Nitric Oxide Induces Rapid, Calcium-Dependent Release of Vesicular Glutamate and ATP From Cultured Rat Astrocytes

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KEY WORDS glia; calcium; inflammation; excitotoxicity; neurodegeneration

ABSTRACT Nitric oxide (NO; 1 μM) or an NO donor (500 μM diethylenetriamine-nitric oxide, DETA-NONOate) caused rapid glutamate and ATP release from cultured rat cortical astrocytes. NO-induced glutamate release was prevented by calcium chelators (EGTA or BAPTA-AM) and an inhibitor of vesicular exocytosis (botulinum neurotoxin C, BoTx-C), but not by a glutamate transport inhibitor, L-trans-pyrrolidine-2,4-dicarboxylate (t-PDC), a cyclooxygenase inhibitor (indomethacin), or an inhibitor of soluble guanylate cyclase 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), and was not induced by mitochondrial respiratory inhibitors (myxothiazol or azide). Similarly to glutamate, NO-induced ATP release was also completely blocked by BAPTA-AM and BoTx-C, suggesting again a vesicular, calcium-dependent mechanism of release. Addition of DETA-NONOate (500 μM) to fura-2–loaded astrocytes induced a rapid, transient increase in intracellular calcium levels followed by a lower, sustained level of calcium entry. The latter was blocked by gadolinium (1 μM), an inhibitor of capacitative Ca2+ entry. Thus, NO appears to cause rapid exocytosis of vesicular glutamate and ATP from astrocytes by raising intracellular calcium levels. Astrocytes activated by lipopolysaccharide/endotoxin and interferon-γ express inducible NO synthase (iNOS) maintained substantially higher extracellular glutamate levels than nonactivated cells or activated cells treated with an iNOS inhibitor (1400W), but the rate of glutamate uptake by these cells was similar. This suggests that NO from inflammatory-activated astrocytes causes release of astrocytic glutamate. NO-induced release of astrocytic glutamate and ATP may be important in physiological or pathological communication between astrocytes and neurons. GLIA 40:312–323, 2002. © 2002 Wiley-Liss, Inc.

INTRODUCTION

Physiologically nitric oxide (NO) derived from neuronal (nNOS) or endothelial (eNOS) NO synthase may act as an intercellular messenger between neurons, astrocytes, and other brain cells (Dinerman et al., 1994; Garthwaite and Boulton, 1995; Prast and Philippu, 2001). Pathologically inducible NO synthase (iNOS) may be induced in glia by inflammatory mediators, and the high level of NO produced may contribute to killing neurons in inflammatory, infectious, ischemic, and neurodegenerative diseases (Loihl and Murphy, 1998; Bolanos and Almeida, 1999; Knott et al., 2000). NO has been implicated in most neurodegenerative diseases (including multiple sclerosis, AIDS dementia, Parkinson’s, Huntington’s, Alzheimer’s, and motor neuron diseases) (Heales et al., 1999; Ignarro, 2000; Bolanos and Almeida, 1999). For example, in Alzheimer’s disease, activated microglia and astrocytes expressing...
iNOS are found in the amyloid plaques surrounded by dead and dystrophic neurites (Wa et al., 1996; Wallace et al., 1997; Lee et al., 1999). β-amyloid can induce cultured glia to express iNOS and kill cocultured neurons via NO (Goodwin et al., 1995; Wisniewski et al., 1998) and anti-inflammatory drugs protect against Alzheimer’s disease (McGeer and McGeer, 1995; McGeer et al., 1996; Lim et al., 2000).

NO and iNOS-expressing glia can kill neurons in culture by excitotoxic mechanisms involving extracellular glutamate (Hewett et al., 1994; Leist et al., 1997; Bal-Price and Brown, 2001). NO is known to cause rapid glutamate release from neurons (Meffert et al., 1994; Trabace and Kendrick, 2000), which has been attributed either to inhibition of mitochondrial respiration followed by reversal of glutamate uptake (Sequeira et al., 1997; McNaught and Brown, 1998; Bal-Price and Brown, 2001), or to a direct action on synaptic vesicle docking/fusion reactions followed by (calcium-independent) vesicular exocytosis (Meffert et al., 1996).

Astrocytes are now known to have a vesicular pool of glutamate (and possibly ATP) that is rapidly exocytosed in response to agonists that raise intracellular calcium (Parpura et al., 1994; Beuzzi et al., 1998; Maenschein et al., 1999; Innocenti et al., 2000; Pasti et al., 2001). The mechanism of calcium-activated exocytosis of vesicular glutamate appears to be similar in astrocytes and neurons (Araque et al., 2000; Mazzanti et al., 2001). During the past few years, it has been shown that by releasing glutamate, astrocytes can modulate synaptic transmission and contribute to certain forms of synaptic plasticity (Mazzanti et al., 2001).

NO has been reported to evoke calcium waves in astrocytes, and endogenous NO may be involved in propagation of such waves (Willmott et al., 2000a, 2000b; Bowman et al., 2001). Calcium waves may propagate between astrocytes via gap junctions or via extracellular mediators and may carry information between astrocytes (Scemes, 2000). There is evidence that the extracellular propagation of calcium waves between astrocytes is also mediated by ATP (Cotrina et al., 1998a; James and Butt, 2001). Nanomolar levels of ATP can act at various purinergic receptors on astrocytes, neurons, microglia, and endothelial cells to increase intracellular calcium (James and Butt, 2001). And increases in intracellular calcium can cause ATP release from neurons and astrocytes (Queiroz et al., 1997). The mechanism of ATP release from astrocytes is unclear, but may, as in neurons, be due to calcium-induced exocytosis of vesicular ATP. We set out to test whether NO could cause glutamate and ATP release from astrocytes and, if so, by what mechanism.

**MATERIALS AND METHODS**

**Astrocyte and Microglial Cultures**

Primary, mixed glial cell cultures were prepared from the cerebral cortex of 7-day-old rats (Wistar) as previously described (Bal et al., 1994). Briefly, cells isolated from cerebral hemispheres were dissociated in Hanks’ balanced salt solution (HBSS) containing 0.25% trypsin (Sigma, Poole, U.K.) and 0.02 mg/ml deoxyribonuclease I (Sigma-Aldrich, Steinheim, Germany) and plated at a density of 0.1 × 10⁶ cells/cm² in 25 or 75 cm² culture flasks (Falcon) in DMEM with 10% of fetal calf serum. For measurements of [Ca²⁺], the cells were grown on coverslips (9 × 22 mm). At confluency (12–14 days in vitro, DIV), primary glial cultures were used to isolate microglial cells as previously described (Taubenrot et al., 1996). Briefly, mixed glial cells (cultured on the coverslips or in the flasks) were shaken to dislodge microglia that were loosely attached to the astrocytes. Microglia were purified by preplating for 30 min into culture flasks (75 cm²) at the density 0.1 × 10⁶ cells/cm² and then the contaminating cells were removed by changing the medium. Microglia were maintained in astrocyte-conditioned medium (medium collected from astrocytic cultures after 2 days and spun down) mixed 1:1 v/v with fresh DMEM (containing 10% of fetal calf serum). The purity of the astrocytic cultures was determined (after isolation of microglia) in sister cultures immunocytochemically with OX-42 (microglial marker, anti-CR3 complement receptor antibody; Serotec, Oxford, U.K.), anti-GFAP antibody (an astrocytic marker; AutogenBioclear, Calne, U.K.), anti-NeuN (neuron-specific nuclear protein; Chemicon, Temecula, CA), and anti-Ox7 (the cell surface molecule Thy1.1, specifically expressed on fibroblasts; Department of Pathology, University of Oxford). The purity of microglial cultures was assessed for the presence of microglia and astrocytes only. The cells were fixed in 4% paraformaldehyde (Sigma) and then incubated with OX-42 or anti-GFAP (all at 1:200 dilutions) and visualized using biotinylated antimouse IgG antibodies (1:200 dilutions), avidin-biotin-horseradish peroxidase complex, and diaminobenzidine tetrahydrochloride (ABC staining system, AutogenBioclear). In the case of Ox-7 and NeuN goat antimouse IgG secondary antibodies were used conjugated to tetra-rhodamine isothiocyanate (IgG-TRITC) or to fluoroescin isothiocyanate (IgG-FITC), respectively. As a positive control for anti-NeuN immunocytochemistry, we used neuronal culture of cerebellar granule cells prepared as described before (Bal-Price and Brown, 2001) and for anti-Thy1.1 fibroblast culture prepared from the panning plates after Schwann cell purification (Cohen and Wilkin, 1995); 99.0% ± 0.8% of the cells in microglial cultures were positive for OX-42, marker for macrophage/microglial cell types (GFAP-positive cells were not present). In astrocyte cultures, 97%–98% of cells were anti-GFAP-positive and only 2%–3% cells were OX-42–positive (microglia). Cells anti-NeuN (neuronal marker) or anti-Thy1.1 (fibroblast marker)–positive were not observed, confirming that neurons or fibroblasts were not present in the astrocytic cultures. Microglial cultures were used for the determination of glutamate release 24 h after plating. Cultures of astrocytes were used between 21 and 28 days. When glutamate release was studied using the fluorometric
method, the microglia or astrocytes were gently trypsinized (0.1%) for 2–3 min (at 37°C), the cells were spun down and resuspended in Krebs-Hepes buffer consisting of 1.5 mM CaCl₂, 5.6 mM glucose, 10 mM HEPES, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.1 mM MgSO₄, 118 mM NaCl, pH 7.4.

Measurements of [Ca²⁺]ᵢ in Astrocyte Cultures

Confluent cultures of astrocytes (21–28 DIV) on coverslips (after shaking off microglia) were incubated with 2 μM fura-2 AM (Molecular Probe, Leiden, The Netherlands; from a stock of 1 mM in anhydrous DMSO) in PBS supplemented with 1 mg/ml bovine serum albumin (fraction V) and 0.025% w/v Pluronic F-127 (to disperse fura-2 AM in solution). After 120 min at 20°C in the dark, coverslips were washed three times with Krebs-Hepes buffer (pH 7.4) containing 1 mg/ml BSA and kept in the dark at 20°C for a further hour. The additional hour was to allow de-esterification of the acetoxymethyl ester of fura-2.

For measurements of intracellular free calcium ([Ca²⁺]ᵢ) in populations of astrocytes, rectangular coverslips with confluent fura-2-loaded cells were mounted vertically in a 4.5 ml optical methacrylate cuvette. The cuvette was mounted in a Hitachi F4500 spectrofluorimeter with the coverslip at a 30° angle to the excitation light path. Cells were excited by light of the appropriate wavelengths (λₑx: 340, 359, and 380 nm) using a monochromator, and emitted fluorescence (λₑm) was collected at 0.2-s intervals at 510 nm. After correction for autofluorescence, calibrations of fluorescence ratios (R₃₄₀/₃₈₀) to cytosolic free [Ca²⁺] were performed using look-up tables created from Ca²⁺ standard solutions (Molecular Probes). The Krebs-Hepes (pH 7.4) bathing the cells was changed by perfusion into the bottom of the cuvette using a peristaltic pump while continuously aspirating medium from just above the coverslip. A circulating water bath maintained the temperature of the perfusate at 37°C. At the perfusion rate used (17 ml/min), the medium was exchanged with a half-time of 9.6 ± 0.3 s (n = 3). The latency (i.e., the interval between switching saline reservoirs and arrival of the new media at the cuvette) was 9 s. All figures have been corrected for this latency. In some experiments, astrocytes were preincubated with 10 μM ODQ (Calbiochem, Nottingham, U.K.) for 10 min before applying 500 μM DETA-NONOate (diethylenetriamine-nitric oxide adduct, also known as NOC-18; RBI, Sigma). In other experiments, 100 μM PTIO (2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl 3-oxide; Sigma), 10 μM verapamil (Calbiochem), or 1 μM gadolinium (Sigma) were perfused together with DETA-NONOate (500 μM).

Activation of Astrocytes in Culture

Cultured astrocytes (21–28 DIV, confluent, after shaking off microglia) were activated by exposure to lipopolysaccharide from Salmonella typhimurium (LPS; 10 μg/ml; Sigma) and interferon-γ (IFN-γ; 100 units/ml; Sigma) in the presence and absence of an iNOS inhibitor (25 μM 1400W; Alexis Biochemicals, Lausen, Switzerland) for 18 h. The medium of astrocytes (DMEM with 10% of FCS; level of glutamate 85.3 ± 5.0 μM) was changed just before addition of LPS and IFN-γ. After 18 h of exposure to LPS/IFN-γ (the activation time), the level of glutamate in the medium was determined by a colorimetric method. Additionally, to determine the capacity of activated astrocytes to remove glutamate from the medium, 100 μM glutamate was added to the medium of activated and nonactivated astrocytes, and the level of glutamate in the medium was measured before and 1, 10, 30 min and 1, 4, and 24 h after addition of glutamate.

Determination of Glutamate in Culture Media

Astrocytes for measurements of glutamate release were cultured in 25 cm² culture flasks for 21–28 DIV. The volume of the medium in the culture flasks was reduced to 2.5 ml just before exposure of astrocytes to 500 μM DETA-NONOate or myxothiazol (2 μM) for various intervals of time (5, 30 min or 4 and 24 h). After this time, the deproteinized medium of cultured astrocytes was assessed for levels of glutamate by a colorimetric method coupled to glutamate dehydrogenase producing a formazan end product using a commercially available kit (Boehringer Mannheim, Germany). In brief, diaphorase, iodonitrotetrazolium chloride (INT) and conditioned culture medium (after deproteinization) were combined (according to the provided protocol) and incubated for 2 min. Then 3.0 U of glutamate dehydrogenase solution (GDH) was added, and the absorbance was measured at 492 nm after 15 min and then every 3 min until the reaction reached steady state. A standard curve was constructed by adding known concentrations of glutamate to culture medium in the range between 1 and 50 μM. A linear relationship between steady-state absorbance and glutamate concentration was observed up to 20 μM of glutamate.

Continuous Assay of Glutamate Release

NO-induced release of glutamate from astrocytes was also assayed by following the conversion of NADP to NADPH using the fluorometric method of Nicholls et al. (1987). Changes in NADH fluorescence were used as an indirect indicator of glutamate levels. Confluent astrocytes (21–28 DIV) were gently tripinized (0.1%), spun down, and resuspended in Krebs-Hepes buffer (2 ml with ~ 4.0 × 10⁶ cells). The astrocytes were equilibrated at 37°C in a water bath for ~ 5 min and then transferred into a temperature-controlled (37°C) and magnetically stirred cuvette in a Shimadzu model RF 15-01 fluorimeter (excitation 340 nm; emission 460 nm). After a few minutes (2–3 min), 1 mM NADP and 65 U of L-glutamate dehydrogenase were added. Ap-
proximately 2–4 min later, glutamate release was initiated by addition of 1, 2, or 4 μM of NO-saturated water at 20°C (2 mM). NO-saturated water was prepared by bubbling oxygen-free nitrogen through distilled water in a glass vial with a rubber seal, then bubbling with NO gas until the water was NO-saturated. Glutamate release was calibrated by adding a known amount of glutamate at the end of each assay. The relationship between glutamate concentration and NADPH fluorescence was tested by adding 5, 10, or 15 μM of glutamate to the assay and was found to be roughly linear over this range. The amplitude of the NADPH fluorescence reached a plateau after 10–20 s.

To determine the effects of indomethacin (10 μM; Sigma) or 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (10 μM; ODQ; Alexis Biochemicals) on NO-induced glutamate release, ODQ or indomethacin was preincubated with astrocytes at 37°C in the water bath for 20 min during the equilibration time. NO-induced glutamate release was also measured after preincubation of astrocytes with 25 μM 1,2-bis(2-aminophenoxo)-ethane-N,N,N′,N′-tetra-acetic acid tetrakis, acetoxyethyl ester (BAPTA-AM; Sigma) for 30 min, 15 or 100 μM L-trans-pyrrrolidine-2,4-dicarboxylate (t-PDC; Sigma) for 1 h, 4 mM EGTA for 1 h, or 1.5 mM butyltinum toxin C (BoTx-C; Calbiochem) for 18 or 24 h in the incubator (37°C; 95% O_2 and 5% CO_2). Results are expressed as the amount (in nmol/10^6 cells) of glutamate (mean ± SD) for three of more separate experiments.

**Measurement of Extracellular ATP Levels**

ATP levels in the medium were determined lumino-metrically (Jade luminometer, Labtech International) using an ATP Bioluminescence Assay Kit (Boehringer Mannheim) according to the provided protocol. Briefly, astrocytes (after removing the microglia, 21–28 DIV, cultured in the flasks 25 cm²) after replacing the DMEM medium with Krebs-Hepes buffer (pH 7.4) were exposed to DETA-NONOate (0.5 mM; Fig. 1.) How-ever, with time, the level of glutamate in the medium of astrocytes exposed to DETA-NONOate (500 μM DETA-NONOate) decreased (after 4 h, 1.85 ± 1.3 μM), disappearing completely after 24 h (Fig. 1.), possibly due to a lower amount of NO released from DETA-NONOate after 24 h, or to downregulation of glutamate release. The release of glutamate was caused by NO released from DETA-NONOate because it was almost completely blocked by 100 μM PTIO (an NO scavenger) and the residual product (DETA-NONOate kept at room temperature, exposed to light for a few days) did not cause any significant glutamate release from astrocytes (data not shown). NO-induced glutamate release from neurons (Bal-Price and Brown, 2001), cultured rat astrocytes growing in 25 cm² flasks were exposed to an NO donor (500 μM DETA-NONOate) and the level of glutamate was determined by an enzyme-coupled assay in the medium at various times after addition of DETA-NONOate. Indeed, DETA-NONOate, even after short-term incubation (5 min), caused substantial glutamate release from cultured astrocytes (extracellular glutamate rose from 0.0 to 6.2 ± 1.7 μM; Fig. 1.) However, with time, the level of glutamate in the medium of astrocytes exposed to DETA-NONOate (500 μM) decreased (after 4 h, 1.85 ± 1.3 μM), disappearing completely after 24 h (Fig. 1.), possibly due to a lower amount of NO released from DETA-NONOate after 24 h, or to downregulation of glutamate release. The release of glutamate was caused by NO released from DETA-NONOate because it was almost completely blocked by 100 μM PTIO (an NO scavenger) and the residual product (DETA-NONOate kept at room temperature, exposed to light for a few days) did not cause any significant glutamate release from astrocytes (data not shown). NO-induced glutamate release from neurons and synaptosomes has been attributed to NO-induced inhibition of respiration (McNaught and Brown, 1998), so we tested whether a specific respiratory inhibitor would also cause rapid glutamate release from astrocytes as it does with neurons and synaptosomes. The release of glutamate was not observed after short-term exposure to 2 μM myxothiazol (a specific mitochondrial inhibitor that we have previously shown to inhibit respiration completely at this concentration; extracellular glutamate in control was 0.0 ± 0.0 μM and after addition of 2 μM myxothiazol was 0.28 ± 0.39, 0.92 ± 0.27, 1.7 ± 2.1 μM after 1, 10, and 30 min of exposure, respectively; n = 3). This suggests that NO-induced rapid release of glutamate from astrocytes is not mediated by inhibition of respiration.

**RESULTS**

**Nitric Oxide Donor Induces Glutamate Release From Astrocytes in Culture**

To determine whether nitric oxide can stimulate glutamate release from astrocytes, as it does in the case of neurons (Bal-Price and Brown, 2001), cultured rat astrocytes growing in 25 cm² flasks were exposed to an NO donor (500 μM DETA-NONOate) and the level of glutamate was determined by an enzyme-coupled assay in the medium at various times after addition of DETA-NONOate. Indeed, DETA-NONOate, even after short-term incubation (5 min), caused substantial glutamate release from cultured astrocytes (extracellular glutamate rose from 0.0 to 6.2 ± 1.7 μM; Fig. 1.) However, with time, the level of glutamate in the medium of astrocytes exposed to DETA-NONOate (500 μM) decreased (after 4 h, 1.85 ± 1.3 μM), disappearing completely after 24 h (Fig. 1.), possibly due to a lower amount of NO released from DETA-NONOate after 24 h, or to downregulation of glutamate release. The release of glutamate was caused by NO released from DETA-NONOate because it was almost completely blocked by 100 μM PTIO (an NO scavenger) and the residual product (DETA-NONOate kept at room temperature, exposed to light for a few days) did not cause any significant glutamate release from astrocytes (data not shown). NO-induced glutamate release from neurons and synaptosomes has been attributed to NO-induced inhibition of respiration (McNaught and Brown, 1998), so we tested whether a specific respiratory inhibitor would also cause rapid glutamate release from astrocytes as it does with neurons and synaptosomes. The release of glutamate was not observed after short-term exposure to 2 μM myxothiazol (a specific mitochondrial inhibitor that we have previously shown to inhibit respiration completely at this concentration; extracellular glutamate in control was 0.0 ± 0.0 μM and after addition of 2 μM myxothiazol was 0.28 ± 0.39, 0.92 ± 0.27, 1.7 ± 2.1 μM after 1, 10, and 30 min of exposure, respectively; n = 3). This suggests that NO-induced rapid release of glutamate from astrocytes is not mediated by inhibition of respiration.

**Fig. 1.** Exposure of rat cortical astrocytes (21–28 DIV) in culture flasks to DETA-NONOate (500 μM; n = 6) induced rapid release of glutamate. Values represent means ± SD.
NO Induces Rapid Calcium-Dependent Release of Vesicular Glutamate From Astrocytes

To study the kinetics and mechanism of NO-induced glutamate release, NO-saturated water was injected into a stirred suspension of astrocytes (~ 4.0 × 10^6 cells in 2 ml of Krebs-Hepes buffer, pH 7.4, at 37°C) and glutamate release was assayed continuously using a fluorometric method (Nicholls et al., 1987). Indeed, after addition of NO saturated water (1, 2, or 4 μM NO), rapid and dose-dependent release of glutamate from astrocytes was observed (Figs. 2A and 3). Glutamate release was observed immediately after NO addition and was at least as fast as the response time of the method (a few seconds), but stopped after 10–20 s, presumably because of the rapid decay of NO in such solutions (Bal-Price and Brown, 2001). In control experiments, in the absence of either L-glutamate dehydrogenase, NADP, or astrocytes, there were no fluorescence changes observed when nitric oxide was added. Glutamate release was mediated by nitric oxide because NO scavenger PTIO (100 μM) abolished it almost completely (Fig. 3). NO did not induce glutamate release from cultured microglia (Fig. 3) using the same conditions and the same number of cells, indicating that glutamate release is relatively specific to astrocytes. Interestingly, NO-induced glutamate release was completely blocked by the preincubation of astrocytes with either an extracellular Ca^{2+} chelator 4 mM EGTA (for 1 h; Figs. 2B and 3) or an intracellular Ca^{2+} chelator 25 μM BAPTA-AM (for 30 min; Figs. 2C and 3). Since in the absence of calcium glutamate release was not observed, these results indicate that the NO-induced glutamate release from astrocytes was calcium-dependent.

As there are studies suggesting that Ca^{2+}-dependent glutamate release from astrocytes can occur by a process resembling neuronal exocytosis, we investigated the effect of BoTx-C (1.5 nM), a specific toxin that blocks the exocytotic release of neurotransmitters in neurons by cleavage of syntaxin (synaptic protein). It has been shown that BoTx-C blocks bradykinin-induced calcium-dependent glutamate release from astrocytes (Jeftinija et al., 1997). To determine whether BoTx-C affects NO-induced glutamate release, astrocytes were incubated with BoTx-C (1.5 nM) for 16 or 24 h before NO addition as prolonged incubation with toxin is necessary to inhibit glutamate release from astrocytes (Bezzi et al., 1998; Pasti et al., 2001). This prolonged incubation with BoTx-C (16–24 h) did not cause any cell death as tested by Hoechst 33342 and propidium iodide staining to assess whether any apoptotic or necrotic cells, respectively, were present. Pretreatment of the astrocytes with BoTx-C for 16 h partly decreased NO-induced glutamate release (data not shown), but 24-h incubation completely blocked it (Figs. 2D and 3). This suggests that syntaxin is required for NO-evoked glutamate release and therefore the release is probably due to exocytosis of vesicular glutamate. NO-induced glutamate release was not significantly affected by preincubation of astrocytes with an inhibitor of the glutamate transporter t-PDC (100 μM for 1 h; Figs. 2E and 3), suggesting that glutamate transporters are not required for NO-induced glutamate release from cultured astrocytes. However, after preincubation with t-PDC, NO-induced glutamate release was not significantly dose-dependent. It is possible that the prolonged incubation with 100 μM t-PDC (1 h) may perturb glutamate pools inside and outside
the cells, but it is clear that the inhibitor does not block NO-induced glutamate release.

To provide further insights into the mechanism of NO-induced glutamate release, astrocytes were also preincubated with 10 μM ODQ (inhibitor of soluble guanylate cyclase) to test whether guanylate cyclase is involved. However, ODQ did not block NO-induced glutamate release from astrocytes, suggesting that guanylate cyclase is not involved (Fig. 3).

It has been reported that glutamate release from astrocytes can be blocked by indomethacin, a cyclooxygenase inhibitor (Bezzi and Volterra, 2001). However, in our studies, preincubation of astrocytes with indomethacin (10 μM) had no effect (Fig. 3), suggesting that generation of prostaglandins is not involved in NO-induced glutamate release from astrocytes. Indomethacin, ODQ, or BoTx-C did not affect the glutamate assays on their own and did not cause any cell death after the preincubation time as estimated by trypan blue staining (data not shown).

Release of glutamate was not observed after addition of 2 mM azide (an inhibitor of cytochrome oxidase; data not shown), suggesting again that NO-induced rapid release of glutamate from astrocytes was not mediated by inhibition of respiration.

NO Induces Rapid Calcium-Dependent Release of Vesicular ATP From Astrocytes

To test whether nitric oxide could also stimulate release of ATP, as it does in the case of glutamate, cultured rat astrocytes (after shaking off microglia) were exposed to 500 μM DETA-NONOate for 2.5, 5, and 10 min and the level of ATP was measured in Krebs-Hepes buffer (pH 7.4). Indeed, DETA-NONOate caused a rapid increase of extracellular ATP and after 10 min was about 10-fold higher (5.8 ± 0.7 nM) than the basal level (0.5 ± 0.4 nM; Fig. 4). The ATP release was caused by nitric oxide since 100 μM PTIO (NO scavenger) prevented it almost completely (Fig. 4). Similarly to NO-mediated glutamate release, ATP release was also entirely calcium-dependent since 30-min preincubation of cultured astrocytes with BAPTA-AM (25 μM) completely blocked the ATP release (Fig. 4). Since these results suggested that the vesicular mechanisms could be involved in NO-mediat-
ated ATP release, the astrocytes were exposed to 
BoTx-C (1.5 nM). Prolonged treatment with BoTx-C
(24–28 h) before exposure to DETA-NONOate (0.5
mM) blocked NO-induced ATP release (Fig. 4). These
results indicate that NO-induced ATP release from
cultured astrocytes was mediated by calcium-depend-
ent vesicular release, as in the case of NO-induced
glutamate release. Exposure of astrocytic culture to
DETA-NONOate (500 μM) for 2.5, 5.0, or 10 min,
BoTx-C (1.5 nM) for 28 h, or BAPTA (25 μM) for 30 min
did not cause any cell death as determined by pro-
pidium iodide and Hoechst 33342 staining.

Nitric Oxide Induces a Rapid Biphasic Increase
in Intracellular Calcium ([Ca2+]i)

To determine whether NO increased intracellular
calcium, astrocytes cultured on coverslips loaded with
fura-2 were perfused with DETA-NONOate (500 μM)
and intracellular calcium levels were monitored. The
mean resting [Ca2+]i, in astrocytes was 38.5 ± 15.3 nM
(n = 15; Fig. 6). Application of 500 μM DETA-NONO-
ate resulted in a rapid, transient (1–3 min) increase in
[Ca2+]i, (to 141.2 ± 17.8 nM; Figs. 5 and 6) followed by
return to a lower, but still elevated, level of calcium
(63.1 ± 8.3 nM) sustained for at least 10 min (Figs. 5
and 6). The observed increase in [Ca2+]i, in astrocytes
was indeed induced by NO and was completely and
rapidly reversible; after addition of 100 μM PTIO (NO
scavenger), the level of [Ca2+]i, dropped to the level
observed before DETA-NONOate application (Figs. 5A
and 6).

To test whether the nitric oxide-induced [Ca2+]i, rise
resulted from calcium influx across the plasma mem-
brane or mobilization from intracellular stores, calcium
was removed from Krebs-Hepes buffer prior to DETA-
NONOate application. In this case, only a transient
increase in [Ca2+]i, was observed after DETA-NONOate
application (Fig. 5E), suggesting that extracellular cal-
cium was required for the generation and maintenance
of the sustained [Ca2+]i, plateau observed during con-
tinued presence of extracellular calcium. The specific
inhibitor of capacitative calcium entry (CCE) 1 μM
gadolinium (Moneer and Taylor, 2002) immediately
blocked the increase in [Ca2+]i, observed after DETA-
NONOate application (Fig. 5B). These results suggest
that the biphasic [Ca2+]i, increase induced by nitric
oxide could be separated into two components: an ini-
tial transient component that is due to calcium mobi-
lization from intracellular stores and a plateau com-
ponent sustained by CCE.

Since several studies have described the expression
of voltage-activated calcium channels in astrocytes, we
tested whether nitric oxide-induced Ca2+ entry could
be partially mediated by voltage-gated Ca2+ channels.
However, 10 μM verapamil (blocker of L-type Ca2+
channels) did not affect the [Ca2+]i, plateau induced by
DETA-NONOate (Figs. 5C and 6), suggesting that NO-
induced Ca2+ entry into astrocytes was not mediated
by voltage-sensitive Ca2+ channels. To study further
the mechanisms of NO-induced [Ca2+]i, increase, the
astrocytes were preincubated for 10 min with 10 μM
ODQ (guanylate cyclase inhibitor) before DETA-
NONOate application to determine whether NO-in-
duced calcium entry into the cells could be mediated by
the cGMP pathway. However, preincubation of astro-
cytes with ODQ did not have any influence on NO-
induced increase of [Ca2+]i, (Figs. 5D and 6), indicating
that the NO-mediated Ca2+ entry is cGMP-indepen-
dent.

Nitric Oxide Released From
LPS/IFN-γ-Activated Astrocytes Causes High
Extracellular Glutamate Levels But Has Little
Effect on Glutamate Uptake

Since exogenously added NO caused rapid glutamate
release, we tested whether endogenously produced NO
from cytokine-activated astrocytes could elevate extra-
cellular glutamate levels.

Astrocytes (21–28 DIV, confluent) were exposed for
18 h to LPS and IFN-γ, which leads to high levels of NO
production from iNOS (Bal-Price and Price, 2001) in
the presence or absence of an iNOS inhibitor 1400W
(25 μM). After 18 h of activation, glutamate levels in
the medium were measured using a colorimetric
method and compared with nonactivated astrocytes.
In the case of activated astrocytes, high levels of gluta-
mate were present in the medium (7.38 ± 1.6 μM) after
18 h of activation as compared with very low level of
glutamate in the medium of control astrocytes (0.43 ±
0.06 μM; Fig. 7). In the presence of the iNOS inhibitor
(25 μM 1400W), glutamate was still present in the
medium but at a lower level (3.72 ± 0.6 μM). These
results are compatible with NO from activated astro-
cytes inducing glutamate release. However, the same
result could be caused by a decreased capacity of ac-
vitated astrocytes for glutamate uptake.

To determine whether nitric oxide produced by the
proinflammatory cytokine-activated astrocytes could
attenuate astrocytic capacity for glutamate uptake,
100 μM exogenous glutamate from stock solution was
added to the astrocytes after 18 h of activation in the
presence and absence of iNOS inhibitor (25 μM 1400W),
and then samples of the medium were taken at various intervals of time (1, 10, 30 min or 1, 4, and
24 h) and glutamate content was measured. In the
control cultures, glutamate concentrations showed a
rapid initial decline; within 30 min glutamate concen-
trations dropped to 6.7 ± 1.46 μM (Fig. 7) and after 1 h
reached values below 1 μM. These data show that
control astrocytes were capable of taking up glutamate
rapidly and efficiently. LPS/IFN-γ-activated astro-
cytes removed glutamate from the medium at a similar
rate to nonactivated (or 1400W-treated) astrocytes.
However, the level of glutamate in the medium of ac-
vitated astrocytes was always higher than in the con-
control culture at each time point both before and after
glutamate addition (Fig. 7). After 24 h, glutamate was still present in the medium of activated astrocytes (7.8 ± 1.5 μM) at concentration similar to that prior the addition of 100 μM glutamate. In the presence of the iNOS inhibitor (1400W), glutamate uptake was again similar, but after 24 h the glutamate level returned to a steady-state level lower than in the absence of 1400W (but higher than in nonactivated astrocytes).

These results suggest that the elevated extracellular glutamate level maintained by activated astrocytes is due to increased glutamate release, with little or no change in glutamate uptake.

**DISCUSSION**

It has previously been shown that NO can cause acute glutamate release from neurons or synaptosomes (Sequeira et al., 1997; McNaught and Brown, 1998; Bal-Price and Brown, 2001), and we have attributed this to NO inhibition of mitochondrial respiration causing a fall in ATP, inhibition of the sodium pump, and reversal of the glutamate transporter. Evidence for this mechanism was that the time, oxygen, and NO dependence of glutamate release and respiratory inhibition were similar, glutamate release was calcium-independent, and specific respiratory inhibitors caused a similar fall in ATP and glutamate release (McNaught and Brown, 1998; Bal-Price and Brown, 2001). Sequeira et al. (1997) found a similar calcium-independent glutamate release from synaptosomes induced by NO, associated with a decrease in ATP/ADP ratio and inhibited by t-PDC (an inhibitor of the glutamate transporter).

In contrast, we find here that NO-induced glutamate release from astrocytes is completely calcium-dependent and is not replicated by respiratory inhibitors. The reason NO does not cause energy depletion-induced glutamate release from astrocytes is probably because although it inhibits astrocytic respiration (Brown et al., 1995), it does not cause a fall in astrocytic ATP (Bal-Price and Brown, 2001), presumably because of the relatively high glycolytic capacity of astrocytes (Pauwels et al., 1985; Peuchen et al., 1997). Our finding that t-PDC did not have any significant effect on NO-induced glutamate release from astrocytes confirms that this release is not mediated by reversal of the glutamate transporter.

In the case of neurons, it has been proposed that NO causes glutamate release by directly modifying the exocytotic machinery of synaptic vesicles. This was based on the finding that neuronal glutamate release induced by NO was calcium-independent but inhibited by butu-
linum neurotoxins A, C, and F and associated with covalent modifications of vesicle proteins (Meffert et al., 1996). However, our finding that NO-induced glutamate (and ATP) release in astrocytes is completely calcium-dependent suggests that such direct mechanisms are not relevant in astrocytes, at least in our conditions.

Astrocytes have a glutamate release mechanism similar to that of neurons, mediated by exocytosis of vesicles loaded with glutamate (Araque et al., 2000; Bezzì and Volterra, 2001). This exocytosis is triggered by increased cytosolic calcium (often in the form of calcium waves) (Bezzì et al., 1998; Araque et al., 2000) and is inhibited by botulinum neurotoxin C or B (Jeftinija et al., 1997; Araque et al., 2000). As we found that NO-induced glutamate and ATP release from astrocytes were calcium-dependent and inhibited by BoTx-C, it seems likely that this release is mediated by vesicular exocytosis.

It has previously been shown that NO induces acute increases of cytosolic calcium (and calcium waves) in astrocytes, possibly via activation of soluble guanylate cyclase (Willmott et al., 2000a). In our present studies, we show that the application of DETA-NONOate to fura-2–loaded astrocytes caused a very rapid increase of \([\text{Ca}^{2+}]_i\), but this was not blocked by ODQ (a soluble guanylate cyclase inhibitor), suggesting that soluble guanylate cyclase was not involved. The discrepancy between our studies and the results described by Willmott et al. (2000a) is possibly due to the fact that in their studies LY83583 was used to block guanylate cyclase activity, but this compound is known also to inactivate nitric oxide (Barbier and Lefebvre, 1992), in contrast to ODQ, which does not affect NO levels. cGMP-independent mechanisms of NO-induced increase in \([\text{Ca}^{2+}]_i\) were also shown in C6 glioma (Bowman et al., 2001) and Bergmann glial cells (Matyash et al., 2001).

Since gadolinium returned the \([\text{Ca}^{2+}]_i\), from the plateau level to the basal level, these results suggest that \(\text{Ca}^{2+}\) entry occurs through CCE mechanism in astrocytes. However, gadolinium can also inhibit L- and N-type voltage-dependent calcium channels that are expressed on some astrocytes (Sontheimer, 1994; Agrawal et al., 2001). \(\text{Ca}^{2+}\) entry across the plasma membrane was not mediated by L-type voltage-sensitive \(\text{Ca}^{2+}\) channels because verapamil did not affect \([\text{Ca}^{2+}]_i\) levels but we cannot rule out that N-type channels contribute to calcium entry. As in other nonexcitable cells exposed to agents mobilizing calcium from endoplasmic reticulum (Matsumoto et al., 1986; Kotlikoff et al., 1987), the initial component of the biphasic \([\text{Ca}^{2+}]_i\) increase observed after application of DETA-NONOate was insensitive to the removal of extracellular \(\text{Ca}^{2+}\), indicating that it was due to mobilization of \(\text{Ca}^{2+}\) from intracellular stores. Depletion of intracellular \(\text{Ca}^{2+}\) could provide a sufficient signal for activation of \(\text{Ca}^{2+}\) entry through the plasma membrane as extracellular \(\text{Ca}^{2+}\) is required for the generation and maintenance of the sustained \([\text{Ca}^{2+}]_i\), plateau during prolonged application of DETA-NONOate. The NO-induced glutamate release from astrocytes found here was fast—faster than the response time of the continuous assay (Fig. 2)—and terminated when the added NO was likely to have disappeared from the medium,
so further NO addition caused more glutamate release. This suggests that the effect was mediated by NO itself, rather than one of its derivatives (peroxynitrite, NO2, NO3, or S-nitrosothiols), as it would take time for these derivatives to be produced from NO. Virtually all rapid, reversible effects of NO are mediated by NO binding to hemeproteins, such as soluble guanylate cyclase or cytochrome oxidase (Xu et al., 1998; Eu et al., 2000). However, neither of these hemeproteins appears to mediate the NO-induced glutamate release from astrocytes as release was not prevented by an inhibitor of soluble guanylate cyclase (ODQ) and release was not induced by an inhibitor of cytochrome oxidase (azide).

Willmott et al. (2000a) found that NO-induced calcium waves in astrocytes were eliminated by high concentrations of ryanodine, suggesting that NO activates ryanodine receptor-mediated calcium release from endoplasmic reticulum. NO can elevate calcium in heart and skeletal muscle by S-nitrosating or oxidizing ryanodine receptors (Eu et al., 2000). This has not been reported in astrocytes, but as astrocytes express the R3 isoform of ryanodine receptors (Matyash et. al, 2002), a possible mechanism by which NO elevates calcium in astrocytes is by S-nitrosating or promoting thiol oxidation of the ryanodine receptor to cause release from the endoplasmic reticulum.

The mechanisms by which ATP is released from astrocytes are still unclear. In our present studies, NO-induced ATP release was entirely calcium-dependent and blocked by BoTx-C, suggesting vesicular exocytosis. Indeed, ATP has been identified as a storage component of astrocytic vesicles released from cultured astrocytes (Maienschein et al., 1999) by receptor-mediated mechanisms (Queiroz et al., 1997). Calcium-dependent ATP release from astrocytes was also reported in response to excitatory amino acids like N-methyl-D-aspartate (NMDA) and kainite (Queiroz et al., 1999). However, other mechanisms of ATP release have been reported, for example, through connection hemichannels (Cotrina et al., 1998b; Stout et al., 2002) or calcium-independent mechanism after stimulation of alpha-amino-3-hydroxy-5-methylisoxazole-4-propio (ATP) receptors (Queiroz et al., 1999).

Recently, it has been shown that ATP released from astrocytes mediates increases in cytosolic calcium and propagation of calcium waves in glia (Cotrina et al., 1998a; James and Butt, 2001). ATP-dependent calcium signaling in astrocytes is mediated by metabotropic purinergic P2Y receptors (coupled to calcium release from internal stores) (Guthrie et al., 1999; Fam et al., 2000). ATP apparently also mediates calcium signaling between astrocytes and microglia through P2X purinergic receptor located on microglia (Verderio and Matteoli, 2001). ATP-mediated increase in intracellular calcium was also observed after the mechanical stimulation of astrocytes in culture (Stout et al., 2002). Interestingly, ATP can also cause a dose-dependent release of glutamate and aspartate from cultured rat astrocytes (Jeremic et al., 2001). In the present studies, we have shown that NO induced both ATP and glutamate release but we have not studied whether the NO-induced ATP release potentiates glutamate release or vice versa, as has been suggested in other studies (Queiroz et al., 1999). However, the NO-induced increase in extracellular ATP observed in our studies is rather low (5.8 ± 0.7 nM), and whether it could play a significant role in intercellular signaling needs further investigation.

Physiologically, in the brain NO is mainly produced by nNOS in subpopulation of neurons, and this nNOS is transiently activated by calcium elevations during neuronal activity (Vincent, 1994; Baader and Schilling, 1996; Baltrons et al., 1997). This suggests the possibility that NO released from active neurons may cause calcium waves in and glutamate (and ATP) release from surrounding astrocytes, and thus might be involved in neuronal-astrocytic communication and could particularly affect synaptic transmission as astrocytes and their processes surround synapses (Parpura et al., 1994; Bezi and Volterra, 2001). eNOS is expressed in some astrocytes and has been suggested to be involved in initiation and propagation of calcium waves (Willmott et al., 2000b), and thus potentially astrocytic eNOS might regulate glutamate and ATP release from astrocytes. Whether neurons or astrocytes locally release sufficient NO to cause significant glutamate or ATP release from astrocytes is unclear, but levels of NO up to 100 nM have been measured by electrode in brain slices induced by electrical stimulation (Shibuki and Kimura, 1997), up to 1 μM NO released from cultured aortic endothelial cells induced by bradykinin (Clementi et al., 1999), and up to 1–4 μM has been measured in rat brain in vivo during ischemia and reperfusion (Malinski et al., 1993). The NO released during ischemia or reperfusion might trigger extensive glutamate release from astrocytes, which could contribute to the known pathological glutamate release in these conditions (Sztakowski and Attwell, 1994; Rossi et al., 2000).

In a wide range of brain pathologies, astrocytes and microglia become activated by inflammatory mediators to express iNOS (Wisniewski et al., 1998; Murphy, 2000). In culture, activated glia can kill cocultured neurons by NO and excitotoxic mechanisms (Chao, 1996; Kingham et al., 1999; Bal-Price and Brown, 2001). Such activated astrocytes and microglia continuously produce an extracellular NO level of about 1 μM (Brown et al., 1995; Bal-Price and Brown, 2001), sufficient to cause substantial glutamate release from astrocytes according to our results here. And indeed we have shown here that activated astrocytes maintain a substantially higher extracellular glutamate level, which appears to be due to enhanced glutamate release rather than reduced uptake. It has previously been reported (Ye and Sontheimer, 1998) that uptake of glutamate into astrocytes is inhibited by NO or inflammatory activation; however, the level of inhibition reported was relatively small (about 30%), such that it would be unlikely to contribute substantially to an elevated extracellular glutamate level. We found no...
obvious inhibition of glutamate uptake by activated astrocytes, but our data are not sufficient to rule out a small inhibition of the order of 30%. According to other data (Patneau and Mayer, 1990), the level of extracellular glutamate we find maintained by activated astrocytes would be sufficient to activate NMDA receptors and therefore might contribute to the mechanisms by which activated glia kill neurons in coculture. Thus, NO-induced release of glutamate from astrocytes potentially might contribute to neuronal death during all the inflammatory, infectious, ischemic, and neurodegenerative diseases where iNOS has been shown to be expressed in glia (Eddleston and Mucke, 1993; Kreutzberg, 1996).

ACKNOWLEDGMENT

The authors thank Dr Edward Bampton for preparing the fibroblast culture.

REFERENCES


