Cellular/Molecular

A Calcium-Induced Calcium Influx Factor, Nitric Oxide, Modulates the Refilling of Calcium Stores in Astrocytes

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The roles of nitric oxide are primarily undefined in astrocytes, cells that are active partners in synaptic transmission. Because nitric oxide synthases are present in astrocytes, we imaged the formation of nitric oxide in cultured murine cortical astrocytes using DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate). We demonstrated that physiological concentrations of ATP induced a Ca^{2+}-dependent production of nitric oxide. We then investigated the roles of nitric oxide in astrocytic Ca^{2+} signaling by exogenous application of a nitric oxide donor and found that nitric oxide induced an influx of external Ca^{2+}. Because these observations raise the possibility that nitric oxide-dependent Ca^{2+} influx could lead to the refilling of internal stores with Ca^{2+}, we directly monitored the Ca^{2+} levels of the cytosol and of internal stores while manipulating nitric oxide. Cultures were coloaded with mag-fluo-4 and X-rhod-1 to differentially load the internal stores and cytosol, respectively. ATP induced a cytosolic increase in Ca^{2+} that results from the IP_3-dependent release of Ca^{2+} from internal stores, detected as a simultaneous reduction in mag-fluo-4 and an increase in X-rhod-1 fluorescence. To monitor store refilling, we measured the recovery of mag-fluo-4 fluorescence after removal of ATP. When nitric oxide signaling was blocked by the nitric oxide scavenger 2-phenyl-4,4,5,5-ketramethyl-imidazoline-1-oxyl-3-oxide or by the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine, fluorescence recovery was significantly reduced. These data suggest that transmitters that induce Ca^{2+} signaling in astrocytes lead to the Ca^{2+}-dependent synthesis of nitric oxide. This in turn stimulates a Ca^{2+} influx pathway that is, in part, responsible for the refilling of internal Ca^{2+} stores.

Key words: **nitric oxide; astrocyte; calcium influx; internal calcium stores; DAF-FM; mag-fluo-4; ATP; tripartite synapse**

Introduction

The endogenous gas nitric oxide (NO) was first discovered as a physiological messenger in vascular smooth muscle relaxation (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Palmer et al., 1987). In the CNS, nitric oxide acts as a membrane-permeant diffusible neurotransmitter that can signal between distant synapses and cells. Nitric oxide has been implicated in neuronal development, synaptic transmission, synaptic plasticity, and in both neuroprotection and neurotoxicity (Lipton et al., 1994; Schuman and Madison, 1994; Jaffrey and Snyder, 1995; Holscher, 1997).

Although there have been many studies on nitric oxide in the nervous system, these studies have focused predominantly on the neuronal synthesis of nitric oxide (Holscher, 1997; Crepel, 1998; Hawkins et al., 1998). Whereas neurons do express nitric oxide synthases, astrocytes, a subtype of glial cell intimately associated with the synapse, express all three forms of nitric oxide synthases (Murphy, 2000). Whether synaptic activity regulates nitric oxide synthesis in astrocytes is, however, undefined.

Astrocytes are active partners in a "tripartite synapse" comprising the presynaptic and postsynaptic terminals along with the synaptically associated astrocyte (Araque et al., 1999; Volterra et al., 2002). In this arrangement, astrocytes respond to neurochemical stimuli by releasing Ca^{2+} from internal stores (Cornell-Bell et al., 1990; Charles et al., 1993; Salter and Hicks, 1995; Venance et al., 1997). The elevation of internal astrocytic Ca^{2+} has many consequences, including the induction of release of the chemical transmitter glutamate (Parpura et al., 1994; Innocenti et al., 2000), which can signal to adjacent neurons (Charles, 1994; Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995; Pasti et al., 1997; Araque et al., 1998a; Bezzi et al., 1998) and modulate neuronal activity (Araque et al., 1998a; Newman and Zahs, 1998; Parpura and Haydon, 2000) and synaptic transmission (Araque et al., 1998b; Kang et al., 1998). Because the activity of neuronal and endothelial nitric oxide synthases are Ca^{2+} dependent (Griffith and Stuehr, 1995), neurotransmitter-induced Ca^{2+} elevations in astrocytes have the potential to lead to the formation of nitric oxide. Because of the intimate interactions between blood vessel endothelia cells, astrocytes, and neurons, astrocyte-derived nitric oxide may provide a means for astrocytes to modulate synaptic activity and to control blood flow in response to synaptic activity.

To explore this possibility, we first tested whether nitric oxide

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is generated during astrocytic Ca^{2+} signaling by imaging nitric oxide in purified murine cortical astrocyte cultures with a fluorescent indicator, DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) (Kojima et al., 1999). We found that physiological concentrations of a natural neuromessenger, ATP, induced Ca^{2+}-dependent nitric oxide production. Addition of a nitric oxide donor, *S*-nitrosol-*N*-acetylpenicillamine (SNAP), induced an influx of external Ca^{2+}. Moreover, by simultaneously imaging Ca^{2+} in cytosol and internal stores, we demonstrated that, by promoting Ca^{2+} influx, nitric oxide facilitates the refilling of internal stores that become partially depleted as a result of Ca^{2+} release during neurotransmitter-induced Ca^{2+} signaling.

Materials and Methods

Purified cortical astrocyte cultures. Purified cortical astrocyte cultures were prepared as described previously (Parpura et al., 1995). Briefly, postnatal 0- to 2-d-old mice were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ). After the brain was removed and placed into Ca^{2+}/Mg^{2+}-free Earle's balanced salt solution (EBSS), pH 7.35 (Invitrogen, Carlsbad, CA), cortices were dissected and incubated for 1 hr at 37°C in EBSS containing papain (20 U/ml), HEPES (10 mM), L-cysteine (0.2 mg/ml), glucose (20 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Tissue was washed once with fresh EBSS and then placed in EBSS containing HEPES (10 mM) and trypsin inhibitor (10 mg/ml) for 5 min to stop further digestion. After rinsing, cortices were mechanically dissociated in culture medium by triturating through sterile serological glass pipettes. Culture medium consisted of phenol red-free modified minimum essential medium [Earle's salts (Invitrogen), containing 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin] supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). Cells were plated into 25 cm^2 Falcon culture flasks (BD Biosciences, Bedford, MA) and grown to confluence at 37°C in a humidified 5% CO_2–95% O_2 atmosphere. Cells were fed every 3–4 d by replacing 50% of the medium with fresh medium. After 7–14 d, the flasks were shaken twice on a horizontal orbital shaker at 260 rpm, first for 1.5 hr and then, after replacement with ice-cold medium, for 18 hr. The remaining adherent cells were enzymatically detached with trypsin (0.1%), pelleted (800 × *g*, 10 min), resuspended in culture medium, and plated onto poly-L-lysine (1 mg/ml; molecular weight, 40,000–100,000)-coated glass coverslips. The cells were used in experiments after 1–4 d, by which time they had grown to confluence.

Nitric oxide measurement. Relative changes in cytosolic nitric oxide concentration in astrocytes were monitored using the fluorescent nitric oxide probe DAF-FM. Cells were loaded with 10 μM DAF-FM diacetate (Molecular Probes, Eugene, OR) for 45 min at room temperature (20–23°C) in normal external saline containing the following (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, and 6 sucrose, pH 7.35, and then deacetylated for 45 min. Although a previous nitric oxide indicator, DAF-2 (4, 5-diaminofluorescein), has been reported to undergo photosensitization (Broillet et al., 2001), we did not find any change in intensity of DAF-FM fluorescence under illumination in the conditions described in these experiments.

Ca^{2+} measurement. Ca^{2+} levels in astrocytes were imaged using fluorescence microscopy. Unless otherwise specified, cultures were incubated at room temperature for 45 min in the presence of fluo-3 AM (10 μg/ml; Molecular Probes) and then de-esterified for 45 min.

Simultaneous measurement of cytosolic and internal store Ca^{2+}. In some experiments in which we simultaneously monitored cytosolic and internal store Ca^{2+} levels, purified cortical astrocyte cultures were first loaded with mag-fluo-4 AM (10 μg/ml; Molecular Probes) at 37°C for 45 min, washed, loaded with X-rhod-1 AM (2.5 μg/ml; Molecular Probes) at room temperature for 45 min, and then allowed to de-esterify for 45 min before imaging.

Image acquisition and processing. In single-dye imaging experiments, coverslips containing dye-loaded cells were visualized using a cooled digital camera (ORCA; Hamamatsu, Hamamatsu City, Japan) attached to a Nikon (Tokyo, Japan) inverted microscope. For experiments using fluo-3, fluo-4, or DAF-FM alone, light from a xenon arc lamp (100 W) was filtered at 480 nm (480DF10; Omega Optical, Brattleboro, VT) and delivered to the sample through a 20 × 0.7 numerical aperture objective. Fluorescent emission was collected through a dichroic mirror (510DRLP; Omega Optical) and filtered with a 515EFLP filter (Omega Optical). Time-lapse images were acquired using either Automation software (Prairie Technologies, Middleton, WI) or Metamorph software (Universal Imaging, West Chester, PA). Frame acquisition interval was 3 sec.

For two-wavelength imaging experiments, dye-loaded cells were excited with 488 and 568 nm laser lines, and fluorescence was imaged in two channels with a confocal microscope (Prairie Technologies) using Confocal version 1.47 software (Prairie Technologies). A 488/568 nm dual dichroic mirror, which can reflect both 488 and 568 nm excitation light, was used as the primary dichroic. A 550DRLP dichroic mirror was used between channels 1 and 2. Emission filters for channels 1 and 2 were 610/75 and 525/50 bandpass, respectively. Frame acquisition interval was 6 sec. Because mag-fluo-4 exhibited some photobleaching in these confocal experiments, we corrected all traces by extrapolation of a linear regression curve that was fit to the control data before addition of ligands. In parallel control experiments using wide-field epifluorescence in which photobleaching was not detected, we confirmed that the use of linear regression curves in confocal studies did not introduce any systematic errors in our results. For example, in this study, we report an overshoot of mag-fluo-4 intensity after store refilling (see Results). This overshoot is not the result of an error introduced by the use of the photo-bleaching correction method because the overshoot was observed when this correction was not applied to cells that exhibited minimal bleaching.

All imaging experiments were performed at room temperature. We used an imaging chamber with a volume of ~150 μl. Normal external saline as described above (Nitric oxide measurement) was continuously perfused at a rate of 1–2 ml/min, unless otherwise specified.

For quantitative studies, the temporal dynamics in fluorescence were expressed as background-subtracted df/F_0 (%), where F_0 represents the fluorescence level of the cells before stimulation, and df represents the change in fluorescence occurring during stimulation of the cell. We report measurements from all of the cells in the imaging fields regardless of their responses to ligands. Statistical differences were established using the Student's t test at $p < 0.05$, $p < 0.01$, and $p < 0.001$. Data are expressed as mean ± SEM.

Photolysis. In some experiments, flash photolysis was used to provide a fast localized NO stimulus. Caged NO (potassium nitrosylpentachlororuthenate, 1 mM; Molecular Probes) was included in the external saline. We used a Noran Odyssey confocal upright microscope together with an integrated wide-field fluorescence illumination pathway and an optical pathway for laser excitation for photolysis (Prairie Technologies). A pulsed nitrogen laser (337 nm, 3 nS) launched into a UV transmitting optical fiber was used for photolysis excitation. A second 635 nm laser was coupled into the same optical fiber for positioning. The illumination spot on the sample was ~6 μm. Step motors were used to move the position of the fiber in the image space. Positioning–shuttering of the laser were all controlled by computer software.

Immunocytochemistry. The purity of astrocytes in the purified cortical astrocyte cultures (>99%) was confirmed by immunocytochemistry. Cultures were fixed with 4% paraformaldehyde in PBS, pH 7.3, at room temperature for 30 min, rinsed with PBS, and then permeabilized with Triton X-100 (0.25% in PBS) for 10 min. The cultures were then incubated in PBS containing 5% BSA, 5% normal goat serum, 0.25% Triton X-100, and 0.02% NaN_3 for 30 min to block nonspecific binding. Cultures were incubated with mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (Sigma, St. Louis, MO) and rabbit anti-microtubule-associated protein 2 (MAP-2) polyclonal antibody (Chemicon, Temecula, CA) for 12 hr at 4°C. Primary antibodies were removed by washing with PBS containing 5% normal goat serum, 0.25% Triton X-100, and 0.02% NaN_3 and then incubated with fluorescent dye-labeled secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG; Molecular Probes) for 2 hr. The coverslips were mounted in *n*-propyl gallate in glycerol onto glass microscope slides

and imaged with a laser scanning confocal microscope (Prairie Technologies). The sensitivity of GFAP and MAP-2 antibodies were confirmed by cocultures of cortical astrocytes and neurons

Chemicals. DAF-FM diacetate, fluo-3 AM, fluo-4 AM, mag-fluo 4AM, X-rhod-1 AM, SNAP, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) AM, and potassium nitrosylpentachlororuthenate (caged nitric oxide I) were purchased from Molecular Probes; 2-aminoethoxy-diphenylborate (2-APB), N^G-monomethyl-L-arginine (L-NMMA), and N^G-monomethyl-D-arginine (D-NMMA) were purchased from Calbiochem (La Jolla, CA); all other chemicals were purchased from Sigma, unless otherwise specified.

Results

ATP induces nitric oxide production in astrocytes

ATP is a critical natural extracellular messenger involved in Ca^{2+} signaling in astrocytes. It induces an astrocytic Ca^{2+} elevation and mediates the propagation of Ca^{2+} waves between astrocytes (Guthrie et al., 1999). Activities of neuronal nitric oxide synthase and endothelial nitric oxide synthase are known to be Ca^{2+} dependent (Griffith and Stuehr, 1995; Gross and Wolin, 1995). Therefore, we asked whether ATP can induce nitric oxide production using the nitric oxide-sensitive fluorescence indicator DAF-FM. Application of 10 μM ATP induced a small but reliable increase of DAF-FM fluorescence (Fig. 1) in 97.5 ± 1.3% of the cells studied (n = 15 experiments, 683 cells). Control experiments confirmed that the increase in DAF-FM fluorescence was attributable to nitric oxide production, because both the membrane-permeant nitric oxide scavenger 2-phenyl-4,4,5,5-ketramethyl-imidazoline-1-oxyl-3-oxide (PTIO) (100 μM, 5 min incubation) (Akaike et al., 1993) (Fig. 1*D*) and the nitric oxide synthase inhibitor L-NMMA (300 μM, 30 min), but not its inactive analog D-NMMA (300 μM, 30 min), blocked the ATP-induced DAF-FM fluorescence increase (Fig. 1*B*,*E*).

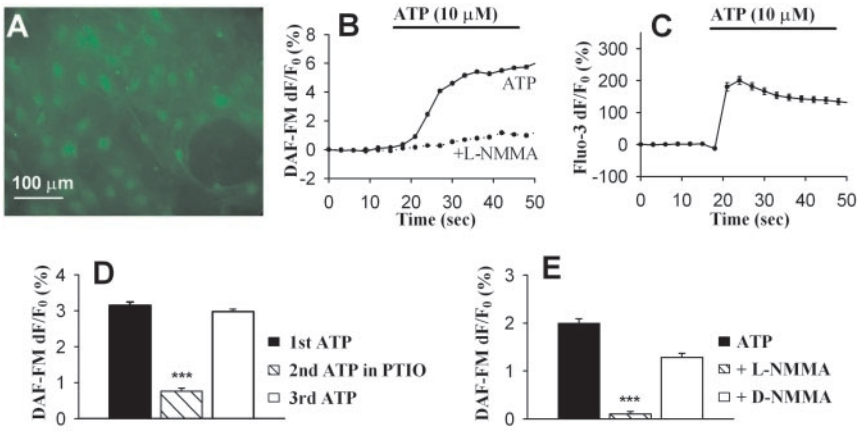

Figure 1. ATP induces nitric oxide production in astrocytes. *A*, An example of a fluorescence image of purified astrocytes loaded with the nitric oxide-sensitive dye DAF-FM. *B*, Typical traces of 10 μM ATP-induced increase of DAF-FM fluorescence in control conditions (solid line) and after treatment with the nitric oxide synthase inhibitor L-NMMA (300 μM, 30 min; dashed line). The data points are averages of all cells in the imaging field in one experiment. Error bars are SEMs. *C*, Ca^{2+} elevation by 10 μM ATP as imaged with Ca^{2+} indicator fluo-3. Data in *C* are from a separate experiment than that shown in *B* and are shown to indicate the time course of changes in cytosolic Ca^{2+} in relation to nitric oxide production. *D*, Summary graph showing that the nitric oxide scavenger PTIO significantly reduced the amplitude of ATP-induced DAF-FM fluorescence increase in astrocytes (n = 171 cells, 4 experiments). Cells were first stimulated with 10 μM ATP for 30 sec, washed for 10 min in normal saline, for 5 min in 100 μM PTIO, and then stimulated again with ATP in PTIO. After washing in normal saline for 15 min, cells were stimulated with ATP for a third time. *E*, Pretreatment with the nitric oxide synthase inhibitor L-NMMA (300 μM, 30 min; n = 244 cells, 4 experiments), but not with its inactive analog D-NMMA (300 μM, 30 min; n = 197 cells, 4 experiments), prevented the increase in DAF-FM fluorescence induced by ATP (control untreated, n = 188 cells, 4 experiments; note that each histogram is from separate experiments performed in parallel on different cells). Statistical differences were established using the Student's *t* test at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Error bars indicate SEMs.

Ca^{2+} is necessary for ATP-induced nitric oxide production

Because the activity of nitric oxide synthases are Ca^{2+} dependent and because ATP induces a Ca^{2+} elevation in astrocytes, it is logical to test whether a Ca^{2+} elevation is required for the ATP-induced nitric oxide production. To test this hypothesis, we chelated the cytosolic Ca^{2+} by incubating astrocytes in BAPTA AM (30 μM, 45 min). In the presence of BAPTA, ATP no longer induced an increase in DAF-FM fluorescence (four experiments each; $p < 0.001$) (Fig. 2*D*). Separate experiments involving imaging with the Ca^{2+} indicator fluo-3 confirmed that BAPTA blocked the Ca^{2+} increase caused by ATP (three experiments each; $p < 0.001$) (Fig. 2*C*). The addition of thapsigargin (10 μM) to cultures, which blocks the Ca^{2+}-ATPase of internal stores and leads to an elevation of cytosolic calcium, caused a 2.97 ± 0.29% (n = 101 cells) increase in DAF-FM fluorescence. These results demonstrate that an increase in internal Ca^{2+} is necessary for ATP-induced nitric oxide production. In addition, when astrocytes were treated with PPADS (50 μM, 5 min), an antagonist for P_2 receptors, the ATP-induced nitric oxide production was also inhibited (n = 150, 4 experiments) (Fig. 2*B*). The effectiveness of PPADS in blocking astrocytic Ca^{2+} elevation

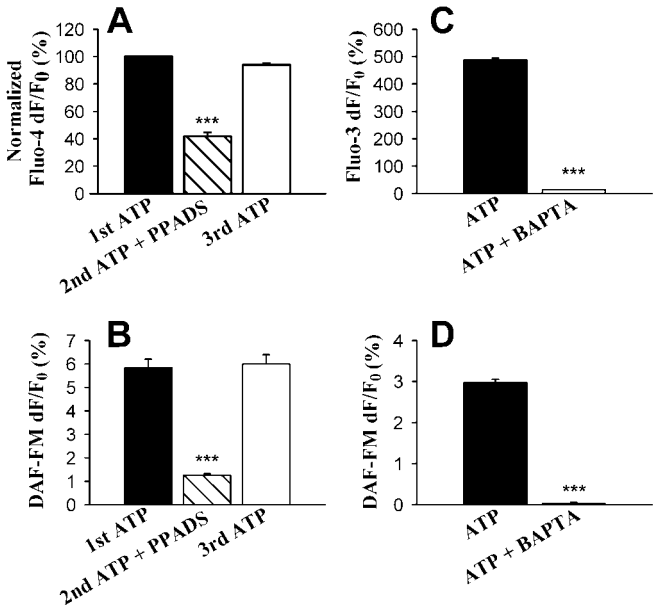

Figure 2. ATP-induced nitric oxide production is P_2 receptor mediated and Ca^{2+} dependent. *A*, Treatment with the P_2 receptor antagonist PPADS (50 μM, 5 min) inhibited the Ca^{2+} elevation caused by ATP (n = 195 cells, 4 experiments). *B*, Treatment with PPADS significantly reduced the amplitude of ATP-induced DAF-FM fluorescence increase (n = 150 cells, 4 experiments). *C*, After cells were incubated with the Ca^{2+} chelator BAPTA (BAPTA AM at 30 μM, 45 min), ATP-induced Ca^{2+} elevation was blocked (n = 242 cells in BAPTA and 316 cells in control conditions, 3 experiments each). *D*, Treatment with BAPTA (BAPTA AM at 30 μM, 45 min) significantly reduced the amplitude of ATP-induced DAF-FM fluorescence increase (n = 240 cells in BAPTA and 174 cells in control conditions, 4 experiments each). Statistical differences were established using the Student's *t* test at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Error bars indicate SEMs.

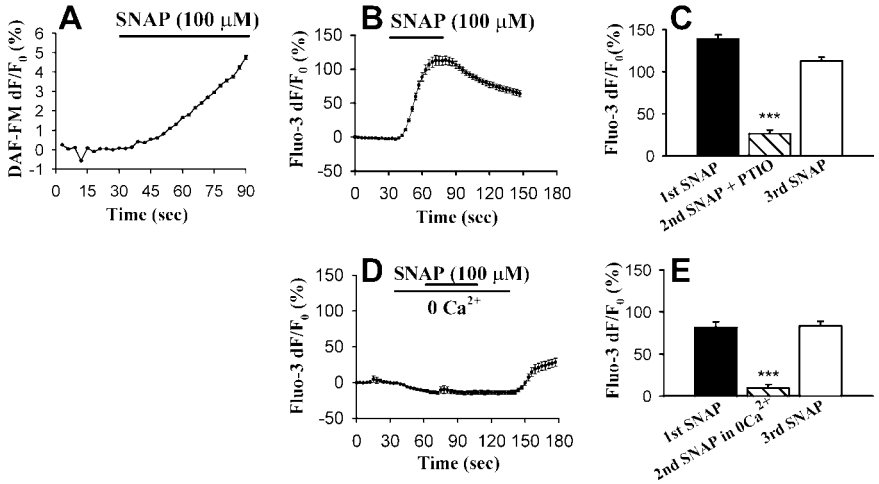

Figure 3. The nitric oxide donor SNAP induces an influx of external Ca^{2+} into astrocytes. *A*, SNAP (100 μM), a nitric oxide donor, caused an increase in cellular DAF-FM fluorescence, confirming that SNAP spontaneously releases nitric oxide in saline. *B*, Typical astrocytic Ca^{2+} increase in response to SNAP. Astrocytes were loaded with fluorescent Ca^{2+} indicator fluo-3. Cells were stimulated by perfusion with SNAP (100 μM, 45 sec). The trace is the average of all of the cells in the imaging field ($n = 31$ cells). Error bars are SEMs. *C*, Nitric oxide is responsible for the SNAP-induced Ca^{2+} increase as the nitric oxide scavenger PTIO inhibited the fluo-3 fluorescence increase caused by SNAP. Three application protocols were used. The first and third SNAP applications were used as internal controls for each cell ($n = 114$ cells, 3 experiments). *D*, After washing with normal saline for 10 min, the same cells as shown in *B* were treated with zero-Ca^{2+} saline for 30 sec and stimulated with SNAP again. This time, little Ca^{2+} change was induced by SNAP. *E*, The summary histogram shows that the second SNAP application in zero-Ca^{2+} saline induced a smaller astrocytic Ca^{2+} increase than the first (control) and third (recovery) applications in normal saline ($n = 84$ cells, 4 experiments). Statistical differences were established using the Student's *t* test at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Error bars indicate SEMs.

was confirmed by Ca^{2+} imaging experiments in which PPADS reversibly inhibited ATP-induced cytosolic Ca^{2+} elevation in astrocytes (Fig. 2*A*). This is consistent with a P_2 receptor-mediated Ca^{2+} elevation stimulating nitric oxide production.

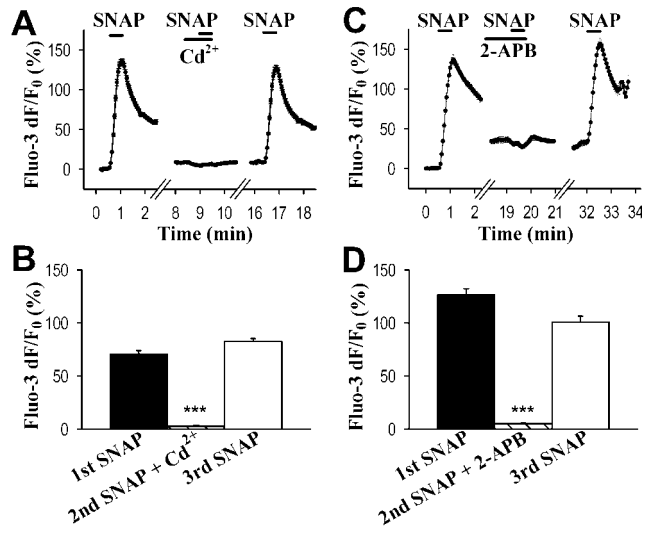

Figure 4. External Cd^{2+} and 2-APB block the SNAP-induced Ca^{2+} elevation in astrocytes, suggesting that nitric oxide induces a Ca^{2+} influx through store-operated channels. *A*, An example of an experiment trace showing that Cd^{2+} (a nonspecific Ca^{2+} channel blocker; 100 μM) blocked SNAP-induced Ca^{2+} influx. The first and third SNAP applications were performed in normal saline, whereas the second SNAP application was performed in the presence of Cd^{2+}. *B*, Summary histogram of the effect of Cd^{2+} on SNAP-induced Ca^{2+} influx ($n = 205$ cells, 3 experiments). *C*, 2-APB (75 μM, 10 min), an IP_3 receptor antagonist and capacitive Ca^{2+} entry inhibitor, reduced SNAP-induced Ca^{2+} increase significantly. Similar protocols were used as in *A*. *D*, Summary histogram of the effect of 2-APB on SNAP-induced Ca^{2+} influx ($n = 96$ cells, 3 experiments). Statistical differences were established using the Student's *t* test at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Error bars indicate SEMs.

Nitric oxide induces a Ca^{2+} influx into astrocytes

The observation that nitric oxide can be produced in astrocytes via a Ca^{2+}-dependent mechanism led to the following question: what are the roles of nitric oxide in astrocytes? Nitric oxide has been shown to modulate Ca^{2+} signaling in other cell types via a cGMP-dependent pathway or via direct nitrosylation of proteins (Willmott et al., 1995, 1996; Guihard et al., 1996; Rooney et al., 1996; Lee, 1997; Volk et al., 1997; Berkels et al., 2000; Loitto et al., 2000; Looms et al., 2001), but its roles in astrocytes are primarily unknown. Because Ca^{2+} excitability is an essential characteristic of astrocytes, we investigated whether nitric oxide can modulate Ca^{2+} signaling in astrocytes.

To test the effects of nitric oxide on astrocyte Ca^{2+} signaling, we applied the nitric oxide donor SNAP, which spontaneously releases nitric oxide (Fig. 3*A*) in saline, to astrocytes and asked whether nitric oxide can induce a Ca^{2+} elevation. Figure 3*B* shows that perfusion with 100 μM SNAP increased Ca^{2+} levels in 96.5 ± 1.5% of the astrocytes ($n = 13$ experiments, 500 cells), an effect that was reversibly blocked by addition of the nitric oxide scavenger PTIO (100 μM; $n = 114$ cells, 3 experiments; $p < 0.001$) (Fig. 3*C*), which confirmed that this Ca^{2+} increase is evoked by the action of nitric oxide, not that of SNAP. Additionally, we performed flash photolysis to photorelease nitric oxide from caged NO (1 mM). Application of one 3 nS, 337 nm UV pulse induced an elevation of astrocytic calcium. Photolysis caused the fluorescence of fluo-3 to increase by 691.4 ± 260.2% ($n = 6$). The increase in intracellular Ca^{2+} was not attributable to UV exposure, because when the same UV pulse was delivered to astrocytes in the absence of the caged compound, astrocytes did not show a Ca^{2+} elevation (fluo-3 df/F_0, 1.8 ± 1.2%; $n = 4$). In another set of control experiments, the NO scavenger PTIO (100 μM) prevented flash photolysis of caged NO from inducing a Ca^{2+} elevation (fluo-3 df/F_0, 7.8 ± 3.2%; $n = 3$), confirming that flash photolysis normally induces a Ca^{2+} signal as a result of the liberation of NO. It is not clear why PTIO more effectively blocked the ability of photoreleased nitric oxide compared with SNAP to elevate astrocytic Ca^{2+}. Regardless of these quantitative differences, the results of these two experiments support the idea that nitric oxide causes a Ca^{2+} elevation in astrocytes.

Whereas the nitric oxide donor SNAP can induce a Ca^{2+} increase in astrocytes, the cGMP pathway agonist 8-Br-cGMP (up to 3 mM) did not induce any detectable change in fluo-3 fluorescence ($n = 4$ experiments) (data not shown), suggesting that the cGMP pathway is not involved in the nitric oxide-induced Ca^{2+} signaling in astrocytes.

To determine whether nitric oxide stimulates Ca^{2+} influx or release from internal stores, we determined the effect of bathing cells in Ca^{2+}-deficient saline. Interestingly, when 100 μM SNAP was applied to the same cells in zero-Ca^{2+} external saline, it no longer induced an increase in intracellular Ca^{2+}; however, when reapplied in the presence of external Ca^{2+}, SNAP could again

induce cytosolic Ca^{2+} elevations (Fig. 3*D*,*E*). This suggests that nitric oxide stimulates a Ca^{2+} influx pathway in astrocytes.

Cadmium (Cd^{2+}), a nonselective Ca^{2+} channel blocker (100 μM), and 2-APB, an IP_3 receptor antagonist and capacitative Ca^{2+} entry blocker (75 μM) (Bootman et al., 2002), also reversibly blocked SNAP-induced Ca^{2+} elevations (Fig. 4). Cd^{2+} reduced the average SNAP-induced change in fluo-3 fluorescence to 2.9 ± 0.5% compared with 70.6 ± 3.4% in the first SNAP application (n = 205 cells, 3 experiments). The average fluo-3 dF/F_0 induced by SNAP in 2-APB-treated cells was 2.9 ± 0.5% compared with 138.7 ± 5.2% in the first SNAP application (n = 96 cells, 3 experiments). In conclusion, these results show that nitric oxide raises astrocytic Ca^{2+} by inducing an influx of extracellular Ca^{2+} and possibly by activating a capacitative Ca^{2+} entry pathway.

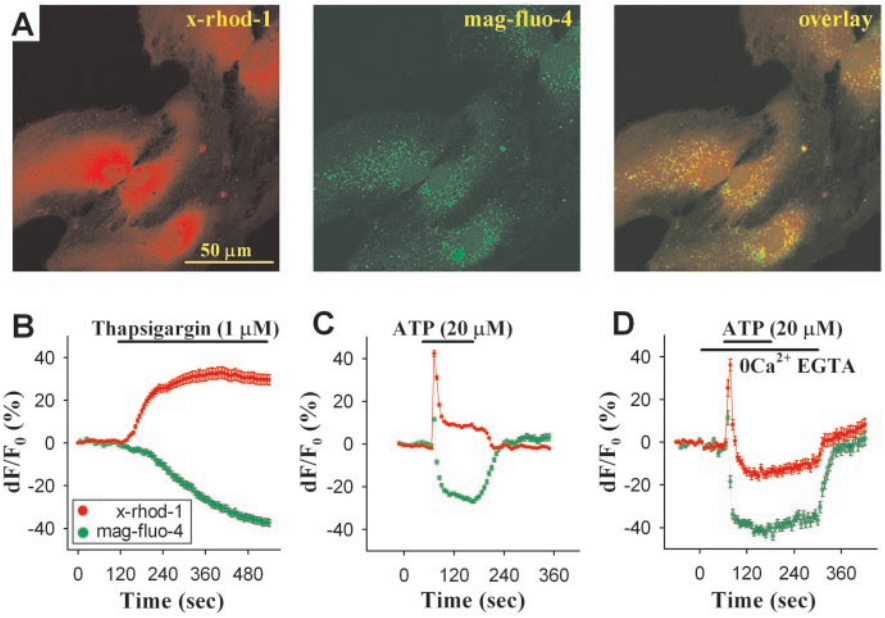

Figure 5. Simultaneous monitoring of astrocytic Ca^{2+} levels in cytosol and internal stores, respectively, by coloading with two fluorescence Ca^{2+} indicators, X-rhod-1 and mag-fluo-4. *A*, An example set of astrocytes coloaded with mag-fluo-4 and X-rhod-1. Cells were first loaded with mag-fluo-4 (mag-fluo-4 AM at 10 μg/ml) at 37°C and then loaded with X-rhod-1 (X-rhod-1 AM at 2.5 μg/ml) at room temperature. X-Rhod-1 and mag-fluo-4 images are shown in red and green, respectively; the third column shows the overlay of these two channels. X-Rhod-1 staining is more homogenous, whereas there are clear punctate patterns in mag-fluo-4 staining. This is consistent with X-rhod-1 and mag-fluo-4 loading into cytosolic and internal store compartments, respectively. *B*, Thapsigargin (1 μM), an irreversible ER Ca^{2+}-ATPase inhibitor that can increase cytosolic Ca^{2+} by depleting ER internal Ca^{2+} stores, led to a decrease in mag-fluo-4 fluorescence and an increase in X-rhod-1 fluorescence at the same time. The results confirm that we were simultaneously monitoring cytosolic and internal stores (mostly ER) of Ca^{2+} with X-rhod-1 and mag-fluo-4. *C*, ATP (20 μM), known to induce cytosolic Ca^{2+} elevation by releasing Ca^{2+} from ER stores, caused a reduction in mag-fluo-4 fluorescence and a biphasic increase in X-rhod-1 fluorescence, showing that the two fluorescence signals indicate cytosolic and internal stores of Ca^{2+}, respectively. *D*, Experiments using zero-Ca^{2+} EGTA external saline with ATP further validate the two-indicator approach for simultaneous monitoring of cytosolic and internal stores of Ca^{2+}. The cells in *C* were again stimulated with ATP, but, in the presence of zero-Ca^{2+} EGTA, the ATP-induced reduction in mag-fluo-4 fluorescence could not recover after the removal of ATP until Ca^{2+} was reintroduced into the bath, and the increase in X-rhod-1 fluorescence lacked an elevated plateau phase, both consistent with the effects of an elimination of Ca^{2+} influx.

Nitric oxide modulates the refilling of internal Ca^{2+} stores

It has been shown that many neurotransmitters (e.g., ATP, glutamate, and norepinephrine) induce an elevation in intracellular Ca^{2+} in astrocytes by causing IP_3-mediated release of Ca^{2+} from internal Ca^{2+} stores (Cornell-Bell et al., 1990; Charles et al., 1993; Salter and Hicks, 1995; Venance et al., 1997), but the mechanism by which these internal Ca^{2+} stores are refilled is primarily undetermined. Capacitative Ca^{2+} entry, or store depletion-induced store refilling, has been intensively investigated in several other cell types (Putney, 1977; Lewis and Cahalan, 1989; Hoth and Penner, 1993; Clapham, 1995; Zhang and McCloskey, 1995; Birnbaumer et al., 1996; Parekh and Penner, 1997; Putney and McKay, 1999; van Rossum et al., 2000; Clapham et al., 2001) but has not been widely studied in astrocytes. Because an ATP-induced Ca^{2+} elevation can induce nitric oxide production in astrocytes and because nitric oxide can induce external Ca^{2+} influx, it is possible that nitric oxide production is responsible for refilling internal Ca^{2+} stores.

Simultaneous monitoring of cytosolic and internal store Ca^{2+}

To test further for a role for nitric oxide signaling in the regulation of Ca^{2+} levels of internal stores, we directly imaged relative Ca^{2+} levels of internal stores using the low-Ca^{2+} affinity fluorescent indicator mag-fluo-4. When cells were incubated with mag-fluo-4 AM at 37°C, loading of this dye into intracellular compartments was facilitated. Because the $K_{d\ (Ca2+)}$ of mag-fluo-4 is 22 μM and the Ca^{2+} level in stores is ~153 μM (Golovina and Blaustein, 2000), whereas cytosolic Ca^{2+} concentration is ~100 nM, this indicator preferentially reports store Ca^{2+} levels. After loading with mag-fluo-4, astrocytes were then loaded with the high-affinity Ca^{2+} indicator X-rhod-1 AM ($K_{d\ (Ca2+)}$ of 700 nM) at room temperature. Because AM dyes are less likely to compartmentalize into internal stores when loaded at room temperature and because the $K_{d\ (Ca2+)}$ of X-rhod-1 is in the operating range of Ca^{2+} in the cytosol, it preferentially reports cytosolic Ca^{2+} levels.

Examples of astrocytes coloaded with mag-fluo-4 and X-rhod-1 are shown in Figures 5*A* and 6. Note that X-rhod-1 staining is more homogenous, whereas mag-fluo-4 staining shows clear punctate patterns in the periphery of cells and is devoid of fluorescence in nuclear regions, consistent with X-rhod-1 and mag-fluo-4 loading into cytosol and internal stores, respectively. To test whether these indicators do indeed report the Ca^{2+} levels in the two different compartments, we applied thapsigargin, an irreversible endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor. In the presence of thapsigargin, Ca^{2+} leaks from the ER, causing cytosolic Ca^{2+} to increase and internal stores to gradually become depleted. As shown in Figure 5*B*, thapsigargin reduced mag-fluo-4 fluorescence and increased X-rhod-1 fluorescence at the same time (n = 4 experiments). These results confirm that we were simultaneously monitoring cytosolic and internal store Ca^{2+} with X-rhod-1 and mag-fluo-4. Although mag-fluo-4 may be loading into other stores and organelles, we will refer to the mag-fluo-4 fluorescence as an indicator of internal Ca^{2+} stores. One concern is whether mag-fluo-4 ($K_{d(Ca2+)}$ of 20 μM) is saturated in internal stores. However, a previous study has demonstrated that this indicator is not saturated in this location (Shmigol et al., 2001), a conclusion that our data support given that we see an overshoot of mag-fluo-4 fluo-

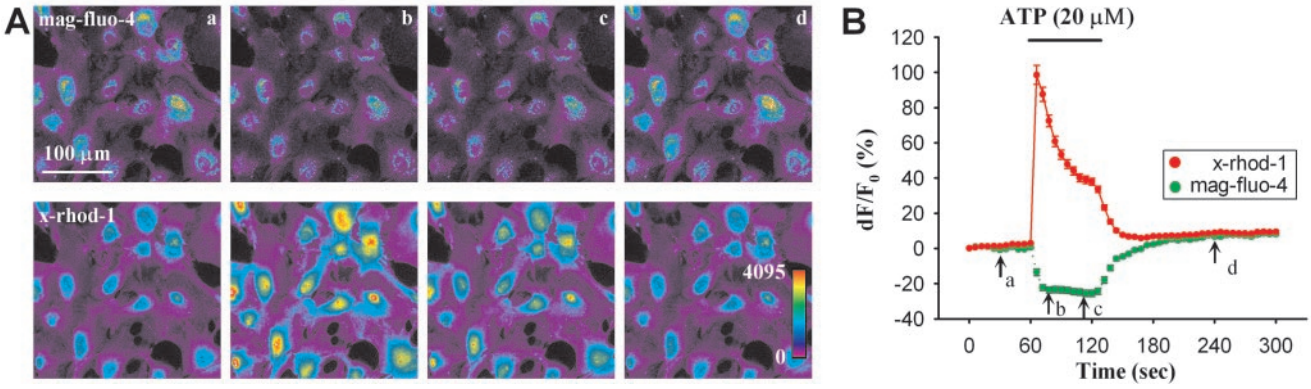

Figure 6. ATP induces release of Ca^{2+} from internal stores and subsequent store refilling. *A*, An example experiment showing that the ATP (20 μM) evoked increase in cytosolic Ca^{2+} results from the release of Ca^{2+} from internal stores. These images are pseudocolor images of mag-fluo-4 and X-rhod-1 fluorescence corresponding to times labeled in the graph in *B* (*a*, 30 sec before ATP application; *b*, 10 sec after starting ATP; *c*, 40 sec after starting ATP; *d*, 2 min after removal of ATP showing full recovery). Note that mag-fluo-4 fluorescence decreased and X-rhod-1 fluorescence increased in response to ATP, but, 2 min after the removal of ATP, both signals returned to their base levels. *B*, Traces showing the average changes in mag-fluo-4 and X-rhod-1 fluorescence of the cells in *A*.

rescence intensity after internal stores refill with calcium (Fig. 6*B*).

ATP is a neurotransmitter that is known to induce Ca^{2+} release from the ER via the IP_3 pathway; therefore, to further confirm the validity of the two-indicator approach in reporting changes in cytosolic and internal stores of Ca^{2+}, we applied ATP to astrocytes. In response to ATP, X-rhod-1 fluorescence exhibited a biphasic increase with a short-latency peak, followed by a plateau, consistent with the known biphasic cytosolic Ca^{2+} increase of ATP. Concurrent with the increase in cytosolic Ca^{2+} was a reduction in the fluorescence of mag-fluo-4, consistent with the reduction of internal stores of Ca^{2+} caused by Ca^{2+} release from stores (Fig. 5*C*). When ATP was then applied to the same cells in the presence of zero-Ca^{2+} 1 mM EGTA, X-rhod-1 fluorescence increased, but this increase lacked an elevated plateau phase, and mag-fluo-4 fluorescence decreased in a larger magnitude than in Ca^{2+}-containing saline (Fig. 5*D*). After washout of ATP, mag-fluo-4 fluorescence did not recover until Ca^{2+} was added back into the saline, consistent with the elimination of Ca^{2+} influx and the refilling of internal store with zero-Ca^{2+} EGTA. These results are all consistent with X-rhod-1 and mag-fluo-4 monitoring cytosolic and internal stores of Ca^{2+}, respectively.

Occasionally, a small initial transient increase in mag-fluo-4 fluorescence was observed in response to ATP (Fig. 5*C*,*D*). This small increase was abolished after cells were treated briefly with BAPTA (BAPTA AM at 30 μM, 20 min; $n = 3$ experiments) (data not shown), suggesting that a small amount of mag-fluo-4 was present in the cytosol. Because we are mainly interested in the magnitude of store depletion and refilling after this transient increase, mag-fluo-4 can still be used as an indicator of internal stores of Ca^{2+} despite this minor contaminating cytosolic signal. Together, these results support the notion that the two indicators, X-rhod-1 and mag-fluo-4, preferentially report changes in cytosolic and internal stores of Ca^{2+}, respectively, and that they can be used for investigating the role of nitric oxide in the regulation of Ca^{2+} store refilling.

Blockade of nitric oxide signaling reduces the refilling of internal Ca^{2+} stores

To investigate the role of nitric oxide in store refilling, we directly visualized ATP-induced store depletion and subsequent store refilling and asked whether nitric oxide modulates store refilling. Figure 6 demonstrates the results from one such experiment using ATP. Both the images and the trace show the ATP (20 μM, 60 sec)-induced cytosolic Ca^{2+} elevation resulting from release of Ca^{2+} from internal stores. Note that the internal stores were able to be fully refilled 2 min after removal of ATP (Fig. 6*B*, *d*). Moreover, because the fluorescence intensity of mag-fluo-4 after refilling exceeded the prestimulus level, it is clear that this calcium indicator is not saturated by the levels of calcium within the internal stores.

The effect of nitric oxide on the refilling of internal stores was then studied by blockade of nitric oxide signaling with the nitric oxide scavenger PTIO. Figure 7 shows that, when cells were pretreated with PTIO (100 μM, 10 min), internal stores were no longer able to refill fully, because mag-fluo-4 fluorescence did not recover to its prestimulation fluorescence intensity. In the presence of PTIO, mag-fluo-4 fluorescence recovered only to 71.2 $\pm$ 5.0% of its initial prestimulus value 2 min after removal of ATP as opposed to 104.5 $\pm$ 7.7% in control conditions ($n = 9$ experiments; $p < 0.01$) (Fig. 7*C*). Consistent with this finding, treatment with the nitric oxide synthase inhibitor L-NMMA, but not its inactive analog D-NMMA, also significantly reduced the refilling percentage of internal stores ($n = 9$, 6, and 7 experiments for control, L-NMMA-treated, and D-NMMA-treated groups, respectively) (Fig. 7*C*). These findings directly support the hypothesis that nitric oxide modulates the refilling of internal Ca^{2+} stores.

Discussion

Using the nitric oxide-sensitive fluorescent indicator DAF-FM, we observed that ATP, an important messenger in astrocytic Ca^{2+} waves, induced the production of nitric oxide in purified astrocyte cultures. Our data are consistent with the notion that the cytosolic Ca^{2+} elevation, rather than the depletion of internal store Ca^{2+}, is responsible for nitric oxide production. Because the P_2 receptor antagonist PPADS and the Ca^{2+}-chelator BAPTA both inhibited the ATP-induced nitric oxide production, this nitric oxide production must be P_2 receptor mediated and Ca^{2+} dependent. To our knowledge, this is the first report to directly visualize nitric oxide production in astrocytes induced by a natural cellular messenger such as ATP. Although we could not differentiate the specific isoforms of nitric oxide synthase with our pharmacological studies, it is likely that Ca^{2+}-dependent nitric

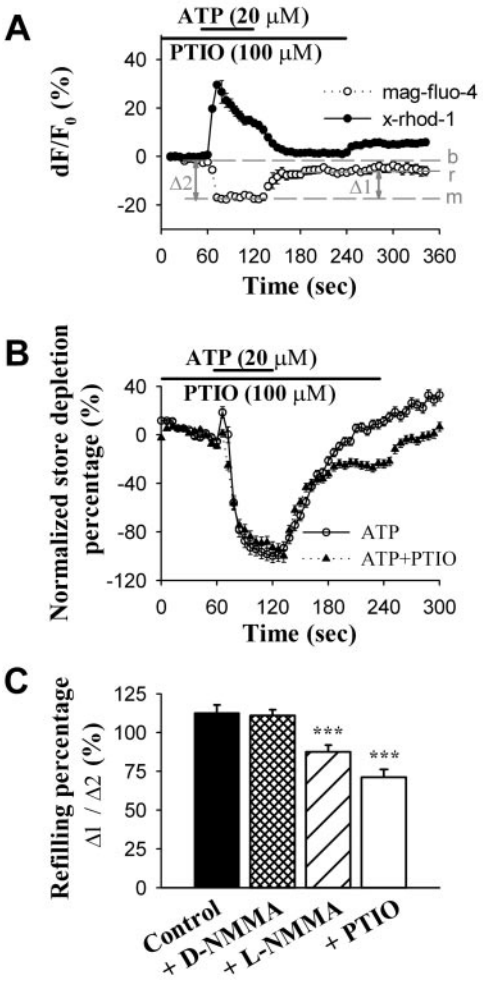

Figure 7. Inhibition of nitric oxide accumulation reduces the refilling of internal Ca^{2+} stores. *A*, Example traces of one experiment showing the ATP-induced elevation of cytosolic Ca^{2+} and depletion of internal store Ca^{2+} and subsequent store refilling in PTIO-treated cells. Note that, unlike control conditions (Fig. 6), internal Ca^{2+} store refilled only partially in the presence of PTIO. The horizontal lines in the graph are artificial lines drawn to indicate the baseline level (b; average of 3 frames immediately before ATP application), maximum decrease (m) and recovered level (r; average of three frames before 2 min post-ATP removal) of mag-fluo-4 df/F_0. $\Delta 1$ and $\Delta 2$ represent the differences between r and m, and b and m, respectively. *B*, The time course of the changes in internal store Ca^{2+} level under control conditions and in PTIO-treated cells, demonstrating that internal stores did not fully refill in the presence of PTIO compared with the control conditions (experiments performed in parallel). The data points represent the average of all cells in all experiments for each group. *C*, Summary histograms showing that store refilling ($\Delta 1/\Delta 2 * 100$) is reduced by the NO scavenger PTIO ($n = 9$ experiments), as well as by the NOS inhibitor L-NMMA ($n = 6$ experiments) but not by its inactive analog D-NMMA ($n = 7$ experiments). Statistical differences were established using the Student's *t* test at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Error bars indicate SEMs.

oxide synthases (endothelial nitric oxide synthases and neuronal nitric oxide synthases) are responsible on the basis of the Ca^{2+} dependence of this ATP-induced production. Nitric oxide has numerous actions in the CNS and is considered a "double-edged sword" because of its neuromodulatory and neuroprotective roles at low concentrations and its neurotoxic roles at high concentrations, usually attributable to the activation of inducible nitric oxide during pathological conditions. Although DAF-FM does not quantitatively measure nitric oxide levels, it is reasonable to speculate that this astrocytic Ca^{2+}-dependent nitric oxide synthesis would be in the physiological range and is therefore involved in the modulation of normal information processing. However, it will be important to validate these studies using brain slice preparations studied at physiological temperatures. Because synaptic activity in brain slices is known to elevate the Ca^{2+} levels of astrocytes (Dani et al., 1992; Porter and McCarthy, 1996), it will be intriguing to determine whether synaptic stimulation cause the synthesis of nitric oxide in this non-neuronal source, as well as in postsynaptic spines (Brenman et al., 1996; Brenman and Bredt, 1997; Burette et al., 2002), and whether astrocyte-derived nitric oxide regulates neurons and the vasculature.

When the nitric oxide donor SNAP was applied to purified astrocyte cultures, it induced a nitric oxide-dependent Ca^{2+} increase in astrocytes. This increase in Ca^{2+} was attributable to an influx of Ca^{2+} from the extracellular space, because both zero-Ca^{2+} external saline and the nonspecific Ca^{2+} channel blocker Cd^{2+} abolished this Ca^{2+} increase. In agreement with our findings, a nitric oxide donor has been shown to elevate astrocytic Ca^{2+} (Bal-Price et al., 2002), and nitric oxide has been shown to trigger a Ca^{2+} influx into Bergmann glial cells (Matyash et al., 2001). In our studies, 8-Br-cGMP, an analog of cGMP, did not induce a detectable change in internal Ca^{2+}, suggesting that the nitric oxide-induced Ca^{2+} increase is mediated by a cGMP-independent pathway. Similarly, nitric oxide can induce a cGMP-independent Ca^{2+} influx in endothelial cells (Berkels et al., 2000). In contrast, another group has reported nitric oxide-induced Ca^{2+} mobilization that is mediated by cGMP kinase-dependent activation of ryanodine receptors (Willmott et al., 2000). However, because they used mixed cultures of astrocytes and neurons, they were unable to confirm that the NO effect that they observed in astrocytes was attributable to NO stimulation of astrocytes or attributable to NO stimulation of adjacent neurons.

The nitric oxide-induced Ca^{2+} influx in astrocytes is blocked by 2-APB, an antagonist of the capacitative Ca^{2+} entry pathway, and by Gd^{3+} (Bal-Price et al., 2002), suggesting that this nitric oxide-induced Ca^{2+} influx is through store-operated Ca^{2+} channels. Because *S*-nitrosylation is emerging as an important specific post-translational modification of numerous proteins (Stamler et al., 2001), including store-operated Ca^{2+} channels (Favre et al., 1998; Ma et al., 1999; van Rossum et al., 2000), nitric oxide might directly modulate store-operated channels and thus induce an influx of Ca^{2+}.

To further understand the consequences of nitric oxide-induced Ca^{2+} influx in astrocytes and to ask whether store-operated Ca^{2+} channels might be a target, we determined whether nitric oxide could modulate the refilling of internal Ca^{2+} stores. We directly imaged changes in internal stores of Ca^{2+} and simultaneously monitored the changes in cytosolic Ca^{2+}. Although membrane-permeant, chemically synthesized ion indicators are not specifically targeted to subcellular compartments, the loading of cells with low-affinity (mag-fluo-4) and high-affinity (X-rhod-1) indicators at high and low temperatures biased these two indicators to report Ca^{2+} levels of store and cytosolic locales, respectively. These two Ca^{2+} indicators reported the simultaneous depletion of internal store Ca^{2+} and the elevation of cytosolic Ca^{2+} in response to thapsigargin and to ATP, demonstrating that they effectively monitor the relative Ca^{2+} levels of these two compartments.

Several mechanisms have been proposed as mediators of the coupling between the depletion of internal Ca^{2+} stores and the stimulation of the Ca^{2+} influx pathway. These include the following: (1) conformational coupling in which the IP_3 receptor is physically coupled to plasma membrane Ca^{2+} influx channels; (2) release of a diffusible Ca^{2+} influx factor from the ER; (3) negative regulation of Ca^{2+} influx channels by cytosolic Ca^{2+};

and (4) an exocytic mechanism in which depletion of stores leads to the insertion into the plasma membrane of vesicles containing Ca^{2+} influx channels (Putney et al., 2001). By chelating nitric oxide through the addition of PTIO or by blocking nitric oxide synthesis with L-NMMA, we identified the presence of an additional pathway in which the elevation of cytosolic Ca^{2+}, which results from store depletion, causes the Ca^{2+}-dependent synthesis of a chemical factor, nitric oxide. This in turn induces the activation of a Ca^{2+} influx pathway that contributes to the refilling of Ca^{2+} stores. Whether this Ca^{2+} influx pathway is mediated by transient receptor potential channels or I_{CRAC} awaits further investigation. It is important to note that this pathway, which we will term the Ca^{2+}-induced Ca^{2+} influx factor, operates in parallel with other mechanisms because the PTIO-sensitive component of Ca^{2+} store refilling represents only a portion of the store-refilling pathway.

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