Modulation of ASIC1a Function by Sigma-1 Receptors: Physiological and Pathophysiological Implications

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Modulation of ASIC1a Function by Sigma-1 Receptors: Physiological and Pathophysiological Implications

by

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DEDICATION

Those who have inspired me throughout my life and scientific career are those that I love the most. This dissertation is dedicated to them - my husband and family. I would like to thank my Mom and Dad for giving me the opportunity to be free, succeed and pursue my education. To my brother, you are the best and I am very proud of you; thank you for always being there and for your support. I would like to give a special thanks to my husband, Roger, for his love and always putting a smile in my face, and for his understanding, support and encouragement in making this dream possible. This accomplishment would have not been feasible if it was not for all of you. Thank you all and I love you all very much. I would also like to dedicate this thesis to my baby, Dukey.
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Modulation of ASIC1a Function by Sigma-1 Receptors: Physiological and Pathophysiological Implications

Yelenis Herrera

ABSTRACT

Acid-sensing ion channels (ASIC) are a class of ligand gated plasma membrane ion channels that are activated by low extracellular pH. During ischemia, ASIC1a are activated and contribute to the demise of neurons. Pharmacological block of ASIC1a provides neuroprotection at delayed time points. However, no endogenous receptors have been implicated in the modulation of ASIC1a activity. The hypothesis presented is that sigma receptor activation inhibits ASIC1a function and ASIC1a-induced [Ca²⁺]ᵢ elevations during acidosis and ischemia, which may be a mechanism by which sigma ligands provide neuroprotection following stroke. This hypothesis is based on the following observations: First, sigma (σ) receptors regulate multiple ion channels that become activated during ischemia. Second, ASIC1a remain functionally active hours beyond the ischemic insult and σ receptors have been shown to be neuroprotective at delayed time points following stroke.

Ratiometric Ca²⁺ fluorometry and whole-cell patch clamp experiments showed that σ-1 receptor activation depresses elevations in [Ca²⁺]ᵢ, and
membrane currents mediated by ASIC1a channels in cortical neurons. Furthermore, most of the elevations in \([\text{Ca}^{2+}]_i\) triggered by acidosis are the result of \(\text{Ca}^{2+}\) channels opening downstream of ASIC1a activation. Stimulation of \(\sigma-1\) receptors effectively suppressed these secondary \(\text{Ca}^{2+}\) fluxes both by inhibiting ASIC1a and the other channels directly.

The signaling cascade linking \(\sigma-1\) receptors and ASIC1a was determined to involve a pertussis toxin-sensitive G protein and A-Kinase Anchoring Protein 150/calcineurin complex, which resulted in a decrease of acid-induced \([\text{Ca}^{2+}]_i\) elevations and ASIC1a-mediated currents. Furthermore, immunohistochemical studies confirmed that \(\sigma-1\) receptors, ASIC1a and AKAP150 colocalize in the plasma membrane of cortical neuron cell bodies and in the dendritic processes of these cells.

Additionally, concurrent exposure to acidosis and ischemia resulted in synergistic potentiation of \([\text{Ca}^{2+}]_i\) dysregulation. Although ASIC1a activation does not induce long-lived priming of synaptic vesicles for release, channel activation does have a temporal effect on ischemia-mediated \([\text{Ca}^{2+}]_i\) increases after ischemia onset. Moreover, presynaptic ASIC1a channels promote synaptic transmission during ischemia by overcoming block of neurotransmission and thus enhance postsynaptic \([\text{Ca}^{2+}]_i\) elevations. \(\sigma-1\) receptor activation decreased ischemia-mediated \(\text{Ca}^{2+}\) dysregulation at pH values of 7.4 - 6.0 and prevented the synergistic interaction between ischemia and acidosis.
CHAPTER 1

INTRODUCTION

Significance

ASIC are proton activated ion channels expressed in the brain and are involved in several physiological functions including neurotransmission and excitability. ASIC are also activated during pathological conditions, like stroke, resulting in Ca²⁺ dysregulation and eventually neurodegeneration. Furthermore, regulation of ASIC function would result in neuroprotection. σ receptors are known to regulate multiple ion channels in neurons, all of which are activated during ischemia. Taken together, these observations demonstrate the importance of studying the regulation of ASIC function by σ receptors under physiological as well as during ischemic conditions and to determine the signaling cascade involved. Results from this study will identify potential therapeutic targets for the treatment of stroke.

Background

Stroke is the third leading cause of death in the industrialized world behind heart disease and cancer, and the major cause of long-term disability. Stroke is a sudden interruption in the blood supply to the brain. Ischemic (embolic) stroke is
the most common type of stroke, accounting for almost 88% of all strokes, and is caused by a clot or other blockage within a cerebral artery. Following a stroke, there are two major regions of injury within the ischemic cerebrovascular bed: the core ischemic region and the ischemic penumbra region (Dirnagl et al., 1999). Within minutes of the ischemic insult, the core area forms. The core region is an area characterized by reduced blood flow, leading to a loss of adequate supply of oxygen and glucose resulting in rapid depletion of cellular energy stores. Ischemia results in neuronal death and supporting cellular elements such as glial cells by necrosis (characterized by cell swelling, cell rupture and activation of inflammatory response) within the severely ischemic core area (Dirnagl et al., 1999).

The penumbra region is an area that is generally defined to be ischemic but still containing viable cerebral tissue. Brain cells within the penumbra, a rim of compromised ischemic tissue lying between tissue that is normally perfused and the area in which infarction is evolving, may remain viable for several days (Dirnagl et al., 1999; Lo et al., 2004). The penumbral region is supplied with blood by collateral arteries. However, even cells in this region will die if reperfusion is not established in a timely manner since collateral circulation is inadequate to maintain the neuronal demand for oxygen and glucose indefinitely. As time goes by, hours and days after the insult, apoptosis in the penumbra results in cell death and expansion of the core (Dirnagl et al., 1999; Lo et al., 2005). Most of research taking place today focuses on the prevention of the apoptotic death that occurs within the penumbra region. This may be achieved by
restoring blood flow to the penumbral zone and/or by disrupting several pathways that are activated resulting in neuronal death.

The thrombolytic agent, tissue plasminogen activator (tPA), is the only drug approved for clinical treatment of acute stroke. tPA cleaves the precursor molecule plasminogen producing the active enzyme plasmin, which in turn dissolves blood clots. Thus, tPA is not a neuroprotective drug. The limitations of tPA arise from the high risk of intracranial hemorrhage associated with its use, and the short therapeutic window in which it must be administered (≤ 3 hours) (Dirnagl et al., 1999). Additionally, tPA can not be administered to hemorrhagic stroke patients or patients with either hypertension or diabetes. Therefore, there is only a very small percentage of people (~ 3%) that are candidates for this drug. The significant inadequacy of current acute stroke therapy has increased the demand for the development of alternative drugs to improve outcome following stroke injury.

A major component of current stroke research focuses on excitotoxicity and less is known about acidotoxicity or the interaction of ASIC1a channel activation and ischemia. Currently, there are no drugs that directly enhance neuronal survival approved for clinical use. Furthermore, drugs that have targeted a single component in the pathobiology cascade of stroke (e.g. NMDA receptors, glutamate receptors and voltage-gated Ca^{2+} channels) have failed in clinical trials.
Pathobiology of Stroke

To better understand neurodegeneration during ischemia, it is important to analyze the pathobiology of stroke. Ischemia is a restriction of blood flow to the brain triggering a series of events that lead to necrotic neuronal death in the affected infarct area (core region) and delayed apoptotic neuronal death in the penumbra. Brain ischemia is associated with glucose and oxygen deprivation, and a cellular switch to anaerobic glycolysis that results in energy failure (Figure 1.1). The accumulation of lactic acid produced by anaerobic glycolysis leads to acidosis in the ischemic region, and acidotoxicity eventually results in cell death (Xiong et al., 2004; Yermolaieva et al., 2004). Dysregulation of intracellular calcium (Ca^{2+}) triggers various events that also result in cell death (Figure 1.1).

Loss of adenosine triphosphate (ATP) production results in membrane dysfunction and a rapid depolarization of neurons promotes Ca^{2+} entry, which in turn evokes the release of neurotransmitters, such as glutamate (Figure 1.1). Glutamate, acting via postsynaptic receptors, elicits depolarization of the neurons and further elevations in intracellular Ca^{2+} (Figure 1.1). At the same time, the depletion of ATP blocks the reuptake of glutamate, which leads to an accumulation of glutamate at synaptic and extrasynaptic sites. Accumulation of glutamate in the extracellular space leads to over-stimulation of N-methyl-D-aspartate (NMDA) receptors resulting in Ca^{2+} overload. This becomes a vicious cycle that leads to ion fluxes into the cell via the activation of Cl^-, Na^+, Ca^{2+} and K^+ channels (Tanaka et al., 1997). The accumulation of extracellular K^+ allows surrounding neurons to depolarize, whereas the buildup of intracellular Na^+
results in cell swelling, or oedema (Figure 1.1). Intracellular Ca\textsuperscript{2+} dysregulation promotes neuronal death by disrupting plasma membrane function via activation of Ca\textsuperscript{2+}-sensitive ion channels (Murai et al., 1997), and by triggering biochemical cascades that lead to proteolysis, lipolysis, and the production of reactive oxygen species (Mattson, 2000) (Figure 1.1). Ca\textsuperscript{2+} overload and energy depletion leads to activation of secondary events including release of inflammatory mediators, expression of pro-apoptotic genes, all of which result in cell death and subsequent expansion of the core (Figure 1.1).

Though not depicted in Figure 1.1, cell death and expansion of the core region is also enhanced by inflammation caused by the immune response. Activated microglia and reactive astrocytes are capable of entering the central nervous system since the blood brain barrier is degraded (Stoll et al., 1998). These cells then exacerbate damage by activating proinflammatory mediators, releasing nitric oxide and glutamate (Heales et al., 1999; Hertz et al., 2001; Bal-Price et al., 2002; Trendelenburg and Dirnagl, 2005). All these events are also detrimental to the neurons and result in subsequent expansion of the core.
Figure 1.1 - Pathobiology of ischemic stroke. Intracellular calcium plays a critical role during ischemia. Ischemia leads to acidosis and acidotoxicity in the ischemic region, which results in further increases in \([\text{Ca}^{2+}]_i\). Calcium dysregulation ultimately leads to the activation of all these events that result in cell death and subsequent expansion of the core region.
Pathobiology of ischemic stroke and acidosis

Ischemia

Energy Failure

Membrane Dysfunction

Oedema

Acidosis

$\uparrow [\text{Ca}^{2+}]_i$

Depolarization

Cell Death

Pro-apoptotic gene expression

Inflammatory Mediators

Degradation of membrane and cytoskeleton

Oxidative damage
Acid-Sensing Ion Channels (ASIC)

Recently, the acid-sensing ion channel has been shown to be a major contributor to ischemic injury in the central nervous system. Physiological extracellular and intracellular pH is maintained at ~ 7.3 and 7.0 through various proton transporting mechanisms. Under pathological conditions, like stroke, the metabolic switch to anaerobic glycolysis produces lactic acid leading to a drop in pH and acidotoxicity in the ischemic region. During ischemia, the pH can drop to as low as 6.0 (Nedergaard et al., 1991). More recent studies imaging brains in rats post-MCAO and patients with cortical ischemic stroke symptoms show that lactate accumulations remain elevated for 3-5 days (Weinstein et al., 2004; Munoz Maniega et al., 2008). The decrease in pH is sufficient to trigger the opening of ASIC and consequently, permitting calcium influx into the neurons (Xiong et al., 2004). Both in vitro and in vivo studies have shown that acidotoxicity aggravates ischemic neuronal injury and a direct correlation has been shown between infarct size and brain acidosis (Xiong et al., 2004; Pignataro et al., 2007). Thus, activation of ASIC has now been unequivocally linked to brain injury resulting from ischemic stroke. It has been suggested that low tissue pH may lead to protein and nucleic acid denaturation, trigger cell swelling via activation of proton exchangers, lead to excitotoxicity by inhibiting astrocyte glutamate reuptake, and damage glial cells (see review (Xiong et al., 2006)). In contrast, mild acidosis has been implicated to be beneficial due to NMDA receptor inhibition by extracellular low pH. Therefore, during ischemia,
other calcium influx pathways may exist independently of NMDA receptor
function.

ASIC are a class of ligand-gated ion channels that are members of the
degenerin/epithelial sodium channel (Deg/ENac) superfamily (Benos and
Stanton, 1999). ASIC are expressed in both peripheral and central nervous
system neurons and become activated as a result of low extracellular pH caused
by ischemia (Xiong et al., 2004; Yermolaieva et al., 2004). Four genes (ASIC1 –
ASIC4) encoding for six ASIC subunits have been cloned, and four of those
subunits can form functional homomultimeric or heteromultimeric channels that
are activated by extracellular protons. Recent stoichiometric studies of the
chicken ASIC1a subtype suggests that 3 subunits are required to form a
functional channel (Jasti et al., 2007). The pH of half-maximal activation (pH_{0.5}) of
these channels and the tissue expression pattern differs between each subtype.

Pertinent to ischemia, ASIC1a has a pH_{0.5} = 6.2, and is mainly expressed
in the brain (Waldmann et al., 1997b; Alvarez de la Rosa et al., 2003). ASIC1a is
a nonselective cation channel that is sodium and calcium permeable and
generates fast activating and inactivating membrane currents (Waldmann et al.,
1997b; Chu et al., 2002; Xiong et al., 2004; Yermolaieva et al., 2004). ASIC1b, a
splice variant of ASIC1a with a unique N-terminal, has a pH_{0.5} = 5.9 (Chen et al.,
1998) and is only expressed in sensory neurons (Chen et al., 1998; Bassler et
al., 2001). ASIC2a is widely expressed in peripheral and central nervous system
with a pH_{0.5} = 4.4 (Garcia-Anoveros et al., 1997; Lingueglia et al., 1997). ASIC2a
channels generate slower activating and inactivating currents compared to the
ASIC1a subtype. ASIC3 has a pH_{0.5} = 6.5, and it is predominantly expressed in neurons of the dorsal root ganglia (Waldmann et al., 1997a), while ASIC4 is highly expressed in the pituitary gland but does not form functional channels on their own (Akopian et al., 2000; Grunder et al., 2000). It is been established that neither ASIC2b nor ASIC4 can form functional homomeric channels, but ASIC2b can associate with other subunits and modulate their activity, and thus biophysical properties of the channels. For example, studies of heteromeric ASIC1a/2a channels have shown that ASIC1a establishes the current amplitude, while ASIC2a influences desensitization, recovery from desensitization, and pH sensitivity of the channel (Askwith et al., 2004).

ASIC contain two transmembrane domains flanked by a large cysteine-rich extracellular loop and short intracellular N- and C- termini (Waldmann et al., 1997b; Alvarez de la Rosa et al., 2000; Saugstad et al., 2004). In the peripheral nervous system, ASIC localize to neurons innervating skin, heart, gut, and muscle, and have also been detected in the eye, ear, taste buds, and bone (see review (Wemmie et al., 2006)). These channels are known to be involved in various processes such as mechanoreception (Price et al., 2000), taste transduction (Ugawa et al., 2003), maintenance of retinal integrity (Ettaiche et al., 2004), and nociception (Allen and Attwell, 2002), particularly in the ischemic myocardium where ASIC are believed to transduce anginal pain (Benson et al., 1999).

ASIC1a have a wide spread distribution in the brain including hippocampus, cerebral cortex, cerebellum, striatum, habenula, amygdale, and
olfactory (Wemmie et al., 2002; Alvarez de la Rosa et al., 2003). The ASIC1a subtype have been localized to dendrites and dendritic spines, axons, and throughout neurons with no preferential distribution to synapses (Wemmie et al., 2003). In the central nervous system, ASIC1a is involved in synaptic plasticity, fear conditioning, and learning and memory (Wemmie et al., 2002; Wemmie et al., 2003). It has been shown that loss of ASIC1a disrupted hippocampal-dependent long term potentiation and spatial memory, impaired cerebellum-dependent learning, and reduced fear response in the amygdala (freezing behavior) (Wemmie et al., 2003). Conversely, overexpression of ASIC1a in the amygdala and different regions of the brain leads to increased ASIC1a currents and enhanced context of fear conditioning (anxiety) (Wemmie et al., 2004). ASIC1 and ASIC1 gene knockout studies have shown that this ion channel is calcium permeable and is involved in acidosis-mediated ischemic brain injury and neuronal death (Xiong et al., 2004; Yermolaieva et al., 2004; Pignataro et al., 2007).

ASIC also belong to the amiloride-sensitive superfamily and studies have shown that inhibition of ASIC by this nonspecific blocker leads to diminished $[\text{Ca}^{2+}]_i$ elevations during acidosis. It has been shown that amiloride dose-dependently blocked ASIC currents in mouse cortical neurons with an $IC_{50}$ of 16.4 µM (Xiong et al., 2004). It has also been demonstrated that amiloride and its derivatives benzamil and ethylisopropylamiloride (EIPA) reversibly blocked proton-activated inward ASIC currents in sensory neurons ($K_d = 10 \mu M$) (Waldmann et al., 1997b). Mutation studies have suggested that residues within
the pore and extracellular domain are critical for amiloride binding (Kleyman et al., 1999). This observation suggests that the sites that interact with amiloride within the channel’s extracellular domain may be in close proximity to residues within the channel’s pore region. Supporting the notion that amiloride blocks the outer pore of ASIC and Deg/ENac include the voltage dependence of block, $k_{on}$ increases, and $k_{off}$ decreases linearly at hyperpolarizing membrane voltages, and, therefore, indicates amiloride senses 20% of the membrane electrical field (Alvarez de la Rosa et al., 2000). Amiloride affinity for binding the mouth of the pore also decreases by increasing extracellular sodium concentrations, indicating a competitive mechanism for the pore binding between amiloride and its permeable cation (Alvarez de la Rosa et al., 2000).

Studies with the venom of the South American tarantula *Psalmopoeus cambridgei* (psalmotoxin 1, PcTx1) have provided great insight about the role of ASIC1a during ischemia. PcTx1 is the first potent and specific blocker of ASIC1a (Escoubas et al., 2000; Pidoplichko and Dani, 2006; Diochot et al., 2007). It has been shown that PcTx1 blocks ASIC1a current in heterologous expression systems ($IC_{50}=0.9$ nM) at pH 6.0 and also at pH 4 or 5 (10 nM PcTX1). In subpopulations of dorsal root ganglia neurons, the toxin is also effective at nM concentrations (Escoubas et al., 2000) as well as in mice cortical neurons (Xiong et al., 2004). In whole animal studies, administration of the venom (500 ng/ml) following middle cerebral artery occlusion (MCAO) produced a significant reduction in infarct size and volume (Xiong et al., 2004). It has been suggested that the mechanism by which PcTx1 inhibits ASIC1a channels is via chronic
desensitization of the channel caused by increased affinity of the channel for protons (Chen et al., 2005). Interestingly, this PcTx1-induced shift of the pH-dependent inactivation of ASIC1a is Ca$^{2+}$-dependent, where increasing extracellular Ca$^{2+}$ results in a decrease of the PcTx1 inhibition.

Acidosis occurs within minutes of stroke onset (mainly affecting the core region but also the inner penumbral zone) (Back et al., 2000) and ASIC1a remain functionally active beyond 4 hrs following the ischemic insult (Pignataro et al., 2007). Additionally, pharmacological blockade of ASIC1a by amiloride or PcTx1 and administration of PcTx1 even 5 hrs after middle cerebral artery occlusion (MCAO) diminishes stroke injury (Xiong et al., 2004; Pignataro et al., 2007). These observations are consistent with studies showing the presence of lactate 72 hours following transient MCAO in rats (Weinstein et al., 2004). It remains to be elucidated whether endogenous receptors could regulate ASIC1a channel function.

ASIC1a Channels, Calcineurin and Scaffolding Proteins

Several extracellular and intracellular modulators of ASIC have been identified. It has been established that various divalent cations (Zn$^{2+}$, Pb$^{2+}$, Ca$^{2+}$) (Baron et al., 2001; Chu et al., 2004; Gao et al., 2004; Wang et al., 2006), lactate (Immke and McCleskey, 2001), serine proteases (Poirot et al., 2004) and redox reagents (Andrey et al., 2005; Chu et al., 2006) may interact with the extracellular domain of ASIC and influence the function of the channels. Other investigators have shown that there is a conserved phosphorylation site within the intracellular
C-terminal domain of ASIC1a for calcium/calmodulin protein kinase II (CaMKII) (Gao et al., 2005), protein kinase C (Baron et al., 2002) and protein kinase A (PKA) (Leonard et al., 2003) to bind. However, phosphorylation of ASIC1a has been shown to potentiate ASIC1a function in neurons (Xiong et al., 2004; Gao et al., 2005).

Calcineurin-dependent dephosphorylation of ASIC1a channels is the only modulator of ASIC1a that results in downregulation of channel function (Chai et al., 2007). Calcineurin, a second messenger, is a serine/threonine phosphatase that is activated by calcium-calmodulin, and a heterodimer consisting of 2 subunits, A and B (Shibasaki et al., 2002; Dodge and Scott, 2003). The calcineurin A subunit has 3 domains consisting of a catalytic domain, calmodulin-binding domain, and an autoinhibitory domain. The regulatory subunit, calcineurin B subunit, encodes the calcium-binding domain. The immunosuppressive drugs, cyclosporine A and FK-506, are well known calcineurin inhibitors. Cyclosporine A-cyclophilin A and FK-506-FKBP form drug-immunophilin complexes that bind to the calcineurin active site heterodimer to inhibit calcineurin activity competitively and thus, covering the catalytic groove (see review (Dumont, 2000; Shibasaki et al., 2002; Martinez-Martinez and Redondo, 2004)).

Calcineurin, also known as PPB2, may exist as free calcineurin in the cytosol but some is also found bound to scaffolding protein A-kinase anchoring protein (AKAP). AKAP is a diverse protein family with more than 50 members which are abundantly expressed in the brain (Feliciello et al., 2001). Neuronal
AKAP150 (rat) and AKAP79 (human) share a high degree of sequence homology, differing primarily in a 9 amino acid repeat sequence insert found only in rodents which has no known function (Dell'Acqua et al., 2006). AKAP150 anchors both kinases (cAMP-dependent Protein Kinase A and Protein Kinase C) and phosphotases (calcineurin) that are inhibited when bound. AKAP150 targets these proteins to specific subcellular sites through various targeting motifs (Dell'Acqua et al., 1998). AKAP150 has 2 domains: (1) a PKA-anchoring domain to interact with hydrophobic residues in the N-terminal of the RII dimmer, forming an amphipathic helix of residues, and (2) a targeting domain that anchors to the plasma membrane via phospholipids (phosphotidylinositol-4,5-biphosphate, PIP2) (see reviews (Dell'Acqua et al., 1998; Diviani and Scott, 2001)). Moreover, AKAP is regulated by calcium-calmodulin.

AKAP150 has been shown to be involved in the regulation of receptor activity, localization, and synaptic structure during synapse formation during development, synaptic plasticity in learning and memory, and neuronal dysfunction and cell death under pathophysiological conditions (Dell'Acqua et al., 2006). In addition to ASIC1a regulation, AKAP150 modulates internalization of AMPA and NMDA receptors during long term potentiation and depression (Rosenmund et al., 1994; Westphal et al., 1999; Colledge et al., 2000; Gomez et al., 2002; Smith et al., 2006) and voltage-gated Ca^{2+} channels function (Oliveria et al., 2007). All these proteins are possible constituents of the signaling cascade involving ASIC1a channels and σ receptors.
Sigma Receptors

Sigma receptors have been shown to regulate multiple ion channel function in neurons. This observation raises the possibility that σ receptors could modulate ASIC1a function. σ receptors were discovered by Martin et al. in 1976, and were thought to be an opioid receptor (Martin et al., 1976). Further studies proved that opioid receptors have a high affinity for the (-) enantiomer of benzomorphans while σ receptors prefer to bind the (+) enantiomer. Moreover, in vitro and in vivo studies suggested that the opioid antagonists, naloxone or naltrexone, do not affect σ receptor-mediated events (Iwamoto, 1981; Brady et al., 1982; Slifer and Balster, 1983; Vaupel, 1983; Katz et al., 1985). Thus, σ receptors were then classified as their own unique receptor type.

Two σ receptor subtypes have been identified on the basis of pharmacology. σ receptors have been shown to bind a range of substances including opiates, antipsychotics, antidepressants, phencyclidine (PCP)-related compounds, and neurosteroids (Walker et al., 1990; Bowen, 2000). σ-1 receptors have a higher affinity for the positive isomer of benzomorphans such as (+)-pentazocine and (+)-SKF-10,047, and the σ-2 receptors have high affinity for ibogaine and its congeners (Bowen, 2000; Vilner and Bowen, 2000). σ ligands like 1,3-di-o-tolyguanidine (DTG) and haloperidol have been shown to be pan-selective and bind to both σ receptor subtypes with high affinity. Evidence suggest that progesterone, neuropeptide Y, peptide YY, and zinc are possible endogenous σ ligands (Su et al., 1988; Roman et al., 1989; Connor and Chavkin, 1992).
To date, only the σ-1 receptor has been cloned (Hanner et al., 1996). The structure of the σ-1 receptor subtype is thought to consist of 2 transmembrane-spanning domains with internal C- and N-terminals (Aydar et al., 2002). While σ-1 receptors do not possess sequence homology to other mammalian proteins, it does have similar homology to fungal sterol isomerases (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1998). The cDNA of σ-1 receptors encodes a protein of 223 amino acids with a molecular weight of 25 kDa. In humans, the σ-1 receptor gene is located on chromosome 9p13, a region commonly associated with psychiatric disorders (Prasad et al., 1998). σ-1 receptors are found in the plasma membrane as well as in intracellular membranes like the endoplasmic reticulum (Hanner et al., 1996; Kekuda et al., 1996; Aydar et al., 2002).

In contrast to σ-1 receptors, the molecular identity of σ-2 receptors remains to be determined. Reports suggest that a splice variant of σ-1 receptors displays σ-2 receptor binding activities (Monassier and Bousquet, 2002). However, studies with σ-1 receptor knockout mouse showed that σ-1 and σ-2 receptors were not isoforms of the same protein because σ-2 effects were still seen in the absence of the σ-1 receptor subtype (Langa et al., 2003). Similar to σ-1 receptors, pharmacological experiments suggest that σ-2 receptors are located in the intracellular membrane of the endoplasmic reticulum and the mitochondria (Bowen, 2000).

σ receptors are ubiquitously expressed in the immune system, the kidney and liver, as well as throughout the central nervous system (Wolfe and De Souza, 1993; Hellewell et al., 1994). Microglia activation is one of the main
contributors of the immune response in the brain. Under physiological conditions, microglia respond to substances released by degenerating neurons by migrating to the site of injury, phagocytosing debris and releasing proinflammatory mediators (Streit et al., 2004). However, under pathological conditions, activated microglia are thought to be detrimental since these cells contribute to the demise of compromised neurons, and thus, exacerbate neurodegeneration (Streit et al., 2004). Therefore, the immune response plays a critical role in pathological conditions like stroke. Sigma receptor activation has been shown to inhibit multiple aspects of microglial activation in vitro, including the ability to rearrange the actin cytoskeleton, migrate, and release cytokine (Hall et al., 2008). Furthermore, in vivo studies also suggest that sigma receptor activation decreases reactive gliosis following stroke (Ajmo et al., 2006).

In the central nervous system, σ receptors are found in brainstem areas that regulate motor function, limbic structures, some predominantly sensory areas, and areas associated with endocrine function (della Puppa and London, 1989; Matsumoto et al., 1989; Matsumoto et al., 1990b; Walker et al., 1990; Elsinga et al., 2004). σ receptors have been implicated in numerous physiological and pathophysiological processes such as learning and memory (Senda et al., 1996; Maurice and Privat, 1997), movement disorders (Matsumoto et al., 1990a), and drug addiction (McCracken et al., 1999a; McCracken et al., 1999b). These receptors are emerging as therapeutic targets for various diseases such as neuropsychiatric disorders and cancer (Casellas et al., 2004; Hayashi and Su, 2004).
σ -2 receptors have been shown to regulate sodium and calcium channels. σ receptor activation blocks several types of calcium channels including N-, T-, P/Q- and R-type in neonatal intracardiac (parasympathetic neurons) and superior cervical ganglia (sympathetic neurons) neurons, and also inhibits voltage-gated sodium channels (Zhang and Cuevas, 2002; Katnik et al., 2006). Studies of mouse and rat hippocampal neurons have demonstrated that σ-2 ligands inhibit voltage-gated calcium channels while σ-1 selective agonists failed to show the same effect, indicating the inhibition is mediated via σ-2 receptors (Fletcher et al., 1995).

In contrast, σ-1 receptors regulate inositol 1,4,5-triphosphate (IP₃) receptors and calcium signaling at the endoplasmic reticulum, mobilization of cytoskeletal adaptor proteins, modulation of nerve growth factor-induced neurite sprouting, modulation of neurotransmitter release and neuronal firing, and modulation of potassium channels as regulatory subunits (see review, (Su and Hayashi, 2003)). σ -1 agonists are known to cause amplification of signal transduction incurred upon activation of the glutamatergic, dopaminergic, IP₃-related metabotropic, or nerve growth factor-related systems (Su and Hayashi, 2003). Immunohistochemistry studies have shown that σ-1 receptor, IP₃ type-3 receptor, and ankyrin B are colocalized in NG-108 cells in perinuclear areas and regions of cell-to-cell communication, suggesting that σ-1 ligands may play a role in cells by controlling the function of cytoskeleton proteins and regulating calcium signaling may represent a site of action for neurosteroids and cocaine (Hayashi and Su, 2001).
σ-1 receptors have been implicated in the regulation of various ion channels. σ-1 receptors are directly coupled to potassium channels in intracardiac neurons and activation of this receptor subtype depresses the excitability of these neurons, blocking parasympathetic input to the heart (Zhang and Cuevas, 2005). In cultured frog melanotrope cells, DTG and (+)-pentazocine have been shown to modulate electrical activity by reducing both a tonic K⁺ current and a voltage-dependent K⁺ conductance through activation of a cholera toxin-sensitive G protein (Soriani et al., 1998; Soriani et al., 1999). In Xenopus oocytes, the signal transduction cascade between Kv1.4 and σ receptors has been shown to be dependent on protein-protein interaction (Aydar et al., 2002). The σ ligand, (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3-PPP), has been shown to depolarize sympathetic neurons of the mouse isolated hypogastric ganglion by inhibiting the M-current, A-current and calcium-dependent K⁺ current (Kennedy and Henderson, 1990).

Activation of sigma receptors regulate multiple ion channels subtypes in neurons (Aydar et al., 2002; Zhang and Cuevas, 2002; Zhang and Cuevas, 2005). This regulation of ion channels may result in neuroprotection during ischemia. It has been suggested that the neuroprotective properties of σ ligands depend in part on their ability to depress elevations in [Ca²⁺], associated with glutamate receptor-mediated excitotoxicity (Klette et al., 1995; Klette et al., 1997; Katnik et al., 2006), neurotransmitter release regulation (Matsuno et al., 1993; Couture and Debonnel, 1998), and modulation of multiple ion channel subtypes. The studies of σ receptor modulation of glutamate evoked changes in
intracellular calcium have resulted in considerable controversy in the literature. There are conflicting reports as to whether σ receptor ligands exert their effects as a result of σ receptor activation (Hayashi et al., 1995) or through non-specific interaction with other targets, in particular, NMDA receptors (Kume et al., 2002). σ receptors and NMDA receptors both bind PCP and related compounds like MK-801 with high affinity (Sircar et al., 1987), and thus, such drugs cannot be used to discriminate between direct and indirect effects. Activation of σ receptors has been shown to be neuroprotective at delayed time points in a rat model of ischemic stroke (Ajmo et al., 2006) and blocks ischemia-induced increases in [Ca^{2+}]_i (Katnik et al., 2006). σ-1 receptor activation has also been shown to prevent neuronal death associated with glutamate excitotoxicity in cultured neurons (Takahashi et al., 1996; Kume et al., 2002), diminish infarct size by blocking nitric oxide production in vivo (Goyagi et al., 2001), inhibit pro-inflammatory factors (TNFα) and activate the expression of anti-inflammatory agents like IL-10 (Bourrie et al., 1995; Bourrie et al., 1996; Bourrie et al., 2002). Stimulation of σ receptors have been found to block voltage-gated calcium and potassium channels (Zhang and Cuevas, 2002; Zhang and Cuevas, 2005) in intracardiac neurons and ionotropic glutamate receptors (Monnet et al., 2003). All of these channel types are involved in the dysregulation of intracellular calcium homeostasis accompanying ischemia, and blocking the function of these channels provides neuroprotection (Schurr, 2004). Thus, σ receptor ligands have been targeted for the treatment of stroke due to the neuroprotective properties
observed in both in vivo and in vitro models of ischemia (Lockhart et al., 1995; Takahashi et al., 1996).

**In Vitro Chemical Ischemia Models**

The role of acidotoxicity, ASIC1a channels and acid-induced Ca\(^{2+}\) dysregulation during ischemia remains to be established. Furthermore, the effects of \(\sigma\) receptor activation on acidotoxicity remain to be determined. It is well known that in response to hypoxia-ischemia, an in vivo model of stroke, the brain relies on anaerobic glycolysis as the source of energy (Vannucci et al., 1994). This is energetically unfavorable and results in rapid depletion of ATP along with subsequent activation of deliterious biochemical cascades. All of these events are similar to those observed in cultured neurons (in vitro): (1) Ca\(^{2+}\) dysregulation due to glutamate accumulation and activation of NMDA receptors (Shalak and Perlman, 2004; Vannucci and Hagberg, 2004), (2) production of free oxygen radicals (Shalak and Perlman, 2004), (3) damage to proteins and lipids (Shalak and Perlman, 2004), (4) mitochondrial dysfunction leading to activation of apoptotic pathways (Northington et al., 2001), and (5) activation of secondary mechanisms including release of proinflammatory mediators (Silver and Miller, 2004; Chew et al., 2006; Fukui et al., 2006; Van Lint and Libert, 2007).

There are several models of in vitro chemical ischemia that are used to mimic the injury profile seen in animal models of stroke and conditions associated with cerebral ischemia in vivo. These models include: (1) oxygen glucose deprivation (Monyer et al., 1989; Laake et al., 1999) and the addition of
either (2) sodium azide (Dawson et al., 1991; Dawson et al., 2002; Katnik et al., 2006; Marino et al., 2007), (3) sodium cyanide (Vornov et al., 1994; Vornov, 1995; Zhang et al., 2000), or (4) 2,4-dinitrophenol (Riepe et al., 1996; Sullivan et al., 2003) in glucose-free solutions. These ischemic models are associated with Ca\(^{2+}\) overload triggered by the excess influx of extracellular Ca\(^{2+}\) through Ca\(^{2+}\)-sensitive ion channels and receptors (Monyer et al., 1989; Goldberg and Choi, 1993; Katnik et al., 2006). All of these models affect the electron transport chain in the mitochondria, which is the site of oxidative phosphorylation in eukaryotes. Oxidative phosphorylation is the main pathway that uses energy released by the oxidation of nutrients to drive the production of adenosine triphosphate (ATP), which supplies energy for metabolism. Inhibition of enzymes, like cytochrome c, during this metabolic process results in energy failure due to the lack of ATP production.

Sodium-azide and sodium-cyanide are both compounds that inhibit oxidative phosphorylation. Azide and cyanide bind to the iron-copper center (when the iron is in the ferric state) of cytochrome c with greater affinity than oxygen. This prevents the reduction of oxygen and stops the electron transfer preventing translocation of protons across the membrane, which disrupts the electrochemical gradient that powers ATP synthase. Therefore, these compounds are inhibitors of the enzyme cytochrome c oxidase in the fourth complex of the electron transport chain and both prevent the aerobic production of ATP as the source of cellular energy. However, the main disadvantage of cyanide is that it is a very toxic and lethal compound to humans since cyanide
interacts with pH and produces hydrogen cyanide, thus making difficult to control its concentration. Thus, azide is a safer compound to handle. In contrast to azide and cyanide, 2,4-dinitrophenol (DNP) uncouples the electron transport from oxidative phosphorylation, which disrupts the proton gradient by transporting protons back across the inner mitochondrial membrane. There are several disadvantages to using oxygen depletion: (1) pre and early post natal rat tissue is resistant to oxygen depletion, (2) difficult to completely remove oxygen and thus requires long incubation to produce significant injury (Rothman, 1984), and (3) requires using a closed-chamber, making patch-clamp experiments difficult. The major advantage of the sodium azide/glucose deprivation model over the oxygen/glucose deprivation model is that it elicits neurochemical responses that are significantly more rapid and robust, thus facilitating the recording of changes in [Ca^{2+}]i increases (Katnik et al., 2006). Disadvantages of this model are that azide has multiple affects including inhibition of cytochrome aa3, superoxide dismutase and DNA synthesis (Varming et al., 1996). Azide breaks down to nitric oxide and thus enhances excitatory neurotransmission (Varming et al., 1996). Moreover, because of the short duration of the application of azide, this only models acute ischemia. This azide/glucose deprivation model has previously been used in rat cortical neurons to study σ receptor inhibition of ischemia-induced [Ca^{2+}]i elevations (Katnik et al., 2006). Moreover, calcium influx through the plasma membrane and not release from the mitochondria or endoplasmic reticulum, accounts for most of the ischemia-mediated [Ca^{2+}]i increases in cortical neurons using this azide model of ischemia (Katnik et al., 2006).
CHAPTER 2

SIGMA-1 RECEPTOR MODULATION OF ASIC1a CHANNELS AND ASIC1a-INDUCED Ca\textsuperscript{2+} INFLUX IN RAT CORTICAL NEURONS

Introduction

Acid-sensing ion channels are a class of ligand-gated channels that are members of the degenerin/epithelial sodium channel superfamily and are expressed in both peripheral and central nervous system neurons (Waldmann et al., 1997b). Thus far, four genes (ASIC1 – ASIC4) and two splice variants of ASIC1 and ASIC2 (a and b) have been cloned (Wemmie et al., 2006) which encode protein subunits that form functional proton-gated homomultimeric or heteromultimeric channels (Wemmie et al., 2006). The pH of half-maximal activation and the tissue expression patterns differ between each channel subtype.

One of the most common ASIC subtypes in the central nervous system (CNS) contains the ASIC1a subunit, which can form homomultimeric or heteromultimeric channels with ASIC2a (Askwith et al., 2004). These channels are activated by \( \text{pH} \leq 7 \) and have a pH of half-maximal activation of \( \sim 6.0 - 6.5 \) (Waldmann et al., 1997b; Hesselager et al., 2004). ASIC1a homomultimeric channels differ from other ASIC subtypes in that they are highly permeable to both Na\textsuperscript{+} and Ca\textsuperscript{2+} ions (Waldmann et al., 1997b; Yermolaieva et al., 2004). This
ASIC subtype has been implicated in a number of physiological processes such as synaptic plasticity, fear conditioning, and learning and memory (Wemmie et al., 2002; Wemmie et al., 2003; Wemmie et al., 2004). ASIC1a has also been shown to be activated following cerebral ischemia, and has been unequivocally linked to neuronal cell death (Xiong et al., 2004; Gao et al., 2005; Pignataro et al., 2007). Transgenic mice deficient in ASIC1a have reduced infarct size in response to middle cerebral artery occlusion (MCAO) relative to wild-type mice (Xiong et al., 2004). Moreover, pharmacological inhibition of ASIC1a with either amiloride or psalmotoxin1, which is selective for homomultimeric ASIC1a channels (Diochot et al., 2007), diminishes ischemic brain injury (Xiong et al., 2004). Several studies have suggested that Ca\(^{2+}\) influx through these channels is a key mechanism leading to neurodegeneration (Xiong et al., 2004; Yermolaieva et al., 2004).

Despite efforts to determine the function of ASIC1a and the role of these channels in ischemia, little is known about endogenous mechanisms which control ASIC1a activity. Thus far, only the NMDA receptor, acting via a Ca\(^{2+}\)-calmodulin kinase II cascade (CaMKII), has been shown to modulate ASIC1a (Gao et al., 2005). Activation of NMDA receptors enhances ASIC1a-mediated currents, which consequently exacerbates acidotoxicity during ischemia (Gao et al., 2005).

σ receptor activation has been shown to modulate multiple cell membrane ion channels in neurons. σ-1 receptors regulate ionotropic glutamate receptors and voltage-gated K\(^+\) channels, whereas σ-2 receptors modulate voltage-gated
Ca\(^{2+}\) channels (Hayashi et al., 1995; Aydar et al., 2002; Zhang and Cuevas, 2002; Zhang and Cuevas, 2005). The inhibition of ionotropic glutamatergic receptors by \(\sigma\) receptors prevents elevations in \([Ca^{2+}]_i\), associated with glutamate-induced excitotoxicity (Klette et al., 1995). All of these voltage-gated ion channels and NMDA receptors have been shown to contribute to the demise of neurons during an ischemic insult. Our laboratory has recently shown that \(\sigma\)-1 receptors inhibit Ca\(^{2+}\) dysregulation evoked by ischemia and that activation of \(\sigma\) receptors is neuroprotective at delayed time points in a rat model of ischemic stroke (Ajmo et al., 2006; Katnik et al., 2006). Interstitial pH in the brain remains low several hours after an ischemic event (Nedergaard et al., 1991), and pharmacological blockade of ASIC1a by amiloride or psalmotoxin1 administered even 5 hours after MCAO has been shown to diminish stroke injury (Simon, 2006). These observations raise the possibility that \(\sigma\) receptors may regulate ASIC1a function and ASIC1a-induced intracellular calcium transients, and provide neuroprotection when stimulated at delayed time points after an ischemic insult.

Experiments were conducted to determine the effects of \(\sigma\) receptors on ASIC-mediated membrane currents and transient \([Ca^{2+}]_i\) elevations. It was determined that \(\sigma\) receptor agonists inhibit acidosis-induced increases in \([Ca^{2+}]_i\) and peak membrane currents in cells expressing homomeric ASIC1a channels. Pharmacological studies demonstrated that the \(\sigma\)-1 receptor subtype was responsible for these effects. Moreover, acidosis was also shown to activate downstream Ca\(^{2+}\) influx pathways (e.g. NMDA and AMPA/kainate receptors and voltage-gated Ca\(^{2+}\) channels, VGCC), and activation of \(\sigma\)-1 receptors also
diminished Ca²⁺ entry via these channels. Therefore, σ-1 receptors couple to ASIC1a channels to inhibit channel function and also block ASIC1a-induced [Ca²⁺]i dysregulation. Our findings suggest that σ-1 receptors represent potential targets for improving outcome of stroke injury and expanding the therapeutic window for ischemic stroke treatment.

**Materials and Methods**

*Primary Rat Cortical Neuron Preparation*

Primary cortical neurons from embryonic (E18) rats were cultured as previously described by our laboratory (Katnik et al., 2006). All procedures were done in accordance with the regulations of the University of South Florida Institutional Animal Care and Use Committee. Briefly, dams were sacrificed, uterus removed, and embryos dissected out and placed in isotonic buffer consisting of (in mM): 137 NaCl, 5 KCl, 0.2 NaH₂PO₄, 0.2 KH₂PO₄, 5.5 glucose, and 6.0 sucrose, titrated to pH 7.4 with NaOH. Cortices were excised and minced, followed by digestion in isotonic buffer containing 0.25% trypsin/EDTA for 10 minutes at 37°C and added to 3X volume of Dulbecco’s modified Eagle’s Medium (DMEM) (Invitrogen Inc, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were counted using a hemocytometer, plated (0.5 X 10⁶ cells/well) on 18-mm pre-treated poly-L-lysine coverslips, and incubated at 37°C under 95% air, 5% CO₂ atmosphere. After 24 hours, the media was replaced with Neurobasal medium supplemented with B27 and 0.5 mM L-glutamine (Invitrogen Inc, Carlsbad, CA)
to minimize astrocyte proliferation in the cultures. Cells were used between 10-21
days in culture.

*Calcium Imaging Measurements*

The effects of acidosis on intracellular Ca\(^{2+}\) concentrations were examined
in isolated cultured cortical neurons using fluorescent imaging techniques.
Cytosolic free-Ca\(^{2+}\) was measured using the Ca\(^{2+}\) sensitive dye, fura-2, as
previously described (Katnik et al., 2006). Cells, plated on poly-L-lysine coated
coverslips, were incubated for 1 hour at room temperature in Neurobasal
(Invitrogen) medium supplemented with B27 (Invitrogen) and 0.5 mM L-
glutamine, or in physiological saline solution (PSS) consisting of (in mM): 140
NaCl, 5.4 KCl, 1.3 CaCl\(_2\), 1.0 MgCl\(_2\), 20 glucose, and 25 HEPES (pH to 7.4 with
NaOH), 330 ± 10 mOsm. Both solutions contained 3 μM acetoxymethyl ester
fura-2 and 0.3 % dimethyl sulfoxide. The coverslips were washed in PSS (fura-2
free) prior to the experiments being carried out. All solutions were applied via a
rapid application system identical to that previously described (Cuevas and Berg,
1998).

A DG-4 high-speed wavelength switcher (Sutter Instruments Co., Novato,
CA) was used to apply alternating excitation light. Fluorescent emission was
captured using a Sensicam digital CCD camera (Cooke Corporation, Auburn
Hills, MI) and recorded with Slidebook 4.02 software (Intelligent Imaging
Innovations, Denver, CO). Slidebook 4.02 software was used to calculate
changes in [Ca\(^{2+}\)]\(_i\) from the intensity of the emitted fluorescence following
excitation with 340 nm and 380 nm light, respectively, using the Grynkiewicz
equation: $[\text{Ca}^{2+}]_i = K_d * \frac{Q (R - R_{\text{min}})}{(R_{\text{max}} - R)}$, where $R$ represents the fluorescence intensity ratio (F340/F380) as determined during experiments, $Q$ is the ratio of $F_{\text{min}}$ to $F_{\text{max}}$ at 380 nm, and $K_d$ is the $\text{Ca}^{2+}$ dissociation constant for fura-2 (224 μM). The system was calibrated using a Fura-2 Calcium Imaging Calibration Kit (Molecular Probes; Eugene, OR) and values for $F_{\text{min}}/F_{\text{max}}$, $R_{\text{min}}$, and $R_{\text{max}}$ were determined.

**Electrophysiology Recordings**

Neurons plated on glass coverslips (as described above) were transferred to a recording chamber mounted on a Zeiss Axiovert 200 and visualized at 400x. Membrane currents were amplified using an Axopatch 200B (Axon Instruments), filtered at 1 kHz, digitized at 5 kHz with a Digidata 1322A (Axon), and acquired using Clampex 8 (Axon). Electrical access was achieved using the amphotericin B perforated-patch method to preserve intracellular integrity of neurons (Rae et al., 1991). An amphotericin B stock solution (60 mg/ml in DMSO) was made daily, kept on ice, light protected, and diluted to 240 μg/ml (0.4% DMSO) in control pipette solution immediately prior to patch clamp experiments. The pipette solution consisted of (in mM): 75 K$_2$SO$_4$, 55 KCl, 5 MgSO$_4$, and 25 HEPES (titrated to pH 7.2 with N-methyl-d-glucamine, 300 ± 5 mOsm). Patch electrodes were pulled from thin-walled borosilicate glass (World Precision Instruments Inc., Sarasota, FL) using a Sutter Instruments P-87 pipette puller (Novato, CA) and had resistances of 1.0–1.5 MΩ. Access resistance ($R_a$) were monitored throughout experiments for stable values ≤ 20 MΩ and were always compensated at 40% (lag, 10 μs). All cells were voltage-clamped at -70 mV.
**Solutions and Reagents**

The control bath solution for all experiments was PSS. In one series of experiments requiring high extracellular K⁺, an additional 34.6 mM of KCl was isosmotically substituted for NaCl. All drugs were applied in PSS (or high K⁺ PSS) using a rapid application system identical to that previously described (Cuevas and Berg, 1998). ASIC activation was induced by applying PSS with a pH of 6.0 (+/- drug) to specifically target ASIC1a (Askwith et al., 2004). Individual cells were exposed to no more than four low pH applications, and no rundown of the responses was observed with this protocol. All chemicals used in this investigation were of analytic grade. The following drugs were used: DTG, opipramol, ibogaine, metaphit, nifedipine, AP5 and PB28 (Sigma-Aldrich, St. Louis, MO); carbetapentane, BD1063, CNQX and PRE-084 (Tocris Bioscience, Ellisville, MO); dextromethorphan (MP Biomedicals, Inc, Solon, OH); psalmotoxin1 (Spider Pharm, Yarnelle, AZ); tetrodotoxin and thapsigargin (Alomone Labs, Jerusalem, Israel); amiloride (Alexis Biochemicals, Lausen, Switzerland); cadmium (Fischer Scientific, Fair Lawn, NJ); and fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR).

**Data Analysis**

Analyses of measured intracellular Ca²⁺ and membrane current responses were conducted using Clampfit 9 (Axon instruments). Fluorescence intensities were recorded from fura-2-loaded neuronal cell bodies. Time-lapse imaging data files collected with SlideBook 4.02 (Intelligent Imaging Innovations, Inc.) were converted to a text format and imported into Clampfit for subsequent analysis. Statistical
analysis was conducted using SigmaPlot 9 and SigmaStat 3 software (Systat Software, Inc.). Statistical differences were determined using paired and unpaired t-tests for within group and between group experiments, respectively, and were considered significant if \( p < 0.05 \). For multiple group comparisons either a 1-way or a 2-way ANOVA, with or without repeat measures, were used, as appropriate. When significant differences were determined with an ANOVA, post-hoc analysis was conducted using a Tukey Test to determine differences between individual groups. For the generation of concentration response curves, data were best fit using a single-site Langmuir-Hill equation.

**Results**

ASIC subtypes are distinguishable by pH sensitivity and ion selectivity, with homomultimeric ASIC1a being the only subtype that is Ca\(^{2+}\) permeable (Yermolaieva et al., 2004). Experiments were carried out to determine the effects of ASIC activation on intracellular Ca\(^{2+}\) transients and to identify the specific ASIC subtype(s) affecting [Ca\(^{2+}\)], in our rat cortical neuron model. Figure 2.1A shows representative traces of [Ca\(^{2+}\)], as a function of time recorded from a single neuron during acidosis (pH 6.0) in the absence (Control) and presence of amiloride (100 µM), and following a 10 minute washout of drug (Wash). The general ASIC inhibitor, amiloride, reversibly blocked ASIC-mediated increases in [Ca\(^{2+}\)], by 88 ± 1 % (Figure 2.1B). Psalmotoxin1 (PcTx1) from the venom of the tarantula *Psalmopoeus cambridgei* has been shown to be a selective blocker of homomultimeric ASIC1a channels (Diochot et al., 2007). Figure 2.1C shows
representative traces of \([\text{Ca}^{2+}]_i\) as a function of time recorded from a neuron prior to (Control), following a 10 - 20 min preincubation in bath applied PcTx1 (500 ng/ml venom protein), and after washout of the toxin (Wash). In identical experiments, PcTx1 produced a statistically significant reversible decrease in ASIC1a-mediated elevations in \([\text{Ca}^{2+}]_i\) (57 ± 2 %) (Figure 2.1D). The effects produced by this concentration of PcTx1 are consistent with results obtained for ASIC1a responses in mouse cortical neurons (Xiong et al., 2004). These data indicate that, in cultured cortical neurons from embryonic rats, acidosis results in elevations in \([\text{Ca}^{2+}]_i\) that are mediated via the activation of homomultimeric ASIC1a channels, as reported for cultured cortical neurons from embryonic mice (Xiong et al., 2004).

Activation of \(\sigma\) receptors has been shown to inhibit numerous plasma membrane ion channels in neurons (Hayashi et al., 1995; Zhang and Cuevas, 2002; Zhang and Cuevas, 2005). Therefore, experiments were carried out to study the effects of \(\sigma\) receptor activation on ASIC1a function using the pan-selective \(\sigma\) receptor agonist, DTG. Figure 2.2A shows representative traces of \([\text{Ca}^{2+}]_i\) recorded from a single neuron during acidosis in the absence (Control) and presence of 100 \(\mu\)M DTG, a concentration previously shown to effectively regulate other ion channels (Zhang and Cuevas, 2002; Zhang and Cuevas, 2005), and following 10 minute washout of the drug (Wash). DTG rapidly and reversibly inhibited the low pH-induced transient increases in \([\text{Ca}^{2+}]_i\). In identical experiments, 100 \(\mu\)M DTG produced a statistically significant decrease (46 ± 3 %) in ASIC1a-mediated elevations in \([\text{Ca}^{2+}]_i\) (Figure 2.2B). Opipramol (10 \(\mu\)M),
another pan-selective $\sigma$-1/$\sigma$-2 agonist, also inhibited ASIC1a-mediated increases in $[\text{Ca}^{2+}]_i$ by 57 ± 1 % (Figure 2.2B). The concentration-response relationship for DTG inhibition of ASIC1a was determined to confirm that the actions of DTG on ASIC1a are consistent with $\sigma$ receptor activation. Increasing DTG concentrations resulted in further depression of ASIC1a-mediated increases in $[\text{Ca}^{2+}]_i$ (Figure 2.2C). A plot of the concentration-response relationship obtained from measurements made in multiple cells is shown in Figure 2.2D. The data were best fit using a single-site Langmuir-Hill equation with an IC$_{50}$ value of 109 µM and a Hill coefficient of 0.9. These values are consistent with the effects of DTG being mediated via activation of $\sigma$ receptors (Zhang and Cuevas, 2002; Zhang and Cuevas, 2005; Katnik et al., 2006). Taken together these results suggest that $\sigma$ receptors depress acid-induced increases in $[\text{Ca}^{2+}]_i$ and modulate ASIC1a function.

$\sigma$ receptor subtype-selective agonists were used to identify the specific $\sigma$ receptor subtype(s) mediating these effects. Representative traces of $[\text{Ca}^{2+}]_i$ as a function of time recorded from two cells during acidosis in the absence (Control) and presence of the $\sigma$-1 selective agonists carbetapentane (CBP) and dextromethorphan (DEX) at the indicated concentrations are shown in Figures 2.3A and 2.3B, respectively. $\sigma$-1 selective agonists blocked the low pH-induced elevations in $[\text{Ca}^{2+}]_i$ in a concentration dependent and reversible manner. Concentration-response plots for mean changes in peak $[\text{Ca}^{2+}]_i$ recorded in identical experiments using the $\sigma$-1 selective ligands CBP, DEX, and PRE-084, are shown in Figure 2.3C. The data were best fit using the Langmuir-Hill equation.
and values obtained for IC$_{50}$ and Hill coefficients were 13.8 μM and 0.7 (CBP), 22 μM and 0.8 (DEX), and 13.7 μM and 0.6 (PRE-084), respectively. Presented for comparison is the best fit to the data obtained for CBP inhibition, via σ-1 receptors, of chemical ischemia-induced increases in [Ca$^{2+}$]$_i$ in cortical neurons (dotted line, IC$_{50}$ = 18.7 μM; Hill coefficient, 0.8) (Katnik et al., 2006). This curve superimposes on the responses to CBP observed in the current study.

To provide further evidence that σ-1 receptors modulate ASIC1a-induced Ca$^{2+}$ elevations experiments were conducted using the irreversible σ antagonist, metaphit, and a selective σ-1 antagonist, BD1063, in combination with the σ-1 agonist, CBP. Cells were exposed to acidosis in the absence and presence of CBP (30 μM), with or without preincubation in metaphit (50 μM; 30 min -1hr, 23°C). CBP decreased the acid-induced elevations in [Ca$^{2+}$]$_i$ in control cells, and this inhibition was lessened by preincubation with metaphit (Figure 2.4A). Figure 2.4B shows results from several similar experiments determining percent inhibition of acid-induced increases in [Ca$^{2+}$]$_i$ observed in the presence of CBP in control neurons (Control) and neurons preincubated with metaphit (MET).

Whereas CBP decreased the elevations in [Ca$^{2+}$]$_i$ evoked by acidosis in control cells by 52 ± 2 %, the σ-1 receptor agonist only reduced the response by 30 ± 3 % in cells preincubated in the irreversible σ receptor antagonist. This >40% decrease in the effects of CBP following metaphit preincubation was statistically significant (p < 0.001). The selective σ-1 antagonist, BD1063, showed more pronounced effects. Figure 2.4C shows representative traces of [Ca$^{2+}$]$_i$ as a function of time recorded from two neurons during acidosis in absence (Control)
and presence of CBP (30 µM), without (PSS) and with co-application in BD1063 (BD1063, 10 nM). In identical experiments, the σ agonist CBP completely loses its ability to block the low pH-induced elevations in [Ca^{2+}] in the presence of the σ antagonist BD1063 when compared to 40% block in control cells (Figure 2.4D). Inhibition of σ-1 receptors by BD1063 significantly blocked the effects of CBP on ASIC1a-mediated [Ca^{2+}] elevations when compared to control cells (p <0.001). These data confirm the effects of σ-1 agonists on ASIC1a mediated increases in [Ca^{2+}], are the result of these compounds acting on σ-1 receptors.

Further experiments were carried out using the σ-2 selective ligands ibogaine and PB28 to determine if the σ-2 receptor subtype also affects ASIC1a function. Figure 2.5A shows representative traces of [Ca^{2+}] as a function of time recorded from two cells during acidosis in the absence (Control) and presence of ibogaine (IBO, left traces) and PB28 (right traces) at the indicated concentrations. The σ-2 ligands inhibited acidosis-evoked increases in mean peak changes in [Ca^{2+}], in a concentration dependent manner (Figure 2.5B). Best fits to the data demonstrated that application of ibogaine and PB28 resulted in inhibition of ASIC1a-mediated increases in [Ca^{2+}], with IC_{50} values of 69 µM and 11 µM, and Hill coefficients of 0.86 and 0.85, respectively. For comparison, the best fit to the data obtained for ibogaine inhibition of I_{Ca}-induced increases in [Ca^{2+}] is presented, which has been shown to be mediated by σ-2 receptors (dashed line; IC_{50} = 31 µM; Hill coefficient, 1.1) (Zhang and Cuevas, 2002). Unlike the similar concentration-response relationship observed for CBP inhibition of ASIC1a and ischemia responses, there is a discrepancy between the
ibogaine block of responses mediated by ASIC1a and by voltage-gated Ca\(^{2+}\) channels (VGCC). To determine if the effects of these σ-2 ligands were mediated by activation of σ-2 receptors, experiments using metaphit were carried out.

PB28 (20 µM) produced an inhibition of ASIC1a-mediated increases in [Ca\(^{2+}\)]\(_i\) in both control (PSS) and metaphit (+MET) treated cells (Figure 2.5C). PB28 blocked ASIC1a-mediated increases in [Ca\(^{2+}\)]\(_i\) by 67 ± 1% and 60 ± 2 % in the absence and presence of metaphit preincubation, respectively (Figure 2.5D). The ~10% reduction in the effects of PB28 produced by metaphit was not statistically significant (p = 0.64). Furthermore, BD1063 (10 nM) failed to block the effects of PB28 (20 µM) on ASIC1a-induced elevations in [Ca\(^{2+}\)]\(_i\) (Figure 2.5D). These results demonstrate that the effects of PB28 on ASIC1a-mediated increases in [Ca\(^{2+}\)]\(_i\) are not mediated by σ-2 receptors since the inhibition of these responses to acidosis by PB-28 is metaphit-insensitive and occurs at concentrations inconsistent with σ-2 receptor activation.

The activation of σ receptors has previously been shown to directly affect Ca\(^{2+}\) release from intracellular stores (Cassano et al., 2006). Thus, experiments were conducted to resolve if σ receptor activation reduces acid-induced increases in [Ca\(^{2+}\)]\(_i\) in part via the inhibition of calcium-induced-calcium release from the endoplasmic reticulum, triggered by Ca\(^{2+}\) influx through the plasma membrane. For these experiments, thapsigargin was used to block the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase, which results in depletion of both ryanodine- and IP\(_3\)-sensitive stores. Figure 2.6A shows representative traces of [Ca\(^{2+}\)]\(_i\) as a function of time recorded from a neuron during acidosis in
the absence (Control) and presence of 100 µM DTG (DTG), while Figure 2.6B shows traces in the absence and presence of DTG following pre-incubation (1 hr, 23°C) in 10 µM thapsigargin (THAP and THAP + DTG, respectively).

Thapsigargin alone did not decrease the elevations in [Ca²⁺], produced by acidosis, and DTG depressed the increases in [Ca²⁺], under both conditions (± thapsigargin preincubation). Analysis of the data collected in identical experiments indicates that preincubation with thapsigargin does not significantly alter the effects of DTG on acid-mediated increases in [Ca²⁺], (Figure 2.6C).

Thus, DTG does not decrease the low pH-induced elevations in [Ca²⁺] by affecting release of calcium from intracellular stores. The fact that depletion of intracellular stores fails to depress increases in [Ca²⁺] evoked by acidosis suggests that Ca²⁺ influx through the plasma membrane accounts for most, if not all, of the increases in [Ca²⁺] evoked by ASIC1a activation.

Simultaneous Ca²⁺ fluorometry and whole-cell patch clamp recordings were performed to study ASIC1a-mediated membrane currents and to determine how much of the observed Ca²⁺ influx is due to Ca²⁺ entry through ASIC1a channels. Cells were voltage-clamped at -70 mV to minimize NMDA receptor and VGCC activation, and thus, isolate ASIC1a currents. Figure 2.7A shows that ASIC1a stimulation by low pH solution (pH 6.0), resulted in a small intracellular Ca²⁺ transient measured in a patched cell ([Ca²⁺] V-Clamp). In contrast, a second cell ([Ca²⁺] Control) in the same field of view, which was not voltage clamped, had a significantly larger increase in [Ca²⁺]. Acidosis also resulted in a large inward current in the patched cell (Figure 2.7A, inset). The cumulative
results from several similar experiments demonstrate that ASIC1a activation of neurons voltage clamped at -70 mV resulted in elevations in [Ca^{2+}], which were an order of magnitude smaller than changes evoked in cells that were not voltage-clamped (Figure 2.7B). These data confirm ASIC1a activation results in minimal Ca^{2+} influx through the ASIC1a channel itself and that the majority of the acid-induced [Ca^{2+}], increases are mediated by downstream Ca^{2+} influx pathways.

To test the possibility that ASIC1a channels promote cell depolarization, cortical neurons were held under current-clamp mode. Figures 2.8A and 2.8C show representative traces of membrane potential as a function of time recorded from a single cell in absence (Control) and presence of amiloride (100 µM) and from a different neuron in the absence (Control) and presence of PcTx1 peptide (50 nM). A summary of the changes in membrane potential recorded from several cells in the absence (Control) and presence of ASIC blockers, amiloride and PcTx1 peptide, are shown in Figures 2.8C and 2.8D. Inhibition of ASIC1a channels by amiloride, but not PcTx1 peptide, produced a significant reduction in changes in membrane potential (Figure 2.8C). Thus, these data suggest that activation of ASIC1a channels depolarizes cortical neurons to potentials capable of activating other Ca^{2+} channels.

Application of protons evoked a rapid depolarization of neurons held under current-clamp mode which, unlike the case in voltage-clamped neurons, was associated with pronounced elevations in [Ca^{2+}]. Thus, ASIC1a channels are likely promoting significant Ca^{2+} influx into the neurons by depolarizing the cells.
To mimic this change in membrane potential evoked upon ASIC1a activation, cells were exposed to high K⁺ (40 mM) extracellular solution. Figure 2.9A shows representative [Ca²⁺]ᵢ traces recorded in response to high K⁺₀ application in the absence (Control) and presence of CBP (100 μM, +CBP). Depolarizing the neurons in this manner evoked robust [Ca²⁺]ᵢ elevations which were blocked by addition of CBP. In identical experiments CBP reduced the high K⁺₀-evoked increases in [Ca²⁺]ᵢ by 83 ± 1% and this decrease was statistically significant (p < 0.001) (Figure 2.9B). Thus, σ receptor activation inhibits Ca²⁺ channels downstream of ASIC1a in addition to the proton-gated channels themselves.

To determine the specific ion channels contributing to the ASIC1a-induced [Ca²⁺]ᵢ influxes, several inhibitors of plasma membrane ion channels were used. Activation of ASIC1a depolarizes these neurons which could stimulate action potential firing and, consequently, synaptic transmission, both of which may elevate [Ca²⁺]ᵢ. Thus, tetrodotoxin (TTX, 500 nM) was used to inhibit voltage-gated Na⁺ channels to prevent the genesis of action potentials. Figure 2.10A shows that application of TTX inhibited ~ 10% of the acid-induced increases in [Ca²⁺]ᵢ, but did not significantly affect CBP (50 µM) modulation of the Ca²⁺ responses, when compared to the PSS group. Because TTX had no effect on the σ-1/ASIC1a interaction, all subsequent experiments included 500 nM TTX in the bath solutions to prevent spontaneous action potentials from contributing to the measured increases in [Ca²⁺]ᵢ. Inhibition of NMDA receptors by AP5 (100 µM) significantly reduced the acid-induced elevations in [Ca²⁺]ᵢ, and co-application with CBP (50 µM) resulted in a further decrease in [Ca²⁺]ᵢ, which was statistically
significant (Figure 2.10A). These results show that ~ 30% of ASIC1a-induced 
$[\text{Ca}^{2+}]_i$ increases are dependent on NMDA receptor activation. Furthermore,
since the effects of AP5 and CBP are less than additive, activation of $\sigma$-1 receptors depresses ASIC1a-evoked increases in $[\text{Ca}^{2+}]_i$ in part by blocking this NMDA receptor-dependent component. Blockade of L-type VGCC by nifedipine (10 µM) significantly inhibited (>75%) acid-induced $[\text{Ca}^{2+}]_i$ elevations, and CBP (50 µM) continued to provide further blockade of the residual increases in $[\text{Ca}^{2+}]_i$ (Figure 2.10A). Cadmium (100 µM), a broad-spectrum blocker of plasma membrane calcium channels, inhibited ~ 90% of the acid-induced increases in $[\text{Ca}^{2+}]_i$, and CBP (50 µM) had no effect on the remaining $\text{Ca}^{2+}$ influx (Figure 2.10A). Thus, voltage-gated $\text{Ca}^{2+}$ channels either directly (influx through the channel) or indirectly (facilitating glutamate release) account for most of the $[\text{Ca}^{2+}]_i$ increases resulting from ASIC1a activation, and $\sigma$-1 receptor activation provides an inhibition similar to that observed with $\text{Cd}^{2+}$.

Further experiments were conducted to determine if AMPA/kainate receptors are involved in the $[\text{Ca}^{2+}]_i$ elevations elicited upon ASIC1a activation. Figure 2.10B shows relative changes in $[\text{Ca}^{2+}]_i$ in the absence (Control) and presence of the AMPA/kainate receptor blocker CNQX (10 µM), and the effects of CBP at two different concentrations (50 µM and 300 µM). Maximal blockade of AMPA/kainate receptors alone produced ~ 40% reduction of the elevations of $[\text{Ca}^{2+}]_i$, and 50 µM CBP did not provide additional, statistically significant block. Increasing the CBP concentration to 300 µM, however, did produce an additional block of the $\text{Ca}^{2+}$ response on top of the effects of CNQX. Figure 2.10B shows
that co-application of 300 µM CBP and CNQX blocked >80% of the acid-induced increases in [Ca$^{2+}$], and this was statistically significant when compared to CNQX alone. Thus, [Ca$^{2+}$] elevations elicited upon ASIC1a activation involve Ca$^{2+}$ influx through both ASIC1a and other Ca$^{2+}$ permeable plasma membrane ion channels expressed in cortical neurons. Importantly, it is also evident that activation of σ-1 receptors results in depression of Ca$^{2+}$ influx through all of these sources, either by inhibiting ASIC1a or the downstream channels themselves.

Two ASIC blockers, amiloride and PcTx1, were used to confirm that the acid-activated inward currents observed in our cortical neuron model were mediated by ASIC1 channels. Figure 2.11A shows representative traces of membrane currents as a function of time recorded from a single cell in absence (Control) and presence of amiloride (100 µM, top traces, i), and from a different neuron in the absence (Control) and presence of PcTx1 (500 ng/ml venom, bottom traces, ii). Figure 2.11B summarizes the normalized peak proton-gated whole-cell currents recorded from several cells in the absence (Control) and presence of amiloride and PcTx1. Amiloride produced a 79 ± 6 % inhibition of acid-activated currents while 500 ng/ml of PcTx1 containing venom produced a 42 ± 11 % reduction in this current. Both reductions were significantly different from control (p<0.01). Experiments were also carried out to confirm that ASIC1a currents were not affected by the channel blockers used in the imaging studies described above. Figure 2.11C shows relative peak proton-gated currents, normalized to control (PSS), during acidosis in the absence (PSS) and presence of the indicated drugs. These data demonstrate that neither TTX, AP5, CNQX,
nifedipine, nor cadmium have any direct effects on ASIC1a channels.

σ receptor activation has been shown to modulate multiple cell membrane ion channels in neurons that are activated following ASIC1a stimulation (Hayashi et al., 1995; Zhang and Cuevas, 2002). This fact raises the possibility that σ-1 receptors only couple to the secondary events that result from ASIC1a activation and not to ASIC1a itself. To investigate if σ receptors affect ASIC1a function, whole-cell patch clamp recordings were performed in the presence of σ agonists. Figure 2.12A shows representative membrane current traces as a function of time recorded from a cell in the absence (Control) and presence of the pan-selective σ agonist DTG (100 µM). Results from several similar experiments show that activation of σ receptors with the DTG inhibited ASIC1a-mediated currents by 53 ± 9 %, and this decrease was statistically significant manner (Figure 2.12B). To determine if the effects of DTG on ASIC1a currents were mediated by σ-1 or σ-2 receptors, the effects of the σ-1 selective agonist, CBP, on whole-cell currents were measured. Figure 2.12C shows representative membrane current traces as a function of time recorded in the absence (Control) and presence of CBP (50 µM). After multiple experiments, analysis of the measured current densities showed that control cells had statistically significant larger current densities than CBP treated cells (Figure 2.12D). In the presence of 50 µM CBP, ASIC1a-mediated currents were decreased by 30 ± 5 % relative to control. In contrast, the σ-2 selective agonist, PB28 (20 µM), failed to inhibit ASIC1a-mediated membrane currents, suggesting σ-2 receptors do not regulate ASIC1a function (Figure 2.12D). Therefore, these results demonstrate that σ-1
receptors functionally couple to ASIC1a channels in cortical neurons as well as to targets downstream of ASIC1a activation.
Figure 2.1 - ASIC1a blockers inhibit proton-evoked increases in $[Ca^{2+}]_i$ in cultured cortical neurons from embryonic (E18) rats. A, Representative traces of $[Ca^{2+}]_i$ as a function of time recorded from a single cell during acidosis in the absence (Control, Wash) and presence of 100 $\mu$M Amiloride (Amiloride). B, Mean change in peak $[Ca^{2+}]_i$ (± SEM) measured in response to low pH solution (pH 6.0) under the indicated conditions ($n = 69$). C, Representative traces of $[Ca^{2+}]_i$ as a function of time recorded from a neuron during acidosis (Control, Wash) and following 20 minutes preincubation in 500 ng/ml Psalmotoxin1 venom (PcTx1). Psalmotoxin1 venom was only present in the pH 7.4 conditioning solution. D, Mean change in peak $[Ca^{2+}]_i$ (± SEM) measured during acidosis under the indicated conditions ($n = 158$). Asterisks denote significant difference from Control and Wash groups in (B) and (D) ($p < 0.001$).
Figure 2.2 - Pan-selective sigma agonists inhibit proton-evoked transient increases in [Ca$^{2+}$]. A, Representative traces of [Ca$^{2+}$] as a function of time recorded from a single neuron during acidosis in the absence (Control, Wash) and presence of 100 µM DTG (DTG). B, Mean change in peak [Ca$^{2+}$] (± SEM) measured during acidosis in the absence (Control, n = 119) and presence of 100 µM DTG (DTG; n = 99) or 10 µM opipramol (OPI, n = 119). Asterisks denote significant difference from Control group (p < 0.001), and pound symbol indicates significant difference from DTG group (p < 0.05). C, Representative traces of [Ca$^{2+}$] as a function of time recorded from a neuron during acidosis in the absence (Control) and presence of 100 µM and 300 µM DTG. D, Concentration-response relationship for DTG inhibition of mean change in peak [Ca$^{2+}$] (± SEM). Values obtained for each cell were normalized to their respective controls (no DTG) (n = 117-124). Line represents a best fit to the data using a single-site Langmuir-Hill equation.
Figure 2.3 - Activation of σ-1 receptors inhibits ASIC1a-induced increases in [Ca^{2+}]_i. Representative traces of [Ca^{2+}]_i as a function of time recorded from two cells during acidosis in the absence (Control) and presence of 10 μM and 100 μM carbetapentane (A, CBP), or in the absence (Control) and presence of 10 μM and 100 μM dextromethorphan (B, DEX). C, Concentration-response relationships for mean change in peak [Ca^{2+}]_i (± SEM) measured during acidosis in the presence of the indicated σ-1 selective ligands DEX, PRE-084, and CBP. Values were normalized to control (absence of σ ligand). Solid and dashed lines represent best fits to the data using single-site Langmuir-Hill equations. Dotted line represents best fit to the data obtained for CBP inhibition of chemical ischemia-induced (4 mM azide in glucose-free PSS) increases in [Ca^{2+}]_i, and is shown for comparison (Katnik et al., 2006). For each condition n > 73.
Figure 2.4 - Inhibition of σ-1 receptors blocks CBP-mediated suppression of proton-evoked increases in [Ca^{2+}]. A, Representative traces of [Ca^{2+}] as a function of time recorded from 2 different neurons during acidosis in the absence (Control) and presence of 30 µM CBP (CBP), without (PSS, left traces) and with 1hr preincubation in 50 µM metaphit (Metaphit, right traces). B, Percent inhibition of [Ca^{2+}] increases (± SEM) by CBP measured during acidosis under control conditions (Control, n = 135) and following metaphit preincubation (MET, n = 113). C, Representative traces of acid-induced increases in [Ca^{2+}] as a function of time recorded from 2 different neurons in the absence (Control) and presence of 30 µM CBP (CBP), without (PSS, left traces) and with co-application of 10 nM BD1063 (BD1063, right traces). D, Percent inhibition of [Ca^{2+}] increases (± SEM) by CBP under control conditions (Control, n = 178) and in the presence of BD1063 (BD1063, n = 116). Asterisks in (B) and (D) denote significant difference from Control groups (p < 0.001).
Figure 2.5 - Sigma-2 receptor ligands inhibit ASIC1a-mediated elevations in 
Ca^{2+}, at concentrations inconsistent with σ-2 mediated effects and in a metaphit-
insensitive manner. A, Representative traces of [Ca^{2+}], as a function of time 
recorded from two neurons during acidosis in the absence (Control) and 
presence of 10 µM and 100 µM ibogaine (IBO, left traces), or in the absence 
(Control) and presence of 1 µM and 10 µM PB28 (right traces). B, Mean change 
in peak [Ca^{2+}], (± SEM) measured during acidosis in the presence of the σ-2 
selective ligands PB28 and IBO. Solid lines represent best fits to the data using 
single-site Langmuir-Hill equations. Dashed line represents best fit to the data 
obtained for IBO inhibition of I_{Ca}, and is shown for comparison (Zhang and 
Cuevas, 2002). For each condition, n > 108 cells. C, [Ca^{2+}], as a function of time 
recorded from 2 different neurons during acidosis in the absence (Control) and 
presence of 20 µM PB28 (PB28), without (PSS) and with (+MET) 1hr 
preincubation in 50 µM metaphit. D, Percent inhibition of [Ca^{2+}], (± SEM) by PB28 
measured during acidosis under control conditions (Control, n = 324), following 
metaphit preincubation (+MET, n = 276) and co-applied with 10 nM BD1063 
(BD1063, n = 139). There is no significant difference between Control and +MET 
groups, (p = 0.64) or Control and BD1063 groups (p = 0.70).
Figure 2.6 - Intracellular Ca\textsuperscript{2+} stores are not involved in DTG modulation of ASIC1a-induced increases in [Ca\textsuperscript{2+}]. A, Representative traces of [Ca\textsuperscript{2+}]\textsubscript{i} as a function of time recorded from a neuron during acidosis in the absence (Control) and presence of 100 µM DTG (DTG). B, Representative traces of [Ca\textsuperscript{2+}]\textsubscript{i} as a function of time recorded from a neuron during acidosis following preincubation in 10 µM thapsigargin (1 hr, 23° C) in the absence (THAP) and presence of 100 µM DTG (THAP + DTG). C, Mean change in peak [Ca\textsuperscript{2+}]\textsubscript{i} (± SEM) measured in response to acidosis with (THAP, n = 346) or without (PSS, n = 216) thapsigargin preincubation in the absence (Control) and presence of 100 µM DTG (DTG). Asterisks denote significant difference from Control group (p < 0.001). There was no significant difference between PSS and THAP groups (p = 0.48).
Figure 2.7 - ASIC1a-mediated [Ca^{2+}]_i increases are membrane potential dependent. A, Representative traces of [Ca^{2+}]_i recorded in response to ASIC1a activation from a neuron electrically accessed using the perforated patch whole-cell configuration and held at -70 mV ([Ca^{2+}]_i V-Clamp), and from a second neuron in the same field of view which was not electrically accessed ([Ca^{2+}]_i Control). Inset, whole-cell current trace recorded simultaneously from the voltage-clamped neuron. Lines above traces indicate application of pH 6.0 solution. Scale bars: 500 pA, 5 sec. B, Mean change in peak [Ca^{2+}]_i (± SEM) measured during acidosis in non voltage-clamped neurons (Control, n = 37) and in voltage-clamped neurons (V-Clamp, n = 4). Asterisks denote significant difference between groups (p < 0.001).
Figure 2.8 - ASIC1a activation promotes membrane depolarization. A, Representative traces of ASIC1a-mediated membrane potential changes as a function of time recorded from a neuron held under current-clamp mode in the absence (Control) and presence of 100 μM amiloride (Amiloride). B, Changes in peak membrane potential (± SEM) recorded from neurons in normal PSS (Control) or in PSS containing 100 μM amiloride (Amiloride, n = 3). Asterisk denotes significant difference from Control group (p < 0.05). C, Representative traces of ASIC1a-mediated membrane potential changes as a function of time recorded from a different cell held under current-clamp mode in the absence (Control) and presence of 50 nM PcTx1 peptide (PcTx1). D, Changes in peak membrane potential (± SEM) recorded from neurons in the absence (Control) or presence of 50 nM PcTx1 peptide (PcTx1, n = 3). No significant difference between Control and PcTx1 groups were noted (p = 0.11).
Figure 2.9 - Sigma-1 receptor activation inhibits Ca$^{2+}$ channels downstream of ASIC1a. A, Representative traces of [Ca$^{2+}$]$\text{i}$ recorded in response to application of extracellular high K$^+$ (40 mM) solution in the absence (Control) and presence of 100 $\mu$M CBP. B, Bar graph of mean change in peak [Ca$^{2+}$]$\text{i}$ (± SEM) measured after multiple experiments (n = 59). Asterisks denote significant difference between groups ($p < 0.001$).
Figure 2.10 - Multiple plasma membrane ion channels downstream of ASIC1a activation contribute to acidosis-evoked [Ca$^{2+}$]$_i$ increases. A, Relative changes in [Ca$^{2+}$]$_i$ (± SEM) during acidosis in the absence (PSS) and presence of 500 nM tetrodotoxin (TTX) alone or TTX (500 nM) co-applied with 100 µM AP5, 10 µM nifedipine or 100 µM cadmium. All combinations were done without (Control) and with 50 µM CBP (CBP). For each condition n > 101. Asterisks denote significant differences from respective control groups ($p < 0.001$), pound symbol from PSS group ($p < 0.01$ for TTX vs. PSS, $p < 0.001$ for all others), and dagger symbols from TTX group ($p < 0.001$). B, Relative changes in [Ca$^{2+}$]$_i$ (± SEM) during acidosis in the absence (Control) and presence of 10 µM CNQX (CNQX) without CBP (PSS) or with CBP at the indicated concentrations. For each condition n > 95. Asterisks denote significant differences from PSS group within Control or CNQX groups ($p < 0.001$), pound symbols indicate significance differences between 50 µM and 300 µM CBP groups within Control or CNQX groups ($p < 0.001$), and daggers denote significant differences between Control and CNQX within PSS ($p < 0.001$) and 300 µM CBP ($p < 0.05$) groups.
Figure 2.11 - ASIC1a blockers inhibit acidosis-mediated currents in voltage-clamped neurons. A, Representative traces of ASIC1a-mediated currents as a function of time recorded from two neurons held at -70 mV in the absence (Control) and presence of 100 \( \mu \text{M} \) amiloride (Amiloride) (i.) or 500 ng/ml Psalmotoxin1 venom (PcTx1) (ii.). B, Relative mean peak proton-gated currents (± SEM) recorded from neurons in normal PSS (Control) or in PSS containing either 100 \( \mu \text{M} \) amiloride (Amiloride, n = 6) or 500 ng/ml Psalmotoxin1 venom (PcTx1, n = 4). Values were normalized to the maximum proton-evoked response recorded for control conditions (no drug) in each cell (I/I_{max}). Asterisks denote significant difference from Control group (p < 0.01). C, Relative peak proton-gated currents (I/I_{max}, ± SEM) recorded in the absence (PSS) and presence of 500 nM tetrodotoxin (TTX, n = 4) alone and TTX with 100 \( \mu \text{M} \) AP-5 (n = 3), 10 \( \mu \text{M} \) CNQX (n = 4), 10 \( \mu \text{M} \) nifedipine (Nif, n = 6) or 100 \( \mu \text{M} \) cadmium (Cd, n = 3) in the bath solution. Cells were voltage-clamped at -70 mV. No significant differences between the groups were noted (p = 0.826).
Figure 2.12 - Sigma receptor agonists inhibit ASIC1a-mediated currents in voltage-clamped neurons. A, Representative traces of ASIC1a currents as a function of time recorded from a single perforated-patched neuron held at -70 mV in the absence (Control) and presence of 100 μM DTG (DTG). B, Mean peak proton-gated current densities (± SEM) measured from neurons held at -70 mV without (Control) or with 100 μM DTG (DTG) in the bath solution (n = 6). C, Representative traces of ASIC1a-mediated currents as a function of time recorded from a voltage-clamped neuron (-70 mV) in the absence (Control) and presence of 50 μM CBP (CBP). D, Mean peak proton-gated current densities (± SEM) recorded from neurons held at -70 mV without (Control) or with 50 μM CBP (CBP, n = 6) or 20 μM PB28 (PB28, n = 4). Asterisks in (B) and (C) denote significant differences from respective Control groups (p < 0.001).
Discussion

Activation of σ receptors depresses membrane currents and elevations in [Ca\(^{2+}\)]\(_i\) mediated by ASIC1a channels in cortical neurons. The pharmacological properties of the receptor involved are consistent with the effects being specifically mediated by the σ-1 receptor subtype. Furthermore, most of the elevations in [Ca\(^{2+}\)]\(_i\) triggered by acidosis are the result of Ca\(^{2+}\) channels opening downstream of ASIC1a activation. Stimulation of σ-1 receptors effectively suppressed these secondary Ca\(^{2+}\) fluxes both by inhibiting ASIC1a and the other channels directly.

ASIC are regulated by various factors such as pH, membrane distention and arachidonic acid, and therefore, function as signal integrators in the CNS (Allen and Attwell, 2002; Lopez, 2002). All of these factors elicit or potentiate ASIC-mediated responses. Information on endogenous mechanisms that inhibit ASIC function is lacking. It has been shown that NMDA receptors modulate ASIC1a function via the activation of a CaMKII signaling cascade, but activation of this pathway results in an increase in currents through ASIC1a (Gao et al., 2005). Thus, our finding that activation of σ receptors depresses ASIC1a-mediated responses is novel. Our conclusion that the responses observed are mediated specifically by ASIC1a is supported by the inhibition produced with the selective ASIC1a channel blocker, PcTx1 (Diochot et al., 2007), and that cultured cortical neurons from embryonic mice deficient in the ASIC1a subunit fail to show increases in [Ca\(^{2+}\)]\(_i\), or membrane currents at the proton concentrations used here (Xiong et al., 2004). ASIC2a and ASIC2b subunits are also expressed in the
CNS, but homomeric ASIC2a channels are activated below pH 5.5, and ASIC2b
does not generate currents in response to low pH (Lingueglia et al., 1997).
Furthermore, neither homomeric ASIC2a nor heteromultimeric ASIC1a/ASIC2a
channels conduct Ca\textsuperscript{2+}, and thus could not account for the changes in [Ca\textsuperscript{2+}]i
observed here (Yermolaieva et al., 2004).

Results from Ca\textsuperscript{2+} imaging experiments suggest that it is specifically the
\(\sigma\)-1 receptor subtype that modulates neuronal responses to ASIC1a activation.
Studies have shown that the affinity of carbetapentane for \(\sigma\)-1 receptors is
>50-fold greater than for \(\sigma\)-2 receptors (Rothman et al., 1991; Vilner and Bowen,
2000). The calculated IC\textsubscript{50} for carbetapentane inhibition of ischemia-evoked
increases in [Ca\textsuperscript{2+}]i via \(\sigma\)-1 receptor activation is 18.7 µM (Katnik et al., 2006),
which is comparable to the 13.8 µM IC\textsubscript{50} for CBP inhibition of ASIC1a-induced
[Ca\textsuperscript{2+}]i increases. Carbetapentane also inhibits epileptiform activity in rat
hippocampal slices via \(\sigma\)-1 receptors with an IC\textsubscript{50} value of 38 µM (Thurgur and
Church, 1998). Similarly, we show that the \(\sigma\)-1 agonists dextromethorphan
(IC\textsubscript{50} = 22 µM) and PRE-084 (IC\textsubscript{50} = 13.7 µM), both of which have >100-fold
greater affinities for \(\sigma\)-1 than \(\sigma\)-2 receptors, block ASIC1a-mediated responses at
concentrations consistent with those reported in the literature.
Dextromethorphan inhibits spreading depression in rat neocortical brain slices
with an IC\textsubscript{50} ~ 30 µM (Anderson and Andrew, 2002), whereas PRE-084 protects
human retinal cells against oxidative stress with an IC\textsubscript{50} ~ 10 µM (Bucolo et al.,
2006). The fact that IC\textsubscript{50} values determined here for carbetapentane,
dextromethorphan and PRE-084 are in the low µM range suggests that it is
unlikely these agonists are affecting ASIC1a activity via σ-2 receptors, since high μM to mM concentrations of these compounds are required to stimulate σ-2 receptors. Moreover, σ-2-selective agonists failed to inhibit ASIC1a-mediated responses at concentrations consistent with σ-2 specific effects.

The strongest evidence that σ-1 receptor activation modulates ASIC1a comes from experiments using the σ antagonists, metaphit and BD1063. Metaphit has been shown to bind irreversibly to σ-1 receptors with an IC_{50} value of 50 μM (Wu, 2003). Preincubation in metaphit blocks σ-1 receptor mediated modulation of voltage-gated K⁺ channels in intracardiac neurons and depression of ischemia-induced elevations in [Ca^{2+}] in cortical neurons (Zhang and Cuevas, 2005; Katnik et al., 2006). Preincubation of cortical neurons in 50 μM metaphit antagonized CBP inhibition of ASIC1a by ~ 40%. BD1063 has been shown to have a higher affinity for σ-1 than σ-2 receptors and attenuates the dystonia produced by DTG in rats in a dose-dependent manner, suggesting this ligand acts as an antagonist at σ sites (Matsumoto et al., 1995). Here we show that CBP is unable to block acid-induced increases in [Ca^{2+}] when co-applied with BD1063. In addition, we found that metaphit fails to inhibit the effects of the σ-2 agonist, PB28, on ASIC1a-mediated responses. Taken together, these data show that increases in [Ca^{2+}] in response to ASIC1a activation are modulated only by σ-1 receptors.

Several studies have suggested that Ca^{2+} influx through ASIC1a channels is a key mechanism leading to cell death (Xiong et al., 2004; Yermolaieva et al., 2004). Depletion of Ca^{2+} from intracellular stores indicates that most, if not all, of
the acid-induced increases in [Ca\(^{2+}\)]\(_i\) is due to plasma membrane influx. However, our results show that multiple ion channels downstream of ASIC1a activation contribute to acidosis-induced elevations in [Ca\(^{2+}\)]\(_i\), including NMDA and AMPA/kainate receptors and VGCC. The activation of NMDA and AMPA/kainate receptors following ASIC1a stimulation was observed even when neuronal conduction was inhibited with tetrodotoxin. This observation suggests a presynaptic localization of ASIC1a, whereby activation of the channel by protons results in synaptic transmission and subsequent activation of postsynaptic glutamatergic receptors. Consistent with this hypothesis, ASIC1a has been found to regulate neurotransmitter release probability in mouse hippocampal neurons (Cho and Askwith, 2008).

σ receptors have been identified in both presynaptic and postsynaptic sites (Gonzalez-Alvear and Werling, 1995; Alonso et al., 2000), and thus may modulate channels in both regions. In the presence of specific inhibitors of ionotropic glutamate receptors, activation of σ-1 receptors with CBP further decreased proton-evoked increases in [Ca\(^{2+}\)]\(_i\), but the effects of CBP and the glutamate channel inhibitors were less than additive. Thus, σ-1 receptors also inhibit Ca\(^{2+}\) entry via NMDA and AMPA/kainate receptors directly by inhibiting these channels and indirectly by depressing ASIC1a activation. Application of the L-type VGCC inhibitor, nifedipine, and the broad-spectrum Ca\(^{2+}\) channel inhibitor, cadmium, blocked ASIC1a-induced increases in [Ca\(^{2+}\)]\(_i\) by >70% and >90%, respectively. This observation indicates that most of the increases in [Ca\(^{2+}\)]\(_i\) produced upon ASIC1a activation is dependent on Ca\(^{2+}\) influx through VGCC.
Co-application of CBP with nifedipine, but not with Cd²⁺, resulted in further reduction in the proton-evoked increases in [Ca²⁺]. The conclusion that Ca²⁺ influx through ASIC1a channels itself contributed only a small fraction to the total observed [Ca²⁺], increases was confirmed with simultaneous Ca²⁺ fluorometry and whole-cell patch clamp recordings. Cells voltage-clamped at –70 mV, which prevents NMDA receptor and VGCC activation, demonstrated minimal acid-evoked elevations in [Ca²⁺]. Taken together, our results show that the increases in [Ca²⁺], evoked by ASIC1a activation are the result of synaptic transmission and subsequent opening of multiple Ca²⁺ channels, and that stimulation of σ-1 receptors downregulates all of these events. However, the fact that activation of σ-1 receptors depressed ASIC1a-mediated currents in cells voltage-clamped at –70 mV indicate that σ-1 receptors are functionally coupled to ASIC1a, and that the depression in acid-evoked increases in [Ca²⁺], is not exclusively the result of σ-1 receptors blocking channels downstream of ASIC1a.

The finding that σ-1 receptors can inhibit ASIC1a channels has significant physiological and pathophysiological implications. It has been proposed that ASIC1a activation may facilitate neurotransmission by compensating for the decrease in excitatory neurotransmission caused by direct inhibition of post-synaptic Na⁺ and Ca²⁺ channels by protons which are released during exocytosis (Krishtal et al., 1987; Zha et al., 2006). Furthermore, the expression levels of ASIC1a have direct effects on the density of dendritic spines in hippocampal neurons (Zha et al., 2006). Thus, σ-1 receptors may influence cell-to-cell signaling in the CNS by affecting ASIC1a activity. One of the consequences of
ASIC1a overexpression in mice is enhanced fear conditioning (Wemmie et al., 2004), whereas stimulation of σ-1 receptors is known to ameliorate conditioned fear stress (Kamei et al., 1997). These observations, coupled with our current report, suggest that σ-1 receptor activation may produce anxiolytic effects via the inhibition of ASIC1a channels.

The inhibition of ASIC1a by σ-1 receptors is a potential component of the neuroprotective properties of σ receptors, since activation of ASIC1a has been shown to contribute to stroke injury (Xiong et al., 2004). Importantly, inhibition of ASIC1a has been shown to be neuroprotective at delayed time points following ischemic stroke (Simon, 2006). Thus, σ-1 receptor-mediated inhibition of ASIC1a may contribute to the enhanced neuronal survival following σ receptor activation 24 hr post-stroke in rats (Ajmo et al., 2006). Furthermore, our data suggests that activation of ASIC1a stimulates the activity of NMDA and AMPA/kainate receptors and VGCC, all of which have been linked to ischemia-induced brain injury. Thus, σ-1 receptor activation may provide further neuroprotection by reducing the activity of these channels which occurs subsequent to ASIC1a stimulation. Consistent with this pleiotropic effect of σ-1 receptors is our observation that σ-1 receptor activation suppressed extracellular high K⁺-induced increases in [Ca²⁺], which would also activate these downstream effectors. In conclusion, σ-1 receptors inhibit ASIC1a channel function and blunt acidosis-evoked ionic fluxes and increases in [Ca²⁺]. Thus, σ-1 receptors can be targeted for therapeutic intervention in pathophysiological conditions involving ASIC1a activation.
CHAPTER 3

SIGMA-1 RECEPTOR ACTIVATION INHIBITS ASIC1a CHANNELS VIA A PERTUSSIS TOXIN SENSITIVE G PROTEIN AND AN AKAP/CALCINEURIN COMPLEX

Introduction

Acid-sensing ion channels (ASIC) are a class of ligand-gated ion channels that are members of the degenerin/epithelial sodium channel (Deg/ENac) superfamily (Waldmann et al., 1997b; Benos and Stanton, 1999). ASIC are expressed in both peripheral and central nervous system neurons. Several extracellular and intracellular modulators of ASIC have been identified. Divalent cations (Zn$^{2+}$, Pb$^{2+}$, Ca$^{2+}$) (Baron et al., 2001; Chu et al., 2004; Gao et al., 2004; Wang et al., 2006), lactate (Immke and McCleskey, 2001), serine proteases (Poirot et al., 2004) and redox reagents (Andrey et al., 2005; Chu et al., 2006) have been shown to interact with the extracellular domain of ASIC and influence the function of these channels. Moreover, there is a conserved phosphorylation site within the intracellular C-terminal domain of ASIC1a that is also the calcium/calmodulin protein kinase II (CaMKII) (Gao et al., 2005), protein kinase C (PKC) (Baron et al., 2002) and protein kinase A (PKA) (Leonard et al., 2003) binding site. Phosphorylation of ASIC1a has been shown to potentiate ASIC1a function in neurons (Xiong et al., 2004; Gao et al., 2005). In contrast, the second
messenger, calcineurin, dephosphorylates ASIC1a channels and results in
downregulation of channel function (Chai et al., 2007).

Dephosphorylation of ASIC1α is mediated through the interaction with
cytoskeletal anchoring protein, A-kinase anchoring protein (AKAP). Interestingly,
AKAP150 and calcineurin have been implicated in the downregulation of both
ASIC1α and ASIC2α channels (Chai et al., 2007). AKAP is a diverse protein
family with more than 50 members which are abundantly expressed in the brain
(Feliciello et al., 2001). Neuronal AKAP150 (rat) and AKAP79 (human) share a
high degree of sequence homology, differing primarily in a 9 amino acid repeat
sequence insert found only in rodents which has no known function (Dell’Acqua
et al., 2006). AKAP150 anchors both kinases (cAMP-dependent Protein Kinase A
and Protein Kinase C) and phosphatases (calcineurin) that are inhibited when
bound. AKAP150 targets these proteins, through a unique targeting motif, to
specific subcellular sites and plasma membrane via association with structural
proteins, membranes, or cellular organelles (Dell'Acqua et al., 1998; Diviani and
Scott, 2001).

AKAP150 has been shown to modulate the internalization of AMPA
receptors, NMDA receptors during long term potentiation and depression
(Rosenmund et al., 1994; Westphal et al., 1999; Colledge et al., 2000; Gomez et
al., 2002; Smith et al., 2006) and voltage-gated Ca^{2+} channel function (Oliveria et
al., 2007). All of these ion channels have shown to be regulated by σ receptor
activation in neurons via various signaling cascade mechanisms. σ receptor
activation has been shown to depress both membrane currents and elevations in
[Ca^{2+}]_i mediated by ASIC1a channels in cortical neurons. Furthermore, most of the elevations in [Ca^{2+}]_i triggered by acidosis were determined to be the result of Ca^{2+} channels opening downstream of ASIC1a activation, and activation of σ-1 receptors effectively suppressed these secondary Ca^{2+} fluxes by both inhibiting ASIC1a and/or the Ca^{2+} channels directly. But the signaling cascade mechanism by which σ-1 receptors regulate ASIC1a channels remains to be elucidated.

σ-1 receptors are directly coupled to potassium channels in intracardiac neurons and activation of this receptor subtype depresses the excitability of these neurons, blocking parasympathetic input to the heart (Zhang and Cuevas, 2005). In cultured frog melanotrope cells, DTG and (+)-pentazocine have been shown to modulate electrical activity by reducing both a tonic K^+ current and a voltage-dependent K^+ conductance through activation of a cholera toxin-sensitive G protein (Soriani et al., 1998; Soriani et al., 1999). In Xenopus oocytes, the signal transduction cascade between Kv1.4 and σ receptors has been shown to be dependent on protein-protein interactions (Aydar et al., 2002).

In this study, experiments were conducted to determine the signaling cascade linking σ-1 receptors to ASIC1a channels and downstream Ca^{2+} channels. σ-1 receptors, ASIC1a and AKAP150 were shown to colocalize not only in the plasma membrane of cortical neuron cell bodies but also in the dendritic processes of these cells. Calcineurin inhibitors, cyclosporin A and FK-506, and the G protein inhibitor pertussis toxin (PTX) diminished the downregulation of ASIC1a by σ-1 receptors, suggesting that σ-1 receptors exert their effect via calcineurin-dependent dephosphorylation of ASIC1a and a PTX-
sensitive G protein. Furthermore, disruption of the actin cytoskeleton or
dissociation AKAP150 from the plasma membrane were shown to diminish σ-1
receptor mediated inhibition of ASIC1a channels. Moreover, whole-cell patch
clamp experiments confirmed that preincubation in PTX or disruption of the
AKAP/calcineurin interaction with VIVIT, prevented σ-1 receptor modulation of
ASIC1a-mediated membrane currents, suggesting σ-1 receptors couple to
ASIC1a channels via a PTX-sensitive G protein and an AKAP150/calcineurin
complex. This is the second report of receptor-mediated functional
downregulation of ASIC1a channels. Thus far, σ-1 receptors are the only
receptors identified to inhibit ASIC1a channel function.

Materials and Methods

Primary Rat Cortical Neuron Preparation

Primary cortical neurons from embryonic (E18) rats were cultured as
previously described by our laboratory (Katnik et al., 2006). All procedures were
done in accordance with the regulations of the University of South Florida
Institutional Animal Care and Use Committee. Cells were used after 10-21 days
in culture.

Calcium Imaging Measurements

The effects of acidosis on intracellular Ca^{2+} concentrations were examined
in isolated cortical neurons using ratiometric calcium imaging. Cytosolic free-Ca^{2+}
was measured using the Ca^{2+} sensitive dye, fura-2. The membrane permeable
ester form of fura-2, fura-2 AM, acetoxymethyl ester (AM), was loaded and
imaged as we have previously described (DeHaven and Cuevas, 2004; Katnik et al., 2006). Briefly, cells plated on coverslips were incubated for 1 hour at room temperature in Neurobasal (Invitrogen) medium supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine, or in physiological saline solution (PSS) consisting of (in mM): 140 NaCl, 5.4 KCl, 1.3 CaCl₂, 1.0 MgCl₂, 20 glucose, and 25 HEPES (pH to 7.4 with NaOH). Both solutions contained 4 μM of fura-2, acetoxymethylester (fura-2 AM) and 0.4 % dimethyl sulfoxide. The coverslips were washed in PSS (fura-2-AM free) prior to the experiments being carried out.

**Electrophysiology Recordings**

ASIC1a-mediated membrane currents were recorded using the protocol previously described by our laboratory. Briefly, neurons plated on glass coverslips were transferred to a recording chamber and membrane currents were amplified, filtered at 1 kHz, digitized at 5 kHz, and acquired using Clampex 8 (Axon). Electrical access was achieved using the amphotericin B perforated-patch method to preserve intracellular integrity of neurons (Rae et al., 1991). An amphotericin B stock solution (60 mg/ml in DMSO) was made fresh everyday, kept on ice, light protected, and diluted to 240 μg/ml (0.4% DMSO) in control pipette solution immediately prior to patching. The control pipette solution consisted of (in mM): 75 K₂SO₄, 55 KCl, 5 MgSO₄, and 10 HEPES (titrated to pH 7.2 with N-methyl-d-glucamine). Patch electrodes were pulled from thin-walled borosilicate glass (World Precision Instruments Inc., Sarasota, FL) using a Sutter Instruments P-87 pipette puller (Novato, CA) and had resistances of 1.0–1.5 MΩ. Access resistances (Rₛ) were monitored throughout experiments for stable
values ≤ 20 MΩ and were always compensated at 40% (lag, 10 µs). All cells were voltage-clamped at -70 mV.

Immunohistochemistry

Cortical neurons plated on coverslips were first rinsed with phosphate buffer saline (PBS) to remove excess media. Cells were fixed by incubating in 95% ethanol and 5% acetic acid at -20°C for 20 minutes, followed by four 2-minute incubations at room temperature in 95%, 75%, 50% and 0% ethanol. Neurons were then permeabilized with PBS solution containing 0.1% Triton X for 15 minutes and rehydrated with 3 washes in PBS and 5 washes in PBS with 0.5% bovine serum albumin (BSA). The cells were then blocked with 2% BSA in PBS for 45 minutes at room temperature. Neurons were incubated in the primary antibodies (ASIC1 guinea-pig polyclonal IgG at 1:500 dilution, AKAP150 goat polyclonal IgG at 1:50 dilution and σ -1 rabbit polyclonal IgG at 1:500 dilution) in 0.5% BSA in PBS solution overnight at 4°C and in the secondary antibodies (Alexa-Fluor 633 goat anti-guinea pig, Alexa-Fluor 555 donkey anti-goat and Alexa-Fluor 488 goat anti-rabbit, all at 1:1000 fold dilutions) for an hour at room temperature. Primary antibodies were purchased from: AKAP150 (Santa Cruz Biotechnology, Santa Cruz, CA) and ASIC1a (Lifespan Biosciences, Seattle, WA). σ-1 receptor antibody was kindly provided by Drs. T. P. Su and Teruo Hayashi (Baltimore, Maryland). Secondary antibodies were purchased from Invitrogen (Eugene, OR): Alexa Fluor 488 anti-rabbit, Alexa Fluor 555 anti-goat, and Alexa Fluor 633 anti-guinea-pig. Coverslips with labeled cells were then
mounted onto a microscope slide using VectaShield Hardset Media with DAPI purchased from Vector Laboratories (Burlingame, CA).

**Confocal Microscopy**

Confocal images of triple labeled neurons were collected using a Leica DMI6000 inverted microscope, TCS SP5 confocal scanner and a 100x/1.4 N.A. Plan Apochromat oil immersion objective (Leica Microsystems, Germany). Laser lines (405 Diode, Argon, HeNe 543, and HeNe 633) were applied to excite stained cells and tunable filters were used to minimize crosstalk between fluorochromes. Image sections at 0.4 μm were acquired with photomultiplier detectors, processed and analyzed using Leica LAS AF software version 1.8.2 (Leica Microsystems, Germany). 2-D images of fluorescence emission for each fluorophore (AF488, green; AF555, red; AF633, purple) were collected for 20-30 optical sections along the z-axis for each field of view. Areas of colocalization for pairs of fluorophores in a single z-section are depicted as white pixels in the merged images and represent pixels within the boundaries drawn in the scatter plot (Figure 3.1). The area underneath the arc represents background pixels which have been eliminated, while pixels outside the two straight lines are pixels with intensities greater than background in only one of the images. The Overlap Coefficient (R), a correlation coefficient relating two images or regions within an image, is defined as

$$R = \frac{\sum_{i} S_{1i} \cdot S_{2i}}{\sqrt{\sum_{i} (S_{1i})^2 \cdot \sum_{i} (S_{2i})^2}}$$

(1)
where $S_{1i}$ is the intensity of pixel $i$ in image 1 and $S_{2i}$ its intensity in image 2 (Manders, 1993). $R$ ranges from 1, complete colocalization, to 0, complete non-colocalization and was calculated for entire images and within regions of interest drawn around individual cells.

**Solutions and Reagents**

The control bath solution for all experiments was PSS. All drugs were applied in this solution using a rapid application system identical to that previously described (Cuevas and Berg, 1998). ASIC activation was induced by applying PSS with a pH of 6.0 (+/- drug) to specifically target ASIC1a. Individual cells were exposed to ≤ 3 low pH applications. No rundown of responses was observed using this protocol. All chemicals used in this investigation were of analytic grade. The following drugs were used: DTG and pertussis toxin (Sigma-Aldrich, St. Louis, MO); carbetapentane and NMDA (Tocris Bioscience, Ellisville, MO); latrunculin-A, cyclosporineA and VIVIT (Calbiochem, San Diego, CA); FK-506 (LC Laboratories, Woburn, MA); tetrodotoxin (Alomone Labs, Jerusalem, Israel); and fura-2 AM (Molecular Probes, Eugene, OR).

**Data Analysis**

Analysis of measured intracellular $Ca^{2+}$ and membrane current responses was conducted using Clampfit 9 (Axon instruments). Imaging data files collected with SlideBook 4.02 (Intelligent Imaging Innovations, Inc.) were converted to a text format and imported into Clampfit for subsequent analysis. Statistical analysis was conducted using SigmaPlot 9 and SigmaStat 3 software (Systat Software, Inc.). Statistical differences were determined using paired and
unpaired t-tests for within group and between group experiments, respectively, and were considered significant if $p < 0.05$. For multiple group comparisons either a 1-way or a 2-way ANOVA was used, as appropriate. When significant differences were determined with an ANOVA, post-hoc analysis was conducted using a Tukey’s Test to determine interaction between individual groups.

Results

To answer whether the signaling cascade linking σ receptors to ASIC channels might involve protein-protein interactions, immunohistochemical fluorescent staining experiments were performed on cortical neurons to determine if σ-1 receptors, ASIC1a channels and AKAP150 colocalize. Figure 3.1A shows fluorescent images of neurons triple labeled for σ-1 receptors(i), AKAP150 (ii) and ASIC1a channels (iii) from the same field of view. The white pixels represent areas in the image where σ-1 receptor and AKAP150 (Figure 3.1B, i) labeling appear together as selected in the scatter plot (Figure 3.1B, ii). The scatter plot maps pixel intensities from the σ-1 receptor labeling versus intensities from the AKAP150 labeling. The overlap coefficient for σ-1 receptor and AKAP150 colocalization calculated using Eq. 1 for the entire image was calculated to be 0.74. Figure 3.1C, i, shows the merged image of σ-1 receptor and ASIC1a labeling with colocalized pixels represented in white and the colocalization scatter plot (Figure 3.1C, ii). The overlap coefficient for σ-1 receptors and ASIC1a channels for the entire image was calculated to be 0.78. After multiple experiments (3 stains with a total of 59 cell bodies and 45 neuronal
processes), the mean overlap coefficient for σ-1 receptor colocalization with AKAP150 and ASIC1a channels for individual cell bodies was calculated to be 0.723 ± .007 and 0.790 ± .008, and for defined dendritic processes to be 0.696 ± .013 and 0.751 ± .009, respectively (Figure 3.1D). These images suggest that σ-1 receptors colocalize with both AKAP150 and ASIC1a in the cell body and in the dendritic processes. The similarity of the pixel patterns in both sets of images (Figure 3.1A, ii and iii) is consistent with previous results showing colocalization of AKAP150 and ASIC1a (Chai et al., 2007).

Reports suggest that calcineurin may be involved in the functional downregulation of ASIC1a and ASIC2a (Chai et al., 2007). Thus, experiments were carried out to determine if calcineurin is a constituent of the signaling cascade linking σ-1 receptors to ASIC1a channels. Figure 3.2A show characteristic traces of [Ca^{2+}] as a function of time recorded from three cells exposed to normal PSS solution (PSS, i) or PSS containing the calcineurin inhibitors FK-506 (1 μM, ii) and cyclosporin A (1 μM, cyclo A, iii), all in the absence and presence of CBP (10 μM). Both calcineurin inhibitors reversed the effects of σ-1 receptor activation on ASIC1a function. A summary of data collected from identical experiments is shown in Figure 3.2B. Bath application of FK-506 or cyclosporin A alone had no effects on control responses. However, following inhibition of calcineurin with either FK-506 or cyclosporin A, CBP blocked ASIC1a-mediated responses significantly less than under control conditions (Figure 3.2B). The percent-inhibition of ASIC1a-mediated responses by CBP was significantly decreased from 45 ± 1% in control cells to 31 ± 1% and
These results suggest σ-1 receptors are functionally linked to ASIC1a via a signaling cascade involving calcineurin. These observations are consistent with reports in the literature which suggest that dephosphorylation of ASIC1a and ASIC2a channels by this phosphatase may be involved in the functional downregulation of ASIC (Chai et al., 2007).

Calcineurin has been shown to be activated by a pertussis toxin-sensitive G protein, specifically G_{i2} (Gromada et al., 2001). Previous reports suggest that σ-1 receptors may couple to ion channels, such as voltage-sensitive K^+ channels, via G proteins (Soriani et al., 1998; Soriani et al., 1999). Experiments were performed to determine whether G proteins were also involved in σ-1 receptor regulation of ASIC1a channels. Figures 3.3A and 3.3B show representative traces of [Ca^{2+}] as a function of time recorded during acidosis in the absence (Control) and presence of CBP (CBP, 50uM) without (A) and with PTX preincubation (200 ng/ml for 24 hrs at 37°C, B). Results from multiple experiments suggest that PTX alone has some effect on ASIC1a-mediated [Ca^{2+}] increases but it does not change the kinetics of the responses (Figure 3.3B). Preincubation in PTX significantly reduced σ-1 receptor mediated inhibition of ASIC1a-induced [Ca^{2+}] increases (Figure 3.3C). While CBP inhibited 41 ± 1% of acid-induced increases in [Ca^{2+}] in control cells, the σ-1 ligand only blocked 26 ± 1% of the increases following PTX treatment. This inhibition was statistically significant (Figure 3.3D). These results suggest that σ-1 receptor
activation inhibits ASIC1a-induced increases in [Ca\(^{2+}\)]\(_i\), via a PTX-sensitive G protein.

Having identified calcineurin and a PTX-sensitive G protein in the signaling pathway linking ASIC1a and \(\sigma\)-1 receptors, it was of interest to determine other constituents of the signaling cascade. The scaffolding protein AKAP150 has been shown to anchor kinases (PKA and PKC) and the phosphatase calcineurin to regulate ion channels and receptors (Dell'Acqua et al., 1998; Dodge and Scott, 2000; Feliciello et al., 2001; Moita et al., 2002; Wong and Scott, 2004; Beene and Scott, 2007). Activation of NMDA due to Ca\(^{2+}\) influx through the receptor has been shown to cause rearrangement of the cytoskeleton resulting in dissociation of AKAP150 from the plasma membrane (Gomez et al., 2002). Figures 3.4A and 3.4B show representative traces of [Ca\(^{2+}\)]\(_i\) as a function of time recorded during acidosis in the absence (Control) and presence of CBP (CBP, 50 \(\mu\)M) without (PSS, A) and with NMDA (10 \(\mu\)M, B) pre-incubation. Pre-incubation in NMDA significantly reduced ASIC1a-mediated elevations in [Ca\(^{2+}\)]\(_i\) (Figure 3.4C). In addition, pre-incubation in NMDA also significantly reduced \(\sigma\)-1 receptor mediated inhibition of the remaining ASIC1a-induced [Ca\(^{2+}\)]\(_i\) increase (Figure 3.4C). While CBP inhibited 44 \(\pm\) 1\% of acid-induced increases in [Ca\(^{2+}\)]\(_i\) in control cells, it only inhibited 40 \(\pm\) 1\% in NMDA treated cells and this inhibition was statistically significant (Figure 3.4D). The small but significant block by NMDA of \(\sigma\)-1 receptor modulation of ASIC1a can probably be attributed to additional effects of Ca\(^{2+}\) overload following preincubation in NMDA which led to increases in basal calcium levels. These
results are consistent with the theory that the scaffolding protein AKAP150 is involved in σ-1 receptors modulation of ASIC1a-induced increases in [Ca^{2+}]_{i}, assuming NMDA receptor activation results in AKAP150 dissociation.

AKAP150 has been shown to associate with the plasma membrane in neurons by binding to the cytoskeleton via f-actin (Dell’Acqua et al., 1998; Dodge and Scott, 2000; Diviani and Scott, 2001; Feliciello et al., 2001; Wong and Scott, 2004). Latrunculin-A, a bioactive 2-thiazolidinone macrolide, reversibly disrupts the actin cytoskeleton resulting in AKAP dissociation away from the membrane (Allison et al., 1998; Sattler et al., 2000; Zhou et al., 2001; Popp and Dertien, 2008). Figures 3.5A and 3.5B show representative traces of [Ca^{2+}]_{i} as a function of time recorded during acidosis in the absence (Control) and presence of CBP (CBP, 50 μM) without (DMSO, A) and with latrunculin-A (5 μM, B) preincubation for 4 hours at 37°C. While latrunculin-A alone did not significantly reduce ASIC1a-mediated [Ca^{2+}]_{i} elevations, disruption of the neuronal cytoskeleton more substantially affected σ-1 receptor mediated inhibition of ASIC1a Ca^{2+} dysregulation (Figure 3.5C). In control cells CBP inhibited 47 ± 1% of acid-induced increases in [Ca^{2+}]_{i}, but only inhibited 29 ± 1% of these increases in latrunculin-A treated cells (Figure 3.5D). This inhibition was statistically significant. Disruption of the cytoskeleton by latrunculin-A was confirmed using phalloidin stain, which detects actin filaments cytochemically (Figure 3.6). Results showed that control cells had preserved cytoskeleton staining around the cell body and along the dendritic processes, while latrunculin-A treated cells
showed staining patterns consistent with retraction of the cytoskeleton from the processes and synapses (Figure 3.6).

Previous studies have demonstrated that the major component of acid-induced increases in neuronal [Ca\(^{2+}\)] is from influx through Ca\(^{2+}\) channels activated by ASIC1a-induced membrane depolarization. To isolate what direct effects disruption of the ω-1/G-protein/calcineurin/AKAP150 protein structure has on ASIC1a function, whole-cell patch clamp experiments using the perforated patch method were performed. Cells were voltage-clamped at -70 mV to prevent NMDA receptor and voltage-gated Ca\(^{2+}\) channel activation, thus isolating ASIC1a channels. In these experiments the pan-selective ω agonist DTG was used instead of the ω-1 selective agonist CBP. Figures 3.7A and 3.7B show superimposed ASIC1a currents evoked by application of low pH solution (pH = 6) in the absence (Control) and presence (DTG) of 100 μM DTG (DTG) without (A) and with (B) pertussis toxin (PTX, 24 hr preincubation at 37 °C, 200 ng/ml). Figure 3.7C shows a bar graph of mean percent inhibition (± SEM) of ASIC1a-mediated currents elicited by bath application of 100 μM DTG in neurons under control conditions and following preincubation in pertussis toxin (PTX, n = 7). Preincubation in PTX significantly decreased the effects of DTG on acid-evoked currents, 10% versus 30% inhibition, respectively (Figure 3.7B). Therefore, in cortical neurons, the effects of ω-1 receptors on ASIC1a currents are dependent on activation of a PTX-sensitive G protein.

VIVIT is peptide that selectively and potently inhibits calcineurin/AKAP interaction by disrupting calcineurin binding to a PxlxlIT-docking motif in AKAP
(Oliveria et al., 2007). Moreover, VIVIT does not affect phosphatase activity (Hogan et al., 2003; Im and Rao, 2004). To elucidate the effects of VIVIT on the signaling cascade linking ASIC1a channels to σ-1 receptors, cortical neurons were voltage-clamped at -70 mV to isolate ASIC1a currents. Figure 3.8A shows representative ASIC1a-mediated currents evoked by low pH solution in the absence (Control) and presence of CBP (CBP, 100 μM). In contrast, Figure 3.8B shows traces of ASIC1a-mediated current during acidosis from a different neuron in the absence (Control), presence of preincubation in the cell permeable peptide VIVIT (VIVIT, 10 nM, 5 minutes) and coapplication of VIVIT and CBP (VIVIT/CBP 100 μM). Analysis of the currents densities shows that while CBP significantly inhibited ASIC1a-mediated currents compared to control cells (Figure 3.8C), VIVIT alone or in combination with CBP had no affect on ASIC1a-mediated currents (Figure 3.8D). Taken together, these data, consistent with Ca²⁺ imaging results, suggest that σ-1 receptors couple to ASIC1a channels via a calcineurin/AKAP complex in addition to a PTX-sensitive G protein.
Figure 3.1 - Sigma-1 receptors colocalize with both ASIC1a channels and AKAP150 in cortical neurons. A, Representative fluorescent images of neurons triple labeled for σ-1 receptors(i), AKAP150 (ii) and ASIC1a channels (iii) in the same field of view. B, From A, merged image of σ-1 receptor and AKAP150. The white pixels represent areas in the image where σ-1 receptor and AKAP150 labeling appear together (B, i) as selected in the scatter plot (B, ii). The scatter plot maps pixel intensities from the σ-1 receptor labeling versus intensities from the AKAP150 labeling. The overlap coefficient for σ-1 receptor and AKAP150 colocalization for the entire image was calculated to be 0.74. C, From A, merged image of σ-1 receptor and ASIC1a labeling with colocalized pixels represented in white (C, i) and the colocalization scatter plot (C, ii). The overlap coefficient for σ-1 receptor and AKAP150 colocalization for the entire image was calculated to be 0.78. All scale bars (A, B, C) are 25 µm. D, Mean overlap coefficient for σ-1 receptor colocalization with AKAP150 and ASIC1a channels for individual cell bodies was calculated to be 0.723 ± .007 and 0.790 ± .008, and for defined dendritic processes 0.696 ± .013 and 0.751 ± .009, respectively.
Figure 3.2 - Calcineurin inhibition prevents \( \sigma-1 \) receptor modulation of ASIC1a.

A, Characteristic traces of \([\text{Ca}^{2+}]_i\) as a function of time recorded from three neurons in the absence (Control) and presence of 10 \( \mu \text{M} \) carbetapentane (CBP) during acidosis in normal PSS (i), or PSS containing 1 \( \mu \text{M} \) FK-506 (ii, FK-506) or 1 \( \mu \text{M} \) cyclosporin A (iii, Cyclo A). B, Mean peak proton-evoked changes in \([\text{Ca}^{2+}]_i\) recorded from neurons in the absence (Control) and presence of 10 \( \mu \text{M} \) carbetapentane (CBP) in normal PSS (n = 184), or PSS containing either 1 \( \mu \text{M} \) FK-506 (n = 216) or 1 \( \mu \text{M} \) cyclosporin A (n = 155). Asterisks denote significant differences from respective control groups (\( p < 0.05 \)), and pound symbols indicate significant differences from the PSS group within CBP (\( p < 0.05 \)). C, Bar graph of mean CBP-induced inhibitions of peak proton-evoked changes in \([\text{Ca}^{2+}]_i\) from the same experiments as B. Control group represents cells exposed to acidosis in PSS alone. Asterisks denote significant differences from control group (\( p < 0.05 \)).
A

i. PSS

ii. FK-506

iii. Cyclo A

B

\[ \Delta [\text{Ca}^{2+}] \text{(nM)} \]

- PSS
- FK-506
- Cyclo A

C

CBP-Induced Inhibition (%)

- Control
- FK-506
- Cyclo A

* Indicates statistical significance.
Figure 3.3 - Sigma-1 receptors inhibit acid-induced elevations in [Ca$^{2+}$]$_i$ via a PTX-sensitive G protein. A, Representative traces of [Ca$^{2+}$]$_i$ as a function of time recorded from a vehicle treated (PSS) neuron during acidosis in the absence (Control) and presence of 50 µM CBP (CBP). B, Representative traces of [Ca$^{2+}$]$_i$ as a function of time recorded from a different neuron during acidosis following preincubation in 200 ng/ml PTX (PTX, 24 hrs at 37° C) in the absence (Control) and presence of 50 µM CBP (CBP). C, Mean changes in peak [Ca$^{2+}$]$_i$ (± SEM) measured in response to acidosis with (PTX, n = 246) or without (PSS, n = 188) PTX preincubation in the absence (Control) and presence of 50 µM CBP (CBP). Asterisks denote significant differences from respective Control groups ($p < 0.05$), pound symbol indicates significant difference between PSS and PTX groups within CBP ($p < 0.05$), and dagger denotes significant difference between PSS and PTX groups within Control ($p < 0.05$). D, Percent inhibitions of [Ca$^{2+}$]$_i$ increases (± SEM) by CBP under control conditions (Control) and in the presence of PTX (PTX). Asterisk denotes significant difference between the groups ($p < 0.05$).
Figure 3.4 - AKAP150 dissociation from the plasma membrane prevents σ-1 receptor modulation of acid-induced elevations in [Ca\textsuperscript{2+}]\textsubscript{i}. A, Representative traces of [Ca\textsuperscript{2+}]\textsubscript{i} as a function of time recorded from a neuron during acidosis (PSS) in the absence (Control) and presence of 50 µM CBP (CBP). B, Representative traces of [Ca\textsuperscript{2+}]\textsubscript{i} as a function of time recorded from a different neuron during acidosis following preincubation in 10 µM NMDA (NMDA, 5 min -1 hr) in the absence (Control) and presence of 50 µM CBP (CBP). C, Mean changes in peak [Ca\textsuperscript{2+}]\textsubscript{i} (± SEM) measured in response to acidosis with (NMDA, n = 398) or without (PSS, n = 198) NMDA preincubation in the absence (Control) and presence of 50 µM CBP (CBP). Asterisks denote significant differences from respective Control groups (p < 0.05), pound symbol indicates significant difference between PSS and NMDA groups within CBP (p < 0.05), and dagger denotes significant difference between PSS and NMDA groups within Control (p < 0.05). D, Percent inhibition of [Ca\textsuperscript{2+}]\textsubscript{i} increases (± SEM) by CBP under control conditions (Control) and in the presence of NMDA (NMDA). Asterisk denotes significant difference between the groups (p < 0.05).
Figure 3.5 - Disruption of the actin cytoskeleton prevents σ-1 receptor inhibition of acid-induced increases in [Ca^{2+}]. A, Representative traces of [Ca^{2+}] as a function of time recorded from a vehicle (DMSO) treated neuron during acidosis in the absence (Control) and presence of 50 µM CBP (CBP). B, Representative traces of [Ca^{2+}] as a function of time recorded from a different neuron during acidosis following preincubation in 5 µM latrunculin A (Latrunculin A, 4 hrs at 37°C) in the absence (Control) and presence of 50 µM CBP (CBP). C, Mean changes in peak [Ca^{2+}] (± SEM) measured in response to acidosis with (Latrunculin A, n = 222) or without (DMSO, n = 276) latrunculin A preincubation in the absence (Control) and presence of 50 µM CBP (CBP). Asterisks denote significant differences from respective Control groups (p < 0.05), pound symbol indicates significant difference between DMSO and latrunculin A groups within CBP (p < 0.05), and dagger denotes significant difference between DMSO and latrunculin A groups within Control (p < 0.05). D, Percent inhibition of [Ca^{2+}] increases (± SEM) by CBP under control conditions (Control) and in the presence of latrunculin A (Latrunculin A). Asterisk denotes significant difference between the groups (p < 0.05).
Figure 3.6 - Latrunculin A preincubation disrupts the actin cytoskeleton. Cells were treated with either vehicle (A, DMSO) or latrunculin A (B, 5 μM) for 4 hours at 37°C. Cells were then stained with phalloidin to detect actin filaments cytochemically (A and B, right images). For comparison, bright field pictures are also shown (A and B, left images). All scale bars are 25 μm.
Figure 3.7 - Sigma receptor-mediated inhibition of ASIC1a currents are blocked by preincubation in pertussis toxin. A and B, Superimposed ASIC1a currents evoked by application of low pH solution (pH = 6) in the absence (Control) and presence of 100 μM DTG (DTG) from cortical neurons without (A) and with preincubation for 24 hrs (37 °C) in 200 ng/ml pertussis toxin (B, PTX). Cells were held at -70 mV. C, Bar graph of mean percent inhibitions (± SEM) of ASIC1a-mediated currents elicited by 100 μM DTG under control conditions (Control) or following preincubation in pertussis toxin (PTX), n = 7. Asterisk denotes significant difference from Control group (p < 0.05).
Figure 3.8 - Sigma-1 receptors couple to ASIC1a channels via an AKAP150/calcineurin complex. ASIC1a currents were elicited in whole-cell perforated patched, voltage-clamped (-70 mV) neurons by a 10 second application of pH 6.0 PSS. A, Representative traces of ASIC1a currents as a function of time recorded from a neuron in the absence (Control) and presence of 100 μM CBP (CBP). B, Representative traces of ASIC1a-mediated currents as a function of time recorded from a different cell in the absence (Control) and presence of 10 nM VIVIT (VIVIT) and during coapplication of VIVIT with 100 μM CBP (VIVIT/CBP). C, Mean peak proton-gated current densities (± SEM) measured from neurons without (Control) or with 100 μM CBP (CBP) in the bath solution (n = 6). Asterisks denote significant difference from respective Control group (p < 0.01). D, Mean peak proton-gated current densities (± SEM) recorded under control conditions, in the presence of VIVIT alone, and in the presence of VIVIT plus CBP (n = 5). There were no significant differences between Control, VIVIT and VIVIT/CBP groups, (p = 0.972).
Discussion

Results from this study suggest the signaling cascade linking $\sigma$-1 receptors to ASIC1a channels in rat cortical neurons involves direct protein-protein interactions involving PTX-sensitive G proteins, calcineurin and AKAP150. $\sigma$-1 receptors are shown to colocalize with both ASIC1a channels and the scaffolding protein AKAP150, not only in regions of the cell body but also along the dendritic processes of these cells. Like previous studies in our laboratory and others, which showed that $\sigma$ receptors affect ion channel function via mechanisms consistent with protein-protein interactions, $\sigma$-1 receptors modulate ASIC1a channels via a pertussis toxin-sensitive G protein-coupled signal transduction cascade. Furthermore, $\sigma$-1 receptors are also shown here to couple to ASIC1a channels via the second messenger calcineurin anchored to AKAP150. This coupling results in decreases in both ASIC1a-mediated currents and concomitant elevations in cytosolic $\text{Ca}^{2+}$ following $\sigma$-1 receptor activation.

$\sigma$ receptors are the only reported instance of receptor mediated downregulation of ASIC activity. Thus far, only the NMDA receptor, acting via a $\text{Ca}^{2+}$-calmodulin kinase II cascade (CaMKII), has been shown to modulate ASIC1a channels (Gao et al., 2005). Activation of NMDA receptors enhances ASIC1a-mediated currents, which consequently exacerbates acidotoxicity during ischemia (Gao et al., 2005). ASIC channels are also regulated in a similar manner by C kinase-1 (PICK-1), which binds to the C-terminus of several ASIC isoforms (Duggan et al., 2002). PICK1 has been shown to promote the stimulation of homomeric ASIC2a and heteromultimeric ASIC3/ASIC2b channels.
by protein kinase C (PKC\(\alpha\)) (Baron et al., 2002; Deval et al., 2004), while several protein kinase C isoforms (PKC\(\beta\)I or PKC\(\beta\)II) have also been shown to inhibit ASIC1 (Berdiev et al., 2002). Protein kinase A phosphorylation of ASIC interferes with the binding of PICK-1 to these channels, and disrupts PICK1-ASIC1 colocalization (Leonard et al., 2003). Furthermore, various signaling molecules have been linked to the regulation of ASIC. Three distinct kinases (CaMKII, protein kinase C and protein kinase A) have all been shown to modulate the function of ASIC, by direct phosphorylation of the channel (Baron et al., 2002; Leonard et al., 2003; Gao et al., 2005). Kinase anchoring proteins such as PICK-1 and AKAP150 have also been shown to bind to ASIC subtypes, and appear to increase currents through these channels by facilitating protein kinase C and protein kinase A phosphorylation of the channels, respectively (Baron et al., 2002; Deval et al., 2004; Chai et al., 2007). Conversely, inhibition of calcineurin has been shown to potentiate currents through ASIC1a and ASIC2a channels, and thus dephosphorylation of the channels by this phosphatase may be involved in downregulation of ASIC (Chai et al., 2007). Our results with the calcineurin inhibitors, cyclosporin A and FK-506, demonstrate that \(\sigma\)-1 receptor-mediated block of ASIC1a is dependent on activation of this phosphatase.

Interestingly, reports have shown that calcineurin may be activated by a pertussis toxin-sensitive G protein (Gromada et al., 2001). Consistent with this observation, our study shows that a pertussis toxin-sensitive G protein is involved in the signaling cascade coupling \(\sigma\)-1 receptors to ASIC1a. It has been proposed that \(\sigma\) receptors regulate ion channel function via protein-protein interactions.
Moreover, inhibition of G proteins either by cell dialysis with GDP-β-S or preincubation in pertussis toxin failed to affect σ-1 and σ-2 receptor modulation of voltage-gated K⁺ and Ca²⁺ channels in intracardiac neurons (Zhang and Cuevas, 2002; Zhang and Cuevas, 2005). To date, nearly all reports of σ receptor modulation of ion channels suggest that the effects involve a membrane-delimited signaling pathway which likely involves a protein-protein interaction. However, σ receptor activation has been shown to stimulate GTPase activity in mouse prefrontal membranes (Tokuyama et al., 1997). A pertussis toxin-sensitive G protein has been implicated in the modulation of NMDA receptors by σ receptors in rat CA3 dorsal hippocampus neurons (Monnet et al., 1994), whereas a cholera toxin-sensitive G protein has been suggested to couple σ-1 receptors to the channels mediating the A-current in frog pituitary melanotopes (Soriani et al., 1999). However, others have argued that σ receptors do not couple to G proteins (Hopf et al., 1996). Our findings demonstrate that σ-1 receptors can modulate ion channel function via activation of a PTX-sensitive G protein, and these results are the first evidence of a receptor coupling to ASIC1a through this mechanism.

AKAP150 has been shown to be involved in the regulation of receptor activity and localization, and in the regulation of synaptic structure during developmental synapse formation, in synaptic plasticity in learning and memory, and neuronal dysfunction and cell death during pathophysiological conditions (Dell’Acqua et al., 2006). Additionally, a calcineurin/AKAP150 complex has been shown to modulate both ASIC1a and ASIC2a function (Chai et al., 2007). Our
immunohistochemical studies suggest that \( \sigma \)-1 receptors colocalize with both ASIC1a channels and AKAP150 in the plasma membrane of the cell body and along the neuronal processes of the cells. Furthermore, the similarity of the distributions of \( \sigma \) receptors colocalized with ASIC1a channels and with AKAP150 is consistent with previous studies showing colocalization of ASIC channels and AKAP (Chai et al., 2007). Disruption of the actin cytoskeleton by chemical agents or by NMDA activation has been shown to result in the redistribution of AKAP150 away from the plasma membrane (Gomez et al., 2002). Our data shows that both of these manipulations significantly prevents \( \sigma \)-1 receptor modulation of ASIC1a-induced \([Ca^{2+}]_i\) elevations. The small but significant block by NMDA of \( \sigma \)-1 receptor modulation of ASIC1a can probably be attributed to additional effects of Ca\(^{2+}\) overload following preincubation in NMDA which led to increases in basal calcium levels.

Previous results in our laboratory have shown that most of the elevations in \([Ca^{2+}]_i\), triggered by acidosis are the result of Ca\(^{2+}\) channels (NMDA and AMPA receptors, voltage-gated Ca\(^{2+}\) channels) opening downstream of ASIC1a activation, and that activation of \( \sigma \)-1 receptors effectively suppresses these secondary Ca\(^{2+}\) fluxes. AKAP150 has been shown to modulate glutamate receptors like the internalization of AMPA receptors and NMDA receptors during long term potentiation or depression (Rosenmund et al., 1994; Westphal et al., 1999; Colledge et al., 2000; Gomez et al., 2002; Smith et al., 2006). In addition, AKAP also regulates voltage-gated Ca\(^{2+}\) channel function (Oliveria et al., 2007). Data presented here shows that \( \sigma \)-1 receptors functionally couple to ASIC1a...
channels via AKAP150, which is consistent with all of these observations. Moreover, these results also raise the possibility that AKAP150 is a component in the modulation of other ion channels (voltage-gated Ca\textsuperscript{2+} channels) and receptors (NMDA and AMPA) by \sigma-1 receptors.

The strongest evidence that \sigma-1 receptors couple to ASIC1a channels via a calcineurin/AKAP150 complex comes from experiments using the VIVIT peptide. Whole-cell patch clamp experiments with VIVIT suggest that when this peptide competes with calcineurin for binding to the PxIxIT-like motif in AKAP150 and thus prevents calcineurin binding to AKAP150, \sigma receptor activation loses its ability to regulate ASIC1a function. The disruption of this interaction prevents \sigma-1 receptors from functionally coupling to ASIC1a channels. Similarly, VIVIT has been implicated in the disruption of calcineurin/AKAP150 modulation of L-type Ca\textsuperscript{2+} channels in hippocampal neurons (Oliveria et al., 2007).

In conclusion, our data show that the signaling cascade between \sigma-1 receptors and ASIC1a channels involves a PTX-sensitive G protein and an AKAP150/calcineurin complex in cortical neurons. This coupling results in decreases in both ASIC1a-mediated membrane currents and concomitant elevations in cytosolic Ca\textsuperscript{2+} following \sigma-1 receptor activation. Moreover, calcineurin may be a potential component of the neuroprotective properties of \sigma receptors. Furthermore, our results also raise the possibility of \sigma-1 receptors coupling to downstream Ca\textsuperscript{2+} channels (voltage-gated Ca\textsuperscript{2+} channels) and receptors (NMDA and AMPA) via a similar signaling cascade. All of these channels and receptors have been implicated in pathophysiological conditions,
such as stroke (Tanaka et al., 1997; Tanaka et al., 1999; Tanaka et al., 2002). σ-1 receptors are the first receptor shown to downregulate ASIC1a channel function and remain as the only receptor identified thus far to negatively modulate ASIC1a channels.
CHAPTER 4

SIGMA-1 RECEPTOR INHIBITION OF INTRACELLULAR Ca^{2+} DYSREGULATION DUE TO SYNERGISTIC INTERACTION BETWEEN ACIDOSIS AND ISCHEMIA

Introduction

Glucose and oxygen deprivation associated with brain ischemia initiates a switch in metabolism from aerobic to anaerobic glycolysis to produce cellular energy. The accumulation of lactic acid, the end product of anaerobic glycolysis, leads to acidosis in the ischemic region, activating Acid-Sensing Ion Channels (ASIC) (Xiong et al., 2004; Gao et al., 2005; Pignataro et al., 2007). Synaptic vesicles contain not only the neurotransmitter glutamate but also protons that are released along with glutamate during neurotransmission. It remains to be elucidated if protons released during neurotransmission also activate ASIC.

ASIC subtypes are distinguishable by the pH of half-maximal activation, Ca^{2+} permeability and tissue expression pattern. The predominant ASIC subtype in the central nervous system (CNS) contains the ASIC1a subunit, which can form homomultimeric or heteromultimeric channels with ASIC2a (Askwith et al., 2004). ASIC1a channels are activated by pH ≤ 7 and have a pH of half-maximal activation of ~ 6.0 - 6.5 (Waldmann et al., 1997b; Xiong et al., 2004), which is similar to the pH seen during an ischemic insult (Nedergaard et al., 1991; Back et
The homomeric ASIC1a channel is the only ASIC subtype that is highly permeable to both Na\(^+\) and Ca\(^{2+}\) ions (Xiong et al., 2004; Yermolaieva et al., 2004).

ASIC1a has been implicated in various neuronal physiological processes such as synaptic plasticity, fear conditioning, and learning and memory (Wemmie et al., 2002; Wemmie et al., 2003; Wemmie et al., 2004). ASIC1a has also been shown to be activated following cerebral ischemia, and has been linked to neuronal cell death (Xiong et al., 2004; Gao et al., 2005; Pignataro et al., 2007). Compared to wild-type mice, transgenic mice deficient in ASIC1a have reduced infarct size in response to middle cerebral artery occlusion (MCAO) (Xiong et al., 2004). Furthermore, pharmacological inhibition of ASIC1a with either the non-selective Na\(^+\) channel blocker amiloride or the homomultimeric ASIC1a selective inhibitor psalmotoxin1 (Diochot et al., 2007), diminishes ischemic brain injury (Xiong et al., 2004). Thus, there is a direct correlation between infarct size, brain acidosis and ASIC activation, suggesting the acidotoxicity occurring during stroke is in part mediated by ASIC1a channels (Xiong et al., 2004).

The demise of neurons during ischemia and acidosis is predicated by intracellular calcium overload (Xiong et al., 2004; Yermolaieva et al., 2004). Our laboratory has shown that σ-1 receptors inhibit Ca\(^{2+}\) dysregulation evoked by ischemia (Katnik et al., 2006) as well as acidosis, and that activation of σ receptors is neuroprotective at delayed time points in a rat model of ischemic stroke (Ajmo et al., 2006). Moreover, σ-1 receptors regulate ionotropic glutamate receptors, voltage-gated K\(^+\) channels and voltage-gated Ca\(^{2+}\) channels (Hayashi
et al., 1995; Aydar et al., 2002; Zhang and Cuevas, 2002; Zhang and Cuevas, 2005). The inhibition of ionotropic glutamatergic receptors by σ receptors prevents elevations in [Ca^{2+}], associated with glutamate-induced excitotoxicity (Klette et al., 1995; Klette et al., 1997). All of these voltage-gated ion channels as well as NMDA receptors have been shown to contribute to the demise of neurons during ischemia (Tanaka et al., 2002). Consistent with this observation, our laboratory has also shown that most of the elevations in [Ca^{2+}]_{i} triggered by acidosis are the result of Ca^{2+} channels (NMDA and AMPA receptors and voltage-gated Ca^{2+} channels) opening following ASIC1a activation, and stimulation of σ-1 receptors effectively suppressed these secondary Ca^{2+} fluxes by inhibiting both ASIC1a and these Ca^{2+} channels directly.

Previous reports have indicated that the glucose-oxygen deprivation model of ischemia potentiates ASIC1a-mediated currents in neurons (Xiong et al., 2004). Moreover, it has also been shown that ischemia enhances ASIC1a currents through phosphorylation of the channel by CaMKII as a result of activation of NMDA receptor (Gao et al., 2005). But how ASIC1a activation affects the responses to ischemia and whether acidosis has any temporal effects during ischemia remains to be determined. Furthermore, it is unknown whether acidosis and ischemia interact in terms of the Ca^{2+} dysregulation produced. Interestingly, ASIC have been shown to remain active for greater than 4 hours following an ischemic insult (Pignataro et al., 2007). Pharmacological blockade of ASIC1a by amiloride or PcTx1 administered even 5 hours after MCAO has been shown to diminish stroke injury (Simon and Xiong, 2006; Pignataro et al., 2007).
These observations raise the possibility that σ receptors may provide neuroprotection when stimulated several hours after an ischemic insult by directly inhibiting ASIC1a channels as well as Ca^{2+} channels opening in response to membrane depolarizations produced by ASIC1a activation.

Experiments were undertaken to determine whether ASIC1a activation and ischemia interact to produce potentiated elevations in \([\text{Ca}^{2+}]_i\) and to determine if activation of σ-1 receptors is able to block this synergistic potentiation. Ratiometric Ca^{2+} fluorometry experiments demonstrated that acidification of the extracellular solution from pH 7.4 to 6.0 produced a potentiation of the ischemia-induced elevations in \([\text{Ca}^{2+}]_i\). Inhibition of ASIC1a channels with either amiloride or PcTx1, significantly decreased ischemia-induced elevations in \([\text{Ca}^{2+}]_i\) at pH values ranging from 7.4 to 6.0, suggesting that homomeric ASIC1a channels are activated during ischemia alone and that these channels contribute to the pH dependence of these \([\text{Ca}^{2+}]_i\) increases. Furthermore, inhibition of synaptic transmission with tetrodotoxin prevented ischemia-evoked increases in \([\text{Ca}^{2+}]_i\), but the effect of tetrodotoxin was overcome by acidification of the extracellular solution (pH 6.0). The selective σ-1 receptor agonist, carbetapentane, significantly decreased ischemia-mediated Ca^{2+} dysregulation at all pH values tested (7.4-6.0), suggesting that activation of σ-1 receptors modulate ischemic- and ASIC1a-activated \([\text{Ca}^{2+}]_i\) increases individually while also effecting the mechanism mediating the synergistic interaction between these two initiators of Ca^{2+} influx pathways in cortical neurons.
Materials and Methods

Primary Rat Cortical Neuron Preparation

Primary cortical neurons from embryonic (E18) rats were cultured as previously described by our laboratory (Katnik et al., 2006). All procedures were done in accordance with the regulations of the University of South Florida Institutional Animal Care and Use Committee. Cells were used after 10-21 days in culture.

Calcium Imaging Measurements

The effects of acidosis and chemical ischemia on intracellular Ca\(^{2+}\) concentrations were examined in isolated cortical neurons using fluorescent imaging techniques. Cytosolic free-Ca\(^{2+}\) was measured using the Ca\(^{2+}\) sensitive dye, fura-2. The membrane permeable ester form of fura-2, fura-2 AM, acetoxymethyl ester (AM), was loaded and imaged as we have previously described (DeHaven and Cuevas, 2004). Cells plated on coverslips were incubated for 1 hour at room temperature in Neurobasal (Invitrogen) medium supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine, or in physiological saline solution (PSS) consisting of (in mM): 140 NaCl, 5.4 KCl, 1.3 CaCl\(_2\), 1.0 MgCl\(_2\), 20 glucose, and 25 HEPES (pH to 7.4 with NaOH). Both solutions contained 3 \(\mu\)M fura-2, acetoxymethyl ester (fura-2 AM) and 0.3 % dimethyl sulfoxide. The coverslips were washed in PSS (fura-2-AM free) prior to experiments being performed.
Electrophysiology Recordings

Na⁺-mediated membrane currents were recorded using a protocol previously described by our laboratory (Dr. Hongling Zhang, unpublished data). Briefly, neurons plated on glass coverslips were transferred to a recording chamber and membrane currents were amplified, filtered at 1 kHz, digitized at 5 kHz, and acquired using Clampex 8 (Axon). Cells were patch-clamped using the conventional dialysis whole-cell configuration and voltage-clamped at -90 mV. Na⁺ currents were activated by stepping cells to -10 mV for 250 msec. The control pipette solution consisted of (in mM): 130 CsCl, 10 NaCl and 10 HEPES (titrated to pH 7.2 with CsOH). The extracellular solution consisted of (in mM): 72 NaCl, 79 TEA-Cl, 5 KCl, 1.4 CaCl₂, 1 MgCl₂, 10 glucose, 5 BaCl₂, 0.1 CdCl₂, and 10 HEPES (titrated to pH 7.4 or 6.0 with TEA-OH). Both the external and internal solutions were modified from other protocols (Dr. Hongling Zhang, unpublished data, and (Mike et al., 2003)). Patch electrodes were pulled from thin-walled borosilicate glass (World Precision Instruments Inc., Sarasota, FL) using a Sutter Instruments P-87 pipette puller (Novato, CA) and had resistances of 2.0 – 5.0 MΩ. Access resistances (Rₛ) were monitored throughout experiments for stable values ≤ 20 MΩ and were always compensated at 40% (lag, 10 µs). All cells were voltage-clamped at -90 mV.

Solutions and Reagents

The control bath solution for all experiments was PSS. All drugs were applied in this solution using a rapid application system identical to that previously described (Cuevas and Berg, 1998). ASIC activation was induced by
applying PSS buffered to pH values of 7.4, 7.0, 6.5 and 6.0 (+/- drug). Chemical ischemia was induced by applying glucose-free PSS containing 4mM azide (+/- drug) and titrated to pH 7.4, 7.0, 6.5, and 6.0. Individual cells were exposed to ≤ 3 ischemic or acidic+ischemic insults to prevent rundown of responses (Katnik et al., 2006). All chemicals used in this investigation were of analytical grade. The following drugs were used: carbetapentane (Tocris Bioscience, Ellisville, MO); psalmotoxin1 venom (Spider Pharm, Yarnelle, AZ); psalmotoxin1 peptide (Peptide International, Louisville, KY); tetrodotoxin (Alomone Labs, Jerusalem, Israel); amiloride (Alexis Biochemicals, Lausen, Switzerland); sodium-azide (Sigma-Aldrich, St. Louis, MO); and fura-2 AM (Molecular Probes, Eugene, OR).

Data Analysis

Analysis of measured intracellular Ca$^{2+}$ responses and Na$^{+}$-current activation was performed using Clampfit 9 (Axon instruments). Imaging data files collected with SlideBook 4.02 (Intelligent Imaging Innovations, Inc.) were converted to a text format and imported into Clampfit for subsequent analysis. Statistical analysis was conducted using SigmaPlot 9 and SigmaStat 3 software (Systat Software, Inc.). Statistical differences were determined using paired and unpaired t-tests for within group and between group experiments, respectively, and were considered significant if $p < 0.05$. For multiple group comparisons either a 1-way or a 2-way ANOVA, with or without repeat measures, were used, as appropriate. When significant differences were determined with an ANOVA, post-hoc analysis was conducted using a Tukey Test to determine differences between individual groups.
Results

The low extracellular pH associated with ischemia can activate ASIC (Xiong et al., 2004; Gao et al., 2005; Pignataro et al., 2007), and ASIC-mediated currents are potentiated during ischemia (Xiong et al., 2004; Gao et al., 2005). During neurotransmission, protons are released along with glutamate but it remains to be elucidated whether these protons can activate ASIC at physiological pH values. Experiments were carried out to determine if ASIC are activated during ischemia at normal physiological pH (7.4) and to determine the effects of pathophysiological pH values (7.0 - 6.0) during ischemia on [Ca\textsuperscript{2+}]i dysregulation. Figures 4.1A and 4.1B show representative traces of [Ca\textsuperscript{2+}]i as a function of time evoked by ischemia at pH 7.4 (A) and pH 6.0 (B) in the absence (Control) and presence of the ASIC blocker amiloride (100 µM). Similar experiments demonstrate that amiloride significantly inhibits ischemia-induced peak elevations in [Ca\textsuperscript{2+}]i by 48% in solutions buffered to pH 7.4 (Figure 4.1C). These results indicate that protons released along with glutamate during neurotransmission under physiological conditions activate ASIC. The application of ischemia + acidosis resulted in more robust peak increases in [Ca\textsuperscript{2+}]i, and these elevations were also inhibited by amiloride (Figure 4.1C). These data suggest that ASIC are activated during ischemia and potentiate ischemia-induced Ca\textsuperscript{2+} dysregulation.

The kinetics of the Ca\textsuperscript{2+} responses to the combination of ischemia + acidosis are noticeably different from those to ischemia or acidosis alone. To compare the kinetics of these responses, a single cell was exposed to acidosis
(pH 6.0), ischemia at pH 7.4, and ischemia at pH 6.0. Figure 4.2A shows representative traces of $[\text{Ca}^{2+}]_i$ as a function of time from a single neuron during 2 minute applications of acidosis (pH 6.0 PSS), ischemia at pH 7.4 (Ischemia), and ischemia at pH 6.0 (Acidosis + Ischemia) separated by 10 minute recovery periods. Acidosis alone produced an initial transient increase in $[\text{Ca}^{2+}]_i$ with a rapid desensitization of the $\text{Ca}^{2+}$ influx resulting in a return to basal levels within seconds of the acid application. In contrast, ischemia alone resulted in smaller initial transient increase in $[\text{Ca}^{2+}]_i$ that decreased to a sustained elevated level which returned to basal levels only upon washout of the ischemia solution. The combination of acidosis + ischemia produced a significant potentiation in the initial transient peak $[\text{Ca}^{2+}]_i$ elevations, which rapidly decreased to an elevated concentration which monotonically increased in the continual presence of the acidosis + ischemia solution. Upon washout of this solution, there was a transient rebound increase in $[\text{Ca}^{2+}]_i$ which then slowly decayed to baseline levels. To quantitate the net elevations in the cytosolic $\text{Ca}^{2+}$ concentration during these stimulations, increases in $[\text{Ca}^{2+}]_i$ were integrated over the time period surrounding the acidosis + ischemia challenge yielding the area under the curve. Figure 4.2B shows that there is a synergistic increase in $[\text{Ca}^{2+}]_i$ in response to the combination of ischemia + acidosis when compared to the responses observed to acidosis and ischemia alone. These results indicate that not only does ischemia enhances ASIC activation, as previously reported (Xiong et al., 2004; Gao et al., 2005), but that acidosis potentiates the response to ischemia. This is
the first report showing the role of acidosis during ischemia and the effects of ASIC activation on ischemia-induced [Ca\textsuperscript{2+}]i elevations.

To better understand this synergistic interaction between acidosis and ischemia, experiments were performed to measure the [Ca\textsuperscript{2+}]i responses to ischemic insults as a function of pH. Four parameters were used to compare these responses: (1) magnitude of the initial [Ca\textsuperscript{2+}]i peak, (2) the steady state level of [Ca\textsuperscript{2+}]i at the end of the ischemia application, (3) the peak magnitude of the rebound transient [Ca\textsuperscript{2+}]i increase following washout of the ischemia solution, and (4) the net Ca\textsuperscript{2+} elevations measured in the cytosol as determined by integrating the area under the [Ca\textsuperscript{2+}]i vs. time curve. Results from multiple experiments show a significant increase in initial [Ca\textsuperscript{2+}]i peak (Figure 4.3A), rebound [Ca\textsuperscript{2+}]i peak (Figure 4.3C) and area (Figure 4.3D) as the pH of the ischemia solution was reduced from 7.4 to 6.0. Interestingly, the steady state level of [Ca\textsuperscript{2+}]i obtained at the end of azide application increased when the pH was reduced from 7.4 to 7.0, but then remained constant as the pH was further reduced to 6.0 (Figure 4.3B). These data suggest that ischemia-induced increases in [Ca\textsuperscript{2+}]i are pH dependent. Furthermore, acidosis significantly affects ischemia-induced [Ca\textsuperscript{2+}]i elevations such that greater synergy is observed as the pH decreased from 7.4 to 6.0.

Psalmotoxin1 (PcTx1) from the venom of the tarantula *Psalmopoeus cambridgei* has been shown to be a selective inhibitor of homomultimeric ASIC1a channels (Diochot et al., 2007). To confirm that ASIC1a channels were mediating this synergistic potentiation of changes in [Ca\textsuperscript{2+}]i during ischemia at low pH
values, cells were preincubated in the absence (PSS, Control) and presence of 
PcTx1 venom (PcTx1) in solutions buffered to the indicated pH values. The 
venom was present in the conditioning solution (PSS pH 7.4) as well as all the 
ischemia solutions (pH 7.4 - 6.0). PcTx1 venom inhibited the initial peak 
elevations in [Ca$^{2+}$], to a statistically significant degree at all pH values tested 
(Figure 4.4A). The venom also blocked the rebound increases in [Ca$^{2+}$], following 
washout of the ischemia solution at pH values lower than 7.0 (Figure 4.4C). 
Interestingly, at the lower pH values, the PcTx1 venom is unable to block 
elevated steady state levels of [Ca$^{2+}$], or the net [Ca$^{2+}$], elevation (area) in 
response to ischemia + acidosis (Figure 4.4B and 4.4D). This observation could 
be explained as a result of PcTx1 inducing a shift in the steady-state 
desensitization of the channel. In contrast, amiloride which directly blocks the 
channel, does inhibit steady state [Ca$^{2+}$], elevations. These results show that 
inhibition of ASIC1a channels prevents acidosis-associated synergistic 
potentiation of [Ca$^{2+}$], dysregulation during ischemia.

As a negative control, PcTx1 venom was heat inactivated to denature all 
proteins. Figures 4.5A and 4.5B show representative traces of [Ca$^{2+}$], as a 
function of time recorded from a neuron during ischemia at pH 7.0 and from a 
second cell during ischemia + acidosis in the absence (Control) and presence of 
heat inactivated PcTx1 venom (h.PcTx1). After multiple experiments, changes in 
initial peak, steady state and rebound [Ca$^{2+}$], and net [Ca$^{2+}$], (area) (± SEM) were 
measured in response to ischemia at pH 7.0 and ischemia at pH 6.0 (Figure 4.5C
and 4.5D). These results show that heat inactivated PcTx1 venom do not significantly affect ischemia-induced [Ca^{2+}]_i elevations at pH 7.0 or 6.0.

Because PcTX venom is a cocktail of toxins (Psalmotoxin 1 (PcTx1), Psalmopeotoxin I (PcFK1), Psalmopeotoxin II (PcFK2), Vanillotoxin 1 (VaTx1), Vanillotoxin 2 (VaTx2), Vanillotoxin 3 (VaTx3)) experiments were performed with pure PcTx1 peptide during acidosis alone (pH 6.0), ischemia at pH 7.0 and ischemia at pH 6.0. Figures 4.6A and 4.6B show representative traces of [Ca^{2+}]_i as a function of time recorded from a neuron during ischemia at pH 7.0 and from a second neuron during ischemia + acidosis in the absence (Control) and presence of synthetic PcTx1 peptide (s.PcTx1). Similar to results from the venom experiments, PcTx1 peptide significantly inhibited changes in initial peak, steady state and rebound [Ca^{2+}]_i, and net [Ca^{2+}]_i (area) (± SEM) measured in response to ischemia pH 7.0 (Figure 4.6C). Furthermore, the peptide inhibited initial and rebound [Ca^{2+}]_i peaks in response to ischemia at pH 6.0 (Figure 4.6D). These data confirm the results shown in Figure 4.4 were due to the activity of PcTx1 and not the other toxins in the venom. Therefore, ASIC1a channels are mediating the synergistic potentiation seen during the combination of ischemia and acidosis.

The synergistic interaction between ischemia and acidosis raises the possibility that ASIC1a channels promote long-lived priming of presynaptic vesicles, and could thus, enhance release of neurotransmitter if stimulated prior to an ischemic event. To test this hypothesis, cells were exposed to acid alone prior to the ischemia application. Figure 4.7A shows representative traces of
[Ca$^{2+}$], as a function of time recorded from a neuron exposed to ischemia (black trace) and from a different cell exposed to a 10 second acid application prior to ischemia (gray trace). Analysis of the data comparing the two ischemia applications suggest that ASIC1a activation alone does not promote long-lived vesicle priming since the mean changes in peak [Ca$^{2+}$], were comparable (Figure 4.7B). These data suggest that ASIC1a activation alone prior to ischemia does not result in greater glutamate release. Furthermore, these data are consistent with results presented in this study and confirm that the synergistic potentiation in Ca$^{2+}$ observed is the result of the combination of ischemia + acidosis and that for the potentiation to occur, the events must take place simultaneously. Consistent with this observation, ischemia potentiates ASIC1a-mediated currents following OGD preincubation (Xiong et al., 2004).

To further substantiate that the concurrent incidence of ischemia and acidosis is required to produce synergistic potentiation of Ca$^{2+}$ dysregulation, the temporal effects of acidosis on the ischemia responses were studied. Figure 4.7C shows representative traces of [Ca$^{2+}$], as a function of time recorded from a cell exposed to ischemia alone (black trace) and from a second neuron exposed to ischemia followed by the combination of ischemia + acidosis (gray trace). Addition of protons resulted in a statistically significant increases in mean changes in [Ca$^{2+}$], in peak, steady state, rebound peak, and net [Ca$^{2+}$], (area) (Figure 4.7D). These data confirm that the synergistic potentiation of Ca$^{2+}$ dysregulation is due to the interaction between ischemia and acidosis. Moreover,
these results indicate that ASIC1a channels can be activated again after the initial ischemic onset, exacerbating ischemia-induced Ca\textsuperscript{2+} dysregulation.

Next we wanted to determine the mechanism responsible for the rebound increase in [Ca\textsuperscript{2+}], following washout of ischemia and acidosis. Figure 4.7E shows representative traces of [Ca\textsuperscript{2+}], as a function of time recorded from a neuron exposed to the combination of ischemia + acidosis (black trace) and from a second cell exposed to the combination of ischemia + acidosis then rapidly switched to ischemia (pH 7.4) alone (gray trace). In identical experiments, populations of cells exposed to these two treatments did not exhibit significant differences comparing mean changes in peak, steady state and net [Ca\textsuperscript{2+}], (area) (Figure 4.7F). In contrast, mean changes in rebound were significantly different (Figure 4.7F). Thus, these results suggest that the relief of proton block of downstream Ca\textsuperscript{2+} channels allows for the influx of Ca\textsuperscript{2+} contributing to the rebound [Ca\textsuperscript{2+}], elevations.

σ receptor activation has been shown to modulate multiple ion channels which are opened following an ischemic insult. Figure 4.8 shows bar graph representation of data from multiple experiments in the absence (Control) and presence of the σ-1 receptor agonist carbetapentane (CBP) during ischemia at the indicated pH values. CBP significantly inhibited the initial transient ischemia-induced [Ca\textsuperscript{2+}], elevations (Figure 4.8A), consistent with previous results showing σ-1 receptor inhibition of ASIC1a-activated Ca\textsuperscript{2+} increases. Similarly, CBP significantly reduced steady state (Figure 4.8B) and rebound (Figure 4.8C) levels of [Ca\textsuperscript{2+}], induced by ischemia at all pH values tested, consistent with σ-1
receptor inhibition of Ca\textsuperscript{2+} channels activated by ASIC1a-mediated depolarization. Likewise, CBP reduced the net [Ca\textsuperscript{2+}], elevations (area), suggesting σ-1 receptor activation not only inhibit Ca\textsuperscript{2+} influx pathways but may also modulate release from intracellular stores and/or extrusion pathways (exchangers and ATPases) (Figure 4.8D). These results show that σ-1 receptor activation prevents the synergistic interaction between acidosis and ischemia. Moreover, the σ-1 selective ligand inhibits ischemia-induced Ca\textsuperscript{2+} dysregulation at all pH values (7.4 - 6.0).

Tetrodotoxin (TTX) is an inhibitor of voltage-gated Na\textsuperscript{+} channels, and thus, subsequent synaptic transmission. The activation of postsynaptic Ca\textsuperscript{2+} channels and receptors following ASIC1a stimulation has been observed even when neuronal conduction is inhibited by TTX, suggesting a presynaptic localization of ASIC1a channels. Therefore, experiments were conducted to determine the effects of presynaptic ASIC1a channels during ischemia in the presence of TTX. Figures 4.9A and 4.9B show representative traces of [Ca\textsuperscript{2+}]\textsubscript{i} as a function of time evoked by ischemia pH 7.4 (A) and ischemia pH 6.0 (B) in the absence (Control) and presence of tetrodotoxin (TTX, 500 nM). Similar to earlier reports, addition of TTX prevented ischemia-induced elevations in [Ca\textsuperscript{2+}]\textsubscript{i} by 69 ± 2% when compared to control responses (Figure 4.9C and 4.9D) (Katnik et al., 2006). While in response to ischemia + acidosis, the effects of TTX on [Ca\textsuperscript{2+}]\textsubscript{i} dysregulation were diminished, reducing only 9 ± 2% of the initial peak increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Figure 4.9C and 4.9D). These data suggest that presynaptic ASIC1a channels promote synaptic transmission during ischemia, and thus, overcome
block of synaptic transmission and enhance postsynaptic [Ca^{2+}]_i increases. This observation is consistent with the hypothesis that ASIC1a channels are located presynapticly and regulate neurotransmitter release probability (Cho and Askwith, 2008).

To confirm that the lack of TTX block of ischemia-induced increases in [Ca^{2+}]_i by acidosis was not due to TTX being pH sensitive, whole-cell voltage-clamp experiments were performed to measure voltage-gated Na^+ currents in the absence and presence of TTX at pH 7.4 and 6.0. Cells were patch-clamped using the conventional dialysis whole-cell configuration and voltage-clamped at -90 mV. Na^+ currents were activated by stepping cells to -10 mV for 250 msec. Figure 4.10A show representative current traces recorded from a single cell in the absence (Control) and presence of TTX (TTX, 500 nM) at pH 7.4 (i) and pH 6.0 (ii). Analysis of the measured current densities shows that TTX significantly inhibits Na^+ currents at pH 7.4 and pH 6.0 (Figure 4.10 B and 4.10C). Consistent with reports in the literature, our data also suggest that Na^+ channels are pH sensitive (Figure 4.10B). Results from recordings of peak inward Na^+ currents from 12 cells showed that TTX (500 nM) inhibited I_{Na} by 96.1 ± 0.9% at pH 7.4 and 96.6 ± 1.0% at pH 6.0 (Figure 4.10C). No significant difference was noted between TTX-evoked inhibition at pH 7.4 and 6.0 (p = 0.68) (Figure 4.10C). In conclusion, our data indicate that TTX inhibition of voltage gated Na^+ currents is not pH sensitive, and thus, confirm that presynaptic ASIC1a channels promote synaptic transmission during ischemia by overcoming block of neurotransmission by TTX and enhancing postsynaptic [Ca^{2+}]_i increases.
Figure 4.1 - ASIC activation contributes to ischemia-evoked [Ca\textsuperscript{2+}]i increases in cultured rat cortical neurons. A, Representative traces of [Ca\textsuperscript{2+}]i as a function of time recorded from a single cell during ischemia at pH 7.4 in the absence (Control) and presence of 100 μM amiloride (Amiloride). B, Representative traces of [Ca\textsuperscript{2+}]i as a function of time recorded from a single neuron during ischemia and acidosis (pH 6.0) in the absence (Control) and presence of 100 μM amiloride (Amiloride). C, Mean changes in peak [Ca\textsuperscript{2+}]i (± SEM) measured in response to ischemia at pH 7.4 (n = 182) and pH 6.0 (n = 166) in the absence (Control) and presence of 100 μM Amiloride. Asterisks denote significant differences from respective Control groups (p < 0.001) and pound symbols indicates significant difference between pH 6.0 and pH 7.4 within Control and Amiloride groups (p < 0.001).
Figure 4.2 - ASIC activation and ischemia interact to produce a synergistic potentiation of elevations in $[\text{Ca}^{2+}]_i$. A, Representative traces of $[\text{Ca}^{2+}]_i$ as a function of time recorded from a single cell during the indicated conditions. B, Quantification of net $[\text{Ca}^{2+}]_i$ elevations from multiple experiments identical to that in (A) ($n = 84$). Net $[\text{Ca}^{2+}]_i$ elevation is calculated by integrating $\Delta[\text{Ca}^{2+}]_i$ over time. Asterisks denote significant differences from the Acidosis group ($p < 0.001$), and pound symbols indicates significant difference from Ischemia group ($p < 0.001$).
Figure 4.3 - Protons potentiate ischemia-induced increases in [Ca^{2+}]. Mean change in initial peak (A), steady state (B) and rebound peak (C) [Ca^{2+}], and net increase in [Ca^{2+}], (area, D) (± SEM) measured in response to ischemia at pH 7.4 (n = 80), pH 7.0 (n = 120), pH 6.5 (n = 120) and pH 6.0 (n = 121). Asterisks denote significant difference from pH 7.4 group (p < 0.05), pound symbols indicate significant differences from pH 7.0 group (p < 0.05), and daggers denote significant differences from pH 6.5 group (p < 0.05).
Figure 4.4 - Inhibition of homomeric ASIC1a channels decreases ischemia + acidosis-induced elevations in [Ca^{2+}]_i at pH values ranging from 7.4 to 6.0. Mean change in initial peak (A), steady state (B) and rebound peak (C) [Ca^{2+}]_i, and net [Ca^{2+}]_i increase (area, D) (± SEM) measured in response to ischemia at pH 7.4 (n = 72), pH 7.0 (n = 87), pH 6.5 (n = 62) and pH 6.0 (n = 215) in the absence (Control) and presence of 500 ng/ml PcTx1 venom (PcTx1). Asterisks denote significant differences from pH 7.4 group (p < 0.05) and pound symbols indicates significant differences from respective Control groups (p < 0.05).
Figure 4.5 - Heat inactivated PcTx1 venom does not inhibit ischemia-induced increases in $[\text{Ca}^{2+}]_i$. Representative traces of $[\text{Ca}^{2+}]_i$ as a function of time recorded from a neuron during ischemia at pH 7.0 (A) and from a second cell during ischemia + acidosis (B) in the absence (Control) and presence of heat inactivated PcTx1 venom (h.PcTx1). Mean changes in initial peak (peak), steady state (SS) and rebound (Rbd) $[\text{Ca}^{2+}]_i$, and net $[\text{Ca}^{2+}]_i$ elevation (area) ($\pm$ SEM) measured in response to ischemia pH 7.0 (C, $n = 79$) and ischemia pH 6.0 (D, $n = 89$). There is no significant difference between any of the groups.
Figure 4.6 - PcTx1 peptide inhibits ischemia-induced elevations in $[\text{Ca}^{2+}]_i$.

Representative traces of $[\text{Ca}^{2+}]_i$ as a function of time recorded from a neuron during ischemia at pH 7.0 (A) and from a second cell during ischemia + acidosis (B) in the absence (Control) and presence of synthetic PcTx1 peptide (s.PcTx1). Mean changes in initial peak (peak), steady state (SS) and rebound (Rbd) $[\text{Ca}^{2+}]_i$, and net $[\text{Ca}^{2+}]_i$ elevation (area) ($\pm$ SEM) measured in response to ischemia pH 7.0 (C, $n = 165$) and ischemia pH 6.0 (D, $n = 171$). Asterisks denote significant difference between groups ($p < 0.001$).
Figure 4.7 - Temporal effects of acidosis on ischemia-induced Ca^{2+} dysregulation. A, Representative traces of [Ca^{2+}]_i as a function of time recorded from a cell during ischemia pH 7.4 (black trace, protocol A) and from a second neuron during a 10 second acid application followed by ischemia pH 7.4 (gray trace, protocol B). B, Mean changes in initial peak (peak), steady state (SS) and rebound (Rbd) peak [Ca^{2+}]_i, (nM), and net [Ca^{2+}]_i elevation (area, nM * min) (± SEM) measured in response to ischemia pH 7.4 (protocol A, n = 81) and acid followed by ischemia pH 7.4 (protocol B, n = 108). Area was calculated using the two minute application of control protocol (A). Asterisks denote significant differences between the groups in peak [Ca^{2+}]_i (p < 0.05), rebound [Ca^{2+}]_i peak (p < 0.001) and area (p < 0.001). C, Representative traces of [Ca^{2+}]_i as a function of time recorded from a cell during ischemia pH 7.4 (black trace, protocol A) and from another neuron during ischemia pH 7.4 followed by ischemia + acidosis (gray trace, protocol C). D, Mean changes in initial ischemia pH 7.4 and ischemia + acidosis peaks (Peak), steady state (SS) and rebound (Rbd) [Ca^{2+}]_i, (nM), and net elevation in [Ca^{2+}]_i (area, nM * min) (± SEM) measured in response to ischemia pH 7.4 (protocol A, n = 49) and ischemia pH 7.4 followed by ischemia + pH 6.0 (protocol C, n = 149). Area was calculated using the two minute application of control protocol (A). Asterisks denote significant differences between the groups (p < 0.001). E, Representative traces of [Ca^{2+}]_i as a function of time recorded from a neuron during ischemia + acidosis (black trace, protocol D) and from a second cell during ischemia + acidosis followed by ischemia pH 7.4 (gray trace, protocol E). F, Mean changes in initial peak (Peak), steady state...
(SS) and rebound (Rbd) [Ca^{2+}]_i (nM), and net [Ca^{2+}]_i elevation (area, nM * min) (± SEM) measured in response to ischemia + acidosis (protocol D, n = 79) and ischemia + pH 6.0 followed by ischemia at pH 7.4 (protocol E, n = 294). Area was calculated using the two minute application of control protocol (D). Asterisks denote significant differences between the groups (p < 0.001).
Figure 4.8 - Sigma-1 receptor activation inhibits ischemia-mediated Ca$^{2+}$ dysregulation at pH values ranging from 7.4 to 6.0. Mean changes in initial peak (A), steady state (B) and rebound peak (C) [Ca$^{2+}$]$_i$ and net [Ca$^{2+}$]$_i$ elevation (area) (D) (± SEM) measured in response to ischemia at pH 7.4 (n = 141), pH 7.0 (n = 110), pH 6.5 (n = 196) and pH 6.0 (n = 181) in the absence (Control) and presence of 50 µM carbetapentane (CBP). Asterisks denote significant differences from pH 7.4 group ($p < 0.05$) and pound symbols indicate significant differences from respective Control groups ($p < 0.05$).
Figure 4.9 - Effects of synaptic transmission inhibition are overcome by presynaptic ASIC1a channels. A, Representative traces of [Ca$^{2+}$]$_i$ as a function of time recorded from a neuron during ischemia (pH 7.4) in the absence (Control) and presence of 500 nM tetrodotoxin (TTX). B, Representative traces of [Ca$^{2+}$]$_i$ as a function of time recorded from a different cell during the combination of ischemia and acidosis (pH 6.0) in the absence (Control) and presence of 500 nM tetrodotoxin (TTX). C, Mean changes in peak [Ca$^{2+}$]$_i$ (± SEM) measured in the absence (Control) and presence of TTX (TTX, 500 nM) in response to ischemia (pH 7.4, n = 126) and ischemia + acidosis (pH 6.0, n = 240). D, Percent inhibition of changes in peak [Ca$^{2+}$]$_i$ by TTX measured during ischemia at pH 7.4 and pH 6.0. Asterisks denote significant differences from respective Control groups ($p < 0.001$).
Figure 4.10 - TTX inhibits Na\(^+\) currents at pH 7.4 and 6.0. A, Representative Na\(^+\) current traces as a function of time recorded from a single cell in the absence (Control) and presence of TTX (TTX, 500 nM) at pH 7.4 (\(i\)) and pH 6.0 (\(ii\)). Cells were patch-clamped using the conventional dialysis whole-cell configuration and voltage-clamped at -90 mV. Na\(^+\) currents were activated by stepping cells to -10 mV for 250 msec. B, Mean peak voltage-gated Na\(^+\) current densities (± SEM) measured from neurons under the indicated conditions (n = 12). Asterisks denote significant difference from respective Control group (\(p < 0.01\)) and pound symbols indicate significant difference between pH 7.4 and pH 6.0 within Control (\(p < 0.01\)). C, Percent inhibition of Na\(^+\) currents (± SEM) by TTX at pH 7.4 and pH 6.0. There was no significant difference between pH 7.4 and pH 6.0 groups, (\(p = 0.683\)).
Discussion

The results from this study demonstrate that in rat cortical neurons, acidosis and ischemia synergistically interact to produce potentiated elevations in [Ca\(^{2+}\)], that are inhibited by \(\sigma-1\) receptor activation. Acidification of the ischemia-inducing extracellular solution to pathophysiological pH values (pH \(\leq 7.0\)) produced elevations in [Ca\(^{2+}\)] that were greater than the sum of the changes evoked by acidosis and ischemia alone. Moreover, the kinetics of these responses were significantly different. Inhibition of ASIC1a channels with either amiloride or PcTx1, significantly decreased ischemia-induced increases in [Ca\(^{2+}\)] at pH values ranging from 7.4 to 6.0, suggesting that homomeric ASIC1a channels are activated during ischemia and that these channels contribute to the pH dependency of these [Ca\(^{2+}\)] increases. While our data indicate that activation of ASIC1a channels does not induce long-lived priming of synaptic vesicles for release, channel activation does have a temporal effect on ischemia-mediated [Ca\(^{2+}\)] increases after ischemia onset. Moreover, relief of proton block of Ca\(^{2+}\) influx pathways mediates the rebound of [Ca\(^{2+}\)] following washout of the acidic ischemia solution. The selective \(\sigma-1\) receptor agonist, carbetapentane, decreased ischemia-mediated Ca\(^{2+}\) dysregulation at all pH values tested (7.4-6.0). TTX was shown to block \(~70\%\) of the initial ischemia-induced [Ca\(^{2+}\)] increases when the external solution was buffered to 7.4, but only produced \(~10\%\) block when the solution was acidified to pH 6.0, suggesting that presynaptic ASIC1a channels promote synaptic transmission which leads to postsynaptic [Ca\(^{2+}\)] increases.
Previous studies have shown that ischemia and the acidosis that accompanies ischemia, produces marked increases in \([\text{Ca}^{2+}]_i\) (Katnik et al., 2006), which is one of the main mechanisms leading to cell death (Tombaugh and Sapolsky, 1993; Murai et al., 1997; Mattson, 2000; Xiong et al., 2004; Yermolaieva et al., 2004). ASIC1a-mediated currents in neurons have also been shown to be potentiated by oxygen-glucose deprivation (Xiong et al., 2004; Gao et al., 2005). Though the effects of ASIC1a currents during ischemia have been established, this study is the first report showing how ASIC1a activation affects the responses to ischemia and how acidosis and ischemia interact with each other to produce a synergistic dysregulation in \([\text{Ca}^{2+}]_i\). Our in vitro model of ischemia using the combination of azide in glucose-free PSS and acidosis (pH 6.0), resembles in vivo models of stroke since similar pH values have been observed during ischemia in whole animal studies (Nedergaard et al., 1991). Together, ischemia and acidosis produced elevations in \([\text{Ca}^{2+}]_i\) that were greater than the sum of the changes evoked by acidosis and ischemia alone. There was also a noticeable difference in the kinetics of the observed \([\text{Ca}^{2+}]_i\) transients resulting from acidosis, ischemia, and ischemia + acidosis. These differences cannot be explained by ischemia potentiating ASIC currents because ASIC1a channels desensitize within seconds. Thus, we conclude the effects seen minutes after the initial insult (steady state, rebound and net \(\text{Ca}^{2+}\) elevations) following ischemia + acidosis are the result of ASIC1a activation potentiating ischemia-mediated \([\text{Ca}^{2+}]_i\) increases. Therefore, ischemia and ASIC1a activation interact to produce potentiated elevations in \([\text{Ca}^{2+}]_i\), dysregulation.
The fact that ASIC1a inhibitors amiloride and PcTx1 reduce the ischemia-induced increases in \([\text{Ca}^{2+}]_i\), when the external solution was buffered to pH 7.4 suggests that the acidosis produced by metabolic inhibition is not responsible for ASIC1a activation. Instead, it is reasoned that protons released from glutamate containing synaptic vesicles during neurotransmission (DeVries, 2001; Traynelis and Chesler, 2001) are the source of ASIC1a channel activation. Therefore, there are two sources of protons associated with ischemia. First, an internal source as glucose and oxygen deprivation initiates a metabolic switch to anaerobic glycolysis to produce cellular energy leading to the accumulation of lactic acid, the end product of anaerobic glycolysis. Second, protons released along with glutamate during synaptic transmission. In our model, ischemia produces increased action potential firing and thus acidification of the synaptic cleft and the accumulation of lactic acid is mimicked by buffering external solutions to pH 6.0. Increased neurotransmission would activate more ASIC1a channels, inducing a feed-forward mechanism which promotes further synaptic transmission and greater \(\text{Ca}^{2+}\) accumulation. These increases in \([\text{Ca}^{2+}]_i\), if of sufficient duration, would produce \(\text{Ca}^{2+}\) overload and eventually lead to cell death. Consistent with these conclusions, the interaction between ischemia and acidosis leads to the synergistic potentiation of \(\text{Ca}^{2+}\) dysregulation, which is what is expected to be seen in vivo following an ischemic stroke (Tombaugh and Sapolsky, 1993; Murai et al., 1997; Mattson, 2000; Xiong et al., 2004; Yermolaieva et al., 2004).
The $[\text{Ca}^{2+}]_i$ responses to ischemia + acidosis were characterized by four parameters, the initial transient peak amplitude, the steady state level just prior to washout of the ischemia + acid solution, the transient rebound amplitude following washout and the net $[\text{Ca}^{2+}]_i$ elevations, calculated by integrating $\Delta[\text{Ca}^{2+}]_i$ for the duration of the response. The strongest evidence that the initial peak in ischemia-induced $[\text{Ca}^{2+}]_i$ elevations reported here is due to activation of homomeric ASIC1a channels is the inhibition produced by both amiloride and the selective ASIC1a channel blocker PcTx1 (Diochot et al., 2007). The steady state $[\text{Ca}^{2+}]_i$ is a function of the activated $\text{Ca}^{2+}$ influx and efflux pathways. Interestingly, the steady state level of $[\text{Ca}^{2+}]_i$ obtained at the end of ischemia applications increased as the pH was reduced from 7.4 to 7.0, but then remained constant as the pH was reduced to 6.0. Previous studies suggest most of the elevations in $[\text{Ca}^{2+}]_i$ triggered by acidosis are the result of $\text{Ca}^{2+}$ channels (NMDA and AMPA receptors and voltage-gated $\text{Ca}^{2+}$ channels) opening in response to ASIC1a mediated membrane depolarization. These results suggest that acidosis results in increased ASIC1a channel opening but the effects of pH are countered by increased $\text{H}^+$ block of other ion channels. The antioxidant MnTMPyP (25 nM) did not significantly affect the transient rebound increase in $[\text{Ca}^{2+}]_i$, suggesting that reactive oxygen species production do not account for the rebound mechanism. In contrast, the transient rebound increase in $[\text{Ca}^{2+}]_i$ observed during washout of acidosis + ischemia solution is due to the removal of a block of $\text{Ca}^{2+}$ influx pathways by protons as the proton concentration is reduced to pH 7.4. Since this feature of the $[\text{Ca}^{2+}]_i$ response is not observed following applications of ischemia
at pH 7.4, it is unlikely that when observed at lower pH values it is due to the washout of the ischemia solution. The transient rebound is expected to be more robust at the lower pH values due to a greater proton block of these Ca\(^{2+}\) channels being relieved.

Conditions which produce large net [Ca\(^{2+}\)]\(_i\), elevations, or Ca\(^{2+}\) overload, are likely to lead to apoptosis. Acidosis alone, while producing a large [Ca\(^{2+}\)]\(_i\) increase, has a very small net Ca\(^{2+}\) elevation because the transient increase is short lived. Likewise, while ischemia produces a maintained elevated [Ca\(^{2+}\)]\(_i\) level in the presence of the ischemia solution, it is only a moderately high level which returns quickly to baseline upon washout, and thus results in a small net elevation. The synergistic interaction of acidosis and ischemia, on the other hand, produces potentiated increases at all times during the agonist-evoked application as well as an extenuated rebound increase and a slower decay back to baseline. All these contribute to produce net elevations in [Ca\(^{2+}\)]\(_i\), which are ~400\% times greater than that for acidosis or ischemia alone. This pronounced Ca\(^{2+}\) overload would be more likely to trigger apoptosis.

PcTx1 from the venom of the tarantula *Psalmopoeus cambridgei* has been shown to be a selective blocker of homomultimeric ASIC1a channels with no effect on other ASIC subtypes (1b, 2a or 3) or voltage-gated channels (Na\(^+\), K\(^+\), Ca\(^{2+}\)) (Diochot et al., 2007). PcTx1 induces a shift in the steady-state desensitization of the channel. At normal pH (7.4) this shift puts ASIC1a channels in the inactive state (Chen et al., 2005). When studying ASIC1a activation, it is sufficient to include the toxin only in the conditioning media
because the on and off rates are slow compared to the rate of channel
desensitization (Chen et al., 2005; Chen et al., 2006). This also avoids the
problem of the peptide being degraded in the low pH solutions. For this study,
however, the venom was included in the conditioning media as well as the test
solutions because the cellular response to ischemia lasted for the duration of the
ischemia application. PcTx1 venom blocks the initial ischemia + acidosis-
mediated \([\text{Ca}^{2+}]_i\) increases as well as rebound and the elevated steady state
levels at pH values > 6.5. Thus, blocking ASIC1a channels significantly
prevented activation of the downstream \(\text{Ca}^{2+}\) channels responsible for the initial
\([\text{Ca}^{2+}]_i\) peak as well as disrupting the synergistic interaction between acidosis and
ischemia. Moreover, experiments performed with a synthetic PcTx1 peptide
provided similar results to those obtained with the venom. Therefore, ASIC1a
channels are mediating the synergistic potentiation seen during the combination
of ischemia and acidosis.

Similar to earlier reports, blockade of voltage-gated \(\text{Na}^+\) channels with
TTX and subsequent synaptic transmission inhibition, prevents ischemia-induced
elevations in \([\text{Ca}^{2+}]_i\) at physiological pH (Katnik et al., 2006). Interestingly, with
the more acidic conditions present during the combination of ischemia and
acidosis at pH 6.0, the inhibition of \([\text{Ca}^{2+}]_i\) increases by TTX were overcome,
most likely by the additional activation of ASIC1a channels. This observation
suggests a presynaptic localization of ASIC1a, whereby activation of the channel
by protons results in synaptic transmission and subsequent activation of
postsynaptic receptors. Consistent with this theory, the activation of postsynaptic
Ca\(^{2+}\) channels and receptors following ASIC1a stimulation during acidosis alone (pH 6.0) are observed even when neuronal conduction is inhibited by TTX. It has been proposed that ASIC1a activation may facilitate neurotransmission by compensating for the decrease in excitatory neurotransmission caused by direct inhibition of postsynaptic Na\(^{+}\) and Ca\(^{2+}\) channels by protons which are released during exocytosis (Krishtal et al., 1987; Zha et al., 2006). Thus, action potential inhibition alone is not sufficient to prevent Ca\(^{2+}\) overload, as ASIC1a channel activation is able to overcome this block of glutamate release by depolarizing the presynaptic membrane to potentials capable of activating voltage-gated Ca\(^{2+}\) channels and thus, evoking synaptic transmission. In addition, these results also raise the possibility of ASIC1a activation resulting in enough Ca\(^{2+}\) influx to promote synaptic transmission and glutamate release. Similarly, heteromeric complexes of alpha 5 and/or alpha 7 subunits of nicotinic receptors have been shown to conduct Ca\(^{2+}\) and thus, resulting in nicotine-induced presynaptic facilitation (Girod et al., 1999).

σ receptors have been identified in both pre and postsynaptic membranes (Gonzalez-Alvear and Werling, 1995; Alonso et al., 2000), modulating ion channels in both regions. σ receptor activation has been shown to modulate the function of multiple ion channels, several of which have been linked to neuronal death following an ischemic insult. Studies have shown that the affinity of carbetapentane for σ-1 receptors is >50-fold greater than for σ-2 receptors (Rothman et al., 1991; Vilner and Bowen, 2000). The concentrations of CBP used in this study are consistent with CBP acting as a σ-1 receptor agonist.
Moreover, similar concentrations of CBP have previously been shown to inhibit ischemia-evoked increases in [Ca^{2+}]_i (Katnik et al., 2006) as well as ASIC1a-induced [Ca^{2+}]_i increases and membrane currents. Unlike the results seen with inhibition of ASIC1a by PcTx1 venom, CBP activation of σ-1 receptors is pH insensitive and significantly inhibited the synergistic increases in [Ca^{2+}]_i produced by ischemia and acidosis as measured by initial peak amplitude, steady state, rebound and net elevations of [Ca^{2+}].

In conclusion, σ-1 receptors inhibit [Ca^{2+}]_i increases following ASIC1a channel activation, ischemia-induced increases in [Ca^{2+}]_i as well as the synergistic potentiation during ischemia + acidosis. Because acidic conditions exist during ischemic episodes, σ-1 receptors should be targeted for development of treatments for stroke and other pathophysiological conditions where ASIC1a channel activation are involved. σ-1 receptor mediated inhibition of Ca^{2+} channels activated by membrane depolarizations produced by ASIC1a channel activation or metabolic inhibition and the ASIC1a channels themselves, may contribute to the enhanced neuronal survival observed following administration of σ receptor agonists 24 hr post-stroke in rats (Ajmo et al., 2006). For these reasons, σ-1 receptors should be targeted for therapeutic intervention during ischemia, expanding the therapeutic window of stroke treatment.

The finding that ASIC1a activation at any point during an ischemic insult results in synergistic [Ca^{2+}]_i increases has significant physiological and pathophysiological implications. Our data indicate that though ASIC1a channels rapidly desensitize, they are able to be re-activated minutes into the ischemic
This suggests that during pathophysiological conditions it is possible to get repeated activation of ASIC1a channels if the extracellular media is buffered back to normal pH for even a short time. Even more dramatic though, is that under ischemic conditions these repetitive activations of ASIC1a channels synergistically interact with ischemia to produce potentiated elevations in peak, steady state, and rebound [Ca^{2+}]_i elevations and net [Ca^{2+}]_i elevation (area), suggesting that to prevent the consequences of Ca^{2+} overload during ischemia it is critical to control ASIC1a activation. Consistent with these results, it has been suggested that the acidosis that occurs in vivo during ischemia exacerbates ischemic injury at later time points after the initial ischemic insult. The finding that ASIC1a activation interacts with ischemia to produce synergistic potentiation of Ca^{2+} dysregulation is a novel finding and suggests that this channel should be targeted even at delayed time points after stroke onset to prevent Ca^{2+} overload and neuronal apoptosis.
CHAPTER 5

DISCUSSION

Conclusions

Stroke remains the 3rd leading cause of death and the leading cause of long term disability in the United States. Ischemic stroke, as a result of an embolus and restriction of blood flow to the brain, is the most common type of stroke accounting for over 80% of these cases. Thus far, only one drug (tissue plasminogen activator, tPA) has been approved by the FDA for the treatment of ischemia. This study has determined how σ-1 receptors modulate ASIC1a channels during physiological and pathophysiological conditions, and also suggest that this regulation could be one of the mechanisms of neuroprotection by σ receptor activation. Thus, σ receptors could be a potential therapeutic target for the treatment of stroke at delayed time points.

ASIC are regulated by various factors such as pH, membrane distention and arachidonic acid, and therefore, function as signal integrators in the CNS (Allen and Attwell, 2002; Lopez, 2002), but these factors elicit or potentiate ASIC-mediated responses. NMDA receptors modulate ASIC1a function via the activation of a CaMKII signaling cascade, but activation of this pathway results in an increase in currents through ASIC1a (Gao et al., 2005). Similarly, ASIC are
also regulated by C kinase-1 (PICK-1), which binds to the C-terminus of several ASIC isoforms (Duggan et al., 2002). PICK1 has been shown to promote the stimulation of homomeric ASIC2a and heteromultimeric ASIC3/ASIC2b channels by protein kinase C (Baron et al., 2002; Deval et al., 2004), while several protein kinase C isoforms have also been shown to inhibit ASIC1 (Berdiev et al., 2002). Protein kinase A phosphorylation of ASIC interferes with the binding of PICK-1 to these channels, and disrupts PICK1-ASIC1 colocalization (Leonard et al., 2003). Three distinct kinases (CaMKII, protein kinase C and protein kinase A) have all been shown to modulate the function of ASIC, by direct phosphorylation of the channel (Baron et al., 2002; Leonard et al., 2003; Gao et al., 2005). Kinase anchoring proteins such as PICK-1 and AKAP150 have also been shown to bind to ASIC subtypes, and appear to increase currents through these channels by facilitating protein kinase C and protein kinase A phosphorylation of the channels, respectively (Baron et al., 2002; Deval et al., 2004; Chai et al., 2007). Conversely, activation of σ receptors depresses ASIC1a-mediated responses and remains the only reported instance of receptor mediated downregulation of ASIC activity.

The responses observed throughout these studies are specifically mediated by ASIC1a channels and this conclusion is supported by the inhibition produced by amiloride and the selective ASIC1a channel blocker, PcTx1 venom as well as the PcTx1 peptide (Diochot et al., 2007). Furthermore, cultured cortical neurons from embryonic mice deficient in the ASIC1a subunit fail to show increases in [Ca\textsuperscript{2+}]\textsubscript{i} or membrane currents at the same proton concentrations
used here (Xiong et al., 2004). ASIC2a and ASIC2b subunits are also expressed
in the CNS, but homomeric ASIC2a channels are activated below pH 5.5, and
ASIC2b does not generate currents in response to low pH (Lingueglia et al.,
1997). In addition, neither homomeric ASIC2a nor heteromultimeric
ASIC1a/ASIC2a channels conduct Ca^{2+}, and thus could not account for the
changes in [Ca^{2+}], observed (Yermolaieva et al., 2004). However, PcTx1 itself
may not be an ideal pharmacological agent for stroke patients because of its
size, stability and inability to cross the blood brain barrier (Xiong et al., 2008).
Therefore, the development of better compounds is undergoing for the treatment
of stroke.

The σ-1 receptor subtype modulates neuronal responses to ASIC1a
activation. Studies have shown that the affinity of carbetapentane for σ-1
receptors is >50-fold greater than for σ-2 receptors (Rothman et al., 1991; Vilner
and Bowen, 2000). The calculated IC_{50} for carbetapentane inhibition of ischemia-
evoked increases in [Ca^{2+}], via σ-1 receptor activation is 18.7 µM (Katnik et al.,
2006), which is comparable to the 13.8 µM IC_{50} for CBP inhibition of ASIC1a-
induced [Ca^{2+}] increases. Carbetapentane also inhibits epileptiform activity in rat
hippocampal slices via σ-1 receptors with an IC_{50} value of 38 µM (Thurgur and
Church, 1998). Similarly, other σ-1 agonists, dextromethorphan (IC_{50} = 22 µM)
and PRE-084 (IC_{50} = 13.7 µM), both of which have >100-fold greater affinities for
σ-1 than σ-2 receptors, blocked ASIC1a-mediated responses at concentrations
consistent with those reported in the literature. Dextromethorphan inhibits
spreading depression in rat neocortical brain slices with an IC_{50} ~ 30 µM
(Anderson and Andrew, 2002), whereas PRE-084 protects human retinal cells against oxidative stress with an IC$_{50}$ ~ 10 $\mu$M (Bucolo et al., 2006). The IC$_{50}$ values determined here for carbetapentane, dextromethorphan and PRE-084 are in the low $\mu$M range and suggests that it is unlikely these agonists are affecting ASIC1a activity via $\sigma$-2 receptors, since high $\mu$M to mM concentrations of these compounds are required to stimulate $\sigma$-2 receptors. Moreover, $\sigma$-2-selective agonists (ibogaine and PB28) failed to inhibit ASIC1a-mediated responses at concentrations consistent with $\sigma$-2 specific effects and in a metaphit-insensitive manner.

Pharmacological studies with the $\sigma$ antagonists, metaphit and BD1063, confirm that $\sigma$-1 receptor activation modulates ASIC1a channels. Metaphit has been shown to bind irreversibly to $\sigma$-1 receptors with an IC$_{50}$ value of 50 $\mu$M (Wu, 2003). Preincubation in metaphit blocks $\sigma$-1 receptor modulation of voltage-gated K$^+$ channels in intracardiac neurons and depression of ischemia-induced elevations in [Ca$^{2+}$]$_i$ in cortical neurons (Zhang and Cuevas, 2005; Katnik et al., 2006). Preincubation of cortical neurons in 50 $\mu$M metaphit antagonized CBP inhibition of ASIC1a by ~ 40%. BD1063 has a higher affinity for $\sigma$-1 than $\sigma$-2 receptors and attenuates the dystonia produced by DTG in rats in a dose-dependent manner, suggesting this ligand acts as an antagonist at $\sigma$ sites (Matsumoto et al., 1995). CBP is unable to block acid-induced increases in [Ca$^{2+}$]$_i$ when co-applied with BD1063, suggesting the effects are mediated by $\sigma$-1 receptors. In addition, metaphit fails to inhibit the effects of the $\sigma$-2 agonist, PB28, on ASIC1a-mediated responses. Taken together, these data show that
increases in [Ca^{2+}] in response to ASIC1a activation are modulated only by σ-1 receptors.

Inhibition of the second messenger calcineurin has been shown to potentiate currents through ASIC1a and ASIC2a channels, and thus dephosphorylation of the channels by this phosphatase may be involved in downregulation of ASIC (Chai et al., 2007). The calcineurin inhibitors, cyclosporin A and FK-506, demonstrate that σ-1 receptor-mediated block of ASIC1a is dependent on activation of this phosphatase (Figure 5.1). Interestingly, reports have shown that calcineurin may be activated by a pertussis toxin-sensitive G protein (Gromada et al., 2001). Consistent with this observation, the effects of σ receptor activation on ASIC1a-mediated [Ca^{2+}] increases are significantly lessened in cortical neurons following preincubation in pertussis toxin, suggesting a pertussis toxin-sensitive G protein is involved in the signaling cascade coupling σ-1 receptors to ASIC1a-mediated responses.

This study demonstrates that σ-1 receptors modulate ASIC1a channel function via activation of a PTX-sensitive G protein, and these results are the first evidence of a receptor coupling to ASIC1a through this mechanism (Figure 5.1). σ receptors regulate ion channel function via protein-protein interactions (Aydar et al., 2002). Moreover, inhibition of G proteins either by cell dialysis with GDP-β-S or preincubation in pertussis toxin failed to affect σ-1 and σ-2 receptor modulation of voltage-gated K^+ and Ca^{2+} channels in intracardiac neurons (Zhang and Cuevas, 2002; Zhang and Cuevas, 2005). To date, nearly all reports of σ receptor modulation of ion channels suggest that the effects involve a
membrane-delimited signaling pathway which could involve a protein-protein interaction. However, σ receptor activation has been shown to stimulate GTPase activity in mouse prefrontal membranes (Tokuyama et al., 1997). A pertussis toxin-sensitive G protein has been implicated in the modulation of NMDA receptors by σ receptors in rat CA3 dorsal hippocampus neurons (Monnet et al., 1994), whereas a cholera toxin-sensitive G protein has been suggested to couple σ-1 receptors to the channels mediating the A-current in frog pituitary melanotopes (Soriani et al., 1999). However, others have argued that σ receptors do not couple to G proteins (Hopf et al., 1996).
Figure 5.1 - Signaling cascade linking ASIC1a channels and sigma-1 receptors. Activation of σ-1 receptors inhibits ASIC1a-induced Ca^{2+} dysregulation via a PTX-sensitive G protein and a calcineurin/AKAP complex, resulting in a decrease in [Ca^{2+}]i elevations. Sigma-1 receptors directly couple to ASIC1a channels via a PTX-sensitive G protein and a calcineurin/AKAP complex.
Sigma-1 Receptor Modulation of ASIC1a-induced Ca$^{2+}$ Dysregulation
AKAP150 is involved in the regulation of receptor activity and localization, and in the regulation of synaptic structure during developmental synapse formation, in synaptic plasticity in learning and memory, and neuronal dysfunction and cell death during pathophysiological conditions (Dell’Acqua et al., 2006). σ-1 receptors colocalize with both ASIC1a channels and AKAP150 in the plasma membrane of the cell body and along the neuronal processes of the cells. Consistent with this finding, a calcineurin/AKAP150 complex has been shown to modulate both ASIC1a and ASIC2a function (Chai et al., 2007). Furthermore, the similarity of the distributions of σ receptors colocalized with ASIC1a channels and with AKAP150 is consistent with previous studies showing colocalization of ASIC channels and AKAP (Chai et al., 2007). Disruption of the actin cytoskeleton by chemical agents or NMDA receptor activation, results in the redistribution of AKAP150 away from the plasma membrane (Gomez et al., 2002), and thus, preventing σ-1 receptor modulation of ASIC1a-induced $[Ca^{2+}]_i$ dysregulation (Figure 5.1).

Depletion of $Ca^{2+}$ from intracellular stores indicates that most, if not all, of the acid-induced increases in $[Ca^{2+}]_i$ is due to plasma membrane influx. Multiple ion channels downstream of ASIC1a activation were shown to contribute to acidosis-induced elevations in $[Ca^{2+}]_i$, including NMDA and AMPA/kainate receptors and VGCC. σ receptors have been identified in both presynaptic and postsynaptic sites (Gonzalez-Alvear and Werling, 1995; Alonso et al., 2000), and thus may modulate channels in both regions. In the presence of specific inhibitors of ionotropic glutamate receptors, activation of σ-1 receptors with CBP
further decreased proton-evoked increases in [Ca\(^{2+}\)], but the effects of CBP and the glutamate channel inhibitors were less than additive. Thus, σ-1 receptors also inhibit Ca\(^{2+}\) entry via NMDA and AMPA/kainate receptors directly by inhibiting these channels and indirectly by depressing ASIC1a activation. Application of the L-type VGCC inhibitor, nifedipine, and the broad-spectrum Ca\(^{2+}\) channel inhibitor, cadmium, blocked ASIC1a-induced increases in [Ca\(^{2+}\)], by >70% and >90%, respectively. This observation indicates that most of the increases in [Ca\(^{2+}\)] produced upon ASIC1a activation is dependent on Ca\(^{2+}\) influx through VGCC. Co-application of CBP with nifedipine, but not with Cd\(^{2+}\), resulted in further reduction in the proton-evoked increases in [Ca\(^{2+}\)].

The activation of NMDA and AMPA/kainate receptors following ASIC1a stimulation was observed even when neuronal conduction was inhibited with tetrodotoxin. This observation suggests a presynaptic localization of ASIC1a, whereby activation of the channel by protons results in synaptic transmission and subsequent activation of postsynaptic glutamatergic receptors. Consistent with this hypothesis, ASIC1a has been found to regulate neurotransmitter release probability in mouse hippocampal neurons (Cho and Askwith, 2008).

AKAP150 has been shown to modulate the internalization of AMPA receptors, NMDA receptors during long term potentiation and depression (Rosenmund et al., 1994; Westphal et al., 1999; Colledge et al., 2000; Gomez et al., 2002; Smith et al., 2006) and voltage-gated Ca\(^{2+}\) channels function (Oliveria et al., 2007). Since most of the elevations in [Ca\(^{2+}\)] triggered by acidosis are the result of Ca\(^{2+}\) channels opening downstream of ASIC1a activation, and
stimulation of \(\sigma\)-1 receptors effectively suppresses these secondary \(Ca^{2+}\) fluxes, these results also raise the possibility that AKAP150 is a constituent in the modulation of these downstream ion channels (e.g. voltage-gated \(Ca^{2+}\) channels) and receptors (e.g. NMDA and AMPA) by \(\sigma\)-1 receptors (Figure 5.1).

Simultaneous \(Ca^{2+}\) fluorometry and whole-cell patch clamp recordings confirmed that \(Ca^{2+}\) influx through ASIC1a channels itself contributed only a small fraction to the total observed \([Ca^{2+}]_i\) increases. Cells voltage-clamped at -70 mV, which prevents NMDA receptor and VGCC activation, demonstrated minimal acid-evoked elevations in \([Ca^{2+}]_i\). Thus, the increases in \([Ca^{2+}]_i\) evoked by ASIC1a activation are the result of synaptic transmission and subsequent opening of multiple \(Ca^{2+}\) channels, and that stimulation of \(\sigma\)-1 receptors downregulates all of these events. However, the fact that activation of \(\sigma\)-1 receptors depresses ASIC1a-mediated currents in cells voltage-clamped at -70 mV indicates that \(\sigma\)-1 receptors are functionally coupled to ASIC1a, and that the depression in acid-evoked increases in \([Ca^{2+}]_i\) is not exclusively the result of \(\sigma\)-1 receptors blocking channels downstream of ASIC1a.

The effects of \(\sigma\) receptor activation on ASIC1a-mediated membrane currents are significantly lessened in cortical neurons following preincubation in pertussis toxin. These results suggest a pertussis toxin-sensitive G protein is involved in the signaling cascade coupling \(\sigma\) receptors to ASIC1a channels (Figure 5.1). Moreover, \(\sigma\)-1 receptors couple to ASIC1a channels via a calcineurin/AKAP150 complex resulting in a decrease in acid-induced membrane currents (Figure 5.1). Whole-cell patch clamp experiments with VIVIT suggest
that when this peptide competes with calcineurin for binding to the PxIxIT-like motif in AKAP150, and thus prevents calcineurin binding to AKAP150, σ receptor activation loses its ability to regulate ASIC1a function. Thus, the disruption of this interaction prevents σ-1 receptors from functionally coupling to ASIC1a channels. Similarly, VIVIT has been implicated in the disruption of calcineurin/AKAP150 modulation of L-type Ca\(^{2+}\) channels in hippocampal neurons (Oliveria et al., 2007).

Pertinent to ischemia, ASIC1a is being studied as a putative target for neuroprotection due to the observation that ASIC1a is involved in neuronal death following ischemic brain injury (Xiong et al., 2004; Yermolaieva et al., 2004). These studies also implicated calcium influx through these channels as a key mechanism leading to neurodegeneration (Waldmann et al., 1997b; Chu et al., 2002; Xiong et al., 2004; Yermolaieva et al., 2004). Transgenic mice deficient in ASIC1a have reduced infarct size in response to middle cerebral artery occlusion, relative to wild-type mice (Xiong et al., 2004; Xiong et al., 2006). The increases in [Ca\(^{2+}\)], that are evoked by acidosis are also absent in these transgenic animals, giving insight into how these channels may contribute to neuronal injury (Xiong et al., 2004; Xiong et al., 2006). Furthermore, it has been shown that either blocking ASIC1a by amiloride or gene knockout of ASIC1a provides additional neuroprotection during oxygen glucose deprivation even in the presence of the NMDA receptor antagonist, memantine (Xiong et al., 2004; Xiong et al., 2006). It has also been established that PcTx1 inhibits ASIC1a via chronic desensitization of the channel (Chen et al., 2005) and also diminishes the
effects of NMDA-induced cell death (Xiong et al., 2004; Gao et al., 2005). Ischemia has also been shown to enhance ASIC1a currents through phosphorylation at Ser478 or Ser479 by calcium/calmodulin protein kinase II (CaMKII), and specific blockade of CaMKII prevented potentiation of the ischemia-induced ASIC1a currents, cytoplasmic calcium dysregulation, and neuronal death (Gao et al., 2005).

Neurons within the penumbra region remain viable for hours after the ischemic insult but are subject to apoptosis if perfusion is not re-established. Previous studies have shown that ischemia and the acidosis that accompanies ischemia, produces marked increases in [Ca$^{2+}$], which is one of the main mechanisms leading to cell death (Tombaugh and Sapolsky, 1993; Murai et al., 1997; Mattson, 2000; Xiong et al., 2004; Yermolaieva et al., 2004). ASIC1a-mediated currents in neurons have also been shown to be potentiated by oxygen-glucose deprivation (Xiong et al., 2004; Gao et al., 2005). Though the effects of ASIC1a currents during ischemia have been established, this study is the first report showing how ASIC1a activation affects the responses to ischemia and how acidosis and ischemia interact with each other to produce a synergistic dysregulation in [Ca$^{2+}$]. Our in vitro model of ischemia using the combination of azide in glucose-free PSS and acidosis (pH 6.0), resembles in vivo models of stroke since similar pH values have been observed during ischemia in whole animal studies (Nedergaard et al., 1991). Together, ischemia and acidosis produced elevations in [Ca$^{2+}$], that were greater than the sum of the changes evoked by acidosis and ischemia alone. There was also a noticeable difference
in the kinetics of the observed \([Ca^{2+}]_i\) transients resulting from acidosis, ischemia, and ischemia + acidosis. These differences cannot be explained by ischemia potentiating ASIC currents because ASIC1a channels desensitize within seconds. Thus, we conclude the effects seen minutes after the initial insult (steady state, rebound and net \(Ca^{2+}\) elevations) following ischemia + acidosis are the result of ASIC1a activation potentiating ischemia-mediated \([Ca^{2+}]_i\) increases. Therefore, ischemia and ASIC1a activation interact to produce potentiated elevations in \([Ca^{2+}]_i\) dysregulation.

The fact that ASIC1a inhibitors amiloride and PcTx1 reduce the ischemia-induced increases in \([Ca^{2+}]_i\) when the external solution was buffered to pH 7.4 suggests that the acidosis produced by metabolic inhibition is not responsible for ASIC1a activation. Instead, it is reasoned that protons released from glutamate containing synaptic vesicles during neurotransmission (DeVries, 2001; Traynelis and Chesler, 2001) are the source of ASIC1a channel activation. Therefore, there are two sources of protons associated with ischemia. First, an internal source as glucose and oxygen deprivation initiates a metabolic switch to anaerobic glycolysis to produce cellular energy leading to the accumulation of lactic acid, the end product of anaerobic glycolysis. Second, protons released along with glutamate during synaptic transmission. In our model, azide produces increased action potential firing and thus acidification of the synaptic cleft and the accumulation of lactic acid is mimicked by buffering external solutions to pH 6.0. Increased neurotransmission would activate more ASIC1a channels, inducing a feed-forward mechanism which promotes further synaptic transmission and
greater Ca\(^{2+}\) accumulation. These increases in [Ca\(^{2+}\)], if of sufficient duration, would produce Ca\(^{2+}\) overload and eventually lead to cell death. Consistent with these conclusions, the interaction between ischemia and acidosis leads to the synergistic potentiation of Ca\(^{2+}\) dysregulation, which is what is expected to be seen in vivo following an ischemic stroke (Tombaugh and Sapolsky, 1993; Murai et al., 1997; Mattson, 2000; Xiong et al., 2004; Yermolaieva et al., 2004).

Several mechanisms are responsible for the different phases of Ca\(^{2+}\) responses to the combination of ischemia and acidosis observed in these neurons. The strongest evidence that the initial peaks associated with ischemia-induced [Ca\(^{2+}\)], elevations reported here are due to activation of homomeric ASIC1a channels is the inhibition produced by both amiloride and the selective ASIC1a channel blocker PcTx1 (Diochot et al., 2007). The steady state [Ca\(^{2+}\)], is a function of the activated Ca\(^{2+}\) influx and efflux pathways. Interestingly, the steady state level of [Ca\(^{2+}\)], obtained at the end of ischemia applications increased as the pH was reduced from 7.4 to 7.0, but then remained constant as the pH was reduced to 6.0. Previous studies suggest most of the elevations in [Ca\(^{2+}\)], triggered by acidosis are the result of Ca\(^{2+}\) channels (NMDA and AMPA receptors and voltage-gated Ca\(^{2+}\) channels) opening in response to ASIC1a mediated membrane depolarization. These results suggest that acidosis results in increased ASIC1a channel opening but the effects of pH are countered by increased H\(^{+}\) block of other ion channels.

The transient rebound increase in [Ca\(^{2+}\)], observed during washout of the acidosis + ischemia solution is most likely due to the removal of a block of Ca\(^{2+}\)
influx pathways by protons as the proton concentration is reduced to pH 7.4. Since this feature of the \([\text{Ca}^{2+}]_i\) response is not observed following applications of azide in pH 7.4 PSS, it is unlikely that when observed at lower pH’s it is due to the washout of azide. Consistent with observations in this study, the transient rebound is expected to be more robust at the lower pH values due to the relief of a greater proton block of these \(\text{Ca}^{2+}\) channels.

Conditions which produce large net \([\text{Ca}^{2+}]_i\) elevations, or \(\text{Ca}^{2+}\) overload, are likely to lead to apoptosis. Acidosis alone, while producing a large \([\text{Ca}^{2+}]_i\) increase, has a very small net \(\text{Ca}^{2+}\) elevation because the transient increase is short lived. Likewise, while ischemia produces a maintained elevated \([\text{Ca}^{2+}]_i\) level in the presence of ischemia, it is only a moderately high level which returns quickly to baseline upon washout, and thus results in a small net elevation. The synergistic interaction of acidosis and ischemia, on the other hand, produces potentiated increases at all times during the agonist-evoked application as well as an extenuated rebound increase and a slower decay back to baseline. All these contribute to produce net elevations in \([\text{Ca}^{2+}]_i\), which are \(\sim400\%\) times greater than that of acidosis or ischemia alone. This pronounced \(\text{Ca}^{2+}\) overload would be more likely to trigger apoptosis, probably too due to endoplasmic reticulum stress.

Blockade of voltage-gated \(\text{Na}^+\) channels by TTX and subsequent synaptic transmission inhibition, prevents ischemia-induced elevations in \([\text{Ca}^{2+}]_i\) at physiological pH values (Katnik et al., 2006). Interestingly, during the combination of ischemia and acidosis at pH 6.0, the inhibition of \([\text{Ca}^{2+}]_i\) increases
by TTX were overcome, most likely by the additional activation of ASIC1a channels. This observation suggests a presynaptic localization of ASIC1a, whereby activation of the channel by protons results in synaptic transmission and subsequent activation of postsynaptic receptors. Consistent with this theory, the activation of postsynaptic Ca\(^{2+}\) channels and receptors following ASIC1a stimulation during acidosis alone (pH 6.0) are observed even when neuronal conduction is inhibited by TTX. It has been proposed that ASIC1a activation may facilitate neurotransmission by compensating for the decrease in excitatory neurotransmission caused by direct inhibition of postsynaptic Na\(^+\) and Ca\(^{2+}\) channels by protons which are released during exocytosis (Krishtal et al., 1987; Zha et al., 2006). Thus, action potential inhibition alone is not sufficient to prevent Ca\(^{2+}\) overload, as ASIC1a channel activation is able to overcome this block of glutamate release by depolarizing the presynaptic membrane to potentials capable of activating voltage-gated Ca\(^{2+}\) channels and thus, evoking synaptic transmission. Thus, even in the absence of glutamate release, ASIC1a would depolarize the cell and subsequently, activate voltage-gated Ca\(^{2+}\) channels to promote neurotransmission. In addition, these results also raise the possibility of ASIC1a activation resulting in enough Ca\(^{2+}\) influx to promote synaptic transmission and glutamate release. Similarly, heteromeric complexes of alpha 5 and/or alpha 7 subunits of nicotinic receptors have been shown to conduct Ca\(^{2+}\) and thus, resulting in acetylcholine-induced presynaptic facilitation (Girod et al., 1999).
The σ-1 selective ligand, CBP, has previously been shown to inhibit ischemia-evoked increases in [Ca^{2+}]_i (Katnik et al., 2006) as well as ASIC1a-induced [Ca^{2+}]_i increases and membrane currents. Unlike the results seen with inhibition of ASIC1a with PcTx1 venom, CBP activation of σ-1 receptors is pH insensitive and significantly inhibited the synergistic increases in [Ca^{2+}]_i produced by ischemia and acidosis as measured by initial peak amplitude, steady state, rebound and net elevations of [Ca^{2+}]_i.

**Overall Significance**

The finding that σ-1 receptors can inhibit ASIC1a channels has significant physiological implications (Figure 5.2). It has been proposed that ASIC1a activation may facilitate neurotransmission by compensating for the decrease in excitatory neurotransmission caused by direct inhibition of post-synaptic Na^+ and Ca^{2+} channels by protons which are released during exocytosis (Krishtal et al., 1987; Zha et al., 2006) (Figure 5.2). Furthermore, the expression levels of ASIC1a have direct effects on the density of dendritic spines in hippocampal neurons (Zha et al., 2006). Thus, σ-1 receptors may influence cell-to-cell signaling in the CNS by affecting ASIC1a activity. One of the consequences of ASIC1a overexpression in mice is enhanced fear conditioning (Wemmie et al., 2004), whereas stimulation of σ-1 receptors is known to ameliorate conditioned fear stress (Kamei et al., 1997). These observations, coupled with our current report, suggest that σ-1 receptor activation may produce anxiolytic effects via the inhibition of ASIC1a channels.
Figure 5.2 - Presynaptic ASIC1a channels under physiological conditions.

Neurotransmitter release leads to acidification of the synaptic cleft, which will lead to decrease neuroexcitability due to the inhibitory effects of protons in the postsynaptic channels. ASIC1a provide a positive feedback by increasing intracellular calcium following activation leading to increased neuroexcitability, and thus, preserving synaptic transmission.
Role of presynaptic ASIC1a channels during nonpathological conditions
The finding that σ-1 receptors can inhibit ASIC1a channels also has significant pathophysiological implications. σ-1 receptors functionally couple to ASIC1a channels via a PTX-sensitive G protein and an AKAP150/calcineurin complex in cortical neurons (Figure 5.1). This coupling results in decreases in both ASIC1a-mediated membrane currents and concomitant elevations in cytosolic Ca\(^{2+}\) following σ-1 receptor activation. Furthermore, these results also raise the possibility of σ-1 receptors coupling to downstream Ca\(^{2+}\) channels (voltage-gated Ca\(^{2+}\) channels) and receptors (NMDA and AMPA) via a similar signaling cascade. All of these channels and receptors have been implicated in pathophysiological conditions. Moreover, calcineurin may be a potential component of the neuroprotective properties of σ receptors. σ-1 receptors are the first receptor shown to downregulate ASIC1a channel function and remain as the only receptor identified thus far to negatively modulate ASIC1a channels.

Further evidence of the significance of σ-1 receptor modulation of ASIC1a channels during pathophysiological conditions was the finding that ASIC1a activation at any point during an ischemic insult results in synergistic [Ca\(^{2+}\)]\(_i\) increases which are σ receptor sensitive (Figure 5.3). First, ASIC1a channels are activated by ischemia alone (pH 7.4), which rapidly desensitize, but are able to be re-activated within less then 1 minute by application of an acidic external solution. This suggests that during pathophysiological conditions it is possible to get repeated activation of ASIC1a channels if the extracellular media is buffered back to normal pH for even a short refractory period. Consistent with this conclusion is the fact that lactate remains elevated and has been shown to
Figure 5.3 - Role of presynaptic ASIC1a channels during pathological conditions.

The combination of ischemia and acidosis interact to produce greater glutamate release leading to a synergistic potentiation of postsynaptic $[\text{Ca}^{2+}]_i$ elevations, resulting in $\text{Ca}^{2+}$ overload, excitotoxicity and eventually cell death. Activation of $\sigma$-1 receptors prevents the synergistic interaction between ischemia and acidosis, and thus, $\text{Ca}^{2+}$ overload.
Role of presynaptic ASIC1a channels during pathological conditions

Acidosis

Ischemia
rebound days after the initial ischemic event (Weinstein et al., 2004; Munoz Maniega et al., 2008). Even more dramatic though, was the observation that under ischemic conditions these repetitive activations of ASIC1a channels synergistically interact with ischemia to produce potentiated elevations in peak, steady state, rebound and net Ca^{2+} elevations in [Ca^{2+}]. These results suggest that in order to prevent the consequences of Ca^{2+} overload during ischemia it is critical to control ASIC1a activation. Equally important is the finding that all phases of these cellular responses are modulated by σ receptor activation. Consistent with these results, it has been suggested that the acidosis that occurs in vivo during ischemia exacerbates ischemic injury at later time points after the initial ischemic insult (Figure 5.3). The finding that ASIC1a activation interacts with ischemia to produce synergistic potentiation in Ca^{2+} dysregulation is a novel finding and suggests that this channel and σ receptors should be targeted even at delayed time points after stroke onset to prevent Ca^{2+} overload and the resulting neuronal apoptosis.

The inhibition of ASIC1a by σ-1 receptors is a potentially important component of the neuroprotective properties of σ receptors, since activation of ASIC1a has been shown to contribute to stroke injury (Xiong et al., 2004). Moreover, σ-1 receptors inhibit [Ca^{2+}] increases following ASIC1a channel activation, ischemia-induced increases in [Ca^{2+}] as well as the synergistic potentiation of these increases observed during ischemia + acidosis insults (Figure 5.3). Importantly, inhibition of ASIC1a has been shown to be neuroprotective at delayed time points following ischemic stroke (Simon, 2006;
Pignataro et al., 2007). Because acidic conditions exist during ischemic episodes, σ-1 receptors should be targeted for development of treatments for stroke and other pathophysiological conditions where ASIC1a channel activation are involved. σ-1 receptor mediated inhibition of ASIC1a channels and Ca$^{2+}$ channels (NMDA and AMPA/kainate receptors and VGCC) activated by membrane depolarizations produced by ASIC1a channel activation or metabolic inhibition and the ASIC1a channels themselves, may contribute to the enhanced neuronal survival observed following administration of σ receptor agonists 24 hr post-stroke in rats (Ajmo et al., 2006). All of these ion channels have been linked to ischemia-induced brain injury. Consistent with this pleiotropic effect of σ receptor activation on neurons, is our observation that σ-1 receptor activation suppresses extracellular high K$^+$-induced increases in [Ca$^{2+}$], which would also activate these downstream effectors. For these reasons, σ-1 receptors can be targeted for therapeutic intervention in pathophysiological conditions involving ASIC1a activation (like ischemia) and expand the therapeutic window of stroke treatment.
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APPENDICES
## Appendix A – Pharmacological Compounds

<table>
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<tr>
<th>Drug Name</th>
<th>Definition and Effective/Inhibitory Concentrations</th>
<th>Mechanism of Action</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Amiloride</td>
<td>Nonspecific ASIC blocker (16 μM)</td>
<td>Binds to residues within the channel’s pore region</td>
<td>(Kleyman et al., 1999; Alvarez de la Rosa et al., 2000; Xiong et al., 2004)</td>
</tr>
<tr>
<td>Psalmotoxin 1 venom or synthetic peptide (PcTx1)</td>
<td>Homomeric ASIC1α channel selective blocker (500 ng/ml, 50 nM)</td>
<td>Increases the affinity of the channel by H⁺ resulting in chromic desensitization of the channel</td>
<td>(Escoubas et al., 2000; Chen et al., 2005; Diochot et al., 2007)</td>
</tr>
<tr>
<td>1,3-di-o-tolyl-guanidine (DTG)</td>
<td>Pan-selective sigma receptor agonist (100 μM, 65 μM)</td>
<td>Binds both sigma receptor subtypes</td>
<td>(Klette et al., 1995; Soriani et al., 1998; Kume et al., 2002; Zhang and Cuevas, 2002; Zhang and Cuevas, 2005; Katnik et al., 2006)</td>
</tr>
<tr>
<td>Carbetapentane citrate (CBP)</td>
<td>Sigma-1 selective agonist (14 μM, 38 μM)</td>
<td>Binds sigma-1 receptors with &gt;50-fold greater affinity than for sigma-2 receptors</td>
<td>(Rothman et al., 1991; Thurgur and Church, 1998; Vilner and Bowen, 2000; Katnik et al., 2006)</td>
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## Appendix A: (Continued)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Action Description</th>
<th>Affinity/Activity Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opipramol</td>
<td>Pan-selective sigma receptor agonist (10 μM)</td>
<td>Binds both sigma receptor subtypes (Muller and Siebert, 1998; Volz et al., 2000; Moller et al., 2001; Holoubek and Muller, 2003; Muller et al., 2004; Volz and Stoll, 2004)</td>
</tr>
<tr>
<td>Dextromethorphan hydrobromide (DEX)</td>
<td>Sigma-1 selective ligand (30 μM)</td>
<td>Binds sigma-1 receptors with &gt;100-fold greater affinity than for sigma-2 receptors (Anderson and Andrew, 2002)</td>
</tr>
<tr>
<td>2-(4-Morpholinethyl) 1-phenylcyclohexane carboxylate hydrochloride (Pre-084)</td>
<td>Sigma-1 selective ligand (10 μM)</td>
<td>Binds sigma-1 receptors with &gt;100-fold greater affinity than for sigma-2 receptors (Bucolo et al., 2006)</td>
</tr>
<tr>
<td>Ibogaine</td>
<td>Sigma-2 selective agonist (31 μM)</td>
<td>Binds sigma-2 receptors with &gt;40-fold greater affinity than for sigma-1 receptors (Vilner and Bowen, 2000; Zhang and Cuevas, 2002)</td>
</tr>
<tr>
<td>1-Cyclohexyl-4-(3-(5-methoxy-1,2,3,4-tetrahydronaphthal en-1-yl)-n-propyl)piperazine dihydrochloride (PB28)</td>
<td>Sigma-2 selective agonist (2 μM)</td>
<td>Potent sigma-2 receptors agonist (Berardi et al., 2004; Cassano et al., 2006)</td>
</tr>
<tr>
<td>Metaphit (MET)</td>
<td>Irreversible pan-selective sigma antagonist (50 μM)</td>
<td>Inhibits both sigma receptor subtypes (Zhang and Cuevas, 2002; Wu, 2003; Zhang and Cuevas, 2005; Katnik et al., 2006)</td>
</tr>
</tbody>
</table>
### Appendix A: (Continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride (BD1063)</td>
<td>Sigma-1 selective receptor antagonist (10 nM, 1 μM) Binds sigma-1 receptors with greater affinity than for sigma-2 receptors</td>
<td>Matsumoto et al., 1995; Hamabe et al., 2000; Nguyen et al., 2005</td>
</tr>
<tr>
<td>Thapsigargin (THAP)</td>
<td>Depletes the ryanodine and IP&lt;sub&gt;3&lt;/sub&gt; sensitive stores (10 μM, 20 μM) Blocks the sarcoplasmic/endoplasmic reticulum Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
<td>DeHaven and Cuevas, 2004; Katnik et al., 2006</td>
</tr>
<tr>
<td>Tetrodotoxin (TTX)</td>
<td>Inhibits voltage-gated Na&lt;sup&gt;+&lt;/sup&gt; channels (200 nM, 400 nM) Blocks action potentials by binding to the pores of the voltage-gated Na&lt;sup&gt;+&lt;/sup&gt; channels</td>
<td>DeHaven and Cuevas, 2004; Katnik et al., 2006</td>
</tr>
<tr>
<td>D-2-Amino-5-phosphonovaleric acid (AP5)</td>
<td>Selective NMDA receptors antagonist (100 μM) Competitively inhibits NMDA receptors active site</td>
<td>Morris, 1989; Steele and Morris, 1999; MacGregor et al., 2003; Katnik et al., 2006</td>
</tr>
<tr>
<td>1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (Nifedipine)</td>
<td>Dihydropyridine calcium channel blocker (5 μM, 10 μM) L-type voltage-gated Ca&lt;sup&gt;2+&lt;/sup&gt; channel blocker</td>
<td>Kim et al., 2008; Tu et al., 2009</td>
</tr>
<tr>
<td>Cadmium</td>
<td>Broad spectrum Ca&lt;sup&gt;2+&lt;/sup&gt; channel blocker (100 μM, 1 mM) Inhibits Ca&lt;sup&gt;2+&lt;/sup&gt; channels</td>
<td>Ryan et al., 2007; Staruschenko et al., 2007</td>
</tr>
<tr>
<td>6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX)</td>
<td>AMPA/kainate receptor antagonist (10 μM) Competitively blocks AMPA and kainate receptors</td>
<td>MacGregor et al., 2003; Marino et al., 2007</td>
</tr>
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## Appendix A: (Continued)

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<tr>
<th><strong>FK-506</strong></th>
<th><strong>Calcineurin inhibitor (1 μM, 10 μM)</strong></th>
<th><strong>Forms immunophilin complex with FKB to bind calcineurin and competitively inhibit its activity by blocking the catalytic groove</strong></th>
<th>(Dumont, 2000; Martinez-Martinez and Redondo, 2004; Chai et al., 2007)</th>
</tr>
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<tbody>
<tr>
<td><strong>Cyclosporin A</strong></td>
<td><strong>Calcineurin inhibitor (1 μM, 30 μM)</strong></td>
<td><strong>Forms immunophilin complex with cyclophilinA to bind calcineurin and competitively inhibit its activity by blocking the catalytic groove</strong></td>
<td>(Martinez-Martinez and Redondo, 2004; Chai et al., 2007)</td>
</tr>
<tr>
<td><strong>Pertussis toxin (PTX)</strong></td>
<td><strong>Inhibits G protein activation (100 ng/ml, 200 ng/ml)</strong></td>
<td><strong>Catalyzes the ADP-ribosylation of the α subunits of G/G_o, preventing the G protein heterotrimers from interacting with receptors</strong></td>
<td>(Locht and Antoine, 1995; Jajoo et al., 2008)</td>
</tr>
<tr>
<td><strong>N-methyl-D-aspartate (NMDA)</strong></td>
<td><strong>NMDA receptor agonist (10 μM)</strong></td>
<td><strong>Selectively activates NMDA receptors and not other glutamate receptors</strong></td>
<td>(Gomez et al., 2002; Watkins and Jane, 2006)</td>
</tr>
<tr>
<td><strong>Latrunculin A</strong></td>
<td><strong>Disrupts the actin cytoskeleton (5 μM)</strong></td>
<td><strong>Disrupts the actin cytoskeleton, resulting in AKAP150 dissociation from plasma membrane</strong></td>
<td>(Allison et al., 1998; Sattler et al., 2000; Zhou et al., 2001; Popp and Dertien, 2008)</td>
</tr>
</tbody>
</table>
Appendix A: (Continued)

| VIVIT | Inhibits calcineurin AKAP150 interaction (1 μM, 10 μM) | Inhibits calcineurin/AKAP150 interaction by disrupting calcineurin binding to a PxIxIT-docking motif in AKAP150 | (Oliveria et al., 2007; Pereverzev et al., 2008) |
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Yelenis Herrera was born in Havana, Cuba. She is the daughter of Silvia and Roberto Herrera. She came to the United States in 1994 at the age of 12 and started to pursue her scientific career. In 2000, she graduated as one of the top students in her class from Suncoast Community High School in West Palm Beach, FL. She continued her education and in 2004, she graduated Cum Laude earning Bachelors degrees in Molecular Biology and Chemistry from Florida Atlantic University in Boca Raton, FL. In 2005, she started her graduate studies in the laboratory of Dr. Javier Cuevas earning a Masters degree in 2007 and a PhD degree in the Spring semester of 2009 in Medical Science from the Department of Molecular Pharmacology and Physiology.