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Components of Astrocytic Intercellular Calcium Signaling

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Abstract

It has become evident that astrocytes play major roles in central nervous system (CNS) function. Because they are endowed with ion channels, transport pathways, and enzymatic intermediates optimized for ionic uptake, degradation of metabolic products, and inactivation of numerous substances, they are able to sense and correct for changes in neural microenvironment. Besides this housekeeping role, astrocytes modulate neuronal activity either by direct communication through gap junctions or through the release of neurotransmitters and/or nucleotides affecting nearby receptors. One prominent mode by which astrocytes regulate their own activity and influence neuronal behavior is via Ca^{2+} signals, which may be restricted within one cell or be transmitted throughout the interconnected syncytium through the propagation of intercellular calcium waves. This review aims to outline the most recent advances regarding the active communication of astrocytes that is encoded by intracellular calcium variation.

Index Entries: Calcium waves; glia; purinoceptors; astrocytes; $InsP_3$ receptors; gap junctions; connexins; ATP; glutamate; cell-cell communication.

Introduction

There are several modes of intercellular communication involving signaling through paracrine, synaptic, and endocrine pathways, as well as direct signal exchange through intercellular gap-junction channels. Although many ions and small molecules may ultimately be involved in relaying intracellular signals, spatial and temporal alterations in the intracellular Ca^{2+} levels in the form of Ca^{2+} spikes, oscillations and waves are certainly major events in signal transduction and cell-cell communication.

Variation in intracellular free-calcium concentration is an ubiquitous event that plays important roles in many cellular processes such as secretion, muscle contraction, gene expression, and metabolism. In the immature central nervous system (CNS), transient variation in cytosolic calcium levels are important for cell proliferation, neuronal migration, motility of axonal and dendritic growht cones, and clustering of the post-synaptic receptors and channels

(1–5). Coordinated endogenous neuronal activity in the form of propagating intercellular calcium waves observed in the immature retina *(6–8),* cortex *(9–11),* and in hippocampal-slice cultures *(12)* is believed to regulate and promote the establishment of neuronal connectivity *(11,13–15),* and calcium waves spreading between glial cells has been proposed not only to be a mechanism by which these cells coordinate their own activity *(16),* but also is thought to modulate neuronal excitability *(17–21).* In addition, it has been suggested that glial Ca^{2+} waves may trigger certain pathological conditions that are manifested as slowly developing and slowly recovering changes in brain activity as seen in spreading depression *(22–25),* which is thought to be related to migraine headaches (*26; see also 27*).

Calcium Signaling Within the Astrocytic Syncytium

The perceived role of glial cells in brain function has dramatically changed over the last decade from that of a passive to an active participant, due to accumulating evidence that these cells express a variety of ion channels and neurotransmitter receptors. Thus, glia are able not only to detect and respond to neuronal activity with fluctuations of intracellular Ca^{2+} concentration but also to control neuronal excitability. (For recent reviews on glial Ca^{2+} signaling, *see* refs. *21,28*).

The first demonstration that neurotransmitters induced Ca^{2+} fluctuations in astrocytes was provided by Cornell-Bell et al. *(29),* Cornell-Bell and Finkbeiner *(22),* and Kim et al. *(30);* the authors observed that in the presence of glutamate, cultured astrocytes displayed three different types of Ca^{2+} responses: (1) a sustained intracellular calcium increase due to the activation of ionotropic receptors, which led to the influx of Na^+ and Ca^{2+}; (2) an oscillatory elevation of intracellular calcium, resultant from the activation of metabotropic receptors inducing synthesis of $InsP_3$ and ultimately to the release of Ca^{2+} from $InsP_3$-sensitive stores; and (3) an intercellular calcium wave that propagated between the cultured cells at a quite constant amplitude and velocity (15 μm/s), extending to about 100 cells.

Similar intercellular calcium waves between astrocytes have been observed propagating in cultured astrocytes following focal mechanical stimulation *(31–38),* following focal or bath application of various neurotransmitters *(31,34,39–43),* and observed in organotypic preparations after neuronal stimulation *(44,45)* and also following mechanical or agonist application *(18).*

More interesting are the observations that not only can astrocytes respond with calcium transients to neuronal activity but that these signals can also convey information, modulating neuronal activity. (For reviews, *see* refs. *21,46,47*). Evidence that astrocytes influence neuronal activity was obtained in hippocampal slices where it was shown that stimulation of astrocytes potentiated inhibitory post-synaptic currents in CA1 pyramidal cells, while blockade of astrocytic calcium signaling with the calcium-chelator BAPTA prevented the potentiation *(48).* In the rat eyecup preparation, it was also shown that calcium waves propagating between glial cells altered the firing rate of retinal neurons whenever these waves reached the ganglion-cell layer *(18);* because the magnitude of such modulation was directly correlated with the amplitude of glial calcium waves and with the activation of neuronal glutamatergic receptors, it was proposed that astrocytes and Muller cells modulate neuronal activity by releasing glutamate in a Ca^{2+}-dependent manner from astrocytes *(18).*

Although most of the evidence that neuronal activity is modulated by glutamate released from astrocytes came from cell-culture preparations *(49–54),* these studies strongly support the new perception of glial function as active participant in CNS information processing. As such, it is plausible to consider that astrocytes

are "excitable"* cells, with Ca^{2+} fluctuations being the signals by which they respond, integrate, and convey information.

Mechanisms for Calcium Wave Initiation

Intercellular Ca^{2+} wave spread is a phenomenon characterized by an increase in cytosolic Ca^{2+} within one cell that is followed by increase of cytosolic calcium levels in neighboring cells in a wave-like, propagating fashion. A brief description of the mechanisms involved in the generation of intracellular calcium transients that are relevant for the understanding of long-range mode of intercellular calcium signaling is presented below.

Neurotransmitters such as acetylcholine, noradrenaline, glutamate, and adenosine nucleotides have been shown to be capable of inducing intracellular calcium transients that are followed by intercellular calcium waves in astrocytes *(see 55,56).* The basic mechanism that leads to an intracellular calcium signal in response to activation of G-protein coupled receptors depends on the activation of phospholipase C, and breakdown of phosphatidylinositol biphosphate (IP_2) to $InsP_3$ which activates Ca^{2+} release from the endoplasmic reticulum (ER) through activation of $InsP_3$ receptors. These intracellular Ca^{2+} signals are spatially and temporally complex events involving the recruitment of elementary Ca^{2+} release sites (Ca^{2+} puffs; *57*) which trigger a propagating intracellular Ca^{2+} wave that spread throughout the cell *(58–60).*

Neurotransmitter-induced intracellular Ca^{2+} waves in astrocytes have been shown to begin at specific subcellular sites (ER), which correlate with the immunolocalized expression of type 2 $InsP_3$ receptors, and then propagate throughout the cell by an amplification mechanism *(61).* Similar observations of conserved initiation sites of Ca^{2+} puffs induced by neurotransmitters and hormones have also been described in a variety of cell types including hepatocytes *(62,63),* oligodendrocytes *(61,64),* and HeLa cells *(65).* It has been suggested that for Ca^{2+} signals to be propagated throughout the cell as a regenerative event, positive-feedback mechanisms should be present; it is likely that the rise in cytosolic Ca^{2+} resultant from the Ca^{2+} puffs provides the necessary positive-feedback mechanism that increases the amount of releasable Ca^{2+}. Amplification mechanisms would include the diffusion of Ca^{2+} released from the elementary sites to neighboring $InsP_3$ receptors, increasing the number of primed $InsP_3$ receptors due to the co-agonistic action of Ca^{2+} on these $InsP_3$ receptors *(66–68),* and the generation of additional $InsP_3$ through the Ca^{2+}-dependent activation of phospholipase C *(34,69).* In astrocytes, the contribution of the Ca^{2+}-induced Ca^{2+} release through activation of ryanodine receptors is believed to be negligible; depletion of ryanodine-sensitive stores does not interfere with the responses induced by glutamate, ATP, and thapsigargin *(70).*

The link between phospholipase C and Ca^{2+} transients is inositol 1,4,5 trisphosphate, which diffuses from its site of production into the cytosol and binds to Ca^{2+}-releasing channels, the $InsP_3$ receptors *(69,71).* Of the three $InsP_3$ receptor isoforms so far described *(see 72),* astrocytes express types 2 and 3 *(61,73).* Each of these $InsP_3$ receptor types displays a different affinity for $InsP_3$ and each is differently modulated by Ca^{2+} and by accessory proteins such as calcineurin *(see 71);* these differences play important roles in the initiation and propagation of all phases of agonist-mediated intracellular Ca^{2+} transients.

Sites of regenerative intracellular Ca^{2+} wave amplification in astrocytes have been described. Calcineurin, a Ca^{2+}-calmodulin activated phosphatase *(74)* that is associated with $InsP_3$ receptors in the cerebellum *(75),* has been

*The term excitable is here used as described for electrically excitable membranes *(54a)*, meaning the presence of a positive feedback mechanism leading to a regenerative sequence.

recently shown to be highly expressed in the astrocytic ER specifically at sites of Ca^{2+} amplification where type 2 $InsP_3$ receptors are expressed *(76,77).* Furthermore, it is also likely that mitochondria contribute to Ca^{2+} amplification during intracellular Ca^{2+} waves, buffering the excess Ca^{2+} at $InsP_3$ receptor microdomains *(77,78)* that otherwise will negatively affect the $InsP_3$ receptor sensitivity to $InsP_3$ *(see 71).* Evidence for such a mitochondrial role in maintaining adequate Ca^{2+} concentration for $InsP_3$ receptor activation include: (1) co-localization of mitochondria at sites of high expression of calcineurin that correspond to the Ca^{2+} amplification sites; and (2) attenuation of norepinephrine-induced increase in intracellular Ca^{2+} levels by the protonophore FCCP, which abolishes the proton gradient across mitochondrial membranes preventing mitochondria Ca^{2+} uptake *(77).*

Once triggered, the intracellular calcium wave is likely to be propagated to neighboring cells. Independently of which of the two routes the signaling messenger may travel (through intercellular gap-junction channels or through extracellular space, *see* below), the mechanism that triggers calcium transients in adjacent cells relies on $InsP_3$ generation and release of stored calcium as described earlier. This implies that the amplitude of calcium transients generated within each cell is dependent on the properties of the Ca^{2+}-releasing machinery. The extent to which calcium signals can spread between cells is likely to be dictated by the effective diffusion properties of the signaling molecules, and the velocity with which the intercellular calcium waves can spread is likely to depend on the restrictions imposed by the routes traveled (gap junction-dependent and -independent pathways).

The Pathways for Intercellular Calcium-Wave Propagation

There are two routes by which calcium waves can be propagated from one cell to the other: (1) through intercellular gap-junction channels, which involves the diffusion of signaling molecules generated in one cell to the neighboring ones where an increase in $[Ca^{2+}]$ levels are induced, and (2) through the extracellular space, which involves the release of pharmacologically active substances from one cell, activating plasmalemmal receptors in neighboring cells *(see 55,56,79).*

The relative contribution of intercellular gap junction-dependent and -independent pathways in the propagation of intercellular calcium waves among astrocytes has been controversial. Some reports indicate that calcium wave propagation between astrocytes relies entirely on the gap junction-mediated pathway *(23,33,34,39,80),* while others support the idea of a pathway involving primarily the extracellular diffusion of ATP *(81–85).* Such divergent views regarding the pathways involved in the propagation of calcium waves between astrocytes may be related in part to the heterogeneous distribution of gap junctions and purinoceptors in the CNS. Astrocytes from different CNS regions display different strengths of gap junction-mediated coupling *(38,42,86–90)* and express different subtypes of purinoceptors *(38,91–93).* Furthermore, under different pathological conditions, the expression levels of connexins and purinergic receptors may be altered, which is likely to affect the relative contribution of these two pathways for calcium signaling between astrocytes. Cultured human astrocytes when exposed to the cytokine interleukine 1β switch the mode of calcium-wave propagation from a gap junction-dependent to a gap junction-independent mechanism due to the downregulation of Cx43 expression levels and upregulation of $P2Y_2$ receptors *(94).* A similar compensatory mechanism for intercellular calcium-wave propagation was observed to occur in cultured spinal-cord astrocytes from Cx43 knockout (KO) mice: gap junction-mediated calcium-wave propagation was evident in wild-type cells, while a gap junction-independent calcium wave propagation mediated by activation of $P2Y_2$ receptors was dominant in Cx43 KO spinal-cord astrocytes *(38).* Such change in

mode of intercellular calcium wave propagation from a gap junction-dependent to a gap junction-independent mechanism was related to the pharmacological switch in P2Y receptors sensitivity to agonists *(38).* Recent data obtained by our laboratory showing that Cx43 KO astrocytes transiently transfected with Cx43 cDNA displayed similar P2Y sensitivity to agonists to that seen in WT astrocytes *(95),* support the idea of a cross-talk between connexins and P2Y receptors to sustain astrocytic intercellular calcium signaling.

Intercellular Ca^{2+} Signaling Through Gap Junctions

Intercellular calcium-wave propagation directly from the cytosol of one cell to that of another requires the presence of gap-junction channels, which allow the diffusion of molecules (Mr < 1000 Da) across cell boundaries. Astrocytes *in situ* and in culture are coupled to each other by gap-junction channels formed mainly by connexin43 (Cx43) *(see 90,96).* Although the heterogeneous distribution of gap junctions in the CNS, the expression levels of at least two connexins (Cx43 and Cx30) and the degree of coupling between cultured astrocytes derived from different brain regions seem to correspond to the expression levels observed in brain *(see 96).* In the brain cortex, Cx43 channels contribute about 95% of total junctional conductance between cultured astrocytes (total junctional conductance in rats 13 nS: *(97);* in mouse, 17 nS: *(88)* with other connexins (Cx30, Cx40, Cx45, and Cx46) supporting the remaining junctional communication *(88–90,99,98).* In the spinal cord, where Cx43 has also been described to be expressed *(100–102),* about 70% of total junctional conductance is contributed by Cx43 channels (total junctional conductance in mouse 3 nS: *(38).*

Such differences in connexin expression and in the strength of coupling are expected to determine the function and regulation of the interconnected astrocytic syncytium. Furthermore, because Cx43 forms nonselective channels, whereas Cx40 and Cx45 are more permissive to the transfer of cations than anions *(103–106),* it is possible that the diffusion of anionic signaling molecules, such as $InsP_3$, would be differently affected by the selective permeabilities of the intercellular channels and by the strength of coupling within the astrocytic syncytium. (In this regard, two different laboratories have recently evaluated the properties of calcium waves propagation between HeLa cells exogenously expressing different connexins, Cx26, Cx32 and Cx43 *[107,108].* Interestingly, the two groups attained different conclusions regarding the selective permeability of these connexins for the diffusion of $InsP_3$; while Niessen et al. *[107],* by measuring the distance of calcium-waves propagation, reported that Cx32 channels were more permeable to $InsP_3$ than channels formed by Cx26 or Cx43, Paemeleire et al. *[108]* observed a correlation between the distance of calcium wave propagation with the amount of connexin expressed but not with the type of connexin. These results highlight the difficulty in interpreting dye permeability or diffusion when an independent experiment of the number of channels [such as electrical conductance] is lacking.)

The molecules that fulfill the size requirements to permeate gap-junction channels (up to 1 kDa) and that have been shown to trigger regenerative Ca^{2+} mobilization are $InsP_3$, Ca^{2+}, and cyclic ADP ribose *(109–116).* The ability of $InsP_3$ to permeate gap-junction channels *(109)* and the low intracellular buffering of this molecule *(117)* suggest that $InsP_3$ may be the main signaling molecule involved in gap junction-mediated communication of Ca^{2+} waves between astrocytes. The observations that calcium waves between cultured astrocytes could be induced by flash photolysis of pressure-injected caged $InsP_3$ but not of caged Ca^{2+}, and that the PLC inhibitor, U73122, blocks calcium-wave propagation *(34,116),* support the hypothesis that $InsP_3$ is the main messenger used by astrocytes to coordinate their activity. Furthermore, the extent to which calcium-waves propagate between cultured astrocytes was shown to be directly proportional to the amount of $InsP_3$

generated in the stimulated cell; progressively longer duration exposures of UV flashes in astrocyte injected with caged $InsP_3$ resulted in a progressively larger radius of intercellular calcium-wave propagation *(45).* Based on mathematical calculations and considering the amount of caged $InsP_3$ injected into one cell, the photolytic efficiency of the UV flash and the distance from the injection point where flash photolysis still caused a calcium waves, it was estimated that an initial intracellular $InsP_3$ concentration of 2.5 μ*M* would be sufficient to initate an intercellular calcium wave *(116,118).*

Intercellular Ca^{2+} Signaling Through the Extracellular Space

The existence of an alternative pathway for communication of the calcium signals that would operate independently from gap junctions, involving the diffusion of signaling molecules through the extracellular space, was first indicated by experiments performed on mast cells *(119).* In these cells, the mechanical stimulation of one cell was shown to induce an increase in the intracellular Ca^{2+} level of the stimulated cell and to trigger the propagation of Ca^{2+} waves to neighboring cells that were not in physical contact with the stimulated cell. In this system, mechanical stimulation or antigen-induced cross-linking of fcε receptors induced the release of ATP from the stimulated cells at a concentration (1–10 μ*M*) that was shown to be sufficient to activate Ca^{2+} responses in neighboring cells in a wavelike propagating event traveling at a velocity of 5–10 μm/s over 320 μm *(119).*

Extracellular signaling mechanisms were also shown to participate in the propagation of Ca^{2+} waves between astrocytes *(37,38,81,120).* Similarly to the noncoupled mast cells, mechanical stimulation *(120),* glutamate application *(121),* or purinergic-receptor stimulation *(84)* were shown to induce ATP release from the stimulated cells. Based on the diffusion properties of ATP and the amount of ATP released from stimulated astrocytes, it was suggested that, in order for this extracellular signal to induce a Ca^{2+} wave capable of propagating to long distances at a constant velocity, it would be necessary to have ATP being sequentially released by each cell participating in the Ca^{2+} wave *(120).* Nevertheless, such waves do not spread forever; using a chemiluminescence dynamic-imaging method to monitor ATP waves, it was shown that ATP released from stimulated astrocytes spread as a radial wave from its origin reaching limited distances *(85).* The blockade of ATP waves by the phospholipase C inhibitor U-73122, and by the purinergic antagonist suramin, indicated that $InsP_3$ or diacylglycerol may induce the release of ATP from astrocytes *(85).*

ATP is not the only compound that is released from astrocytes; several observations have suggested that glutamate is released during calcium signaling *(17,21,49,52,122).* Using an enzyme-linked assay in which NAD^+ is reduced to NADH by the enzyme L-glutamic dehydrogenase when in the presence of glutamate, Innocenti et al. *(53)* imaged extracellular waves of glutamate in cultured astrocytes. These extracellular glutamate waves were shown to propagate at a velocity (about 25 μm/s) similar to that observed for the intercellular calcium waves; the extracellular glutamate waves were shown to be abolished by altering intracellular calcium levels either by the addition of thapsigargin or BAPTA *(53).* These observations indicated that, differently from the extracellular ATP waves, glutamate waves are dependent on changes in intracellular Ca^{2+} concentration; i.e, glutamate is released from astrocytes by a Ca^{2+} dependent-mechanism *(53,54).*

Concluding Remarks

It is evident from this brief review that in the last decade several interesting aspects of astrocytic calcium signaling have been disclosed, especially with the advance of new and fast imaging techniques, which have revealed that astrocytes release neuro-active compounds (ATP and glutamate) in a wave-like fashion.

Although these extracellular waves are potentially interesting from the standpoint of neuroglia interactions, there are several unresolved questions regarding this mode of glial communication. For instance, it would be interesting to know whether ATP and glutamate are released from astrocytes following flash photolysis of caged $InsP_3$ or Ca^{2+}, respectively, rather than by mechanical stimulation, a stimulus that may damage the cells which *per se* would induce the release of these compounds. Even considering the lack of cell damage, it is not yet clear which pathways are involved in ATP and glutamate release from astrocytes. It has been hypothesized that ATP is released through chloride channels/anion transporters or through gap-junction hemichannels *(84,123).* Evidence favoring the release of ATP through chloride channels/anion transporters came from data indicating that the channel/transport inhibitors NPBB, SITS, and furosemide reduced the extent to which intercellular calcium waves propagate between astrocytes *(84);* while indication for a gap-junction hemichannel pathway for ATP release came from the observation that C6 glioma cells transfected with either Cx43 or Cx32 plasmids released more ATP than the parental nontransfected cells *(84,123).* It is, however, unlikely that gap-junction hemichannels contribute to ATP release. Although there are two reports suggesting the presence of functional Cx43 hemichannels in mammalian systems, openings of these channels are reported to occur under special conditions such as absence of extracellular calcium or following metabolic inhibition *(124,125).* Moreover, it remains speculative that under ischemic conditions, opening of connexin hemichannels will favor the release of ATP.

One intriguing aspect of glial calcium-signaling studies is the dichotomized emphases given to the role of the extracellular messengers: ATP as the inter-astrocytic signaling molecule and glutamate as a glial-neuron messenger. Both neurons and astrocytes express purinergic and glutamatergic receptors *(see 126,127),* and as described earlier, both agents participate in the propagation of intercellular calcium waves between astrocytes. It is tempting to speculate that the release of ATP from astrocytes would modulate the release and action of glutamate at the synapses, similarly to what has been described in rat hippocampus *(128),* where ATP acts in a synergistic way in the induction of long-term potentiation (LTP) *(129–131).*

Another open question regards the extent to which these extracellular waves spread *in situ.* The experiments performed to evaluate extracellular waves of ATP and glutamate, although pioneering, have been analyzed in cell cultures, conditions where there is no constraint for diffusion of molecules in the extracellular medium; however, the extracellular space (ECS) in tissues is generally quite restricted. In the CNS, the ECS makes up about 20% of total brain volume. Such limited ECS is expected to reduce the extent to which extracellular-mediated waves propagate; the composition, dimension, anisotropy, and the geometry of the ECS have been shown to affect the movement of substances in the CNS *(132).* Furthermore, because the diffusion properties of the ECS can change during neuronal activity and under pathological conditions *(133,134),* the distribution of signaling molecules (ATP, glutamate) within the brain may be affected under these conditions thus altering the direction of wave propagation.

It is important to realize that intercellular communication between neurons and glia is not only restricted to the diffusion of active compounds released from astrocytes affecting neuronal activity; more than five years ago, gap-junctional communication was proposed to be the pathway-mediating signal transmission between astrocytes and neurons *(23),* and recently the presence of functional gap junctions between neurons and astrocytes have been confirmed in co-cultures by electrophysiological techniques and by dye-transfer assays showing diffusion of injected Lucifer Yellow from neurons to astrocytes *(135).* In the locus cerulus of neonatal and adult rat-brain slices, evidence has also been provided recently for

gap-junctional communication between neurons and glia *(136).*

This is an exciting time for the glial field; as answers to these questions and the understanding of languages of astrocytic communication become translatable, new workers in the field as well as neuroscience textbooks are expected to acknowledge that astrocytes may well play housekeeping functions in the CNS, but also actively support and modulate neuronal information integration and processing.

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