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Research Article

EP_2 receptor stimulation promotes calcium responses in astrocytes via activation of the adenylyl cyclase pathway

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Abstract. Astrocytes are a heterogeneous population of cells that are endowed with a great variety of receptors for neurotransmitters and neuromodulators. Recently prostaglandin E_2 has attracted great interest since it is not only released by astrocytes but also activates receptors coupled to either phospholipase C or adenylyl cyclase. We report that EP_2 receptor stimulation triggers cAMP production but also causes release of Ca^{2+} from intracellular stores. This effect is shared by other receptors similarly coupled to adenylyl cyclase and elicited by direct stimulation of the enzyme or application of cAMP analogues. However, the stimulation of the Ca^{2+} response by cAMP is not mediated by protein kinase A, since a specific antagonist of this kinase had no effect. Such a cross-talk between cAMP and Ca^{2+} was not observed in all astrocytes. It might therefore reflect a specific resource of either a subpopulation or astrocytes in a specific functional state.

Keywords. Prostaglandin E_2, butaprost, sulprostone, isoproterenol, forskolin, cAMP.

Introduction

The arachidonic acid pathway has long been recognized as one of the main mechanisms for the induction of pain and inflammation. In particular, cyclooxygenase 2 (COX2), the inducible isoform of cyclooxygenase, is known to be rapidly up-regulated at inflammation sites giving rise to pro-inflammatory prostanoids [1, 2]. One of the products of this arachidonic acid metabolism, prostaglandin E_2 (PGE2), is an important mediator of a number of effects in the central nervous system, including pain sensation, sleep-wake cycle, and temperature regulation. Brain PGE2 has recently attracted fresh attention since it was reported to play an important role in intercellular communication. PGE2 can be released not only by activated microglia, where it is supposed to perform an autocrine/paracrine feedback loop able to influence the same activation state of the cells [3], but also by astrocytes. In fact, chronic stimulation promotes specific phenotypical changes in astrocytes, referred to as 'activation' [4], that include COX2 induction with ensuing release of PGE2 [5]. Astrocyte-derived PGE2 can, in turn, act back on microglia [6]. Therefore, PGE2 has an important role in the signaling interplay between microglia and astrocytes under physiological and pathological conditions. In addition, PGE2 has been suggested to play a role also in the functional coupling between neurons and astrocytes, exerting a stimulatory influence on neuronal activity [7, 8], and to have either a neuroprotective [9–12] or a neurotoxic [13] effect, depending on its concentration. In astrocytes, PGE2 was reported to increase the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by acting on a number of cognate receptors [14–16], and to mediate the release of both glutamate [17, 18] and neurotrophic factors [19].

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On the other hand, a $[Ca^{2+}]_i$ increase in astrocytes can be sufficient to release PGE2 [17, 20] and to determine vasodilation of arterioles [21].

This body of findings points toward a loop of auto/paracrine effects, with PGE2 that, once released, can promote $[Ca^{2+}]_i$ elevation in astrocytes, via receptor activation, thereby amplifying the responses, also involving other mediators [17, 22]. More obscure is the effect of chronic PGE2 treatment, which has been proposed to either stimulate [23, 24] or inhibit [25] astrocyte proliferation. All these effects of PGE2 in astrocytes rely on the expression of the whole family of EP receptors [26] and the activation of two main intracellular signaling pathways. In fact, PGE2 can determine the production of either cyclic adenosine-3′,5′-monophosphate (cAMP), via EP_2 and EP_4 receptors [27], or inositol-1,4,5-trisphosphate (IP_3)-mediated $[Ca^{2+}]_i$ increases via EP_1 and EP_3 receptors [16].

In this study, we investigated the signal transduction pathways activated by PGE2 in astrocytes. Using selective agonists for EP receptors, we provide evidence for a cross-talk between the two main pathways, with cAMP elevations able to promote Ca^{2+} release from intracellular stores.

Materials and methods

Materials. Cell culture media and reagents, if not otherwise stated, were from Cambrex (East Rutherford, NJ, USA). Culture flasks were from Nalge Nunc (Rochester, NY, USA) and multiwell plates from Corning (Corning, NY, USA). Sulprostone, butaprost and anti-COX2 (murine) polyclonal antibody were from Cayman (Ann Arbor, MI, USA). Forskolin, 8-bromo-adenosine-3′,5′-cyclic-monophosphate (8Br-cAMP), and fura-2/AM were from Calbiochem (La Jolla, CA, USA). The Epac1 activator 8-CPT-2′-O-Me-cAMP was from Biaffin (Kassel, Germany). Recombinant rat interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) were from R&D Systems (Minneapolis, MN, USA). PGE2, isoproterenol and other chemicals for general use were from Sigma-Aldrich (St Louis, MO, USA).

Cell culture. The animal use procedures were approved by the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute. Primary cultures of cortical astrocytes were established from 1–2-day-old Sprague-Dawley rats according to a standard procedure [28]. Briefly, cortices were freshly dissected, cut into small pieces and washed in Hanks' balanced salt solution supplemented with 10 mM HEPES/Na pH 7.4, 12 mM $MgSO_4$, 50 U/ml penicillin and 50 µg/ml streptomycin. Tissue dissociation was performed with trypsin (2.5 mg/ml trypsin type IX, in presence of 1 mg/ml DNase; 10 min at 37 °C) in two subsequent steps and terminated by 1 : 1 dilution in serum-containing medium. After centrifugation (100 *g*, 10 min) cells were plated in 75-cm^2 flasks with Eagle's minimum essential medium supplemented with 10% donor horse serum, 33 mM glucose, 2 mM glutamax (Gibco, Grand Island, NY, USA), 50 U/ml penicillin and 50 µg/ml streptomycin, and kept at 37 °C in an humidified 5% CO_2 atmosphere. The medium was changed the day after plating and every 3–4 days. When cell cultures reached confluence (10–15 days), astrocytes were obtained by shaking flasks at 230 rpm for 24 h at 37 °C and re-plating attached cells on poly-L-lysine-coated (150 µg/ml) glass coverslips for videoimaging, or on plastic multiwells for biochemical experiments. Cells were used within 1 week after re-plating. This experimental condition produced cultures highly enriched in glial cells that could be classified as type I astrocytes based on their flat morphology (> 95%). The characterization of our cultures with antibodies against specific markers for the various astrocytic phenotypes (such as GFAP, A2B5 and O4) confirmed that our shaking protocol ensures the removal of most of O2A precursors as well as type II astrocytes making their presence negligible (< 1%).

Videoimaging of $[Ca^{2+}]_i$. Sub-confluent astrocytes plated on poly-L-lysine-coated glass coverslips were washed in Krebs-Ringer solution buffered with HEPES (KRH) containing: 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 6 mM glucose, 25 mM HEPES/Na pH 7.4. For dye loading the cells were incubated for 45 min at room temperature with 4 µM fura-2/AM in KRH solution supplemented with 0.02% pluronic F-127. After washing, the coverslips were mounted in a recording chamber placed on the stage of an inverted Axiovert 135 TV microscope (Zeiss, Oberkochen, Germany). The cells were alternately excited at 340 and 380 nm by a modified CAM-230 dual wavelength fluorimeter (Jasco, Tokyo, Japan) and the fluorescence images were captured by a low-light level CCD camera (ISIS-Photonic Science, Robertbridge, UK) with 2-s delay between the 340/380 couple of images. The Openlab software (Improvision, Coventry, UK) was used to control the protocol of acquisition and to perform the analysis of the data. Drugs were applied directly in the chamber in an appropriate volume (1:10 dilution) to ensure rapid mixing. No renewal of the solution in the chamber was performed. The experiments in the absence of extracellular Ca^{2+} were performed in KRH containing no Ca^{2+} and supplemented with 0.1 mM EGTA added immediately before the beginning of the acquisition. Only cells with astrocytic morphology were analyzed.

Fura-2 data analysis. The fluorescence values of $[Ca^{2+}]_i$ (340/380 fura-2 measurements) are expressed as ratio. At least 20 coverslips (40 for butaprost stimulation) with

5–25 cells per field of observation were analyzed. For the simultaneous measurement of $[Ca^{2+}]_i$ in multiple fields on the same coverslip, we used the visiting point feature of a DeltaVision setup (Applied Precision, Issaquah, WA, USA). More than 800 cells (20–40 per microscopic field, 5 fields per experiment, 4–6 experiments per agonist) were analyzed.

For each set of experiments either the number or the percentage of responsive cells are indicated. Assessment of the presence/absence of an effect was based on comparison of the amplitude of signal shifts, following the application of a stimulus, with the fluctuations of the baseline. The effect was considered to be present when the response amplitude exceeded three times the baseline RMS (root-mean-square departure). Mean 'time to peak' and mean 'time for 50% decay', were used to describe the kinetics of $[Ca^{2+}]_i$ transients.

In a series of experiments, an evaluation of $[Ca^{2+}]_i$ was performed by treating cells with the Ca^{2+} ionophore ionomycin. The relation between 340/380 and $[Ca^{2+}]$ was linear for ratio values from ~0.5 to ~4.0 (corresponding to an estimated $[Ca^{2+}]_i$ of ~95 nM and ~1100 nM, respectively).

Intracellular cAMP measurement. Intracellular cAMP levels were determined by an enzyme-immunoassay kit (Amersham Biosciences, Uppsala, Sweden). Astrocytes cultured in 96-well microplate were washed with KRH and incubated with the specific agonist for 10 min at room temperature in 100 µl of the same buffer containing 0.1 mM 3-isobutyl-1-methylxanthine. After removal of the extracellular buffer, the reaction was terminated by addition of lysis reagent and cAMP detected according to manufacturer's instructions.

Western blotting. Astrocytes, grown in 6-well multiwell plates, were treated with cytokines (10 ng/ml IL-1β and 30 ng/ml TNFα, for 24 h) in the presence of 10% donor horse serum, washed twice with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer pH 7.4) and lysed for 15 min at 4 °C with 300 µl/wells of lysis buffer (PBS supplemented with 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 10 mM EDTA/Na pH 8, and chymostatin, leupeptin, antipain, pepstatin, 10 µg/ml each). Lysates were centrifuged for 15 min at 10 000g at 4 °C, the supernatants were collected and their total protein content analyzed by the micro BCA reagent (Pierce, Rockford, IL, USA). About 25 µg protein was separated by SDS-PAGE and transferred onto an nitrocellulose membrane. After overnight blocking at 4 °C in PBS containing 0.2% Tween-20 and 5% skimmed milk, the membrane was incubated for 2 h with anti-COX2 antibody and, after washing, with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA, USA). COX2 protein was then revealed on auto-radiographic films by Super Signal West Substrate enhanced chemiluminescence (Pierce).

PGE2 release. For detection of extracellular PGE2, 50 µl of culture media from control and cytokine-activated astrocytes were properly diluted and their PGE2

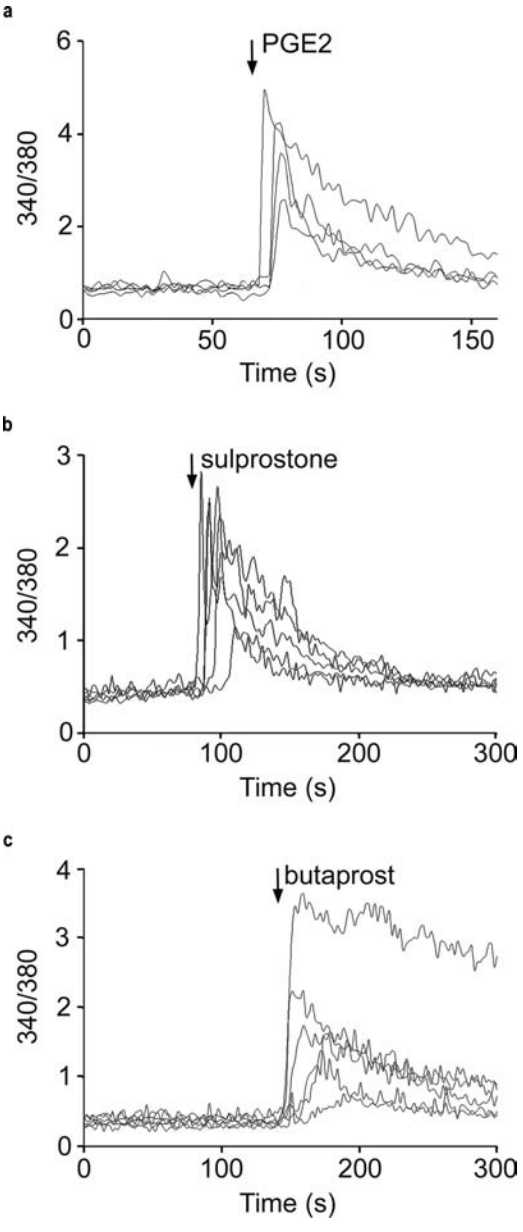

Figure 1. $[Ca^{2+}]_i$ elevations induced by PGE2 and selective agonists of EP receptors in rat cortical astrocytes. PGE2 stimulation (1 µM) promotes $[Ca^{2+}]_i$ increases in astrocytes (*a*). In this and the following figures, individual traces represent the $[Ca^{2+}]_i$ responses measured by fura-2 videomicroscopy in single cells from the same field in a representative experiment. Variations in $[Ca^{2+}]_i$ over time are represented by the ratio between the fluorescence intensities at 340 and 380 nm excitation wavelengths. The temporal analyses show the $[Ca^{2+}]_i$ responses to 100 nM sulprostone, an agonist of the EP_1-EP_3 receptors (*b*), and 100 nM butaprost, an agonist of the EP_2 receptor (*c*).

content quantified by the use of PGE2 EIA Kit-Monoclonal (Cayman, Ann Arbor, Michigan, USA), according to manufacturer's instructions.

Results

Rat cortical astrocytes were cultured *in vitro* and their $[Ca^{2+}]_i$ was monitored by fura-2 video-imaging. Figure 1 shows the typical $[Ca^{2+}]_i$ increases that were observed when astrocytes were stimulated by 1 μM PGE2 (Fig. 1a). The responses (revealed in 262 cells out of 312) were rapid and transient, thereby suggesting an involvement of intracellular Ca^{2+} stores. Indeed, when PGE2 was administered in the absence of extracellular calcium the responses were maintained (data not shown). We then investigated the nature of the receptors responsible for the observed PGE2-induced $[Ca^{2+}]_i$ increase. An agonist of both EP_1 and EP_3 receptors was first employed since these two receptors are known to be coupled with IP_3 production, and thus to be potentially responsible for $[Ca^{2+}]_i$ release from intracellular stores. In line with this prediction, specific stimulation of these receptors with 100 nM sulprostone produced $[Ca^{2+}]_i$ increases (in 240 cells out of 325) that were undistinguishable from those elicited by PGE2 (Fig. 1b). We next used butaprost, an agonist specific for the EP_2 receptors, *i.e.* those coupled to cAMP production. Unexpectedly, astrocytes exposed to 100 nM butaprost, a concentration known to selectively activate EP_2 receptor, induced (in 115 cells out of 530) $[Ca^{2+}]_i$ elevations (mean ratio value = 280% over basal) that were comparable to those observed after stimulation of EP_1 and EP_3 receptors (Fig. 1c). However, the kinetics of the two responses were slightly different with the butaprost-induced $[Ca^{2+}]_i$ responses somewhat slower in the rising phase (mean time to peak = 15 s) and more sustained (mean time for 50% decay = 90 s) than those induced by sulprostone (mean time to peak = 3 s; mean time for 50% decay = 35 s). Intracellular cAMP was determined in astrocytes after 10 min exposure to PGE2 and butaprost. Both agonists were able to promote a concentration-dependent cAMP increase in astrocytes, although PGE2 was more efficient, possibly because of the involvement of EP_4 receptors (Fig. 2). Even at maximal stimulation,

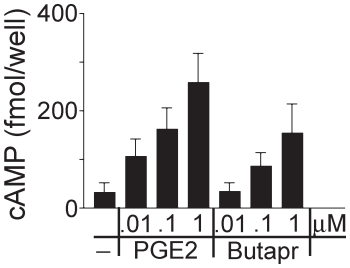

Figure 2. Concentration dependence of cAMP increases in cortical astrocytes with PGE2 and butaprost. cAMP levels were determined in cells after 10 min without (–) or with stimulation by increasing concentrations of PGE2 or butaprost ($n = 4$, bars represent SD).

cAMP levels were one order of magnitude lower than those obtained with forskolin, a direct activator of adenylyl cyclase (not shown).

The effect of butaprost on $[Ca^{2+}]_i$ homeostasis could be explained by either a promiscuous coupling of the EP_2 receptor with heterotrimeric G proteins other than G_s, or a cAMP-mediated mechanism. Interestingly, a $[Ca^{2+}]_i$ response was observed even in 62 astrocytes (out of 212) exposed to isoproterenol, a drug that stimulates G_s-mediated cAMP increase by acting on the β-adrenergic receptors (Fig. 3a). To evaluate whether elevation of cAMP was *per se* able to induce $[Ca^{2+}]_i$ elevations, astrocytes were treated with either forskolin or 8Br-cAMP, a membrane-permeant cAMP analog (Fig. 3b, c). A $[Ca^{2+}]_i$ increase was observed under both experimental conditions (88 cells out of 283 for forskolin and 22 cells out of 180 for 8Br-cAMP). The responses were unaffected (82 cells analyzed) by a pretreatment with 10 μM H89, a specific protein kinase A inhibitor (Fig. 4a). Moreover, the responses to butaprost, isoproterenol and forskolin were retained in the absence of extracellular Ca^{2+}, indicating they were mainly sustained by Ca^{2+} release from internal stores (Fig. 4b–d).

Since in the above experiments we observed heterogeneity in the responsiveness to the different protocol of stimulation, $[Ca^{2+}]_i$ changes were monitored in multiple fields after a single stimulation (see Materials and methods for details). While sulprostone was consistently able to induce $[Ca^{2+}]_i$ responses in more than 50% of the cells in each microscopic field (mean percentage of respond-

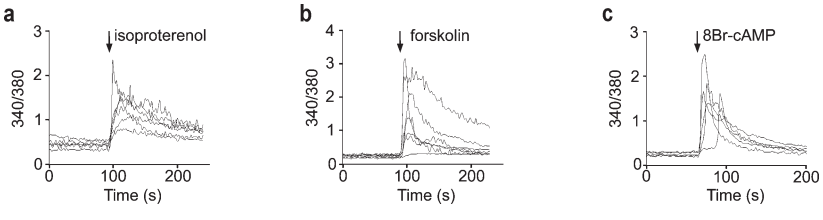

Figure 3. $[Ca^{2+}]_i$ elevations induced by cAMP-modulating agents in astrocytes. Cells were analyzed after stimulation with 1 μM isoproterenol (*a*), 1 μM forskolin (*b*), or 100 μM 8Br-cAMP (*c*).

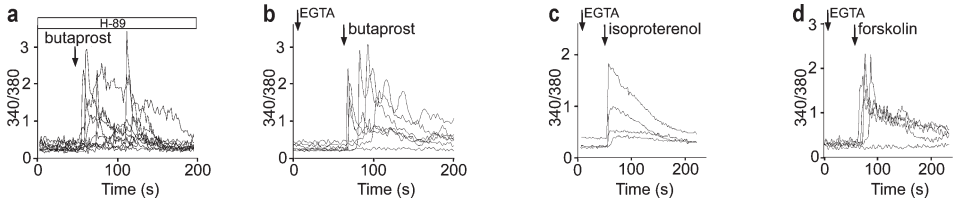

Figure 4. Role of protein kinase A and intracellular Ca^{2+} stores in cAMP-mediated $[Ca^{2+}]_i$ elevations in astrocytes. Cells were pretreated with 10 μM protein kinase A inhibitor H-89 for 30 min and then stimulated with 100 nM butaprost (*a*). In the subsequent panels cells were kept in the absence of extracellular Ca^{2+} (100 μM EGTA added) and stimulated with 100 nM butaprost (*b*), 1 μM isoproterenol (*c*), or 1 μM forskolin (*d*).

ing cells ~60%), after butaprost, cell responsiveness was reduced to ~20%, and never exceeded ~30% in a single field. $[Ca^{2+}]_i$ responses to forskolin were also observed in ~20% of the cells. In few experimental fields, which were not included in the statistical analysis, forskolin-induced $[Ca^{2+}]_i$ responses were present in more than 90% of the cells. However, the onset of the responses was variable, with kinetics compatible to the involvement of soluble mediators (such as ATP) released by stimulated astrocytes, a mechanism that has been reported to account for the propagation of Ca^{2+} waves (see for instance [29]).

We further investigated whether Epac1, a protein reported to link cAMP increases to phospholipase Cε activation [30–32], was involved. When cells were exposed to 10 μM 8-CPT-2′-O-Me-cAMP, a direct activator of Epac1, a $[Ca^{2+}]_i$ response was observed in a fraction of astrocytes (about 10%, 20 cells out of 160). Most of the responsive cells (~80%) were responsive also to butaprost stimulation (Fig. 5).

Since COX2-dependent PGE2 release was proposed to promote an autocrine/paracrine amplification of $[Ca^{2+}]_i$ responses in astrocytes [22], we investigated whether the observed variability could be ascribed to the presence of a subset of cells expressing COX2. We exposed our cultures for 24 h to a mix of IL-1β and TNFα (10 ng/ml and 30 ng/ml, respectively), a protocol that is known to bring astrocytes to a state of activation characterized by increased COX2 expression [33]. As expected, we obtained a strong elevation in COX2 expression accompanied by increased PGE2 release (Fig. 6a). However, this treatment changed neither the percentage of responding cells nor the intensity of $[Ca^{2+}]_i$ responses mediated by butaprost, isoproterenol or forskolin (data not shown), thereby suggesting that a mechanism of PGE2-mediated amplification does not play a major role. In line with this indication, no increase in PGE2 release was detected when activated astrocytes were exposed to agents that are known to mobilize $[Ca^{2+}]_i$ in almost all the astrocytes in the culture, such as ATP and glutamate (Fig. 6b). However, we cannot rule out that, under different experimental conditions, *i.e.* when release of PGE2 may act in an autocrine/paracrine

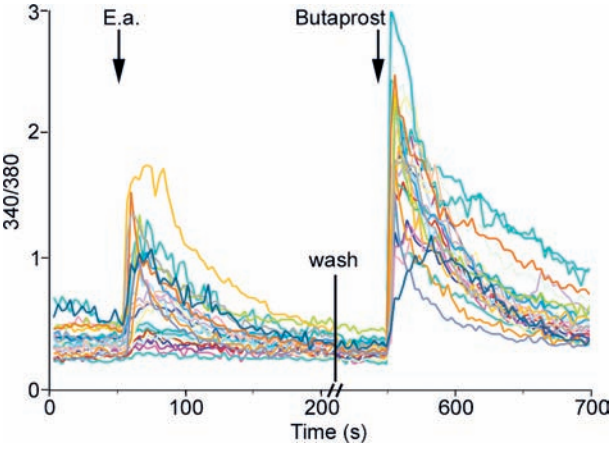

Figure 5. Epac activation induces $[Ca^{2+}]_i$ elevations in astrocytes. Cells were stimulated with 10 μM 8-CPT-2′-O-Me-cAMP (E.a.) and, after washing and a 5-min wait, with 100 nM butaprost. Traces, representing single cells, are in various colors to better appreciate variability and the relationship between the responses to the two stimulations.

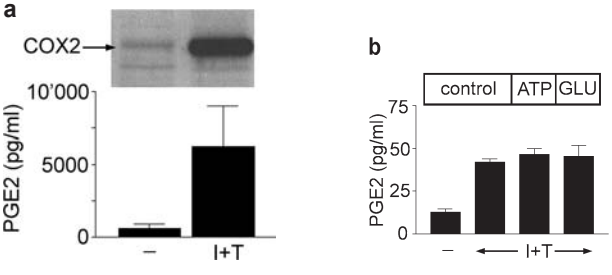

Figure 6. COX2 induction and PGE2 release in resting and activated astrocytes. (*a*) COX2 induction (measured in the cell lysate) and total production of PGE2 (determined in the culture medium) were detected after 24 h in the absence (–) or presence (I + T) of IL-1β and TNFα (10 ng/ml and 30 ng/ml, respectively). (*b*) Cells in resting (–) or activated (I + T) condition were washed and then total PGE2 release in the medium was measured within 3 min under control condition or stimulation with either 100 μM ATP (ATP) or 50 μM glutamate (GLU). In both panels the bars represent the SD of at least four independent determinations.

fashion, cAMP-mediated Ca^{2+} responses could be modulated by astrocyte activation.

Discussion

The PGE2 receptors are widely expressed in the central nervous system and the effects of PGE2 largely depend on the nature of the target cells, as well as the receptor subtype that is stimulated. In the last few years, astrocytes have attracted considerable attention because of their capability not only to respond to, but also to release neurotransmitters and neuromodulators [34]. This dual nature of astrocytes is well represented by PGE2, since this molecule is released under conditions of both acute and chronic stimulation, but also acts as an autocrine and paracrine stimulus [22]. Taking into account the close association of astrocytes with both neurons and endothelial cells of the blood-brain barrier, it is evident that PGE2 plays extremely important roles. The aim of the present work was the definition of the intracellular signaling pathways activated in astrocytes after PGE2 exposure.

PGE2 is reported to raise levels of cAMP and $[Ca^{2+}]_i$, independently, via the activation of specific receptors that are known to be expressed in astrocytes [26]: EP_2 and EP_4, coupled to adenylyl cyclase; or EP_1 and EP_3, coupled to phospholipase C. Here we show that, in a fraction of cells, stimulation of the EP_2 receptors promotes not only cAMP but also $[Ca^{2+}]_i$ increases. An uneven responsiveness is not surprising since astrocytes are reported to be a heterogeneous population [35], endowed with different signaling machineries [36]. Such heterogeneity might also be determined by the functional state of the cells. In fact, it is well known that the time in culture, the degree of confluence, and the exposure to various animal sera, are able to influence the gene expression programs of cultured astrocytes. In particular, the activation state of astrocyte cultures was reported to be important to determine heterogeneity [33]. However, our data show that the heterogeneity of cAMP-dependent $[Ca^{2+}]_i$ responses in astrocytes was not affected by treatment with cytokines, a protocol widely used to mimic the microglial-dependent activation. Whatever its origin, heterogeneity can, to some extent, explain contradictory results obtained with PGE2-stimulated astrocytes [25]. For instance, PGE2 was reported to induce cAMP increases in type II (star morphology) but not in type I (flat morphology) astrocytes [37]. In contrast, our cortical astrocytes in culture, classified as type I, responded to PGE2 (and butaprost) with cAMP increases.

More unexpected was the evidence that stimulation of the EP_2 receptors, coupled to adenylyl cyclase, also promoted a $[Ca^{2+}]_i$ increase. This effect was not specific for PGE2, since other conditions that increase cAMP, via receptor stimulation such as isoproterenol but also calcitonin gene-related peptide (S. Morara, personal communication) are able to induce $[Ca^{2+}]_i$ responses. Moreover, direct activation of adenylyl cyclase by forskolin or exposure to cAMP analogues also promoted $[Ca^{2+}]_i$ elevation. An increase in cAMP had already been proposed to account for $[Ca^{2+}]_i$ responses in isoproterenol-stimulated cortical astrocytes [38], but the $[Ca^{2+}]_i$ increase was ascribed to a Ca^{2+} influx from the extracellular space. In our experimental conditions, we demonstrate unambiguously that the cAMP-induced $[Ca^{2+}]_i$ increase is sustained by release of Ca^{2+} from intracellular stores. To account for such a discrepancy, it should be considered that the concentrations used in the previous study were 100-fold higher than those employed by us, a condition that might trigger activation of additional mechanisms. It remains to be explained how cAMP can lead to $[Ca^{2+}]_i$ increase. We cannot rule out the occurrence of a promiscuous coupling of EP_2 with G proteins other than G_s, as reported for dopamine or adenosine receptors [39–41]. However, our data strongly suggest that the effect of butaprost on $[Ca^{2+}]_i$ is a consequence of cAMP elevation. More precisely, it appears to be a direct effect of cAMP, since the lack of effect of H89, a specific protein kinase A inhibitor, rules out the participation of this enzyme in the process.

While the cross-talk involving Ca^{2+}-dependent cAMP modulation is well established [42], the effects of cAMP elevations on $[Ca^{2+}]_i$ is more obscure. In this study, we investigated the possibility that the activation was due to the involvement of the recently identified phospholipase Cε, an enzyme that was reported to be responsible for the cAMP-induced Ca^{2+} release from intracellular stores [43]. Our hypothesis was fully supported by the evidence that a direct activator of Epac-1, a protein reported to link cAMP increases to phospholipase Cε activation, was able to induce Ca^{2+} responses in astrocytes and that most of the responsive cells were also responsive to butaprost.

It is difficult to put this unexpected coupling between EP_2 receptor activation and Ca^{2+} signaling in a physiological perspective. It is firmly established that astrocytes possess a form of excitability that is based on calcium signaling [44], and that is triggered by the activation of a variety of ionotropic and metabotropic receptors for neurotransmitters and modulators [45]. This high degree of complexity makes it impossible to describe the synaptic signals as a linear sequence of events. Rather, they should be considered in terms of 'parallel processing' and 'cross-talk' between and within different pathways. This is particularly true under conditions of moderate stimulation in which calcium elevations can be due to the convergence of intracellular signals but can also be responsible for their integration. In fact, on the one hand, calcium release from internal stores can be sustained by the activation of various metabotropic receptors and, on the other hand, the same calcium elevations can exert a synergistic effect on key effectors such as phospholipase C and protein

kinase C. In this respect, the heterogeneous expression of receptor coupled to distinct signaling pathway can represent for astrocytes one of the way to respond to various physiological as well as pathological conditions.

In conclusion, it is widely recognized that Ca^{2+} signals play a fundamental role in a host of functions, and this is particularly relevant for astrocytes since neuronal activity is critically dependent on their chemical excitability. Accordingly, a concomitant activation of cAMP and Ca^{2+} signals can concur to finely modulate the release of transmitters such as glutamate and ATP, which can ultimately contribute to synaptic integration of information [46]. Finally, in view of the interrelationship between COX2 expression, with ensuing PGE2 release, and neurodegeneration [22, 26, 47], we expect that the interplay between these two signaling pathways might influence not only the way astrocytes modulate synaptic activity, but also the fine balance between neuroprotective and neurotoxic action of astrocytes.

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