Mechanisms of Calcium Wave Initiation and Propagation in Astrocytes

Charles Emmet Stout

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Mechanisms of Calcium Wave Initiation and Propagation in Astrocytes

by

Charles Emmet Stout

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology

March 2002
Each person whose signature appears below certifies that this dissertation in their opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABSTRACT OF THE DISSERTATION

Mechanisms of Calcium Wave Initiation and Propagation in Astrocytes

By

Charles Emmet Stout

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University, June, 2000
J. Mailen Kootsey Ph.D., Chairperson
Andrew Charles M.D., Co-Chairperson

Glial cells display propagated waves of cytoplasmic calcium (Ca\textsuperscript{2+}) mobilization that spread outward from an initiating cell to involve hundreds to thousands of surrounding cells. This intercellular communication system is thought to initiate and coordinate Ca\textsuperscript{2+} dependent processes associated with vascular control, gut motility, uterine contraction, and synaptic plasticity. Not surprisingly, aberrant Ca\textsuperscript{2+} signaling is thought to play a featured role in the pathophysiologicals associated with dysfunction of these processes. This suggests that elucidation of the mechanism of Ca\textsuperscript{2+} waves will lead to rational therapeutic modalities for a variety of clinically significant entities.

Early studies revealed that Ca\textsuperscript{2+} waves in glia require the expression and function of the gap junction protein connexin43 (Cx43), the function of phospholipase C (PLC) and the mobilization of Ca\textsuperscript{2+} from inositol-1,4,5-trisphosphate (IP\textsubscript{3}) dependent stores. This lead to the hypothesis that Ca\textsuperscript{2+} waves are mediated by mobilization of IP\textsubscript{3} in the stimulated cell that then diffuses to surrounding cells through gap junctions composed of Cx43 where it initiates Ca\textsuperscript{2+} mobilization from IP\textsubscript{3} dependent stores. Subsequently, this hypothesis was confounded by the discovery that Ca\textsuperscript{2+} waves are mediated by ATP diffusion in the extracellular space. This confusion arose because Cx43 channels are most studied in the gap junction configuration and no role could easily be ascribed to an
intercellular channel in an extracellular paracrine signaling system. Cx43 channels also exist, however, in a hemichannel configuration that gates between the cytoplasm and the extracellular space.

The main discovery of this dissertation is that it is the hemichannel configuration that is required for Ca\(^{2+}\) waves. Specifically, Cx43 hemichannels mediate the release of ATP into the extracellular space to initiate and sustain glial Ca\(^{2+}\) waves. Further, it is demonstrated that diacylglycerol mobilization is associated with hemichannel activation indicating a new role of PLC activity in Ca\(^{2+}\) wave propagation. Finally, modulation of this ATP release pathway by several agents, including those with clinical significance, is demonstrated to be directly correlated with modulation of Ca\(^{2+}\) wave propagation suggesting that modulation of ATP release represents a viable target for rational therapeutic treatment of disorders that arise from aberrant Ca\(^{2+}\) signaling.
CHAPTER 1
INTRODUCTION

Calcium (Ca$^{2+}$) plays a critical role in cellular physiology and pathology in the central nervous system (CNS). Ca$^{2+}$ is a potent modulator of a vast array of enzymes that regulate cellular events including neurotransmitter release (Zucker, Delaney et al. 1991; Nicoll, Castillo et al. 1994; Simpson, Challiss et al. 1995; Matthews 1996; Capogna 1998; Verkhratsky and Petersen 1998), metabolism (Siesjo 1990; Walz 1992), cell contraction (Ebashi and Endo 1968; Wilkins 1990; Alborch, Salom et al. 1995; Mayberg 1998; Rapoport 2000), gene expression (Ghosh, Ginty et al. 1994; Bito, Deisseroth et al. 1997; Bading 1999; Carafoli, Genazzani et al. 1999), plasticity (Verkhratsky and Petersen 1998; Vesce, Bezzi et al. 1999), and differentiation (Schwantke, Le Bouffant et al. 1985; Kelly 1991; Koizumi, Bootman et al. 1999). Changes in cytoplasmic calcium concentration ([Ca$^{2+}$]$_i$) occur with complex temporal and spatial patterns both within cells and between cells. Therefore, it is no surprise that [Ca$^{2+}$]$_i$ is involved in coordinating the activity of groups of cells to orchestrate these and other complex tissue functions (Smith 1992; Cooper 1995; Verkhratsky, Orkand et al. 1998; Verkhratsky and Steinhauser 2000).

The development of technology to visualize changes in [Ca$^{2+}$]$_i$ using video imaging techniques has prompted an intense research effort to characterize Ca$^{2+}$ signaling dynamics at the single cell level, as well as in homologous and heterologous cell groups. To date, these studies have elucidated two classes of rapid dynamic fluctuations in cellular Ca$^{2+}$. At the single cell level, repetitive Ca$^{2+}$ transients or “oscillations” in [Ca$^{2+}$]$_i$ occur with varying frequency either spontaneously (Charles, Merrill et al. 1991; Fatatis and Russell 1992) or in response to activators like (non-inclusive) mechanical stimulation.
(Charles, Merrill et al. 1991), glutamate (Cornell-Bell, Finkbeiner et al. 1990; Jensen and Chiu 1990; Charles, Merrill et al. 1991), adenosine trisphosphate (ATP) (Kastritsis, Salm et al. 1992; Reetz, Wiesinger et al. 1997), or low extracellular Ca$^{2+}$ ([Ca$^{2+}$]e) (Zanotti and Charles 1997). In groups of cells, increases in [Ca$^{2+}$], are propagated from cell to cell as intercellular Ca$^{2+}$ "waves". Intercellular Ca$^{2+}$ waves also occur in response to a wide variety of stimuli including mechanical stimulation (Charles, Merrill et al. 1991) and glutamate stimulation (Cornell-Bell, Finkbeiner et al. 1990). Individual cell Ca$^{2+}$ oscillations and propagated intercellular Ca$^{2+}$ waves can coexist in the same cell preparation – i.e. cells that show asynchronous Ca$^{2+}$ oscillations can be stimulated to produce an intercellular Ca$^{2+}$ wave (Charles, Merrill et al. 1991). On the other hand, Ca$^{2+}$ oscillations in some cases initiate intercellular Ca$^{2+}$ waves (Zanotti and Charles 1997).

Three sets of cellular machinery have been firmly linked to intercellular calcium signaling in several cell systems including astrocytes. These are connexin channels (Charles, Naus et al. 1992; Enkvist and McCarthy 1992; Finkbeiner 1992; Venance, Piomelli et al. 1995), an extracellular purinergic signaling system (Hassinger, Guthrie et al. 1996; Charles 1998; Cotrina, Lin et al. 1998; Cotrina, Lin et al. 1998; Guthrie, Knappenberger et al. 1999), and phospholipase C (Charles, Dirksen et al. 1993; Venance, Stella et al. 1997; Zanotti and Charles 1997; Muyderman, Nilsson et al. 1998). This indicates that any proposed mechanism must rely on these cellular systems.

Connexin channels are typically studied in the gap junction configuration. In this configuration, connexin channels provide a small aqueous pore between the cytoplasms of two contacting cells. The relatively recent discovery that connexins can exist as functional hemichannels gating between the cytoplasm and the extracellular space could
potentially resolve some of the conflicting data that indicates that intercellular calcium waves in astrocytes are mediated by an extracellular signaling system, yet require the expression and function of connexin channels. Specifically, it seems very plausible to suggest that the purinergic signal released by the stimulated cell(s), namely ATP, could be released through connexin hemichannels.

Astrocytes, the most abundant cell type in the nervous system, display a wide range of intercellular signaling systems including glutamate signaling (Araque, Li et al. 2000; Innocenti, Parpura et al. 2000), nitric oxide signaling (Willmott, Wong et al. 2000), osmotic signaling (Scemes and Spray 1998), and intercellular calcium signaling (Cornell-Bell, Finkbeiner et al. 1990; Charles, Merrill et al. 1991; Cornell-Bell and Finkbeiner 1991). The presence of all these intercellular signaling mechanisms has led to the hypothesis that the astrocytic compartment comprises a unique information processing system in the CNS. Of these intercellular signaling systems, intercellular calcium signaling most directly links the astrocytic and the neuronal signaling compartments. This has resulted in calcium signaling being featured in contemporary theories of neurobiology which suggest that astrocytes are directly involved with the higher functions of the brain (Smith 1992; Froes and de Carvalho 1998; Newman and Zahn 1998; Bikson, Ghai et al. 1999; Vesce, Bezzi et al. 1999; Carmignoto 2000; Laming, Kimelberg et al. 2000; Verkhratsky and Steinhauser 2000; Walz 2000).

Characterization of the intercellular calcium signaling capabilities of astrocytes should provide important information about the role of astrocytes in nervous system function and dysfunction. The goal of the research described in this dissertation is to characterize the mechanism(s) of \( \text{Ca}^{2+} \) signaling in astrocytes. Specifically, the
experimental aims are designed to examine the role of connexin hemichannels and ATP release as fundamental components of the mechanism of intercellular signaling in astrocytes.

The results presented here include: 1) Demonstration of the presence of connexin hemichannels in astrocytes; 2) Demonstration that connexin hemichannels are opened by stimuli, including mechanical stimulation, that initiate intercellular calcium signaling in astrocytes; 3) Demonstration that hemichannel activity is modulated by flufenamic acid, gadolinium, calcium, magnesium (Mg$^{2+}$), and quinine; 4) Demonstration that hemichannel activity is directly correlated with ATP release and the extent of Ca$^{2+}$ waves; 5) Evidence that the signaling cascade leading from mechanical stimulation to hemichannel gating involves PLC activation; 6) Evidence that ATP released via connexin hemichannels acts as an intercellular signaling messenger in astrocytes and 7) Evidence that clinically relevant modulation of extracellular Mg$^{2+}$ affects intercellular signaling in astrocytes by modulating ATP release through connexin hemichannels. Taken together, these data suggest that intercellular calcium signaling in astrocytes can occur by ATP release through connexin hemichannels. This novel result has the advantage of explaining current seemingly contradictory data concerning the mechanism of calcium signaling in astrocytes. These data also demonstrate novel regulatory mechanisms of connexin43 (Cx43) hemichannels that extend the original goals for this research and present new avenues for hemichannel research. Finally, the elucidation of this mechanism of calcium signaling presents several therapeutic targets for disorders linked to intercellular calcium signaling.
Background
Calcium Signaling

Single Cell Calcium Oscillations

Dynamics

$\text{Ca}^{2+}$ oscillations refer to the mobilization of $\text{Ca}^{2+}$ in a single cell that occurs spontaneously or in response to stimulating agents. The characteristics of these oscillations vary with the stimulating agent (Table 1). There are, however, a few characteristics that seem to be noted in most studies. "Low" concentrations of agonist typically induce oscillations that have a frequency proportional to agonist concentration and are often preceded by a "short" biphasic $\text{Ca}^{2+}$ transient (Salm and McCarthy 1990; Inagaki, Fukui et al. 1991; Fatatis, Holtzclaw et al. 1994; Manning and Sontheimer 1997; Bernstein, Behnisch et al. 1998). Higher concentrations of agonist typically elicit single biphasic events consisting of an initial transient followed by a sustained increase in $[\text{Ca}^{2+}]_i$ above baseline (Salm and McCarthy 1990; Inagaki, Fukui et al. 1991; Fatatis, Holtzclaw et al. 1994; Yagodin, Holtzclaw et al. 1995; Manning and Sontheimer 1997; Bernstein, Behnisch et al. 1998).

Mechanics

Currently, there is not an accepted mechanism for $\text{Ca}^{2+}$ oscillations in astrocytes. It is possible, though, to identify some of the components of the oscillatory machinery. For example, oscillatory behavior is reported to be sensitive to phospholipase C (PLC) inhibitors (Ubl and Reiser 1997; Zanotti and Charles 1997). Furthermore, oscillations are
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<td>Spontaneous</td>
<td>Frequency proportional to confluency; Can initiate Ca(^{2+}) waves; Independent of [Ca(^{2+})](_e)</td>
<td>(Charles, Merrill et al. 1991; Fatatis and Russell 1992)</td>
</tr>
<tr>
<td>Mechanical Stimulation</td>
<td>Occurs in the absence of [Ca(^{2+})](_e)</td>
<td>(Charles, Merrill et al. 1991)</td>
</tr>
<tr>
<td>Low [Ca(^{2+})](_e)</td>
<td>Sensitive to thapsigargin, U73122, divalent cations; Initiates Ca(^{2+}) waves; Biased by perfusion;</td>
<td>(Zanotti and Charles 1997)</td>
</tr>
<tr>
<td>Neurons</td>
<td>Frequency modulated by neuronal activity;</td>
<td>(Pasti, Voltterra et al. 1997)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Sensitive to phorbol ester, partially dependent on [Ca(^{2+})](_e); Initiates Ca(^{2+}) waves</td>
<td>(Jensen and Chiu 1990; Charles, Merrill et al. 1991; Cornell-Bell and Finkbeiner 1991; Jensen and Chiu 1991; Nakahara, Okada et al. 1997)</td>
</tr>
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<td>ATP</td>
<td>Biphasic - 1(^{st}) phase independent of [Ca(^{2+})](_e), 2(^{nd}) phase partially dependent on [Ca(^{2+})](_e); Frequency proportional to [ATP](_e); Modulated by medium osmolarity</td>
<td>(Kastritsis, Salm et al. 1992; Reetz, Wiesinger et al. 1997; Bernstein, Behnisch et al. 1998)</td>
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<td>Histamine</td>
<td>Asynchronous oscillations in type-2 astrocytes</td>
<td>(Inagaki, Fukui et al. 1991)</td>
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<tr>
<td>5-hydroxytryptamine</td>
<td>Two types of oscillations; low-amplitude and baseline spiking</td>
<td>(Nilsson, Hansson et al. 1991; Nilsson, Hansson et al. 1991)</td>
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<tr>
<td>Hypo-Osmotic Treatment</td>
<td>Initiates Ca(^{2+}) waves; Sensitive to Suramin</td>
<td>(Scemes and Spray 1998)</td>
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<td>Biphasic; PLC dependent; Sensitive to nexin-1; Partially sensitive to [Ca(^{2+})](_e)</td>
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<td>Biphasic - 1(^{st}) phase independent of [Ca(^{2+})](_e), 2(^{nd}) phase partially dependent on [Ca(^{2+})](_e); Sensitive to BAPTA</td>
<td>(Manning and Sontheimer 1997)</td>
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<td>Oxytocin</td>
<td>Independent of [Ca(^{2+})](_e); Insensitive to cadmium; Sensitive to thapsigargin</td>
<td>(Di Scala-Guenot, Mougnot et al. 1994)</td>
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<td>Vasoactive Intestinal Peptide</td>
<td>Synergistic with (\alpha)-adrenergic receptors; Sensitive to thapsigargin</td>
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<td>Norepinephrine</td>
<td>Biphasic - 1(^{st}) phase independent of [Ca(^{2+})](_e), 2(^{nd}) phase partially dependent on [Ca(^{2+})](_e); Both sensitive to thapsigargin</td>
<td>(Salm and McCarthy 1990; Nilsson, Hansson et al. 1991; Yagodin, Holtzclaw et al. 1995)</td>
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sensitive to Ca\textsuperscript{2+} store depletion by agents such as thapsigargin (Wood, Wing et al. 1993; Di Scala-Guenot, Mougnot et al. 1994; Fatatis, Holtzclaw et al. 1994; Yagodin, Holtzclaw et al. 1995; Ubl and Reiser 1997; Zanotti and Charles 1997; Stix and Reiser 1998). Finally, Ca\textsuperscript{2+} oscillations are not only present but are potentiated by removal of extracellular Ca\textsuperscript{2+} (Zanotti and Charles 1997). These data indicate that oscillations in [Ca\textsuperscript{2+}]\textsubscript{j} occur by mobilization and sequestration of Ca\textsuperscript{2+} from inositol-1,4,5-trisphosphate (IP\textsubscript{3}) sensitive cytoplasmic calcium stores.

These data suggest that Ca\textsuperscript{2+} oscillations occur by “oscillations” in PLC activity. This is consistent with results from several other non-excitable cell types where intracellular IP\textsubscript{3} concentration ([IP\textsubscript{3}]\textsubscript{i}) oscillations are associated with [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. For example, direct visualization of changes in [IP\textsubscript{3}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{i} has been achieved simultaneously in Madin-Darby canine kidney epithelial cells, demonstrating a direct one-to-one correlation between Δ[IP\textsubscript{3}]\textsubscript{i} and Δ[Ca\textsuperscript{2+}]\textsubscript{i} (Hirose, Kadowaki et al. 1999). Further, each Δ[IP\textsubscript{3}]\textsubscript{i} slightly precedes the corresponding Δ[Ca\textsuperscript{2+}]\textsubscript{i}, demonstrating the sequential activity of PLC and Ca\textsuperscript{2+} mobilization.

The biphasic Ca\textsuperscript{2+} transients that often precede Ca\textsuperscript{2+} oscillations initiated by “low” agonist concentrations or that occur solely in response to higher agonist concentrations have different characteristics. The initial phase is usually reported as being sensitive to thapsigargin and insensitive to Ca\textsuperscript{2+} channel blockers and short-term extracellular Ca\textsuperscript{2+} removal (Kastritsis, Salm et al. 1992; Manning and Sontheimer 1997; Reetz, Wiesinger et al. 1997; Bernstein, Behnisch et al. 1998). Largely because of these characteristics, it is commonly held that this initial Ca\textsuperscript{2+} transient is the result of intracellular IP\textsubscript{3} dependent Ca\textsuperscript{2+} mobilization. On the other hand, the sustained or second phase of the biphasic
response depends on Ca\(^{2+}\) influx across the plasma membrane. As such, it is sensitive to Ca\(^{2+}\) channel blockers and extracellular Ca\(^{2+}\) removal (Salm and McCarthy 1990; Nilsson, Hansson et al. 1991; Yagodin, Holtzclaw et al. 1995; Thorlin, Eriksson et al. 1998).

There are other possible mechanisms of Ca\(^{2+}\) oscillations. All data examined regarding astrocytes, however, specifically indicate that PLC activity oscillations are involved. For example, the metabotropic glutamate receptor (mGluR5) selective agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate (1S,3R- ACPD) elicits Ca\(^{2+}\) oscillatory behavior that is blocked by either the protein kinase C (PKC) inhibitor, bisindolylmaleimide I, or the PKC activator phorbol 12-myristate 13- acetate (PMA) (Nakahara, Okada et al. 1997). This involves serine phosphorylation of the mGluR5 receptor (Nakanishi, Nakajima et al. 1998) altering its activation of PLC—i.e., under-damped negative feedback, a feedback pattern known to cause oscillations in many systems.

Thus, it is clear that PLC activity and intracellular IP\(_3\) sensitive stores are critical elements of the Ca\(^{2+}\) oscillatory machinery in astrocytes. This is important since Ca\(^{2+}\) wave initiation in astrocytes requires PLC activity (Zanotti and Charles 1997; Muyderman, Nilsson et al. 1998). This suggests the possibility that oscillations that initiate intercellular waves do so by a similar mechanism as mechanical stimulation — i.e. PLC activation. It is also clear that Ca\(^{2+}\) signaling in astrocytes involves ATP release (see below). This leads to the intriguing hypothesis that PLC activation initiates both spontaneous and stimulation induced intercellular Ca\(^{2+}\) signaling by opening the channel that mediates ATP release (i.e. connexin hemichannel; see below).
**Intercellular Ca\(^{2+}\) Waves**

Dynamics

The term "Ca\(^{2+}\) wave" refers to increases in [Ca\(^{2+}\)]\(_i\) that are propagated from cell to cell in a wave-like pattern (Figure 1). Limited intercellular Ca\(^{2+}\) waves (e.g. 3-10 cells) may occur spontaneously. More extensive intercellular Ca\(^{2+}\) waves (those numbering up to hundreds of cells) occur in response to a variety of stimuli, including (non-inclusive) mechanical stimulation (Charles, Merrill et al. 1991), glutaminergic agonists (Cornell-Bell, Finkbeiner et al. 1990; Charles, Merrill et al. 1991), focal electrical stimulation (Nedergaard 1994; Guthrie, Knappenberger et al. 1999), focal ionomycin stimulation (Venance, Stella et al. 1997), low [Ca\(^{2+}\)]\(_e\) (Zanotti and Charles 1997), and ATP (Reetz, Wiesinger et al. 1997; Guthrie, Knappenberger et al. 1999). Ca\(^{2+}\) waves initiated by these activators exhibit distinct characteristics suggesting that either the mechanism of initiation or the mechanism(s) of propagation is/are distinct. Detailed comparison of glutamate-induced and mechanically induced Ca\(^{2+}\) waves highlights some of these differences.

Glutamate exposure initiates an initial increase in [Ca\(^{2+}\)]\(_i\) in astrocytes that may be communicated as a wave; this has been referred to as a "spatial spike". Then, after a delay of 30-60 seconds, intercellular Ca\(^{2+}\) waves propagate from multiple single-cell foci to surrounding cells (Kim, Rioult et al. 1994). In contrast, mechanical stimulation of a Single astrocyte induces a wave of increasing [Ca\(^{2+}\)]\(_i\) that travels from the point of stimulation to involve the entire stimulated cell. After a brief delay (0.5-1 s), the wave is communicated to neighboring cells at points of intercellular contact (Charles, Merrill et al. 1991). Both glutamate-induced and mechanically stimulated waves propagate from
Mechanically Initiated Glial Calcium Wave

Figure 1 – Stimulation-Induced Intercellular Ca\(^{2+}\) Wave in Astrocytes

Purified cortical astrocytes respond to mechanical stimulation of a single cell with a propagated wave of Ca\(^{2+}\) mobilization. Cells are loaded with a Ca\(^{2+}\) indicator dye (fura2) by incubation with membrane permeable AM ester form (fura2-AM) for 30 minutes. Cells are then rinsed (3x) and loaded onto the microscope as described in Materials and Methods. A single cell is stimulated by brief perturbation with a micropipette of the cell membrane.
a single cell to multiple surrounding cells at a velocity of 10-20 μm/sec. Under both conditions, the waves are communicated at sites of cell-to-cell contact, and the increase in [Ca^{2+}], often travels as an intracellular wave within individual cells (Cornell-Bell, Finkbeiner et al. 1990; Charles, Merrill et al. 1991). Ca^{2+} waves in response to glutamate, however, require extracellular Ca^{2+} and are communicated without pause at the cell borders (Cornell-Bell and Finkbeiner 1991; Kim, Rioul et al. 1994). In contrast, mechanically induced Ca^{2+} waves show delays at the borders between cells and do not require extracellular Ca^{2+} (Charles, Merrill et al. 1991). Thus, although the temporal and spatial characteristics of the glutamate-induced and mechanically induced Ca^{2+} waves are superficially similar, they involve different mechanisms of initiation and/or propagation.

Mechanics

Multiple characteristics of astrocytic Ca^{2+} waves, including their spatial pattern and velocity (10-20 μm/sec), are consistent with the diffusion of a signaling molecule(s). The radial pattern of spread suggests that the signal is either extracellular or that an intracellular signal propagates through intercellular channels such as gap junction channels. As of this writing, three sets of cellular machinery have been shown to be critically involved in initiating and/or propagating the signal molecule(s) in astrocytes. These include PLC, connexin channels, and the purinergic paracrine signaling system. Inhibition of any of these typically results in a major reduction or complete inhibition of the extent of Ca^{2+} wave propagation. These data have lead to two competing hypotheses that seemed to be irreconcilable, i.e. the IP_3 diffusion hypothesis and the ATP paracrine hypothesis (Figure 4).
Calcium Waves Require Phospholipid Mobilization

Figure 2 - Role of Phospholipids in Intercellular Ca\(^{2+}\) Signaling

Intercellular Ca\(^{2+}\) signaling is dependent on Ca\(^{2+}\) mobilized from IP\(_3\) dependent stores. Inhibition of any step in this pathway blocks intercellular Ca\(^{2+}\) wave propagation, including: 1) PLC inhibition, 2) IP\(_3\) receptor inhibition; or 3) intracellular Ca\(^{2+}\) store depletion.
a. Phospholipase C is Required for Calcium Wave Propagation

Multiple studies indicate that astrocytic Ca^{2+} waves involve activation of PLC and the release of intracellular Ca^{2+} mediated by the intracellular messenger IP3 (Figure 2). Inhibition of PLC activity by 1-[6-((17beta-3-methoxyestra-1,3, 5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122) leads to rapid (<30s) inhibition of Ca^{2+} waves (Zanotti and Charles 1997; Muyderman, Nilsson et al. 1998). Further, blocking the IP3 receptor (IP3r) using heparin leads to inhibition of Ca^{2+} wave propagation (Newman and Zahs 1997) as does emptying cellular Ca^{2+} stores with thapsigargin (Yagodin, Holtzclaw et al. 1995; Newman and Zahs 1997; Simpson and Russell 1997; Zanotti and Charles 1997; Cotrina, Lin et al. 1998; Harris-White, Zanotti et al. 1998; Laskey, Roth et al. 1998; Newman and Zahs 1998; Innocenti, Parpura et al. 2000). In contrast, Ca^{2+} wave propagation is relatively insensitive to dantrolene (Charles, Dirksen et al. 1993), Ca^{2+} channel blockers (Fatatis and Russell 1992; Yagodin, Holtzclaw et al. 1995), and short-term removal of extracellular Ca^{2+} (Fatatis and Russell 1992; Zanotti and Charles 1997). These results show that the [Ca^{2+}]_{e} transients associated with Ca^{2+} wave propagation are mobilized by IP3 from intracellular stores.

b. Connexin Channels are Necessary for Calcium Wave Propagation

In astrocytes, and several other cell types, Ca^{2+} wave propagation depends on connexin expression (Charles, Naus et al. 1992; Lynn, Marotta et al. 1995; Naus, Bechberger et al. 1997; Cotrina, Lin et al. 1998; Blomstrand, Aberg et al. 1999; Naus, Bani-Yaghoub et al. 1999) and connexin channel function (Finkbeiner 1992; Venance, Piomelli et al. 1995; Muyderman, Nilsson et al. 1998; Grafstein, Liu et al. 2000) (Figure 3). For example, the predominant connexin expressed in fetal cortical astrocytes is Cx43
Calcium Waves Require Connexin Channels

Figure 3 - Role of Connexin Channels in Intercellular Ca\(^{2+}\) Signaling

Intercellular Ca\(^{2+}\) signaling is dependent on connexin channel expression and function. Cell types that do not express connexin channels fail to propagate intercellular Ca\(^{2+}\) waves. In cells that express connexins, connexin channel inhibitors such as n-chain alcohols block intercellular Ca\(^{2+}\) waves.
(Dermietzel, Hertberg et al. 1991) and expression of this protein confers the ability of C6 glioma cells to exhibit propagated Ca\(^{2+}\) waves (Charles, Naus et al. 1992). Similarly, other connexins have been examined, such as connexin32 (Cx32), which also restore propagated Ca\(^{2+}\) waves in connexin-deficient cell lines (Cotrina, Lin et al. 1998).

In addition, pharmacological inhibition of connexin channels invariably block Ca\(^{2+}\) wave propagation in astrocytes (Finkbeiner 1992; Venance, Piomelli et al. 1995; Nadal, Fuentes et al. 1997; Muyderman, Nilsson et al. 1998; Grafstein, Liu et al. 2000) and other cell lines (Sanderson, Charles et al. 1990; Charles, Kodali et al. 1996; Young, Ennes et al. 1996; Zimmermann and Walz 1997; Miura, Boyden et al. 1998), indicating that connexin channel function is also necessary for Ca\(^{2+}\) wave propagation. These considerations show that connexin channels formed by Cx43 are critical for the fullest extent of Ca\(^{2+}\) wave propagation in astrocytes as well as many other cell types.

**IP\(_3\) Diffusion Hypothesis**

These studies, completed early in the study of Ca\(^{2+}\) signaling, suggested that intercellular Ca\(^{2+}\) waves occur primarily via diffusion of IP\(_3\) or other signaling molecules through gap junctions (Figure 4). Evidence for this *IP\(_3\) Diffusion Hypothesis* for intercellular propagation of Ca\(^{2+}\) waves included observations that:

1) Intercellular propagation of Ca\(^{2+}\) waves are correlated with the expression of connexins (the proteins that comprise gap junctions) (personal observation) (Charles, Naus et al. 1992; Naus, Bechberger et al. 1997; Naus, Bani-Yaghoub et al. 1999).

2) Inhibitors of gap junctions inhibit Ca\(^{2+}\) waves propagation.
Proposed Mechanisms of Calcium Wave Propagation

Figure 4 - Proposed Mechanisms of Ca$^{2+}$ Signaling in Astrocytes

Two mechanisms have been proposed for intercellular Ca$^{2+}$ wave propagation in astrocytes: 1) The "IP$_3$ diffusion hypothesis" where signal propagation is the result of IP$_3$ diffusion into surrounding cells, from the stimulated cell, through gap junction channels; and 2) The "ATP paracrine hypothesis" where signal propagation is the result of ATP, released from the stimulated cell, which diffuses to surrounding cells in the extracellular space.
3) The propagation of Ca\(^{2+}\) waves in some protocols is not altered by perfusion of the extracellular medium.

4) Microinjection of IP\(_3\) into a single cell could initiate Ca\(^{2+}\) signals in some adjacent cells (Haller, Lindschau et al. 1996; Haller, Maasch et al. 1998; Haller, Lindschau et al. 1999).

c. Calcium Wave Propagation Depends on the Purinergic Signaling System

More recent studies, however, have shown that intercellular Ca\(^{2+}\) signaling in astrocytes clearly involves an extracellular signaling molecule, and that this molecule is ATP (Hassinger, Guthrie et al. 1996; Charles 1998; Cotrina, Lin et al. 1998; Guthrie, Knappenberger et al. 1999). These studies are summarized as follows:

1) Delivery of IP\(_3\) via perfusion through a patch pipette does not initiate intercellular Ca\(^{2+}\) signaling in Cx43 expressing (personal observation) and Cx32 expressing (Chanson, Mollard et al. 1999) cells.

2) Ca\(^{2+}\) waves in astrocytes propagate across cell free zones (Hassinger, Guthrie et al. 1996; Charles 1998) and are biased by movement of the bathing media (Hassinger, Guthrie et al. 1996; Charles 1998).

3) Ca\(^{2+}\) waves are reduced by inhibitors of the purinergic signaling system including apyrase (Guthrie, Knappenberger et al. 1999), suramin (Cotrina, Lin et al. 1998; Guthrie, Knappenberger et al. 1999) and pyridoxal phosphate-6-azophenyl-2'4'-disulphonic acid (PPADS) (Cotrina, Lin et al. 1998).

4) Ca\(^{2+}\) waves in astrocytes are associated with the release of ATP (Guthrie, Knappenberger et al. 1999), as are multiple stimuli that evoke Ca\(^{2+}\) waves, including purinergic stimulation (UTP) (Cotrina, Lin et al. 1998), and
Calcium Waves are Mediated by Extracellular ATP

Purinergic Receptor (P2) - Inhibited by Suramin, PPADS

Figure 5 - Role of Purinergic Paracrine Pathway in Intercellular Ca\textsuperscript{2+} Signaling

Intercellular Ca\textsuperscript{2+} wave propagation is dependent on ATP paracrine signaling. Purinergic receptor inhibitors like suramin or PPADS reduce the extent of Ca\textsuperscript{2+} wave propagation. It is also reduced by treatment with the ATP degrading enzyme apyrase. Finally, Ca\textsuperscript{2+} waves can traverse a cell free zone and are biased by media perfusion.
glutamate stimulation (Queiroz, Gebicke-Haerter et al. 1997; Queiroz, Meyer et al. 1999).

**ATP Paracrine Hypothesis**

These studies led to the formation of the *ATP paracrine hypothesis* (Figure 4). This links astrocytes to the CNS purinergic signaling system. Originally, purinergic signaling in the CNS was referred to as the “purinergic nerve hypothesis” and was quite controversial due to the idea that a cell’s “energy metabolism” would be “compromised” if ATP were released from the cell (Burnstock 1977; Burnstock, Cocks et al. 1978). This objection and others have completely faded over the last 20 years as the purinergic signaling system has been systematically dissected and explored (Neary and Norenberg 1992; Chen, Levy et al. 1995; Illes, Nieber et al. 1996; Christofi, Guan et al. 1997; Lalo and Kostyuk 1998). The discovery that astrocytes display propagated purinergic signals is another link between glial cells and neuronal function, and thus the higher functions of the brain.

**Connexin Hemichannels**

In total, these data create a paradox in that the fullest extent of Ca$^{2+}$ wave propagation is mediated by an extracellular propagation system, yet Ca$^{2+}$ wave propagation relies on Cx43, which is typically considered an intercellular channel (Figure 6a). The simplest resolution to this involves the discovery that connexin proteins form functional hemichannels (also referred to as connexons and hemi-gap junctions) that gate between the cytoplasm and the extracellular space (Figure 6b) (Musil and Goodenough 1991; Liu, Li et al. 1995; Li, Liu et al. 1996). This suggests the distinct possibility that hemichannels play a central role in ATP release. Consistent with this, it has been
Connexin Channel Configurations

A. Gap junctions are formed when two connexons or hemichannels from adjacent cells align forming an ion channel between the cytoplasms of the two cells. These intercellular channels are permeable to ions and small molecules (MW <1kD).

B. Hemichannels are formed from six connexin proteins that oligomerize in the membrane in a hexameric array forming an ion channel that gates between the cytoplasm and the extracellular space. Like gap junction channels, hemichannels are permeable to ions and small molecules (MW < 1kD).

Artwork modified from (Kumar and Gilula 1996).
demonstrated that Cx43 and Cx32 expression facilitates ATP release several-fold (Cotrina, Lin et al. 1998). While it could be argued that this facilitation does not necessitate direct release by the channel, the permeation properties of hemichannels (Li, Liu et al. 1996; Zhang, McBride et al. 1998; Zhang, Gao et al. 2000) and the critical dependence of Ca^{2+} wave propagation on connexin channels suggest that the most logical explanation is direct involvement in ATP release.

Hemichannels have been documented to occur in several cell types, including cardiac myocytes (John, Kondo et al. 1999), urinary bladder epithelial cells (Vanoye, Vergara et al. 1999), Cx43 transfected cells (Li, Liu et al. 1996), rat lens fiber cells (Eckert, Donaldson et al. 1998) and Novikoff cells (Liu, Li et al. 1995; Li, Liu et al. 1996; Liu, Paulson et al. 1997; Moreno 1998). One of the first discoveries was that hemichannels are [Ca^{2+}]_e sensing channels – i.e. they are inhibited by extracellular Ca^{2+} (Sarthy, Johnson et al. 1982). For example, treatment with zero [Ca^{2+}]_e initiates dye-uptake (Sarthy, Johnson et al. 1982; Vanoye, Vergara et al. 1999). Evidence that this occurs through hemichannels includes: 1) Dye-uptake is restricted to dyes that are known to permeate gap junction channels (i.e. MW < 1kD) (Liu, Li et al. 1995; Li, Liu et al. 1996; Liu, Paulson et al. 1997); 2) Dye-uptake is inhibited by agents that block gap junctions (Liu, Li et al. 1995; Li, Liu et al. 1996; Liu, Paulson et al. 1997); and 3) Dye-uptake is dependent on connexin expression (Li, Liu et al. 1996; Vanoye, Vergara et al. 1999).

The electrophysiologic characteristics of various hemichannels have been examined in some detail by expression in Xenopus oocytes. Xenopus oocytes, injected with connexin mRNA, exhibit membrane currents when treated with zero [Ca^{2+}]_e that are blocked by
connexin channel inhibitors (Trexler, Bennett et al. 1996; Barrio, Capel et al. 1997; Pfahnl, Zhou et al. 1997; Zhang, McBride et al. 1998; Pfahnl and Dahl 1999). This system has provided a convenient system for screening other potential modulators of hemichannels. These studies have revealed that hemichannels are blocked by gadolinium (Gd$^{3+}$) (Zhang, McBride et al. 1998), acid (Takahashi and Copenhagen 1996; Peracchia and Wang 1997; Zhang, Zhang et al. 1998; Trexler, Bukauskas et al. 1999; Zampighi, Loo et al. 1999; Quist, Rhee et al. 2000) and flufenamic acid (Zhang, McBride et al. 1998; Zhang and Hamill 2000). In contrast, hemichannels are potentiated by quinine (Malchow, Qian et al. 1994; Dixon, Takahashi et al. 1996; White, Deans et al. 1999; Al-Ubaidi, White et al. 2000). Some agents seem to have only a very little or no effect on hemichannels, including DIDS (Dixon, Takahashi et al. 1996; Bruzzone, Guida et al. 2001). Finally, consistent with the fact that hemichannels are permeable to anionic dyes, ion substitution studies indicate that hemichannels are permeable to large anions and cations (Zhang, McBride et al. 1998; Zhang and Hamill 2000).

Relatively little information exists about the electrical characteristics of Cx43 composed hemichannels. The sole report that exists indicates that Cx43 composed hemichannels are inhibited by depolarization (Moreno 1998). Also, they exhibit two conducting states similar to Cx43 composed gap junctions. Importantly, the cellular messengers involved in gating have not been examined. The hypothesis that hemichannels are involved in Ca$^{2+}$ signaling would necessitate that hemichannels be opened by stimuli that initiate intercellular Ca$^{2+}$ waves such as mechanical stimulation.
Table 2 - Characteristics of Heterocellular Calcium Signaling in the Central Nervous System

<table>
<thead>
<tr>
<th>Type of Interaction</th>
<th>Characteristics</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Astrocyte to Astrocyte</td>
<td>Coordination of cooperative glial function; Long-range glial networks; Associated with rapid extension of filopodia; Rhythmic glial cell contraction and neuroligand release; Integrate and propagate signals; Extracellular ionic sensing; Glial scar formation; Organization of vimentin filament networks; Formation of spiral-waves indicating self-organizing criticality; Edemolytic Effects; $[\text{K}^+]_e$ clearance</td>
<td>(Cornell-Bell, Finkbeiner et al. 1990; Charles, Merrill et al. 1991; Cornell-Bell, Thomas et al. 1992; Cooper 1995; Verkhratsky and Kettenmann 1996; Inagaki, Goto et al. 1997; Nadal, Fuentes et al. 1997; Newman and Zahs 1997; Zanotti and Charles 1997; Harris-White, Zanotti et al. 1998; Jung, Cornell-Bell et al. 1998; Simard, Couldwell et al. 1999; Walz 2000)</td>
</tr>
<tr>
<td>Astrocyte to Endothelial</td>
<td>Blood brain barrier interactions</td>
<td>(Leybaert, Paemeleire et al. 1998)</td>
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<tr>
<td>Endothelial to Astrocyte</td>
<td>Blood brain barrier interactions</td>
<td>(Leybaert, Paemeleire et al. 1998)</td>
</tr>
<tr>
<td>Neuron to Astrocyte</td>
<td>Astrocyte networks, in situ, respond to neuronal network activity with propagated waves; Neuronal activity initiates concurrent Ca$^{2+}$ mobilization in astrocytes; Facilitated by NE release;</td>
<td>(Dani, Chernjavsky et al. 1992; Murphy, Blatter et al. 1993; Charles 1994; Dani and Smith 1995; Duffy and MacVicar 1995)</td>
</tr>
<tr>
<td>Astrocyte to Neuron</td>
<td>Increased neuronal activity; Rhythmic glial cell contraction and ion/neuroligand release modulating neuronal activity; Modulation of the frequency in miniature synaptic currents</td>
<td>(Charles 1994) (Nedergaard 1994; Parpura, Basarsky et al. 1994; Cooper 1995; Hassinger, Atkinson et al. 1995; Araque, Parpura et al. 1998; Araque, Sanzgiri et al. 1998; Trudeau, Fang et al. 1998; Araque, Sanzgiri et al. 1999; Sanzgiri, Araque et al. 1999)</td>
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Calcium Signaling Physiology

No definitive role for glial $\text{Ca}^{2+}$ waves has been clearly established. However, given that an increase in $[\text{Ca}^{2+}]_i$ modulates ion channels (Baudry and Lynch 1979), triggers the release of neurotransmitters (Zucker, Delaney et al. 1991; Matthews 1996; Capogna 1998; Bacci, Verderio et al. 1999; Koizumi, Bootman et al. 1999), modulates metabolic enzymes (Siesjo 1990; Walz 1992; Morley, Hogan et al. 1994; Simpson, Challiss et al. 1995), and controls gene expression (Ghosh, Ginty et al. 1994; Bito, Deisseroth et al. 1997; Hardingham, Cruzalegui et al. 1998; Bading 1999), it is easy to speculate that glial $\text{Ca}^{2+}$ waves may provide spatial and temporal coordination of these events (Table 2).

Coordination of Cellular Function

$\text{Ca}^{2+}$ dependent enzymes regulate nearly every aspect of cell function, suggesting that $\text{Ca}^{2+}$ waves coordinate these functions in spatially localized groups of cells. For example, the activity of typical protein kinase C (PKC) isoforms would be upregulated in a group of cells responding to a $\text{Ca}^{2+}$ wave as a result of mobilization of $[\text{Ca}^{2+}]_i$ and DAG. This spatially organized activation of PKC would lead to the modulation of several processes that PKC is known to regulate such as metabolism (Itoh et al. 1989; Lim and Zaheer 1995; Mangoura et al. 1995; Pearce et al. 1988; Shafit-Zagardo et al. 1988), neurotransmitter release (Dekker, De Graan et al. 1991; Majewski and Musgrave 1995; Majewski, Kotsonis et al. 1997; Majewski and Iannazzo 1998; Vaughan, Walker et al. 1998), cell division (Schwantke, Le Bouffant et al. 1985), and Cx43 composed gap junction mediated intercellular communication (Enkvist and McCarthy 1992; Konietzko 1992).
and Muller 1994; Scemes and Spray 1998). Alternately, purinergic receptor activation in a group of cells participating in a Ca$^{2+}$ wave could result in mobilization of cyclic-adenosine monophosphate (cAMP) causing upregulation of protein kinase A (PKA). Coordination of PKA activity in a group of cells may also synchronize several cellular processes.

**Regulation of Cell Growth and Tissue Organization**

The extent of Ca$^{2+}$ wave propagation is nearly always modulated by changes in growth rate induced by tumor agents or transfection with growth-altering genes. For example, the growth rate of C6 glioma cells is reduced by overexpression of Cx43 (Naus, Bechberger et al. 1991) while the extent of Ca$^{2+}$ wave propagation is increased (Charles, Naus et al. 1992). This suggests that Ca$^{2+}$ signaling may be involved in glial cell proliferation. This is supported by the fact that the growth rate in these cells has been directly correlated with Ca$^{2+}$-dependent PKC expression (Cook, Morash et al. 1996; Sharif and Sharif 1999; Slepko, Patrizio et al. 1999) and activity (Couldwell, Uhm et al. 1991; Couldwell, Antel et al. 1992). This indicates a key role for Ca$^{2+}$ signaling in growth control. Consistent with this, suramin, a commonly used non-specific inhibitor of purinergic receptors, was originally classified and is still studied as an anti-cancer agent (Hensey, Boscoboinik et al. 1989; Olson, Polk et al. 1994; Takano, Gately et al. 1994; Schrell, Gauer et al. 1995; Housley 1998; Bernsen, Rijken et al. 1999), and is currently being investigated for clinical use (Hutson, Tutsch et al. 1998).

**Response to and Modulation of Extracellular Ionic Conditions**

Ca$^{2+}$ signaling may be involved in the regulation of the extracellular ionic environment. For example, by activating Ca$^{2+}$-activated potassium (K$^+$) channels, Ca$^{2+}$
waves may coordinate the regulation of extracellular potassium concentration ([K⁺]ₑ) by localized groups of glial cells (Vera, Sanchez-Abarca et al. 1996; Wang, Tymianski et al. 1997; Velasco, Tabernero et al. 2000). In this context, it is interesting that astrocytes respond to changes in [K⁺]ₑ bathing solutions with vigorous Ca²⁺ signaling events (unpublished observations). Another way that Ca²⁺ waves may be involved in the homeostasis of the extracellular environment is by modulation of gap junction coupling. Both [Ca²⁺]ᵢ (Vera, Sanchez-Abarca et al. 1996; Wang, Tymianski et al. 1997) and [ATP]ₑ (Enkvist and McCarthy 1992) modulate coupling mediated by gap junctions and would therefore regulate the glial syncytium that has long been implicated in facilitating [K⁺]ₑ regulation (Tse, Fraser et al. 1992; Enkvist and McCarthy 1994; Chen, Backus et al. 1997; Froes and de Carvalho 1998).

Ca²⁺ signaling may also serve as an ionic sensor in both physiologic and pathologic conditions. For example, low [Ca²⁺]ₑ initiates Ca²⁺ oscillations and Ca²⁺ waves in astrocytes (Zanotti and Charles 1997). This low [Ca²⁺]ₑ condition occurs in vivo during excessive neuronal activity, ischemia, or hypoglycemia suggesting that some of the CNS manifestations associated with low [Ca²⁺]ₑ may be a result, at least in part, of a dysfunction of Ca²⁺ signaling. Ca²⁺ signals generated in astrocytes, for example, can be propagated to neurons (Murphy, Blatter et al. 1993; Charles 1994; Nedergaard 1994; Cooper 1995; Newman and Zahs 1998). This could be part of the reason that low [Ca²⁺]ₑ initiates seizing (Bragdon, Kojima et al. 1992; Bikson, Ghai et al. 1999) since low [Ca²⁺]ₑ initiates spontaneous signaling in astrocytes (Zanotti and Charles 1997).

Preliminary results suggest that intercellular Ca²⁺ signaling is modulated by [Mg²⁺]ₑ. Specifically, raising extracellular Mg²⁺ inhibits spontaneous calcium signaling.
This suggests the possibility that some of the changes in organ physiology that accompany changes to $[\text{Mg}^{2+}]_e$ may result from modulation of intercellular $\text{Ca}^{2+}$ signaling. These include: 1) Tetany (Reyes and Leary 1983; Kingston, Al-Siba'i et al. 1986; Durlach, Bac et al. 1997), which is associated with low $[\text{Mg}^{2+}]_e$ and 2) Neuromuscular, autonomic, and pupillary paralysis, which is associated with hypermagnesemia (Krendel 1990; Whang 1997; Martin 1998). Since calcium signaling is directly related to neuronal activation, an inverse relationship between $[\text{Mg}^{2+}]_e$ and intercellular calcium signaling would suggest that $[\text{Mg}^{2+}]_e$ and neuronal activity would be inversely related via glial calcium signaling. This would be consistent with these and other clinical observations.

**Intercellular Communication with Other Cell Types**

$\text{Ca}^{2+}$ wave propagation proceeds across many cell-type boundaries throughout the body. In the CNS, $\text{Ca}^{2+}$ wave propagation has been noted between (non-inclusive) astrocytes and neurons (Murphy, Blatter et al. 1993; Charles 1994; Nedergaard 1994; Cooper 1995; Newman and Zahs 1998) and between astrocytes and endothelial cells (Leybaert, Paemeleire et al. 1998) (Figure 7). Given the close association between neuronal $[\text{Ca}^{2+}]_e$ and nervous system transmission, it seems evident that $\text{Ca}^{2+}$ wave propagation represents a link between glial cells and information processing and propagation by neurons (Smith 1992; Froes and de Carvalho 1998; Verkhratsky and Steinhauser 2000). This has specifically been shown to include the initiation and modulation (Kang, Jiang et al. 1998; Araque, Sanzgiri et al. 1999), by astrocytes, of electrotonic transmission in neuronal circuits. Conversely, neuronal activity has been shown to initiate $\text{Ca}^{2+}$ signaling in astrocytes (Dani, Chernjavsky et al. 1992; Murphy,
suggesting bidirectional information flow and processing mechanisms.

Ca$^{2+}$ signaling between glial cells and endothelial cells is likely to be involved in the control of blood flow and control of metabolic transfer between the cells. For example, it has recently been demonstrated that nitric oxide potentates astrocytic Ca$^{2+}$ signaling (Willmott, Wong et al. 2000). This could be one mechanism of interaction between endothelial cell function and astrocytic Ca$^{2+}$ signaling. In addition, endothelial cells release ATP in response to mechanical stimulation (i.e. a model of shear stress and/or vessel distention) (unpublished data) suggesting that purinergic paracrine Ca$^{2+}$ signaling occurs bidirectionally between these cell types. Interestingly, if ATP were released by hemichannels, both of these forms of heterocellular signaling would be modulated by [Mg$^{2+}$]e. Nitric oxide (NO) signaling, Ca$^{2+}$ signaling, Mg$^{2+}$ and endothelial cells are also implicated in migraine research, and Mg$^{2+}$ treatment is a rational, tested treatment for acute migraine relief (Mauskop 1996; Mauskop 1998).
Intercellular Ca\textsuperscript{2+} signaling is dependent on connexin channels and ATP paracrine signaling. This suggests the possibility that astrocytes release ATP through connexin hemichannels. Released ATP activates surrounding astrocytes as well as endothelial cells and neurons to initiate coordinated tissue-heterocellular responses.
Conclusions and Hypothesis

These considerations indicate that Ca\(^{2+}\) signaling plays a central role in multiple aspects of cellular and tissue physiology and pathology in the CNS. An increased understanding of the basic mechanisms of Ca\(^{2+}\) signaling in astrocytes may lead to new insights into these roles. The goal of this work is to further characterize the mechanisms of Ca\(^{2+}\) signaling in astrocytes, and to further define its potential functional roles. The general model for these studies is succinctly stated as follows: ATP released through connexin hemichannels (Figure 7) can mediate intercellular calcium signaling in astrocytes.

This general model yields multiple specific testable hypotheses that have not been directly investigated and documented in the literature at the time of this writing, including:

1) Astrocytes express functional connexin hemichannels.
2) Connexin hemichannels are opened by stimuli that initiate Ca\(^{2+}\) waves.
3) Hemichannel activators and inhibitors modulate ATP release.
4) Hemichannel activators and inhibitors modulate intercellular Ca\(^{2+}\) signaling in astrocytes.

Completion of protocols addressing these specific testable hypotheses will yield significant data for or against the proposed general model of intercellular calcium signaling in astrocytes.

Main Aims of Dissertation Research

The aims of this project are to use multiple techniques, including patch clamping, dye flux measurements, ATP measurements, and video imaging of [Ca\(^{2+}\)]\(_{e}\) to test the
hypotheses listed above. The experimental design for testing each hypothesis is summarized below:

**Aim 1: Demonstration of Connexin Hemichannels in Astrocytes**

Sub Aim A – Electrophysiology recordings of connexin hemichannel currents.
1. Measure changes in membrane currents initiated by changes to a zero $[\text{Ca}^{2+}]_e$ bathing solution.
2. Determine if inhibitors of connexin hemichannels modulate these currents.

Sub Aim B - Dye flux measurements in astrocytes.
1. Characterize the uptake of low molecular weight dyes by astrocytes under conditions known to open connexin hemichannels.
2. Characterize the release of low molecular weight dyes under conditions known to open connexin hemichannels.

**Aim 2: Measurement of Stimulation Induced ATP Release via Hemichannels**

Sub Aim A – ATP release occurs via connexin hemichannels.
1. Measure ATP released by astrocytes in response to conditions known to activate connexin hemichannels.
2. Determine if inhibitors of connexin hemichannels inhibit ATP release.

Sub Aim B – Test whether hemichannel mediated ATP release requires PLC activity and, if so, whether $[\text{Ca}^{2+}]_i$, DAG and/or IP$_3$ are involved.
1. Measure ATP release in response to mechanical stimulation in the presence of thapsigargin and BAPTA.
2. Measure ATP release in response to mechanical stimulation in the presence of PLC inhibitors.

**Aim 3: Intercellular $\text{Ca}^{2+}$ Signaling in Astrocytes Occurs via ATP Release Through Connexin Hemichannels**

Sub Aim A – Test whether mechanically induced $\text{Ca}^{2+}$ waves are regulated by hemichannel modulators as follows.
1. Measure the extent of $\text{Ca}^{2+}$ wave propagation in the presence of hemichannel inhibitors.

Sub Aim B – Test whether spontaneous $\text{Ca}^{2+}$ signaling is regulated by hemichannel modulators as follows.
1. Measure spontaneous Ca$^{2+}$ signaling in the presence of hemichannel modulators.
CHAPTER 2
INTERCELLULAR CALCIUM SIGNALING IN ASTROCYTES VIA ATP RELEASE THROUGH CONNEXIN HEMICHANNELS

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Abstract

Astrocytes are capable of widespread intercellular communication via propagated increases in intracellular Ca^{2+} concentration. We have used patch-clamp, dye flux, ATP assay, and Ca^{2+} imaging techniques to show that one mechanism for this intercellular Ca^{2+} signaling in astrocytes is the release of ATP through connexin channels ("hemichannels") in individual cells. Astrocytes showed low Ca^{2+}-activated whole-cell currents consistent with connexin hemichannel currents that were inhibited by the connexin channel inhibitor flufenamic acid (FFA). Astrocytes also showed molecular weight-specific influx and release of dyes, consistent with flux through connexin hemichannels. Transmembrane dye flux evoked by mechanical stimulation was potentiated by low Ca^{2+}, and was inhibited by FFA and Gd^{3+}. Mechanical stimulation also evoked release of ATP that was potentiated by low Ca^{2+} and inhibited by FFA and Gd^{3+}. Similar whole cell currents, transmembrane dye flux, and ATP release were observed in C6 glioma cells expressing connexin43, but were not observed in parent C6 cells. The connexin hemichannel activator quinine evoked Ca^{2+} signaling in astrocytes and in C6 cells expressing connexin43. The propagation of intercellular Ca^{2+} waves in astrocytes was also potentiated by quinine and inhibited by FFA and Gd^{3+}. Release of ATP through connexin hemichannels represents a novel signaling pathway for intercellular communication in astrocytes and other non-excitable cells.
Introduction

Astrocytes respond to a variety of stimuli with increases in intracellular calcium concentration \([\text{Ca}^{2+}]_i\) (Finkbeiner 1993; Charles 1998). \text{Ca}^{2+} signaling in astrocytes may be limited to individual cells, or may occur as a "wave" of increased \([\text{Ca}^{2+}]_i\), that is propagated from one cell to surrounding cells. Initial studies focused on gap junctions as the pathway for intercellular communication of \text{Ca}^{2+} waves in astrocytes. \text{Ca}^{2+} wave propagation has been correlated with the expression of connexins (the proteins that comprise gap junctions) in multiple cell types. For example, C6 glioma cells express low levels of connexins and do not exhibit intercellular \text{Ca}^{2+} wave propagation. Expression of connexin43 (Cx43, the predominant connexin in astrocytes) in C6 cells is correlated with both intercellular dye-transfer (Zhu, Caveney et al. 1991; Naus, Zhu et al. 1992) and propagated \text{Ca}^{2+} waves (Charles, Naus et al. 1992; Cotrina, Lin et al. 1998). Studies with other cell types have shown similar correlation of \text{Ca}^{2+} wave propagation with connexin expression (Toyofuku, Yabuki et al. 1998). In addition, pharmacological inhibitors of connexin channels have been shown to inhibit intercellular \text{Ca}^{2+} wave propagation in astrocytes (Finkbeiner 1993; Venance, Premont et al. 1998). These data show that connexin channel expression and function play a central role in \text{Ca}^{2+} wave propagation.

Recent studies have also clearly shown that the intercellular propagation of \text{Ca}^{2+} signals in astrocytes involves the diffusion of an extracellular messenger, namely ATP (Guthrie, Knappenberger et al. 1999; Cotrina, Lin et al. 2000). \text{Ca}^{2+} waves in astrocytes induced by mechanical or electrical stimuli are associated with ATP release, and intercellular \text{Ca}^{2+} waves are blocked by purinergic receptor antagonists (Guthrie, Knappenberger et al. 1999; Wang, Haydon et al. 2000). Multiple other stimuli that evoke
astrocyte signaling, including glutamate and UTP, also evoke release of ATP (Cotrina, Lin et al. 1998; Queiroz, Meyer et al. 1999).

The requirement for connexins and the involvement of ATP as a messenger in astrocyte Ca\(^{2+}\) wave propagation seem to paradoxically indicate both gap junction and extracellular messenger mediated intercellular communication. However, in addition to docking with connexins in neighboring cells, connexins form "hemichannels" or "connexons" that exist independently within an individual cell (Liu, Li et al. 1995; Li, Liu et al. 1996; Hofer and Dermietzel 1998; John, Kondo et al. 1999; Zampighi, Loo et al. 1999). Similar to other membrane channels, connexin hemichannels are transmembrane channels that connect the cytoplasm and the extracellular space. Astrocytes in culture have been reported to express connexin hemichannels that allow transmembrane dye flux (Hofer and Dermietzel 1998). These channels may provide a pathway for release of small signaling molecules, such as ATP, to the extracellular space. Roles for both an extracellular messenger and connexins in intercellular Ca\(^{2+}\) signaling could therefore be reconciled by the hypothesis that the ATP that mediates Ca\(^{2+}\) waves is released through connexin hemichannels.

Consistent with this hypothesis, Cotrina et al. (1998) reported that ATP release from C6 glioma cells is increased in direct correlation with the level of forced connexin expression. While these studies raised the possibility of a role for connexin channels in ATP release, they also found that ATP release was not inhibited by concentrations of octanol that were sufficient to inhibit gap junction channels as evidenced by dye coupling. Although this result would seem to contradict a direct role for connexin hemichannels in ATP release, it is possible that inhibitors of gap junction coupling may
not have the same effects or potency as inhibitors of connexin hemichannels. Bruzzone et al. (2000) reported that transmembrane flux of NAD$^+$ occurs via connexin43 hemichannels, indicating that the hemichannels comprised of the primary astrocyte connexin are permeable to nucleotides. In the present study, we have used dye transfer and electrophysiological techniques to confirm the presence of connexin hemichannels in astrocytes. We have then used Ca$^{2+}$ imaging and ATP measurements to show that the extent of intercellular Ca$^{2+}$ waves and ATP release in astrocytes is correlated with the function of connexin hemichannels.
Experimental Procedures

Cell Culture

Mixed glial cultures from postnatal day 1-5 mouse pups were prepared using the methods described previously (Charles, Merrill et al. 1991). To prepare purified astrocyte cultures, flasks were shaken daily for 3 days for 1 hour on a shaker at 37°C, and displaced cells were removed. Astrocytes were passaged onto poly-L-lysine-coated glass coverslips, 35 mm diameter culture dishes, or culture flasks at 50,000 cells/cm² and were maintained in growth medium (DMEM with 10% fetal calf serum, penicillin and streptomycin).

A line of C6 glioma cells stably expressing connexin43 (Cx43-13 clone) that has been described in previous studies (Zhu, Caveney et al. 1991; Charles, Naus et al. 1992) was used along with non-transfected C6 cells to examine the functional roles of connexin43. C6 cells were passaged from flasks onto glass coverslips or 35 mm culture dishes, and maintained in the same medium as described for astrocytes. Cells were grown for 1-3 days to a confluence of 70-90% prior to experimentation.

Measurement of [Ca²⁺]

[Ca²⁺] was measured using a fluorescence imaging system that has previously been described in detail (Charles, Merrill et al. 1991). In brief, cells on glass coverslips were loaded with fura2 by incubation in 5 µM fura2-AM for 30 min. Coverslips were then placed on a Nikon inverted microscope and excited with a mercury lamp through 340 and 380 nm bandpass filters, and fluorescence at 510 nm was recorded through a 20X objective with a SIT camera to a video tape recorder. Video images were digitized
using an Axon Image Lightning board and Image WorkBench software, and ΔF was calculated on a pixel-by-pixel basis as previously described.

Mass Mechanical Stimulation and Collection of Extracellular Samples

Mouse cortical astrocytes plated on 35 mm plastic petri dishes (grown for 7 - 10 days) were rinsed with control Hanks Balanced Salt Solution (HBSS) and allowed to equilibrate (25 min). Then each plate was rinsed (3x) with control HBSS (1 mM EGTA, 1mM free Mg$^{2+}$, 1.3 mM free Ca$^{2+}$) and bathed (1.5 mL) for 5 minutes at which time a sample was collected (500 μL; control spontaneous release sample) and rapidly frozen. At this point the medium was changed to treatment HBSS. A sample was taken at 4.5 minutes, after which mass mechanical stimulation was achieved by dropping ~110 μg of glass microBeads (30-50 μM diameter) through the ~3 mm of medium onto the cells. This type of stimulation has been shown to initiate multi-focal calcium waves by mechanical stimulation of individual cells (Guthrie, Knappenberger et al. 1999). All direct comparisons were made in sister cultures plated to the same density.

ATP Measurement and Analysis

ATP was measured using a luciferin/luciferase bioluminescence assay (Molecular Probes) and a fluorescence plate reader (Wallac VictorV). Calibration [ATP] curves were obtained in corresponding HBSS (i.e. matched [Mg$^{2+}$] and [Ca$^{2+}$]). The difference between [ATP]$_e$ in the sample taken after mass mechanical stimulation (5 minutes time point) and the sample taken just prior to mechanical stimulation (4.5 minute time point) was calculated as the change in [ATP]$_e$ (Δ[ATP]$_e$) for each plate under treatment HBSS.
\( \Delta [\text{ATP}]_e \) was not significant with control medium exchange without mechanical stimulation with microbeads (data not shown).

**Dye and LDH Release Measurement**

For dye release assays, cells were gently rinsed (3x; 1.5 mL) with control HBSS and then incubated with control HBSS supplemented with Calcein Blue AM (10 \( \mu \)M), for 30 minutes. Some control plates were also loaded with Oregon Green BAPTA 1 (OG) by supplementing the dye loading solution with Oregon Green BAPTA 1 AM (5 \( \mu \)M, Molecular Probes, Eugene OR). Cells were then rinsed (3x; 1.5mL) and incubated for an additional 30 minutes to allow for deesterification, after which they were placed in treatment HBSS (1.5 mL). After 4.5 minutes a sample (500 \( \mu \)L for this and all subsequent samples) was collected as a baseline. To control for fluorescence of components of treatment HBSS (e.g. Ca\(^{2+}\) or Mg\(^{2+}\)), 250 \( \mu \)L of treatment HBSS was added to baseline samples. Mass mechanical stimulation was then performed by adding medium containing microBeads (110 \( \mu \)g in 500 \( \mu \)L of HBSS) to the cells. After 40 seconds, medium samples were collected. The extracellular solution was then replenished and the cells were lysed using a cell scraper. This was followed by the final sample collection. All collections were immediately put on ice and rapidly frozen.

Samples were measured with a fluorescence plate reader (Wallac Victor\( V \)), using 350/450 (Calcein blue) or 450/535 (Oregon Green) filter sets. Percent dye released (%DR) by stimulation was calculated as follows:

\[
\% \text{DR} = \frac{I_{\text{stim}} - I_{\text{spr}}}{I_{\text{lysate}} - I_{\text{bckg}}} + \frac{I_{\text{stim}} - I_{\text{spr}}}{}
\]
where \( I_{\text{stim}} \) is measured intensity of the sample collected after mechanical stimulation. \( I_{\text{spr}} \) is the measured intensity of the spontaneous release sample (i.e. first collected sample). \( I_{\text{lysat}} \) is the measured intensity of the sample collected after the cells are lysed and \( I_{\text{bckg}} \) is the measured intensity of the treatment solution.

Samples collected as described above were also analyzed for lactate dehydrogenase (LDH) using a coupled enzymatic colorimetric assay in 96 well plates (Cytotox 96, Promega, Madison WI). Cell lysate samples were used as positive controls for LDH.

Electrophysiology

Membrane currents were measured using the whole-cell configuration. The bath solution consisted of Hank’s Balanced Salt Solution (\( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) free) supplemented with HEPES (10 mM), EGTA (1 mM), \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) (pH 7.3 with NaOH). The pipette solution contained (in mM): 140 KCl, 10 EGTA, and 10 HEPES (pH 7.2 with KOH). Pipette tips were fire-polished using a heated platinum filament to a tip resistance of 3-5 M\( \Omega \). Patch-clamp recordings were performed and analyzed using an Axopatch 200B amplifier and pClamp (Axon Instruments). All data was collected at 10 kHz and analog filtered at 5 kHz.
Results

Low Ca\(^{2+}\)-Activated Hemichannel Currents in Glial Cells

We used the whole-cell configuration of the patch clamp technique to determine if astrocytes display low [Ca\(^{2+}\)]\(_e\) activated currents consistent with connexin hemichannels. In extracellular medium containing normal (1.3 mM) [Ca\(^{2+}\)], astrocytes displayed variable inward and outward voltage-activated currents. The inhibition of the inward and outward currents by Cs\(^{2+}\) and Ba\(^{2+}\) was consistent with these currents being primarily due to inward rectifier and voltage-activated K\(^+\) channels (data not shown) as has been previously described in astrocytes (Nowak, Ascher et al. 1987; Clark and Mobbs 1994). Low [Ca\(^{2+}\)]\(_e\)-activated currents were more easily quantified in cells with low levels of baseline currents (Figure 1).

Perfusion of astrocytes with medium containing no added Ca\(^{2+}\) (with 1 mM EGTA) resulted in a baseline current at a holding potential of −50 mV, as well as a significant increase in the amplitude of both inward and outward currents evoked by voltage steps from this holding potential (Figure 1). The average ratio of the maximum steady-state inward current in low [Ca\(^{2+}\)]\(_e\) vs. normal [Ca\(^{2+}\)]\(_e\) was 1.56 ± .17 (n=5). Currents returned to baseline when the extracellular solution was exchanged for control solution. Approximately 60% of isolated astrocytes showed low [Ca\(^{2+}\)]\(_e\)-activated currents Flufenamic acid (FFA) has been reported to block currents through connexin hemichannels expressed in oocytes with high potency (Zhang, McBride et al. 1998), and to inhibit Cx43 mediated intercellular communication (Harks, de Roos et al. 2001).
Figure 8 - Connexin Hemichannel Currents in Astrocytes

A. Whole-cell currents recorded in an isolated cultured astrocyte in response to 20 mV voltage steps between -120 and 40 mV from a holding potential of -50 mV. In solution containing 1 mM Ca\textsuperscript{2+}, this cell showed low levels of voltage-activated currents. Replacing the extracellular bath solution with a low [Ca\textsuperscript{2+}]\textsubscript{e} solution immediately increased linear leak currents. Addition of flufenamic acid (50 μM) in low [Ca\textsuperscript{2+}]\textsubscript{e} solution inhibited currents to near control levels.

B. Current-voltage plot shows current amplitudes averaged from 3 different cells in response to low [Ca\textsuperscript{2+}]\textsubscript{e} solution with or without FFA. Baseline currents at the holding potential were subtracted to obtain voltage-activated current values for each cell. Standard errors for each average value ranged from 100-400 pA (not shown).
Application of 50 μM FFA immediately abolished low-[Ca\(^{2+}\)]\(_e\) activated currents (average ratio of maximum current in low [Ca\(^{2+}\)]\(_e\) with FFA vs. normal [Ca\(^{2+}\)]\(_e\) was 0.97 ± 0.02, n=3). The significant inhibition of low-[Ca\(^{2+}\)]\(_e\) activated currents (p<.02) by FFA was partially reversible (~70%; data not shown) with washout. C6 cells expressing connexin43 (C6 Cx43 cells, n=6), but not untransfected C6 cells (n=10), showed FFA-sensitive whole-cell currents similar to those observed in primary astrocytes (data not shown).

Mechanical Stimulation Induced Dye Uptake Through Hemichannels

Transmembrane flux of low-molecular weight dyes has been used to indicate the presence of connexin hemichannels in multiple cell types including astrocytes (Li, Liu et al. 1996; Hofer and Dermietzel 1998). We examined the pattern of Lucifer Yellow (LY) and Rhodamine Dextran (MW 10 kD) (RD) flux into astrocytes under conditions of normal and low [Ca\(^{2+}\)]\(_e\). Cells maintained in HBSS with normal [Ca\(^{2+}\)] containing LY (1 mg/ml) and RD (0.5%) showed little uptake of either dye. When cells were exposed to nominally Ca\(^{2+}\) free medium containing the same concentrations of LY and RD, they showed small but significant uptake of LY, but not of RD (Fig 2A). Similar uptake of LY in astrocytes has been previously reported (Hofer and Dermietzel 1998). Subsequent exposure of cells to Ca\(^{2+}\) free medium containing no dye caused release of the LY (Fig 2B).

To determine if uptake of LY was activated in association with intercellular Ca\(^{2+}\) waves, we observed the pattern of LY uptake in the region of a mechanically stimulated cell. Mechanical stimulation of a single cell reproducibly elicits an intercellular Ca\(^{2+}\)
Figure 9 - Dye Uptake in Astrocytes

Image shows a field of astrocytes that were bathed in low $[\text{Ca}^{2+}]_c$ (HBSS with no added $\text{Ca}^{2+}$) solution with Lucifer Yellow (LY, 1 mg/ml) and Rhodamine Dextran (RD, 0.5 %) for 5 minutes, then washed with normal $[\text{Ca}^{2+}]_c$ solution. Cells showed uptake of LY but not RD (not shown) in low $[\text{Ca}^{2+}]_c$ solution ($n=10$). There was no uptake of Lucifer Yellow in normal $[\text{Ca}^{2+}]_c$ solution (not shown). Image dimensions are 200 X 250 µm. B. Same field of cells shown in A. after 3 minute exposure to low $[\text{Ca}^{2+}]_c$ solution (with no dye). Exposure to low $[\text{Ca}^{2+}]_c$ solution medium caused release of dye that had been taken up in A. C. Effect of mechanical stimulation on dye uptake. A single cell in a different field (asterisk) was mechanically stimulated in low $[\text{Ca}^{2+}]_c$ (HBSS with no added $\text{Ca}^{2+}$) solution containing LY (1 mg/ml) and RD (1 mg/ml). After 5 minutes, cells were washed with normal $[\text{Ca}^{2+}]_c$ solution containing no dye. LY uptake was significantly increased in the stimulated cell and immediately surrounding cells. There was no uptake of RD (not shown). No cell-cell spread of LY fluorescence was observed after washing of LY from the bathing solution. This experiment is representative of 8 different experiments.
wave that is propagated from the stimulated cell to neighboring cells. Coverslips of mouse astrocytes were bathed in HBSS containing LY and RD and single cells were mechanically stimulated using a glass micropipette. Mechanical stimulation resulted in the uptake of Lucifer Yellow in the stimulated cell as well as in immediately adjacent cells, suggesting that this uptake occurred in association with an intercellular Ca\textsuperscript{2+} wave (Figure 2C; n = 12). The uptake of LY by both the stimulated cell and the surrounding cells was greatly potentiated by removal of extracellular Ca\textsuperscript{2+}. Although it is possible that some cell-cell spread of the LY occurred due to diffusion through gap junctions, we did not observe cell-cell spread of dye after rinsing of LY from the medium, suggesting that such diffusion was minimal. There was no uptake of RD in either the stimulated cell or surrounding cells, indicating that there was not non-specific disruption of the cell membrane.

Dye Release through Hemichannels

To further characterize dye flux, plates of cells were loaded with a connexin channel-permeable dye (Calcein Blue, approximate MW 400) and a connexin channel-impermeable dye (Oregon Green 1 BAPTA 488, approximate MW 1100) by incubating cells with the respective AM ester for 20 minutes. Dye-loaded cells were mechanically stimulated by dropping glass microbeads (30-50 \textmu M in diameter) through the medium onto the cells. This mass mechanical stimulation has been found to initiate multi-focal calcium waves without causing non-specific disruption of the cell membrane (Guthrie, Knappenberger et al. 1999).

Mass mechanical stimulation of astrocytes initiated significant release of Calcein blue but not Oregon Green or LDH. Mechanical stimulation evoked release of 7.8% of
intracellular Calcein blue (± 1.6 % SE, n=16) and 0.13 % of Oregon Green (± .25 % SE, n=8). There was no detectable release of LDH in response to mechanical stimulation under any condition (n=32, cell lysates used as positive control). Calcein release was modulated by connexin channel modulators (Figure 3). Low [Ca^{2+}]_e medium evoked dye release in unstimulated cells (not shown). Dye release evoked by mass mechanical stimulation was potentiated by low [Ca^{2+}]_e medium and was inhibited by FFA, and Gd^{3+}, both of which have been identified as potent inhibitors of connexin hemichannels (Zhang, McBride et al. 1998) (Figure 3).

Identical stimulation of C6 glioma cells, which express very low levels of connexins, evoked a significantly reduced level of Calcein blue release (2.86 ± 1.3%, n=8), whereas C6 glioma cells expressing connexin43 showed levels of Calcein blue (9.6 ± 1.3 %, n=16) release that were comparable to or higher than that shown by astrocytes. As with primary astrocytes, Calcein blue release from C6 Cx43 cells was potentiated by low [Ca^{2+}]_e medium and was inhibited by FFA and Gd^{3+} (Figure 3). C6 Cx43 cells did not release Oregon Green in response to mechanical stimulation (not shown).

**ATP Release**

Mechanical stimulation initiates rapid release of ATP from astrocytes (Guthrie, Knappenberger et al. 1999; Wang, Haydon et al. 2000). We found that ATP release evoked by mechanical stimulation with glass microbeads could be detected in as little as 10 seconds following stimulation. [ATP]_e increased for the first ~40 seconds after mechanical stimulation and remained elevated throughout the time-frame of the
Figure 10 - Astrocytes and C6 Glioma Cells Expressing Cx43 Release Low Molecular Weight Dye

Each bar represents the average percentage intracellular Calcein blue (loaded as AM ester) released under the specified condition vs. control conditions. For C6 cells, control conditions represent C6 cells expressing Cx43 in normal medium. Both 50 μM FFA (n=8, p<.03) and 50 μM Gd³⁺ (n=8, p<.02) significantly inhibited mechanically stimulated Calcein blue release by astrocytes. Calcein blue release was significantly potentiated in low [Ca²⁺]ᵢ medium (n=16, p<.03). C6 glioma cells, which show very low levels of connexin expression, showed significantly less release of Calcein blue than did C6 cells expressing Cx43 (n=8, p<.01). As with primary astrocytes, Calcein blue release by C6 cells expressing connexin43 was inhibited by 50 μM FFA (n=8, p<.03) and Gd³⁺ (n=8, p<.02) and potentiated by low [Ca²⁺]ᵢ (n=8, p<.03). Error values represent maximum variation in standard error of ratio [(condition + S.E.)/(control – S.E.) – condition/control].
measurement (5 minutes; data not shown). We took samples at 30 seconds to reflect ATP release associated with intercellular Ca$^{2+}$ waves.

Treatment with low [Ca$^{2+}$]$_e$ medium significantly potentiated the ATP release induced by mechanical stimulation (Figure 4). Conversely, the connexin channel inhibitors FFA and Gd$^{3+}$ significantly inhibited mechanically induced ATP release. Since FFA is a chloride channel blocker, we also examined the effects of another chloride channel blocker DIDS, which does not inhibit connexin channels with high potency. DIDS did not inhibit ATP release by astrocytes (Figure 4).

Cotrina et al. (1998) previously reported that ATP release evoked by UTP was correlated with connexin expression in C6 cells. We found that mechanically-induced ATP release was also correlated with connexin43 expression. Non-transfected C6 cells showed very low levels of ATP release in response to mass mechanical stimulation, whereas C6 cells expressing connexin43 showed ATP release that was potentiated by low Ca$^{2+}$, and inhibited by FFA but not DIDS, and Gd$^{3+}$ (Figure 4).

Calcium Signaling via Hemichannel Mediated ATP Release

We have previously reported that intercellular Ca$^{2+}$ waves are correlated with the level of connexin expression in C6 cells, and that reduction of [Ca$^{2+}$]$_e$ elicits intracellular and intercellular Ca$^{2+}$ signaling in astrocytes (Zanotti and Charles 1997). Based upon the results described above, this effect could be explained by “unblocking” of connexin hemichannels with subsequent ATP release. Quinine has been reported to activate connexin hemichannels in multiple preparations (Malchow, Qian et al. 1994; Dixon, Takahashi et al. 1996; Guthrie, Knappenberger et al. 1999; White, Deans et al. 1999).
Figure 11 - Astrocytes and C6 Glioma Cells Expressing Cx43 Release ATP

Each bar represents the average mechanically stimulated ATP release under the specified conditions vs. control conditions. For C6 cells, control conditions represent C6 cells expressing Cx43 in normal medium. In primary astrocytes, ATP release was significantly potentiated by low [Ca²⁺]e (p<.02) and significantly inhibited by FFA (p<.0005) and Gd³⁺ (p<.005) but not by the Cl⁻ channel blocker DIDS. C6 cells expressing Cx43 also showed release of ATP that was inhibited by FFA (p<.001) but not by DIDS, whereas parent C6 cells that express low levels of connexins showed minimal ATP release (p <.01). n=8 for each experiment, error bars represent maximum variation in standard error of ratio [(condition + S.E.)/(control – S.E.) – condition/control].
Bath application of quinine evoked multifocal intercellular \( \text{Ca}^{2+} \) waves in astrocytes (Figure 5, n=5 coverslips). These intercellular \( \text{Ca}^{2+} \) waves occurred within 3-5 minutes following application of quinine, and immediately ceased upon washout of quinine. Quinine also evoked \( \text{Ca}^{2+} \) oscillations and limited intercellular \( \text{Ca}^{2+} \) waves in C6 Cx43 cells, but did not evoke \( \text{Ca}^{2+} \) signaling in parent C6 cells (Figure 5, n=5 coverslips each).

The \( \text{Ca}^{2+} \) signaling responses of C6 Cx43 cells to quinine were inhibited (but not abolished) by apyrase or by the purinergic receptor antagonist pyridoxal- phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), indicating a role for ATP release in this response. In the presence of 50 U/L apyrase, 100 \( \mu \)M quinine evoked a significant (>100 nM) increase in \([\text{Ca}^{2+}]_i\) in an average of 14 \( \pm \) 4 \% of C6 Cx43 cells compared with an average response of 81\( \pm \)8 \% of cells after washout of apyrase (n=320 cells in 4 experiments). In the presence of 10 \( \mu \)M PPADS, 100 mM quinine evoked a significant increase in \([\text{Ca}^{2+}]_i\) in an average of 30 \( \pm \) 8 \% of C6 Cx43 cells compared with 78 \( \pm \) 6\% of controls in the absence of PPADS (n=300 cells in 3 experiments).

Brief deformation of the membrane of a single cell initiates an increase in \([\text{Ca}^{2+}]_i\) in the stimulated cell that is propagated in a wave-like manner to surrounding cells. The extent of \( \text{Ca}^{2+} \) wave propagation was significantly decreased by FFA 50 \( \mu \)M (Figures 6 and 7) n=8, \( p<.0005 \) and Gd\(^{3+} \) (28 \( \pm \) 6 \%, n=8, \( p<.005 \)) but not DIDS (77 \( \pm \) 22\%, n=8). Conversely, the extent of wave propagation was significantly increased in medium containing quinine (198 \( \pm \) 28\%, n=10, \( p<.0001 \)).
Figure 12 - Activation of Ca\textsuperscript{2+} Signaling in Astrocytes by Quinine

Raster plots show change in fura fluorescence (~[Ca\textsuperscript{2+}]) vs. time in 20 cells in fields of (A) primary astrocytes, (B) C6 cells expressing Cx43, and (C) untransfected C6 cells. Each row in the plot shows ΔF vs. time for an individual cell. Bath application of 100 μM quinine reversibly evoked Ca\textsuperscript{2+} oscillations and intercellular Ca\textsuperscript{2+} waves in the majority of astrocytes or C6 cells expressing connexin43, but not in untransfected C6 cells.
A. Astrocytes

B. C6 – Cx43

C. C6

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Discussion

Astrocytes, like multiple other cell types, are capable of widespread communication via intercellular Ca\(^{2+}\) waves. While initial studies identified gap junctions as a pathway for communication of Ca\(^{2+}\) waves, more recent studies have demonstrated an extracellular signaling pathway involving release of ATP and activation of purinergic receptors (Guthrie, Knappenberger et al. 1999; Cotrina, Lin et al. 2000). Our results provide evidence that connexin hemichannels provide a pathway for this ATP release.

First, we found that astrocytes express functional connexin hemichannels as evidenced by whole-cell patch clamp studies. Activation by low [Ca\(^{2+}\)]\(_e\) is characteristic of currents through connexin hemichannels (Zhang, McBride et al. 1998; Zampighi, Loo et al. 1999). The low [Ca\(^{2+}\)]\(_e\) - activated currents we observed in astrocytes are similar to those observed through connexin hemichannels expressed in Xenopus oocytes (Zampighi, Loo et al. 1999). Like connexin channel currents in oocytes, the low-Ca\(^{2+}\) activated currents in astrocytes were reversibly inhibited by flufenamic acid (Zhang, McBride et al. 1998; Zhang, Gao et al. 2000). Although currents through connexin43 hemichannels have not been reported in Xenopus oocyte models, our results indicate that expression of connexin43 in C6 cells results in the appearance of currents in single cells that are consistent with currents observed through gap junctions comprised of connexin43.

Transmembrane flux of low-molecular weight dyes is a commonly used method for assessing the presence and function of connexin hemichannels. Consistent with previous reports (Hofer and Dermietzel 1998), we found that astrocytes show uptake of...
Figure 13 - Effect of Flufenamic Acid on Ca\(^{2+}\) Wave Propagation

A. Ca\(^{2+}\) wave induced by mechanical stimulation of a single astrocyte in the presence of FFA (50 μM). The increase in [Ca\(^{2+}\)]\(_i\) is propagated from the stimulated cell to only a few surrounding cells. Color scale indicates change in fura fluorescence, which is proportional to change in [Ca\(^{2+}\)]\(_i\). Image dimensions are 450 X 500 μm. B. Ca\(^{2+}\) wave induced by mechanical stimulation of a different cell in the same field following washout of FFA. The increase in [Ca\(^{2+}\)]\(_i\) is propagated over a larger area to a greater number of cells. The extent of the Ca\(^{2+}\) wave following washout of FFA is similar to that observed prior to addition of FFA (not shown).
Figure 14 - Effect of Connexin Hemichannel Modulators on Ca$^{2+}$ Wave Propagation

Each bar represents the average area of intercellular Ca$^{2+}$ waves induced by mechanical stimulation of a single cell under the specified condition relative to the control conditions. The extent of Ca$^{2+}$ wave propagation was significantly decreased by FFA 50 μM (n=8, p<.0005) and Gd$^{3+}$ (n=8, p<.005) but not DIDS (n=8). Conversely, the extent of wave propagation was significantly increased in medium containing quinine (n=10, p<.0001). Error bars represent maximum variation in standard error of ratio $\frac{(\text{condition} + \text{S.E.}) - (\text{control} - \text{S.E.})}{\text{condition/control}}$. 

Error bars represent maximum variation in standard error of ratio $\frac{(\text{condition} + \text{S.E.}) - (\text{control} - \text{S.E.})}{\text{condition/control}}$. 

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Calcein blue or Lucifer yellow, both low molecular weight dyes, but not the high molecular weight dye Rhodamine dextran. In addition, we observed release of AM-ester loaded dyes in response to removal of extracellular Ca\textsuperscript{2+}. Mechanical stimulation of astrocytes also evoked both uptake and release of low-molecular weight dyes, but not release of higher molecular weight dyes (Oregon Green Bapta 1) or LDH. Mechanical stimulation evoked release of Calcein blue from C6 Cx43 cells, but not from non-transfected C6 cells. Dye release was inhibited by flufenamic acid and Gd\textsuperscript{3+}, both of which are potent inhibitors of connexin hemichannels. In summary, the size selectivity, activation by removal of extracellular Ca\textsuperscript{2+}, correlation with connexin43 expression in C6 cells, and inhibition by connexin channel inhibitors are all consistent with transmembrane dye flux via connexin hemichannels.

Since intercellular Ca\textsuperscript{2+} signaling induced by mechanical stimulation and other stimuli has been associated with ATP release (Cotrina, Lin et al. 1998; Guthrie, Knappenberger et al. 1999; Wang, Haydon et al. 2000), we examined the role of connexin hemichannels in mechanically induced ATP release. Cotrina et al. (1998) previously reported a correlation of UTP-induced ATP release with connexin expression in glial cells. We found a similar correlation of mechanically induced ATP release with connexin expression in C6 glioma cells. We also found that low extracellular Ca\textsuperscript{2+} and quinine evoke spontaneous ATP release and potentiate mechanically induced ATP release from astrocytes, while inhibitors of connexin hemichannels including FFA and Gd\textsuperscript{3+} all inhibited mechanically induced ATP release. Based upon these results, we conclude that mechanically induced release of ATP involves connexin hemichannels.
Modulators of connexin hemichannels had consistent effects on intercellular Ca$^{2+}$ signaling in astrocytes. We have previously reported that reduction of extracellular Ca$^{2+}$ evokes intercellular Ca$^{2+}$ signaling in astrocytes (Zanotti and Charles 1997) - this finding can now be explained by the "unblocking" of connexin hemichannels and subsequent ATP release to mediate intercellular signaling. Consistent with this hypothesis, the connexin hemichannel activator quinine evoked intercellular Ca$^{2+}$ signaling in astrocytes, and mechanically induced intercellular Ca$^{2+}$ waves were potentiated by quinine. Conversely, the extent of intercellular Ca$^{2+}$ wave propagation was significantly reduced by the same concentrations of connexin hemichannel inhibitors that inhibited whole cell currents, transmembrane dye flux, and release of ATP.

Cotrina et al. (1998) raised the possibility of connexin hemichannels as a pathway for release of ATP and intercellular Ca$^{2+}$ signaling. However, their observations that octanol did not block ATP release at concentrations sufficient to block dye coupling seemed to contradict this possibility. Our results indicate that there is a consistent correlation between modulation of connexin hemichannel currents, low molecular weight dye release, and ATP release. It is possible that inhibitors of gap junctions such as octanol act with different potency and specificity on intercellular gap junction channels vs. individual connexin hemichannels. Quinine represents an example of an agent with distinct effects on connexin hemichannel function vs. gap junctional coupling; whereas quinine activates connexin hemichannels, it does not have a parallel effect on gap junctional conductance (Malchow, Qian et al. 1994; Dixon, Takahashi et al. 1996; Zanotti and Charles 1997; White, Deans et al. 1999).
We used flufenamic acid and Gd\textsuperscript{3+} as inhibitors of connexin hemichannels, because both have been shown to inhibit connexin hemichannels expressed in oocytes with high potency (30), and neither evoked increases in [Ca\textsuperscript{2+}]. By contrast, we found that other traditional gap junction inhibitors such as octanol, glycyrrhetinic acid, or oleamide all evoked Ca\textsuperscript{2+} signaling that included elevations of baseline Ca\textsuperscript{2+}, Ca\textsuperscript{2+} oscillations, and intercellular Ca\textsuperscript{2+} waves (not shown). The mechanism for the activation of Ca\textsuperscript{2+} signaling by these agents is not known. Regardless of the mechanism, however, these increases in [Ca\textsuperscript{2+}]i confounded interpretation of their effects regarding a role for connexin channels.

Although both FFA and Gd\textsuperscript{3+} also inhibit other channels as well as connexin channels, their effects with multiple different assays of connexin channel function support the interpretation that their effects in the present study are primarily due to connexin channel inhibition. In addition, we found that other Cl\textsuperscript{-} channel blockers such as DIDS that do not inhibit connexin hemichannels (Dixon, Takahashi et al. 1996; Bruzzone, Guida et al. 2000) did not inhibit intercellular Ca\textsuperscript{2+} signaling or ATP release. Quinine is also well known to have effects on other ion channels. However, the activation of Ca\textsuperscript{2+} signaling only in C6 cells expressing connexin43, and not in non-transfected C6 cells, is consistent with activation of connexin hemichannels, as has previously been reported for multiple types of connexins.

The correlation of results of electrophysiological studies, dye flux measurements, ATP release measurements, and imaging of intercellular Ca\textsuperscript{2+} signaling provides strong evidence for the hypothesis that intercellular Ca\textsuperscript{2+} waves in astrocytes can occur by ATP release through connexin hemichannels. The parallel results obtained only with C6 cells
expressing connexin43, and not with non-transfected C6 cells, corroborates this hypothesis. A primary role for connexin hemichannels in intercellular Ca\textsuperscript{2+} signaling does not exclude other potential intercellular pathways, such as gap junctions. It is also possible that connexin expression is associated with upregulation of some other pathway for ATP release. However, ATP release via connexin hemichannels is the most direct explanation for our results, and provides a direct mechanism which reconciles both a role for connexins and a role for extracellular ATP in intercellular Ca\textsuperscript{2+} waves.

Astrocyte Ca\textsuperscript{2+} waves have been implicated in a variety of physiological and pathological processes. They have been shown to modulate synaptic signaling between neurons, suggesting a role in synaptic plasticity (Araque, Parpura et al. 1998; Kang, Jiang et al. 1998; Parpura and Haydon 2000). Haydon and colleagues have provided evidence that this modulation of synaptic function is mediated by astrocytic release of glutamate, which they have shown to occur in association with Ca\textsuperscript{2+} waves (Araque, Sanzgiri et al. 1998; Innocenti, Parpura et al. 2000; Parpura and Haydon 2000). Astrocyte Ca\textsuperscript{2+} waves have also been shown to modulate the response of the retinal neurons to light stimulation (Newman and Zahs 1998). A role for astrocyte Ca\textsuperscript{2+} waves has also been proposed in migraine headache and the spread of seizures (Charles 1998). The identification of connexin hemichannels as a pathway for stimulus-evoked release of small molecules raises the possibility that in addition to ATP, other intercellular messengers may also be released via this pathway. Further investigation of this mechanism of intercellular signaling may provide an opportunity for greater understanding of the functional significance of Ca\textsuperscript{2+} waves in astrocytes and other cell types.

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CHAPTER 3
MODULATION OF CALCIUM SIGNALING IN ASTROCYTES BY EXTRACELLULAR DIVALENT CATIONS

GLIA
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Modulation of Ca^{2+} Waves by Divalent Cations

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Abstract

Glial cells display propagated waves of cytoplasmic Ca\(^{2+}\) mobilization that spread outward from an initiating cell to involve hundreds to thousands of surrounding cells. These Ca\(^{2+}\) waves are mediated by extracellular waves of increased [ATP], termed ATP waves. Recently, we reported that the ATP release occurs through connexin hemichannels. Consistent with this, we demonstrated a direct correlation between the extent of calcium wave propagation and hemichannel activity by pharmacological modulation of hemichannels using inhibitors like Gd\(^{3+}\) and flufenamic acid and hemichannel activators like quinine.

In addition to these exogenous modulators, hemichannels are modulated by divalent cations including Ca\(^{2+}\) and Mg\(^{2+}\) that are endogenous to the glial environment. Previously we reported that Ca\(^{2+}\) signaling in glial cells is modulated by divalent cations which now seems likely to be a result of the regulation of hemichannel-mediated ATP release. Here we explore this possibility finding that hemichannel activity in astrocytes is inversely proportional to both extracellular Mg\(^{2+}\) and Ca\(^{2+}\) concentration. The modulation of hemichannel activity by divalent cations is inversely related to ATP release, the frequency of spontaneous Ca\(^{2+}\) signaling events and the extent of mechanical stimulation-induced Ca\(^{2+}\) waves.

These findings provide further evidence that ATP released through hemichannels mediates calcium waves, and suggest that Mg\(^{2+}\) and Ca\(^{2+}\) are critical regulators of Ca\(^{2+}\) signaling in glial cells. Ca\(^{2+}\) signaling, divalent cations, glial cells and purinergic signaling have all been implicated in the pathophysiology of several types of CNS dysfunction, including seizure and migraine. We propose that the convergence of these
data is the regulation of $\text{Ca}^{2+}$ signaling, in part by regulation of ATP release through hemichannels.
Introduction

It is well known that the extracellular concentrations of magnesium ([Mg$^{2+}$]$_e$) and calcium ([Ca$^{2+}$]$_e$) play an important role in physiological and pathological processes of the nervous system. Decreases in [Mg$^{2+}$]$_e$ are associated with increased excitability in the CNS (Reyes and Leary 1983; Kingston, Al-Siba'i et al. 1986; Whang 1997), and with the pathological phenomena of seizures and migraines (Kingston, Al-Siba'i et al. 1986; Mauskop 1996; Whang 1997; Mauskop 1998) while increases in [Mg$^{2+}$]$_e$ are associated with neuronal hypoactivity (Krendel 1990; Whang 1997; Martin, el-Sabban et al. 1998). These effects of extracellular divalent cations have been traditionally ascribed to effects on the neuronal NMDA receptor channel, or neuronal Ca$^{2+}$ channels.

Recent studies, however, show that astrocytes also play an important role in modulating excitability in the CNS (Smith 1992; Froes and de Carvalho 1998; Newman and Zahs 1998; Laming, Kimelberg et al. 2000). In part, this new understanding of astrocytic function is derived from the discovery of propagated waves of cytoplasmic Ca$^{2+}$ mobilization (Charles, Merrill et al. 1991; Finkbeiner 1992). These “Ca$^{2+}$ waves” occur either spontaneously (Fatatis and Russell 1992; Harris-White, Zanotti et al. 1998) or in response to activators like mechanical stimulation (Charles, Merrill et al. 1991), glutamate stimulation (Cornell-Bell, Finkbeiner et al. 1990; Charles, Merrill et al. 1991; Enkvist and McCarthy 1994), or purinergic stimulation (Guthrie, Knappenberger et al. 1999; Cotrina, Lin et al. 2000). Astrocytic Ca$^{2+}$ waves have been shown to cross into neuronal cells and modulate (Newman and Zahs 1998; Araque, Parpura et al. 1999) or even initiate (Araque, Parpura et al. 1998; Araque, Sanzgiri et al. 1999) neuronal activity.
The mechanism of Ca$^{2+}$ wave propagation in astrocytes involves extracellular diffusion of signal molecule(s), i.e. an extracellular communication system (Hassinger, Guthrie et al. 1996; Charles 1998) mediated by ATP (Guthrie, Knappenberger et al. 1999). Recently, we reported evidence that the ATP release associated with Ca$^{2+}$ waves occurs through connexin43 (Cx43) hemichannels (Stout, Constantin et al. 2002).

Consistent with this hypothesis, we found that Ca$^{2+}$ waves are inhibited by agents known to inhibit hemichannel activity including flufenamic acid and Gd$^{3+}$ (Zhang, McBride et al. 1998; Zhang and Hamill 2000). In contrast, we found that Ca$^{2+}$ wave propagation is increased in the presence of hemichannel activators including zero [Ca$^{2+}$]$_e$ (DeVries and Schwartz 1992; Ebihara and Steiner 1993; Liu, Li et al. 1995; Li, Liu et al. 1996) and quinine (Malchow, Qian et al. 1994; Dixon, Takahashi et al. 1996; White, Deans et al. 1999; Al-Ubaidi, White et al. 2000).

Extracellular divalent cations have been shown in several preparations to modulate connexin hemichannel activity (DeVries and Schwartz 1992; Ebihara and Steiner 1993; Malchow, Qian et al. 1993; Liu, Li et al. 1995; Li, Liu et al. 1996). Previously, we reported that the [Ca$^{2+}$]$_e$ affects Ca$^{2+}$ signaling in astrocytic cultures by a mechanism sensitive to suramin, i.e. the extracellular communication system (Zanotti and Charles 1997). Here we characterize the effect of [Mg$^{2+}$]$_e$ and [Ca$^{2+}$]$_e$ on spontaneous Ca$^{2+}$ signaling, measures of Ca$^{2+}$ wave propagation, hemichannel activity, and mechanical stimulation-induced ATP release. We report that there is an inverse relationship between extracellular divalent cations and measures of intercellular calcium signaling. This is correlated with a parallel effect on Cx43 hemichannel activity, ATP release, and stimulation-induced hemichannel activity. These findings strongly favor the
hypothesis that ATP released through connexin hemichannels mediates glial Ca$^{2+}$ signaling. Given the link between astrocytic Ca$^{2+}$ signaling and neuronal excitability, modulation of this astrocytic signaling system by extracellular divalent cations could represent an extra-neuronal site whereby extracellular divalent cations act to regulate neuronal excitability.
Experimental Procedures

Solutions

Solutions were constructed from Ca\(^{2+}\) and Mg\(^{2+}\) free Hank's balanced salt solution (HBSS) (Sigma) supplemented with EGTA (1 mM), CaCl\(_2\), and MgCl\(_2\) to yield the desired free [Ca\(^{2+}\)] and [Mg\(^{2+}\)]. Total CaCl\(_2\) and MgCl\(_2\) were calculated using MaxChelator (Donald Bers 1994) (http://www.stanford.edu/~cpatton/maxc.html).

Cell Culture

Glia from postnatal day 1-5 mouse pups were obtained using the methods described previously (Charles, Merrill et al. 1991). Briefly, the cortical hemispheres were removed, cleaned, and dissociated by trypsinization (0.2%; Life Technologies, Gaithersburg, MD) and mechanical trituration. Cells were plated on poly-L-lysine-coated glass coverslips, in culture flasks, or in 24-well plates (2 cm\(^2\)/well) at 50,000 cells/cm\(^2\) and were maintained in growth medium [MEM (Life Technologies), 10% fetal calf serum (HyClone, Logan, UT), penicillin and streptomycin (Life Technologies), essential amino acids (Life Technologies), and nonessential amino acids (Life Technologies)] with one to two media changes per week. Cultures were used at 5-15 days in vitro. All experiments were performed at room temperature.

Astrocytes passaged from confluent monolayers were used in several experiments. After enzymatic treatment [0.05% trypsin + 0.53 mM EDTA (Life Technologies)] at 37°C for 10 min, astrocytes were washed off the culture surface, plated onto glass coverslips as described above, and used after 5-15 d. No differences were observed between passaged and primary cultured astrocytes.
Measurement of \([\text{Ca}^{2+}]_i\)

\([\text{Ca}^{2+}]_i\) was measured using a fluorescence imaging system that has previously been described in detail. In brief, cells on glass coverslips will be loaded with fura2 by incubation in 5 \(\mu\)M fura2-AM for 30 min. Cells were then washed and maintained in normal medium for 20-30 minutes prior to experimentation. Coverslips were then placed on a Nikon Diaphot inverted microscope and excited with a mercury lamp through 340 and 380 nm bandpass filters, and fluorescence at 510 nm will be recorded through a 20X or 40X objective with a SIT camera to a videotape recorder. Digitized images (Axon WorkBench 2.1) were subjected to background subtraction and shading correction, after which \(\Delta F\) was calculated on a pixel-by-pixel basis as previously described. In some cases, cells were chosen at random within the field of view and the intensity was recorded continuously using Axon WorkBench 2.1. This data was imported into RasterPlot for visualization of spontaneous \(\text{Ca}^{2+}\) signaling events as previously described. Data acquisition and analysis software were written by Dr. Michael Sanderson.

**Mass Mechanical Stimulation and Macrocollection of Extracellular Message Samples**

Purified mouse cortical astrocytes plated on 2 mL plastic petri dishes (grown for 7–10 days) were rinsed (3x; 1.5 mL) with control HBSS (HEPES, EGTA, 1mM; free Mg\(^{2+}\), 1 mM; free Ca\(^{2+}\), 1.3 mM) and allowed to equilibrate (25 min). For ATP release assays each plate was subsequently rinsed (3x; 1.5 mL) with control HBSS and bathed (1.5 mL) for 4.5 minutes at which time a sample was collected (50 \(\mu\)L; control spontaneous release sample), put on ice (immediately), and rapidly frozen (within 10 minutes). Another sample (50 \(\mu\)L) was collected at 5 minutes. At this point the medium
was changed to treatment HBSS (3x rinse; 1.5 mL). Then a sample was taken both at 4.5 minutes (50 µL; treatment spontaneous release sample) and at 5 minutes (50 µL; mechanical stimulation sample). In all plates but controls, mass mechanical stimulation was achieved by dropping ~110 µgms of glass µBeads (30-50 µM diameter) through the ~3 mm of medium onto the cells immediately after the 4.5 minute collection. This type of stimulation has been shown to initiate multifocal calcium waves by gentle mechanical stimulation of individual cells by µBeads (Guthrie, Knappenberger et al. 1999). All direct comparisons were made in sister cultures plated to the same density. The sample size for each treatment or control was 8 plates with most experiments being repeated three different times.

For dye release assays, cells were gently rinsed (3x; 1.5 mL) with control HBSS and then incubated with control HBSS supplemented with Calcein Blue AM (5 µM) for 20 minutes. Some control plates were also loaded with Oregon Green BAPTA 1 by supplementing the dye loading solution with Oregon Green BAPTA 1 AM (5 µM). After this the cells were rinsed (3x; 1.5mL) and incubated for an additional 20 minutes to allow for deesterification. Next the cells were rinsed with treatment HBSS (3x; 1.5 mL) to replace the bathing solution (1.5 mL) with treatment HBSS. After 4.5 minutes a sample (50 µL) was collected as a baseline. Next mass mechanical stimulation of each plate was accomplished by dropping µBeads (110 µgms) through the medium onto the cells. After 40 seconds of stimulation (i.e. 5 minutes 10 seconds into the experiment), samples of the effluent were collected (500 µL). Finally, the extracellular solution was replaced by HBSS supplemented with 10µg/ml saponin and the cells were lysed using a cell scraper.
This was followed by the final sample collection (500 μL). All collections were immediately put on ice and rapidly frozen within 10 minutes.

**ATP Measurement and Analysis**

ATP was measured using a luciferin/luciferase bioluminescence assay (Molecular Probes) and a luminometer (Monolight 1500; Analytical Luminescence Lab). Luminescent values were converted to [ATP] by means of a standard calibration using known [ATP] in corresponding HBSS (i.e. matched [Mg²⁺] and [Ca²⁺]). The difference between [ATP]ₐ in the sample taken after mass mechanical stimulation (5 minutes time point) and the sample taken just prior to mechanical stimulation (4.5 minute time point) was calculated as the change in [ATP]ₐ (Δ[ATP]ₐ) for each plate. Δ[ATP]ₐ was not significant in plates that were not mechanically stimulated by μBeads (data not shown). Possible changes in Δ[ATP]ₐ were tested using a standard t-Test.

**Dye Release Measurement**

Samples were measured using a microfluorometer (Fluorolite 1000, Rev. 2; Dynex Technologies). To measure the Calcein Blue, a 350/450 filter set was used (ex/em). In cases where Oregon Green BAPTA 1 needed to be measured, a 450/535 filter set was used (ex/em). In each case, treatment HBSS was also measured as a background. Percent dye released (%DR) by stimulation was calculated as follows.

\[ \%DR = \frac{I_{stim} - I_{sr}}{I_{Total} - I_{bg}}, \]

where \( I_{stim} \) is measured intensity of the sample collected after mechanical stimulation. \( I_{sr} \) is the measured intensity of the spontaneous release sample (i.e. first collected sample). \( I_{Total} \) is the total loaded dye which equals \( I_{lysate} + I_{stim} - I_{sr} \) where \( I_{lysate} \) is the measured
intensity of the sample collected after the cells are lysed. $I_{bg}$ is the measured intensity of the treatment solution. Possible differences were tested using the standard t-Test.

**Measurement of Calcium Wave Extent**

$Ca^{2+}$ waves were initiated by a brief gentle perturbation of the membrane of a single cell using a micropipette mounted on a micromanipulator. For each treatment, 8-16 waves initiated sequentially in control HBSS followed by 16 sequentially initiated $Ca^{2+}$ waves in treatment HBSS followed by 8-16 $Ca^{2+}$ waves initiated in control HBSS were digitized using Imaging Workbench 2.2 (Axon Tech, Union City, CA) for measurement. The diameters of these digitized waves were measured in two perpendicular dimensions, one of which was the largest for each individual wave and calibrated by means of a micrometer. The average of the two measurements was taken as a measure of the extent of $Ca^{2+}$ wave propagation for each recorded wave. In order to better represent the changes noted visually, this measure of diameter was converted to an approximation of the area using $\pi \times (\text{diameter}/2)^2$. Potential changes in the extent of $Ca^{2+}$ wave propagation were tested using the standard t-Test.

**Electrophysiology**

Membrane currents were measured using the whole-cell configuration. The bath solution consisted of (mM) 130 NaCl, 4 KCl, 5 CsCl, 2 TEACl, 1 EGTA, 2.3 CaCl$_2$ (1.3 Free), 1 MgCl$_2$ and 10 HEPES (pH 7.3 with NaOH). The pipette solution contained (in mM): 140 K-MES, 5 CsCl, 2 TEACl, 10 EGTA, 1 CaCl$_2$, 1 MgCl$_2$ and 10 HEPES (pH 7.2 with KOH). Pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL; Cat # TW150F-4) using a Kopf Model 730 puller. Patch-
clamp recordings were performed using an Axopatch 200A or B amplifier, the data was stored and analysed using Axon instruments (Foster City, CA) software (pClamp) on a pentium PC. Pipette tips were fire-polished using a heated platinum filament to a tip resistance of 3-5 MΩ. The access resistance was less then 15 MΩ in all experiments as determined by the compensation circuitry of the amplifier. A low resistance 100 mM KCl bridge was used to connect the ground Ag/AgCl electrode to the bath. All data was collected at 10 kHz and analog filtered at 5 kHz.
Results

Extracellular Magnesium Modulates Calcium Signaling in Astrocytes

Modulation of Mechanical Stimulation Induced Calcium Signaling by [Mg$^{2+}$]$_e$

Mechanical stimulation of a single cell results in a wave of Ca$^{2+}$ mobilization that spreads an average of 458 μm +/-29 in each direction under control conditions (i.e. 1 mM [Mg$^{2+}$]$_e$, 1.3 mM [Ca$^{2+}$]$_e$). There is an inverse correlation between [Mg$^{2+}$]$_e$ and the extent of Ca$^{2+}$ wave propagation. This occurs when Ca$^{2+}$ waves are initiated in the same cell field (Figure 15a and 15b) or in separate cell fields (Figure 16; n = 27; N = 3; p = 4.2e-7). In the measurements depicted, Mg$^{2+}$ concentration was changed from 1 mM to 0 mM to 5 mM and finally back to 1 mM. Between 5 and 10 stimulations were recorded at each [Mg$^{2+}$]$_e$ per plate. There was no statistical difference between the 1 mM [Mg$^{2+}$]$_e$ concentrations (data not shown; p = 0.51). In other trials, the order of treatment was different with no notable difference in the finding.

During the course of the experiments it was noted that calcium wave propagation occurs much more frequently between non-contacting cells in sparsely plated cultures in zero [Mg$^{2+}$]$_e$ solutions (data not shown) highlighting the fact that Mg$^{2+}$ treatments modulate calcium signaling by regulating the extracellular signaling system.
Modulation of Spontaneous Calcium Signaling by \([\text{Mg}^{2+}]_e\)

Purified astrocyte cultures routinely display two types of spontaneous changes in \([\text{Ca}^{2+}]_i\). These have been termed "primary" events which are spontaneous changes in \([\text{Ca}^{2+}]_i\), in a single cell (i.e. single cell oscillations) and "secondary" events which are intercellular \(\text{Ca}^{2+}\) waves that sometimes are initiated by "primary" events (Zanotti and Charles 1997). In a cell field which displays spontaneous oscillations, changing from 5 mM \([\text{Mg}^{2+}]_e\) to 0 mM \([\text{Mg}^{2+}]_e\) results in an increase in the number of "secondary" events (Figure 15c; \(N = 5\)). Changing \([\text{Mg}^{2+}]_e\) concentration inconsistently resulted in changes to the frequency of primary events. It was not clear which factor(s) were responsible for this variability (data not shown). The second type of change involves changes in baseline \([\text{Ca}^{2+}]_i\). There is an inverse relationship between \([\text{Mg}^{2+}]_e\) and \([\text{Ca}^{2+}]_i\). Changing \([\text{Mg}^{2+}]_i\) from 1 mM to 5 mM caused a decrease in fluorescence. This effect is qualitatively similar to that seen by changing \([\text{Ca}^{2+}]_e\) (Zanotti and Charles 1997).

Next we tested to see whether low \([\text{Mg}^{2+}]_e\) activated \(\text{Ca}^{2+}\) signaling requires the expression of connexin43. To do this we used the connexin deficient C6 glioma cell line and the C6/Cx43 glioma cell line, constructed by forcing C6 glioma cells to express Cx43 (Zhu, Caveney et al. 1991). Changing from 5 mM to 1 mM \([\text{Mg}^{2+}]_e\) initiated spontaneous primary and secondary events in C6/Cx43 glioma cells. In contrast, C6 glioma cells exhibited very few, if any, primary \(\text{Ca}^{2+}\) signaling events and no secondary events were noted (\(n=6\); data not shown).
Figure 15 – Modulation of Calcium Signaling by Extracellular Divalent Cations.

Ca\(^{2+}\) waves elicited by mechanical stimulation in 5mM (A) and 1 mM (B) [Mg\(^{2+}\)]\(_e\) in the same cell field showing inhibition of the extent of calcium wave propagation by high Mg\(^{2+}\). Inhibition was more pronounced in high Ca\(^{2+}\) (data not shown). There is an inverse relationship between mechanically elicited Ca\(^{2+}\) wave propagation and [Mg\(^{2+}\)]\(_e\) (Figure 16; N = 3; n = 27; p = 4.2e-7).

(C) Spontaneous Ca\(^{2+}\) signaling visualized using Raster plots show changes in fura fluorescence (~[Ca\(^{2+}\)]) vs. time in 33 cells in fields of primary astrocytes. Each row in the plot shows ΔF vs. time for an individual cell. Switching from 5 mM to 0 mM [Mg\(^{2+}\)]\(_e\) reversibly evoked Ca\(^{2+}\) oscillations and intercellular Ca\(^{2+}\) waves in the majority of astrocytes (N = 5).
Mechanical Stimulation Causes a Rapid Increase in [ATP]ₐ

The Effect of [Mg²⁺]ₐ on Stimulation-Induced Changes in [ATP]ₐ

Recently it has been demonstrated that ATP is the messenger involved in the extracellular communication system that is critical for successful Ca²⁺ wave propagation in astrocytes (Guthrie, Knappenberger et al. 1999; Cotrina, Lin et al. 2000). Consistent with this, it was found that mass mechanical stimulation of a plate of astrocytes, accomplished by dropping glass μBeads through the media onto the cells, resulted in a rapid increase in [ATP]ₐ (<30 seconds). We found that ATP release was readily detected within 10 seconds of mass mechanical stimulation (data not shown). Upon mechanical stimulation, [ATP]ₐ increased for 2 minutes and remained elevated through the duration of the measurement (4.5 minutes). This is consistent with other reports where the return to baseline of [ATP]ₐ is found to take approximately 20 minutes (Grygorczyk and Hanrahan 1997). All further protocols involved ATP collection at the 30-second time point to best assess the pattern of ATP release associated with a Ca²⁺ wave.

Since [Mg²⁺]ₐ modulated the extent of Ca²⁺ wave propagation, we tested its effect on mechanically induced increases in [ATP]ₐ. In control HBSS, we found that mass mechanical stimulation caused a marked increase in [ATP]ₐ (3 independent trials, i.e. N=3; 8 samples per trial, i.e. n =8). This increase was substantially decreased in the presence of HBSS containing 5 mM [Mg²⁺]ₐ (N=3, n=8). In contrast, the increase in [ATP]ₐ elicited by mechanical stimulation was potentiated by bathing cells in Mg²⁺ free HBSS (N=3; n=8). Figure 16 illustrates the inverse relationship between changes in [Mg²⁺]ₐ and mechanically induced increases in [ATP]ₐ (p = 0.001; single factor
Dye Release – Mechanical stimulation of astrocytes preloaded with a hemichannel permeable dye (e.g. Calcein Blue) initiates dye release that is sensitive to $[\text{Mg}^{2+}]_e$. In contrast, mechanical stimulation fails to initiate release of co-loaded hemichannel impermeable dyes (e.g. Oregon Green BAPTA; data not shown). One of 2 independent trials ($N=2; n=8$ per treatment; $p = 0.0056$, single factor ANOVA). ATP Release – Mechanical stimulation of astrocytes initiates ATP release that is sensitive to $[\text{Mg}^{2+}]_e$. One of 3 independent trials ($N=3; n=8$ per treatment; $p = 0.0001$, single factor ANOVA). Calcium Wave Extent – Mechanical stimulation of single cells by means of a polished micropipette initiates calcium waves that are sensitive to $[\text{Mg}^{2+}]_e$. One of 3 independent trials ($N = 3; n = 27; p = 4.2e-7$, single factor ANOVA).
ANOVA). The same relationship was noted in each of three independent trials although the baseline release varied between 14 nM and 40 nM. Thus, all direct comparisons described are between sister cultures.

**The Effect of \([\text{Ca}^{2+}]_e\) on Mechanically-Induced Changes in [ATP]_e**

Previously, we reported that treatment with a zero \([\text{Ca}^{2+}]_e\) solution induces spontaneous \(\text{Ca}^{2+}\) transients and alters \(\text{Ca}^{2+}\) wave propagation patterns, suggesting that \([\text{Ca}^{2+}]_e\) also interacts with the extracellular signaling pathway (Zanotti and Charles 1997). Recently, we reported that connexin hemichannels mediate ATP release in astrocytes suggesting that part of the effect of extracellular divalent cations in modulating \(\text{Ca}^{2+}\) signaling is a result of modulation of hemichannel mediated ATP release. Consistent with this, we found that mechanically initiated ATP release was potentiated in zero \([\text{Ca}^{2+}]_e\) solutions. To further examine the role of \([\text{Mg}^{2+}]_e\) in ATP release, we measured ATP release in the absence of \([\text{Ca}^{2+}]_e\) in 1mM and 5 mM \([\text{Mg}^{2+}]_e\). We found that increasing \([\text{Mg}^{2+}]_e\) largely inhibited mechanically induced increases in [ATP]_e (Fig 17; 

\[ p = 0.0002 \]) Further, the increase in ATP release in low \([\text{Ca}^{2+}]_e\) was greater than for low \([\text{Mg}^{2+}]_e\) (N=1; n=8 per treatment; p=0.012; student’s t-Test).

\([\text{Mg}^{2+}]_e\) Modulates Hemichannel Activity in Astrocytes

**\([\text{Mg}^{2+}]_e\) Modulates Low-[\text{Ca}^{2+}]_e Activated Hemichannel Currents**

Hemichannels are inhibited by divalent cations. Thus, removal of divalent cations from the extracellular space opens hemichannels, increasing membrane conductance (Li, Liu et al. 1996). Previously, we have reported that removal of extracellular \(\text{Ca}^{2+}\) increases membrane conductance as a result of hemichannel opening (Stout, Constantin et al. 2002). Briefly, removal of extracellular \(\text{Ca}^{2+}\) initiates ~1.5 fold increase in
Figure 17 – Low [Ca$^{2+}$]$_{e}$ Potentiated Hemichannel Function and ATP Release are Inhibited by High [Mg$^{2+}$]$_{e}$.

Dye Release – Mechanical stimulation of astrocytes preloaded with a hemichannel permeable dye (e.g. Calcein Blue) initiates dye release that is potentiated by low [Ca$^{2+}$]$_{e}$. This is inhibited by high [Mg$^{2+}$]$_{e}$. One of 2 independent trials (N=2; n=8 per treatment; p = 0.00013, student’s t-Test). ATP Release – Mechanical stimulation of astrocytes initiates ATP release that is potentiated by low [Ca$^{2+}$]$_{e}$. This is also inhibited by high [Mg$^{2+}$]$_{e}$. One of 2 independent trials (N=2; n = 8 per treatment; p = 0.0075, student’s t-Test).

Control = 1.3 mM free [Ca$^{2+}$]$_{e}$ and 1 mM [Mg$^{2+}$]$_{e}$. Low [Ca$^{2+}$]$_{e}$ = zero added calcium. Control [Mg$^{2+}$]$_{e}$ = 1 mM [Mg$^{2+}$]$_{e}$. High [Mg$^{2+}$]$_{e}$ = 5 mM [Mg$^{2+}$]$_{e}$. There is 1 mM EGTA is all solutions.
membrane conductance in single astrocytes. Here we report that raising $[\text{Mg}^{2+}]_e$ to 5 mM inhibits this increase (Figure 18). The inhibition reduces maximal inward currents to $1.24^{+/-} 0.2$ times background from the 1.5 fold increase.

**Mechanical Stimulation Induced Dye-Uptake is Modulated by $[\text{Mg}^{2+}]_e$**

Another method commonly used to assay connexin hemichannels involves dye flux across the membrane. It is commonly held that this dye flux is restricted by the size of connexin hemichannel pores to <1 kD and is increased by removal of extracellular Ca$^{2+}$ to such an extent that it is readily measured by standard microfluorimetry techniques (Lal, John et al. 1995; Li, Liu et al. 1996; Trexler, Bennett et al. 1996; Vanoye, Vergara et al. 1999). This technique has been used to demonstrate the presence of hemichannels in astrocytes (Hofer and Dermietzel 1998). Further, dye flux is inhibited by extracellular divalent cations (Li, Liu et al. 1996; Ramanan, Brink et al. 1999), flufenamic acid (Stout, Constantin et al. 2002), and Gd$^{3+}$ (Zhang, McBride et al. 1998; Zhang and Hamill 2000).

We have reported that mechanical stimulation initiates hemichannel channel activity that mediates the flux of large anions, including ATP and Calcein Blue, from astrocytes (Stout, Constantin et al. 2002).

These considerations prompted us to examine the effect of extracellular divalent cations on dye flux in astrocytes. Astrocytes were loaded with either the Cx43 hemichannel permeable dye Calcein Blue (MW ~ 280 D) or the hemichannel impermeable dye Oregon Green (MW ~ 1.4 kD) by incubation with the respective AM ester for 20 minutes. Subsequently, cells were incubated for an additional 20 minutes to
Figure 18 - Effect of $\text{Mg}^{2+}$ on Hemichannel Currents

Treatment with zero $[\text{Ca}^{2+}]_{e}$ initiates an increase in membrane currents that is inhibited by raising $[\text{Mg}^{2+}]_{e}$ to 5 mM. A. Control currents (1 mM $[\text{Mg}^{2+}]_{e}$; 1.3 mM $[\text{Ca}^{2+}]_{e}$). B. Zero $[\text{Ca}^{2+}]_{e}$ induced currents (1 mM $[\text{Mg}^{2+}]_{e}$). C. Inhibition of currents by raising $[\text{Mg}^{2+}]_{e}$ to 5 mM.
insure deesterification. Dye-loaded astrocytes were subjected to mass mechanical stimulation by dropping μBeads (diameter = 30 - 60 uM) through ~3 mm of medium onto the cells. This type of stimulation has been shown to initiate multifocal calcium waves in astrocytes (Guthrie, Knappenberger et al. 1999). Samples were collected before and after stimulation and the amount of dye release was measured as the difference between these two values divided by the total amount of loaded dye (measured by lysing the cell after the assay using 0.5% TritonX-100).

We found an inverse relationship between Calcein Blue dye release from astrocytes and [Mg$^{2+}$]$_e$ ($N=2$; $n=8$ per treatment; $p = 0.0056$, single factor ANOVA; figure 16). Previously, we have shown that zero [Ca$^{2+}$]$_e$ initiates spontaneous calcium signaling in astrocytes (Zanotti and Charles 1997) by potentiating the release of large anions, namely ATP, through hemichannels. These data prompted us to examine whether [Mg$^{2+}$]$_e$ would affect this increase in astrocyte excitability. Similar to our previous finding, we found that zero Ca$^{2+}$ treatment potentiated mechanical stimulation induced Calcein Blue release. We also found that raising [Mg$^{2+}$]$_e$ (to 5 mM) abrogated this response to zero [Ca$^{2+}$]$_e$ treatment ($N=2$; $n=8$ per treatment; $p = 0.00013$, student’s t-Test; Figure17).
Discussion

Extracellular Magnesium Affects Calcium Signaling in Astrocytes

Various types of stimulation cause astrocytes to release ATP into the medium that evokes Ca\(^{2+}\) responses in surrounding astrocytes. This forms a critical part of an extracellular communication system capable of mediating Ca\(^{2+}\) waves; Ca\(^{2+}\) waves can propagate across a cell-free zone in astrocyte cultures (Hassinger, Guthrie et al. 1996; Charles 1998). This extracellular communication system is necessary for the full extent of Ca\(^{2+}\) wave propagation as demonstrated by the fact that Ca\(^{2+}\) waves in astrocytes are biased by gentle perfusion of the bathing medium during a Ca\(^{2+}\) wave (Charles 1998). The amount of ATP released to mediate Ca\(^{2+}\) signaling is increased by connexin expression (Cotrina, Lin et al. 1998) since hemichannels constitute a major ATP release channel (Stout, Constantin et al. 2002).

Hemichannel activity is routinely found to be inversely correlated with [Ca\(^{2+}\)]\(_e\) (DeVries and Schwartz 1992; Ebihara and Steiner 1993; Liu, Li et al. 1995; Li, Liu et al. 1996; John, Kondo et al. 1999; Bruzzone, Guida et al. 2001; Lal and Lin 2001). This includes both hemichannel currents (DeVries and Schwartz 1992; Ebihara and Steiner 1993; Dixon, Takahashi et al. 1996; Eckert, Donaldson et al. 1998; Zhang, McBride et al. 1998; Pfahnl and Dahl 1999) and dye-flux (Liu, Li et al. 1995; Li, Liu et al. 1996; Liu, Paulson et al. 1997; John, Kondo et al. 1999; Bruzzone, Guida et al. 2000; Kondo, Wang et al. 2000) – two commonly used assays of hemichannel function. Other ions, however, have also been found to regulate hemichannel activity including Gd\(^{3+}\), La\(^{3+}\), and Mg\(^{2+}\). This would suggest that tissue events, like intercellular Ca\(^{2+}\) signaling, would be inhibited by treatment with these polyvalent cations. Consistent with this Gd\(^{3+}\) (100 µM) inhibits
spontaneous and stimulated intercellular Ca$^{2+}$ signaling in astrocytes (Zanotti and Charles 1997). This prompted us to examine the effect of Mg$^{2+}$ on hemichannel function, ATP release, and intercellular signaling in astrocytes.

To examine the effect of Mg$^{2+}$ on hemichannel activity, we used both the whole-cell patch clamp technique and dye-flux assays. We found that Mg$^{2+}$ inhibits hemichannels in astrocytes as demonstrated by electrophysiologic and dye release assays. Treatment of astrocytes with zero [Ca$^{2+}$]$_e$ initiates currents that are inhibited by raising [Mg$^{2+}$]$_e$ to 5 mM. Further, we found an inverse relationship between mechanically initiated Calcein Blue release and [Mg$^{2+}$]$_e$. Consistent with our previous findings, zero [Ca$^{2+}$]$_e$ caused an increase in stimulated Calcein Blue release. Here, we report that this increase is also inhibited by treatment with 5 mM [Mg$^{2+}$]$_e$.

ATP released through Cx43 hemichannels mediates glial calcium waves (Stout, Constantin et al. 2002). This suggests that regulation of hemichannel activity by divalent cations would modulate ATP release. Consistent with this, we found that mechanically induced ATP release is inversely correlated with [Mg$^{2+}$]$_e$ and 5mM [Mg$^{2+}$]$_e$ inhibits zero [Ca$^{2+}$]$_e$ potentiated ATP release. Strictly speaking, it is not completely possible to determine the relative contribution of ATP degradation, which is increased by complexing with divalent cations, versus decreased ATP release caused by high [Mg$^{2+}$]$_e$ concentrations. Given that hemichannel inhibition, using agents with no known connection to ecto-ATPases, can nearly completely inhibit ATP release and high Mg$^{2+}$ substantially decreases hemichannel activity (see above), it seems likely that a inhibition of ATP release is a major factor in these results.

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Finally, we examined the relationship between \([\text{Mg}^{2+}]_e\) and intercellular \(\text{Ca}^{2+}\) signaling. The inverse relationship between \([\text{Mg}^{2+}]_e\) and the extent of calcium wave propagation could be a result of: 1) Decreased release of ATP, 2) Increased clearance – i.e., ATP degradation by ecto-ATPases or increased uptake, 3) Decreased purinergic receptor activation by complexed ATP or, 4) a combination of the three. The present study demonstrated that a decreased release was a major contributor. However, since purinergic receptor activation initiates further ATP release (Cotrina, Lin et al. 1998), increased degradation and decreased receptor activation due to complexing by \(\text{Mg}^{2+}\) would also decrease subsequent ATP release – i.e., total ATP release. The concerted action of \(\text{Mg}^{2+}\) at each of the control points in decreasing [ATP]e indicates that \(\text{Mg}^{2+}\) is a very significant regulator of extracellular purinergic signaling.

Spontaneous \(\text{Ca}^{2+}\) signaling in astrocytes has been shown to be modulated by (non-inclusive) \([\text{Ca}^{2+}]_e\), [ATP]e and glutamate. We report that \([\text{Mg}^{2+}]_e\) alters the frequency of \(\text{Ca}^{2+}\) oscillations that initiate intercellular \(\text{Ca}^{2+}\) waves. Specifically, we found a inverse relationship between the number and extent of spontaneous and zero \(\text{Ca}^{2+}\) stimulated calcium waves and \([\text{Mg}^{2+}]_e\). As described above, this likely occurs as a result of activation at several points in the extracellular communication pathway. Given the exact correlation between these treatments and their effects on hemichannel activity, we hypothesize that a major portion of these effects is a result of modulation of the ATP release step.

Implications of the Modulation of Astrocytic Calcium Signaling by \([\text{Mg}^{2+}]_e\)

The functions of astrocytic \(\text{Ca}^{2+}\) signaling are only beginning to be understood. It is clear, however, that astrocytes are critical elements in synaptic formation and function
(Bacci, Verderio et al. 1999) and that synaptic function is modulated by Ca\textsuperscript{2+} signaling (Araque, Sanzgiri et al. 1999). This suggests that agents that affect astrocytic Ca\textsuperscript{2+} signaling will in turn modulate synaptic activity. Further, in so much as astrocytic Ca\textsuperscript{2+} signaling has been shown capable of initiating synaptic transmission (Araque, Sanzgiri et al. 1998), agents that aberrantly activate astrocytic Ca\textsuperscript{2+} signaling could very well initiate surrounding neurons, resulting in synchronized neuronal discharges.

In this regard, it is very interesting that several treatments that activate seizure type activities (including zero Ca\textsuperscript{2+}, zero Mg\textsuperscript{2+}, and E-field exposure) \textit{in situ} activate spontaneous Ca\textsuperscript{2+} signaling events in astrocytes. While it is certain that the mechanism of this phenomenon involves direct action on the neuron (i.e. charge screening and NMDA receptor regulation), it seems likely that an additional site of action is the astrocytic compartment, especially since astrocytes outnumber neurons by more than 5 to 1. For example, zero Ca\textsuperscript{2+} treatment blocks synaptic function yet results in synchronized neuronal discharges. It seems very plausible to suggest that this synchronization is partially the result of the coordinated activation by a group of astrocytes in response to glial Ca\textsuperscript{2+} signaling.

The data reported here that zero [Mg\textsuperscript{2+}]_e treatment (and our previously reported data of zero [Ca\textsuperscript{2+}]_e treatment) results in increased spontaneous intercellular Ca\textsuperscript{2+} waves and increased extent of induced Ca\textsuperscript{2+} waves suggest that these treatments would cause an increase in the number of neurons with synchronized Ca\textsuperscript{2+} transients and/or activity. We hypothesize the effects of Mg\textsuperscript{2+} on the astrocytic purinergic communication system are important in regulating neuronal synchronization, neuronal discharges and synaptic
function, directly implicating astrocytes in the physiology and pathophysiology of phenomena such as seizures, migraine, and hypotonicity.
CHAPTER 4
RESULTS RESERVED FOR FUTURE PUBLICATIONS

Role of PLC Activation in Mechanically Initiated ATP Release

To investigate the possible role of cellular signaling messengers in ATP release, we examined the effects of pharmacological compounds that modulate phospholipase C, PKC, and increases in \([\text{Ca}^{2+}]\), generated by the release of \(\text{Ca}^{2+}\) from intracellular stores. Mechanically induced ATP release was inhibited by the PLC inhibitors U73122 and neomycin (Figure 19a; \(n=8\)/treatment; \(N=2\); \(p = 0.004\) and \(p = 0.012\), respectively).

Mechanically initiated ATP release, however, was not blocked by thapsigargin, BAPTA, or zero \([\text{Ca}^{2+}]_e\) suggesting that mechanically stimulated ATP release does not rely on extracellular or intracellular \(\text{Ca}^{2+}\). In fact, thapsigargin treatment in the presence of zero \([\text{Ca}^{2+}]_e\) did not inhibit mechanically elicited ATP release (data not shown). This suggested the possibility that the other product of PLC, namely DAG, was involved in ATP release. We found that acute stimulation with OAG, a membrane permeable analog of DAG, initiated ATP release in the absence of mechanical stimulation with \(\mu\)Beads (Figure 19b).
Figure 19 - Role of Cellular Messengers in Hemichannel-Mediated ATP Release

A. Baseline samples were collected, then plates of cells were mechanically stimulated and samples were collected 30 seconds afterward. ATP release (i.e. $\Delta [\text{ATP}]_e$) was calculated as the difference of these two measurements. There were 8 plates per treatment and each treatment had its own vehicle control of 8 plates. Only U73122 and neomycin are significantly different than control ($p = 0.004$ and $p = 0.012$, respectively). B. Baseline samples were collected, then OAG (50 μM) was added to treatment plates (n=8) and vehicle was added to control plates (n=8). After 30 seconds, samples were again collected and $\Delta [\text{ATP}]_e$ calculated as the difference between these two measurements. OAG stimulation initiated significantly more ATP release than vehicle control ($p = 0.008$).
Role of DAG in Calcium Signaling

The finding that the DAG analog OAG (20 μM) initiated ATP release prompted us to examine the effects of OAG on spontaneous Ca^{2+} signaling. We discovered that acutely added OAG (20 μM; Figure 12) initiated spontaneous Ca^{2+} signaling in purified mouse astrocytes. This effect was rapidly reversible, and occurred repeatedly after repetitive washing and re-addition of the OAG. In addition, OAG initiated calcium signaling relies on connexin expression since OAG was found to initiate spontaneous Ca^{2+} signaling in C6/Cx43 glioma cells but not C6 glioma cells (data not shown). In total, these results suggest that DAG induces ATP release through Cx43 hemichannels that initiate oscillations and intercellular Ca^{2+} signaling.
Figure 20 - OAG Initiates Ca\(^{2+}\) Signaling Events

Rasterplot of a field of purified cortical astrocytes loaded with a calcium indicator dye (fura2). Recording starts in control Hank’s balanced salt solution (HBSS). After 5 minutes the bath was changed to HBSS plus vehicle (VC; DMSO 0.2%). After incubation for an additional 5 minutes the bath was changed to HBSS plus OAG (20 µM). Multiple oscillations and intercellular signaling events are evident. OAG was washed out and re-added as shown (N = 5).
Effect of Ethanol on Calcium Signaling and ATP Release

Ethanol inhibits PLD activity in astrocytes and other cell types (Desagher, Cordier et al. 1997; Kotter and Klein 1999; Kotter and Klein 1999). It has also been reported to inhibit PLC (Hudspith, John et al. 1985; Katsura, Ohkuma et al. 1994; Pandey 1996). PLD catalyzes the production of the phosphatidic acid, which can be rapidly converted to DAG by phosphatidic acid phosphohydrolase. Activation of PLD by several types of stimulation can occur (Arcaro and Wymann 1993; Barker, Caldwell et al. 1998; Falasca, Logan et al. 1998; Rameh, Rhee et al. 1998; Barker, Lujan et al. 1999) via a phosphoinositide 3-kinase dependent pathway (Reinhold, Prescott et al. 1990; Bonser, Thompson et al. 1991; Carrasco-Marin, Alvarez-Dominguez et al. 1994). Consistent with this, it has been reported that phosphoinositide 3-kinase inhibitors, such as wortmannin, often inhibit PLD activity (Bonser, Thompson et al. 1991; Carrasco-Marin, Alvarez-Dominguez et al. 1994; Ding and Badwey 1994). In light of this, it is interesting to find that wortmannin has been shown to inhibit ATP release (Feranchak, Roman et al. 1998; Feranchak, Roman et al. 1999) and products of phosphoinositide 3-kinase directly initiate ATP release (Feranchak, Roman et al. 1999).

To further examine the role of DAG produced by other enzymes in ATP release from astrocytes, mechanically initiated changes in [ATP]_e were measured in the presence of ethanol (1% v/v). It was found that ethanol substantially decreases ATP release (Figure 18; n = 8; p = 0.007). Further, the extent of mechanically initiated Ca^{2+} wave propagation was decreased (data not shown).
Figure 21 - Ethanol Inhibits ATP Release

Mechanical stimulation-induced ATP release is blocked by ethanol (1% v/v) (n = 8; p = 0.007). First, baseline samples were collected and then plates of cells were mechanically stimulated and samples were collected 30 seconds afterward and Δ[ATP]e was calculated as the difference of these two measurements.
Effect of Staurosporine on ATP Release

Results presented here demonstrate that DAG initiates ATP release. It is well established, however, that connexin43 channels in both the gap junction configuration (Enkvist and McCarthy 1992; Konietzko and Muller 1994) and the hemichannel configuration are inhibited by PKC activation (Liu, Paulson et al. 1997; Ngezahayo, Zeilinger et al. 1998; Zhang, McBride et al. 1998; Jedamzik, Marten et al. 2000). This suggests that DAG opening of connexin hemichannels occurs via a PKC-independent mechanism. To test this, mechanically initiated increases in [ATP]e were measured in the presence of the PKC inhibitor staurosporine (300 nM). This treatment was found not to decrease mechanically induced ATP release significantly (Figure 19; n = 8; p = 0.206).
Figure 22 - Effect of PKC Inhibition on ATP Release

Effect of staurosporine (300 nM; 15-minute incubation) on mechanically induced ATP release. First, baseline samples were collected and then plates of cells were mechanically stimulated and samples were collected 30 seconds afterward and Δ[ATP]e calculated as the difference of these two measurements. Staurosporine (300 nM; 15 minute incubation) failed to significantly affect mechanically induced ATP release (n = 8; p = 0.206).
Effect of Cyclosporine on Mechanically Initiated ATP Release

There are several anion channels that could possibly be involved in ATP release. One such channel is p-glycoprotein, which mediates the transport of drugs across the cell membrane. We examined the role of p-glycoprotein in mediating mechanically induced ATP release using cyclosporine, a inhibitor of p-glycoprotein function (Demeule, Laplante et al. 1998; Saha, Yang et al. 1998; Tsuji 1998; Demeule, Laplante et al. 1999). Cyclosporine also inhibits calcineurin (Liu, Farmer et al. 1991; Swanson, Born et al. 1992; Batiuk, Pazderka et al. 1995; Halloran, Helms et al. 1999), a Ca^{2+} dependent phosphatase. Pretreatment of astrocytes for 6 hours with cyclosporine (10 μM), along with treatment during the time frame of the mechanical stimulation assay, did not inhibit mechanically induced ATP release significantly (Figure 20; n = 8; p = 0.360).
Figure 23 - Effect of Cyclosporine on ATP Release

Effect of cyclosporine (10 μM; 6 hour treatment) on mechanically induced ATP release. First, baseline samples were collected and then plates of cells were mechanically stimulated and samples were collected 30 seconds afterward and Δ[ATP]e was calculated as the difference of these two measurements. Cyclosporine (10 μM; 6 hour treatment) failed to significantly affect mechanically induced ATP release (n = 8; p = 0.360).
CHAPTER 5
CONCLUSIONS AND COMMENTS

Characteristics of Astrocytic Hemichannels

Functional hemichannel expression is typically demonstrated by: 1) Stimulation-induced dye-uptake or release (MW<900) that is dependent on connexin expression (Sarthy, Johnson et al. 1982; Liu, Li et al. 1995; Alves, Coutinho-Silva et al. 1996; Liu, Paulson et al. 1997; Kim, Kam et al. 1999); 2) Increased membrane conductance induced by divalent cation removal from the extracellular space that is dependent on connexin expression (Ebihara, Berthoud et al. 1995; Pfahnl and Dahl 1998; Trexler, Bukauskas et al. 1999; Zampighi, Loo et al. 1999); and 3) Susceptibility of both dye-flux and increased membrane conductance to connexin channel inhibitors including flufenamic acid (Zhang, McBride et al. 1998), Gd$^{3+}$ (Zhang, McBride et al. 1998), Mg$^{2+}$ (Li, Liu et al. 1996; Ramanan, Brink et al. 1999), and n-chain alcohols (Donaldson and Kistler 1992; Li, Liu et al. 1996; John, Kondo et al. 1999). This report documents each of these criteria in glial cells providing the most conclusive evidence to date for functional hemichannel expression by astrocytes.

Hemichannel Mediated Dye-Release

Several dye-uptake and release protocols were examined during the course of this study. Each assay was tested and found to be an assay of hemichannel activity. In early studies hemichannel activity initiated by low [Ca$^{2+}$]$_e$ was found to be potentiated by mechanical stimulation of single cells. This was followed by the discovery that mechanical stimulation could initiate hemichannel activity in the presence of physiologic [Ca$^{2+}$]$_e$. This was one of the first important discoveries of this research. Previous to this finding hemichannels had only been reported to be activated by low [Ca$^{2+}$]$_e$ treatment
(Ebihara 1992; Malchow, Qian et al. 1993; Li, Liu et al. 1996; Quist, Rhee et al. 2000) and metabolic inhibitors (John, Kondo et al. 1999; Kondo, Wang et al. 2000). Therefore, this discovery represented the first known physiologic stimulus to activate connexin hemichannels.

This finding prompted the development of a rapid dye-flux assay activated by mass mechanical stimulation, which was a modification of the μBead assay developed by Guthrie et al (Guthrie, Knappenberger et al. 1999). This assay was used to study the mechanism of mechanical stimulation induced opening of hemichannels. Since mechanical stimulation is the most commonly used stimulation in calcium signaling assays, data garnered from this assay was taken as a measure of hemichannel activity during the initiation and propagation of calcium waves. The conclusions from these studies include: 1) There is an increase in hemichannel activity associated with calcium wave initiation; 2) Hemichannels, upon activation by mechanical stimulation, are permeable to metabolic sized anions; 3) Increased hemichannel activity during a Ca^{2+} wave can be blocked by FFA, Gd^{3+}, and high [Ca^{2+}]_{e}; 4) Hemichannel activity is blocked by agents that inhibit PLC and 5) Hemichannel activity is activated by low [Ca^{2+}]_{e}, DAG analogs and quinine.

Taken together, these data and analysis suggest that the mechanically elicited increase in hemichannel activity that initiates and accompanies Ca^{2+} waves occurs by a mechanism that depends on mobilization of DAG via PLC activation. This raises two pertinent questions: 1) What is the mechanism of mechanical stimulation induced activation of PLC and 2) What is the mechanism of DAG induced hemichannel activation? The answer to the first question is not particularly germane to this research.
However, this has been examined by others who have demonstrated that it likely occurs by a mechanism involving β-integrins and tyrosine phosphorylation of PLC (Berk, Corson et al. 1995; Glogauer, Arora et al. 1997; Schmidt, Pommerenke et al. 1998; Millward-Sadler, Wright et al. 1999; Meyer, Alenghat et al. 2000; Millward-Sadler, Wright et al. 2000; Millward-Sadler, Wright et al. 2000; Salter, Wallace et al. 2000).

The answer to the second question concerning how DAG mobilization activates hemichannel activity is more pertinent since it is directly related to connexin channel activation. There are basically three possibilities: 1) DAG acts directly by binding to the hemichannel itself to promote an “ATP permeable conformation”; 2) DAG acts indirectly through other cellular proteins that ultimately interact with Cx43 to facilitate an ATP permeable configuration; or 3) A combination of the first two where DAG has both direct and indirect modes of action.

While no direct evidence exists as yet that DAG binds directly to Cx43, there are several reasons to suspect that it occurs. First, recent reports demonstrate that DAG activates other channels by directly binding to the channel protein. For example, DAG has been found to directly bind and activate UNC-13 (Orita, Naito et al. 1997; Lackner, Nurrish et al. 1999), a protein that forms intercellular communication channels (i.e. gap junction like channels) in invertebrates and is involved in transmitter release (Aravamudan, Fergestad et al. 1999; Richmond, Davis et al. 1999; Tokumaru and Augustine 1999). Second, Cx43 contains a near match, although admittedly not exact, to the DAG binding consensus sequence (personal observation). Thus it is possible that DAG activates Cx43 hemichannels by binding directly to the constituent Cx43 protein.
subunits. It should be possible to test whether DAG binds Cx43 directly by several different assays.

The second possibility is that DAG activates hemichannels indirectly by binding to a different cellular protein that either initiates a cascade of events or directly acts on Cx43 hemichannels. At the time of this writing, it is not possible to definitely identify this putative protein. However, it is possible to conclude that it is not PKC. First, the time frame for phosphorylation of Cx43 in cells stimulated by PKC activators is longer than for stimulation-induced hemichannel opening. Second, phosphorylation on Cx43 channels by PKC typically is reported to reduce hemichannel activity (Liu, Li et al. 1995; Li, Liu et al. 1996; Liu, Paulson et al. 1997; Jedamzik, Marten et al. 2000). Third, direct demonstration of Cx43 hemichannel permeation by large anions has revealed that phosphorylation largely reduces large anion permeability in cell-free preparations (Kim, Kam et al. 1999) and in monolayer preparations (Liu, Paulson et al. 1997). Finally, activation of PKC by TPA stimulation (15 min) inhibits calcium wave propagation in astrocytes (Enkvist and McCarthy 1992; Muyderman, Nilsson et al. 1998), which, as demonstrated here, is hemichannel dependent. The obvious conclusion is that DAG opens hemichannels before PKC becomes activated by binding to another protein or binding site in the cell – possibly even Cx43. In contrast, the subsequent activation of PKC likely acts as negative feedback so hemichannels do not stay open for prolonged periods of time.

Several lines of evidence gathered recently suggest that the protein activated by DAG could be a phosphatase. These include: 1) Phosphatases have kinetics about ten times faster than protein kinases for catalyzing reactions on Cx43 (Shah 1999); 2) Cx43
exists primarily in the phosphorylated state in vivo in the brain (Hossain, Murphy et al. 1994); 3) Mechanical stimulation initiates a rapid dephosphorylation of Cx43 (Stout 1999); 4) Dephosphorylated Cx43 hemichannels are much more permeable to large anions (Kim, Kam et al. 1999); and 4) Treatment with agents that initiate dephosphorylation of Cx43 rapidly initiate dye-release (data not shown). These data strongly indicate that DAG activates a phosphatase that increases hemichannel permeability to large anions by dephosphorylation of the constituent Cx43 protein. In fact, DAG (and other phospholipids such as phosphatidic acid) has been found to be potent activators of several phosphatases such as SHP-1 (Frank, Keilhack et al. 1999).

Finally, it is not possible to exclude a combination of events where DAG acts both directly on Cx43 and indirectly through other cellular cascades. However, given the strong regulation of Cx43 hemichannel by phosphorylation and the dependence of hemichannel activation on DAG mobilization, it seems most reasonable to suggest that they are linked together where one activates the other, i.e. DAG mobilization activates dephosphorylation which increases hemichannel activity.

**Electrophysiology**

From the earliest reports, removal of extracellular divalent cations was found to increase hemichannel activity. Electrophysiology protocols were designed to exploit this characteristic of hemichannels whereby membrane conductance was measured before and after extracellular divalent removal (Barrio, Capel et al. 1997; Eckert, Donaldson et al. 1998; Ebihara, Xu et al. 1999). An increase in membrane conductance was taken as one indication of the presence of hemichannels. While the kinetics of hemichannels in Xenopus oocytes have been studied for many years, only recently has there been reports
attempting to detail the biophysical characteristics of hemichannels in mammalian cells (Vanoye, Vergara et al. 1999; Kondo, Wang et al. 2000; Valiunas and Weingart 2000; Kamermans, Fahrenfort et al. 2001; Valiunas 2002). These studies are so new and have such divergent results, both amongst themselves and with the data garnered in Xenopus oocytes, that there is no consensus as to what the kinetics of mammalian hemichannels actually are.

There are at least three variants represented in the literature. The first is a current activated by depolarization (Moreno 1998; Pfahnl and Dahl 1998; Ebihara, Xu et al. 1999; Vanoye, Vergara et al. 1999). This is the current found in most instances in the Xenopus oocyte preparation. The second is a current that is inhibited by hyperpolarization which would superficially make it seem to be like the first current described but has kinetics different enough to suggest that is a distinct current (Valiunas 2002). The third is a current that looks very much like a gap junction current, i.e. being inhibited to a steady-state by both hyper- and depolarization (Zampighi, Loo et al. 1999; Valiunas and Weingart 2000).

The electrophysiology assays completed for this dissertation were primarily aimed and demonstrating an increased conductance attributable to hemichannel activation. Very little work was done on examining the kinetics of hemichannels in astrocytes. Through the course of the experiments, however, data was collected that suggests that Cx43 hemichannels may mediate each of the types of currents listed above. Specifically, each of the types of currents described were recorded at different times and with different conditions indicating that these currents could represent Cx43
hemichannels in different states and/or in different conditions (i.e. different pipette solutions and bath solutions) (data now shown).

In nearly all cases reported in the literature, all these types of currents are found to be sensitive to $[\text{Ca}^{2+}]_e$. However, hemichannels in purified preparations are not sensitive to divalent cations (Rhee, Bevans et al. 1996; Ahmad, Iriondo et al. 1998; Kim, Kam et al. 1999; Bruzzone, Guida et al. 2000). This raises the question of whether divalent cations act directly or indirectly on hemichannels. Several lines of evidence suggest that the action of divalent cations on hemichannels is indirect including: 1) Lack of inhibition of purified hemichannels by divalent cations (Rhee, Bevans et al. 1996; Ahmad, Iriondo et al. 1998; Kim, Kam et al. 1999; Bruzzone, Guida et al. 2000); 2) Inhibition of purified hemichannels by divalent cations when calmodulin is present; 3) Activation of single-channel activity in cell-attached preparations by switching the bath to low $[\text{Ca}^{2+}]_e$, i.e. the channels being recorded, which are inside the pipette, are not exposed to the change in $[\text{Ca}^{2+}]_e$ but respond with increased activity (Valiunas and Weingart 2000); and 4) Differences in the $k_{1/2}$ of $[\text{Ca}^{2+}]_e$ for activation of hemichannels in different preparations. Alternately, if there are several states for connexin hemichannels as suggested above, it could be that a subset of the total states are sensitive to calcium whereas the rest are not. If this were true the primary requirement to explain all available data would be that purified hemichannels are primarily in the state(s) not sensitive to calcium.

These questions and others suggest that this aspect of research is the most interesting for long-term study. At least two reasons account for this: 1) Compared to neurons, there is little information about the characteristics of astrocyte membrane channels and 2) Connexin hemichannels composed of Cx43 have several unique
regulatory domains. For example, Cx43 composed gap junction channels have multiple conducting states, each with unique permeability and conductance levels (Donaldson, Roos et al. 1994; Moreno, Rook et al. 1994; Moreno, Saez et al. 1994; Bukauskas and Peracchia 1997; Christ and Brink 1999). Two main states have been reported to occur in hemichannels (Moreno 1998) which would suggest that switching between these two states would change the permeability and the regulatory characteristics of the hemichannels. As previously suggested, the changes between these phosphorylation states is likely responsible for the various kinetic profiles reported for hemichannels. Examining how these changes between the states occur and how this affects ionic flow across the membrane could yield significant insight into how astrocytes function in situ.

**Characteristics of Hemichannel Mediated ATP Release**

Many cell types release ATP into the extracellular space that functions as both an autocrine and a paracrine messenger. This purinergic signaling has been implicated in several forms of tissue pathology including seizure (Wieraszko and Seyfried 1989), cystic fibrosis (Cheung, Wang et al. 1998; Linsdell and Hanrahan 1998; Chinet, Fouassier et al. 1999), migraine (Burnstock 1996) and altered growth rate (Agren, Ponten et al. 1971; Rathbone, Middlemiss et al. 1992; Ciccarelli, Di Iorio et al. 1994). Partly due to these considerations, the mechanism(s) of ATP release has received considerable attention in recent years.

For example, cystic fibrosis conductance regulator (CFTR) Cl⁻ channel expression (Prat, Reisin et al. 1996) and activity (Schwiebert, Egan et al. 1995) have been shown to potentiate basal ATP release, leading to the suggestion that CFTR mediates ATP release (Prat, Reisin et al. 1996). It was subsequently noted, however, that the pore
characteristics of the CFTR channel would exclude ATP, and multiple laboratories have demonstrated that CFTR is not directly responsible for ATP release (Reddy, Quinton et al. 1996; Grygorczyk and Hanrahan 1997; Watt, Lazarowski et al. 1998; Hazama, Shimizu et al. 1999; Hazama, Fan et al. 2000).

Clearly, a better understanding of the mechanism of ATP release would provide a better foundation for these areas of research as well as for Ca\textsuperscript{2+} signaling research. This report documents that mechanically induced ATP release exhibits three main characteristics: 1) Dependence on hemichannel expression and function, 2) Sensitivity to PLC inhibition; and 3) Potentiation by DAG mobilization. Taken together, these data strongly suggest that ATP release occurs through hemichannels upon mobilization of DAG via PLC activation.

The demonstration that Cx43 hemichannel expression and activity are associated with ATP release lead to the most important and provocative finding of this research concerning the mechanism of ATP release, i.e. that Cx43 hemichannels are “ATP release channels”. This conclusion is supported by the fact that connexin hemichannels have the permeation characteristics necessary to mediate ATP flux (Li, Liu et al. 1996; Zhang, McBride et al. 1998; Zhang, Gao et al. 2000). In fact, ATP flux through Cx43 gap junction channels has been directly demonstrated (Goldberg, Lampe et al. 1998). It should be noted, however, that demonstration of ATP flux through hemichannels remains to be tested but all current evidence suggests that the outcome of this experiment will be positive.

Demonstration of the hemichannel as an ATP release channel allows for some interesting analysis of other current data. For example, forced CFTR expression variably
potentiates ATP release in various cell types (Grygorczyk and Hanrahan 1997) but the CFTR channel is not directly responsible for releasing ATP. Instead, it has been demonstrated that CFTR potentiates another channel ultimately responsible for ATP release (Grygorczyk and Hanrahan 1997; Jiang, Mak et al. 1998; Watt, Lazarowski et al. 1998; Hazama, Shimizu et al. 1999; Hazama, Fan et al. 2000). Recently it has been reported that CFTR channel activation potentiates connexin channel function (Chanson, Scerri et al. 1999). Further, Cl⁻ channel inhibitors block this potentiation. This suggests the possibility that CFTR expression could potentiate ATP release by facilitating its release through hemichannels. This could account for the discrepant results concerning the effects of forced CFTR expression on ATP release that have caused controversy (Devidas and Guggino 1997) regarding its role in ATP release.

The second important finding of this research is that ATP release is dependent on PLC activation. This is not surprising given that fact that ATP is released through hemichannels whose activity is regulated by PLC activity. Further, it is the mobilization of DAG (or one of its metabolites), not IP₃ or subsequent Ca²⁺ mobilization, that makes PLC activation a critical part of mechanical stimulation induced ATP release. Several lines of evidence lead to this conclusion, including: 1) OAG (50 μM), a DAG analog resistant to degradation, can initiate ATP and dye release; 2) OAG (20 μM) initiates Ca²⁺ oscillations and intercellular Ca²⁺ waves; and 3) OAG (20 - 50 μM) reversibly activates membrane conductance oscillations (MacVicar, Crichton et al. 1987).

DAG is not only produced by activation of PLC but also indirectly by activation of phospholipase D (PLD). PLD catalyzes the production of phosphatidic acid that can be rapidly converted to DAG by phosphatidic acid phosphohydrolase. Consistent with
this and the association between DAG and ATP release, ethanol (1% v/v) which blocks
PLD in astrocytes (Desagher, Cordier et al. 1997; Kotter and Klein 1999; Kotter and
Klein 1999), inhibits ATP release (see results section). This result strongly indicates that
DAG mobilization, regardless of its source, is capable of initiating ATP release. Since
PLC and PLD have different modes of activation and regulation, it seems likely that
several pathways for hemichannel opening would exist, each with its own unique control
points.

This association between DAG and ATP release can be found in other cell types
and protocols. For example, it has been shown that many types of stimulation result in
PLD activation (Reinhold, Prescott et al. 1990; Bonser, Thompson et al. 1991; Carrasco-
Marin, Alvarez-Dominguez et al. 1994) and PLC activation (Arcaro and Wymann 1993;
Barker, Caldwell et al. 1998; Falasca, Logan et al. 1998; Rameh, Rhee et al. 1998;
Barker, Lujan et al. 1999) via a phosphoinositide 3-kinase dependent pathway.
Consistent with this, it has been reported that phosphoinositide 3-kinase inhibitors, such
as wortmannin, often inhibit PLD activity (Bonser, Thompson et al. 1991; Carrasco-
Marin, Alvarez-Dominguez et al. 1994; Ding and Badwey 1994).

This could explain why wortmannin has been shown to inhibit ATP release
(Feranchak, Roman et al. 1998; Feranchak, Roman et al. 1999). This hypothesis is
strengthened by the fact that products of phosphoinositide 3-kinase can directly initiate
ATP release (Feranchak, Roman et al. 1999). Although this initiation of ATP release has
not been tested for sensitivity to PLD inhibitors, these considerations make it is easy to
speculate that the ATP release is a result of subsequent DAG production by PLD and
phosphatidic acid phosphohydrolase activation.
Taken together, these data and conclusions indicate that ATP is released through hemichannels in response to mobilization of DAG. Therefore, multiple pathways that ultimately lead to DAG production could, under the right conditions, initiate ATP release and cause intercellular Ca\(^{2+}\) signaling in astrocytes. It is interesting to note that direct inhibition of these pathways by drugs with well characterized CNS effects such as ethanol and halothane inhibit ATP release. The well-documented effects of these drugs on neurotransmission and/or cognition reinforce the idea that Ca\(^{2+}\) signaling is involved in the higher functions of the brain.
Characteristics of Ca\textsuperscript{2+} Signaling

Mechanically Initiated Ca\textsuperscript{2+} Signaling

Perhaps the most important finding of this whole dissertation is that there is a three-way correlation between modulation of 1) hemichannel function, 2) ATP release, and 3) the extent of Ca\textsuperscript{2+} wave propagation. Multiple agonists where used (both activators and inhibitors) which acted at several different points in the cascade of events associated with hemichannel gating and intercellular calcium signaling. The strength of the correlation that emerged from the data overwhelmingly supports the hypothesis that calcium wave propagation can occur by ATP released through connexin hemichannels. Thus, previously published data indicating that connexin channels are required for calcium wave propagation and data demonstrating that calcium wave propagation is mediated by ATP release into the extracellular space can now be reconciled.

This prompts the question of what role, if any, intercellular gap junction communication plays in the mechanism of calcium signaling in astrocytes. The fullest extent of Ca\textsuperscript{2+} waves relies on extracellular ATP, but could there be a finite amount of intercellular, gap junction mediated signal transport? There are indications for and against this hypothesis. This question will most likely not be answered satisfactorily until a method for directly visualizing “labeled” IP\textsubscript{3} is devised and its flux from cell to cell can be monitored during the time frame of a calcium wave. This is very critical. There are methods for observing IP\textsubscript{3} passage between cells using a protocol involving radio-labeled IP\textsubscript{3}, two cell populations, and a cell sorter (Niessen, Harz et al. 2000). The time frame of this passage, actual bulk amount of IP\textsubscript{3} transport per cell, and its temporal relationship to
calcium wave propagation, however, cannot be measured. Thus it is not currently possible to address this question adequately.

Regardless, as delineated here and elsewhere (Cotrina, Lin et al. 1998; Guthrie, Knappenberger et al. 1999; Cotrina, Lin et al. 2000), the fullest extent of calcium wave propagation in astrocytes is a result of an extracellular purinergic signaling system in astrocytes. This has several implications regarding the role of purinergic signaling in the CNS. For example, the purinergic nerve hypothesis states that neurons release ATP that functions as a neurotransmitter. The fact that astrocytes respond to this release with a propagated signal is one of the growing number of discoveries that show that astrocytes are integral elements in higher brain functions.

Spontaneous Ca\(^{2+}\) Signaling

The principal findings of this research concerning spontaneous Ca\(^{2+}\) signaling include: 1) Reduction of \([\text{Mg}^{2+}]_e\) can initiate spontaneous Ca\(^{2+}\) oscillations and intercellular Ca\(^{2+}\) waves; 2) Quinine (100 \(\mu\)M) activates spontaneous Ca\(^{2+}\) oscillations and Ca\(^{2+}\) waves; 3) OAG (20 \(\mu\)M), a membrane permeable DAG analog, initiates spontaneous Ca\(^{2+}\) oscillations and Ca\(^{2+}\) waves; and 4) Dependence of all these activators on connexin channel expression. The fact that these same treatments initiate and/or potentiate hemichannel activity and ATP release implicates hemichannel activation and ATP release in the initiation of spontaneous Ca\(^{2+}\) signaling, i.e. the mechanism of spontaneous Ca\(^{2+}\) signaling is very similar to the mechanism of mechanically initiated Ca\(^{2+}\) signaling.

Several things are consistent with this including: 1) Bath application of ATP initiates calcium oscillations in astrocytes (unpublished observations); 2) Spontaneous
calcium oscillations induced by quinine, DAG and low [Ca\(^{2+}\)]\(_e\) (and other treatments) are blocked by suramin (unpublished observations); 3) Gentle perfusion of the extracellular medium during low [Ca\(^{2+}\)]\(_e\) induced oscillations biases spontaneously generated calcium waves (Zanotti and Charles 1997); and 4) Spontaneous Ca\(^{2+}\) oscillations and waves require PLC activity (Zanotti and Charles 1997). Taken together, these data and analysis strongly suggest that the mechanism of spontaneous Ca\(^{2+}\) signaling occurs via the paracrine action of ATP released through Cx43 hemichannels.

The finding that quinine activates spontaneous Ca\(^{2+}\) signaling is particularly significant because of its action on other channels. For example, quinine is significant because it inhibits other potential ATP release channels such as CFTR channels but activities hemichannels (Malchow, Qian et al. 1994; Dixon, Takahashi et al. 1996; White, Deans et al. 1999; Al-Ubaidi, White et al. 2000). In fact, quinine activates hemichannels while having no effect on gap junction channels (Malchow, Qian et al. 1994; Dixon, Takahashi et al. 1996; White, Deans et al. 1999; Al-Ubaidi, White et al. 2000) further implicating hemichannels, not gap junction channels, in Ca\(^{2+}\) signaling. It also definitively shows that hemichannels and gap junction channels have at least some modes of regulation that are unique to the configuration of the connexin channel, i.e. hemichannel versus intercellular gap junction channel.

It is also interesting to consider OAG since DAG, its natural analog, is produced by several enzymatic reactions, not just by PLC activation (as noted above). This suggests the possibility that differences in the characteristics of Ca\(^{2+}\) wave propagation could reflect different mechanisms of DAG mobilization, i.e. activation of PLC or PLD. There are several data that correlate with this hypothesis. For example, it is clear that
several of the types of stimulation that initiate calcium signaling events initiate ATP release including glutamate (Queiroz, Gebicke-Haerter et al. 1997; Queiroz, Meyer et al. 1999), mechanical stimulation (Guthrie, Knappenberger et al. 1999), UTP (Cotrina, Lin et al. 1998) and electrical (Hamann and Attwell 1996; Westfall, Todorov et al. 1996; Ferguson, Kennedy et al. 1997; Vizi, Liang et al. 1997) stimulation. Differences in the mechanism to mobilize DAG by these activators could account for subtle differences noted in Ca$^{2+}$ signaling initiated by these activators. It should be possible to test this hypothesis by characterizing the kinetics of ATP release initiated by these activators.

**Calcium Signaling Physiology and Pathophysiology**

Calcium Signaling in the Astrocytic Compartment

The fact that the regulation of cellular enzymatic processes by cytoplasmic Ca$^{2+}$ is so ubiquitous leads to the rather interesting dilemma of determining what functions of the brain [Ca$^{2+}$], and Ca$^{2+}$ signaling are not involved with rather than the converse. As such, the criteria should be rather stringent so as not to include functions where calcium signaling plays only a minor role. Previously, the correlation between mechanical stimulation induced Ca$^{2+}$ signaling events in astrocytes and tissue function was arguably most directly relevant to pathologic events such as head trauma. However, the delineation that the mechanism of Ca$^{2+}$ wave propagation is largely similar among various modes of stimulation indicates that the findings can be applied on a broader range of cellular events in the astrocytic compartment.

The basic finding of this research that Ca$^{2+}$ wave propagation is critically dependent on hemichannels suggests that previously examined functions associated with connexin channels may be organized, initiated and/or synchronized by Ca$^{2+}$ signaling. It
should be noted that studies involving either gap junction channels or hemichannels might ultimately be relevant because, as delineated in this report, the functions of these channels are easily confused. This is mainly due to the fact that common inhibitors like heptanol or PKC over-activation inhibit both types of channels and because molecular manipulations affect both channel configurations. In the CNS this would include volume regulation (Scemes and Spray 1998), \([K^+]_e\) regulation (Granda, Tabernero et al. 1998; Blomstrand, Aberg et al. 1999; Velasco, Tabernero et al. 2000), and modulation of the glial signaling compartment (Fujita, Nakanishi et al. 1998; Giaume and Venance 1998; Blomstrand, Aberg et al. 1999; Blomstrand, Giaume et al. 1999).

One goal of this report was to tender convincing arguments that astrocytes are involved in the higher functions of the brain. However, it should not be ignored that there are time-honored data and analysis that suggest that astrocytes contribute to CNS function by nurturing and regulating the environment of neurons. This report and others by this laboratory document several ways that this occurs and is coordinated by calcium signaling in astrocytes. This includes sensing and/or regulation of \([Ca^{2+}]_e\) (Zanotti and Charles 1997), \([ATP]_e\) and \([K^+]_e\) (report in progress).

Conversely, it can be seen that neurons act as support cells for astrocytic signaling. For example, it has recently been demonstrated that a primary mechanism of regulation of extracellular divalent cations in the CNS involves the Ca\(^{2+}\) sensing receptor (CaR) (Ye, Ho-Pao et al. 1997; Baum and Harris 1998; Brown, Chattopadhyay et al. 1998). Neurons express this receptor, which is activated by polyvalent cations including Ca\(^{2+}\) and Mg\(^{2+}\), much more robustly that astrocytes (Rogers, Dunn et al. 1997). This indicates that neurons play a central role in regulating \([Ca^{2+}]_e\) (and \([Mg^{2+}]_e\)) in the CNS.
The demonstration that \([\text{Ca}^{2+}]_e\) and \([\text{Mg}^{2+}]_e\) modulate \(\text{Ca}^{2+}\) signaling in astrocytes shows that neurons support signaling functions in astrocytes.

**Heterocellular Calcium Signaling in the CNS**

Clearly, one of the primary roles of \(\text{Ca}^{2+}\) signaling involves signal propagation between different cell populations. There are multiple locations in the CNS where dissimilar cell types make contact, suggesting a plethora of heterocellular \(\text{Ca}^{2+}\) signaling events each with specific functions. Many of these heterocellular \(\text{Ca}^{2+}\) signaling events have been directly demonstrated. The findings of this research provide a basis for the further clarification of the signaling mechanisms responsible and suggest some novel functions for these events.

**Astrocytic/Endothelial Interactions**

\(\text{Ca}^{2+}\) signaling occurs bidirectionally between astrocytes and endothelial cells (Paemeleire and Leybaert 2000). This is particularly interesting since endothelial cells routinely receive and generate multiple types of stimuli known to initiate \(\text{Ca}^{2+}\) signaling, including shear mechanical stimulation, nitric oxide (NO) generation, exposure to purines, and exposure to serum proteins. In like manner, astrocytes routinely receive multiple types of stimulation that initiate \(\text{Ca}^{2+}\) signaling including exposure to purines, glutamate, acethocholine, and serotonin. The bidirectional nature of stimulus input and signal propagation suggest that the astrocyte/endothelial boundary exhibits continuous calcium signaling events *in vivo*.

The results of this research suggest the possibility that this bidirectional signal flow may occur by the same mechanism as intercellular \(\text{Ca}^{2+}\) signaling in astrocytes, i.e. by means of the extracellular ATP mediated signaling system. Consistent with this, mass
mechanical stimulation initiates ATP release from endothelial cells (data not shown) and intercellular signaling is inhibited by the non-specific purinergic antagonist suramin (Paemeleire and Leybaert 2000). Currently, however, no data exist that indicate whether endothelial cells release ATP through hemichannels and, if so, whether this release is initiated by DAG mobilization. It seems likely, though, since endothelial cells express Cx43 (Pepper, Montesano et al. 1992; Polacek, Lal et al. 1993; Little, Beyer et al. 1995), a connexin that is routinely found to form functional hemichannels. It should be noted that endothelial cells also express Cx40 (Pepper, Montesano et al. 1992; Polacek, Lal et al. 1993; Little, Beyer et al. 1995), and this connexin could also be involved in forming functional hemichannels.

Astrocytic/Neuronal Interactions

The discovery of hemichannels in astrocytes may provide clues to some unresolved questions. For example, it has been noted that Cx43 is highly expressed in astrocytes and this expression is often disproportionate to the number of gap junction channels. Furthermore, a similar discrepancy has been noted in cultured neurons that show no detectable coupling, yet have substantial amounts of Cx43 mRNA (Simburger, Stang et al. 1997). Formation of functional hemichannels may account for a portion of this, which would suggest that there are more functional hemichannels than gap junction channels. Consistent with this, several reports where a connexin-GFP fusion protein are expressed show extensive diffuse staining in the membrane, suggesting that the bulk of connexin protein actually exists in the form membrane channels (hemichannels) (Holm, Mikhailov et al. 1999; Jordan, Solan et al. 1999).
Hemichannels could be important in signaling and bulk transport of metabolic molecules between astrocytes and neurons. The main characteristics of hemichannels, including permeability to relatively large anions and modulation of its activity by second messengers, are suitable to this role. For example, it has been demonstrated that connexin32 hemichannels are involved in glutamate release. As demonstrated here, hemichannels are critical to purinergic signaling in astrocytes and, in like manner, may be a part of neuronal ATP release. This demonstrates that hemichannels play a critical role in communication between the neuronal circuits and the astrocytic signaling network.
Final Remarks

It is safe to say that, at the time of this writing, the prescribed role of glial cells in contemporary theories of neurologic function has completely changed. Glial cells, which used to be thought of as simple support cells, are now proposed to be equal to neurons in providing the higher functions of the brain. Many of these theories that are being developed around this idea feature glial calcium waves as the cornerstone argument of this radical departure from past theories of CNS function. Indeed, given the numerical and volumetric superiority of glial cells to neurons (nearly 10:1 and 3:1 respectively) and the numerical equality of neurons and glial cells at each synapse, it is justified to question whether neurons will always enjoy the recognition of being the most important cell type in the CNS. Perhaps, if the study of glial biology continues to provide profound and novel data, glial cells may ultimately be featured as the main cell type responsible for the higher functions of the brain with neurons relegated to the relatively unprestigious role of information transfer elements, i.e. similar to information buses in electronic circuits. This may be overstating things somewhat but it is true that neurons are well suited to for this function.

These considerations indicate that the most important contribution of this research will likely be in utilizing the technologies, theories, and molecular agents derived herein as a beginning point for in situ or in vivo studies directed at exploring the relationship between the astrocytic signaling system and signaling in neuronal networks. It seems reasonable to suggest that it will ultimately be possible to divide many clinical syndromes into those arising from astrocyte pathology versus those that are the result of neuronal dysfunction. For example, seizure could be divided into types arising from dysfunction
of neuronal elements versus types arising from aberrant calcium signaling in astrocytes.

It will be exciting to begin to explore Ca\textsuperscript{2+} signaling events \textit{in situ} and \textit{in vivo} to characterize some of these possibilities and propose rational therapeutic modalities based on the resulting advances in cellular neuroscience.
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