

# Vesicular ATP Is the Predominant Cause of Intercellular Calcium Waves in Astrocytes

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Brain astrocytes signal to each other and neurons. They use changes in their intracellular calcium levels to trigger release of transmitters into the extracellular space. These can then activate receptors on other nearby astrocytes and trigger a propagated calcium wave that can travel several hundred micrometers over a timescale of seconds. A role for endogenous ATP in calcium wave propagation in hippocampal astrocytes has been suggested, but the mechanisms remain incompletely understood. Here we explored how calcium waves arise and directly tested whether endogenously released ATP contributes to astrocyte calcium wave propagation in hippocampal astrocytes. We find that vesicular ATP is the major, if not the sole, determinant of astrocyte calcium wave propagation over distances between  $\sim 100$  and  $250 \mu\text{m}$ , and  $\sim 15$  s from the point of wave initiation. These actions of ATP are mediated by P2Y1 receptors. In contrast, metabotropic glutamate receptors and gap junctions do not contribute significantly to calcium wave propagation. Our data suggest that endogenous extracellular astrocytic ATP can signal over broad spatiotemporal scales.

## INTRODUCTION

Astrocytes are an integral part of the brain, where they form connections with blood vessels, other glia, and neurons (Haydon, 2001). In contrast to neurons, which exhibit electrical excitability, astrocytes display “calcium excitability” (Cornell-Bell et al., 1990), which is manifest as transient or prolonged elevations in intracellular calcium levels ( $[\text{Ca}^{2+}]_i$ ). These can be spontaneous or triggered in response to specific neurotransmitters (Cornell-Bell et al., 1990). Spontaneous intracellular calcium transients  $[\text{Ca}^{2+}]_i$  have been described in cultured astrocytes (Cornell-Bell et al., 1990; Charles et al., 1991), acute brain slices where the cellular architecture remains virtually intact (Porter and McCarthy, 1996), as well as in vivo in the cortex (Hirase et al., 2004; Tian et al., 2005, 2006; Wang et al., 2006). In acute slices and in cultured astrocytes, waves of elevated  $[\text{Ca}^{2+}]_i$  can pass between astrocytes (Charles et al., 1991; Newman and Zahs, 1997; Guthrie et al., 1999), and are often referred to as intercellular calcium waves. Several pathways have been proposed to mediate calcium waves, including diffusion of  $\text{Ca}^{2+}$  and/or inositol triphosphate through membrane gap junctions between adjoining astrocytes (Scemes et al., 2000). Additionally, it has been suggested that astrocytic release of ATP or glutamate into the extracellular space, and subsequent activation of their

respective receptors on neighboring astrocytes may also underlie calcium wave propagation (Fellin et al., 2006). However, it remains unclear if ATP, gap junctions, and glutamate contribute equally to calcium wave propagation, or if any one mechanism predominates (Charles, 1998; Scemes and Giaume, 2006).

In the present experiments we studied hippocampal cultures because astrocyte calcium transients are well described in this model system (Charles, 1998; Fields and Burnstock, 2006). To trigger astrocyte calcium waves reproducibly and on demand we used a minimal mechanical stimulus (MS), which has been used extensively in the past (Charles, 1998; Koizumi et al., 2003; Chen et al., 2005). We then experimentally explored the mechanisms underlying calcium wave propagation.

## MATERIALS AND METHODS

### $\text{Ca}^{2+}$ Imaging

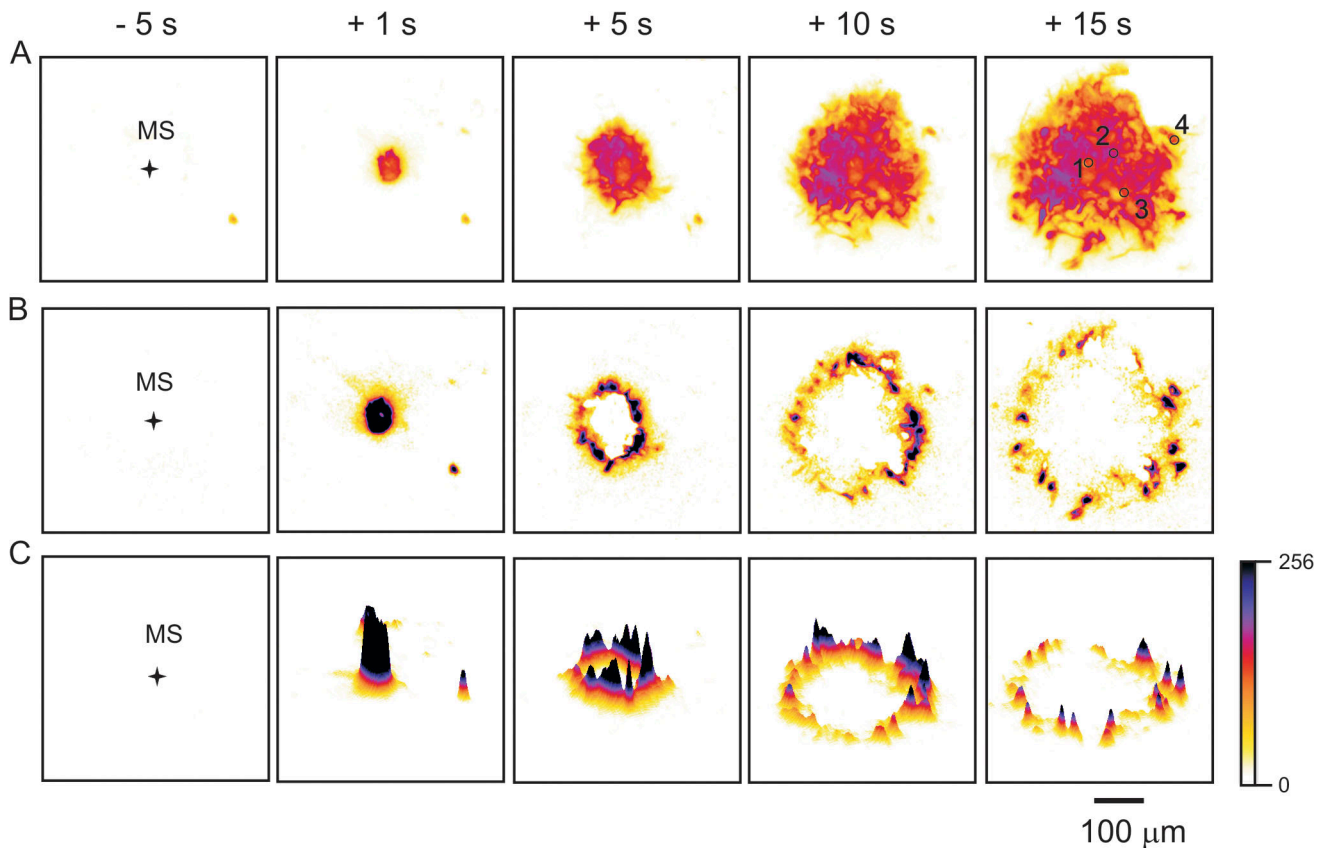
Mixed rat hippocampal neuron-astrocyte cultures were prepared and maintained in 35-mm glass bottom dishes as previously described (Granseth et al., 2006; Bowser and Khakh, 2007). Physiological saline comprised (in mM) 125 NaCl, 5 KCl, 1  $\text{MgCl}_2$ , 10 D-glucose, 2  $\text{CaCl}_2$ , 25 HEPES. Cells were loaded with  $5 \mu\text{M}$  Fluo-3/AM (Molecular Probes) as previously described (Bowser and Khakh, 2004; Bowser and Khakh, 2007). The setup used for imaging has also been described. In brief, monolayers of Fluo-3-loaded astrocytes were excited with  $490 \pm 5\text{-nm}$  light, and the emission was collected through a 510-nm-long pass filter. Images were acquired at 2 Hz. We specifically chose regions that lacked

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Abbreviations used in this paper: CBX, carbenoxolone; FFA, flufenamic acid; FWHM, full width at half maximal height; MS, mechanical stimulation.



**Figure 1.** MS evokes astrocyte calcium waves. (A) A series of images taken at the indicated times in seconds before and after MS at the point indicated by the star. (B) Wavefront images created by subtracting the previous image in the series from the current one. The result is a concentric circle of astrocyte activation that spreads outward from the site of MS. (C) 3D projection of the pixel intensity in the image series presented in B. Note the decreasing intensity of the wavefront with time indicating that the calcium levels diminish with increasing distance from the site of MS.

neuronal processes and somata to simplify the analysis and interpretation of the experiments. This can be seen in the images in Figs. 1 and 3, where only sheets of flat astrocytes were imaged. The camera gain (Princeton Instruments cooled I-PentaMAX camera; Roper Scientific) was adjusted for each astrocyte culture to provide the best signal to noise images, and so comparisons between astrocytes can only be reported as  $\Delta F/F$ . We used a well-established mechanical stimulation (MS) protocol to trigger calcium waves in astrocytes (Charles, 1998). A central astrocyte was touched from above with a fine (1–2  $\mu\text{m}$ ) blunt-ended pipette to induce a calcium response in the central initiating cell. This protocol has been used extensively in the past and produces reproducible calcium waves that spread intercellularly to neighboring astrocytes. In most experiments the bath was not perfused, for reasons that will become clear from the results. In the cases where bath perfusion was used, flow rate was  $\sim 4\text{--}5$  ml/min in the direction indicated by the arrows in the figures. For these experiments we used a custom made local perfusion device (Fisher et al., 2004).

#### Chemicals

All chemicals used were from Tocris, Sigma-Aldrich, Invitrogen, or Molecular Probes Invitrogen. The names of the chemicals are abbreviated as adenosine 5'- $\beta$ -thio-diphosphate (ADP $\beta$ S), carbenoxolone (CBX), flufenamic acid (FFA), and 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP).

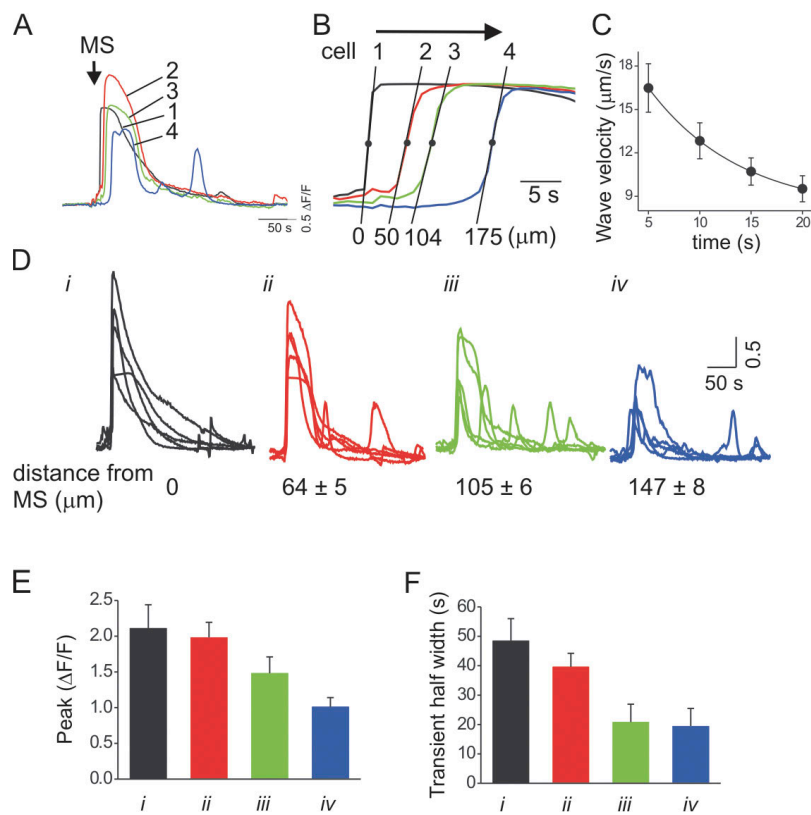
#### Analysis

Images were analyzed using Image J, Metamorph, and Corel-Draw12. Data were analyzed using Origin 6.1 (Origin Lab Corp.) and Graphpad Instat 3.06 for Windows. Data in the text and graphs are shown as mean  $\pm$  SEM from  $n$  determinations as indicated.

## RESULTS

#### Initial Observations

We loaded astrocytes with the calcium indicator dye Fluo-3 to measure and track fluctuations within their  $[\text{Ca}^{2+}]_i$  levels (Bowser and Khakh, 2004; Bowser and Khakh, 2007). We used a gentle mechanical stimulus (Chen et al., 2005) (MS; see Materials and Methods) to trigger  $[\text{Ca}^{2+}]_i$  transients in a central astrocyte, and then monitored  $[\text{Ca}^{2+}]_i$  changes in the surrounding astrocytes within a region with a diameter of  $\sim 0.5$  mm. MS always resulted in  $[\text{Ca}^{2+}]_i$  elevations in the central astrocyte ( $n = 37$ ) and this was always followed by the secondary appearance of increased  $[\text{Ca}^{2+}]_i$  levels in surrounding astrocytes ( $n = 37$ ). These secondary increases in astrocyte  $[\text{Ca}^{2+}]_i$  levels radiated out in all directions from the central astrocyte, up to



**Figure 2.** Properties of astrocyte calcium waves. (A) Calcium transients from individual astrocytes as indicated in Fig. 1 A. (B) Zoomed in calcium transients from A to emphasize the latency time differences between transients. The distances of the astrocytes from the site of MS are indicated. (C) Plot of the velocity of the wave at 5, 10, 15, and 20 s post-stimulus. Plot indicates deceleration of the wave with time. (D) Examples of calcium transients from five representative astrocytes (individual experiments) at distances (*i-iv*) from the stimulated cell as indicated in μm. (E) Calcium transient peak amplitude for astrocytes of increasing distance from the initiating cell. Each bar represents 40 individual astrocytes. (F) Full-width half-maxima of the astrocytes at distance groups indicated (*i-iv*; *n* = 40).

an average radius of  $180 \pm 12 \mu\text{m}$  ( $n = 19$ , range of  $120\text{--}235 \mu\text{m}$ ), covering an area of  $\sim 39,413 \mu\text{m}^2$ . An example of such a radiating calcium wave is presented in Fig. 1 A, before and at various time points after MS. From the images in Fig. 1 A we determined the wave front by image subtraction (Fig. 1 B) and rendered this as a 3D projection of the astrocyte fluorescence intensity (Fig. 1 C). By visual examination it was clear that the peak  $[\text{Ca}^{2+}]_i$  levels of individual astrocytes were smaller as the distance from the site of MS increased, such that by a radius of  $\sim 200 \mu\text{m}$  the change in  $[\text{Ca}^{2+}]_i$  for astrocytes was minimal. We analyzed images such as those presented in Fig. 1 to quantify key parameters of the astrocyte calcium wave, and then explored the underlying mechanisms.

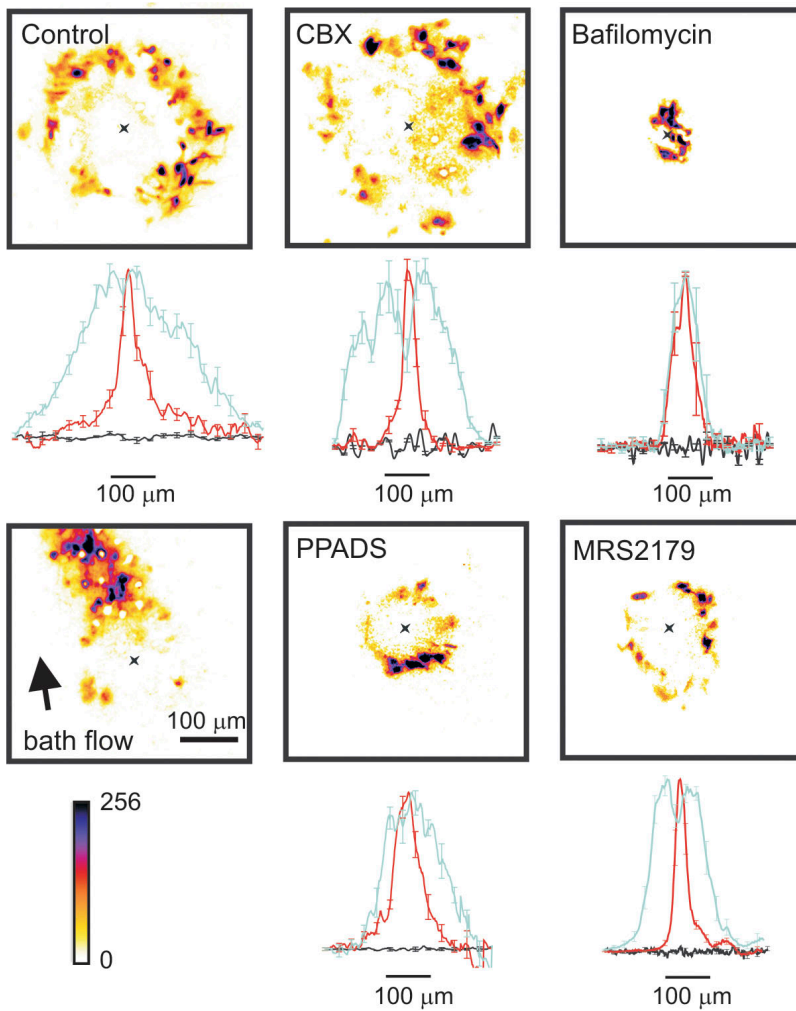
Why does the calcium wave cease after  $\sim 15$  s and a radius of  $\sim 200 \mu\text{m}$ ? To make inroads into this issue we measured the peak change in  $[\text{Ca}^{2+}]_i$  levels for astrocytes at increasing distances from the MS site (the astrocytes highlighted in Fig. 1 A are plotted in Fig. 2 A). Such traces were normalized to the peak in order to examine the latency for the calcium wave to arrive at sites of increasing distance from the site of MS (Fig. 2 B). As expected for a radiating wave, the  $[\text{Ca}^{2+}]_i$  changes were delayed for astrocytes at increasing distances from MS (Fig. 2 B). From such data we calculated the wave velocity, which slowed significantly with time after MS (Fig. 2 C). We next plotted the peak  $\Delta F/F$  for five representative astrocytes at increasing distances from MS. These data show that the peak change in  $[\text{Ca}^{2+}]_i$  levels decreases

with increasing distance from MS, a trend that was true for all experiments (Fig. 2 E;  $n = 37$ ). Finally, we measured the calcium transient half width at increasing distances from MS, and found that astrocytes further away from MS displayed significantly briefer calcium transients (Fig. 2 F;  $n = 37$ ). Overall the analysis presented in Fig. 2 suggests that after MS, the astrocyte calcium wave ceases after  $\sim 200 \mu\text{m}$  because (a) the wavefront velocity decreases, (b) the peak change in  $[\text{Ca}^{2+}]_i$  diminishes with distance, and (c) the duration of the  $[\text{Ca}^{2+}]_i$  transients in each astrocyte decreases. Presumably, for distances  $> 200 \mu\text{m}$ , the peak change in  $[\text{Ca}^{2+}]_i$  for individual astrocytes are too small and brief to allow the calcium wave to propagate.

We performed a specific set of experiments to determine if neuronal activity and action potential firing contributed to astrocyte calcium waves after MS. Thus we measured the full width at half maximal height (FWHM) of MS-evoked astrocyte calcium waves with and without TTX ( $1 \mu\text{M}$ ) to block sodium channels in neurons. We found that the FWHM of astrocyte calcium waves 15 s after MS was  $213 \pm 41 \mu\text{m}$  ( $n = 5$ ) in the control experiments, and  $220 \pm 57 \mu\text{m}$  in the presence of TTX ( $n = 6$ ). Thus we suggest that neuronal action potential firing does not contribute to astrocyte calcium waves in response to MS.

#### A Diffusible Extracellular Signal Is Responsible for the Calcium Waves

Having established a reproducible protocol to trigger calcium waves in astrocytes (Figs. 1 and 2), we next



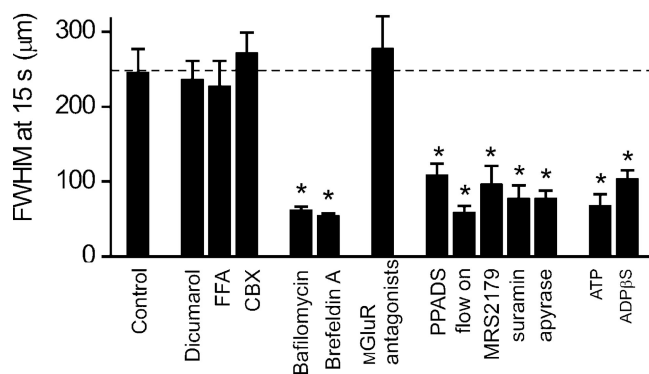
**Figure 3.** Representative subtraction images and analysis of astrocyte calcium waves. The top panels show wavefront images of astrocyte calcium waves at +15 s following MS. The bottom panels show average ( $\pm$ SEM for  $n$  experiments as indicated in the text) line profiles of calcium waves before (black traces), 1 s after (red traces), and 15 s after MS (blue traces). Note that the calcium wave spreads significantly under control conditions and when gap junctions are blocked (CBX; 100  $\mu$ M), but not when P2Y1 receptor antagonists are applied to the bathing medium (PPADS; MRS2179; 30  $\mu$ M), or when bafilomycin (2  $\mu$ M) is used to prevent vesicular release. For the line profiles we chose lines for the widest dimension and estimated the FWHM from these. This was appropriate because the calcium wave propagated in a radial manner. However, in the case of the bath flow experiments the calcium wave is not radial, because it is carried in the direction of flow, resulting in a tail of fluorescence in the direction of flow. We could not use the same method to determine the FWHM for the bath flow experiments, because this would result in an artificially extended asymmetric FWHM. To get around this we estimated the FWHM for the bath flow experiments from line profiles approximately at right angles to the direction of flow. We present the numerical analysis for these experiments in the bar graph in Fig 4. The star indicates the site of MS in each image. The horizontal scale bar indicates the distance in the X-Y dimensions in micrometers.

explored the mechanisms. In so doing we chose to compare images of  $[Ca^{2+}]_i$  levels, immediately before ( $-1$  s), and after MS ( $+1$  s and  $+15$  s; Fig. 1). To provide a measure of the spread of the calcium wave we present images at  $+15$  s after MS, and line intensity profiles across the calcium wave at  $-1$ ,  $+1$ , and  $+15$  s after MS (Fig. 3). From these line profiles we calculated the full width at half maximal height of the calcium wave (FWHM; Figs. 3 and 4) in micrometers. In the figures, the FWHM are shown as normalized to the peak of the calcium wave  $+1$  s after MS, thus there is no scale for the Y axis for the graphs in Fig. 3.

We began by determining if blockers of gap junctions affected the calcium waves. This may be expected if the wave is propagated by an intracellular mechanism, in which case blockade of gap junctions should reduce the FWHM of the calcium wave. Astrocytes were incubated for at least 30 min in FFA (50  $\mu$ M;  $n = 8$ ), CBX (100  $\mu$ M;  $n = 8$ ), or dicumarol (Dic, 10  $\mu$ M;  $n = 13$ ), before MS. The concentrations of FFA, CBX, and dicumarol were chosen on the basis of past work, where these molecules have been shown to be specific gap junction blockers (Abdelmohsen et al., 2005). We found that

neither FFA, CBX, nor dicumarol produced any significant effect on the FWHM of the calcium wave at  $+15$  s after MS (Figs. 3 and 4). These data strongly argue against an intracellular mechanism as the cause of astrocyte calcium waves. We next considered an extracellular mechanism. We hypothesized that if the calcium wave is propagated by the release of an extracellular signal, then increasing directional solution flow should carry the signal in the direction of flow, resulting in asymmetric astrocyte calcium increases following MS. To test for this we placed a local fast perfusion device within  $\sim 5$  mm of the imaging site, and flowed solution over the astrocytes at 4–5 ml/min. We verified that solution flow was directional by monitoring the flow of the neutral dye Fast Green (0.01% solution). Remarkably, MS resulted in increased intracellular astrocyte calcium levels with a tail in the direction of solution flow ( $n = 19$  from 14 fields of view). Taken together, the data with gap junction blockers (Figs. 3 and 4) and the directional flow experiments (Fig. 3) provide strong evidence that MS results in the release of a diffusible messenger into the extracellular space that can be carried by directional solution flow.





**Figure 4.** Bar graph of astrocyte calcium wave FWHM. The bars show mean  $\pm$  SEM from at least seven experiments as described in the text. The treatment groups were compared with the control using unpaired Student's *t* tests, and significance was declared at  $P < 0.05$  for the bars indicated by the asterisk.

How might the diffusible messenger be released into the extracellular space? Astrocytes undergo spontaneous and calcium-evoked vesicle exocytosis (Bowser and Khakh, 2007), and we next determined if vesicles were involved in astrocyte calcium wave propagation. To inhibit vesicular release, astrocytes were incubated in either bafilomycin A (2  $\mu$ M;  $n = 9$ ), an inhibitor of the vesicular  $H^+$ -ATPase, or brefeldin A (2  $\mu$ g/ml;  $n = 6$ ), an inhibitor of vesicular trafficking, for  $\sim 60$  min before MS (Abdipranoto et al., 2003; Coco et al., 2003). Under these conditions MS evoked an increase in astrocyte calcium in only a few astrocytes adjacent to the MS site. However, it failed to evoke a calcium wave, and the FWHM at +15 s after MS was significantly reduced (Figs. 3 and 4). These data suggest that vesicular release underlies propagation of calcium waves by a diffusible extracellular signal over a distance scale of  $>100 \mu$ m from the site of MS.

#### ATP Is Responsible for the Calcium Waves

Astrocytes express metabotropic glutamate receptors and are known to release glutamate (Pasti et al., 2001). Moreover, in our recent experiments we monitored calcium-evoked exocytosis of glutamatergic vesicles (Bowser and Khakh, 2007). Might glutamate exocytosis be responsible for astrocyte calcium wave propagation? To test for this we incubated astrocytes in a cocktail of antagonists (Bowser and Khakh, 2004) known to block metabotropic glutamate receptors (MPEP and LY367385; 100  $\mu$ M, antagonists of mGluR<sub>5</sub> and mGluR<sub>1a</sub> receptors respectively) and then examined the FWHM of calcium waves after MS (Figs. 3 and 4). Surprisingly, we found that blockers of mGluRs had no significant effect on the astrocyte calcium waves ( $n = 8$ ; Fig. 4).

A paper in 1972 explicitly proposed a role for ATP as a signaling molecule (Burnstock, 1972). Consistent with this suggestion, subsequent work has identified both metabotropic and ionotropic ATP receptors on cells,

including astrocytes (Fields and Burnstock, 2006). We next considered a role for ATP in astrocyte calcium wave propagation. We first used PPADS and suramin (10  $\mu$ M) as broad spectrum ATP receptor antagonists and found that they significantly inhibited calcium wave propagation for distances  $>100 \mu$ m from MS ( $n = 8$ ; Figs. 3 and 4). One needs to exercise caution in using PPADS to study astrocyte mechanisms (Fellin et al., 2006) because this broad spectrum antagonist blocks many ATP receptors, and may even block glutamate responses, as has been previously well noted (Humphrey et al., 1995; Khakh et al., 2001). Because astrocytes express P2Y1 receptors (Gallagher and Salter, 2003; Bowser and Khakh, 2004), we next investigated their role in calcium wave propagation; we used the selective P2Y1 receptor antagonist 2'-deoxy-N6-methyladenosine 3', 5'-bisphosphate (MRS2179) (Moro et al., 1998). In experiments where astrocytes were incubated in MRS2179 (10  $\mu$ M,  $n = 11$ ) for 30 min, MS did not evoke propagating calcium waves. This suggests that endogenously released ATP acting via P2Y1 receptors is responsible for calcium wave propagation. To explore this possibility further we incubated astrocytes with the ectonucleotidase apyrase (10 U/ml;  $n = 9$ ) to degrade endogenously released ATP (Buell et al., 1996). In astrocyte cultures treated with apyrase we measured no propagated calcium waves, and the degree of inhibition of the FWHM was identical to that with MRS2179 (Fig. 4). As a final test for the role of ATP in calcium wave propagation we incubated the cultures with ATP (10  $\mu$ M;  $n = 7$ ) or ADP $\beta$ S (10  $\mu$ M;  $n = 14$ ) to saturate/desensitize P2Y1 receptors. Under these circumstances we measured no astrocyte calcium waves in response to MS (Fig. 4).

## DISCUSSION

The main findings of the present study are that (a) MS-evoked astrocyte calcium waves are mediated by an intercellular mechanism, (b) the waves rely on a diffusible messenger released from vesicles into the extracellular space, and (c) that the messenger responsible for calcium waves is ATP acting via P2Y1 receptors.

A role for ATP in signaling between cells in the nervous system has been appreciated for over three decades (Burnstock, 1972; Fields and Burnstock, 2006). In previous work, hippocampal astrocytes displayed elevated  $[Ca^{2+}]_i$  levels in response to electrical field stimulation of afferent fibers in acute brain slices (Porter and McCarthy, 1996; Charles, 1998; Bowser and Khakh, 2004). Moreover, in our work on the stratum radiatum we suggested that astrocyte calcium waves can spread to  $\sim 200 \mu$ m from the stimulating electrode, and that ATP may be involved in this process (Bowser and Khakh, 2004). Where might this ATP come from? There is evidence for astrocyte ATP release via membrane pores such as those formed by gap junctions (Arcuino et al., 2002;

Bennett et al., 2003; Suadicani et al., 2004) and P2X<sub>7</sub> channels (Suadicani et al., 2006). Astrocytes are also known to display calcium-triggered vesicular exocytosis and express the proteins associated with transmitter release (Maienschein et al., 1999; Bezzi et al., 2004; Zhang et al., 2004; Chen et al., 2005; Bowser and Khakh, 2007), but there are opposing views about whether ATP is released by exocytosis (Queiroz et al., 1999; Coco et al., 2003). Thus although the study of astrocyte calcium waves is a mature field (Charles, 1998), the role of ATP in this process is controversial and debated (Fellin et al., 2006). From the published literature it remained unclear if calcium wave propagation in hippocampal astrocytes was due to vesicular exocytosis, if it involved ATP, or if it was entirely or only partly due to ATP (Charles, 1998; Fam et al., 2000; Fellin et al., 2006). In the present study we returned to this issue. We sought to provide a mechanistic basis for our past work (Bowser and Khakh, 2004) and to clarify the role of ATP in calcium waves. This is an important issue because brain microglia, astrocytes, and neurons all express a plethora of metabotropic and ionotropic ATP receptors (Illes and Alexandre Ribeiro, 2004; Fields and Burnstock, 2006), and thus astrocyte calcium waves mediated by ATP have the potential to affect signaling in large volumes of brain tissue (Fields and Burnstock, 2006).

It is interesting to note that we found no role for glutamate acting on metabotropic glutamate receptors in calcium wave propagation (Figs. 3 and 4). In this regard, our data for hippocampal astrocytes are similar to previous modeling and experimental studies on spinal cord astrocytes (Bennett et al., 2005, 2006). This may be portentous. Often synaptic responses in brain neurons mediated by endogenously released ATP are outweighed by those mediated by glutamate (Edwards et al., 1992; Nieber et al., 1997; Pankratov et al., 1998; Khakh, 2001; Mori et al., 2001; North, 2002; Pankratov et al., 2002, 2007). However, based on the data presented in this and previous studies, ATP appears to dominate over glutamate with respect to astrocyte signaling (Koizumi et al., 2003; Bowser and Khakh, 2004; Bennett et al., 2005; Bennett et al., 2006; Fields and Burnstock, 2006).

In summary, the data presented here for astrocyte cultures extends our previous work in acute brain slices (Bowser and Khakh, 2004) and suggests that ATP is the predominant cause of intercellular calcium waves. From this perspective, in future work it will be important to determine if astrocytic ATP contributes to calcium waves in vivo, and to determine its ability to synchronize neurons and astrocytes over extended spatiotemporal scales (Fellin et al., 2004; Wirkner et al., 2007).

We thank Dr. Aude Derevier for help with cell culture.

This work was supported by the Medical Research Council.

Angus C. Nairn served as editor.

Submitted: 9 March 2007

Accepted: 26 April 2007

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