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μ-Opioid Receptor-Induced Ca^{2+} Mobilization and Astroglial Development: Morphine Inhibits DNA Synthesis and Stimulates Cellular Hypertrophy through a Ca^{2+}-Dependent Mechanism

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Abstract

Morphine, a preferential μ opioid receptor agonist, alters astroglial development by inhibiting cell proliferation and by promoting cellular differentiation. Although morphine affects cellular differentiation through a Ca^{2+}-dependent mechanism, few studies have examined whether Ca^{2+} mediates the effect of opioids on cell proliferation, or whether a particular Ca^{2+} signal transduction pathway mediates opioid actions. Moreover, it is uncertain whether one or more opioid receptor types mediates the developmental effects of opioids. To address these questions, the present study examined the role of μ opioid receptors and Ca^{2+} mobilization in morphine-induced astrocyte development. Morphine (1 μM) and non-morphine exposed cultures enriched in murine astrocytes were incubated in Ca^{2+}-free media supplemented with < 0.005, 0.3, 1.0, or 3.0 mM Ca^{2+} ($[Ca^{2+}]_o$), or in unmodified media containing Ca^{2+} ionophore (A23187), nifedipine (1 μM), dantrolene (10 μM), thapsigargin (100 nM), or L-glutamate (100 μM) for 0–72 h. μ-Opioid receptor expression was examined immunocytochemically using specific (MOR1) antibodies. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured by microfluorometric analysis using fura-2. Astrocyte morphology and bromodeoxyuridine (BrdU) incorporation (DNA synthesis) were assessed in glial fibrillary acidic protein (GFAP) immunoreactive astrocytes. The results showed that morphine inhibited astroglial growth by activating μ opioid receptors. Astrocytes expressed MOR1 immunoreactivity and morphine's actions were mimicked by the selective μ agonist PL017. In addition, morphine inhibited DNA synthesis by mobilizing $[Ca^{2+}]_i$ in developing astroglia. At normal $[Ca^{2+}]_o$, morphine attenuated DNA synthesis by increasing $[Ca^{2+}]_i$; low $[Ca^{2+}]_o$ (0.3 mM) blocked this effect, while treatment with Ca^{2+} ionophore or glutamate mimicked morphine's

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actions. At extremely low $[Ca^{2+}]_o$ (<0.005 mM), morphine paradoxically increased BrdU incorporation. Although opioids can increase $[Ca^{2+}]_i$ in astrocytes through several pathways, not all affect DNA synthesis or cellular morphology. Nifedipine (which blocks L-type Ca^{2+} channels) did not prevent morphine-induced reductions in BrdU incorporation or cellular differentiation, while thapsigargin (which depletes IP_3-sensitive Ca^{2+} stores) severely affected inhibited DNA synthesis and cellular differentiation—irrespective of morphine treatment. However, dantrolene (an inhibitor of Ca^{2+}-dependent Ca^{2+} release) selectively blocked the effects of morphine. Collectively, the findings suggest that opioids suppress astroglial DNA synthesis and promote cellular hypertrophy by inhibiting Ca^{2+}-dependent Ca^{2+} release from dantrolene-sensitive intracellular stores. This implies a fundamental mechanism by which opioids affect central nervous system maturation.

Keywords

Cell division; Endogenous opioid system; μ-Opioid receptors; Neural development; Intracellular calcium; Dantrolene; Nifedipine; Thapsigargin; fura-2

1. Introduction

Endogenous opioid peptides and opioid receptors are present during development and can modify nervous system maturation through both direct and indirect mechanisms [24,51]. Opioids can profoundly inhibit the growth of neurons and glia.

Astrocytes are important in mediating opioid-induced maturational changes in the nervous system. Not only do astrocytes themselves express endogenous opioid peptides during development [22,36,49,50,53], but opioids inhibit astrocyte proliferation and promote premature morphologic differentiation in vitro [22,55,57] and in vivo [45,64]. Opioid drugs with abuse liability, such as heroin or morphine, affect glial development by disrupting the normal interactions between endogenous opioids and opioid receptors. Depending on the parameter measured, morphine is as potent as the endogenous opioid Met-enkephalin in affecting astrocyte growth [23,55]. Morphine is particularly efficacious at inducing changes in morphologic differentiation including increases in astrocyte area and cell processes [55,57]. Recently, diffuse, reactive astrocytosis, with regressive astrocytic changes, has been noted in the postmortem brains of chronic (non-HIV-infected) i.v. heroin abusers [19]. Despite findings that astroglia are a target for opioid action, little is known about the mechanisms by which opioids affect astroglial function.

Although opioids are predominately inhibitory in their action [12], it has been shown that opioids can also be excitatory [11,63]. Crain and Shen report that nanomolar amounts of opioids are excitatory, while micromolar concentrations of the same opioids are inhibitory in modulating the action potential of dorsal root ganglia neurons [11] Opioid actions are complex and differ depending on the particular opioid receptor type and its coupling to particular intracellular effectors [12,25–27,40]. μ- And δ-opioid receptors are reported to preferentially mediate potassium conductance, whereas κ opioid receptors preferentially affect Ca^{2+} conductance in this system [11,37,62].

There is emerging evidence that opioid receptors are “promiscuous” in their interactions with particular intracellular effectors, especially in developing cells. Both excitatory and inhibitory opioid actions have been directly or indirectly linked to changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Opioid receptor activation may increase $[Ca^{2+}]_i$ through several pathways. For example, in neuroblastoma × glioma (NG108-15) hybrid cell lines, δ opioid receptor agonists can increase $[Ca^{2+}]_i$ through the G-protein coupled phospholipase C-dependent production of inositol 1,4,5-trisphosphate (IP_3), or through L-type or ω-conotoxin GVIa-sensitive voltage-gated Ca^{2+} channels [25,26]. Opioids elevate $[Ca^{2+}]_i$ in neuroblastoma X dorsal root ganglia hybrid cells through activation of $G_{\alpha i2}$ [58].

In astrocytes or C6 glioma cells, opioids appear to increase $[Ca^{2+}]_i$ by two separate pathways [4,15,57]. The ability of the dihydropyridine, nifedipine, to block κ opioid agonist-induced increases in $[Ca^{2+}]_i$ has implicated L-type Ca^{2+} channels as mediating κ opioid receptor increases in $[Ca^{2+}]_i$ in type 1 astrocytes[15]. Alternatively, the endogenous κ opioid ligand, dynorphin A (1–17), attenuates vasopressin-induced mobilization of $[Ca^{2+}]_i$ through an IP_3-dependent process in pituicytes (GFAP-immunoreactive cells in the neurohypophysis) [6]. Moreover, in a *Xenopus* oocyte translation system functionally expressing μ, δ, or κ opioid receptors, all three opioid receptor types can mobilize $[Ca^{2+}]_i$ by signaling Ca^{2+} release from internal stores [28]. Despite some advances, the mechanism(s) by which opioids mobilize intracellular calcium in astrocytes during development are incompletely understood.

We previously reported that continuous morphine exposure (72 h) causes Ca^{2+}-dependent increases in astrocyte size and shape which are similar to reactive changes/cellular hypertrophy[57]. The importance of Ca^{2+} in cell proliferation additionally prompted us to explore whether opioids inhibit astroglial DNA synthesis by affecting Ca^{2+} homeostasis. Our results suggest that Ca^{2+}-dependent Ca^{2+} release from dantrolene sensitive intracellular stores mediates the effects of opioids on astrocyte development.

2. Materials and Methods

2.1. Drugs and compounds

Morphine sulfate was obtained from Sigma Chemical Co. (St. Louis, MO) and (−)-naloxone was obtained from E.I. Dupont (Wilmington, DE). H-Tyr-Pro-Phe (N-Me)-D-Pro-NH_2 (PL017) was obtained from Chiron (Chiron Mimotopes Peptide Systems, San Diego, CA). Dantrolene; thapsigargin; Ca^{2+} ionophore, A23187; L-glutamate; and nifedipine were obtained from Sigma.

2.2. Cell culture

Primary cultures, enriched in astrocytes were obtained from 1- to 2-day-old Swiss-Webster mice (ICR strain, Harlen Sprague Dawley, IN) as previously described [55,57]. Briefly, using aseptic technique, cells were isolated from the cerebral hemispheres of mouse pups killed by ether anesthesia and decapitation. Sterile 16 mm diameter, plain glass coverslips were coated with poly-L-lysine, placed into 22 mm diameter wells, and seeded with 5×10^5 cells in 1 mL of culture media. Culture media consisted of Dulbecco’s modified Eagle’s medium (DMEM) (1.8 mM $CaCl_2$) supplemented with 0.5% glucose, 0.06% Na_2CO_3, and 5% fetal

bovine serum (FBS) (KC Biological, Lenexa, KS). Cultures were incubated at 35°C in 5% CO_2/95% air at high humidity and examined at 6 to 9 days in vitro. The serum-containing culture medium supports the growth of flat, polyhedral (type 1) astrocytes; this astroglial type is developmentally sensitive to opioids [23]. In these cultures, the greatest number of type 1 astroglia express μ, δ and κ opioid receptors after day 5 in vitro (Hauser, unpublished), and that the inhibitory effect of opioids on astroglial division is maximal at 4–9 days in vitro. Typically, the cells in these cultures reach confluence on day 12.

2.3 Pharmacological manipulation of opioids and Ca^{2+}

Independent preparations of cells were distributed across treatment groups. In acute fura-2 studies, the agonists, morphine or PL017, were added to the cultures where indicated. Naloxone was added 20 min prior to the addition of agonist. Extracellular Ca^{2+} was removed by 4-fold-rinses with media containing 0 [Ca^{2+}] and 1 mM EGTA, 5 min prior to the addition of an agonist. In studies where astrocyte development was assessed, the media was replaced with basal media containing Ca^{2+} (<0.005, 0.3, 1.0, or 3.0 mM) with or without morphine sulfate (1 μM) 72 h before harvesting. in addition, some cultures were continuously treated 6 or 48 h prior to harvesting with culture media plus: Ca^{2+} ionophore A23187 (1.0 μM), glutamate (100 μM), nifedipine (1 μM), dantrolene (10 μM); or thapsigargin (100 nM) [9]. Nifedipine and thapsigargin are initially dissolved in DMSO and serially diluted to reduce the final concentration of DMSO in the media to < 0.1% as previously described [9]; nifedipine is protected from exposure to light. Media and drugs were changed daily.

2.4. μ-Opioid receptor immunocytochemistry

Cultures were fixed for 10 min in 3% paraformaldehyde in Sorenson's phosphate buffer, pH 7.2, at 4 °C. Rabbit-anti-μ-opioid receptor (MOR1) antibodies [1] were diluted 1:5000 in phosphate buffered saline (PBS, pH 7.2) containing 0.1% Triton-X 100 and 0.1% crystalline bovine serum albumin (Calbiochem, San Diego, CA). Cultures were incubated with MOR1 antibodies for 24 hr at 4 °C on an orbital shaker at 40–60 rpm. Secondary, biotinylated goat-anti-rabbit antibodies conjugated to an avidin-peroxidase (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) were used as directed to detect MOR1 primary antibodies. Nickel-intensified DAB consisting of 2.5% nickel ammonium sulfate, 0.35% DAB, and 0.012% H2O2 in 0.1 M sodium acetate (pH 6.0) was used as a substrate for peroxidase. Preabsorbed controls were included to assure specificity of the MOR1 antibodies.

2.5. Intracellular Ca^{2+}

The procedures for fura-2 fluorescence imaging have been described before [57]. Briefly, cells were grown on 35 mm diameter plastic dishes with glass bottoms (MatTek Co., MA). The cells were loaded at 37°C for 45 min with 4 or 10 μM fura-2/AM (Molecular Probes, Eugene, OR) in culture media containing 2% DMSO. The loaded cells were then washed 3 times with DMEM at 35°C and incubated for about 45 min in 5% CO_2/95% air at 35°C to allow for complete hydrolysis of the fura-2/AM. Determinations of free $[Ca^{2+}]_i$ were made in 1 mL of DMEM (or Ca^{2+}-free DMEM with 1 mM EGTA), containing 10 or 25 mM Hepes buffer at pH 7.2: Repeated measures of $[Ca^{2+}]i$ levels were made at intervals from 1 to 20 seconds in the same cells before and at varied intervals after the addition of opioids.

Opioids and/or Ca^{2+} agents were dissolved in DMEM before adding to the cultures. To block drug effects, cells were pretreated for 20 min (unless noted otherwise) with the opioid antagonist naloxone, nifedipine, thapsigargin, or 0 $[Ca^{2+}]_o$ + 1 mM EGTA. In some experiments, cells were grown on 25 mm diameter glass coverslips and imaged in a perfusion chamber (RC-21; Warner Instrument Corp., Hamden, CT) which allowed continuous monitoring of cells while exchanging drug solutions. Cells were perfused at a rate of about 1 mL/min (100 μL bath volume) using a syringe pump (MD-1001; Bioanalytical Systems, W. Lafayette, IN). The cultures were viewed using an inverted Nikon microscope with an oil immersion, fluoro 40x, N.A. 1.3 objective and an intensified (Hamamatsu C2400, Hamamatsu City, Japan) CCD camera (Dage 72, Dage-MTI, Michigan City, IN). Intracellular Ca^{2+} levels were determined from the ratio of the fluorescence using two different excitation wavelengths (340 nm and 380 nm) as described before [20]. A computer-controlled optical filter wheel (Lambda 10; Sutter Instrument Co./Axon Instruments, Novato, CA) was used to change excitation filters. Imaging Research Corporation M1 or M4 imaging systems (St. Catharines, Ontario) with fura-2 ratiometric software were used to acquire and process the images. Measurements were taken from the glial cytoplasm, away from peripheral processes but not over the nucleus [57]. Values represent the average $[Ca^{2+}]_i$ level therein. Cultures were processed for GFAP immunocytochemistry (see below) and re-examined to confirm that the cells were GFAP-immunoreactive (astrocytes).

2.6. Combined BrdU and GFAP Immunocytochemistry

To determine whether opioids affect DNA synthesis, cultures were treated with 5-bromodeoxyuridine (BrdU; Sigma) (50 mg/mL) for 6 h immediately before harvesting. At 4 or 6 days in vitro, cells were fixed *in situ* on the coverslips on which they were grown for 30 min in ice-cold Zamboni's fixative with 3% paraformaldehyde and then post-fixed in 70% ethanol at 4°C overnight. A sequential double-labeling immunocytochemical procedure was used to detect BrdU and GFAP in the same cells.

To detect incorporated BrdU, double-stranded DNA was partially denatured by treating cultures with 0.07 M NaOH for 1 h. Mouse-anti-BrdU monoclonal antibodies (Chemicon, Temecula, CA) were diluted 1:1000 (w/v) in PBS (phosphate buffered saline) at pH 7.4 in the presence of 0.1% Triton-X 100 and 1% crystalline grade BSA (Calbiochem). Tissues were incubated with anti-BrdU antibodies for 24 h at 4°C on an orbital shaker at 40–60 rpm. Biotinylated donkey-anti-mouse secondary antibodies conjugated to avidin-peroxidase were used to detect BrdU antibodies (Vectastain ABC kit). The reaction yields an insoluble, blue-black product within the cell nucleus by incubating for 4–6 min in nickel-intensified DAB.

Rabbit-anti-GFAP antibodies (Chemicon, Temecula, CA) were used at a 1:1000 dilution overnight at 4°C to detect GFAP. Biotinylated, goat-anti-rabbit secondary antibodies conjugated to avidin-peroxidase were used to detect GFAP antibodies as directed (Vectastain ABC kit). Peroxidase-conjugated GFAP antibody complexes were visualized by the brown reaction with 0.06% DAB, 0.02% hydrogen peroxide in 50 mM Tris buffer, pH 7.6. The DAB reaction was observed using an inverted microscope and stopped when signal-to-noise is optimal (e.g., 4–8 min). The black BrdU product in the nucleus and brown

GFAP product in the cytoplasm did not overlap and were discernible in the same cell. To demonstrate the BrdU and GFAP immunoproducts in the same cells using black and white photography, a blue-green acetate filter (#858 Roscolene; Rosco, Port Chester, NY) was used to enhance the GFAP product, while a dark copper-brown filter (#819 Roscolene) was used to attenuate the GFAP product compared to the BrdU product. There was no specific immunoreactivity when primary BrdU or GFAP antibodies were eliminated from the reaction.

2.7. Astroglial size and shape

The area, perimeter, and form factor of individual flat, polyhedral (type 1) GFAP-positive cells were determined using computer-assisted image analysis (M1; Imaging Research Corporation) and an Olympus Vanox microscope with a 40x objective as previously described [57]. Form factor is an index of cell shape [$(4\pi)(\text{area})/(\text{perimeter}^2)$]; its value is highest for circles, but decreases with complex shapes (increased size/number of processes). Glia pooled from 2–3 mice were maintained as independent preparations of cells ($n = 1$) and distributed across treatment groups as separate cultures. Twenty cells were randomly sampled from each of at least $n = 6$ independent preparations of cells (at least 120 cells per group).

2.8. Percentage of astroglia incorporating BrdU

Individual astrocytes were manually counted using an Olympus Vanox microscope with a 40x objective and a calibrated 10 X 10 square-lattice eyepiece reticle. About 600 randomly selected, type 1 astrocytes were sampled from defined areas (μm^2) in each culture with the observer unaware of treatment history. Astrocytes in multi-cell clusters were not counted. Flat, polyhedral (type 1) astroglia were identified by using morphologic and immunological criteria. The labeling index was determined by the formula: [BrdU labeled astrocytes/(BrdU-labeled astrocytes + non-BrdU-labeled astrocytes)]. Glia pooled from 2–3 mice were maintained as independent preparations of cells ($n = 1$) and distributed across treatment groups. Determinations were made from at least $n = 6$ independent preparations of cells in each experiment, and all experiments were replicated at least twice.

2.9. Statistics

Data were reported as the mean ± SE. Experimental differences were tested using analysis of variance (ANOVA) and subsequent *post hoc* comparisons were made using Newman-Keuls test (General ANOVA programs, Statistica, StatSoft, Tulsa, OK). Student's *t* test was used to assess the effect of manipulating Ca^{2+} with or without morphine (or L-glutamate). Differences were considered significant if $P < 0.05$.

3. Results

3.1. μ-Opioid receptor immunocytochemistry

At 6 to 9 days in vitro, 95–97% of the flat, polyhedral cells in our cultures are GFAP immunoreactive identifying them as type 1 astrocytes (Hauser and Stiene-Martin, unpublished). Using MOR1 antibodies, one-third to one-half of the type 1 astrocytes expressed μ-opioid receptor immunoreactivity (Fig. 1). Punctate patterns of MOR1

immunoreactivity were associated with the cytoplasm and/or plasma membrane, and were especially prominent in the juxtanuclear cytoplasm which may be associated with the endoplasmic reticulum or with the endosomal compartment. Fainter, more diffuse patterns of immunoreactivity occasionally outlined the plasma membrane or adjacent cytoplasm of some astrocytes. The levels of immunoreactivity differed greatly among type 1 astrocytes, some cells contained high levels of MOR1 immunoreactivity, whereas others lacked immunoproduct. Immunoreactive product was absent in preabsorbed controls.

3.2. Intracellular Ca^{2+}

Morphine (100 nM) treatment increased $[Ca^{2+}]_i$ in a subpopulation of astrocytes (Fig. 2a). $[Ca^{2+}]_i$ increases were similarly observed following the addition of the selective μ opioid receptor agonist PL017 (Fig. 2b), and prolonged increases in $[Ca^{2+}]_i$ were seen with higher agonist concentrations or prolonged exposure. Pretreatment with equimolar concentrations of naloxone prevented PL017 or morphine (data not shown) induced increases in $[Ca^{2+}]_i$, suggesting that the effect was mediated through opioid receptors (2c). Morphine, a less-selective μ agonist, typically activated $[Ca^{2+}]_i$ in the same astrocytes as PL017, a highly selective μ agonist, suggesting that both drugs affected μ sites (2d).

In a subset of astrocytes, morphine-induced increases in $[Ca^{2+}]_i$ were caused by Ca^{2+} release from IP_3-sensitive intracellular stores, rather than Ca^{2+} influx across the plasma membrane. When Ca^{2+} influx was prevented by removing $[Ca^{2+}]_o$, PL017 treatment increased $[Ca^{2+}]_i$ (2e). Blocking L-type Ca^{2+} channels with nifedipine also did not prevent morphine-induced increases in $[Ca^{2+}]_i$ (Fig. 3a). However, morphine-induced increases in $[Ca^{2+}]_i$ were prevented when IP_3-sensitive intracellular Ca^{2+} stores were depleted by thapsigargin pretreatment (3b).

3.3. Astroglial size and shape

We have previously shown that morphine-induced elevations in $[Ca^{2+}]_i$ cause increases in astrocyte size and in the elaboration of cytoplasmic processes [57]. To explore the role of particular Ca^{2+} activation pathways in mediating these changes, Ca^{2+} was manipulated pharmacologically and morphine-induced changes were assessed morphometrically (Figs. 4; 5a,b).

Although morphine can increase $[Ca^{2+}]_i$ through several different mechanisms, only the activation of some Ca^{2+} pathways resulted in changes in cellular morphology at 48 h (Figs. 4; 5a,b). For example, morphine alone caused a significant 94% increase in astrocyte area ($P < 0.01$), while significantly reducing form factor by 26% ($P < 0.05$). Dantrolene treatment prevented morphine-induced alterations in astroglial morphology, while dantrolene alone had no effect on cell size or shape compared to controls. In contrast, morphine-induced increases in astroglial morphology were unaffected by nifedipine. Lastly, thapsigargin treatment caused significant decreases in astrocyte area (44% without morphine; 47% with morphine) and marked reductions in form factor (37% without morphine; 47% with morphine) compared to control values--irrespective of whether morphine was co-administered.

3.4. Percentage of astroglia incorporating BrdU

BrdU and GFAP immunoproducts were readily discernible in the same cell (Fig. 6).

In the presence of unmodified cell culture medium, morphine caused a 24% reduction in BrdU incorporation by astrocytes compared to control values. In media containing 1.0 or 3.0 mM $[Ca^{2+}]_o$, morphine also caused a significant suppression in the proportion of astroglia incorporating BrdU (Fig. 7). However, at low $[Ca^{2+}]_o$ (0.3 mM), morphine did not suppress BrdU incorporation (Fig. 7). Treatment with Ca^{2+} ionophore A23187 significantly reduced BrdU incorporation by 26–27%--irrespective of morphine treatment; whereas treatment with glutamate (100 μM), which also increases intracellular Ca^{2+} in type 1-like astrocytes (Fig. 7) [10,18], significantly inhibited BrdU incorporation by 13% compared to non-glutamate-treated cultures. At extremely low <0.005 mM $[Ca^{2+}]_o$ (0 Ca^{2+}), DNA synthesis was reduced compared to astrocytes in unmodified culture medium. Surprisingly, at <0.005 mM $[Ca^{2+}]_o$, morphine treatment caused a paradoxical 52% increase in BrdU incorporation compared to non-morphine treated astrocytes (Fig. 7). Growth could not be assessed in the complete absence of extracellular Ca^{2+} (0 $[Ca^{2+}]_o$ + 1 mM EGTA), because without $[Ca^{2+}]_o$ astrocytes detach from the cell culture dish within 30–40 min.

To assess how opioid-induced changes in $[Ca^{2+}]_i$ affected DNA synthesis, particular Ca^{2+} activation pathways were blocked and morphine-induced changes in BrdU incorporation were assessed at 6 and 48 h (Fig. 8a,b).

At 6 h, morphine treatment did not affect BrdU incorporation by type 1 astrocytes (Fig. 8a). In addition, neither dantrolene-induced inhibition of Ca^{2+} -dependent Ca^{2+} release nor thapsigargin-induced depletion of IP_3-dependent Ca^{2+} stores affected BrdU incorporation by astroglia at 6 h following exposure (Fig. 8a). Furthermore, BrdU incorporation was unaffected by blocking L-type Ca^{2+} channels with nifedipine (Fig. 8a).

In contrast to 6 h, at 48 h, the manipulation of some Ca^{2+} pathways significantly affected the ability of morphine to reduce BrdU incorporation in type 1 astrocytes ($P < 0.05$) (Fig. 8b). Most notably, dantrolene treatment prevented morphine-induced decreases in BrdU incorporation by astroglia, while dantrolene alone had no effect on BrdU incorporation compared to untreated controls. DNA synthesis was also unaffected by administering nifedipine alone. Notably, however, nifedipine did not prevent morphine-induced reductions in the proportion of astroglia incorporating BrdU. When co-administered with nifedipine, morphine significantly reduced BrdU incorporation by 34% compared to cultures treated with nifedipine alone ($P < 0.05$) or by 44% compared to untreated controls ($P < 0.01$). Although nifedipine appeared to enhance the inhibitory effect of morphine, combined treatment with morphine and nifedipine did not differ markedly from treatment with morphine alone. Lastly, irrespective of whether morphine was given, prolonged (48 h) thapsigargin treatment caused a near cessation of BrdU incorporation by astroglia ($P < 0.025$).

4. Discussion

Identifying the particular opioid receptor types that affect cellular growth is of fundamental importance toward understanding the mechanisms by which opioids affect neural maturation. Previous studies have shown that morphine inhibits astroglial proliferation while causing marked cellular hypertrophy [23,55,57]. More recent evidence shows that the cellular hypertrophy is caused by increased $[Ca^{2+}]_i$ and is likely to be mediated by the activation of μ- or κ-opioid receptors [57]. The present study further supports a role for μ opioid receptors in mediating the effects of morphine on cellular development by showing that (i) μ opioid receptors are expressed by astrocytes, (ii) μ receptor activation mobilizes $[Ca^{2+}]_i$, and (iii) the mobilization of $[Ca^{2+}]_i$ inhibits DNA synthesis and causes cellular hypertrophy. In addition, the present study identifies a particular Ca^{2+}-dependent pathway, i.e., Ca^{2+}-dependent Ca^{2+}-release, as a critical step in mediating the developmental perturbations associated with opioid exposure. This suggests a sequence of events by which opioids inhibit cellular growth consisting of the activation of μ opioid receptors, mobilization of $[Ca^{2+}]_i$, and amplification of that signal through Ca^{2+}-dependent Ca^{2+} release.

The present study provides immunocytochemical and functional evidence that astrocytes express μ opioid receptors. However, there has been controversy regarding whether astrocytes express opioid receptors. Recent evidence suggests that primary astrocytes can express multiple opioid receptor types *in vitro* [13–15,21,31,43,60]. Using ribonuclease protection and/or reverse transcriptase-PCR assays, astrocytes have been shown to express μ, δ, and κ opioid receptor mRNAs [43,60]. In contrast, several earlier studies failed to identify either μ opioid receptors, or other non-μ opioid receptor types, in cultured astrocytes [13,14,46,61]. The reason for these inconsistencies is uncertain, but probably result from differing culture conditions, including differences in the availability of cytokines and/or growth factors, e.g., interleukin-1β, which can induce opioid receptor expression in astrocytes [44], or differences in opioid receptor expression among astrocytes derived from different brain regions and times during development [43,54]. In studies examining glial proliferation, we have noted that astroglia gradually lose their response to opioids with progressive development in vitro. The loss occurs irrespective of whether astroglia are subcultured to a lower density to promote growth, and presumably coincides with the loss of functional opioid receptors [23] (Hauser, unpublished). Therefore, when assessing the opioid receptor expression by astrocytes it is important to consider the potential impact of species (and strain) differences, regional and developmental differences in the brain, and varied culture conditions.

Although μ opioids affect astrocyte development in the present study, we cannot exclude the possibility that other opioid receptor types might also affect astrocyte development through a similar Ca^{2+}-dependent (or alternative) mechanism. This suggestion is prompted by findings that δ or κ opioid receptor activation increases $[Ca^{2+}]_i$ in subsets of astrocytes [15,21,60], and by findings that selective κ opioid agonists can inhibit astrocyte proliferation [21]. In addition, because only 30–50% of the astrocytes in our cultures appear to express μ opioid receptors, the mean values reported herein are likely to underestimate the developmental changes within this subpopulation, i.e., the developmental changes occurring within the μ receptor expressing subpopulation are likely to be quite profound. The extent to

which κ- or δ-opioid receptor expressing astrocyte subpopulations overlap or interact with μ receptor expressing subpopulations is uncertain and warrants further study.

Opioids increase intracellular Ca^{2+} in astrocytes

The cellular mechanisms underlying Ca^{2+} mobilization and/or maintenance of steady state levels are diverse [16]. These include: alterations in Ca^{2+} influx through voltage- or ligand-gated Ca^{2+} channels [16], Ca^{2+} extrusion from the cell (e.g., via Ca^{2+}-ATPase or the Na^{+}/Ca^{2+} exchanger within the plasma membrane), Ca^{2+} binding proteins, or changes in the release or sequestration of Ca^{2+} from internal storage sites [5,42]. Ca^{2+} increases in astrocytes can result from increased extracellular K^{+}, mechanical deformation, the stimulation of specific cell surface receptors, or by intercellular signaling through gap junctions [10,18,30,47,48,52]. Ca^{2+} increases can be mediated by influx through L or T type Ca^{2+} channels [52], or by IP_3, Ca^{2+}, or eicosonoid-induced (ryanodine-sensitive) release from intracellular stores [8]. Intercellular Ca^{2+} signaling among astrocytes has been proposed as a mechanism of long-distance communication between glia, and may be fundamentally important in normal nervous system function or following injury [18,30,52].

There is an absolute requirement for Ca^{2+} for entry into and exit from mitosis [41]. Cells deprived of Ca^{2+} cannot advance from the G_1/S phase of the cell division cycle [35,41.] Ca^{2+}-dependent protein kinases are central in regulating astrocyte proliferation and differentiation [32]. Therefore, it was not surprising that manipulating $[Ca^{2+}]_i$ affected DNA synthesis in astroglia. There was an optimal range of $[Ca^{2+}]_i$ which favored DNA synthesis in astroglia and opioids inhibited DNA synthesis within this range. However, at abnormally high or low Ca^{2+} concentrations, respectively, the effect of morphine on DNA synthesis was negated or no longer inhibitory. For example, morphine had no additive effect on DNA synthesis in the presence of calcium ionophore. High $[Ca^{2+}]_i$ levels resulting from ionophore treatment potentially saturated Ca^{2+} calmodulin-dependent effectors regulating cell replication and/or were cytotoxic through indirect mechanisms. ParadoxicalIy, at very low $[Ca^{2+}]_o$, morphine increased the rate of DNA synthesis. Under these conditions, morphine might have compensated for decreased $[Ca^{2+}]_o$ by mobilizing Ca^{2+} from intracellular stores. Thus, our results suggest that, depending on the starting concentration, relative increases in $[Ca^{2+}]_i$ can increase or decrease the rate of DNA synthesis in astrocytes. Similar observations have been noted in other neural cell types. For example, at optimal concentrations Ca^{2+} permits plasticity and survival, but at extreme low or high concentrations, respectively, Ca^{2+} promotes stasis or death [29,33,34].

Opioid-dependent declines in astroglial proliferation are likely to be mediated through release of Ca^{2+} from intracellular stores. In the presence of very low $[Ca^{2+}]_o$, opioids can increase $[Ca^{2+}]_i$ and can affect DNA synthesis. Conversely, if IP_3-sensitive Ca^{2+} release or Ca^{2+}-dependent Ca^{2+} release are prevented, opioids no longer have any differential effect on DNA synthesis compared to non-opioid treated astrocytes. Similarly, other investigators report that extracellular Ca^{2+} is not necessary to evoke the anti-proliferative effects of excitatory amino acids such as glutamate in cultured astrocytes [38]. In these studies, proliferation is inhibited through increased IP_3 turnover without stimulating $^{45}Ca^{2+}$ influx [38]. Since glutamate substantially increases $[Ca^{2+}]_i$ in astroglia [18,30,52], it is likely that

Ca^{2+} release from internal stores accompanies changes in inositol phospholipid hydrolysis in the above studies. Moreover, although opioids are reported to cause increases in astrocyte $[Ca^{2+}]_i$ by opening L-type Ca^{2+} channels [4,15], continuous nifedipine treatment (even at 10 μM concentrations) does not prevent morphine-induced reductions in BrdU incorporation or alterations in cell size and shape. Apparently, the influx of Ca^{2+} through L-type Ca^{2+} channels is not a mechanism by which opioids affect astrocyte proliferation or differentiation.

Our findings suggest that the release of Ca^{2+} from IP_3-dependent stores is a requirement for DNA synthesis, as well as the maintenance of cellular morphology, in astrocytes. This is consistent with findings that thapsigargin-induced depletion of IP_3-sensitive intracellular Ca^{2+} stores causes cell cycle arrest [35,41], and agrees with findings that opioids affect DNA synthesis by affecting IP_3 turnover in fetal brain cell aggregates [2–4]. Moreover, $[Ca^{2+}]_i$ mobilization is associated with altered IP_3 turnover and coincides with altered DNA synthesis by C6 glioma cells [4]. Thapsigargin depletes IP_3-sensitive Ca^{2+} stores by inhibiting endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and by causing leakage of Ca^{2+} from these sites [59]. Whether IP_3-induced increases in $[Ca^{2+}]_i$ mediate opioid-dependent reductions in DNA synthesis cannot be addressed in the present study based on BrdU incorporation studies alone, since prolonged thapsigargin treatment (48 h) caused a near cessation in DNA synthesis and dramatic alterations in morphology--irrespective of morphine treatment. However, the ability of thapsigargin to attenuate opioid-dependent rises in $[Ca^{2+}]_i$ shows that opioids can release Ca^{2+} through an IP_3-dependent mechanism, but do not provide a causal link between the opioid-dependent inhibition of IP_3-induced Ca^{2+} release and alterations in astroglial growth or differentiation.

Importantly, the ability of dantrolene to block the effects of morphine on BrdU incorporation and cellular morphology provides novel insight to the mechanism by which opioids inhibit astrocyte development. Dantrolene blocks Ca^{2+}-dependent Ca^{2+} release from internal stores [39]. Normally, Ca^{2+}-dependent Ca^{2+} release is thought to be important in amplifying intracellular Ca^{2+} signals in astroglia [8], and/or may potentiate IP_3-dependent Ca^{2+} release [17]. Our findings suggest that opioids affect cell growth and differentiation by amplifying intracellular Ca^{2+} signals. Opioids may potentiate Ca^{2+} signals evoked by opioid or non-opioid stimuli. Lastly, dantrolene does not block Ca^{2+} signaling through gap junctions [8], and therefore does not block morphine's action by preventing intercellular signaling among astrocytes. Conversely, intercellular signaling may permit opioid-induced Ca^{2+} signals to be propagated to astrocytes that lack opioid receptors.

During development, astroglia can affect the proliferation, migration, differentiation and survival of neurons and neuronal progenitors [7]. In the adult central nervous system, astrocytes function to preserve the ionic and metabolic extracellular milieu, are involved in intercellular signaling, and provide key neural responses to injury [47,48]. It has been proposed that endogenous opioids [56], as well as opiate drugs [23,55], affect neural development by inhibiting the genesis of astrocytes. Presumably, a component of the neurobehavioral defects seen in the offspring of opiate-dependent mothers results from the impact of opiate drugs on astroglia [23,55,57]. Thus, findings that opioids can affect astrocyte growth and differentiation

through changes in Ca^{2+} homeostasis suggest a basic mechanism by which opioids modulate nervous system development.

Acknowledgments

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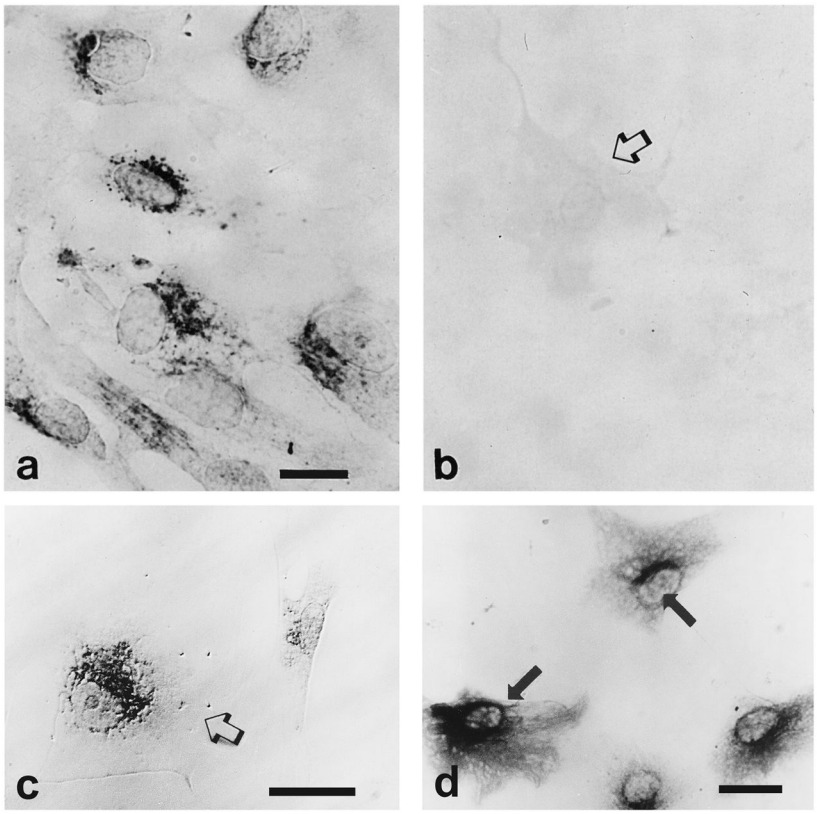

Fig. 1.

Brightfield photomicrographs of μ-opioid receptor (MOR1) immunoreactive type 1 astrocytes at 6 days *in vitro*. MOR1 immunoreactivity was typically punctate with some faint, diffuse immunoreactivity occasionally outlining the plasma membrane and/or adjacent cytoplasm (a,c) Intense immunostaining was often associated with the juxtanuclear cytoplasm (c). The intensity of MOR1 immunoreactivity varied among individual astrocytes; some cells lacked immunoproduct (not clearly shown here). MOR1 immunoreactivity was absent in preabsorbed controls (b). Greater than 95% of the flat, polyhedral cells were glial fibrillary acidic protein-immunoreactive (d). Scale bars: 10 μm (a and b); 25 μm (c), 25 μm (d). Unmodified brightfield optics (a,b,d); differential interference contrast optics (c). Arrows = astrocytes.

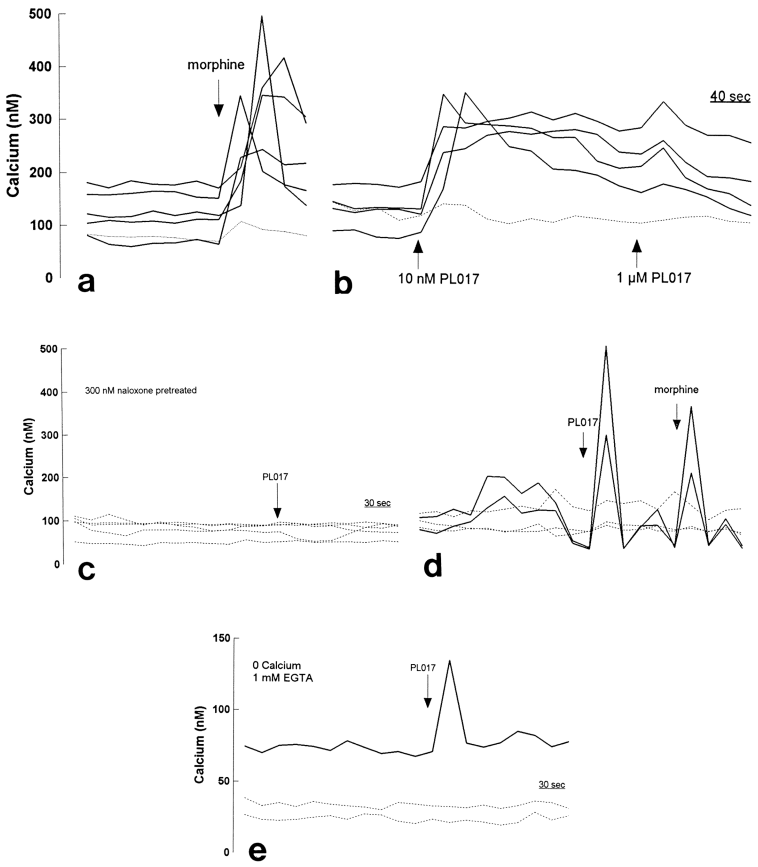

Fig. 2.

Effect of morphine and/or PL017 on $[Ca^{2+}]_i$ in individual type 1 astrocytes (a–e). Morphine (1 μM) (a), or the selective μ opioid receptor agonist PL017 (b), increased $[Ca^{2+}]_i$ in some astrocytes, while naloxone pretreatment prevented PL017-induced increases in $[Ca^{2+}]_i$ (c). Morphine and PL017 increased $[Ca^{2+}]_i$ in the same cells (d). PL017 treatment increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (0 $[Ca^{2+}]_o$ and 1 mM EGTA) in some cells (e). Drugs were added at 100 nM concentrations, except where noted. Responsive astrocyte (------), non-responsive astrocyte (-------). The scale is identical in a–b, and in c,d–e.

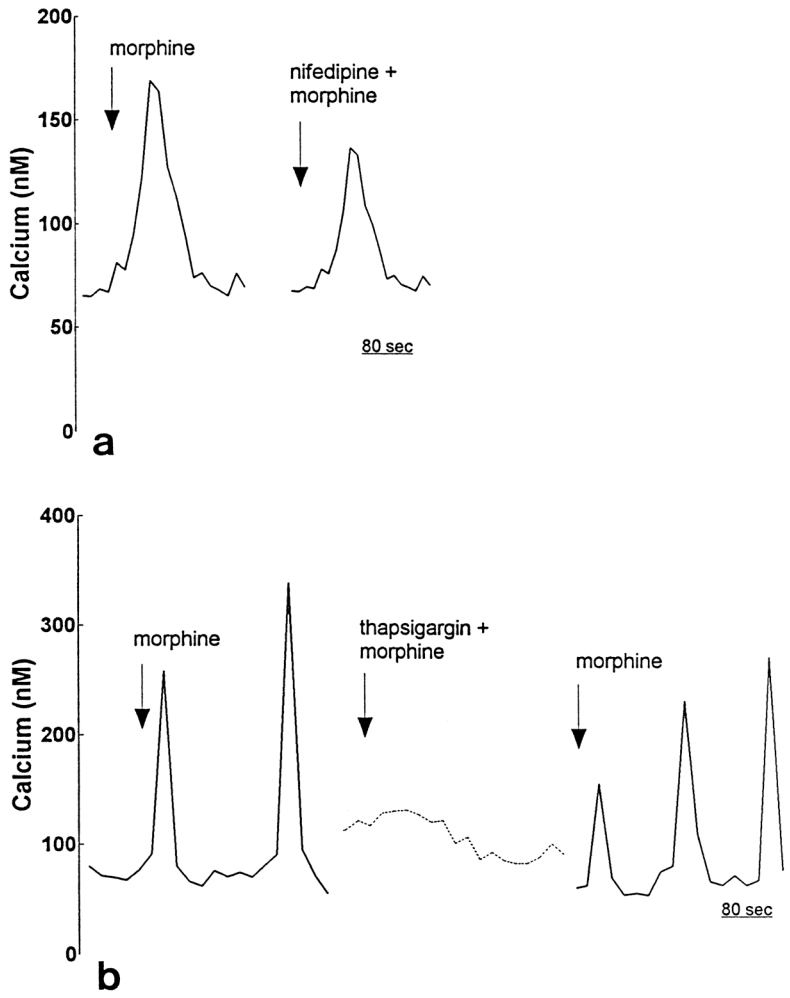

Fig. 3.

Effect of nifedipine or thapsigargin on morphine-induced increases in $[Ca^{2+}]_i$ in individual type 1 astrocytes. Nifedipine (1 μM) treatment did not prevent morphine-induced increases in $[Ca^{2+}]_i$ in some astrocytes (a). In contrast, thapsigargin treatment reversibly blocked morphine-induced increases in $[Ca^{2+}]_i$ (b). Drug concentrations were 100 nM—except where noted. Responsive astrocyte (-------), non-responsive astrocyte (-------).

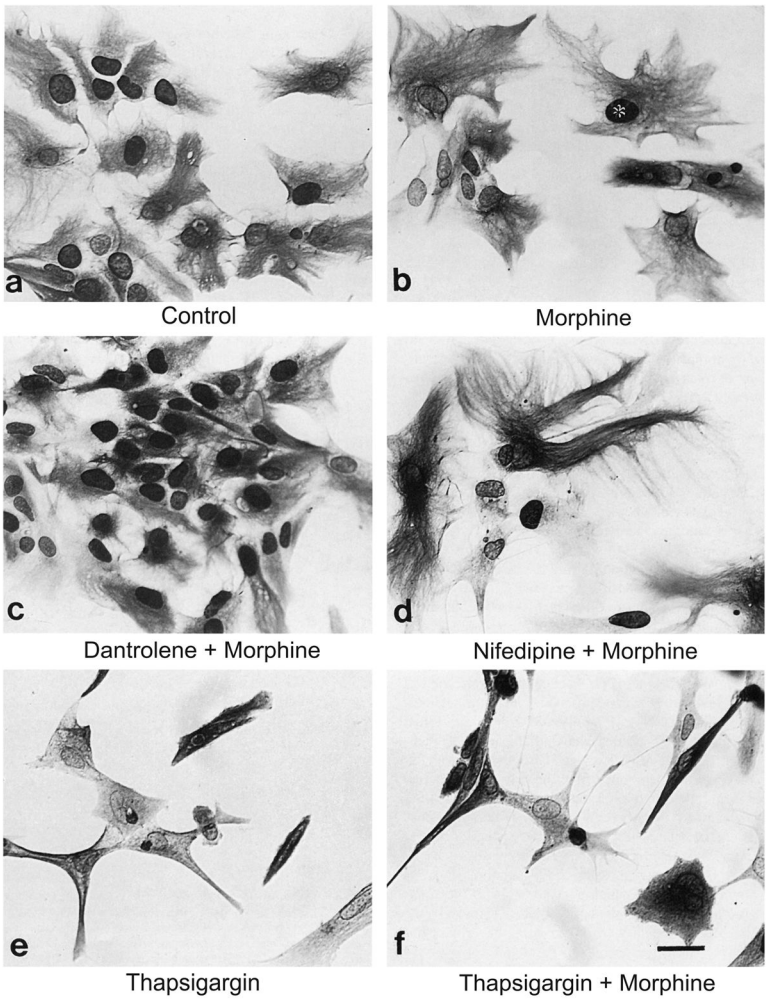

Fig. 4.

Brightfield photomicrographs showing the effects of morphine, and/or drugs affecting $[Ca^{2+}]_i$, on astrocyte morphology at 48 h following continuous drug exposure (a–f). Compared to untreated controls (a), morphine treatment caused cellular hypertrophy (b). Dantrolene treatment prevented morphine-induced cellular hypertrophy (c), while nifedipine did not prevent morphine-dependent changes in the appearance of individual cells (d). Thapsigargin treatment caused astrocytes to assume an abnormal morphology—irrespective of morphine treatment (e,f); a blue-green filter was used to enhance photographic contrast; scale bar = 25 μm.

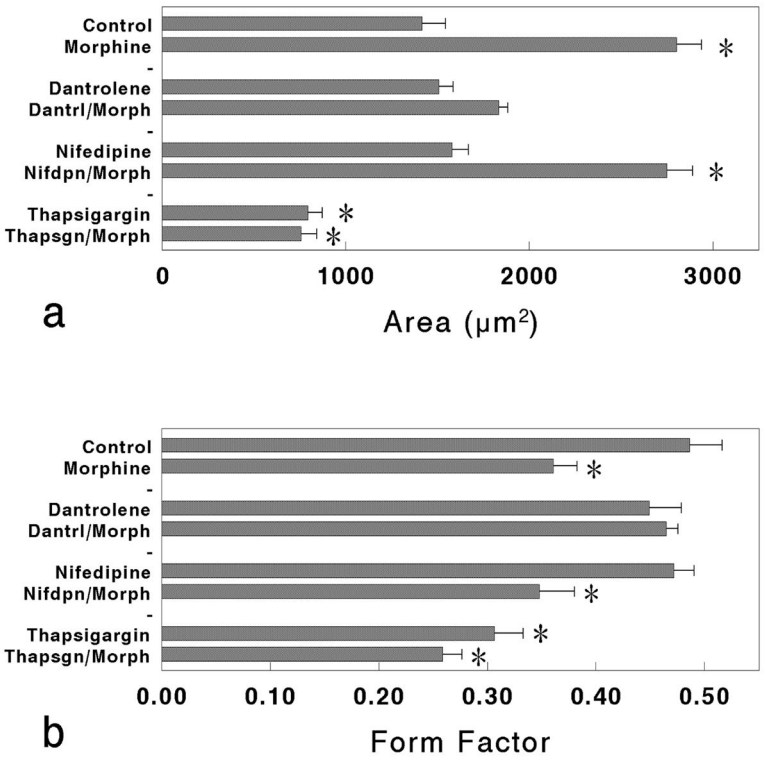

Fig. 5.

Effects of morphine, and/or drugs affecting $[Ca^{2+}]_i$, on the area (a) and form factor (b) of astrocytes at 48 h following continuous drug exposure. Compared to untreated controls, morphine treatment caused significant increases in the area (a) and in the abundance of cytoplasmic processes (indicated by significant decreases in form factor) (b). Measurements of form factor decrease with increasingly complex shape; i.e., form factor is greatest for circles. Treatment with dantrolene (Dantrl; 10 μM), but not nifedipine (Nifdpn; 1 μM), prevented morphine-induced changes in astrocyte morphology. Thapsigargin treatment (Thapsgn; 100 nM) caused significant decreases in area and form factor compared to untreated controls—irrespective of morphine treatment. $^*P < 0.05$ vs. non-morphine treated controls.

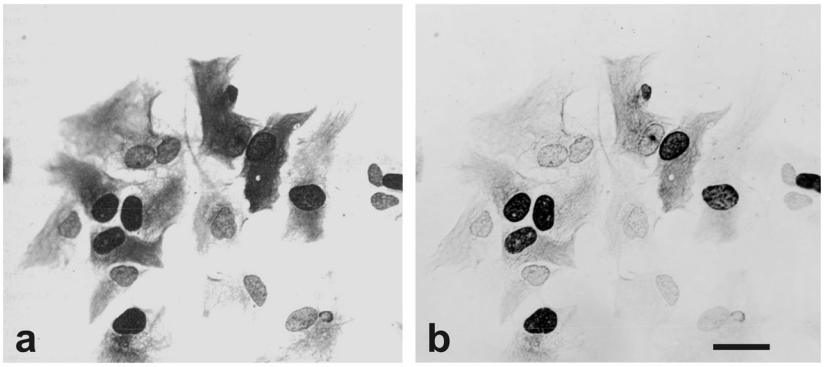

Fig. 6.

Brightfield photomicrographs showing combined bromodeoxyuridine (BrdU) and glial fibrillary acidic protein (GFAP) immunoreactivity in the same cells. To better distinguish the two products with black and white photography, a blue-green filter is used to enhance the copper-brown (DAB) GFAP immunoproduct (a); whereas a copper-brown filter is used to attenuate the GFAP immunoproduct relative to the black-blue BrdU immunoproduct (b). (a) and (b) are the same field. Scale bar = 25 μm.

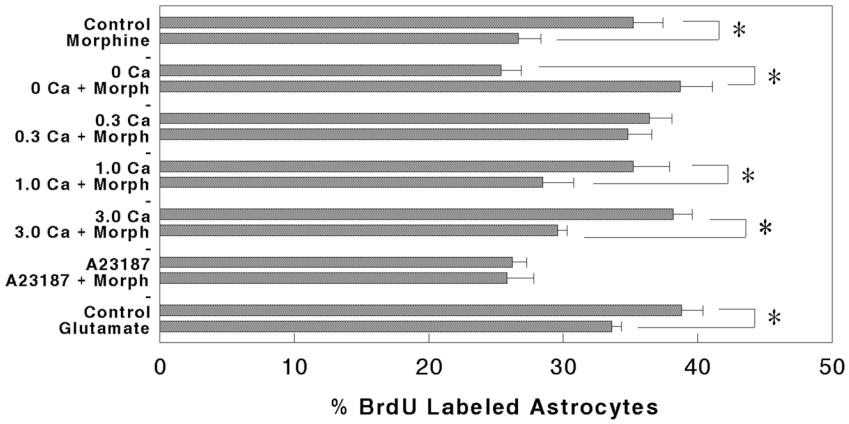

Fig. 7.

Effect of manipulating Ca^{2+} on morphine-induced suppression of bromodeoxyuridine (BrdU) incorporation by type 1 astrocytes. In the presence of unmodified culture medium (Control), or medium with near-normal (1.0 mM) or high (3.0 mM) $[Ca^{2+}]_o$, morphine significantly decreased BrdU incorporation into astrocytic DNA. In contrast, at low (0.3 mM) $[Ca^{2+}]_o$, or in the presence of 1.0 mM $[Ca^{2+}]_o$ and Ca^{2+} ionophore A23187 (1 μM), morphine no longer affected BrdU incorporation. Paradoxically, when $[Ca^{2+}]_o$ levels were exceedingly low (< 0.005 mM), morphine treatment significantly increased the proportion of astrocytes incorporating BrdU compared to non-morphine treated controls. Treatment with glutamate (100 μM), which elevates $[Ca^{2+}]_i$ in astroglia, also significantly reduced BrdU incorporation. The ability of morphine to inhibit BrdU incorporation by astrocytes was dependent on $[Ca^{2+}]_o$. *$P < 0.05$ vs. non-morphine (or non-glutamate) treated controls.

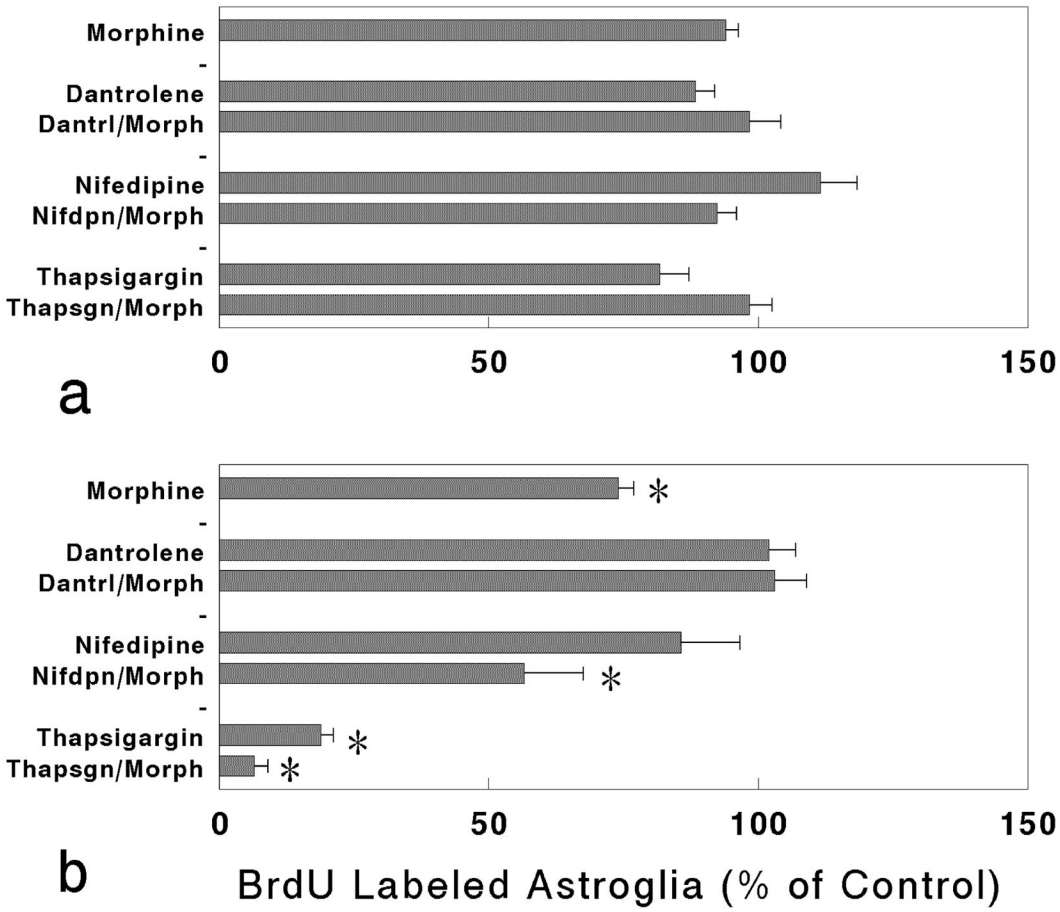

Fig. 8.

Effect of manipulating intracellular calcium ($[Ca^{2+}]_i$) on morphine-dependent decreases in bromodeoxyuridine (BrdU) incorporation in type 1 astrocytes at 6 h (a) and 48 h (b). At 6 h, continuous treatment with morphine alone, or drugs affecting $[Ca^{2+}]_i$, did not affect the percentage of astrocytes incorporating BrdU (a). In contrast, at 48 h following continuous exposure, dantrolene treatment (Dantrl; 10 μM) prevented morphine-induced decreases in BrdU synthesis compared to control values. Dantrolene alone did not affect on the rate of BrdU synthesis. Nifedipine treatment (Nifdpn; 1 μM) did not prevent morphine-induced declines in BrdU incorporation compared to control values, while nifedipine alone had no affect on BrdU uptake. Thapsigargin treatment (Thapsgn; 100 nM) alone, or in the presence of morphine, significantly reduced the proportion of astrocytes incorporating BrdU. *$P <$ 0.05 vs. non-morphine treated control.