Characteristics of calcium signaling in astrocytes induced by photostimulation with femtosecond laser

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Abstract. Astrocytes have been identified to actively contribute to brain functions through Ca^{2+} signaling, serving as a bridge to communicate with neurons and other brain cells. However, conventional stimulation techniques are hard to apply to delicate investigations on astrocytes. Our group previously reported photostimulation with a femtosecond laser to evoke astrocytic calcium (Ca^{2+}) waves, providing a noninvasive and efficient approach with highly precise targeting. In this work, detailed characteristics of astrocytic Ca^{2+} signaling induced by photostimulation are presented. In a purified astrocytic culture, after the illumination of a femtosecond laser onto one cell, a Ca^{2+} wave throughout the network with reduced speed is induced, and intracellular Ca^{2+} oscillations are observed. The intercellular propagation is pharmacologically confirmed to be mainly mediated by ATP through P_2Y receptors. Different patterns of Ca^{2+} elevations with increased amplitude in the stimulated astrocyte are discovered by varying the femtosecond laser power, which is correspondingly followed by broader intercellular waves. These indicate that the strength of photogenerated Ca^{2+} signaling in astrocytes has a positive relationship with the stimulating laser power. Therefore, distinct Ca^{2+} signaling is feasibly available for specific studies on astrocytes by employing precisely controlled photostimulation. © *2010 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.3454390]

Keywords: Ca^{2+} elevation; Ca^{2+} wave; astrocyte; femtosecond laser; photostimulation.

1 Introduction

The astrocyte is a large non-neuronal population in the brain. During the last two decades, an understanding of these cells has been promoted by numerous evidences that they participate in brain functions through Ca^{2+} signaling. They have intimate contact with neurons and the cerebrovascular system, stretching their processes onto synapses and blood vessel cells to form the so-called structure of endfeet.[1] Within these microdomains, astrocytes respond to synaptic transmitters with intracellular Ca^{2+} elevation, and in turn, release various gliotransmitters acting on the neurons and blood vessels.[2,3] While bidirectional communications between astrocytes and neurons and the bridge role in neurovascular coupling[4–6] help us to recognize the essential functions of astrocytes in brain physiology, delicate investigations have been constrained due to the lack of a noninvasive, efficient stimulation with spatial specificity and precision.

There are already several conventional ways to provoke Ca^{2+} increase in astrocytes. However, special devices are required to provide mechanical or electrical stimulus, and agents are introduced for uncaging or pharmacological applications. These methods are complex to carry out and make it hard to achieve site-specific stimulation. Comparatively, we have previously introduced a NIR femtosecond laser to precisely activate astrocytes and developed photostimulation.[7] The femtosecond laser with ultrashort pulse width and high peak intensity has been increasingly utilized for delicate manipulations on cells. Due to nonlinear effects, the interaction between femtosecond lasers and biological substances is highly localized, facilitating selective nanosurgery on subcellular structures.[8–12] This noninvasive optical tool has thus opened new doors for various artificial nanoprocessing in living cells, and even *in vivo*.[13–17]

Accordingly, for photostimulation of astrocytes,[7] an 800-nm femtosecond laser was focused onto the upper membrane of one cell. During the short (milliseconds) illuminating time, multiphoton effects including photochemistry were expected to occur, resulting in a transient photoporation on the membrane, which was verified by the fluorescence of FM 1-43, a membrane-biding dye, and entry of a membrane-impermeable dye PI. The Ca^{2+} ion then flew over into the cytoplasm through photoporation due to the much higher extracellular concentration. This intracellular signal would soon be magnified by the process of Ca^{2+}-induced Ca^{2+} release (CICR), leading to remarkable Ca^{2+} elevation and intercellu-

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lar Ca^{2+} wave. We have also shown that photogenerated astrocytic Ca^{2+} signaling could be reproduced by repetitive stimulation. With the highly localized nanodissection of the femtosecond laser, noncontact photostimulation was verified to be compatible with cell viability.

As photostimulation of astrocytes provides a noncontact, nondisruptive, and reproducible way for high targeting precision to activate astrocytes, the purpose of this work is to further characterize photogenerated astrocytic Ca^{2+} signaling.

2 Materials and Methods

2.1 Cell Cultures

Preparation of purified astrocytes was described before.[7] Briefly, cells were extracted from the cortex of postnatal 1- to 3-day-old Wistar rats, and cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum.

2.2 Solution and Drugs

The experimental solution, termed Hepes-buffered solution (HBS), contained (mM): 145 NaCl, 3 KCl, 10 HEPES, 3 $CaCl_2 \cdot 2H_2O$, 2 $MgCl_2 \cdot 6H_2O$, and 8 glucose.

Carbenoxolone (100 μM), octanol (500 μM), suramin (100 μM), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (30 μM), and Reactive Blue 2 (RB-2) (2 μM) were all diluted in HBS.

2.3 Calcium Imaging and Photostimulation

Before the experiments, cells were loaded with a Ca^{2+}-sensitive dye Fluo-3/AM, diluted in HBS, to reach a final concentration of 4 μM, at 37 °C for 30 min. The confocal laser scanning system, FluoView1000 (Olympus, Japan), for Ca^{2+} imaging and photostimulation has been described in detail.[7] In brief, fluorescence of Fluo-3 was relayed into a photomultiplier tube (PMT) before and after photostimulation with a femtosecond laser, which was generated by a Ti:sapphire laser (Mai Tai, Spectra-Physics, Newport Corporation, Santa Clara, California). Generally, the femtosecond laser beam (800 nm, ∼90 fs, 80 MHz) was exposed onto the cell membrane for a duration of 1 to 4 ms and with a laser power of 15 to 60 mW after a $20\times$ (NA 0.75) objective. Images were acquired at ∼1 frame/s by FluoView 1.5 software (Olympus) on a PC. Normalized fluorescence ($\Delta F/F$) inside the cell was calculated as the Ca^{2+} increase in Image-Pro Plus 5.0 (Media Cybernetics, Bethesda, Maryland).

3 Results

3.1 Intercellular Propagation and Intracellular Oscillations in Astrocytes

In the astrocyte network, the femtosecond laser was targeted to one cell. After illumination for 2 ms, intracellular Ca^{2+} elevations indicated by increased Fluo-3 fluorescence was observed in the cells successively away from the stimulating point, forming a radial intercellular propagation as called a Ca^{2+} wave [Fig. 1(a)]. This wave would gradually slow down along with the spreading time [Fig. 1(c)], and finally fade away, going more than 200 μm in radius. Normalized fluorescence in some cells [indicated by the circles in Fig. 1(a)]
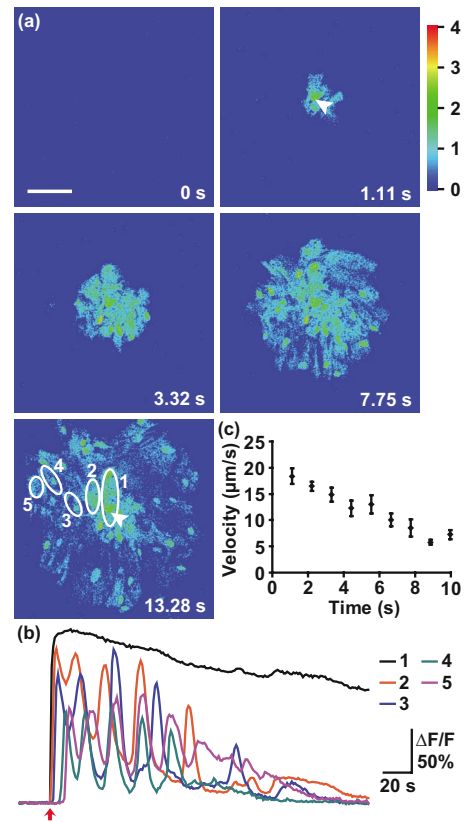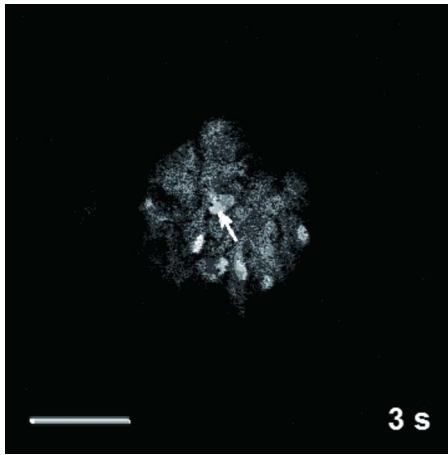

Fig. 1 Intercellular Ca^{2+} wave and intracellular Ca^{2+} oscillations in astrocytes induced by photostimulation. (a) Propagation of Ca^{2+} elevations after photostimulation of an astrocyte, indicated by arrowheads. The color bar represents pseudocolor of normalized fluorescence ($\Delta F/F$). Scale bar, 100 μm. (b) Time courses of fluorescence change in the five cells marked by the circles in (a). The arrow indicates the onset of photostimulation. (c) Velocity (mean±SEM, $n=10$) of the Ca^{2+} wave against spreading time. (Color online only.)

including the stimulated one (cell 1, marked by an arrowhead) and some others (cells 2 through 5) were calculated, as presented in Fig. 1(b). The changes in cells 2 through 5 all showed several more peaks after the initial Ca^{2+} increase. These Ca^{2+} oscillations were actually often ($n=28/46$) observed in most cells involved in the spreading wave, lasting for 142.8 ± 14.1 s, but rarely ($n=7/46$) in the stimulated one (see also Video 1).

3.2 Mechanism of Photogenerated Calcium Wave

Two main mediators in the astrocytic Ca^{2+} wave have previously been confirmed: intracellular diffusion of inositol trisphosphate (IP_3) through gap junctions, and release of extracellular messenger ATP.[5,18,19] We next pharmacologically testified the underlying mechanisms of photogenerated intercellular Ca^{2+} waves in astrocytes.

As shown in Fig. 2, carbenoxolone (carben) and octanol, both blockers of gap junctions, had modest effects ($p > 0.05$) on the spreading. Comparatively, the propagation was dramatically reduced ($81.3 \pm 2.9\%$, $p < 0.001$) by suramin, a nonselective antagonist of purinergic receptors (P_2Rs). This depression indicated the involvement of ATP, which is known to activate both ionotropic receptors (P_2XRs)

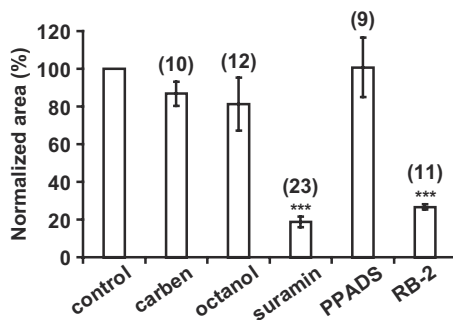**Video 1** Intercellular Ca²⁺ wave and intracellular Ca²⁺ oscillations in astrocytes induced by photostimulation. The femtosecond laser target is indicated by a white arrow. Scale bar, 100 μm (MPEG, 5.0 MB). [URL: http://dx.doi.org/10.1117/1.3454390.1].

Fig. 3 Potentiated astrocytic Ca²⁺ signaling by repetitive photostimulation with increased femtosecond laser power: 18, 21, and 24 mW. (a) Three different Ca²⁺ elevation patterns in the stimulated cell (from top to bottom): LS-, S-, and H-type. (b) Widened Ca²⁺ waves following the corresponding patterns of Ca²⁺ elevations in (a). Scale bar, 100 μm.

and metabotropic receptors (P_2YRs). The contribution of these two types of receptors in the wave was then examined by application of their antagonists, PPADS and RB-2, respectively. The results showed that the Ca²⁺ wave continued normally ($100.8 \pm 15.9\%$) in the presence of PPADS, but diminished greatly ($73.4 \pm 1.4\%$, $p < 0.001$) in RB-2. Therefore, astrocytic Ca²⁺ waves induced by a femtosecond laser were suggested to be predominantly mediated by ATP through the activation of P_2YRs.
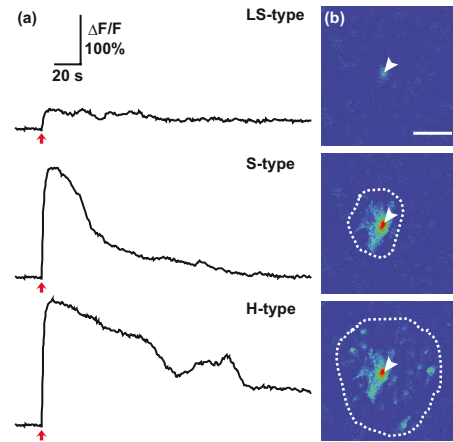
3.3 *Femtosecond Laser Intensity-Dependent Astrocytic Calcium Signaling*

Actually, photogenerated Ca²⁺ responses in the astrocytic network were related to the femtosecond laser intensity. Different patterns of Ca²⁺ elevation in the stimulated cell were discovered by varying the femtosecond laser power, and correspondingly affecting the consequent Ca²⁺ wave.

Repetitive photostimulations with increasing laser power on an astrocyte were carried out, if only intracellular Ca²⁺ concentration in the field of view turned to basal level. Several trials ($n = 16$) showed that the Ca²⁺ response patterns in the stimulated cell were classified into three groups, as illus-

trated in Fig. 3(a), in which the femtosecond laser power was 18, 21, and 24 mW, respectively: 1. LS-type, with a modest Ca²⁺ elevation (amplitude=$49.4\% \pm 7.6\%$); 2. S-type, with a significant Ca²⁺ spike (amplitude=$224.8\% \pm 35.9\%$) and rapid recovery; and 3 H-type, also with a remarkable Ca²⁺ elevation (amplitude=$250.2\% \pm 41.9\%$), but a slow decaying phase, which could not fully recover within 4 min. The statistical result [Fig. 4(a)] showed increased normalized amplitude of Ca²⁺ elevations for these three patterns as to the one for H-type. For S- and H-types, the duration of Ca²⁺ elevations was 81.8 ± 10.0 s and 163.6 ± 24.6 s, respectively, which was calculated as the time between photostimulation and when $\Delta F/F$ was decreased to 1/e of the peak value.

Moreover, following the responses of the three patterns in the stimulated astrocyte, the intercellular Ca²⁺ wave covered distinct ranges, respectively [Figs. 3(b) and 4(b)]. While there was little propagation elicited by the LS-type response, the S-type evoked a wave just spreading to the neighborhood (70.6 ± 27.1 μm in radius), and the wave area following H-type was dominantly the largest, reaching 231.7 ± 126.9 μm in radius.

In brief, photogenerated Ca²⁺ signaling in astrocytes, both the Ca²⁺ elevation in the stimulated cell and the spreading

Fig. 2 Pharmacological effects on photogenerated Ca²⁺ wave (carben: carbenoxolone). Data were presented as mean±SEM. Bracketed numbers indicate the numbers of experiments for each drug. Student's t-test: ***, $p < 0.001$.
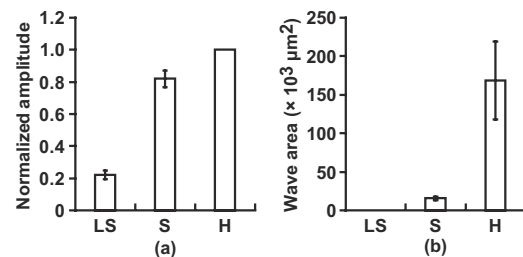

Fig. 4 Statistical results ($n = 16$) of astrocytic responses under the LS-, S-, and H-type of Ca²⁺ elevations in the stimulated cell. (a) Normalized amplitude to the one for H-type. (b) Ca²⁺ wave area.

across others, was demonstrated to have a positive relationship with the femtosecond laser intensity.

4 Discussions and Conclusion

Since photostimulation with a NIR femtosecond laser has previously been proposed to be a novel effective way to evoke Ca^{2+} signaling in astrocytes, here, the characteristics of photogenerated Ca^{2+} signaling were revealed in detail.

After the femtosecond laser was targeted onto one astrocyte, intracellular Ca^{2+} elevation was immediately elicited and then succeeded in other cells, forming a concentric propagation [Fig. 1(a)]. Furthermore, Ca^{2+} oscillations in nonstimulated cells were observed in 60.9% of the waves ($n=46$), but less likely (15.2%) in the stimulated one [Fig. 1(b) and Video 1]. Regarding this, it was interesting to discover that in HeLa cells irradiated by a femtosecond laser, hyperpolarization following the initial depolarization of membrane potential was observed.[20] As astrocytes can have varying resting membrane potentials,[21] hyperpolarization might also be involved in this work, resulting in the depression of further Ca^{2+} excitability in the stimulated astrocyte, which requires further validation. This difference in Ca^{2+} oscillations in the stimulated and nonstimulated astrocytes has also been reported in a study employing mechanical stimulation and suggesting the distinct causes of intracellular and intercellular signaling.[22] We have previously demonstrated that the photogenerated Ca^{2+} elevation in the stimulated cell resulted from photoporation on the membrane, which allowed extracellular Ca^{2+} influx and triggered release of intracellular Ca^{2+} stores.[7] Hence, the mechanism of intercellular propagation should be figured out.

Much evidence has proven the contribution of both gap junction and ATP to astrocytic Ca^{2+} waves.[5,18,19] In our study, suramin and RB-2 both significantly depressed the photogenerated intercellular propagation in astrocytes, proving that ATP be mainly involved through the activation of P_2YRs (Fig. 2). Note that the spreading was not fully blocked by the two ATP receptor antagonists (suramin, 81.3%; RB-2, 73.4%). This incomplete inhibition has also been observed in another study performing photostimulation on HeLa cells,[23] and other papers employing conventional stimulations on astrocytes.[18,24,25] We thus considered this was related to the competitive property of these drugs, but not an artifact attributable to photostimulation on astrocytes. Additionally, the gap junction blockers also affected the Ca^{2+} wave, though slightly, indicating that gap junction might partially play a role. Actually, several studies found that the contribution of gap junction in intercellular propagation might be facilitating ATP release, and connexin-deficient cells released 5- to 15-fold more ATP after expressing connexin proteins.[24,25] It was even reported that ATP released from gap junction channels in astrocytes should serve as one of the intercellular signaling pathways.[26] Taken together, our results suggested that the photogenerated astrocytic Ca^{2+} wave was predominantly mediated by the extracellular messenger ATP, in partial collaboration with gap junctions.

Photogenerated Ca^{2+} signaling, including Ca^{2+} elevation in the stimulated astrocyte and Ca^{2+} wave, was shown to be potentiated with increased femtosecond laser power (Figs. 3 and 4). Three patterns of intracellular Ca^{2+} elevations with increased amplitude in the stimulated cell were defined, which corresponded to distinct extents of intercellular waves. With regard to the former, it was suggested that a femtosecond laser with greater power should result in larger or longer poration on the membrane, leading to more Ca^{2+} influx and release from stores. As a result, the total fluorescence increase inside the cell would be stronger. However, the fluorescence change in the stimulated cell would finally reach the maximum and could not be enhanced any more, even if the laser power was further increased (data not shown). This was probably because of the saturation of intracellular Ca^{2+} capacity or Ca^{2+} binding capacity of Fluo-3. And in this condition, the Ca^{2+} response in the stimulated cell was biphasic with an initial increase and a long-lasting plateau (data not shown). This sustained high level of intracellular Ca^{2+} concentration could hardly recover to baseline in the end, which might indicate that the cell had suffered from laser-induced injury. Indeed, if the femtosecond laser intensity was even higher, cell bubbling and cell death would be induced,[7] which, as well as the long-lasting Ca^{2+} plateau, should be avoided in our experiments.

On the other hand, it is known that an important regulated response to Ca^{2+} signaling in astrocytes is Ca^{2+}-dependent release of transmitters, including ATP.[27–29] It was found that there was a threshold of intracellular Ca^{2+} concentration for ATP release from the astrocytes.[30] Therefore, it was suggested that LS-type was not able to induce an intercellular wave due to its inefficiency in triggering ATP release from the stimulated astrocyte. And comparatively, during the high intracellular Ca^{2+} level for S- and H-types, abundant ATP was released from the stimulated cell to act on others, leading to a Ca^{2+} wave. As ATP release in the intercellular signaling of astrocytes was reported to be restricted to a point source, i.e., in a nonregenerative model,[31] the propagation was dependent on the diffusion of ATP derived from the stimulated cell. Thus, the spreading would eventually vanish until there was little ATP to act on distant cells. Notably, with larger femtosecond laser power, broader intercellular propagation was supposed to be related to more and longer ATP release from the stimulated cell, in which the intracellular Ca^{2+} elevation level was enhanced (Figs. 3 and 4). Accordingly, these results suggested that the quantity of ATP release from astrocytes was dependent on the intracellular Ca^{2+} level.

As before, physiological activities of astrocytes and the range of recruited cells could be well controlled by varying the femtosecond laser intensity. For instance, low stimulating laser power is only recommended to provoke Ca^{2+} elevation of LS-type when signaling in a single astrocyte is explored; studies on communications among neighboring astrocytes require limited Ca^{2+} waves; and while the function of the astrocytic network is exploited, large femtosecond laser power is demanded to evoke widespread signaling. Therefore, as photostimulation is efficient to activate astrocytes with high feasibility and target precision, the finely controlled femtosecond laser could provide Ca^{2+} signaling in an intensity-dependent manner for various specific studies on astrocytes.

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