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Glutamate-mediated cytosolic calcium oscillations regulate a pulsatile prostaglandin release from cultured rat astrocytes

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SUMMARY

The synaptic release of glutamate evokes in astrocytes periodic increases in the intracellular calcium concentration ($[Ca^{2+}]_i$), due to activation of metabotropic glutamate receptors (mGluRs). The frequency of these $[Ca^{2+}]_i$ oscillations is controlled by the level of neuronal activity, indicating that they represent a specific, frequency-coded signaling system of neuron-to-astrocyte communication. We recently found that neuronal activity-dependent $[Ca^{2+}]_i$ oscillations in astrocytes are the main signal that regulates the coupling between neuronal activity and blood flow, the so-called functional hyperemia. Prostaglandins play a major role in this fundamental phenomenon in brain function, but little is known about a possible link between $[Ca^{2+}]_i$ oscillations and prostaglandin release from astrocytes. To investigate whether $[Ca^{2+}]_i$ oscillations regulate the release from astrocytes of vasoactive prostaglandins, such as the potent vasodilator prostaglandin E_2 (PGE_2), we plated wtHEK293 cells - constitutively responding to PGE_2 with $[Ca^{2+}]_i$ elevations - onto cultured astrocytes, and used them as biosensors of prostaglandin release. After loading the astrocyte-HEK cells co-cultures with the Ca^{2+} indicator Indo-1, in confocal microscope experiments we found that mGluR-mediated $[Ca^{2+}]_i$ oscillations triggered spatially and temporally coordinated $[Ca^{2+}]_i$ increases in the sensor cells. This response was absent in a clone of HEK cells unresponsive to PGE_2, and recovered after transfection with the $InsP_3$-linked prostanoid receptor EP_1. We conclude that $[Ca^{2+}]_i$ oscillations in astrocytes regulate prostaglandin releases that retain the oscillatory behaviour of the $[Ca^{2+}]_i$ changes. This finely tuned release of PGE_2 from astrocytes provides a coherent mechanistic background for the role of these glial cells in functional hyperemia.

INTRODUCTION

Since their early description, astrocytes were supposed to assist neurons in their function but, probably due to their inability to fire action potentials, they were never thought to actively participate in information processing in the brain. However, recent observations revealed their ability to respond to the synaptic release of neurotransmitters with $[Ca^{2+}]_i$ elevations (Porter & McCarthy, 1995; Pasti *et al.*, 1997), mainly mediated by the activation of group I metabotropic glutamate receptor (mGluR) (Pasti *et al.*, 1997), and GABAb receptors (Kang *et al.*, 1998), both linked to intracellular calcium mobilization. Neurotransmitter-mediated calcium elevations were proposed to represent a calcium-based form of excitability in astrocytes (Smith, 1994).

An important property of astrocyte $[Ca^{2+}]_i$ oscillations triggered by synaptically released glutamate is that they are tunable in frequency according to the level of neuronal activity: an increase in the intensity of stimulation applied to neuronal afferents results in a parallel increase in the frequency of $[Ca^{2+}]_i$ oscillations in astrocytes (Pasti *et al.*, 1997). Glutamate-mediated neuron-to-astrocyte signaling may thus represent a refined communication system which allows neurons to transfer to astrocytes information on the level of their activity (Carmignoto, 2000). What type of functional event in the brain may depend on this neuron-to-astrocyte signaling? We recently identified a precise functional significance for this pathway in the control of cerebral microcirculation. We found that mGluR-mediated $[Ca^{2+}]_i$ oscillations, activated in astrocytes by synaptically released glutamate, are central for the coupling between neuronal activity and blood flow, the so-called functional hyperemia (Zonta *et al.*, 2003). Evidence has also been obtained indicating that arachidonic acid metabolites of the cyclooxygenase (COX) pathway play a major role in the astrocyte control of vascular tone.

Astrocytes are well known to represent a major source of prostanoids in the brain, under both physiological and pathological conditions (Bezzi *et al.*, 1998; Hirst *et al.*, 1999), but whether or not $[Ca^{2+}]_i$ oscillations can regulate PG release from these cells and what spatio-temporal properties this release have, are issues which are not defined yet. The principal aim of the present study was to investigate whether mGluR-mediated $[Ca^{2+}]_i$ oscillations in astrocytes can control the pulsatile release from these cells of a prostanoid with vasodilating properties, such as the prostaglandin (PG) E_2.

In view of the wide array of PG actions in the brain, the characterization of the rules governing PG release from these glial cells has additional motives of interest. The synthesis of prostaglandin in these cells as well as in other cell types, depends on the enzymatic activity of COX on its substrate arachidonic acid, which is released from phospholipids by the Ca^{2+}-dependent enzyme phospholipase A_2. The Ca^{2+} dependency of PG production raises the question of whether a cell integrates the Ca^{2+} signals generating a sustained PG synthesis and release, or retains their temporal coding resulting in a release that faithfully follows the kinetics and general pattern of the $[Ca^{2+}]_i$ changes. All the studies designed to analyze the synthesis and/or release of COX products in eucaryotic cells failed to provide information about the spatio-temporal features of this release, since they were performed either on cell homogenates (Seregi *et al.*, 1987; Amruthesh *et al.*, 1993), or through off-line measurements in cell population (Bezzi *et al.*, 1998). Our experimental approach allows us to monitor the process of PG release at the level of single, living cells. We here provide evidence that activation of mGluRs controls the release of PGs from astrocytes, and that this release is pulsatile and synchronous with mGluR-mediated $[Ca^{2+}]_i$ oscillations.

METHODS

Cell cultures.

All experimental procedures were in strict accordance with the Italian and EU regulation on animal welfare, and previously authorized by the Italian Ministry of Health. Neonatal Wistar rats were deeply anaesthesized by a cocktail of xilazina, tiletamina and zolazepan and then sacrificed by cervical dislocation. Primary cultures of cortical astrocytes were prepared as previously described (Pasti *et al.*, 1995). Fourteen days after plating, cultured cells were subjected to 15 hrs of continuous shaking and then incubated for 5 min with 0.2% trypsin. Detached cells were collected and replated on poly-L-lysine coated 24-mm-diameter coverslips. In all experiments, 2-weeks-old astrocyte secondary cultures were used. The absence of contaminating neurons and microglia in these cultures was assessed by immunocytochemistry with anti-200-kDa neurofilament antibody to identify neurons (Vitadello & Denis-Donini, 1990), and by specific staining of microglial cells with a FITC-labeled lectin from Lycopersicon Esculentum (Sigma) (Boucsein *et al.*, 2000), while astrocytes were identified using anti-GFAP antibody (Boehringer). Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were co-transfected with either the cDNAs encoding for the NMDAR subunits 1–2A and GFP (Pasti *et al.*, 2001), in the presence of 2 mM kynurenic acid and 500 μM ketamine, or the cDNA of the human EP_1 receptor (Funk *et al.*, 1993) (kindly provided by Dr. Mark Abramovitz, Dept. of Biochemistry & Molecular Biology, Merck Frosst Canada Inc.) and EGFP. These HEK cells express neither ionotropic nor metabotropic glutamate receptors (Pasti *et al.*, 2001). HEK cells were trypsinized after 7–9 hrs of transfection and replated on astrocyte cultures. For confocal microscope experiments, after 1–3 days, co-cultures were incubated with the Ca^{2+} indicator Indo-1 AM (5 μM) at 37°C for 40-50 min in the presence of 0.04% pluronic and 200 μM sulfinpyrazone. Clones of HEK cells were obtained by limiting dilution from wtHEK cells, and a clone which did not display any $[Ca^{2+}]_i$ increase upon stimulation with PGE_2 was isolated (HEK^U). L-quisqualate and 1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD) were from Tocris Cookson Ltd. (Bristol, UK). PGE_2, SC19220 and AH6809 were from BioMol (Plymouth Meeting, PA); indomethacin was from Sigma.

Confocal microscopy.

Digital fluorescence microscopy (Nikon, RCM8000) was used for monitoring the change in Indo-1 emission after cell loading with Indo-1 AM (Molecular Probes, Eugene, OR) as previously described (Pasti *et al.*, 2001). After excitation at 351 nm wavelength, the emitted light was separated into its two components (405 and 485 nm), and the ratio (R405/485) was displayed as a pseudocolor scale. During experiments, cultured cells were continuously perfused (1.5-3 ml/min) with an extracellular solution, consisting of (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1 Na_3PO_4, 5.5 glucose, 10 HEPES and 0.2 sulfinpyrazone, at pH 7.4 with NaOH, at 32°C. Sampling rate was 2s. Less than 6s of delay between $[Ca^{2+}]_i$ increases in the astrocyte and those in the adjacent transfected HEK cell were considered indicative of temporal correlation.

Electrophysiology

Standard procedures for pipette preparation and patch-clamp recordings in the whole-cell configuration were used. During experiments cells were continuously perfused with

the same extracellular solution (without sulfinpyrazone) used for confocal microscope experiments. The recording pipette contained (in mM): 145 K-gluconate , 2 $MgCl_2$, 5 EGTA, 2 Na_2ATP, 0.2 NaGTP and 10 HEPES at pH 7.2 with KOH. Two patch-clamp amplifiers (Axopatch 200B, Axon Instrument) were grounded to a common ground point and were used for voltage-clamp recordings. Current signals were filtered at 1 KHz, digitized at 5 KHz by the interface Digidata1200A and analyzed by PCLAMP-8 software (Axon Instrument). Hyperpolarizing and depolarizing voltage pulses (100 ms duration) from a holding potential of –70 mV were delivered every 2 s. Simultaneous dual patch-clamp recordings were performed from pairs of cells consisting of an astrocyte and a nearby GFP fluorescent HEK cell transfected with the prostaglandin receptor EP1.

RESULTS

Astrocyte stimulation triggers D-AP5 insensitive responses in NMDAR-transfected HEK cells.

To investigate the release of specific molecules from astrocytes, we recently developed an experimental model based on Human Embryonic Kidney (HEK293) cells transiently transfected with the specific receptor for the molecule under study (Pasti *et al.*, 2001). Co-transfection with a green fluorescent protein (GFP) ensures identification of the cells expressing the receptor. When plated on cultured astrocytes, each transfected HEK cell acts as a biosensor of the release of the molecule of interest. This release can be revealed in the sensor cell by monitoring, through Ca^{2+} imaging techniques, the $[Ca^{2+}]_i$ changes and/or, through patch clamp recordings, the current(s) that may result from the activation of the transfected receptor. After loading of the HEK-astrocyte cultures with a Ca^{2+} indicator, it is thus possible to simultaneously monitor both the $[Ca^{2+}]_i$ increases in the astrocyte and, indirectly through the $[Ca^{2+}]_i$ rise in the HEK cell, the release event. Detailed information can therefore be obtained on the relationship between the spatio-temporal features of the $[Ca^{2+}]_i$ elevations in astrocytes and the process of release.

By employing HEK cells transfected with the ionotropic glutamate receptor NMDA (NR-GFP HEK cells) (Pasti *et al.*, 2001), we previously showed that $[Ca^{2+}]_i$ oscillations triggered by L-quisqualate, an agonist of both mGlu and AMPA receptors, regulate a SNARE protein-dependent pulsatile release of glutamate from astrocytes. However, in 36.2% (17 of 47) of responsive NR-GFP HEK cells, the NMDAR antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5), failed to block $[Ca^{2+}]_i$ elevations in sensor cells (Fig.1A). To investigate whether astrocytes release another molecule that acts on receptors endogenously expressed in sensor cells, HEK cells solely transfected with GFP (GFP-HEK) were plated on astrocytes. A number of GFP-HEK cells (27.7%, 5 of 18) showed $[Ca^{2+}]_i$ elevations upon astrocyte stimulation, demonstrating that a subpopulation of HEK cells (and thus also of NR-GFP HEK cells) is equipped with a receptor sensing an astrocyte-derived molecule other than glutamate.

Among the various Ca^{2+} mobilizing compounds that can be released by activated astrocytes, PGs appear as good candidates. In fact, astrocytes can release PGE_2 upon activation of glutamatergic receptors (Bezzi *et al.*, 1998). An initial observation that supports our hypothesis is that a subpopulation of wild type (wt) HEK cells (43.6%, 48 of 110) display $[Ca^{2+}]_i$ elevations when directly stimulated with PGE_2. To get further insights into the nature of the molecule released by astrocytes, we used the COX

inhibitor indomethacin to block the synthesis of PGs. Cultures, in which GFP-HEK cells displayed $[Ca^{2+}]_i$ increases correlated with those induced by L-quisqualate in astrocytes, were incubated for 40 min with 5 μM indomethacin. Under these conditions, no $[Ca^{2+}]_i$ elevations were observed in GFP-HEK cells upon a second challenge with L-quisqualate (n=4, Fig.1B). L-quisqualate triggered in astrocytes $[Ca^{2+}]_i$ increases of comparable amplitude (0.39 ± 0.03 and 0.42 ± 0.04, before and after indomethacin, respectively, mean R405/485 change ± SEM; n=27; Fig. 1C).

Astrocyte stimulation fails to trigger D-AP5 insensitive responses in HEK^U cell clone.

To obtain evidence that a COX product is released from astrocytes and is responsible for the $[Ca^{2+}]_i$ increases observed in GFP-HEK cells, we isolated by limiting dilution a clone of PGE_2-unresponsive HEK (HEK^U) cells. These cells were transfected with the NMDAR and then plated on astrocytes. In contrast to what observed in wt HEK cells, $[Ca^{2+}]_i$ elevations triggered in NMDAR-transfected HEK^U (n=15) by astrocyte stimulation were regularly blocked by D-AP5 (Fig.1D), suggesting that D-AP5-insensitive responses observed in wt HEK cells were due to a molecule acting on a PG receptor.

EP_1-transfected HEK^U cells recover the response to astrocyte stimulation.

To directly prove that glutamate receptor-mediated $[Ca^{2+}]_i$ oscillations regulate the release of a PG from astrocytes, HEK^U were transfected with the specific PGE_2 receptor EP_1 (EP_1R), and with the enhanced form of GFP (EGFP). Transfected HEK^U (EP_1-EGFP HEK^U), either plated alone or on astrocytes, showed $[Ca^{2+}]_i$ elevations upon stimulation with PGE_2 (Fig.2A, panel *b*). Panel *a* of figure 2A shows three EP_1-EGFP HEK^U plated on cultured astrocytes, easily recognizable when illuminated at the excitation wavelength of EGFP (488nm). The same cells are visible among the astrocytes in panels *b* and *c*, when illuminated at the excitation wavelength of Indo-1 (351nm). Stimulation with 10 μM L-quisqualate (panel *c*) triggered repetitive $[Ca^{2+}]_i$ increases in the astrocyte labeled 1, as well as in other astrocytes. The adjacent EP_1-EGFP HEK^U (labeled 2) also displayed two successive $[Ca^{2+}]_i$ elevations, temporally correlated with those observed in the astrocyte (Fig.3A and 3B; see also movies available as Additional Information). Noteworthy, also the other two EP_1-EGFP HEK^U visible in the field raised their $[Ca^{2+}]_i$ in temporal correlation with $[Ca^{2+}]_i$ increases occurring in nearby astrocytes (see movies). Similar results were obtained using the mGluR agonist *t*-ACPD. Of a total of 148 EP_1-EGFP HEK^U analyzed, 57 exhibited clear responses to astrocyte stimulation. In 84% of the responding EP_1-EGFP HEK^U, $[Ca^{2+}]_i$ elevations were temporally correlated with those of adjacent astrocytes. When L-quisqualate triggered in astrocytes one $[Ca^{2+}]_i$ peak followed by a sustained plateau, in most of the responsive HEK cells (15 of 19, 78.9%) a single $[Ca^{2+}]_i$ peak was observed. In figure 2C the amplitudes of the $[Ca^{2+}]_i$ peaks in stimulated astrocytes are grouped according to the presence (success) or absence (failure) of the response in nearby EP_1-EGFP HEK^U cells. With respect to low-amplitude, high-amplitude $[Ca^{2+}]_i$ peaks more efficiently triggered the release.

To investigate the possible existence of gap junctional coupling between astrocytes and EP_1-EGFP HEK^U cells, we performed whole-cell, patch clamp recordings from pairs of cells consisting of an astrocyte and an adjacent EP_1-EGFP HEK^U cell. Voltage pulses, which were always applied to either the astrocyte and the EP_1-EGFP HEK^U cell, were never observed to spread to the other cell (Supplementary Fig.1A). In all pairs (n=5),

the stimulation protocol was repetitively applied for at least 20 min. In three pairs the voltage steps were also applied after stimulation with 20 μM *t*-ACPD (Supplementary Fig.1B). We never observed signs of electrical coupling between the astrocyte and the EP_1-EGFP HEK^U cell.

The commercially available EP_1R antagonists SC19220 and AH6809 (10-100 μM), failed to inhibit the $[Ca^{2+}]_i$ response of EP_1-EGFP HEK^U to astrocyte stimulation. However, these antagonists, applied at the highest concentration (100 μM), were able to block the response of EP_1-EGFP HEK^U to the direct stimulation with PGE_2 only at PGE_2 concentration not higher than 1 nM. Consequently, they could not be considered reliable pharmacological tools to effectively block the EP_1 receptors.

To prove that the presence of the PG receptor EP_1 is essential to HEK^U cells for sensing the molecule released from astrocytes, we compared the percentage of EP_1-EGFP HEK^U responding to astrocyte stimulation, with that of HEK^U not transfected with EP_1 receptor (EGFP HEK^U). When plated on astrocytes, EGFP HEK^U failed, with one exception, to respond to astrocytic oscillations (n=74, Fig.3A). Consistently, inhibition of PG synthesis with indomethacin blocked $[Ca^{2+}]_i$ elevations in EP_1-EGFP HEK^U following astrocyte stimulation, while washout of the inhibitor allowed the recovery of the response (n=5, Fig.3B).

Run down of the system is unlikely to account for this lack of response in EP_1-EGFP HEK^U cells since in cultures not incubated with indomethacin two subsequent challenges with either L-quisqualate or *t*-ACPD, triggered comparable responses in the great majority (18 of 21; 86%) of EP_1-EGFP HEK^U cells. Also noteworthy is that in nine EP_1-EGFP HEK^U cells we performed a 3rd challenge and found responses in 8 cells.

DISCUSSION

PG release from astrocytes is pulsatile.

Over the last years, several experimental evidences demonstrated that the stimulation of mGluRs induces Ca^{2+}-dependent production of arachidonic acid metabolites including PGE_2 (Dumuis *et al.*, 1990; Bezzi *et al.*, 1998), but the kinetics and spatial aspects of such a release at the single cell level could not be determined since prostanoids were biochemically measured in the supernatant of cell cultures. Our experimental approach allows to investigate the process of PG release at the single cell level. We used HEK cells transfected with the specific PGE_2 receptor EP_1 as biosensors of PG release from astrocytes. To stimulate $[Ca^{2+}]_i$ elevations in astrocytes we could used agonists of GluRs such as L-quisqualate and *t*-ACPD since HEK cells express neither ionotropic nor metabotropic glutamate receptors (Pasti *et al.*, 2001). The key finding of the present work is that the release of a PG, possibly PGE_2, from activated astrocytes is oscillatory in nature, and synchronous with the oscillations of $[Ca^{2+}]_i$.

The first hint that $[Ca^{2+}]_i$ oscillations in astrocytes could lead to the pulsatile release of a prostanoid, in turn capable of inducing $[Ca^{2+}]_i$ rises in the adjacent reporter cell, came from the observation that upon astrocyte stimulation, NMDAR-transfected HEK cells showed $[Ca^{2+}]_i$ increases insensitive to the NMDAR antagonist D-AP5 (Pasti *et al.*, 2001). $[Ca^{2+}]_i$ elevations triggered by astrocyte stimulation were also observed in HEK cells not expressing the NMDAR and were found to be abolished after cell

preincubation with the COX inhibitor indomethacin. However, we previously showed that the release of glutamate from astrocytes depends on PG formation (Bezzi *et al.*, 1998). Therefore, it could not be excluded that the synthesis and/or the release of the factor that causes D-AP5-insensitive responses in NMDAR-transfected HEK cells were also dependent on a COX product. In other words, the results from indomethacin experiments does not provide unambigous evidence that a PG is the factor that directly acts in the sensor cell to trigger the Ca^{2+} response. Further difficulties for the identification of the prostanoid nature of the molecule released from astrocytes, depend on the fact that the available antagonists of EP_1 receptors, such as SC19220 and AH6809 (Funk *et al.*, 1993), are largely unsatisfactory. In fact, at the highest concentration that we could use (100 μM), these inhibitors antagonized the action of PGE_2, applied directly to EP_1-expressing HEK cells (EP_1-EGFP HEK^U), only when the PG was used at concentration 10^5 fold lower. The failure of these antagonists to inhibit the $[Ca^{2+}]_i$ response of EP_1-EGFP HEK^U to astrocyte stimulation could simply indicate that the local PG concentration is higher than 1 nM.

It was the use of HEK^U (a PGE_2 unresponsive HEK cell clone) that ultimately allowed us to directly show that the ability of wt HEK cells to respond to astrocyte stimulation depends on the presence of a PG receptor, and thus on the release from astrocytes of a PG. Indeed, the response observed in wt HEK cells was absent in HEK^U cells, but it was restored after transfection of these cells with the EP_1 receptor.

The $[Ca^{2+}]_i$ elevations in EP_1-EGFP HEK^U cells in response to astrocyte stimulation could not be attributed to gap junction communication between astrocytes and the sensor cell. In prolonged patch-clamp recordings from pairs of cells consisting of an astrocyte and an adjacent EP_1-EGFP HEK^U, we never observed evidence for electrical coupling between these two cells. These observations confirm results from previous experiments in which Lucifer Yellow included in a patch pipette was not found to diffuse from patched HEK cells to sorrounding astrocytes or from patched astrocytes to HEK cells (Pasti *et al.*, 2001).

The $[Ca^{2+}]_i$ elevations observed in the sensor HEK cells were found to be oscillatory. Two possible mechanisms could be envisaged: i) a single episode of PG release occurs upon astrocyte stimulation, and the evoked $[Ca^{2+}]_i$ oscillations in HEK cells are due to an intrinsic feature of EP_1R activation; ii) PG release from astrocytes is pulsatile and drives $[Ca^{2+}]_i$ oscillations in the sensor HEK cells. Two lines of evidence argue in favour of the second possibility: i) when high doses of mGluR agonists were used, the $[Ca^{2+}]_i$ change in the astrocytes was composed by a single $[Ca^{2+}]_i$ peak followed by a sustained plateau; under these conditions, in most of the HEK cells only a single episode of $[Ca^{2+}]_i$ increase was observed; ii) the great majority (84%) of $[Ca^{2+}]_i$ elevations observed in the sensor cells were temporally correlated with a $[Ca^{2+}]_i$ peak in a nearby astrocyte. Taken together, these observations suggest that either the activation of phospholipase A_2 and/or the release process faithfully follow the kinetics and general pattern of the $[Ca^{2+}]_i$ changes. While the frequency of $[Ca^{2+}]_i$ oscillations controls the timing of the release, the amplitude of each $[Ca^{2+}]_i$ peak controls its efficacy. Last but not least, PG release from astrocytes is extremely fast, given that the delay between the $[Ca^{2+}]_i$ peak of the astrocyte and that in the sensor cells can be as short as 2 s.

In a number of EP_1-EGFP HEK^U cells (16%), $[Ca^{2+}]_i$ elevations were not temporally correlated with $[Ca^{2+}]_i$ elevations in nearby astrocytes. These responses may be due to the release of PGs from astrocytic processes which could not be visualized because they were on a different focal plane. Alternatively, these uncorrelated $[Ca^{2+}]_i$ elevations may

be due to slow diffusion of PGs released from astrocytes located faraway from the sensor cells. The consequent delayed activation of EP_1 receptors may account for the lack of correlation.

Astrocytes can display $[Ca^{2+}]_i$ oscillations in response to the synaptic release of the inhibitory neurotransmitter GABA (Kang *et al.*, 1998), raising the possibility that $[Ca^{2+}]_i$ elevations triggered in astrocytes by signaling molecules other than glutamate might also trigger PG release. Additional experiments are necessary to address this issue.

Possible functional roles of PG release from astrocytes.

PGs are known to affect diverse phenomena in the CNS, such as neuronal transmission (Sekiyama *et al.*, 1995; Borgland *et al.*, 2002), vascular tone control (Narumiya *et al.*, 1999) and inflammation (Davies *et al.*, 1984), as well as to be involved in neurodegenerative diseases, including prion infections, HIV-dementia and Alzheimer's disease (Griffin *et al.*, 1994) (Williams *et al.*, 1997) (Combs *et al.*, 2000; Yasojima *et al.*, 2001). Our finding that PG release from astrocytes is regulated by the frequency of $[Ca^{2+}]_i$ oscillations provides a novel perspective for identifying and characterizing the involvement of astrocytic PG release in one or more of these PG actions.

Given that the frequency of $[Ca^{2+}]_i$ oscillations in astrocytes is controlled by neuronal activity (Pasti *et al.*, 1997), PG release from astrocytes could also be under the direct control of neurons. This view finds a strict functional correlation with our recent data describing a distinct astrocyte role in the control of cerebral microcirculation. We found that the coupling between neuronal activity and blood flow, the so-called functional hyperemia, mainly depends on mGluR-mediated $[Ca^{2+}]_i$ oscillations in astrocytes and on a COX product. Our present finding suggests that the COX product mediating vasodilation is released from astrocytes. Indeed, the same mGluR-mediated oscillations that control neuronal activity-dependent vasodilation, control the pulsatile release from cultured astrocytes of a PG with vasodilating properties. The PGs that can activate the PG receptor subtype in sensor cells, i.e. PGE_2 and, with a lower affinity, PGI_2 and PGE_1 (Funk *et al.*, 1993) (Narumiya *et al.*, 1999), are in fact powerful vasodilators.

We recently provided evidence for a central role of astrocytes in functional hyperemia, i.e. the tight coupling between neuronal activity and blood flow (Zonta *et al.*, 2003). An important implication of the observations here reported is related to the time course of functional hyperemia. While there is a general agreement on the strict relationship existing between the timing for neuronal activity and that for the blood flow response, the molecules and mechanism(s) governing this temporal correlation are largely unknown. Indeed, the dilation of cerebral arterioles - that guarantees a blood flow increase in the region of high neuronal activity - is maintained as long as high neuronal activity is maintained. When synaptic activity returns to resting conditions, the blood flow in that region also returns to basal levels. We propose that the timing of this process is regulated by astrocyte $[Ca^{2+}]_i$ oscillations. The frequency and the duration of $[Ca^{2+}]_i$ oscillations in astrocytes, which are dynamically regulated by neuronal signals - set up the timing of PG release from these cells. This provide a coherent mechanistic background that may account for the timing of blood vessel responses to high neuronal activity. In other words, the pulsatility of PG release together with the the short halflife of this molecule, ensures that the dilating action of PG triggered by a $[Ca^{2+}]_i$ peak will soon vanish unless another $[Ca^{2+}]_i$ peak provides novel PG.

The dependence on frequency and duration of $[Ca^{2+}]_i$ oscillations may represent a general rule governing PG release. In view of the wide array of PG action and of the oscillatory nature often characterizing $[Ca^{2+}]_i$ signals in PG-producing cells, our observation is functional to a better understanding of the PG role in the brain.

FIGURE LEGENDS

Figure 1. Activated astrocytes release a compound different from glutamate, most probably a prostanoid. *A,* Kinetics of $[Ca^{2+}]_i$ changes, expressed as variations in the 405/485 ratio (R), in one astrocyte (black area) and in an adjacent NR-GFP HEK cell (gray area) upon two successive astrocyte stimulation with 10μM L-quisqualate. Note that the response of the HEK cell is not blocked by 50 μM D-AP5. Black arrows indicate the onset and the end of stimulation. *B,* Kinetics of R405/485 changes in another astrocyte and in an adjacent GFP-HEK cell upon two successive astrocyte stimulation with 20μM L-quisqualate. The second challenge with L-quisqualate was performed after cell incubation with indomethacin. *C,* Amplitudes of L-quisqualate-induced $[Ca^{2+}]_i$ peaks from individual astrocytes before (Ctrl) and after (Indom.) incubation with indomethacin. *D,* Histogram showing the percentage of HEK cells displaying D-AP5-insensitive $[Ca^{2+}]_i$ responses, for transfected wtHEK cells (NR-GFP HEK cells, n=65) and for transfected HEK cells from the PGE_2 unresponsive clone (NR-GFP HEK^U cells, n=15).

Figure 2. $[Ca^{2+}]_i$ oscillations in astrocytes trigger correlated $[Ca^{2+}]_i$ elevations in PGE_2-sensor cells. *A,* Three EP_1-EGFP HEK cells are easily identified among astrocytes by their fluorescence at 488 nm excitation wavelength (*a*) and by their clear $[Ca^{2+}]_i$ increase in response to 100 nM PGE_2 (*b*). The sequence of pseudocolor images in *c* shows $[Ca^{2+}]_i$ changes induced by 10 μM L-quisqualate in one astrocyte (spot 1) and in one EP_1-EGFP HEK cell (spot 2). Timing of frame acquisition is reported at bottom right of each frame. Bar, 10 μm. *B,* Kinetics of R405/485 variations from astrocyte 1 (red area) and EP_1-EGFP HEK cell 2 (green area) upon L-quisqualate stimulation. *C,* Values of the astrocyte $[Ca^{2+}]_i$ peaks (n=80), that failed (failure) or succeeded (success) to trigger a correlated $[Ca^{2+}]_i$ elevation in the sensor cells. Only astrocytes displaying an oscillatory pattern comprised of both low- and large-amplitude $[Ca^{2+}]_i$ peaks were considered (n=21). The mean values of the R405/485 changes from the two groups are significantly different (0.35±0.03 vs 0.49±0.03; $p<0.001$, one-way ANOVA).

Figure 3. Prostanoid nature of the compound released by activated astrocytes. *A,* Histogram showing the percentage of HEK^U cells displaying $[Ca^{2+}]_i$ responses upon astrocyte stimulation, for EP_1-EGFP HEK^U cells (n=148) and for EGFP HEK^U cells (n=74). *B,* Kinetics of R405/485 changes in one astrocyte (black area) and in one adjacent EP_1-EGFP HEK cell (grey area) upon three successive stimulation with *t*-ACPD 20μM. The second challenge was performed after 40 min incubation with indomethacin, and the third after 30 min washout of the inhibitor.

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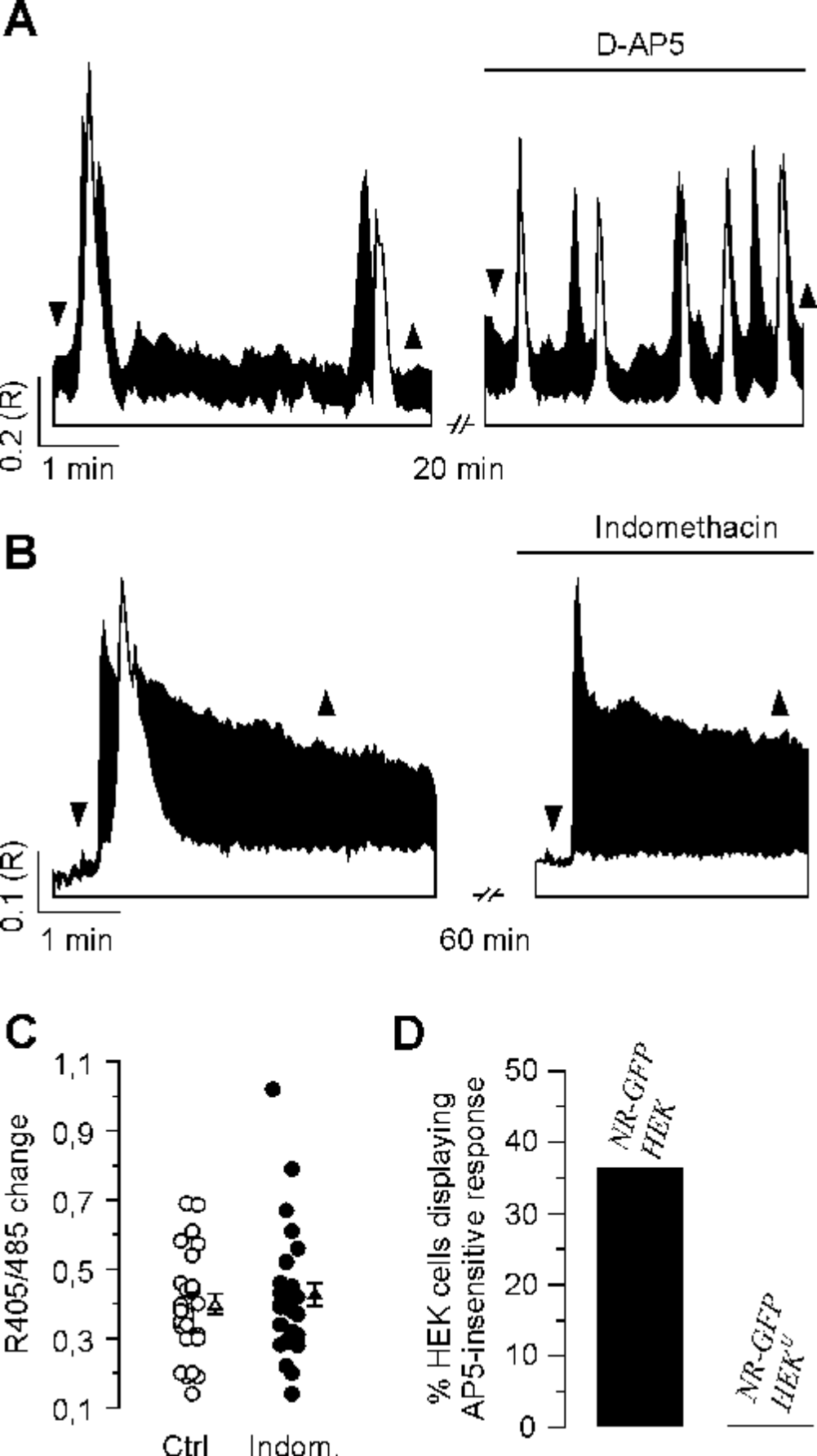
A
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B
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0.1 (R)
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C
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50
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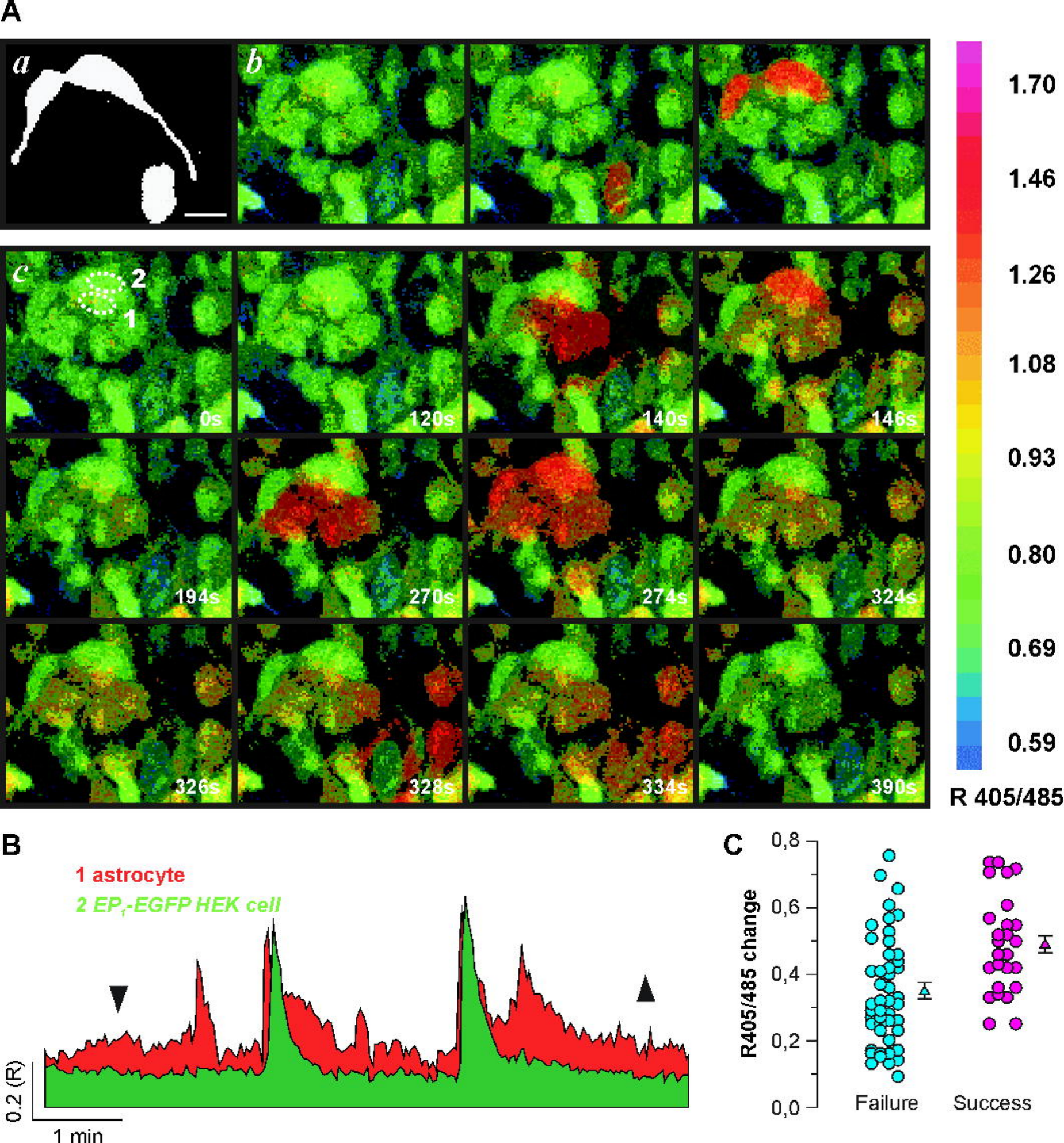
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1.70
1.46
1.26
1.08
0.93
0.80
0.69
0.59
R 405/485
B
1 astrocyte
2 EP$_1$-EGFP HEK cell
0.2 (R)
1 min
C
R405/485 change
0,8
0,6
0,4
0,2
0,0
Failure
Success

A

responsive HEK cells (%)
0
10
20
30
40
50
EP2-EGFP HEK
EGFP HEK

B

Indomethacin
0.2 (R)
2 min
40 min
40 min