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

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The synaptic release of glutamate evokes in astrocytes periodic increases in $[Ca^{2+}]_i$, due to the 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 signalling 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 hyperaemia. 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 of vasoactive prostaglandins, such as the potent vasodilator prostaglandin E_2 (PGE_2), from astrocytes, we plated wild-type human embryonic kidney (HEK)293 cells, which respond constitutively to PGE_2 with $[Ca^{2+}]_i$ elevations, onto cultured astrocytes, and used them as biosensors of prostaglandin release. After loading the astrocyte–HEK cell co-cultures with the calcium indicator Indo-1, confocal microscopy revealed 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 that are 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 hyperaemia.

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Since they were first described, astrocytes were thought 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 have revealed their ability to respond with $[Ca^{2+}]_i$ elevations to the synaptic release of neurotransmitters (Porter & McCarthy, 1996; Pasti *et al.* 1997), mainly mediated by the activation of group I metabotropic glutamate receptors (mGluRs; Pasti *et al.* 1997) and $GABA_B$ receptors (Kang *et al.* 1998), both of which are 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 signalling may thus represent a refined communication system that 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 signalling? 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 to the coupling between neuronal activity and blood flow, the so-called functional hyperaemia (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). However, whether or not $[Ca^{2+}]_i$ oscillations can regulate prostaglandin (PG) release from these cells and what spatiotemporal properties this release possesses, are issues that have yet to be resolved. 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 PGE_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 PG 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 calcium-dependent enzyme phospholipase A_2. The calcium dependency of PG production raises the question of whether a cell integrates the calcium signals, generating a sustained PG synthesis and release, or retains their temporal coding, resulting in a release that follows faithfully the kinetics and general pattern of the $[Ca^{2+}]_i$ changes. All of the studies designed to analyse the synthesis and/or release of COX products in eukaryotic cells have so far failed to provide information about the spatiotemporal 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 populations (Bezzi *et al.* 1998). Our experimental approach allowed us to monitor the process of PG release at the level of single, living cells. We provide here 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 performed in strict accordance with the Italian and EU regulation on animal welfare, and were authorized by the Italian Ministry of Health. Neonatal Wistar rats were deeply anaesthetized by a cocktail of xilazina, tiletamina and zolazepan and then killed by cervical dislocation. Primary cultures of cortical astrocytes were prepared as described previously (Pasti *et al.* 1995). Fourteen days after plating, cultured cells were subjected to 15 h of continuous shaking and then incubated for 5 min with 0.2 % trypsin. Detached cells were collected and replated on poly-L-lysine-coated coverslips that were 24 mm in diameter. In all experiments, 2-week-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 fluorescein-isothiocyanate-labelled lectin from *Lycopersicon esculentum* (Sigma; Boucsein *et al.* 2000). Astrocytes were identified using an antibody raised against glial fibrillary acidic protein (Boehringer). Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA, USA) were co-transfected with either the cDNAs encoding the NMDA receptor (NMDAR) subunits 1–2A and green fluorescent protein (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), which was kindly provided by Dr Mark Abramovitz (Department of Biochemistry and Molecular Biology, Merck Frosst Canada), and the enhanced form of GFP (EGFP). These HEK cells express neither ionotropic glutamate receptors nor mGluRs (Pasti *et al.* 2001). HEK cells were trypsinized after 7–9 h of transfection and replated on astrocyte cultures. For confocal microscope experiments, after 1–3 days, co-cultures were incubated with the calcium 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 wild-type HEK (wtHEK) cells, and a clone that 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 (Bristol, UK). PGE_2, SC19220 and AH6809 were from BioMol (Plymouth Meeting, PA, USA); indomethacin was from Sigma.

Confocal microscopy

Digital fluorescence microscopy (Nikon, RCM8000) was used to monitor the change in Indo-1 emission after cell loading with Indo-1 AM (Molecular Probes, Eugene, OR, USA) as described previously (Pasti *et al.* 2001). After excitation at a wavelength of 351 nm, the emitted light was separated into its two components (405 and 485 nm), and the ratio (R405/485) was displayed as a pseudocolour scale. During experiments, cultured cells were perfused continuously (1.5–3 ml min^{-1}) with an extracellular solution consisting of (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. The sampling rate was 2 s. Less than 6 s of delay between $[Ca^{2+}]_i$ increases in the astrocyte and those in the adjacent transfected HEK cell were considered to be indicative of temporal correlation.

Electrophysiology

Standard procedures were used for pipette preparation and patch-clamp recordings in the whole-cell configuration. During experiments, cells were perfused continuously with the same extracellular solution (without sulfinpyrazone) as was used for confocal microscope experiments. The recording pipette contained (mM): 145 potassium 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 Instruments) 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 analysed 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 comprising an astrocyte and a nearby GFP-fluorescent HEK cell transfected with the PG receptor EP_1.

RESULTS

Astrocyte stimulation triggers D-aminophosphonopentanoic acid (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 HEK293 cells transiently transfected with the specific receptor for the molecule under study (Pasti *et al.* 2001). Co-transfection with 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 calcium-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 calcium 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 spatiotemporal 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 have shown previously that $[Ca^{2+}]_i$ oscillations triggered by L-quisqualate, an agonist of both mGlu and AMPA receptors, regulate a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (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-AP5 failed to block $[Ca^{2+}]_i$ elevations in sensor cells (Fig. 1*A*). 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 %, five 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 calcium-mobilizing compounds that can be released by activated astrocytes, PGs appear to be 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 wtHEK cells (43.6 %, 48 of 110) display $[Ca^{2+}]_i$ elevations when directly stimulated with PGE_2. To

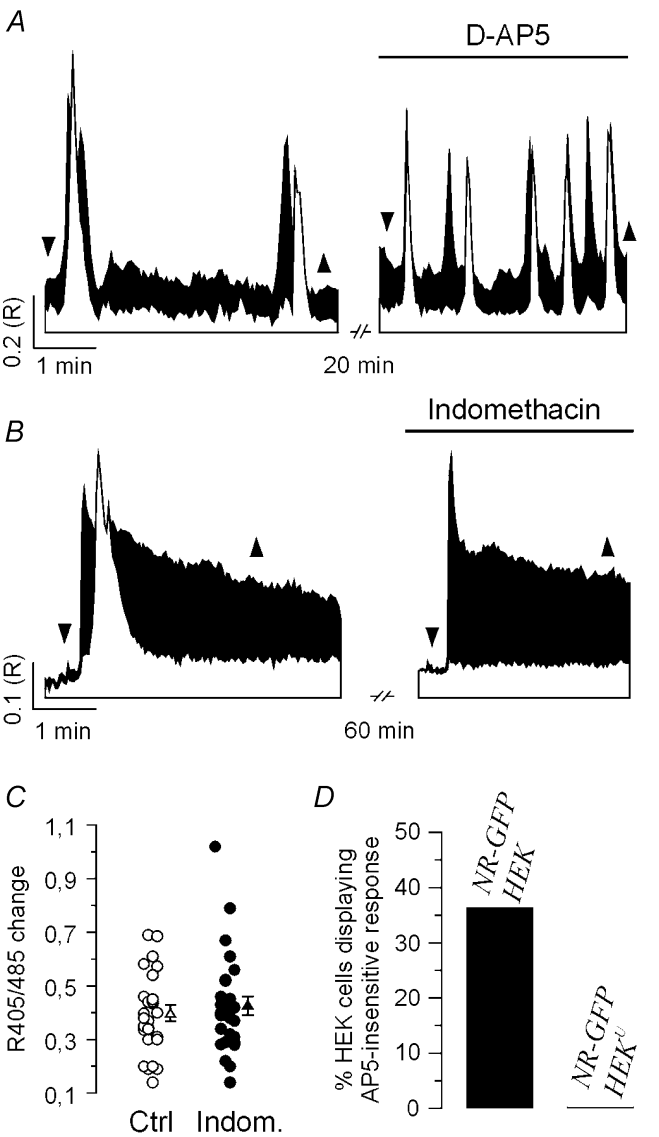

Figure 1. Activated astrocytes release a compound different from glutamate, most probably a prostanoid

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 (white area) upon two successive astrocyte stimulations with 10 μM 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 stimulations with 20 μM L-quisqualate. The second challenge with L-quisqualate was performed after incubation of the cell 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, $n = 15$).

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. 1*B*). L-quisqualate triggered in astrocytes $[Ca^{2+}]_i$ increases of comparable amplitude (mean ± S.E.M. change in R405/485 ($n = 27$): 0.39 ± 0.03 and 0.42 ± 0.04, before and after indomethacin, respectively; Fig. 1*C*).

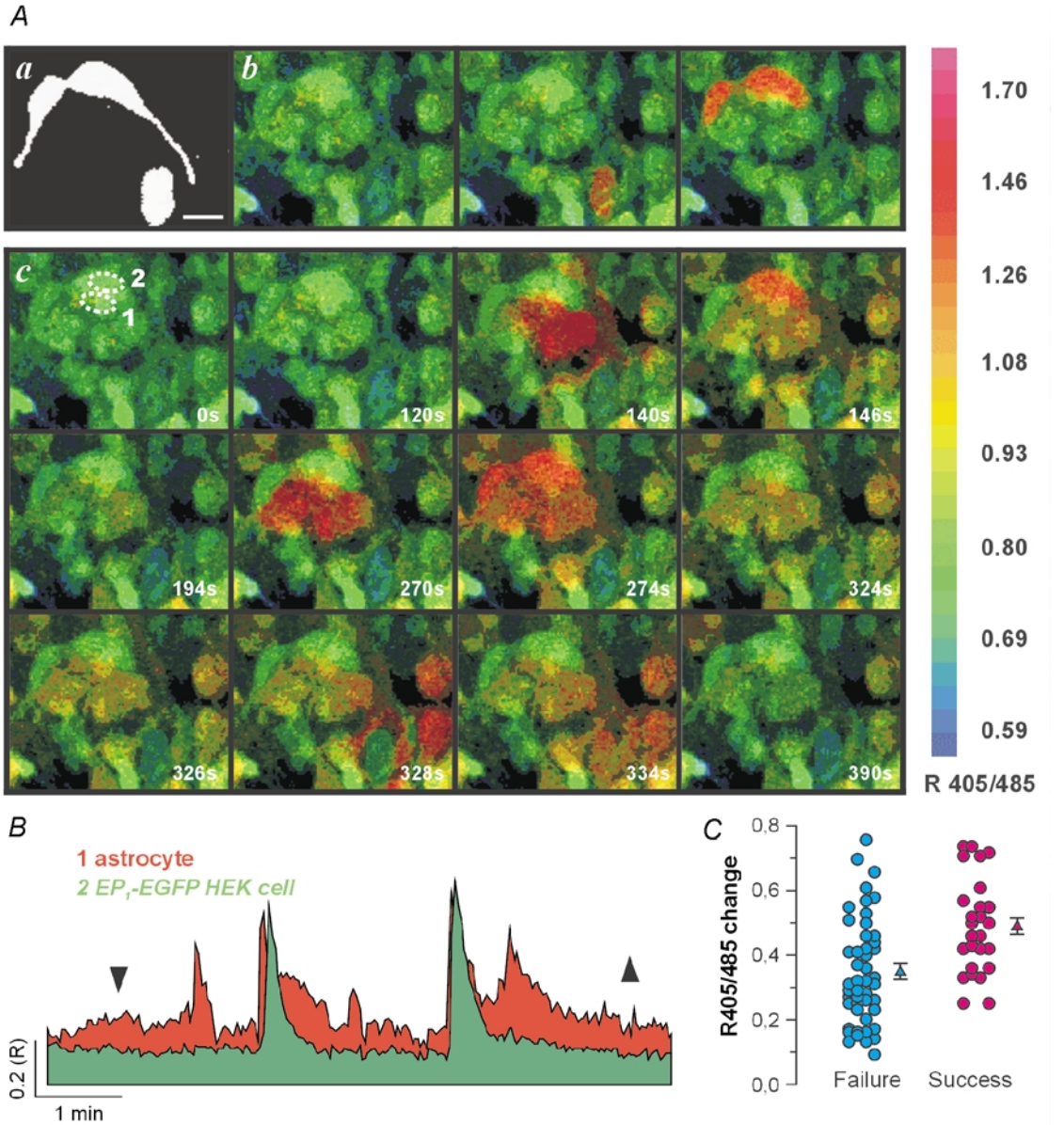

Figure 2. $[Ca^{2+}]_i$ oscillations in astrocytes trigger correlated $[Ca^{2+}]_i$ elevations in PGE_2-sensor cells

A, three EP_1-EGFP HEK cells are easily identified among astrocytes by their fluorescence at an excitation wavelength of 488 nm (*a*) and by their clear $[Ca^{2+}]_i$ increase in response to 100 nM PGE_2 (*b*). The sequence of pseudocolour 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). The timing of the frame acquisition is reported at the 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 comprising 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).

Astrocyte stimulation fails to trigger D-AP5-insensitive responses in the 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, HEK^U cells. These cells were transfected with the NMDAR and then plated on astrocytes. In contrast to what observed in wtHEK cells, $[Ca^{2+}]_i$ elevations triggered in NMDAR-transfected HEK^U cells ($n = 15$) by astrocyte stimulation were regularly blocked by D-AP5 (Fig. 1*D*), suggesting that the D-AP5-insensitive responses observed in wtHEK cells were due to a molecule acting on a PG receptor.

EP$_1$-transfected HEK^U cells recover the response to astrocyte stimulation

To prove directly that glutamate-receptor-mediated $[Ca^{2+}]_i$ oscillations regulate the release of a PG from astrocytes, HEK^U cells were transfected with the specific PGE_2 receptor EP$_1$ (EP$_1$R), and with EGFP (EP$_1$-EGFP HEK^U). EP$_1$-EGFP HEK^U cells, either plated alone or on astrocytes, exhibited $[Ca^{2+}]_i$ elevations upon stimulation with PGE_2 (Fig. 2*A*, panel *b*). Panel *a* of Fig. 2*A* shows three EP$_1$-EGFP HEK^U cells plated on cultured astrocytes, easily recognizable when illuminated at the excitation wavelength of EGFP (488 nm). The same cells are visible among the astrocytes in panels *b* and *c*, when illuminated at the excitation wavelength of Indo-1 (351 nm). Stimulation with 10 μM L-quisqualate (panel *c*) triggered repetitive $[Ca^{2+}]_i$ increases in the astrocyte labelled 1, as well as in other astrocytes. The adjacent EP$_1$-EGFP HEK^U cell (labelled 2) also displayed two successive $[Ca^{2+}]_i$ elevations, which were temporally correlated with those observed in the astrocyte (Fig. 3*A* and *B*; see also movies available as Supplementary Material). In addition, the other two EP$_1$-EGFP HEK^U cells visible in the field raised their $[Ca^{2+}]_i$ in temporal correlation with $[Ca^{2+}]_i$ increases occurring in nearby astrocytes (see Supplementary Material). Similar results were obtained using the mGluR agonist *t*-ACPD. Of the 148 EP$_1$-EGFP HEK^U cells that were analysed, 57 exhibited clear responses to astrocyte stimulation. In 84 % of the responding EP$_1$-EGFP HEK^U cells, $[Ca^{2+}]_i$ 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 Fig. 2*C*, 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 the low-amplitude $[Ca^{2+}]_i$ peaks, 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 comprising an astrocyte and an adjacent EP$_1$-EGFP HEK^U cell. Voltage pulses, which were always applied to either the astrocyte or the EP$_1$-EGFP HEK^U cell, were never observed to spread to the other cell (Supplementary Fig. 1*A*). In all pairs ($n = 5$), the stimulation protocol was applied repetitively for at least 20 min. In three pairs, the voltage steps were also applied after stimulation with 20 μM *t*-ACPD (Supplementary Fig. 1*B*). We never observed signs of electrical coupling between the astrocyte and the EP$_1$-EGFP HEK^U cell.

The commercially available EP$_1$R antagonists SC19220 and AH6809 (10–100 μM) failed to inhibit the $[Ca^{2+}]_i$ response of EP$_1$-EGFP HEK^U cells 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 concentrations not higher than 1 nM. Consequently, they could not be considered reliable pharmacological tools to effectively block the EP$_1$ receptors.

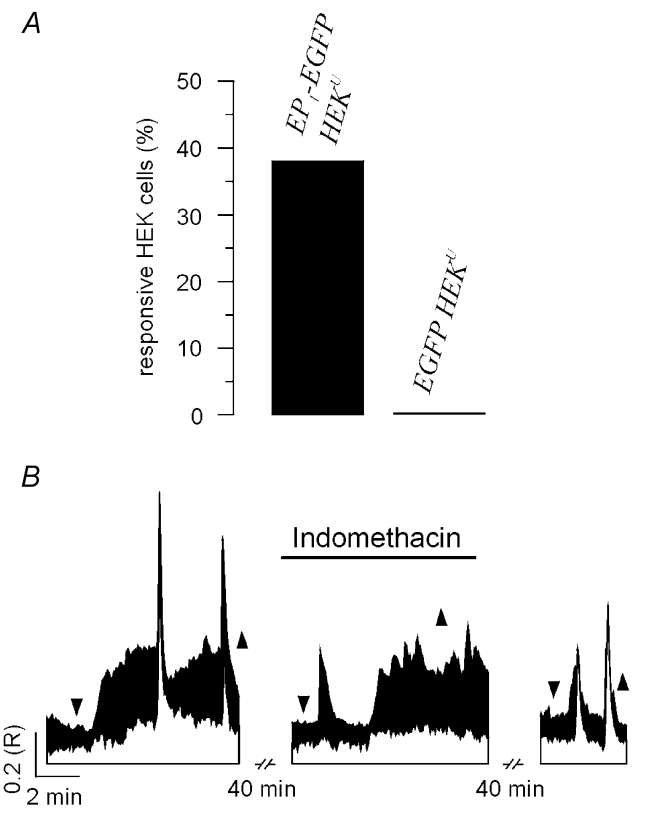

Figure 3. Prostanoid nature of the compound released by activated astrocytes

A, histogram showing the percentage of HEK^U cells displaying $[Ca^{2+}]_i$ responses upon astrocyte stimulation, for EP1-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 EP1-EGFP HEK cell (white area) upon three successive stimulations with *t*-ACPD 20 μM. The second challenge was performed after a 40 min incubation with indomethacin, and the third 30 min after washout of the inhibitor.

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 cells responding to astrocyte stimulation, with that of HEK^U cells not transfected with the EP_1 receptor (EGFP HEK^U). When plated on astrocytes, EGFP HEK^U cells failed, with one exception, to respond to astrocytic oscillations ($n = 74$, Fig. 3*A*). Inhibition of PG synthesis with indomethacin consistently blocked $[Ca^{2+}]_i$ elevations in EP_1-EGFP HEK^U cells following astrocyte stimulation, while washout of the inhibitor allowed the recovery of the response ($n = 5$, Fig. 3*B*).

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 third challenge and found responses in eight cells.

DISCUSSION

PG release from astrocytes is pulsatile

Over the last few years, accumulating experimental evidence has demonstrated that the stimulation of mGluRs induces the calcium-dependent production of arachidonic acid metabolites including PGE_2 (Dumuis *et al.* 1990; Bezzi *et al.* 1998). However, the kinetics and spatial aspects of such a release at the single-cell level could not be determined since prostanoids were measured biochemically in the supernatant of cell cultures. Our experimental approach has allowed us 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 used agonists of glutamate receptors such as L-quisqualate and *t*-ACPD, since HEK cells express neither ionotropic glutamate receptors nor mGluRs (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 is 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, which is 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 NMDARs and were found to be abolished after cell preincubation with the COX inhibitor indomethacin. However, we have shown previously 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 was also dependent on a COX product. In other words, the results from indomethacin experiments do not provide unambiguous evidence that a PG is the factor that acts directly on the sensor cell to trigger the calcium response. Further difficulties regarding 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-EGFP HEK^U cells, only when the PG was used at a concentration 10^5-fold lower. The failure of these antagonists to inhibit the $[Ca^{2+}]_i$ response of EP_1-EGFP HEK^U cells to astrocyte stimulation could simply indicate that the local PG concentration is higher than 1 nM.

It was the use of HEK^U cells (a PGE_2-unresponsive HEK cell clone) that ultimately allowed us to show directly that the ability of wtHEK cells to respond to astrocyte stimulation depends upon the presence of a PG receptor, and thus on the release from astrocytes of a PG. Indeed, the response observed in wtHEK 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 comprising an astrocyte and an adjacent EP_1-EGFP HEK^U cell, we never observed any evidence of 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 surrounding 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 underlie this characteristic: (1) 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 or (2) 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: (1) when high doses of mGluR agonists were used, the $[Ca^{2+}]_i$ change in the astrocytes comprised 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, and (2) 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 that could not be visualized because they were on a different focal plane. Alternatively, these uncorrelated $[Ca^{2+}]_i$ elevations may be due to a slow diffusion of PGs released from astrocytes located far away 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 signalling 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, human immunodeficiency virus-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 role for astrocytes in the control of cerebral microcirculation. We found that the coupling between neuronal activity and blood flow, the so-called functional hyperaemia, depends mainly on mGluR-mediated $[Ca^{2+}]_i$ oscillations in astrocytes and on a COX product. Our present finding suggests that the COX product mediating vasodilatation is released from astrocytes. Indeed, the same mGluR-mediated oscillations that control neuronal-activity-dependent vasodilatation, 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 hyperaemia (i.e. the tight coupling between neuronal activity and blood flow; Zonta *et al.* 2003). An important implication of the observations reported here is related to the time course of functional hyperaemia. While there is a general agreement on the strict relationship that exists 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 dilatation of cerebral arterioles, which guarantees a blood flow increase in the region of high neuronal activity, is maintained as long as a high level of 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 short half-life 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 the 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.

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Supplementary material

The online version of this paper can be found at:

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and contains a figure entitled:

Astrocytes and EP1-EGFP HEK^U cells are not electrically coupled,

and three movies entitled:

Time-lapse movies displaying the correlation between $[Ca^{2+}]_i$ elevations in the astrocytes and in each EP1-EGFP HEK^U cell shown in Fig. 2 of the manuscript.