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Research Overview

Identification of a Novel P2 Receptor Associated With Cyclooxygenase-2 Upregulation and Reactive Astrogliosis

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Strategy, Management and Health Policy				
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ABSTRACT Astrocytes respond to trauma and ischemia with reactive astrogliosis. Although beneficial under certain conditions, excessive gliosis may be detrimental and contribute to neuronal death in neurodegenerative diseases. To evaluate the hypothesis that ATP may act as a trigger of reactive gliosis, we tested α,βmethyleneATP (α,βmeATP) in an in vitro experimental model (rat brain astrocytic cultures), where astrogliosis can be quantified as elongation of astrocytic processes, an event that reproduces one of the main hallmarks of in vivo gliosis. α,βmeATP induced a concentration-dependent elongation of astrocytic processes, an effect which was counteracted by the P2 receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS). Signaling studies revealed that α,βmeATP-induced gliosis is mediated by a G-protein-coupled receptor (a P2Y receptor) characterized by an "atypical" pharmacological profile and coupled to an early release of arachidonic acid. In an earlier study we showed that challenge of cells with α,βmeATP also resulted in upregulation of inducible cyclooxygenase-2 (COX-2), whose activity has been reported to be pathologically elevated in neurodegenerative diseases characterized by inflammation and astrocytic activation. Upregulation of COX-2 by α,βmeATP was causally related to reactive astrogliosis in vitro, since the selective COX-2 inhibitor NS-398 prevented both purine-induced elongation of astrocytic processes and the associated increase in COX-2 protein levels. Preliminary data on the putative receptor-to-nucleus pathways responsible for purine-induced gliosis suggest that upregulation of COX-2 may occur through the protein kinase C / mitogen-activated protein kinase system and may involve the formation of AP-1 transcription complexes. We speculate that antagonists selective for this novel P2Y receptor subtype may represent a new class of neuroprotective agents able to reduce neurodegeneration by counteracting the inflammatory events contributing to neuronal cell death. Drug Dev. Res. 53:148–157, 2001.

Key words: ATP; inflammatory gliosis; neurodegenerative diseases

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INTRODUCTION

Besides their well-known role in supporting neuronal metabolism, astroglial cells are now recognized to play highly specific roles in the central nervous system (CNS) both during development and adulthood [Abbracchio et al., 1995]. During neurogenesis, radial astrocytes, whose fibers attain enormous length in fetal brain, provide guidance to migrating neuroblasts. After completion of neuronal migration and specification of brain areas, these cells are believed to differentiate into mature astrocytes which express receptors, uptake sites, and synthesizing enzymes for a variety of neurotransmitters and polypeptidic growth factors [Abbracchio et al., 1995]. Hence, in the healthy adult brain astroglial cells directly participate in neurotransmission. Another major feature of astroglial cells in mature brain is their ability to both proliferate and differentiate towards a more specialized phenotype ("reactive astrocyte") following different kinds of insults, including acute trauma, hypoxia and seizures, and chronic neurodegeneration and demyelination [Hatten et al., 1991; Ridet et al.; 1997; Neary and Abbracchio, 2001]. Reactive astrogliosis is characterized by profound morphological and functional changes, such as cellular hypertrophy, generation and elongation of cellular processes (stellation), increased expression of the astrocyte intermediate filament protein, glial fibrillary acidic protein (GFAP), and cellular proliferation. There is still debate as to the functional significance of this reaction. Activated (reactive) astrocytes acquire the capability of synthesizing and releasing neurotrophins and pleiotrophins [Neary et al., 1996a], which may contribute to axonal regrowth and guidance in the remodeling of damaged neuronal circuitries and in functional recovery [ibid] during the posttraumatic phase. Moreover, the morphological changes typical of reactive astrocytes (e.g., the elongation of cellular processes) are believed to contribute to isolate the damaged brain area from the surrounding healthy cells, hence limiting the propagation of the "necrotic" wave from the site of injury to neighboring cells. Activated astrocytes also release a variety of potentially toxic metabolites, including nitric oxide and arachidonic acid metabolites, which may contribute to irreversibly kill damaged cells in the necrotic areas, hence rerouting energy and resources to other cells which still retain a possibility for functional recovery [Neary et al., 1996a]. On this basis, it is evident that excessive astrogliosis, a typical feature of several neurodegenerative diseases characterized by a marked inflammatory component (i.e., ischemia, trauma, Alzheimer's and Parkinson's diseases) [Ohtsuki et al., 1996; Dolan et al., 1998; Ho et al., 1999; Blom et al., 1997], may be detrimental and even contribute to brain damage. Dysregulation of astrogliosis may indeed result in propagation of toxic metabolites to healthy cells and in loss of "selective" killing. Moreover, an excess of reactive astrocytes may lead to the formation of the "gliotic scar" that inhibits neurite regrowth and remyelination. It is therefore crucially important to identify the endogenous factors which regulate reactive astrogliosis in order to develop novel pharmacological agents which will enable the control of this phenomenon.

EFFECT OF ATP AND ITS ANALOGS ON ASTROGLIAL CELLS: IN VITRO AND IN VIVO STUDIES

Among the various endogenous agents that have been implicated in the onset and maintenance of reactive astrogliosis, ATP and related nucleotides play a major role. Massive amounts of ATP are released in hypoxic or injured brain areas as a consequence of both release from the cytosolic ATP pool and nucleic acid degradation from dying cells. This leads to a notable elevation in local ATP concentrations which could last for days (or even weeks) after the toxic stimulus [Neary et al., 1996a; Abbracchio et al., 1999; Neary and Abbracchio, 2001].

The effects of extracellular ATP are mediated by two receptor classes, ligand-gated ion channels (P2X receptors) and G-protein-coupled receptors (P2Y receptors) [Dubyak, 1991; Abbracchio and Burnstock, 1994, 1998; Ralevic and Burnstock, 1998]. In the brain, both P2X and P2Y receptors are present [Abbracchio, 1997]. While P2X receptors have been in general implicated in fast neuroneuronal communication [Chessel et al., 2001], P2Y receptors have been mainly found on astroglial cells [Abbracchio, 1997; Neary and Abbracchio, 2001].

A role for these receptors in the induction and maintenance of reactive astrogliosis is supported by the demonstration that the typical hallmark events of this reaction can be reproduced in astrocytic cultures in vitro by application of ATP or its analogs. In rat cortical and striatal astrocytes, stellation and proliferation as well as increased GFAP protein and mRNA levels have been observed [Neary and Norenberg, 1992; Neary et al., 1994a,b; Abbracchio et al., 1994; Bolego et al., 1997; for review, see Neary and Abbracchio, 2001]. In chick astrocytes, both GTP and ATP induced proliferation [Rathbone et al., 1992; Kim et al., 1991]. In human fetal astrocytes, ATP stimulated mitogenesis [Neary et al., 1998]. The ability of hydrolysis-resistant ATP analogs to reproduce these effects [Abbracchio et al., 1994; Bolego et al., 1997], together with the relatively slow formation of AMP and adenosine from ATP by astrocytic ectonucleotidases [Lai and Wong, 1991; Neary et al., 1994a], seem to rule out a role for nucleosides (and P1 receptors) in these experimental models and indicate that these effects are likely mediated by stimulation of P2 receptors. As mentioned above, reactive astrocytes acquire the ability of synthesizing neurotrophins and pleiotrophins which play a crucial role in neurite regeneration after injury. An additional evidence suggesting a role for nucleotides in reactive gliosis is represented by

the demonstration that, in astrocytic cultures, these compounds promote the synthesis and release of polypeptidic growth factors and act synergistically with them on target cells [Neary and Abbracchio, 2001].

However, the above in vitro evidence has been recently challenged by the finding that only a small percentage of acutely isolated astrocytes responded to ATP stimulation with a calcium signal [Kimelberg et al., 1997]. This raised the criticism that expression of P2Y receptors in culture may not reflect the in vivo situation, hence challenging the hypothesis that nucleotides participate in reactive gliosis in vivo. However, while astroglial P2Y receptors mediating cell differentiation and proliferation may couple to transduction mechanisms that do not involve calcium signaling [Bolego et al., 1997; Centemeri et al., 1997; Neary et al., 1999a] (for this important point, see also below), the low calcium response in the acutely isolated astrocytes may depend on rapid P2Y receptor desensitization as a consequence of ATP release during preparation. Mild perturbations such as fluid flows have indeed been shown to evoke a 10-fold increase in ATP release from astrocytes [Shitta-Bey and Neary, 1998]. Moreover, a number of other studies indicate the presence of P2Y receptors in vivo. Functional P2Y responses occur in glial cells in freshly prepared slices obtained from Bergmann glia [Kirischuk et al., 1995], corpus callosum [Bernstein et al., 1996], and retina [Sugioka et al., 1996; Newman and Zahs, 1997].

UTP-stimulated calcium waves in astrocytes in hippocampal slices [Neary et al., 1999b] and P2Y receptors were identified both in neurons and glia by in situ hybridization [Webb et al., 1998]. Even more important, microinfusion into rat nucleus accumbens of the P2Y receptor agonist 2-methyl-thio-ATP, as well as of other ATP analogs, increased GFAP immunoreactivity, astrocyte hypertrophy and proliferation. These effects were reduced by the antagonists reactive blue 2 and PPADS, confirming a role for P2 receptors in mediating astrogliosis in vivo [Franke et al., 1997, 1999]. Thus, from both in vitro and in vivo studies it is clear that release of purines and pyrimidines from damaged or dying cells could play an important role in brain response to injury and activation of the gliotic reaction.

We have developed in our laboratory an in vitro model of reactive astrogliosis (primary astrocytes obtained from either corpus striatum or cortex of 7-day-old rats) (Fig. 1). Astrocytic cultures are initially established in complete medium for 24 h and then shifted to serum-free medium for an additional 24 h before challenge with potentially gliotic factors, such as basic fibroblast growth factor (bFGF), ATP analogs, and others, in the absence or presence of inhibitors of specific transduction pathways and/or enzymes (e.g., the COX inhibitors acetylsalicylic acid, ASA, and NS-398; see "short protocol" in Fig. 1). After challenge, cultures are washed and maintained in drug-free medium up to day 5, when cells are fixed, stained with an anti-GFAP antibody, and analyzed by fluorescence microscopy (Fig. 1). Occasionally, after the 2-h challenge and removal of the gliotic agent(s), cultures are placed in serum-free medium still containing the COX inhibitors and maintained in this medium up to the end of the experiment (see "long protocol" in Fig. 1). At day 5, analysis is performed by quantifying the mean length of GFAP-positive astrocytic processes by means of a computerized image analysis system. Activated astrocytes show elongation of GFAP-positive processes, an event that reproduces in vitro one of the main hallmarks of reactive astrogliosis in vivo (see micrographs in Fig. 1). The morphological analysis is paralleled by biochemical evaluations performed at different times in culture aimed at assessing the role of specific proteins/enzymes and/or transductional pathways in the induction of reactive gliosis (Fig. 1; for more details, see also below) [Brambilla et al., 1999]. In initial experiments, we have demonstrated that a classic gliotic trigger such as bFGF induces a marked and concentration-dependent elongation of astrocytic processes [Abbracchio et al., 1994, 1995], which could be prevented by the concomitant exposure to tyrosine kinase receptor antagonists such as genistein [Bolego et al., 1997], hence validating the model for the identification of novel as-yet uncharacterized gliotic agents.

REACTIVE ASTROGLIOSIS BY ATP IS MEDIATED BY A NOVEL ATYPICAL P2 RECEPTOR

We have therefore utilized the above-described experimental protocol to characterize ATP-induced reactive astrogliosis. We have exposed rat primary astrocytic cultures, obtained from either corpus striatum or cortex, to the relatively hydrolysis-resistant ATP analogs α,βmethyleneATP (α,βmeATP) and β,γmethyleneATP (β,γmeATP). In a similar way to bFGF, a 2-h challenge of cultures with these compounds resulted in a marked concentration-dependent elongation of GFAP-positive astrocytic processes [Abbracchio et al., 1995; Bolego et al., 1997]. Other nucleotides, including the natural nucleotide ATP, also reproduced this effect. However, it became immediately evident that astroglial cells also expressed other P2 receptors distinct from the gliosis-inducing receptor (e.g., P2Y receptors linked to calcium mobilization from intracellular stores via activation of phospholipase C (PLC), and likely involved in cell-to-cell communication) [Centemeri et al., 1997]. All these receptors were obviously activated by ATP, whereas α,βmeATP seemed to behave as a selective agonist for the receptor mediating reactive astrogliosis. On this basis, to dissect the effects evoked by the P2 gliotic receptor from those induced by the other P2 receptors expressed in these cells, α,βmeATP was utilized in all subsequent

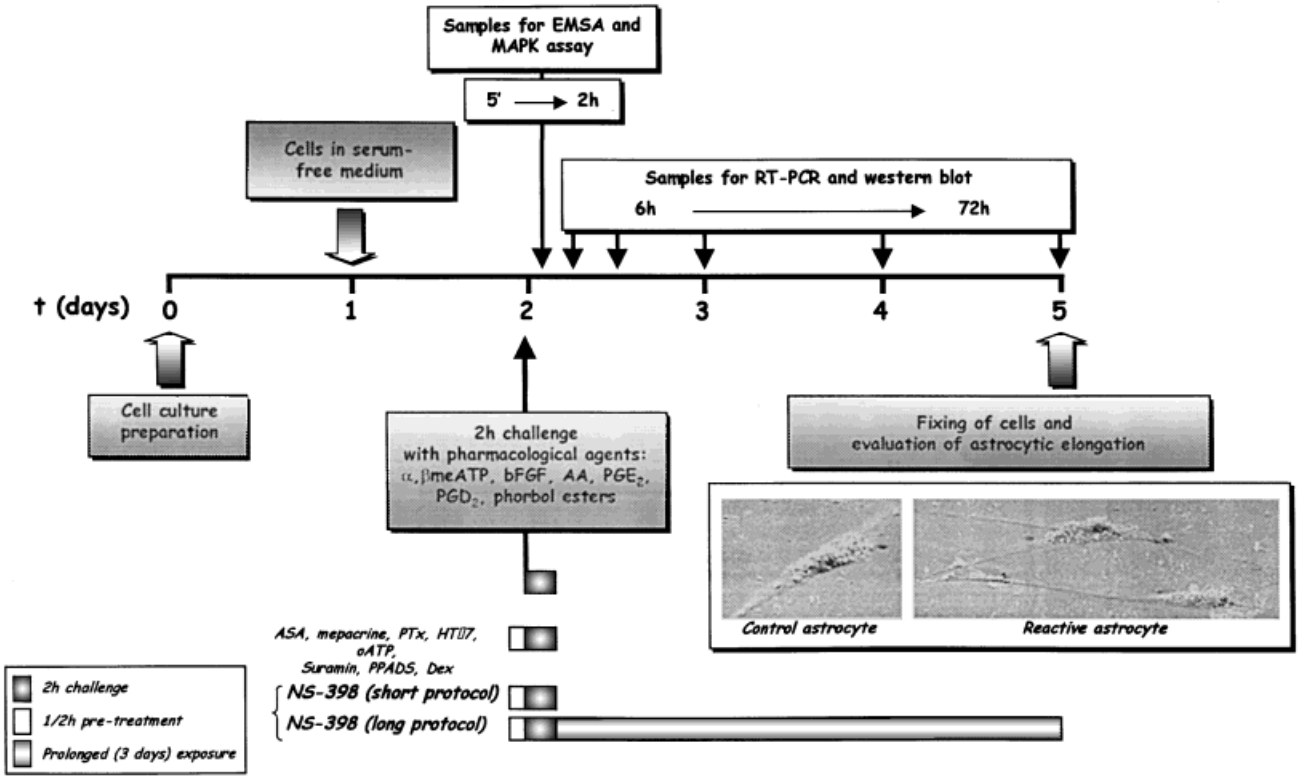

Fig. 1. Experimental protocols for seeding, treatment, and processing of rat brain primary astrocytes. Cells are initially established in complete medium, placed in serum-free medium at day 1, and challenged with either purine analogs (e.g., α,βmeATP) or the other indicated agents at day 2. At different times samples are collected for electrophoresis mobility shift assay (EMSA), Western blotting, and RT-PCR experiments [see also Brambilla et al., 1999].

experiments. A specific role for P2 receptors was confirmed by the fact that both suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) could completely prevent the astrocytic elongation induced by α,βmeATP.

In order to assess the nature of the gliotic P2 receptor (i.e., whether it belonged to the P2X or P2Y family), experiments were performed with pertussis toxin (PTx), an inhibitor of α subunits of Gi and Go families of G-proteins. Pretreatment with PTx fully prevented α,βmeATP-induced effects [Bolego et al., 1997], suggesting that a G-protein-coupled P2Y receptor may be responsible for α,βmeATP-induced gliosis. This was further confirmed by the demonstration that oxidized ATP (oATP), a P2X receptor antagonist, did not modify α,βmeATP-induced effects [Brambilla et al., 2000a], ruling out a role for ligand-gated receptors.

The signaling system utilized by the gliotic P2 receptor was hence characterized. This receptor did not seem to share any of the transduction mechanisms of the other known P2Y receptors, which have been demonstrated to either stimulate phospholipase C or adenylyl cyclase [King et al., 2000]. Activation of this receptor by α,βmeATP did not result in either changes of intracellular Ca^{2+} concentrations (see above) [Bolego et al., 1997], which ruled out the involvement of phospholipase C, or in modulation of adenylyl cyclase activity [ibid], hence ruling out a role for cAMP. Activation of this receptor resulted in an early release of arachidonic acid (AA), suggesting coupling to phospholipase A_2 (PLA_2 [Bolego et al., 1997]). The importance of this transduction pathway in α,βmeATP-induced effects was further confirmed by the ability of mepacrine, a PLA_2 inhibitor, to prevent both purine-induced AA release and the associated elongation of astrocytic processes [ibid]. Moreover, the exogenous addition of AA reproduced the typical hallmarks of gliosis in these cells [Bolego et al., 1997]. This peculiar transduction pathway, together with the rather atypical pharmacological profile of this receptor (high sensitivity to α,βmeATP and β,γmeATP, which is quite unusual for a P2Y receptor) suggests that we may be dealing with a novel P2Y receptor subtype specifically associated with the induction of reactive gliosis. This novel astroglial receptor shows striking similarities with another P2Y receptor, still not identified in the periphery, which mediates relaxation of intestinal smooth muscle. This receptor has high potency for α,βmeATP and its response is not blocked by Evans blue, a widely utilized P2Y antagonist [Bultmann

et al., 1996; Ralevic and Burnstock, 1998]. It would be interesting to assess whether the atypical astroglial and intestinal receptors are indeed the same one.

The possible relevance of the astroglial receptor to human diseases characterized by reactive gliosis has been recently suggested by the demonstration that a similar gliotic effect is induced by α,βmeATP in human astrocytoma cells (ADF cells) [Brambilla et al., 2000a].

CYCLOOXYGENASE-2 AS A POSSIBLE TARGET FOR THE GLIOTIC P2 RECEPTOR

After demonstrating that the activation of the novel gliotic P2 receptor leads to the rapid release of AA, we focused our attention on inducible, mitogen-activated cyclooxygenase-2 (COX-2). This choice was based on the following evidence:

1) AA is a substrate for both constitutive COX-1 and inducible COX-2 [Wu, 1996] for the synthesis of prostanoids (mainly prostaglandin E_2, PGE_2, in astrocytes, see also below) [Blom et al., 1997].
2) Either AA "per se" or its metabolites have been reported to induce the transcription of the COX-2 gene [Barry et al., 1999; Tordjmann et al., 1995; Takahashi et al., 1994]. In the case of AA, this may occur through the activation of an arachidonate-sensitive protein kinase C (PKC), which would activate mitogen-activated protein kinases (MAPKs), that may in turn activate/phosphorylate a variety of transcription factors [Neary and Abbracchio, 2001]. The analysis of the COX-2 promoter on human chromosome 1 has indeed highlighted the presence of consensus sequences for several transcription factors (Fig. 2), some of which are directly activated by MAPKs. ATF-2 and CREB, for instance, after being phosphorylated by MAPKs (specifically by the stress-activated protein kinase/c-jun-N-terminal kinase, SAPK/JNK, or by the p38 kinase), bind to the CRE consensus element (Fig. 2), while c-Jun and c-Myc, after phosphorylation by the extracellular signal-regulated kinase (ERK1/2) or by SAPK/JNK and consequent dimerization, bind to the AP-1 consensus site [Force and Bonventre, 1998]. Interestingly, to further support the involvement of this signaling pathway in α,βmeATP-induced gliosis, we have previously shown that exposure of striatal astrocytes to α,βmeATP results in the nuclear accumulation of Jun and Fos proteins [Bolego et al., 1997], which represent the main components of AP-1 transcription complexes [Neary et al., 1996b]. An example of this effect is shown in Figure 3. In our experimental model, significant nuclear accumulation of Fos and Jun proteins was detected as early as 30 min after challenging cultures with either α,βmeATP or bFGF. For both agents, induction peaked at 60 min and returned to basal values within 180–240 min [Bolego et al., 1997]. To support a specificity for this effect, suramin antagonized α,βmeATP- (but not bFGF-) induced accumulation of Fos and Jun immunoreactivity in nuclei [Bolego et al., 1997].
3) Excessive COX-2 activation has been demonstrated in a variety of neurological disorders including acute (e.g., ischemia) [Ohtsuki et al., 1996; Planas et al., 1995] as well as chronic (e.g., Alzheimer's disease, pain and inflammation) [Dolan et al., 1998; Ho et al., 1999] neurodegenerative disorders, characterized by a marked inflammatory component and activation of astroglial cells [Ohtsuki et al., 1996; Ho et al., 1999; Blom et al., 1997].

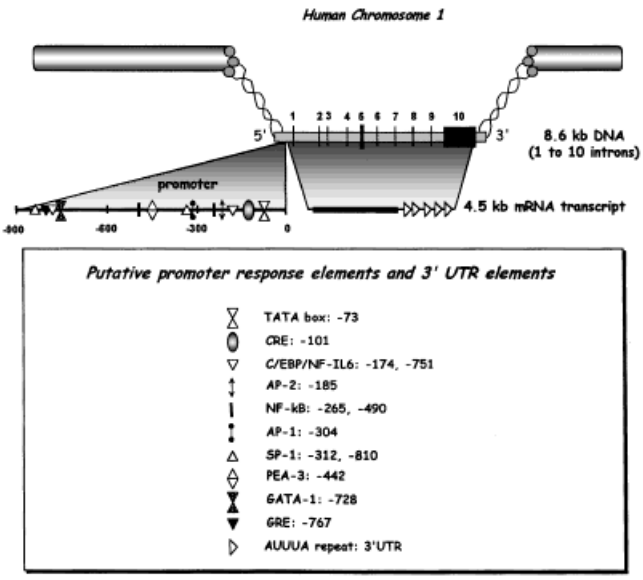

Fig. 2. Diagram of the human COX-2 gene, mRNA and promoter sequence. 3′ UTR: 3′ untranslated region on mRNA transcript.

On this basis, we verified whether astrocytic elongation by α,βmeATP may occur through upregulation of the COX-2 gene. Challenge of cells with α,βmeATP resulted, 24 h later, in a significant upregulation of COX-2 protein expression, as determined by Western blot analysis with a selective anti-COX-2 antibody [Brambilla et al., 1999]. The COX-2 protein level was still elevated 48 h after challenge and returned to basal values 72 h after challenge with the purine analog [Brambilla et al., 1999]. To support a specific role for the P2 receptor in this effect, PPADS inhibited both α,βmeATP-induced gliosis and the

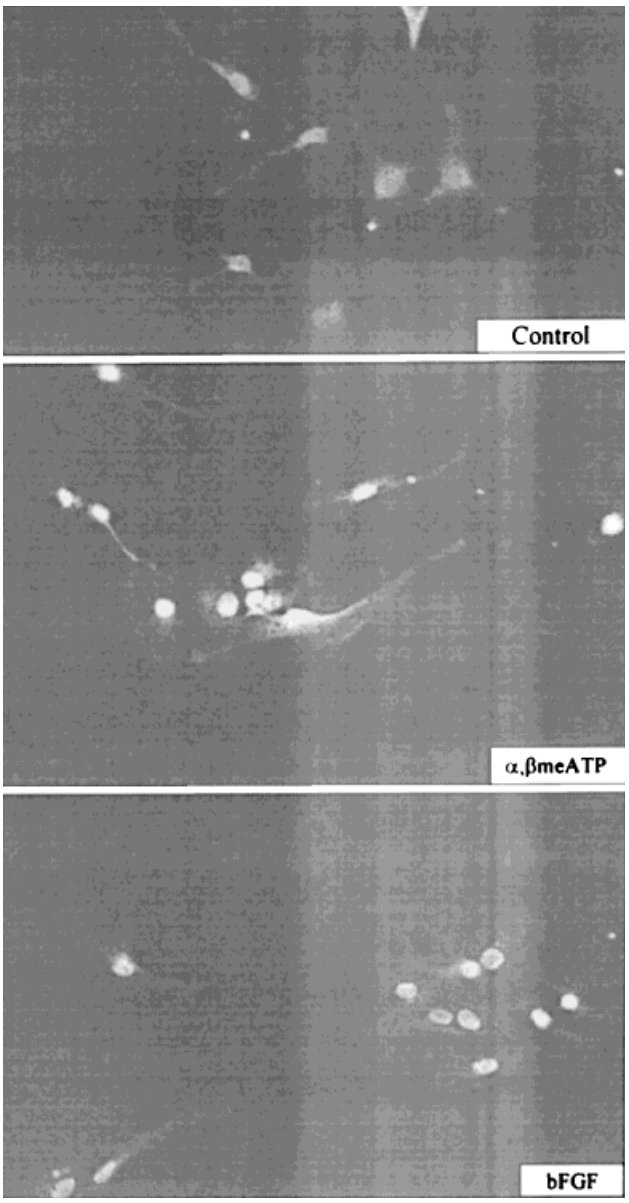

Fig. 3. Nuclear accumulation of the Jun protein induced by α,βmeATP and bFGF in rat brain primary astrocytes. At day 2 in culture, cells were exposed to either medium alone (control), or 10 μM α,βmeATP, or 1 ng/ml bFGF. After 1 h cells were washed, fixed, immunostained for the Jun protein, and analyzed under a fluorescence microscope (reproduced with permission from Bolego et al. [1997]).

associated COX-2 upregulation [Brambilla et al., 2000a]. In order to assess the actual importance of this event in purine-induced gliosis, we utilized the "long exposure protocol" described in Figure 1, i.e., after challenge of cells with α,βmeATP and agonist removal, to fully and persistently inhibit the enzyme as soon as it was synthesized, cultures were placed in serum-free medium containing graded concentrations of the selective COX-2 blocker NS-398. Under these experimental conditions, NS-398 concentration-dependently abolished α,βmeATP-induced astrocytic elongation, suggesting the existence of a causal relationship between COX-2 upregulation and purine-induced astrogliosis [Brambilla et al., 1999]. Other nonselective inhibitors of COX-2 (e.g., dexamethasone) also abolished purine-induced reactive astrogliosis. Since Western blot experiments with the anti-COX-2 antibody suggested low, but significant, constitutive expression of COX-2 also in control unstimulated astrocytes, we designed specific experiments aimed at testing a possible role for constitutive COXs in α,βmeATP-induced astrogliosis. Cultures were exposed to NS-398 to selectively block constitutive COX-2, or to ASA, which irreversibly inhibits both COX-1 and COX-2 immediately before and throughout challenge with the purine analog ("short protocol," Fig. 1). ASA was used at a concentration (1 mM) that would ensure full abolition of the contribution made by all constitutively expressed COXs. After challenge, cells were washed, placed in drug-free medium, and grown for a further 3 days before morphological analysis. Under these conditions no inhibition of α,βmeATP-induced astrogliosis was detected [Brambilla et al., 1999], ruling out a role for constitutive COXs and suggesting that any effect seen after treatment with the purine analog must result from synthesis of new enzyme. Data with NS-398 suggest that this is indeed COX-2. Interestingly, NS-398 could not only prevent α,βmeATP functional effects, but also abolish the associated upregulation of COX-2 protein expression [Brambilla et al., 2000a]. This is consistent with the recent demonstration that, in endothelial cells and fibroblasts, ASA and sodium salicylate at therapeutic concentrations equipotently block upregulation of COX-2 mRNA and protein expression induced by interleukin lβ and PKC activation [Xu et al., 1999]. In this study, salicylate inhibited COX-2 promoter activity in a concentration-dependent manner and also inhibited nascent COX-2 transcript synthesis with no effect on COX-2 mRNA stability [Xu et al., 1999]. These findings are in line with the concept that not all the pharmacological actions of nonsteroidal antiinflammatory drugs (NSAIDs) can be explained by inhibition of COX activity. They suggest that NSAIDs may also modulate the expression of these enzymes at a transcriptional level, a novel mechanism that may significantly contribute to their antiinflammatory properties.

As previously mentioned, exposure of cultures to AA elicits the formation of reactive astrocytes [Bolego et al., 1997]. Since PGE_2 and PGD_2 represent the main functionally important COX-2 products in the brain [Blom et al., 1997; Minghetti et al., 1997], we tested these prostaglandins for their ability to trigger reactive astrogliosis. The exogenous addition of the inflammatory COX-2 metabolite PGE_2 (but not PGD_2) mimicked the effects evoked by the activation of the P2 gliotic receptor, resulting in statistically significant and concentration-de-

pendent elongation of astrocytic processes. This suggests that PGE_2 is likely to represent the main AA metabolite involved in α,βmeATP-induced reactive gliosis.

Globally, the data presented above support the involvement of COX-2 in ATP-induced astrogliosis, a finding that may have intriguing implications for neurodegenerative diseases where excessive COX-2 activation and reactive gliosis play a major role. We speculate that antagonists selective for the novel P2 receptor responsible for this effect may represent a new class of antineurodegenerative agents of potential interest in both acute and chronic neurological diseases. By reducing the extent of gliosis evoked by ATP, such antagonists may indeed counteract the inflammatory events contributing to neurodegeneration and slow down the progression of these diseases. The hope of developing selective antagonists reasonably devoid of side effects is supported by the peculiar and atypical pharmacological and biochemical profile of this receptor, which makes it different from the other known P2 receptor subtypes.

SIGNALING PATHWAYS INVOLVED IN PURINE-INDUCED REACTIVE ASTROGLIOSIS

We are currently working at elucidating the receptor-to-nucleus cascade responsible for ATP-induced gliosis. We believe that the characterization of this transductional pathway may disclose further molecular targets for the pharmacological modulation of reactive astrogliosis, and hence contribute to the identification of novel therapeutic strategies for neurodegenerative diseases (Fig. 4).

Data from both our laboratory and other authors suggest that an intracellular pathway possibly involved is the PKC/MAPK/AP-1 system. This is based on the following evidence:

1) As mentioned above, the stimulation of the novel gliotic receptor results in an early release of AA, which may hence activate a *calcium-independent arachidonate-sensitive PKC* (Fig. 4). The involvement of PKC is supported by our previous demonstration that PKC activators (i.e., phorbol esters) can mimic the gliotic response induced by purine analogs in our model and, conversely, inhibitors of this enzyme (i.e., H7) completely abolish α,βmeATP-induced effects [Bolego et al., 1997]. Moreover, PKC participates in the activation of MAPKs [Neary and Abbracchio, 2001] (see also below). The conclusion that a calcium-insensitive PKC may be involved upstream of MAPK is supported also by results obtained in cortical astrocytes, where ATP signaling to ERK was unaffected by chelation of intracellular calcium, or by inhibition of PLC by U-73122 [Neary et al., 1999a]. Moreover, in these cells, P2Y receptors recruit a calcium-independent PKC isoform, PKCδ [ibid].
2) Signals from PKC may merge on *MAPKs*, which, upon activation, translocate to the nucleus where they activate transcription factors and complexes, thereby leading to the up- and downregulation of genes involved in cell function (e.g., the COX-2 and GFAP genes, Fig. 4). The involvement of this intracellular pathway in purine-induced gliosis is supported by previous data. In rat and human astrocytes, stimulation of P2Y receptors activate ERK [Neary and Zhu, 1994; Neary, 1996; King et al., 1996; Neary et al., 1998, 1999a]. In these cells, these receptors have also been associated with mitogenesis, another hallmark of reactive astrogliosis. Importantly, inhibition of the ERK cascade also blocked ATP-induced mitogenesis in these cells [Neary et al., 1998; 1999a], indicating a crucial role for ERKs in purine and pyrimidine receptor-mediated signaling. Preliminary data suggest that activation of ERK1/2 may as well play a role in α,βmeATP-induced gliosis in our experimental model [Brambilla et al., 2000b; in preparation].
3) MAPKs can activate the *AP-1 transcription complex*, which may in turn participate in COX-2 upregulation [Fletcher et al., 1991]. The evidence involving Fos and Jun proteins in our experimental model has been summarized above (see also Fig. 3) and is consistent with data from other authors. In both astrocytes [Priller et al., 1998] and microglia [Priller et al., 1995], the P2Y receptor agonist ADPβS stimulated a rapid and transient increase in mRNA levels for *c-fos, c-jun, junB*, and *Tis11*. In rat cortical astrocytes, extracellular ATP and UTP stimulated formation of functional AP-1 transcription complexes composed of members of the Fos and Jun families of proteins [Neary et al., 1996b]. AP-1 binding to DNA was partially blocked

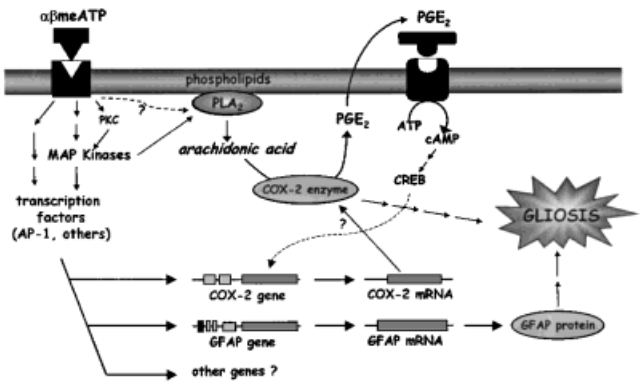

Fig. 4. Putative mechanisms at the basis of P2 receptor-mediated reactive astrogliosis. See text for details.

by inhibition of PKC and ERK1/2, thereby confirming that both kinases are located upstream of the AP-1 complex formation [Neary et al., 1996b, 1999b]. The involvement of AP-1 in our experimental model will be confirmed by measuring the DNA binding of AP-1 complexes (as well as of other transcription factors) by means of electrophoretic mobility shift assay (EMSA) in nuclear extracts obtained from control and α,βmeATP-treated cells.

UNANSWERED QUESTIONS AND FUTURE PERSPECTIVES

There are several unanswered questions that we also plan to take into consideration in the near future. For example, it is not known at present whether the novel gliotic receptor is directly coupled to PLA_2 (via a yet-to-be-determined G-protein), or whether activation of PLA_2 is secondary to MAPK activation. In fibroblasts, PLA_2 is activated by ERK1/2 [Lin et al., 1993; Nemenoff et al., 1993], which is followed by PLA_2-stimulated AA release, production of PGE_2, and elevation in cAMP levels due to the stimulation of specific extracellular PGE_2 receptors linked to adenylate cyclase. This pathway, which, in these cells, has been implicated in P2Y receptor-induced proliferation [Huang et al., 1991] may also be operative in astrocytes and participate in purine-induced reactive gliosis (Fig. 4). It is not known whether and how induction of COX-2 is related to induction of the GFAP gene, which plays a major role in the morphological changes associated with reactive gliosis (Fig. 4). Many other genes are likely to be activated by challenges of cells with purine analogs. For example, initial studies with DNA arrays have demonstrated that genes coding for proteins in a number of functional categories, including extracellular signaling and communication, intracellular transducers, DNA binding, cytoskeleton and motility, oncogenes and tumor suppressors, and stress responses, are up- or downregulated after treatment of rat cortical astrocyte cultures with ATP [Neary, 2000]. Additional experiments are needed to address these important issues before the intracellular cascade evoked by the gliotic P2 receptor is fully elucidated. More important, the molecular structure of the novel atypical receptor responsible for α,βmeATP-induced gliosis has not yet been determined. The cloning of this novel receptor is expected to represent an important step in the understanding of the gliotic effects induced by ATP, and will indeed represent the "sine qua non" condition to synthesize and develop selective ligands for this receptor subtype.

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