Astrocyte Ca^{2+} waves trigger responses in microglial cells in brain slices

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ABSTRACT

Pathologic impacts in the brain lead to a widespread activation of microglial cells far beyond the site of injury. Here, we demonstrate that glial Ca^{2+} waves can trigger responses in microglial cells. We elicited Ca^{2+} waves in corpus callosum glial cells by electrical stimulation or local adenosine triphosphate (ATP) ejection in acute brain slices. Macroglial cells, but not microglia, were bulk-loaded with Ca^{2+}-sensitive dyes. Using a transgenic animal in which astrocytes were labeled by the enhanced green fluorescence protein (EGFP) allowed us to identify the reacting cell populations: the wave activated a Ca^{2+} response in both astrocytes and non-astrocytic glial cells and spread over hundreds of micrometers even into the adjacent cortical and ventricular cell layers. Regenerative ATP release and subsequent activation of metabotropic purinergic receptors caused the propagation of the glial Ca^{2+} wave: the wave was blocked by the purinergic receptor antagonist Reactive Blue 2 and was not affected by the gap junction blocker octanol, but enhanced in Ca^{2+} free saline. To test whether microglial cells respond to the wave, microglial cells were labeled with a dye-coupled lectin and membrane currents were recorded with the patch-clamp technique. When the wave passed by, a current with the characteristics of a purinergic response was activated. Thus, Ca^{2+} waves *in situ* are not restricted to astrocytic cells, but broadly activate different glial cell types.

Key words: mouse • astrocyte • ATP-release • calcium-wave • microglia • brain slice

For a long time, glial cells in general have been considered passive elements in the brain but are now regarded as elements that sense and respond to neuronal activity (1). In particular, astrocytes express a large variety of transmitter and hormone receptors as sensors for synaptic activity and, furthermore, can communicate over long distances, namely via waves of intracellular Ca^{2+} elevation (2, 3). These Ca^{2+} waves travel within the astrocytic cell populations and have been characterized in primary cultures, slice cultures, and in the isolated retina (2, 4–6). Two different mechanisms have been described to explain how the Ca^{2+}-wave propagates within an astrocytic network in culture: A) the diffusion of second messengers via gap junctions between highly coupled astrocytes with subsequent Ca^{2+}-release from intracellular stores (6) or B) the release of the transmitter adenosine triphosphate (ATP) from astrocytes into the

extracellular space followed by purinergic receptor activation on neighboring cells, which, in turn, leads to elevation of internal calcium (7, 8). Few experiments have been carried out so far on astrocytic Ca^{2+} wave propagations in living, acutely isolated brain tissue. Newman and Zahs (9) established that an astrocytic Ca^{2+} wave can propagate within the intact retina, and they showed that the wave recruits Müller cells, a radial glial cell with some astrocyte features. In this preparation, the wave spreads via ATP release and concomitant purinergic receptor activation (4). The mechanisms leading to ATP release are yet unknown. ATP release seems to be Ca^{2+} independent (7), whereas the release of glutamate depends on increased cytosolic Ca^{2+} levels (10, 11). Toxins that interfere with the SNARE-complex block the glutamate release, a finding that suggests a vesicular release mechanism comparable with synaptic exocytosis (12). Consequently, intracellular calcium changes in astrocytes can influence neuronal activity (13–15).

Although astrocytic calcium waves are discussed mostly as a mean by which astrocytes influence neuronal signal transmission, they would also meet the requirements for a mechanism signaling pathological events in the brain over large distances. There is evidence that glial cells can sense pathologic events, which have occurred hundreds of micrometers away. After brain injury, microglial cells are activated several millimeters away from the lesion site (16, 17). Microglial cells are the major immunocompetent element of the central nervous system (CNS) and react with a complex and graded response to any type of CNS injury (18). The initial signal that triggers microglial activation is still unknown. Cultured cells show an activated phenotype after challenge with various pathology-related substances, such as bacterial cell wall components (19) and complement fragments (20), but also after incubation with ATP (21). Thus, ATP is a candidate for signaling injury to microglia, because these cells, at least in culture, express several subtypes of purinergic receptors (22). Stimulation of microglial purinergic receptors in culture leads to the release of tumor necrosis factor α (TNF-α) and modulation of interleukin (IL)-1beta release (21, 23).

In the present study, we have found conditions to trigger Ca^{2+} waves in acute slices of brain tissue, and we have shown that the wave, carried by sequential ATP-release, leads to a global response in glia, including microglial cells.

MATERIALS AND METHODS

Animals, preparation of brain slices, and calcium recordings

For slice preparation, 5- to 8-day old mice (either glial fibrillary acidic protein (GFAP)/EGFP transgenic FVB/N mice (17) or nontransgenic NMRI mice) were decapitated and their brains were removed. Frontal slices of 250–300 μm thickness were cut using a vibratome (Vibracut, FTB Feinwerktechnik, Bensheim, Germany) in ice-cold bicarbonate buffer. Slices were stored at room temperature in gassed buffer for at least 20 min before staining.

Slices were incubated with the Ca^{2+} indicator dyes Fluo-4-acetoxymethylester (10 μM Fluo-4-AM, Molecular Probes, Eugene, OR) or Calcium Orange-acetoxymethylester (10 μM Calcium Orange-AM, Molecular Probes) in bicarbonate buffer at 37°C and 5% CO_2 for 40 to 60 min or at room temperature for 90–120 min. Slices were transferred to a perfusion chamber on an upright microscope (Axioskop FS, Zeiss, Oberkochen, Germany) and fixed in the chamber by using a U-shaped platinum wire with a grid of nylon threads. Slices were superfused with gassed

bicarbonate buffer at a flow rate of 4 to 6 ml/min. Substances were applied by changing the perfusate. Intracellular Ca^{2+} changes were detected by using either a confocal laser scanning microscope (Sarastro 2000, Molecular Dynamics, Sunnyvale, CA) or a cooled CCD camera (SensiCam, PCO, Kelheim, Germany). We used the 488 nm band of an Ar+/Kr+-laser or a monochromator set to 488 nm (Till Photonics, München, Germany), respectively, as a light source for fluorophore excitation. Due to the scanning rate of the confocal microscope, the sampling rate was limited to 0.25 Hz, whereas with the CCD-camera 1 Hz was achieved. Images were stored on a PC and processed with conventional software (ImagePro, Media Cybernetics, Silver Spring, MD. For better illustrating the moving front of a Ca^{2+} wave, we subtracted from each image the pixel values of the previous image. This procedure results in a series of images, which highlights only those regions, that have changed from one image to the next and thus shows the newly responding cells. Electrical stimulation was accomplished with a conventional glass electrode filled with bath solution. The pipettes had a resistance of ~1 Mohm, which corresponds to a tip opening of ~15 μm. The tip of the pipette was placed on top of the slice, with the pipette only gently touching the upper cell layer. After the positioning of the pipette the slice was allowed to recover from mechanical stress for at least 2 min. Chemical stimulation was performed by ejecting ATP-containing solution from a micropipette with a tip opening of ~5 μm. Before the induction of the wave, the slice was superfused with Ca^{2+} free solution for 2 min. Then, the bath perfusion was stopped. The wave was elicited by applying 10 Hz stimulation for 4 s. This stimulation paradigm was chosen as it induced solid responses. In some cases, however, shorter stimulation—even only a few pulses—was also effective in eliciting a wave. The Ca^{2+} wave could be triggered repetitively within the same area. Time between stimulations was at least 5 min, and slices were superfused with Ca^{2+} containing solution during recovery times.

Electrophysiological recordings

We used the patch-clamp technique in the whole cell configuration for recording membrane currents. Electrodes with a resistance of 6–8 MOhm were pulled and filled with pipette solution. Because cell somata were located approximately 10–30 μm underneath the slice surface, we applied pressure to the pipette while it was advanced towards the cell. Uncompensated currents were recorded with conventional electronics (EPC-7 or EPC9-amplifier, HEKA electronics, Lambrecht/Pfalz, Germany).

To identify microglial cells, slices were stained with Texas Red-coupled lectin from *lycopersicon esculentum* (tomato lectin, Sigma, Deisenhofen, Germany). The lectin was added to the Fluo-4-AM containing bicarbonate buffer. Tomato lectin labeled the microglial cells while the slices were loaded with the Ca^{2+}-sensor for 45 min at 37°C in carbogen atmosphere (5% CO_2, 95% O_2).

Solutions and electrodes

The standard bath solution contained 134 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM K_2HPO_4, 10 mM glucose, and 2 mg/l phenol red as pH indicator. By gassing the solutions with carbogen, we could then adjust the pH to 7.4–7.5. The pipette solution for patch clamp recordings contained 130 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM Na-ATP, 5 mM ethylene glycol-bis(ß-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM N-(2-hydroxyethyl) piperazine-2'-(2-ethanesulphonic acid) (HEPES). Ca^{2+} activity of the pipette solution was ~11 nM. All experiments were carried out at room temperature. Recording and

stimulation pipettes were fabricated from borosilicate capillaries (Hilgenberg, Malsfeld, Germany). Drugs were applied to the bath solution in the following concentrations (µM): Reactive Blue 2 (30), Suramin (100), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (50), octanol (500), Cd^{2+} (100), Tetrodotoxin (TTX) (1), ((+)-alpha-methyl-4-carboxy-phenylglycine) (MCPG) (50), thapsigargin (20).

RESULTS

Ca^{2+} waves can be triggered in acute brain slices

To record Ca^{2+} signals from glial cells, we incubated brain slices of young (P5 – P8) mice with the Ca^{2+}-sensitive dye Fluo-4-AM (10 µM). To select for glial cells, we focused on the corpus callosum, because this structure, as a white matter tract, contains no neuronal cell bodies and thus all Fluo-4 labeled somata were glial. To elicit a Ca^{2+} wave, we placed a pipette with a tip diameter of about 15 µm onto the surface of the slice and stimulated it electrically (4 sec. at 10 Hz). Already during the stimulation period, we recorded a Ca^{2+} increase in cells close (10–20 µm) to the stimulation pipette. With a delay, cells in increasing distance from the pipette responded and this event was reminiscent of a spreading wave. Measuring the onset of the Ca^{2+} response with respect to the distance of the cell indicated the speed by which the wave traveled, namely at 13.9 +/– 1.8 µm/s (n=26). In some cases, the wave spread slightly more rapidly in the orientation of the axonal tracts. The Ca^{2+} signal propagated to the border of our observation area, which indicated that the wave extended over a distance of at least half a millimeter. The Ca^{2+} response of a given cell lasted for about 1 min, except for cells located in the close vicinity of the stimulation pipette in which the Ca^{2+} increase lasted for about 2 min. The wave could be evoked up to four times within the same region, if the slice was allowed to recover for about 5 min between stimulations. Local ATP ejection from a patch pipette also evoked a Ca^{2+} wave with similar features (data not shown). Cellular depolarization by extracellular application of buffer with high potassium concentrations was not sufficient to elicit a wave: local ejection of elevated $[K^+]$ (100 mM) from a patch pipette induced only a transient Ca^{2+} elevation restricted to the site of application (data not shown).

The phenomenon of the spreading Ca^{2+} wave was not confined to the corpus callosum: cells in the adjacent cortical layers also responded with an increase in Ca^{2+}. The wave spread with a similar velocity within the cortical layers compared with the corpus callosum (Fig. 1).

Intracellular calcium stores give rise to enhanced cytosolic Ca^{2+} levels

To test for the source of Ca^{2+}, we compared responses in Ca^{2+} containing and Ca^{2+} free solution. The wave spread even further and recruited more cells in Ca^{2+} free solution than in normal buffer, which suggests that Ca^{2+} influx from the extracellular space does not cause the cytosolic Ca^{2+} transient. We therefore performed most of our experiments in nominally Ca^{2+} free solution. Complete depletion of internal Ca^{2+} stores abolished the Ca^{2+} wave as could be shown by an experimental paradigm, which leads to a depletion of Ca^{2+} stores (24) in cultured astrocytes, namely the application of ATP in the presence of thapsigargin, a blocker of Ca^{2+} uptake into the endoplasmic stores. The first application of ATP triggers an intracellular Ca^{2+} increase, but the refilling of stores is prevented by thapsigargin, so that a second application no longer leads to a significant Ca^{2+} increase. After eliciting a Ca^{2+} wave, we applied this depletion protocol.

Subsequently, still in the presence of thapsigargin, electrical stimulation did not elicit a Ca^{2+} wave, but the signal was restricted to the close proximity of the stimulation pipette (n=3) (Fig. 2).

Different cell types react with a Ca^{2+} signal during wave propagation

To identify the cells that participate in the wave, we made use of a transgenic animal in which astrocytes are labeled by the enhanced green fluorescent protein (EGFP) (17). We have used the red-shifted fluorescent Ca^{2+} sensor Calcium Orange, which allowed for imaging of EGFP fluorescence and intracellular Ca^{2+} simultaneously. As shown in Figure 3A, the wave propagated both in EGFP positive as well as in EGFP negative cells, which indicated that both astrocytes and non-astrocytic cells participate in the wave. To characterize the responding cells further, we studied the membrane current pattern with the patch clamp technique after we established that the cells were part of the Ca^{2+} wave. We recorded two different current patterns in response to voltage steps in de- and hyperpolarizing direction (Fig. 3B): cells with a linear current-voltage curve exhibited passive membrane currents with no sign of voltage activation and without the typical tail currents of the oligodendrocytes: this is the current pattern typical for astrocytes (25). A second population of cells was characterized by the lack of inward rectifyer currents, a prominent delayed rectifying outward current and small sodium inward currents. This current pattern is characteristic for glial precursor cells (26). Thus, the wave propagates within astrocytes and glial precursor cells. As can be seen in Figure 1, the wave spreads from the corpus callosum also to the ventricular wall, most likely to ependymal cells. These cells also display a strong Ca^{2+} signal.

ATP released into the extracellular space is the carrier of the Ca^{2+} wave

The spread of the Ca^{2+} wave was influenced strongly by the bath perfusion: as can be seen in Figure 4, the wave travels radial from the stimulation pipette if the bath perfusion is stopped. With a rapid perfusion, as used in most physiological recording configurations, the Ca^{2+} increase was restricted to cells close to the pipette. A slow flow of buffer led to wave propagation predominantly in the direction of the bath flow, indicating that a diffusible substance mediates the wave (Fig. 4A). In cultured astrocytes, Ca^{2+} signals spread either due to propagation via gap junctions or through release of glutamate or ATP and consecutive activation of glutamatergic or purinergic receptors (2, 6, 27). To test for the involvement of purinergic receptors, we compared the propagation of the Ca^{2+} wave in the presence and absence of Reactive Blue 2, an antagonist for purinergic receptors. When slices were incubated for 2 min with Reactive Blue 2 (30 µM), stimulation resulted only in a local Ca^{2+} increase in the close vicinity of the stimulation pipette (n=5) compared with the control (Fig. 4B). A similar result was obtained with suramin (100 µM) (n=5), another antagonist of purinergic receptors (data no shown). To exclude the involvement of glutamatergic receptor activation, we carried out stimulations in the presence of 50 µM MCPG (n=6) and 50 µM CNQX (n=4) in different sets of experiments. The presences of these blockers did not alter the wave (data not shown). In addition, we performed experiments in the presence of 100 µM glutamate to desensitize all glutamate receptors. Even in the presence of glutamate in the bath, a wave could be elicited (data not shown) (n=3).

To exclude that electrical activity of axons is involved in the propagation of the wave, we incubated slices with 1 µM TTX and 100 µM Cd^{2+}, thus blocking the generation of action

potentials and synaptic release, respectively (n=4). The Ca^{2+} wave was not affected, indicating that the wave spread independent of neuronal activity (Fig. 5A). To test for wave propagation via gap junctions, slices were incubated for 2 min with the gap junction blocker octanol (500 µM) (n=3). In acute retina preparations, octanol has been shown to block gap junctional coupling between astrocytes (28). Wave propagation was not affected by that treatment (Fig. 5B).

The Ca^{2+} wave triggers a response in microglia

Because microglial cells do not accumulate the calcium dyes, we analyzed the potential participation of microglial in the Ca^{2+} wave with electrophysiological methods. The cells were identified by specific staining with tomato lectin (lectin from *lycopersicon esculentum*, coupled to Texas Red) (29), which does not interfere with the physiological properties of microglial cells (30). Double labeling with a Ca^{2+} sensor and tomato lectin confirmed that microglia did not take up the Ca^{2+} dye. We approached the tomato lectin-labeled cells with the patch pipette and recorded membrane currents while eliciting a Ca^{2+} wave by electrical stimulation 50-100 µm away from the patch pipette. As shown in Figure 6, microglia showed the transient induction of an outwardly rectifying current when the Ca^{2+} wave passed the cell. Of the 13 microglia investigated, 5 responded with this type of current to a passing wave. The induced current corresponds to currents observed in cultured microglia after stimulation with ATP (22). This finding suggests that microglial cells can sense the activation of other glial cells and participate in the global glial activity represented by the Ca^{2+} wave.

DISCUSSION

Glial cells lack the ability to generate action potentials and thus cannot communicate via propagating electrical activity. Yet the astrocytic form for transferring information within their cellular network is mediated by Ca^{2+} signaling. Ca^{2+} waves that have been described in astrocyte cultures and mechanisms underlying this form of glial cell communication are due to either propagation via gap junctions (5) or ATP release/activation of purinergic receptors (31). Waves of Ca^{2+} through astrocytic networks have been found in slice cultures (32) and in the isolated retina (4).

Our experiments demonstrate that such waves can be activated in brain tissue and can spread over hundreds of micrometers from their site of origin. This response is not restricted to astrocytes, but it spreads to microglia and glial progenitor cells, as identified by their current patterns in response to de- and hyperpolarizing voltage jumps (26, 30), and can thus be viewed as a global glial wave. Our data indicate that, as in the retina, the propagation of the Ca^{2+} wave is mediated by release of ATP and subsequent activation of purinergic receptors and involves Ca^{2+} release from cytoplasmic stores. Unlike in culture, gap junctions are not an essential prerequisite for the wave to propagate in our experiments. Expression of connexin 43, a gap junction protein subunit, in microglia has been shown four days after a stab wound lesion (33). In acute slices, however, we have not observed any dye-coupling between microglial or between microglia and any other cell type (30). Thus, gap junctional coupling is not a possible mechanism to signal to and from microglia in the unlesioned brain.

We assume that there is a propagation of ATP release; that is, that ATP is released from one cell and triggers the release of ATP from neighboring cells. We think that it is unlikely that ATP is

released only from the initially stimulated cells because the amount of ATP released from ~10 cells would not be sufficient to trigger large Ca^{2+} responses in cells located 250 µM away. The amplitude of the microglial response hints to a local concentration ~100 µM ATP, because the response can be mimicked with this concentration (unpublished results). Moreover, the initial trigger does not destroy the cells in the close vicinity of the stimulation pipette, as the wave can be initiated several times from the same group of cells. Our finding that the wave spreads better in the direction of the axonal pathways hints to the fact that ATP also spreads within the slice and not only along its surface.

The initial signal that communicates CNS injury to microglia is unknown. Astrocytic Ca^{2+} waves and concomitant ATP-release could communicate such an injury to uninjured areas and could lead to the activation of microglial cells via purinergic receptor activation. As a response to a focal ischemic lesion, for instance, widespread microglial activation occurs, even in tissue that shows no sign of damage (16). Microglial cells are even activated during spreading depression (34). Purinergic receptors are expressed prominently by microglial cells (35, 22), and stimulation of purinergic receptors in cultured microglia leads to release of TNF-α and modulation of IL-1beta release, two cytokines well established to be involved in pathologic cascades.(21, 23, 36). It has been shown that cytokines themselves can influence ATP release from astrocytes (37) and thereby influence wave propagation (38). Moreover, essentially all types of glial cells, namely astrocytes (39), oligodendrocytes and their progenitors (40), and microglial cells (22) express various types of purinoreceptors and this receptor family can be viewed as the backbone of a common glial communication system.

ACKNOWLEDGMENT

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Fig. 1
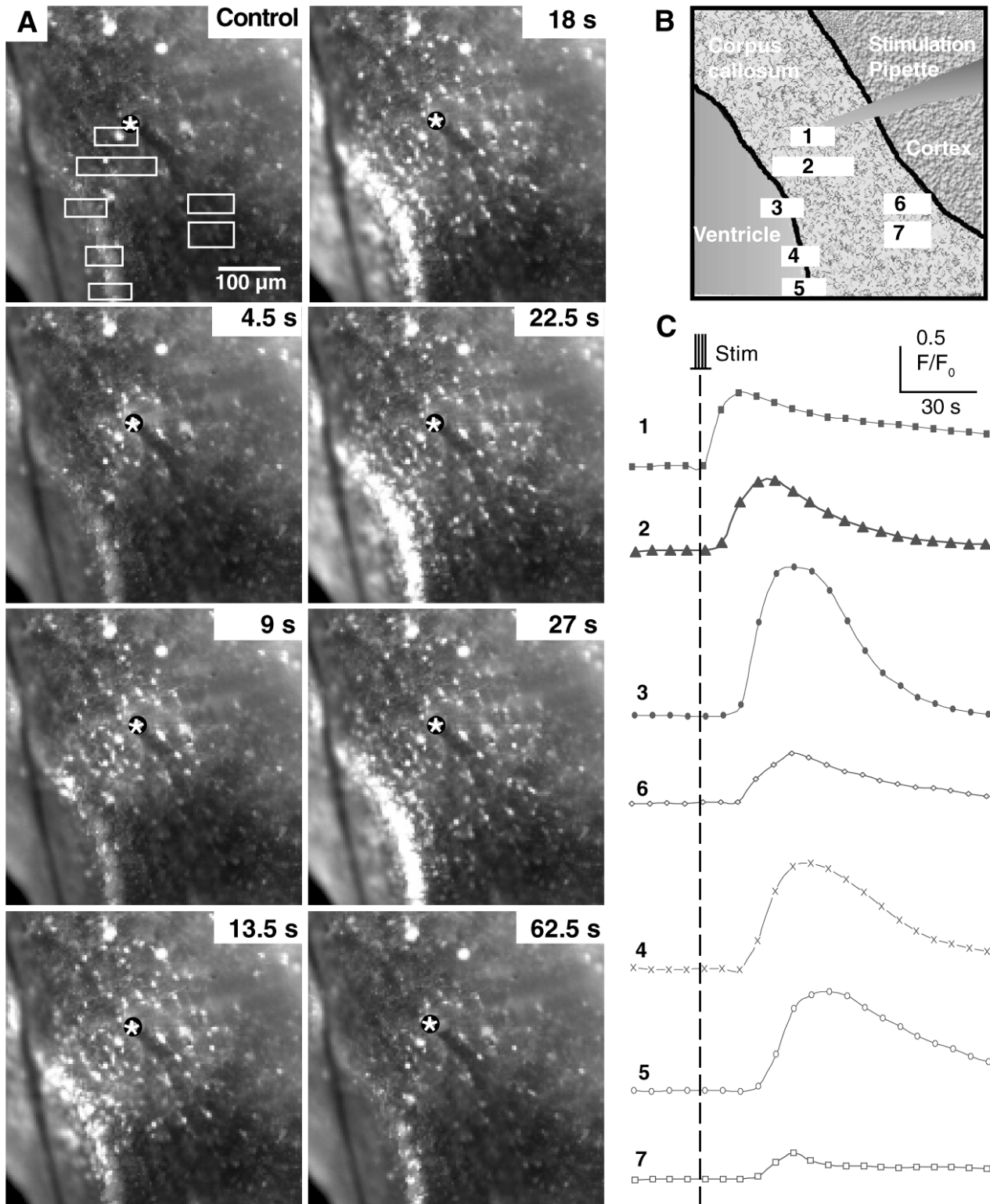
Figure 1. Propagation of Ca^{2+} waves in an acute brain slice. A series of fluorescence images just before (control) and at defined times (as indicated) after electrical stimulation illustrate the spread of the Ca^{2+} signal within a slice **(A)**. The position of the stimulation electrode is marked by the asterix. The cartoon in **(B)** outlines the anatomical structures of the fluorescence images. Moreover, the transient changes in Ca^{2+} were measured at the indicated areas **(B)** and are displayed in **(C)**. In area 1, close to the stimulation pipette, the increase in fluorescence (F/F$_o$) occurred right after stimulation (indicated by a vertical line). At more distant areas, the delay amounted to several seconds. Note that cells in the ventricle wall respond with an intense signal.

Fig. 2
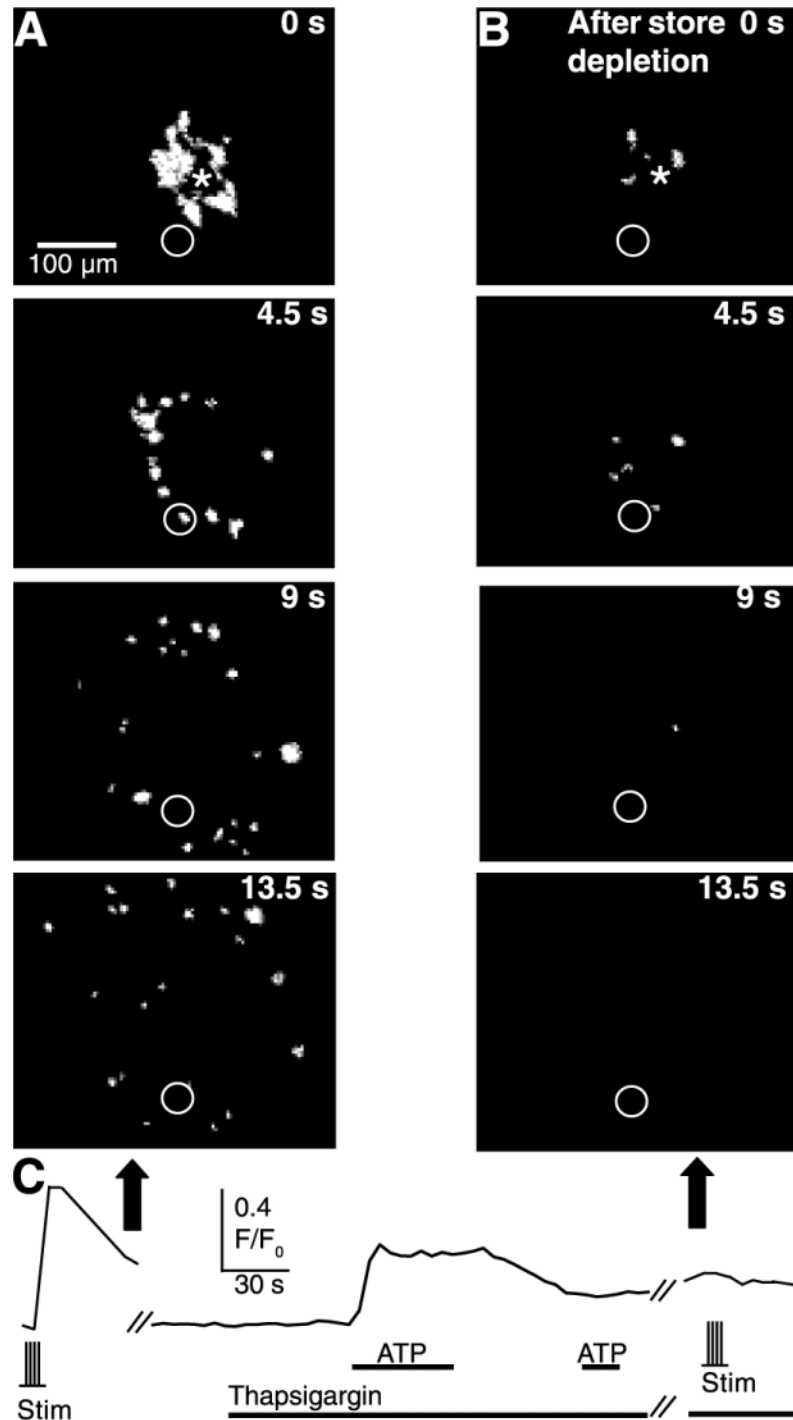
Figure 2. The calcium signal is abolished after depletion of internal calcium stores. A) A Ca^{2+} wave was elicited under control conditions. By subtracting consecutive images only the newly responding cells are displayed. The first image was recorded briefly after stimulation and subsequent ones with a delay as indicated. B) Internal calcium stores were depleted by application of thapsigargin (1 µM), and two subsequent ATP applications still in the presence of thapsigargin. After this paradigm electrical stimulation did no longer trigger the induction of a Ca^{2+} wave as shown in the series of images in (B). Calcium traces of the area outlined by the white circle in (A) and (B) are displayed in (C). In addition, the responses to ATP and to thapsigargin are shown which is not illustrated in (A) or (B).

Fig. 3
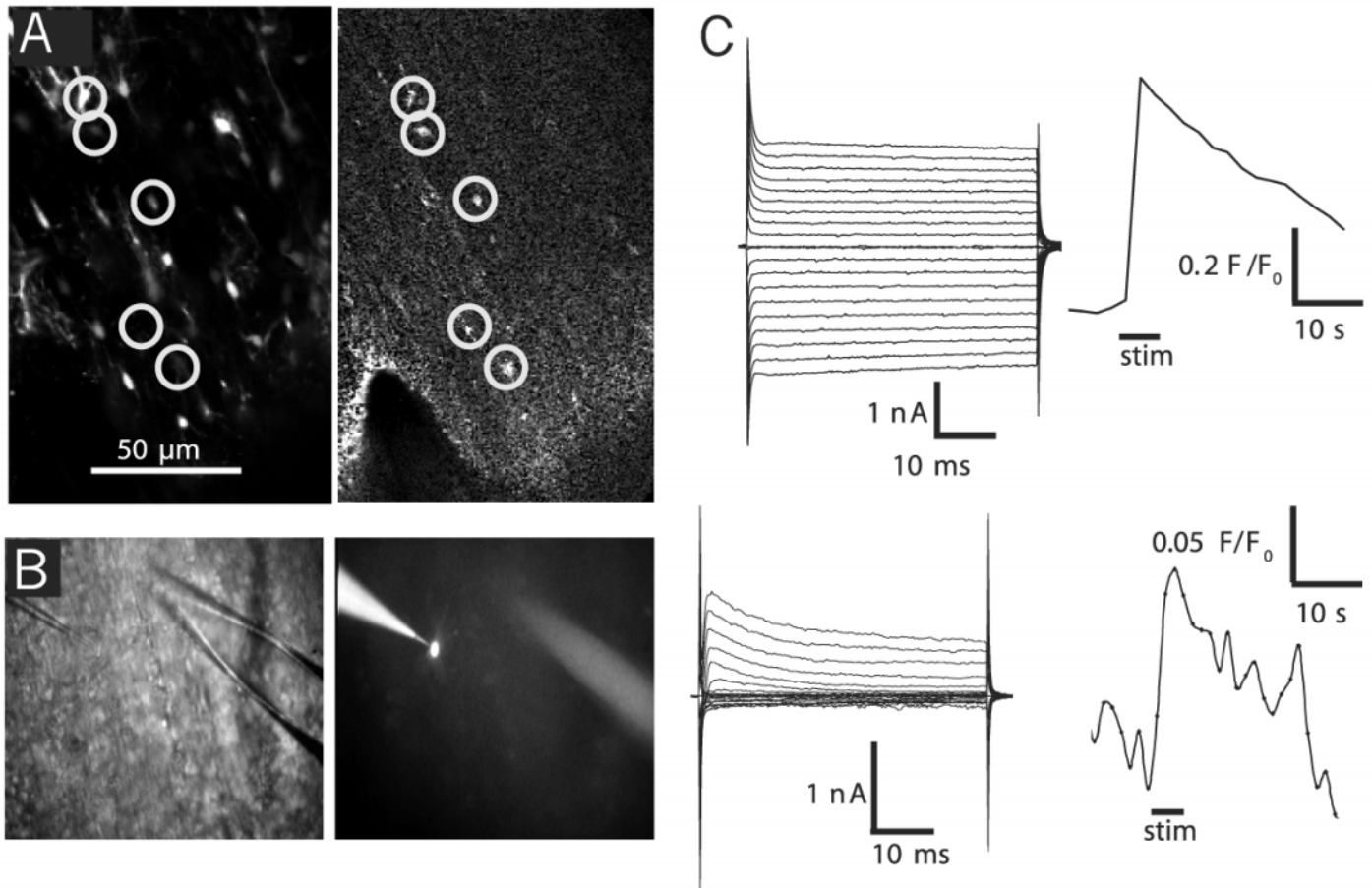
Figure 3. Different cell populations participate in the Ca^{2+} wave. A) To identify astrocytes, we obtained slices obtained from a transgenic animal in which astrocytes are labeled by the expression of the enhanced green fluorescent protein. The fluorescent micrograph on the left shows the intrinsic fluorescence of the EGFP-labeled astrocytes. On the right, the same area is displayed with filters for detection of Calcium Orange and thus for recording changes in intracellular Ca^{2+}. Electrical stimulation elicited a fluorescence increase in cells highlighted by circles. Note that not all of the responsive cells are astrocytes. **B)** After eliciting a Ca^{2+} wave, membrane currents were recorded from a responding cell. The micrograph on the upper left shows the arrangement of the recording (left) and stimulation pipette (right) as visualized with conventional optics. On the upper right, a fluorescence image for detecting Lucifer Yellow staining is displayed. Note the Lucifer Yellow filled cell and the recording pipette. **C)** The traces display currents activated in response to de- and hyperpolarizing voltage steps (from –160 to +20 mV, 10 mV increment) from a holding potential of –70 mV. On the lower right, the fluorescence response (F/F$_0$) of the Ca^{2+} dye Fluo-4 is displayed in response to stimulation. Current responses and fluorescence response of a different cell similar as described in (**B**). Note the activation of outward currents with depolarization.

Fig. 4
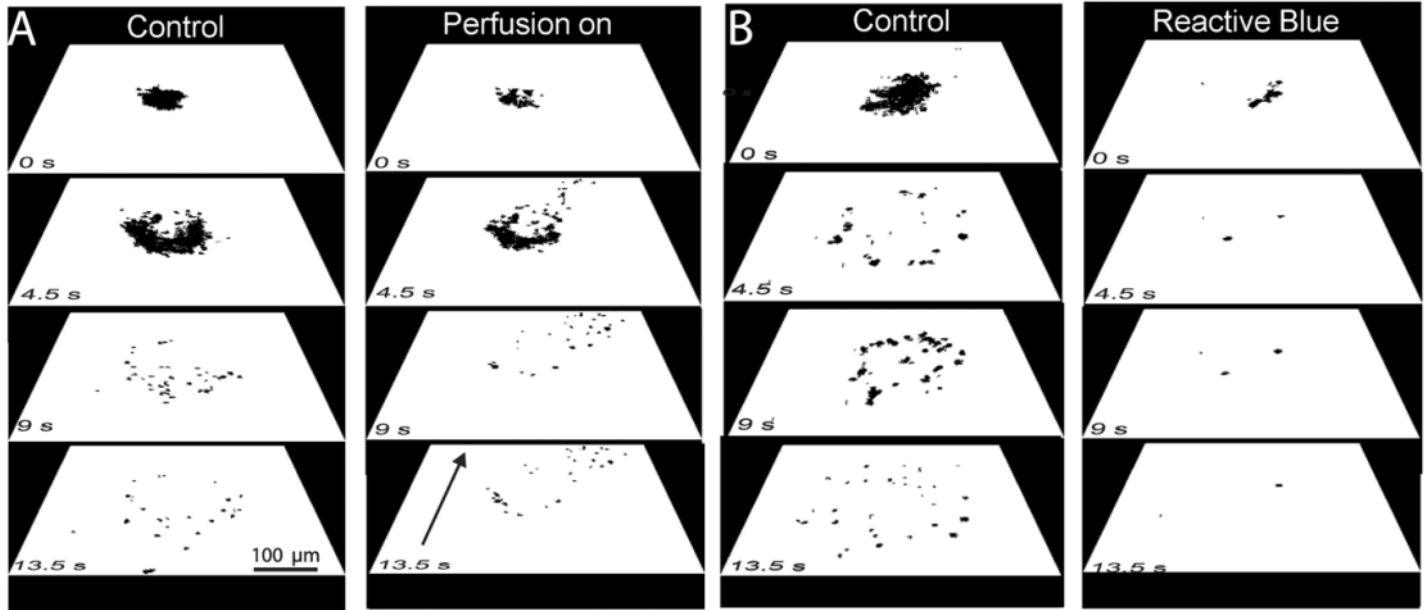
Figure 4. Characteristics of the Ca^{2+} wave: effect of bath perfusion and Reactive Blue 2. To illustrate the propagation of the Ca^{2+} wave, only those cells are displayed that were newly recruited to the population of Ca^{2+} responsive cells. This was achieved by subtracting the previous image from the one, which was displayed. The black areas represent the population of responsive cells at the times indicated. The first image shows the cells responding close to the pipette briefly after stimulation (0 s). In **(A)**, two responses from the same field are shown, with no bath perfusion (left) and one with a slow perfusion into the direction of the arrow (right). In **(B)**, a stimulation elicited a Ca^{2+} wave in control conditions (left), whereas only a local response was observed after applying the purinergic receptor antagonist Reactive Blue 2 (right).

Fig. 5
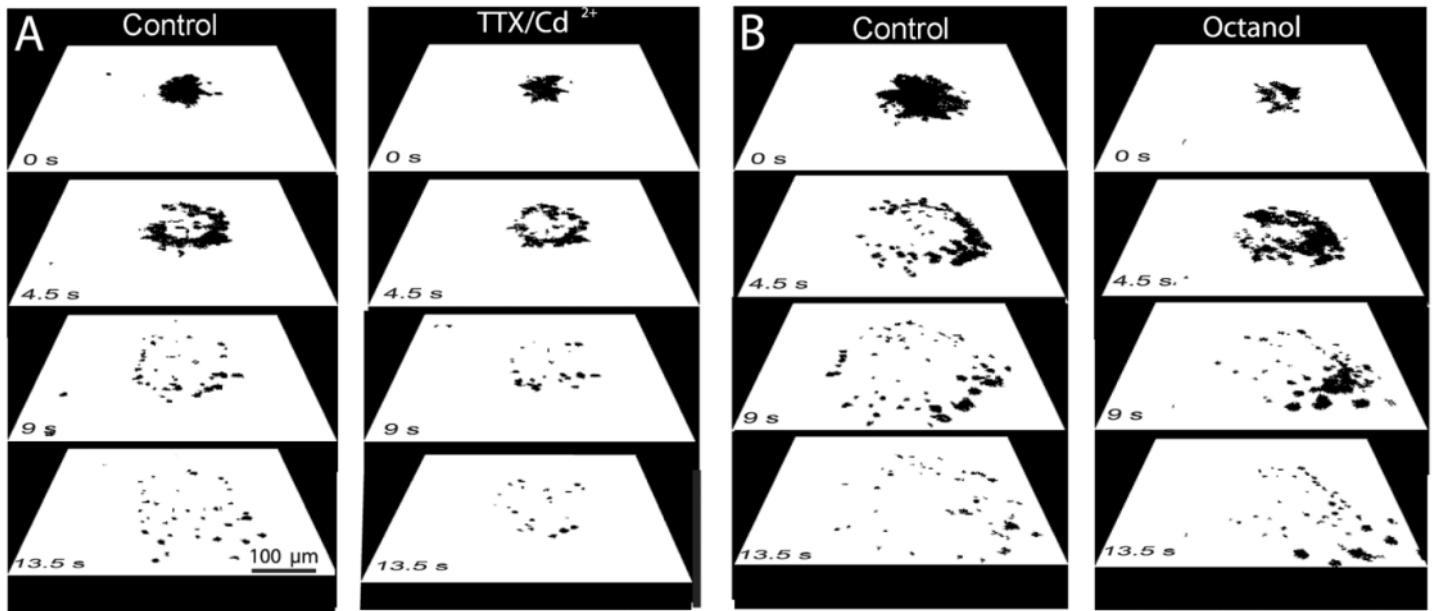
Figure 5. The wave spreads independently from neuronal activity and does not propagate via gap junctions. As in **Figure 4**, two responses from the same fields are shown. **A)** Stimulation elicited a Ca^{2+} wave in control conditions (left), and the response was still present after superfusing the slice with 1 µM TTX and 100 µM Cd^{+} for 2 min before stimulating a second time (right). **B)** A stimulation-triggered response in control conditions (left) was compared to a response after the slice was superfused with 500 µM Octanol for 5 min. The spread of the wave was neither markedly affected by TTX/Cd^{2+} nor by octanol.

Fig. 6
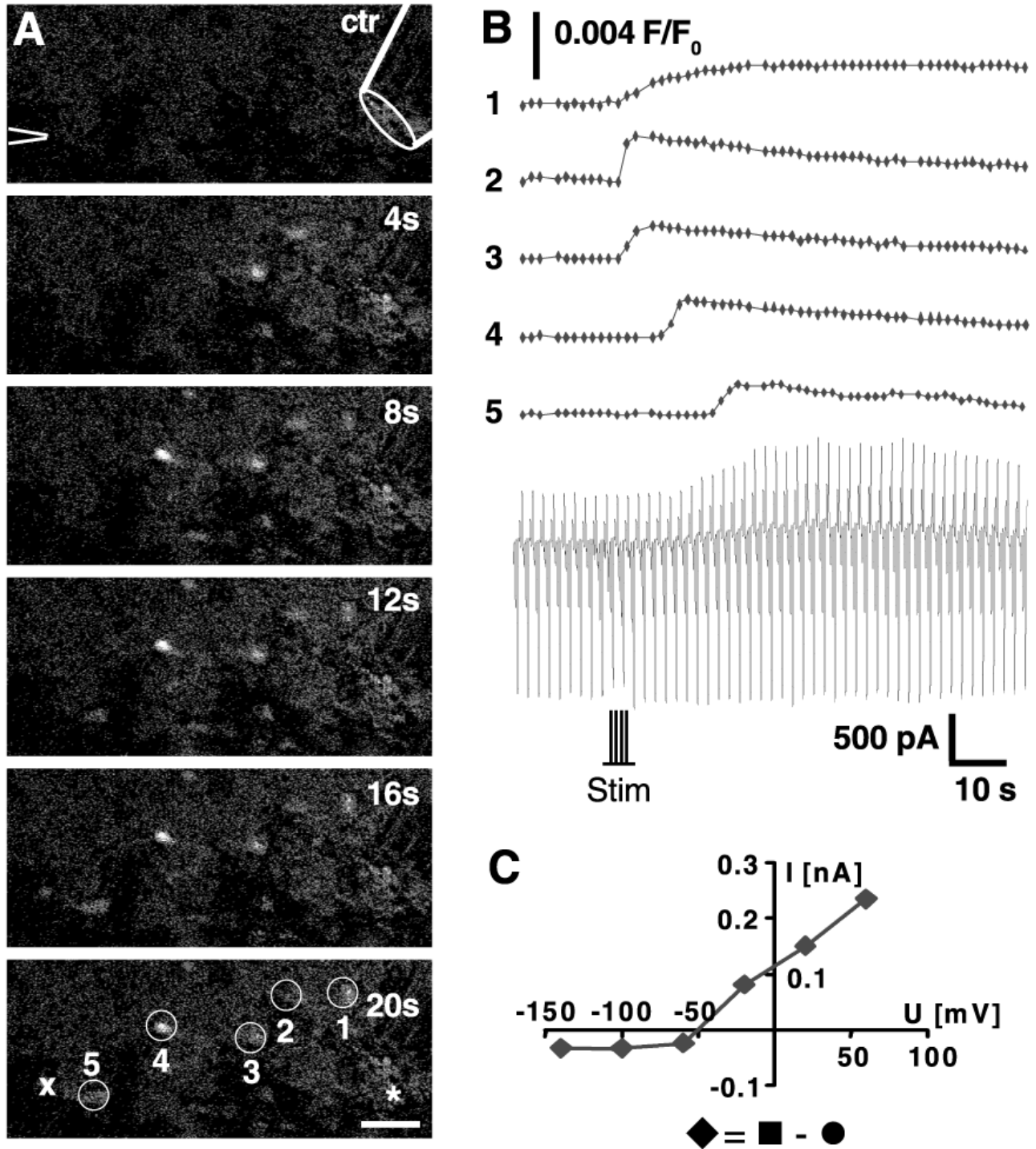
Figure 6. Microglial cells sense and respond to the calcium wave. The series of images in **(A)** illustrate the spread of a Ca^{2+} wave elicited by electrical stimulation. At the same time, membrane currents were recorded from a microglial cell. The position of the recording and stimulation pipette is indicated in the first image. The bar denotes 20 µm and the time after stimulation is indicated in each image recorded prior to stimulation (control). In **(B)**, the transient changes in Ca^{2+} from five cells (indicated in the last image in **A**) are displayed in combination with the membrane currents recorded from the microglial cell. The membrane of the microglial cell was clamped at -20 mV, and repetitively clamped to a series of de- and hyperpolarizing values. This procedure allowed us to construct current-to-voltage curves at 2-second intervals. It is evident that, with a delay in stimulation, outward currents increased in amplitude. In **(C)**, the current voltage curve of the stimulation-induced current is displayed showing features of an outward rectifying K^+ channel.