DAF signal that is more diffuse indicating NO diffusion. NO electrode measurements just outside of these cells in the ganglion cell layer show Ca\(^{2+}\)-activated NO signals reaching about 200 nM (Eldred and Blute, 2005). These observations suggest that the concentration within an NO producing (and diffusion limiting) amacrine cell would be substantially higher. To evaluate our perfused NO concentrations, we measured NO using an NO electrode positioned near the perfusion outlet and find that injections of 30-50 uL of NO-bubbled solutions produce NO concentrations ranging from 100’s of nanomolar to low μM (Hoffpauir et al., 2006). By using NO-bubbled solutions we can achieve the relatively high levels of NO that are likely to occur in NO-producing amacrine cells or their immediate synaptic partners.

**Implications of altering internal Cl\(^-\)**

Alterations of plasma membrane Cl\(^-\) gradients have previously been demonstrated in developing neurons. The shift in Cl\(^-\) gradient across the plasma membrane neurons shifts the signaling properties of GABAergic synapses during development (Payne et al., 2003b). NKCC which is a Cl\(^-\) accumulator function early in development transporting Cl\(^-\) into the cytosol, thereby rendering GABAergic inputs excitatory (Kaila et al., 1989). Later in neuronal development KCC is highly expressed and moves Cl\(^-\) out of the cytosol, making the E\(_{\text{Cl}^-}\) more negative and rendering GABAergic inputs inhibitory. This crucial shift in cytosolic Cl\(^-\) levels determines the nature of synaptic signaling at GABAergic synapses. This result points to a major finding that the synaptic strength is not exclusively determined by neurotransmitters but is also due to the distribution of Cl\(^-\) across the plasma membrane.
The popular notion that GABA is exclusively an inhibitory neurotransmitter in adult neurons has recently been challenged. Changes in intracellular Cl\(^-\) shifts the chloride concentration gradient rendering the reversal potential for GABA receptors ready for action potential generation. (Glykys et al., 2014a) used the cytosolic Cl\(^-\) indicator Clomeleon and addressed the influence of the cation-chloride cotransporters (CCCs) KCC2 and the NKCC1 on the distribution of Cl\(^-\) in brain slices. Their results demonstrated that it was impermeant organic anions rather than the CCCs that were key in the determination of cytosolic Cl\(^-\). This implies that the regulation of intracellular Cl\(^-\) is more complicated than previously appreciated.

Altered cytosolic Cl\(^-\) concentrations have been implicated in neurological diseases (Kahle et al., 2008) such as neuronal trauma and brain disorders (De Koninck, 2007). In a report by (Cooper and Przebinda, 2011) the term chloride-opathy was defined to be pathological changes in the Nernst potential for Cl\(^-\). These chloride-opathies are capable of producing aberrant Cl\(^-\) currents.

**Candidate Cl\(^-\) transporters involved in NO mediated Cl\(^-\) release**

The physiological link between NO-dependent Cl\(^-\) release and the channels and transporters involved remains unknown. Candidate AOs include endosomes, synaptic vesicles, lysosomes and the Golgi. Interestingly, an anion channel has been identified (GOLAC 1 & 2) that supports Cl\(^-\) and ATP flux into the Golgi lumen which in turn facilitates luminal acidification by the organelle's H\(^+\)-ATPase (Nordeen et al., 2000, Thompson et al., 2006)

There is strong evidence that the CIC family of Cl\(^-\) transporters has multiple members (ClCs 3-7) that are expressed on internal membranes including synaptic vesicles (ClC3) endosomes (ClCs 4 and 5) and late endosomes and lysosomes ClCs 6 and 7 (Weinert et al., 2010). The Gleason lab has both immunocytochemistry and single cell PCR evidence that all
internal ClC subtypes (ClC 3-7) are expressed in retinal amacrine cells. Unfortunately, there is a dearth of effective pharmacological inhibitors of ClCs. This has compelled us to utilize molecular biology methods to knock down (miRNA) or knock out (CRISPER/CAS 9) the ClCs to determine their involvement in the NO-dependent release of internal Cl⁻. Once knockdown (or out) is achieved, we will determine if there is a correlation between the silenced gene and an impairment of NO-dependent Cl⁻ release.

The cystic fibrosis transmembrane regulator (CFTR) is an additional candidate Cl⁻ transporter. The gene encoding this Cl⁻ channel was initially identified by positional cloning and the involvement of mutant CFTR in cystic fibrosis was characterized (Riordan et al., 1989). CFTR mediated Cl⁻ transport is dependent upon cAMP binding to and activating protein kinase A to initiate phosphorylation of the channel. CFTR has a been reported to participate in the process of acidification of lysosomes in retinal pigment epithelium (Liu et al., 2012). We have immunocytochemical and single cell PCR evidence that CFTR is expressed in retinal amacrine cells. Additionally I have demonstrated that the effect of NO on cytosolic Cl⁻ was blocked when CFTR activity was inhibited using CFTR specific blockers (Data not shown). This physiological evidence together with the reported lysosomal localization makes CFTR a strong candidate for involvement in NO-dependent Cl⁻ release.

**Alternative methods in studying intracellular Cl⁻**

From imaging studies, is it known that transmembrane chloride gradients are dynamic and regionally different across the dimensions of individual neurons. For example, retinal bipolar cells have a standing Cl⁻ gradient with their dendrites having relatively high cytosolic Cl⁻ but the axon having relative low Cl⁻ (Vardi et al., 2000). One of the limitations of my study is that the electrophysiological techniques used provide no spatial information on the NO-
dependent Cl\(^-\) release. To provide spatial information live cell Cl\(^-\) imaging could be implemented.

The first indicators used for Cl\(^-\) imaging were the quinolinium dyes: 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ), N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) and 6-methoxy-N-ethylquinolinium iodide (MEQ). All three dyes operate through the mechanism of collisional quenching in which the dyes fluorescence gets quenched through interaction with Cl\(^-\) ions. We have used MQAE dye for our preliminary experiments because it has a low pH sensitivity and we know that cytosolic pH changes co-occur with Cl\(^-\) release (McMains and Gleason, 2011a). The results of these experiments will provide a qualitative picture of the spatial characteristics of Cl\(^-\) release, cytosolic recovery of Cl\(^-\) levels and store refilling. We will also gain insight into the types of cellular activities that will have an impact on the duration of the Cl\(^-\) signal. For example, we would predict that high levels of GABAergic or glycinergic synaptic activity would speed recovery back to resting Cl\(^-\) levels. The main drawback of this method is the UV excitation of these dyes which is damaging to the cells during imaging.

To avoid the difficulties with UV excitation, genetically encoded Cl\(^-\) reporter proteins were developed based on modifications of green fluorescent protein. These have the advantage of being photo-stable and can be targeted to specific subcellular compartments. The first was Clomeleon (Kuner and Augustine, 2000a) which was not useful to us because the fluorescence is pH-sensitive. The newest member of this family is the ClopHensor, based on a Cl\(^-\) sensitive GFP variant. ClopHensor can be used to measure changes in Cl\(^-\) and pH simultaneously but separately in live cells (Arosio et al., 2010). This would be particularly useful in this project since the NO-dependent Cl\(^-\) release from internal stores is pH dependent. This would serve as a powerful tool in elucidating Cl\(^-\) as well as pH dynamics at a spatial level.
Much remains to be discovered about the mechanisms underlying the NO-dependent release of Cl\(^-\) from AOs. Is the Cl\(^-\) coming from just one type of compartment or do all AOs contribute? Ongoing experiments in the lab are exploring the contribution of synaptic vesicles as a source of Cl\(^-\). What are the Cl\(^-\) transporters involved in release of internal Cl\(^-\)? We have some candidates but this remains to be worked out. It is also important to discover the plasma membrane transporters and internal transporters involved in recovery of cytosolic Cl\(^-\) and refilling of the Cl\(^-\) store, respectively. Another gap in our understanding is the mechanism by which NO exerts its effect on the Cl\(^-\) store. This question will become more tractable when we know the organelle(s) and transporters involved so that we can directly assess the effects of NO on each of these targets. Together, these studies will add a new dimension to our understanding of the regulation of Cl\(^-\) in neurons.

References


APPENDIX

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