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Extracellular ATP induces stellation and increases glial fibrillary acidic protein content and DNA synthesis in primary astrocyte cultures

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Abstract A number of factors appear to be involved in the proliferative and hypertrophic processes which characterize reactive astrocytosis. We have investigated the possibility that ATP, an agent that is released by injured cells following tissue destruction, may be one such factor. For this purpose, we utilized primary cultures of astrocytes derived from cerebral cortices of neonatal rats to study the effect of extracellular ATP on properties associated with astrogliosis. Light microscopic studies disclosed marked stellation of astrocytes after 30–60 min of exposure to 100 μM – 1 mM ATP. In addition, the content of the astrocyte-specific intermediate filament, glial fibrillary acidic protein (GFAP), was increased 35–40 % following 60-min exposure to ATP; this effect persisted for 1–3 days of exposure to 100 μM ATP. [^{3}H]Thymidine incorporation increased progressively from 1–3 days; a 3.6-fold increase in DNA synthesis was observed following 3 days of exposure to 1 mM ATP, suggesting stimulation of cellular proliferation. These findings show that high micromolar to low millimolar concentrations of extracellular ATP reproduce several features associated with reactive gliosis and suggest that extracellular ATP may be involved in the activation of astrocytes following CNS injury.

Key words Astrocytes · Gliosis · Extracellular ATP Glial fibrillary acidic protein · Stellation

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Reactive gliosis represents the hyperplastic and proliferative response of astrocytes to tissue injury such as found in trauma, stroke, demyelinative disorders and other conditions. Several factors have been implicated in the initiation of reactive gliosis including growth factors (EGF, FGF, PDGF), cytokines (interleukin-1, tumor necrosis factor), myelin basic protein and others (for reviews see [15, 26]). One such factor we have been exploring is ATP, an agent that is released by injured cells following tissue destruction [4, 10]. ATP has been reported to have mitogenic activity in several types of cells, including fibroblasts [13], T lymphocytes [33], neuroblastoma cells [35], and chick astrocytes [31]. Here we report that exposure of astrocytes to extracellular ATP leads to stellation and an increase in glial fibrillary acidic protein (GFAP) content, as well as an increase in DNA synthesis. As these properties are characteristic of reactive astrocytes, these findings have implications for mechanisms of gliosis. Parts of this work have been presented in abstract form [24].

Materials and methods

Cell culture

Astrocytes were obtained from neonatal rat cortices and maintained in primary culture as previously described [11], except that (a) the culture media was changed twice weekly, and (b) for some experiments fetal calf serum was replaced by horse serum (Gibco) after 1 week in culture. At least 95 % of the cell population are astrocytes, as determined by glial-specific markers. Cultures were treated with ATP (Sigma) for the time periods and concentrations indicated in the text; for treatment times longer than 1 day, the media (± ATP) was changed daily. Studies were conducted with cells maintained in culture for 3–5 weeks.

Cell staining

For stellation studies, cultures were maintained in DMEM/fetal calf serum, and ATP was applied as indicated in the text. Cells were fixed in 10 % neutral formalin, stained with May-Grunwald/

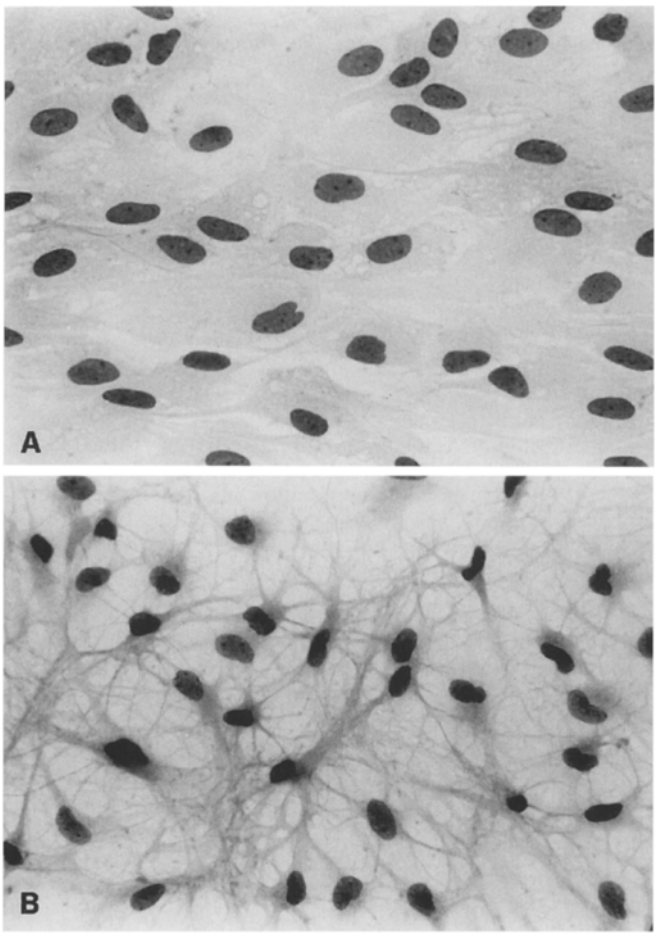

Fig. 1 A, B ATP-induced stellation. Primary astrocyte cultures were prepared as described in Methods. In the experiment shown here, cultures were maintained in DMEM containing fetal calf serum for 3 weeks. **A** A representative untreated plate; **B** ATP-treated (final concentration, 100 μM; 60 min). Cultures were stained as described in Methods. Application of extracellular ATP resulted in the formation of numerous fine, thread-like processes

Giemsa and examined by light microscopy. Stellation was characterized qualitatively by the presence of fine, thread-like processes.

GFAP content

Studies were conducted by the addition of ATP to the DMEM culture media containing horse serum. Following treatment with ATP at the times and concentrations indicated in the text, cultures were rinsed quickly in a calcium-free salt solution (145 mM NaCl, 3 mM KCl, and 20 mM HEPES, pH 7.4), harvested from petri dishes in a solution consisting of 0.15 % SDS, 2 mM EDTA (disodium salt), and 50 mM TRIS, pH 8.0, and homogenized. The homogenates were centrifuged for 15 min at 4 °C in a Sorvall SS/34 rotor at 15,000 rpm and the supernatants were stored at −70 °C prior to assay. GFAP content was determined by ELISA as previously described [25] using antibodies to GFAP kindly supplied by Dr. L. Eng, VA Medical Center, Palo Alto, CA. Assays were conducted in triplicate. Protein concentration of the supernatants were determined by the method of Lowry et al. [17].

DNA synthesis

Confluent cultures containing the normal concentration of serum were used in these experiments; growth was diminished due to contact inhibition. [^{3}H]Thymidine (New England Nuclear, 50–90 Ci/mmol) incorporation was determined following incubation for 18–24 h in 0.2 μCi [^{3}H]Thymidine/ml culture media. Cells were rinsed in ice-cold physiological saline solution (PSS), fixed in 10 % trichloroacetic acid (TCA) for 30 min on ice and washed once in ice-cold 5 % TCA. Cells were rinsed three times in ice-cold PSS and then lysed in 1 % SDS, 0.3 M NaOH. Aliquots were counted in a liquid scintillation spectrometer.

ATP breakdown

Cultures were treated with 100 μM or 1 mM ATP containing 2 μCi [α-^{32}P]ATP (New England Nuclear), and aliquots were removed at 0.5, 1, and 18 h and stored frozen. The samples, as well as an undegraded control sample (an aliquot of [α-^{32}P]ATP containing an equivalent amount of radioactivity as added to the cultures), were applied to polyethyleneimine-coated thin layer chromatography plates and nucleotides were separated by development in 1.4 M LiCl as described by Randerath and Randerath [29]. The nucleotides were identified by comparison to standards. Radioactive spots were detected by autoradiography and quantitated by scintillation counting. The extent of ATP breakdown and the levels of nucleotide products formed were calculated by comparison to the amount of radioactivity in the undegraded control sample; adenosine was estimated by the difference between the total radioactivity in the undegraded control sample and the amount of radioactivity in ATP, ADP, and AMP.

Results

Stellation

Addition of extracellular ATP to astrocyte cultures led to a marked increase in process formation (Fig. 1). In the untreated cells (Fig. 1A), the astrocytes exhibited a polygonal outline and processes were rarely observed. By contrast, virtually all of the ATP-treated cultures had cells with numerous, fine, and elongated processes (Fig. 1B). During the course of our studies, we examined the effect of ATP on cells derived from 18 different seedings ranging in age from 3–5 weeks. Stellation was noted in 25 of 28 separate experiments. ATP was effective in inducing morphological transformation in cultures maintained in either fetal calf serum or horse serum. Process formation was usually observed in discrete foci, suggesting heterogeneity among astrocytes responding to extracellular ATP. The optimum ATP concentration was 100 μM; stellation was also observed at 1 mM ATP, although the effects often appeared to be diminished as compared to 100 μM. No consistent differences were noted in cultures treated with 1 or 10 μM ATP versus controls. Stellation was studied over a time period of 5 min to 3 days. The optimum time for stellation was 30–60 min. An increase in the number of processes was observed as early as 10 min. ATP-induced stallation appeared to be reversible as the effect was diminished following 1 day of treatment and no effect was seen at 3 days.

GFAP content

Since GFAP is present abundantly in astrocytic processes, we studied the effect of extracellular ATP on

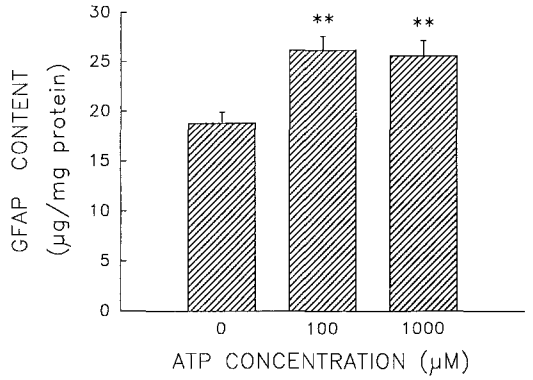

Fig. 2 Effect of extracellular ATP on GFAP content. Cultures were treated with 100 μM or 1 mM ATP for 1 h, and GFAP was quantitated by ELISA, as described in Methods. Significant increases in GFAP content were observed at both concentrations of ATP ($P < 0.01$)

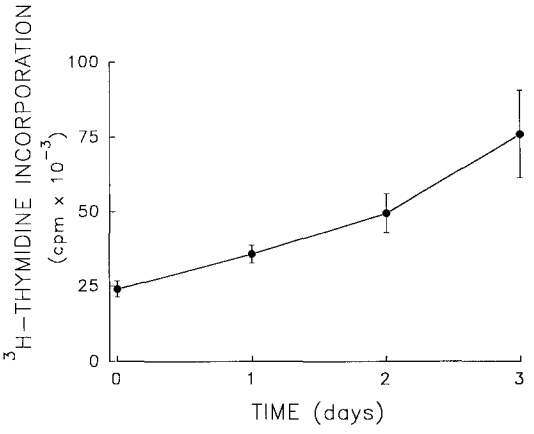

Fig. 3 Time course of effect of extracellular ATP on DNA synthesis. Cultures were treated with 1 mM ATP for 1–3 days (media changed daily) followed by an incubation in [^{3}H]thymidine for 18 h. [^{3}H]Thymidine incorporation was determined as described in Methods. The values were obtained from four separate experiments. Treatment with ATP resulted in a progressive increase in [^{3}H]thymidine incorporation, with a three-fold increase found after 3 days exposure to ATP

GFAP content, which was quantitated by ELISA. As shown in Fig. 2, exposure of astrocytes for 1 h to 100 μM or 1 mM ATP resulted in a 35–40 % increase in GFAP content. The data were obtained from eight independent experiments consisting of untreated cultures (total $n = 23$) and cultures treated with 100 μM or 1 mM ATP (total $n = 16$ and 23, respectively). An analysis of variance revealed an overall significant difference ($F_{2,59} = 8.62$; $P = 0.0005$). Planned comparisons (Bonferroni t-tests) revealed that both the 100 μM and 1 mM ATP-treated cultures were significantly different from the control ($P < 0.01$), while the two groups treated with ATP were not significantly different from each other. No consistent differences were found at 1 and 10 μM ATP (data not shown). In untreated cultures, the GFAP content ranged from 9.4 to 28.7 μg GFAP/mg protein with a mean ± SEM of 18.8±1.14.

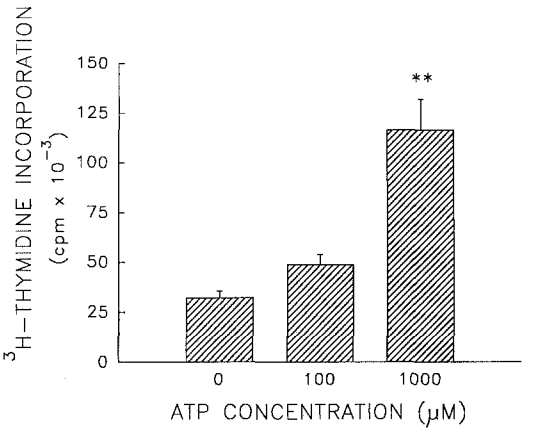

Fig. 4 Effected of ATP concentration on DNA synthesis. Cultures were treated for 3 days with 100 μM or 1 mM ATP, as indicated in Fig. 3. [^{3}H]Thymidine incorporation was determined as described in Methods. The values were obtained from five separate experiments. A significant increase (3.6-fold) was found with 1 mM ATP ($P < 0.001$)

GFAP content was also studied following a 1- and 3-day exposure to extracellular ATP. Treatment for 1 day with 100 μM ATP resulted in 34±6.7 % increase in GFAP content ($n = 6$, $P < 0.01$), while 3-day treatment gave a 65.2±17.9 % increase in GFAP ($n = 5$, $P < 0.05$). In experiments conducted with 1 mM ATP, cultures treated for 1 and 3 days were not statistically different from controls, although a trend of increased GFAP content was observed (21.1±11.1 % at 1 day and 29.7±12.9 % at 3 days).

DNA synthesis

The effect of extracellular ATP on DNA synthesis was determined by incorporation of [^{3}H]thymidine. As shown in Fig. 3, exposure of astrocytes for 1 to 3 days to 1 mM ATP followed by an 18-h incubation in [^{3}H]thymidine resulted in a progressive increase in [^{3}H]thymidine incorporation. A 3.6-fold increase in [^{3}H]thymidine incorporation was observed at 1 mM ATP, whereas a much smaller increase (51 %) was noted at 100 μM ATP (Fig. 4). The data were obtained from five independent experiments consisting of untreated cultures (total $n = 14$) and cultures treated with 100 μM or 1 mM ATP (total $n = 11$ and 14, respectively). An analysis of variance revealed an overall significant difference ($F_{2,36} = 19.7$; $P < 0.0001$). Planned comparisons (Bonferroni t-tests) revealed that the 1 mM ATP-treated cultures were significantly different from the control ($P < 0.001$) and from the 100 μM ATP-treated cultures ($P < 0.001$), while the 100 μM ATP-treated cells were not significantly different from the control.

Breakdown of ATP

Following 30-min exposure of extracellular ATP to astrocyte cultures, over 80–90 % of the initial ATP was

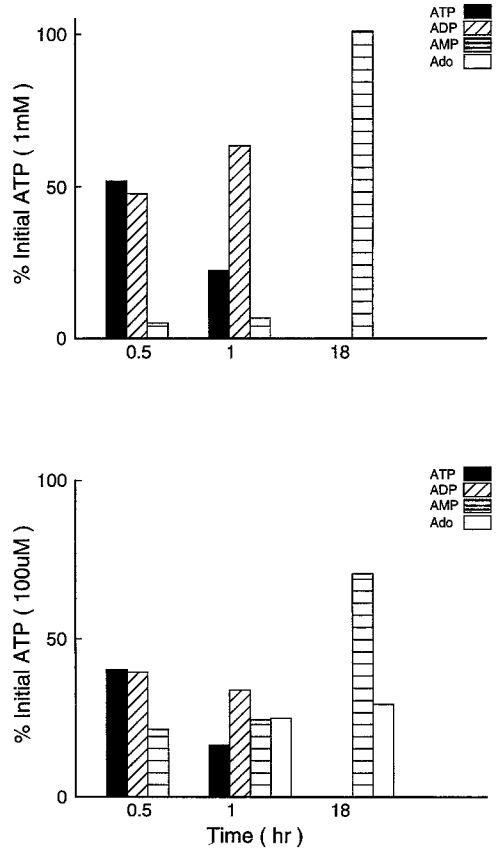

Fig. 5 Breakdown of extracellular ATP. Cultures were treated with 100 μM or 1 mM ATP containing 2 μCi [α-^{32}P]-ATP, aliquots were removed at 0.5, 1, and 18 h, and nucleotides were separated as described in Materials and Methods. Similar results were obtained in a second experiment using cultures from a different seeding. Extracellular ATP was converted to ADP, but the formation of AMP was relatively slow

present as ATP or ADP. At 60 min, 85 % of the initial 1 mM ATP remained as ADP and ATP, while at 100 μM initial ATP, 50 % remained as ADP and ATP (Fig. 5). Following 18 h of exposure to astrocytes, most of the ATP was converted to AMP or adenosine (Fig. 5).

Discussion

The studies presented here indicate that application of extracellular ATP to cultured rat astrocytes results in stellation, an increase in GFAP content and an increase in DNA synthesis. Previous reports have shown that ATP can increase [^{3}H]thymidine incorporation in several types of cells. In chick astrocytes, a 2-fold increase was observed [31] following 16- to 24-h incubation with 100 μM ATP, while greater increases were reported in human T lymphocyte Jurkat cells (20-fold; 500 μM ATP for 18 h [33]) and human astrocytoma cells (5- to 10-fold; 1 mM ATP for 16–24 h [31]). In Swiss 3T6 cells and in neuroblastoma cells (50 μM; 24–72 h) 2- to 5-fold increases were seen, respectively [35]. We have now shown that application of ATP can also induce stellation in astrocytes and that this change occurs within 10–30 min. This suggests that an early response of astrocytes to ATP involves a change in cellular phenotype. Because of the rapidity of the response, it seems reasonable to speculate that an early step in the transformation from polygonal to stellated shape may involve retraction of the cellular membrane and rearrangement of cytoskeletal elements to form thin, elongated fibers. In addition, application of extracellular ATP brought about an increase in GFAP content. This may reflect stimulation of protein synthesis or an inhibition of degradation, although the latter possibility appears less likely because of the relatively long half-life of GFAP [5].

The concept that ATP can act extracellularly has gained support through a large number of studies showing that ATP (a) regulates numerous biological functions, (b) is released from several types of cells, and (c) binds to specific puringergic receptors located on the surface of many cells (for reviews see [3, 10]). For example, it is now recognized that extracellular ATP can affect several biological processes including neurotransmission (central and peripheral), cardiac function, muscle contraction and relaxation, platelet aggregation, vascular tone, secretion of hormones and other factors, immune responses and cell growth. In the nervous system there is considerable evidence that ATP is released following electrical stimulation [36–38] and can activate synaptic currents [6]. The concentration of ATP released by neurons into the synaptic cleft appears to range from low micromolar during normal neuronal transmission to high micromolar upon repetitive excitation [32]. Endothelial cells are also adjacent to astrocytes, and ATP can be released from these cells as well [23, 28]. Because the intracellular concentration is in the millimolar range, extracellular levels in the high micromolar or low millimolar range would be expected following tissue damage [10].

Two major classes of purinergic receptors have been distinguished by Burnstock [2] based in part on relative potencies of adenine nucleotides and nucleosides which can be formed by ectonucleotidases. In general, P1 receptors are more responsive to adenosine and AMP than to ATP and ADP, whereas P2 receptors are more responsive to ATP and ADP than to AMP and adenosine. In astrocytes, both P1 [12, 18, 19, 34] and P2 [1, 9, 14, 20, 21, 27] purinergic receptors have been reported.

The results of our studies (Fig. 5) as well as that of others [16] on the breakdown of ATP following addition to astrocyte cultures provides information on which type(s) of purinergic receptor(s) may mediate the various astrocytic responses. After 30-min exposure, 80–90 % of the initial ATP is present as ATP or ADP. The relatively slow conversion of ADP to AMP confirms the report of Lai and Wong, who studied the metabolism of extracellular ATP by cultured astrocytes [16]. These findings suggest that P2 receptors mediate stellation because process formation could be observed

as early as 10 min after application of ATP. The ATP-evoked increase in GFAP content may also involve P2 receptors because at the 30-min time point the main products are ATP and ADP, and sufficient additional time would be needed to synthesize GFAP to explain the increase observed at 1 h. The ATP-induced increase in DNA synthesis may involve activation of P1 receptors following breakdown of ATP to AMP and adenosine because neither ATP nor ADP were detected after 18 h, while several days were required before DNA synthesis was substantially increased. This is consistent with the work of Rathbone and colleagues [31], who reported a stimulation of DNA synthesis in astrocytes by adenosine as well as ATP. However, we cannot exclude the involvement of P2 or a combination of P2 and P1 receptors since the effect is dependent on the time in the cell cycle when purinergic receptor stimulation activates DNA synthesis.

As reported here, extracellular application of ATP induced stellation, an increase in GFAP content as well as DNA synthesis. Because these properties are among the most characteristic of reactive astrocytes, this suggests that ATP may be a contributing factor to reactive gliosis. Additional support for this hypothesis comes from a report in which it was noted that microinjection of ATP into rat brain led to an increase in immunoreactive-GFAP fibers [30]. A number of agents including growth factors and cytokines have been reported to increase following CNS injury (for reviews see references [15, 26]), and it is possible that some agents may act in concert to generate the gliotic response. In this regard, we have recently found that application of basic fibroblast growth factor (bFGF), a growth factor known to increase following brain injury [7, 8], together with ATP, results in a synergistic stimulation of DNA synthesis in astrocyte cultures [22]. Subsequent studies suggest that ATP exerts a priming effect on bFGF-induced DNA synthesis because application of ATP for a brief period followed by treatment with bFGF also results in enhanced DNA synthesis in astrocytes (Neary et al., unpublished observations). Thus, in addition to functioning under normal conditions as a neurotransmitter, extracellular ATP may also be involved in the gliotic response to CNS injury when extracellular levels reach pathological levels.

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