Intercellular Calcium Signaling in Astrocytes via ATP Release through Connexin Hemichannels

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Astrocytes are capable of widespread intercellular communication via propagated increases in intracellular Ca²⁺ concentration. We have used patch clamp, dye flux, ATP assay, and Ca²⁺ imaging techniques to show that one mechanism for this intercellular Ca²⁺ signaling in astrocytes is the release of ATP through connexin channels (“hemichannels”) in individual cells. Astrocytes showed low Ca²⁺-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 Ca²⁺ and was inhibited by FFA and Gd³⁺. Mechanical stimulation also evoked release of ATP that was potentiated by low Ca²⁺ and inhibited by FFA and Gd³⁺. 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 quinidine evoked ATP release and Ca²⁺-signaling in astrocytes and in C6 cells expressing connexin43. The propagation of intercellular Ca²⁺ waves in astrocytes was also potentiated by quinidine and inhibited by FFA and Gd³⁺. Release of ATP through connexin hemichannels represents a novel signaling pathway for intercellular communication in astrocytes and other non-excitable cells.

Astrocytes respond to a variety of stimuli with increases in intracellular calcium concentration [Ca²⁺],¹ (1, 2). Ca²⁺ signaling in astrocytes may be limited to individual cells or may occur as a “wave” of increased [Ca²⁺], that is propagated from one cell to surrounding cells. Initial studies focused on gap junctions as the pathway for intercellular communication of Ca²⁺ waves in astrocytes. Ca²⁺ 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 Ca²⁺ wave propagation. Expression of connexin43 (Cx43, the predominant connexin in astrocytes) in C6 cells is correlated with both intercellular dye transfer (3, 4) and propagated Ca²⁺ waves (5, 6). Studies with other cell types have shown similar correlation of Ca²⁺ wave propagation with connexin expression (7). In addition, pharmacological inhibitors of connexin channels have been shown to inhibit intercellular Ca²⁺ wave propagation in astrocytes (1, 8). These data show that connexin channel expression and function play a central role in Ca²⁺ wave propagation.

Recent studies (9, 10) have also clearly shown that the intercellular propagation of Ca²⁺ signals in astrocytes involves the diffusion of an extracellular messenger, namely ATP. Ca²⁺ waves in astrocytes induced by mechanical or electrical stimuli are associated with ATP release, and intercellular Ca²⁺ waves are blocked by purinergic receptor antagonists (9, 11). Multiple other stimuli that evoke astrocyte signaling, including glutamate and UTP, also evoke release of ATP (5, 12).

The requirement for connexins and the involvement of ATP as a messenger in astrocyte Ca²⁺ wave propagation seem to indicate paradoxically both gap junctional 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 (13–17). 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 (13). 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 Ca²⁺ signaling could therefore be reconciled by the hypothesis that the ATP that mediates Ca²⁺ waves is released through connexin hemichannels.

Consistent with this hypothesis, Cotrina et al. (5) reported that ATP release from C6 glioma cells is increased in direct correlation with the level of forced connexin expression. Although 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 junctional coupling may not have the same effects or potency as inhibitors of connexin hemichannels.

Bruzzone et al. (29) reported that transmembrane flux of NAD⁺...
occurs via connexin43 hemichannels, indicating that the hemichannels composed 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 (18). To prepare purified astrocyte cultures, flasks were shaken daily for 3 days for 1 h on a shaker at 37 °C, and displaced cells were removed. Astrocytes were passaged onto poly-L-lysine-coated glass coverslips or 35-mm culture dishes, or culture flasks at 50,000 cells/cm$^2$ and were maintained in growth medium (Dulbecco's modified Eagle's medium 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 (4, 6) 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 [Ca$^{2+}$]$_i$**—[Ca$^{2+}$]$_i$ was measured using a fluorescence imaging system that has been described previously in detail (18). In brief, cells on glass coverslips were loaded with fura2 or incubated 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 ×20 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 described previously.

**Membrane currents**—Membrane currents 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 3 times (30 s) with control HBSS (1 mM EGTA, 1 mM free Mg$^{2+}$, 1.3 mM free Ca$^{2+}$) and bathed (1.5 ml) for 5 min, and 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 min, after which mass membrane stimulation was achieved by dropping ~110 μg of microglass microbeads (30–50 μm diameter) through the ∼3-mm medium onto the cells. This type of stimulation has been shown to initiate multifocal calcium waves by mechanical stimulation of individual cells (9). All direct comparisons were made for 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 Victor). Calibration (ATP) curves were obtained in corresponding HBSS (i.e., matched [Mg$^{2+}$] and [Ca$^{2+}$]). MeSO$\_4$ increased luminescence measurements, so assays involving ligands dissolved in MeSO$\_4$ required calibration including MeSO$\_4$. The difference between [ATP]o in the sample taken after mass mechanical stimulation (5-min time point) and the sample taken just prior to mechanical stimulation (4.5-min time point) was calculated as the change in [ATP]o (Δ(ATP)$_o$) for each plate under treatment HBSS. Δ(ATP)$_o$ 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 (3 times; 1.5 ml) with control HBSS and then incubated with control HBSS supplemented with Calcein Blue AM (10 μM) for 30 min. 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, Molecular Probes, Eugene OR). Cells were then rinsed (3 times; 1.5 ml) and incubated for an additional 30 min to allow fixation and permeabilization of the cells, after which they were placed in treatment HBSS (1.5 ml). After 4.5 min a sample (500 μl for this and all subsequent samples) was collected as a base line. To control for fluorescence of components of treatment HBSS (e.g. Ca$^{2+}$ or Mg$^{2+}$), 250 μl of treatment HBSS was added to base-line samples. Mass mechanical stimulation was then performed by adding medium containing microbeads (110 μg in 500 μl of HBSS) to the cells. After 40 s, medium samples were collected. The extracellular solution was then replenished, and the cells were lysed using a 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), using 350/450 (Calcein Blue) or 490/535 (Oregon Green) filter sets. Percent dye released (%DR) by stimulation was calculated as shown in Equation 1,

\[
\text{%DR} = \frac{[I_{\text{stim}} - I_{\text{ref}}]}{[I_{\text{stim}} - I_{\text{base}}]} + [I_{\text{stim}} - I_{\text{ref}}]
\]

where $I_{\text{stim}}$ is measured intensity of the sample collected after mechanical stimulation. $I_{\text{ref}}$ is the measured intensity of the spontaneous release sample (i.e., first collected sample). $I_{\text{base}}$ is the measured intensity of the sample collected after the cells are lysed, and $I_{\text{base}}$ 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 (Cytox, 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 Hanks' balanced salt solution (Ca$^{2+}$- and Mg$^{2+}$-free) supplemented with HEPES (10 mM), EGTA (1 mM), Ca$^{2+}$ and Mg$^{2+}$ (pH 7.3 with NaOH). The pipette solution contained the following: 140 KCl, 10 HEPES, 10 EGTA, and 2 Mg$^{2+}$ (pH 7.2 with KOH). Pipette tips were fire-polished using a heated platinum filament to a tip resistance of 3–5 meqms. Patch clamp recordings were performed and analyzed using an Axopatch 200B amplifier and pClamp (Axon Instruments). All data were collected at 10 kHz and analog-filtered at 5 kHz.

**RESULTS**

**Low Ca$^{2+}$-activated Currents**—We used the whole-cell configuration of the patch clamp technique to determine whether astrocytes display low [Ca$^{2+}$]$_i$-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 Ca$^{2+}$ 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 described previously in astrocytes (19, 20). Low [Ca$^{2+}$]$_i$-activated currents were more easily quantified in cells with low levels of base-line currents (Fig. 1).

Perfusion of astrocytes with medium containing no added Ca$^{2+}$ (with 1 mM EGTA) resulted in a base-line 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 (Fig. 1). The average ratio of the maximum steady-state inward current in low [Ca$^{2+}$], versus normal [Ca$^{2+}$], was 1.56 ± 0.17 (n = 5). Currents returned to base line when the extracellular solution was exchanged for control solution. Approximately 60% of isolated astrocytes showed low [Ca$^{2+}$]$_i$-activated currents. Flueneanic acid (FFA) has been reported to block currents through connexin hemichannels expressed in oocytes with high potency (21) and to inhibit Cx43-mediated intercellular communication (22). Application of 50 μM FFA immediately abolished low [Ca$^{2+}$]$_i$-activated currents (average ratio of maximum current in low [Ca$^{2+}$], with FFA versus normal [Ca$^{2+}$], was 0.97 ± 0.02, n = 3). The significant inhibition of low [Ca$^{2+}$]$_i$-activated currents (p < 0.02) by FFA was partially reversible (~70%; data not shown) with washout. C6 cells expressing connexin43 (C6 Cx43 cells, n = 6), but not transfected C6 cells (n = 10), showed FFA-sensitive whole-cell currents similar to those observed in primary astrocytes (data not shown).

**Dye Uptake**—Transmembrane flux of low molecular weight dyes has been used to indicate the presence of connexin hemichannels in multiple cell types including astrocytes (13, 14). We examined the pattern of Lucifer Yellow (LY) and rhodamine dextran (RD) (10 kDa) flux into astrocytes under conditions of normal and low [Ca$^{2+}$]$_i$. 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 reported previously (13). Subsequent exposure of cells to Ca\(^{2+}\)-free medium containing no dye caused release of the LY (Fig. 2B).

To determine whether 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\(^{2+}\) 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\(^{2+}\) wave (Fig. 2C; n = 12). The uptake of LY by both the stimulated cell and the surrounding cells was greatly potentiated by removal of extracellular Ca\(^{2+}\). 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 nonspecific disruption of the cell membrane.

**Dye Release**—To characterize dye flux further, plates of cells were loaded with a connexin channel-permeable dye (Calcein Blue, approximate M, 400) and a connexin channel-impermeable dye (Oregon Green 1 BAPTA 488, approximate M, 1100) by incubating cells with the respective AM ester for 20 min. Dye-loaded cells were mechanically stimulated by dropping glass microbeads (30–50 μm in diameter) through the medium onto the cells. This mass mechanical stimulation has been found to initiate multifocal calcium waves without causing nonspecific disruption of the cell membrane (9).

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% S.E., n = 16) and 0.13% of Oregon Green (±0.25% S.E., n = 8). There was no detectable release of LDH in response to mechanical stimulation under any condition (n = 32, cell lysates were used as positive control). Calcein release was modulated by connexin channel modulators (Fig. 3). Low [Ca\(^{2+}\)] solution (with no dye) 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 [Ca\(^{2+}\)], (HBSS with no added Ca\(^{2+}\)) solution containing LY (1 mg/ml) and RD (1 mg/ml). After 5 min, cells were washed with normal [Ca\(^{2+}\)] 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 eight different experiments.
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**Fig. 3.** 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 versus control conditions. For C6 cells, control conditions represent C6 cells expressing Cx43 in normal medium. Both 50 μM FFA (n = 8, p < 0.03) and 50 μM Gd3+ (n = 8, p < 0.02) significantly inhibited mechanically stimulated Calcein Blue release by astrocytes. Calcein Blue release was significantly potentiated in low [Ca2+]i medium (n = 16, p < 0.03). C6 glioma cells, which show very low levels of connexin expression, showed significantly less release of Calcein Blue than did C6 cells expressing Cx43 (C6 Cx43 cells, n = 8, p < 0.01). As with primary astrocytes, calcine release by C6 Cx43 cells was inhibited by 50 μM FFA (n = 8, p < 0.03) and Gd3+ (n = 8, p < 0.02) and potentiated by low [Ca2+]i, (n = 8, p < 0.03). Error values represent maximum variation in standard error of ratio (condition + S.E./control − S.E.) − condition/control.

**Fig. 4.** Astrocytes and C6 glioma cells expressing Cx43 release ATP. Each bar represents the average mechanically stimulated ATP release under the specified conditions versus control conditions. For C6 cells, control conditions represent C6 Cx43 cells in normal medium. In primary astrocytes, ATP release was significantly potentiated by low [Ca2+]i, (p < 0.02) and significantly inhibited by 50 μM FFA (p < 0.0005) and 50 μM Gd3+ (p < 0.005) but not by the Cl– channel blocker 100 μM DIDS. C6 Cx43 also showed release of ATP that was inhibited by 50 μM FFA (p < 0.001) and 50 μM Gd3+ (p < 0.001) but not by 100 μM DIDS and potentiated by low [Ca2+]i, (p < 0.02), whereas parent C6 cells that express low levels of connexins showed minimal ATP release (p < 0.01). n = 8 for each experiment; error bars represent maximum variation in standard error of ratio (condition + S.E./control − S.E.) − condition/control.

FFA and by Gd3+, which has also been shown to inhibit connexin hemichannels with high potency (21) (Fig. 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 with exogenously expressed connxin43 (C6 Cx43 cells) showed levels of Calcein Blue (9.6 ± 1.3%, n = 16) release that were comparable with or higher than that showed by astrocytes. As with primary astrocytes, Calcein Blue release from C6 Cx43 cells was potentiated by low [Ca2+]i, medium and was inhibited by FFA and Gd3+ (Fig. 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 (9, 11). We found that ATP release evoked by mechanical stimulation with glass microbeads could be detected in as little as 10 s following stimulation. [ATP], increased for the first ~40 s after mechanical stimulation and remained elevated throughout the time frame of the measurement (5 min; data not shown). We took samples at 30 s to reflect ATP release associated with intercellular Ca2+ waves.

Treatment with low [Ca2+]i, medium significantly potentiated the ATP release induced by mechanical stimulation (Fig. 4). Conversely, the connexin channel inhibitors FFA and Gd3+ significantly inhibited mechanically induced ATP release (Fig. 4). Because 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 (Fig. 4).

Cotrina et al. (5) reported previously 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 Cx43 cells showed ATP release that was potentiated by low Ca2+, and inhibited by FFA but not DIDS, and Gd3+ (Fig. 4). The inhibitory effect of FFA was concentration-dependent. In the presence of 25 μM FFA, ATP release was 69 ± 9% of control release, whereas in the presence of 50 or 100 μM FFA ATP release was 10 ± 6% of control release (no significant difference between the effect of 50 and 100 μM FFA).

Quinine has been reported to activate connexin hemichannels in multiple preparations (23–25). Quinine evoked a con-
FIG. 5. Activation of Ca^{2+} signaling in astrocytes by quinine. Raster plots show change in fura fluorescence (−[Ca^{2+}]) versus time in 20 cells in fields of primary astrocytes (A), C6 Cx43 cells (B) and un-transfected C6 cells (C). Each row in the plot shows ΔF versus time for an individual cell. The line tracing at the top shows a representative cell. Bath application of 100 μM quinine reversibly evoked Ca^{2+} oscillations and intercellular Ca^{2+} waves in the majority of astrocytes or C6 Cx43 cells but not in untransfected C6 cells.

concentration-dependent release of ATP from C6 cells expressing connexin43, in a concentration range (50–500 μM) similar to that observed to activate connexin hemichannels (23–25). ATP concentrations measured 1 min following quinine exposure were 24, 28, and 33 μM for 50, 100, and 500 μM quinine, respectively (n = 8 each, p < 0.012 by single factor analysis of variance).

Calcium Signaling—We have reported previously that intercellular Ca^{2+} waves are correlated with the level of exogenous connexin43 expression in C6 cells (6), and that reduction of [Ca^{2+}], elicits intracellular and intercellular Ca^{2+} signaling in astrocytes (26). Based upon the results described above, this effect could be explained by “unblocking” of connexin hemichannels with subsequent ATP release. Bath application of quinine evoked multifocal intercellular Ca^{2+} waves in astrocytes (Fig. 5, n = 5 coverslips). These intercellular Ca^{2+} waves occurred within 3–5 min following application of quinine and immediately ceased upon washout of quinine. Quinine also evoked Ca^{2+} oscillations and limited intercellular Ca^{2+} waves in C6 Cx43 cells but did not evoke Ca^{2+} signaling in parent C6 cells (Fig. 5, n = 5 coverslips each). The 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 units/liter apyrase, 100 μM quinine evoked a significant (>100 nM) increase in [Ca^{2+}], in an average of 14 ± 4% of C6 Cx43 cells compared with an average response of 81 ± 8% of cells after washout of apyrase (n = 320 cells in four experiments). In the presence of 10 μM PPADS, 100 μM quinine evoked a significant increase in [Ca^{2+}], in an average of 30 ± 8% of C6 Cx43 cells compared with 78 ± 6% of controls in the absence of PPADS (n = 300 cells in three experiments). As reported by others previously (5, 27), we found that both C6 cells and C6Cx43 cells showed increases in [Ca^{2+}], in response to bath application of ATP with similar sensitivity. In agreement with previous reports, we found that the ATP concentration at which 50% of cells responded was 2 μM for both C6 and C6Cx43 cells (n = 3 coverslips each).

Brief deformation of the membrane of a single cell initiates an increase in [Ca^{2+}], in the stimulated cell that is propagated in a wave-like manner to surrounding cells (18) and Fig. 6. The extent of Ca^{2+} wave propagation was significantly decreased by 50 μM FFA and 50 μM Gd3+ but not DIDS. Conversely, the extent of wave propagation was significantly increased in medium containing quinine (Fig. 6C).

DISCUSSION

Astrocytes, like multiple other cell types, are capable of widespread communication via intercellular Ca^{2+} waves. Although initial studies (1, 6) identified gap junctions as a pathway for communication of Ca^{2+} waves, more recent studies (9, 10) have demonstrated an extracellular signaling pathway involving release of ATP and activation of purinergic receptors. 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+}], is characteristic of currents through connexin hemichannels (17, 21). The low [Ca^{2+}],-activated currents we observed in astrocytes are similar to those observed through connexin hemichannels expressed in Xenopus oocytes (17). Like connexin channel currents in oocytes, the low Ca^{2+}-activated currents in astrocytes were reversibly inhibited by similar concentrations of flufenamic acid (21, 28). Although currents through connexin43 hemichannels have not been reported in 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 composed 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 (13), we found that astrocytes show uptake of 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^{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 Gd3+, both of which are potent inhibitors of connexin hemichannels. In summary, the size selectivity, activation by removal of extracellular Ca^{2+}, correlation with connexin43 ex-
pression in C6 cells, and inhibition by connexin channel inhibitors are all consistent with transmembrane dye flux via connexin hemichannels.

Because intercellular Ca\(^{2+}\) signaling induced by mechanical stimulation and other stimuli has been associated with ATP release (5, 9, 11), we examined the role of connexin hemichannels in mechanically induced ATP release. Cotrina et al. (5) reported previously 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\(^{2+}\) and quinine evoke spontaneous ATP release and potentiate mechanically induced ATP release from astrocytes and C6 glioma cells expressing connexin43, whereas inhibitors of connexin hemichannels including FFA and Gd\(^{3+}\) 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 reported previously that reduction of extracellular Ca\(^{2+}\) evokes intercellular Ca\(^{2+}\) signaling in astrocytes (26). 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 ATP release and intercellular Ca\(^{2+}\) signaling in astrocytes and C6 cells expressing connexin43, and mechanically induced intercellular Ca\(^{2+}\) waves in astrocytes 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. (5) 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 versus individual connexin hemichannels. Quinine represents an example of an agent with distinct effects on connexin hemichannel function versus gap junctional coupling; although quinine activates connexin hemichannels, it does not have a parallel effect on gap junctional conductance (23–25).

We used flufenamic acid and Gd\(^{3+}\) as inhibitors of connexin hemichannels, because both have been shown to inhibit connexin hemichannels in oocytes with high potency (21), and neither evoked increases in [Ca\(^{2+}\)]\(_i\). Flufenamic acid has also been reported to be a potent and reversible blocker of gap junctions (22). By contrast, we found that other traditional gap junction inhibitors such as octanol, glycyrrhetinic acid, or oleamide all evoked Ca\(^{2+}\) signaling that included elevations of baseline Ca\(^{2+}\), Ca\(^{2+}\) oscillations, and intercellular Ca\(^{2+}\) waves (not shown). The mechanism for the activation of Ca\(^{2+}\) signaling by these agents is not known. Regardless of the mechanism, however, these increases in [Ca\(^{2+}\)]\(_i\) confounded interpretation of their effects regarding a role for connexin channels.

Although both FFA and Gd\(^{3+}\) 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
connexion channel inhibition. In addition, we found that other Cl− channel blockers such as DIDS that do not inhibit connexion hemichannels (25, 29) did not inhibit intercellular Ca2+ signaling or ATP release. Quinine is also well known to have effects on other ion channels. However, the activation of Ca2+ signaling only in C6 cells expressing connexin43, and not in non-transfected C6 cells, is consistent with the activation of connexion hemichannels, as has been reported previously (23–25) for multiple types of connexins.

The correlation of results of electrophysiological studies, dye flux measurements, ATP release measurements, and imaging of intercellular Ca2+ signaling provides strong evidence for the hypothesis that intercellular Ca2+ waves in astrocytes can occur by ATP release through connexion hemichannels. The parallel results obtained only with C6 cells expressing connexion43, and not with non-transfected C6 cells, corroborates this hypothesis. A primary role for connexion hemichannels in intercellular Ca2+ signaling does not exclude other potential intercellular pathways, such as gap junctions. It is also possible that connexion expression is associated with up-regulation of some other pathway for ATP release. However, ATP release via connexion hemichannels is the most direct explanation for our results and provides a direct mechanism that reconciles both a role for connexins and a role for extracellular ATP in intercellular Ca2+ waves.

Astrocyte Ca2+ 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 (30–32). Haydon and colleagues (30, 31, 33) 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 Ca2+ waves. Astrocyte Ca2+ waves have also been shown to modulate the response of the retinal neurons to light stimulation (34). A role for astrocyte Ca2+ waves has also been proposed in migraine headache and the spread of seizures (2). The identification of connexion hemichannels as a pathway for stimulus-evoked release of small molecules by our studies and others (29) 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 Ca2+ waves in astrocytes and other cell types.

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