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Enteric Glia Exhibit P_{2U} Receptors that Increase Cytosolic Calcium by a Phospholipase C-Dependent Mechanism

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Abstract: Calcium signaling in fura-2 acetoxymethyl ester-loaded enteric glia was investigated in response to neuroligands; responses to ATP were studied in detail. Carbachol (1 m*M*), glutamate (100 μM), norepinephrine (10 μM), and substance P (1 μM) did not increase the intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured enteric glia. An increasing percentage of glia responded to serotonin (4%; 100 μM), bradykinin (11%; 10 μM), and histamine (31%; 100 μM), whereas 100% of glia responded to ATP (100 μM). ATP-evoked calcium signaling was concentration dependent in terms of the percentage of glia responding and the peak $[Ca^{2+}]_i$ achieved; responses were pertussis toxin insensitive. Based on responsiveness of enteric glia to purinergic agonists and peak $[Ca^{2+}]_i$ evoked, ATP = UTP > ADP > β,γ-methyleneadenosine 5′-triphosphate ≫ 2-methylthioadenosine 5′-triphosphate = α,β-methyleneadenosine 5′-triphosphate = AMP = adenosine, suggesting a glial P_{2U} receptor. Depletion of D-*myo*-inositol 1,4,5-trisphosphate-sensitive calcium stores by thapsigargin (10 μM) abolished glial responses to ATP. Similarly, calcium responses were decreased 92% by U-73122 (10 μM), an inhibitor of phospholipase C, and 93% by the phorbol ester phorbol 12-myristate 13-acetate (100 n*M*), an activator of protein kinase C. Thus, cultured enteric glia can respond to neurotransmitters with increases in $[Ca^{2+}]_i$. Our data suggest that glial responses to ATP are mediated by a P_{2U} receptor coupled to activation of phospholipase C and release of intracellular calcium stores. **Key Words:** Myenteric plexus—ATP—Purinergic receptors—Signal transduction.
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The enteric nervous system lies within the intestinal wall, uninterrupted from the esophagous to the internal anal sphincter. Composed of neurons, glia, and occasional fibroblasts, the enteric nervous system is involved in the regulation of intestinal smooth muscle contractility, absorptive-secretory epithelium, and blood flow (Jabbur et al., 1988). Enteric glia, which outnumber enteric neurons 2:1, display morphological and molecular similarities to CNS astrocytes: They have irregular shapes, do not synthesize a basal lamina, and have an abundance of glial fibrillary acidic protein (GFAP) (Gershon and Rothman, 1991). Traditionally, enteric glia are described as a homogeneous population of cells whose primary function is supporting neurons in the continuously moving intestinal wall (Gabella, 1981).

Evidence has accumulated challenging the concept that glial cells of the CNS and PNS are merely passive supports for neurons. Voltage-dependent ion channels similar to those observed in neurons have been demonstrated in glial cells (Barres et al., 1990). Studies both in vitro and in situ have shown functional neuroligand receptors linked to second messenger systems on astrocytes, oligodendroglia, and Schwann cells (Murphy and Pearce, 1987; Barres, 1991; Kastritsis et al., 1992; Kastritsis and McCarthy, 1993). Furthermore, both neuron–glial signaling (Dani et al., 1992) and glial–neuron signaling (Nedergaard, 1994) have been reported.

We postulated that enteric glia, like glia in the CNS and PNS, possess signal transduction systems that can be stimulated by neuroactive substances. We examined calcium signaling evoked by selected neuroligands in fura-2 acetoxymethyl ester (fura-2/AM)-loaded enteric glia. Furthermore, we investigated a receptor subtype and intracellular mechanism of action for ATP, which stimulated calcium fluxes in all enteric glia examined. We report that cultured enteric glia (a) respond to select neurotransmitters with increases in cytosolic calcium concentration, (b) are pharmacologically heterogeneous in responses to neuroligands, and (c) possess a P_{2U} receptor for ATP linked to phospholipase C (PLC) activation with release of intracellular calcium stores.

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Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium concentration; fura-2/AM, fura-2 acetoxymethyl ester; GFAP, glial fibrillary acidic protein; MAP, microtubule-associated protein; α,β-MeATP, α,β-methyleneadenosine 5′-triphosphate; β,γ-MeATP, β,γ-methyleneadenosine 5′-triphosphate; 2-MeSATP, 2-methylthioadenosine 5′-triphosphate; NSE, neuron-specific enolase; PBS, phosphate-buffered saline; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; RB2, reactive blue 2.

MATERIALS AND METHODS

Reagents

ATP, ADP, AMP, adenosine, UTP, α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP), β,γ-methyleneadenosine 5′-triphosphate (β,γ-MeATP), collagenase, trypsin-EDTA 1×, trypsin inhibitor, thapsigargin, L-glutamic acid, penicillin/streptomycin solution, rabbit anti-GFAP, mouse anti-microtubule-associated protein (MAP), and gel mount were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Methylthioadenosine 5′-triphosphate (2-MeS-ATP), reactive blue 2 (RB2), phorbol 12-myristate 13-acetate (PMA), pertussis toxin, bradykinin, and histamine were from Research Biochemicals International (Natick, MA, U.S.A.). Suramin was from Calbiochem (La Jolla, CA, U.S.A.). Thapsigargin epoxide and 4α-PMA were from LC Laboratories (Woburn, MA, U.S.A.). The U-73122 compound was a kind gift from Dr. Stephen K. Fisher (University of Michigan). The U-73343 compound was from Biomol (Plymouth Meeting, PA, U.S.A.). Duncan–Hartley guinea pigs were from Simonsen Labs (Gilroy, CA, U.S.A.). Hanks' balanced salt solution, medium 199, and fetal bovine serum were from GibcoBRL (Grand Island, NY, U.S.A.). NU-serum was from Collaborative Research (Bedford, MA, U.S.A.). Fura-2/AM and fura-2 free acid were from Molecular Probes (Eugene, OR, U.S.A.). Rabbit anti-human fibronectin, fluorescein-conjugated goat anti-rabbit IgG (whole molecule), Texas red-conjugated goat anti-mouse IgG,A,M, and fluorescein-conjugated goat F(ab′)$_2$ fragment to mouse IgG (whole molecule) were from Cappel Organon Teknika Corp. (Durham, NC, U.S.A.). Rabbit anti-neuron-specific enolase (NSE) and rabbit anti-bovine brain S-100 were from Dako (Carpenteria, CA, U.S.A.). Biotinylated goat anti-rabbit IgG (H + L) and streptavidin fluorescein were from Amersham (Arlington Heights, IL, U.S.A.). Normal goat serum was from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Myenteric plexus isolation

The use and care of the Duncan–Hartley guinea pigs in the following protocols have been approved by the University Committee on the Use and Care of Animals (approval no. 5182A). Dispersed primary cultures of guinea pig myenteric plexus were prepared on collagen-coated coverslips. The taenia coli from 1 day-old male Duncan–Hartley guinea pigs were removed and placed in Hanks' balanced salt solution plus 0.1% collagenase (type V) for 16–20 h at 4°C. After a 35-min incubation at 37°C, the muscle layers of the taenia coli were separated from the myenteric plexus using a dissecting microscope. The plexus was trypsinized for 30 min at 37°C using trypsin-EDTA solution, triturated with siliconized flamed Pasteur pipettes of decreasing tip diameter, and plated on collagen-coated coverslips. Cultures were exposed to complete medium 199 plus 5% NU-serum and 0.001% trypsin inhibitor (type I-S from soybean). Penicillin/streptomycin solution was added for the first 24 h at a 2% concentration. Antimitotic agents were not added. The cultures were incubated at 37°C with 95% O_2/5% CO_2, and medium was changed every other day.

Loading and cell preparation for imaging

Cultured plexus was incubated at 37°C in fresh warmed medium containing 4 μM fura-2/AM for 30 min. Loaded coverslips were washed, resuspended in control buffer (pH 7.40, containing 118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, 15 mM $NaHCO_3$, 11 mM glucose, 0.9 mM NaH_2PO_4, and 0.8 mM $MgSO_4$), and placed in an aluminum superfusion chamber. The superfusion rate of the control buffer and experimental solutions was 1 ml/min at 37°C. For calcium-free conditions, $CaCl_2$ was removed from the buffer, and 1 mM EGTA was added.

Calcium level measurements

A Zeiss (Rockville, MD, U.S.A.) Axiovert inverted microscope and Attofluor digital imaging system were used for single cell intracellular calcium concentration ($[Ca^{2+}]_i$) determinations. $[Ca^{2+}]_i$ was calculated from the ratios of the fluorescence intensities of fura-2 at 334 and 380 nm wavelengths with an emission wavelength of 500 nm (Grynkiewicz et al., 1985). Calibration of the system was performed daily using fura-2 free acid and the following two-point standardization equation: $[Ca^{2+}]_i = K_D\,[(R - R(\mathrm{Lo}))/(R(\mathrm{Hi}) - R)]\beta$, where K_D is the dissociation constant of the Ca^{2+}/fura-2 complex (225 nm), R is the F_{334}/F_{380}, i.e., the fluorescence at 334 nm excitation divided by the fluorescence at 380 nm excitation, R(Lo) is the ratio at zero calcium (1 mM EGTA), R(Hi) is the ratio at high calcium (1 mM $CaCl_2$), and β is the F_{380}(zero Ca^{2+})/F_{380}(saturating Ca^{2+}). Frames were not averaged to obtain images. A ratio pair was taken every 1.5 s. Calibration was done in the same superfusion chamber using a saturating Ca^{2+} level of 2 mM and a zero Ca^{2+} value of 4 mM EGTA.

Immunocytochemical staining

Immediately following a calcium imaging experiment, coverslips were fixed in methanol (100% at −20°C), air-dried, and stored at −40°C. For staining, coverslips were allowed to come to room temperature, rehydrated with phosphate-buffered saline (PBS) for 5 min and then blocked for 20 min in PBS/5% fetal bovine serum with 10 drops of normal goat serum. After the blocking solution was removed, primary antibodies were immediately applied and allowed to incubate overnight at 4°C.

Staining for NSE, fibronectin, and S-100 was performed as follows: Coverslips were washed twice for 5 min in PBS, incubated with biotinylated anti-rabbit IgG (1:100) for 1 h at room temperature, and washed twice again in PBS. Coverslips were then incubated with streptavidin-fluorescein isothiocyanate (NSE and fibronectin, 1:100 dilution for both) or streptavidin-Texas red (S-100, 1:100) for 1 h at room temperature.

Staining for MAP2 and GFAP was performed as follows: Coverslips were washed twice for 5 min in PBS and then incubated with goat anti-mouse-Texas red (1:20) in blocking solution (for MAP2) or goat anti-mouse-fluorescein isothiocyanate fragment [F(ab′)$_2$; 1:600] in blocking solution (for GFAP) for 30 min at 37°C.

After a final washing in PBS, coverslips were mounted with gel mount and stored in the dark at 4°C until photographed using a Leitz Orthoplan microscope. Negative controls were performed with normal mouse serum or normal rabbit serum as appropriate.

Data presentation and calculation

Results are presented as mean ± SEM values. The significance level was defined as $p < 0.05$ by Student's t test. A glial cell was judged to have responded to a ligand if its $[Ca^{2+}]_i$ increased by at least 100% over baseline values. Experiments were performed on days 2–4 in vitro, and glia examined were free from overlying myenteric neurons.

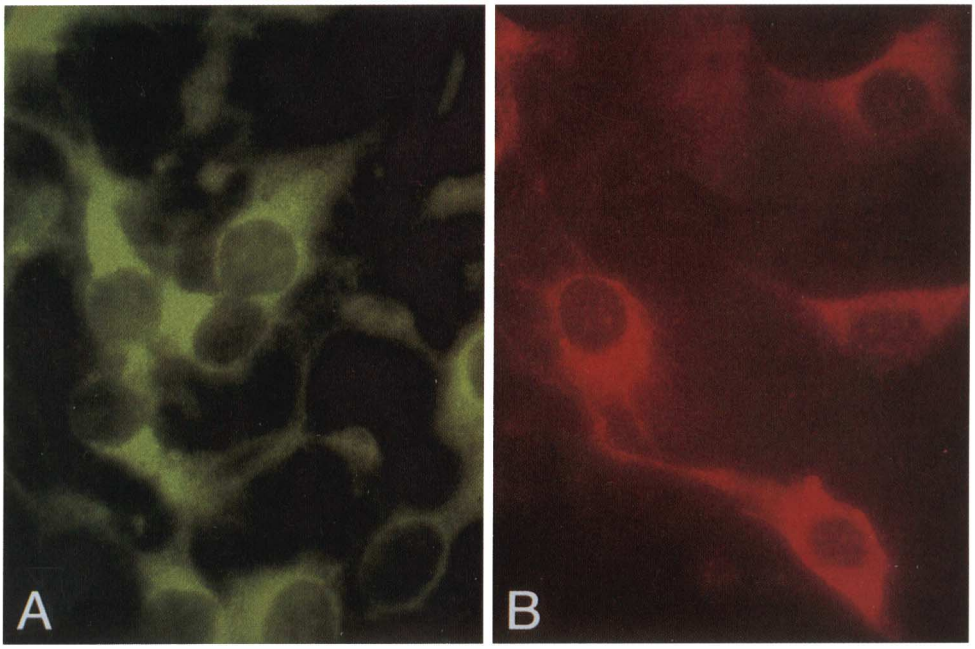

FIG. 1. Myenteric plexus cultures from days 2–4 in vitro were incubated with anti-GFAP (primary) and goat anti-mouse fluorescein isothiocyanate fragment (secondary) or anti-S-100 (primary) and streptavidin-Texas red (secondary). Glia stained positively for GFAP (**A**) and S-100 (**B**). (×250). Myenteric neurons were unstained.

Experiments using inhibitors/antagonists were done as follows: An initial 60-s superfusion of ATP was given, followed by a 300-s resting perfusion with buffer alone, followed by a second superfusion of ATP plus inhibitor/antagonist. Thus, each responding glial cell served as its own control.

In antagonist/inhibitor studies, percent inhibition was calculated using the equation $100 - 100(x)$, where x equals the change in calcium level ($\Delta\ Ca^{2+}$) of the second exposure divided by the $\Delta\ Ca^{2+}$ level of the first exposure.

During experiments, enteric glia were differentiated from neurons morphologically as well as via depolarization-induced $[Ca^{2+}]_i$ increases. Under 40× phase contrast 2–4 days postplating, enteric glial cells are flat with a centrally placed nucleus and wide surrounding cytoplasm. In contrast, myenteric neurons appear round, compact, and phase bright (Saffrey et al., 1991). In addition, at the end of each experiment, coverslips were superfused with 55 m*M* KCl. Enteric glial cells have not been shown to possess voltage-gated calcium channels (Broussard et al., 1993) and thus do not respond to KCl superfusion. In contrast, myenteric neurons have L- and N-type calcium channels (Hirning et al., 1990) and increase $[Ca^{2+}]_i$ in response to depolarization.

RESULTS

Immunocytochemical characterization of enteric glia

Glia in myenteric plexus cultures from days 2–4 postplating were characterized immunocytochemically using standard glial stains. Cells with distinct glial morphology on light microscopy stained positively for GFAP and S-100 (Fig. 1). Glia stained negatively for fibronectin, indicating a lack of contaminating fibroblasts (Bannerman et al., 1988). Furthermore, glia were negative for NSE, an isoenzyme specific to neurons of both the CNS and PNS (Scheuermann et al., 1989), and MAP2, a nontubulin protein associated with microtubules and specific for neurons (Olmstead, 1986). Myenteric neurons in these cultures stain positively for both NSE and MAP2 (Mulholland et al., 1994). In these dispersed enteric plexus cultures at days 2–4 postplating, enteric glia comprise the majority of cells (~70%), with the remainder of cells being enteric neurons (25%) and fibroblasts (5%) (Saffrey et al., 1991).

Neuroligand-evoked calcium signaling in enteric glia

Fura-2/AM-loaded enteric glia from days 2–4 postplating were exposed to neuroligands in a calcium-containing buffer individually for 60 s from an eight-well perfusion chamber. The exposures to agonists were separated by continuous buffer perfusion for 300 s. Results were consistent whether ATP was added either before or after other ligands. Over 10 coverslips were examined with a different sequence of agonist addition, as described by Kastritsis and McCarthy (1993), to minimize effects, if any, of heterologous receptor desensitization. The agonists chosen (carbachol, glutamate, norepinephrine, substance P, serotonin, bradykinin, histamine, and ATP) have been linked to the hydrolysis of polyphosphoinositides in CNS glial cultures (for reviews, see Murphy and Pearce, 1987; Barres, 1991).

Baseline $[Ca^{2+}]_i$ of enteric glia was 48 ± 1 (n

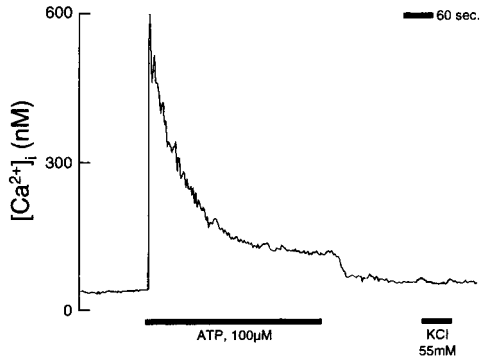

FIG. 2. Typical enteric glial response to ATP superfusion (100 μM, 250 s) in a calcium-containing buffer. KCl superfusion (55 mM, 60 s) does not increase $[Ca^{2-}]_i$ in enteric glia; this lack of response in addition to morphology differentiates glia from myenteric neurons during experiments. This tracing is typical of 139 glial responses.

= 243). Neither spontaneous nor agonist-induced calcium oscillations were seen, in contrast to fura-2/AM-loaded astroglia (Salm and McCarthy, 1990; Kastritsis et al., 1992). None of 80 enteric glia examined responded to carbachol, glutamate, norepinephrine, or substance P with calcium fluxes. One of 23 glia (4%) responded to serotonin (100 μM), whereas nine of 81 glia (11%) responded to bradykinin (10 μM) and 25 of 81 (31%) responded to histamine (100 μM). ATP (100 μM) consistently evoked calcium transients in 100% of glia examined (81 of 81). ATP-induced transients in calcium-containing buffer were biphasic, with a sharp peak followed by a sustained elevation maintained during the presence of the agonist (Fig. 2). This sustained elevation appeared to require continued agonist exposure, as it was not observed with shorter agonist exposures (60 s), as will be evident in succeeding figures. Because cultured enteric glia universally responded to ATP, we further investigated a receptor subtype and intracellular signal transduction mechanism mediating ATP's action.

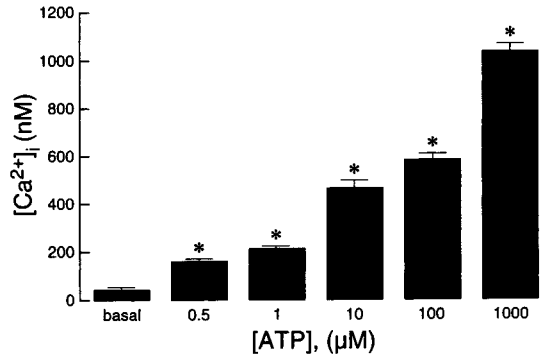

FIG. 3. Concentration-dependent increases in peak $[Ca^{2+}]_i$ evoked by ATP in enteric glia. The total number of glia examined was 352; each glial cell was exposed to a single ATP concentration. * $p < 0.05$ versus basal value by Student's t test.

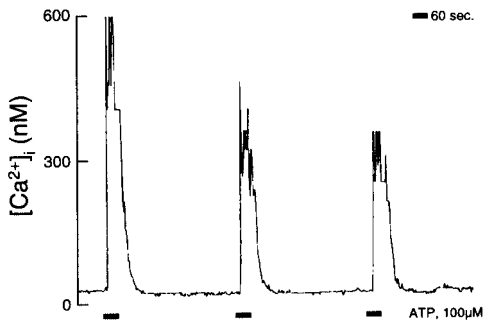

FIG. 4. Effect of multiple exposures to ATP. Repetitive superfusion of enteric glia with ATP (100 μM, 60 s) in calcium-containing buffer, separated by 300 s of buffer perfusion, evoked decreasing peak $[Ca^{2+}]_i$ responses. This tracing is typical of 68 glial responses.

ATP-evoked concentration-dependent calcium transients in enteric glia

Glia did not respond to ATP at a concentration of 0.01 μM. Eleven percent of cells responded to 0.5 μM ATP, 45% of cells responded to 1.0 μM ATP, and 100% of glia responded to ATP at concentrations of 10, 100, and 1,000 μM (total glia examined = 352). Responses were judged to be an increase in $[Ca^{2+}]_i$ by at least 100% over basal levels during ATP exposure. As the ATP concentration increased, the stimulated peak $[Ca^{2+}]_i$ increased as well (Fig. 3). In single glial cells, repetitive ATP exposures (0.5–1,000 μM) given between 300-s rest intervals of control buffer produced progressive decreases in $[Ca^{2+}]_i$ responses (Fig. 4). There was a 41% decrease in peak $[Ca^{2+}]_i$ between the first and second ATP exposures in calcium-containing buffer (n = 68).

Enteric glial purinoceptor

ATP cogeners and related nucleotides were used to characterize an enteric glial purinoceptor. Each purinergic receptor agonist was superfused for 250 s at 100 μM, and each glial cell was exposed to only one agonist. Based on the percentage of enteric glia responding with Ca^{2+} fluxes, the rank order of potency was UTP = ATP > ADP > β,γ-MeATP $\gg$ 2-MeS-ATP = α,β-MeATP = AMP = adenosine (Fig. 5). In responding glia, peak $[Ca^{2+}]_i$ levels achieved by these ligands at 100 μM were as follows: UTP = 1,123 ± 43 nM (n = 68) > ATP = 583 ± 26 nM (n = 139) > ADP = 223 ± 26 nM (n = 65) > β,γ-MeATP = 138 ± 10 nM (n = 49). Both the percentage of glia responding to purinergic agonists and the peak $[Ca^{2+}]_i$ achieved by those ligands are consistent with action at a glial P_{2U} (Dubyak, 1991) or "nucleotide" (O'Connor et al., 1991) or P_{2Y2} (Abbracchio and Burnstock, 1994) receptor.

Two P_2 receptor antagonists were used to confirm agonist studies. Suramin (300 μM), a trypanocidal agent that has been shown to antagonize both P_{2X}- and P_{2Y}-mediated responses (Dunn and Blakely, 1988),

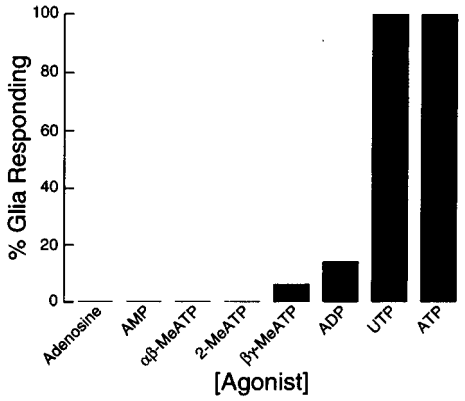

FIG. 5. Each purinergic receptor agonist was superfused for 250 s at 100 μM. Glia were judged to respond to an agonist if $[Ca^{2+}]_i$ increased by at least 100% over baseline values. The total number of glia examined was 385; each glial cell was exposed to only one agonist.

did not inhibit ATP-evoked calcium transients (n = 41; Fig. 6A). RB2, an anthraquinone sulfonic acid derivative and specific P_{2Y} antagonist (Bean, 1992), also did not reduce ATP-mediated transients (n = 96; Fig. 6B).

Enteric glia respond to ATP in the absence of extracellular calcium

To identify the source(s) of calcium responsible for ATP's action, glia were superfused with ATP (100 μM) in calcium-free buffer plus EGTA. One hundred percent of the glia responded with a peak $[Ca^{2+}]_i$ of 621 ± 21 nM (n = 48). Figure 7 shows a typical tracing of a glial response to ATP in the absence of extracellular calcium. The sustained elevation or plateau following the sharp peak in calcium-containing buffer is abolished in calcium-free buffer. These observations suggest that an influx of extracellular calcium occurs during the plateau phase.

ATP and thapsigargin induce release of identical calcium stores in enteric glia

To quantify the intracellular calcium store(s) released by ATP, glia were superfused with thapsigargin followed by ATP or by ATP followed by thapsigargin. Thapsigargin, a sesquiterpene lactone, is a highly selective and potent inhibitor of endoplasmic reticulum Ca^{2+}-ATPases, resulting in release of intracellular stores of calcium (Thastrup et al., 1990). Of 89 glia exposed to thapsigargin, 89 responded with increases in $[Ca^{2+}]_i$, and none responded to the subsequent ATP superfusion (Fig. 8A). Fifty-two of 52 glia responded to ATP (data not shown) following exposure to thapsigargin epoxide, a negative control for thapsigargin (Christensen et al., 1992). This implies that toxicity from thapsigargin is not the reason for lack of glial response to subsequent ATP superfusion.

ATP superfused repetitively in a calcium-free buffer plus EGTA generated small increases in $[Ca^{2+}]_i$ on

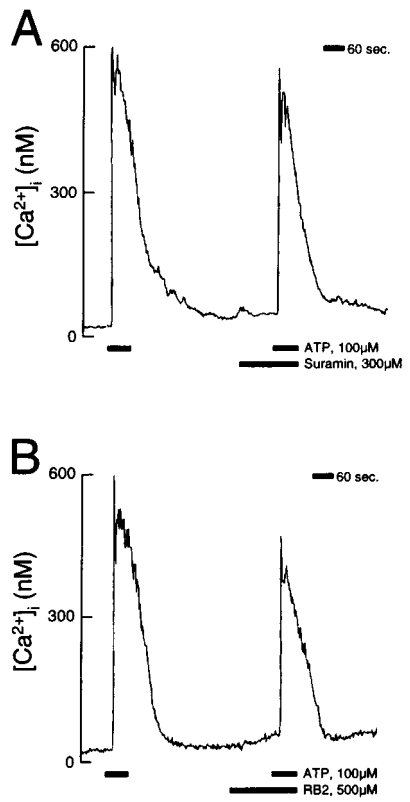

FIG. 6. Neither suramin (300 μM) nor RB2 (500 μM) inhibited ATP-evoked calcium fluxes in enteric glia. Glia were superfused with ATP (100 μM) for 60 s, followed by 300 s of buffer, followed by suramin (**A**) or RB2 (**B**) alone for 60 s and then the inhibitor plus ATP for 60 s. These tracings are typical of 41 (A) and 96 (B) glia.

the second and third exposure. None of 44 glia responded to thapsigargin superfused after three successive ATP exposures (Fig. 8B). These data suggest that (a) an initial exposure to ATP in a calcium-free buffer induces release of nearly all the intracellular calcium sensitive to ATP; (b) thapsigargin and ATP induce release of identical intracellular calcium stores; and (c)

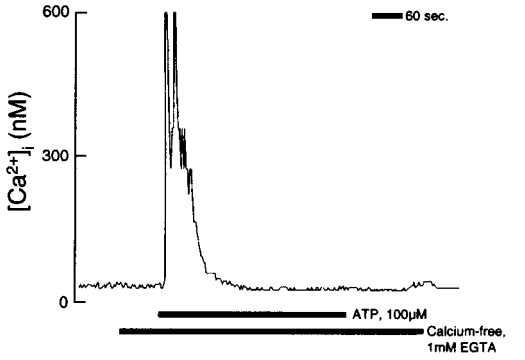

FIG. 7. Glial response to ATP (100 μM, 250 s) in a calcium-free buffer plus 1 mM EGTA. This tracing is typical of 48 cells.

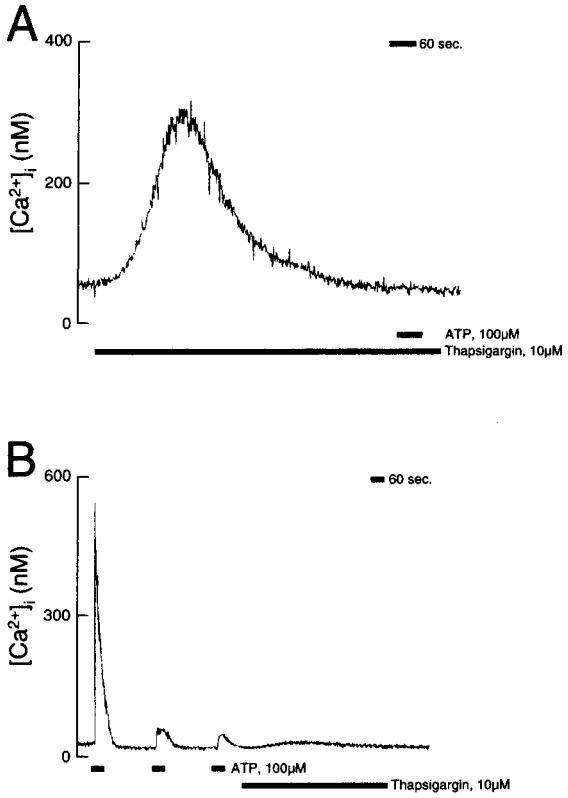

FIG. 8. A: Thapsigargin (10 μM) superfused for 500 s in calcium-free buffer evoked calcium fluxes in enteric glia. Subsequent exposure to ATP (100 μM, 60 s) did not generate responses in these cells. **B:** ATP (100 μM, 60 s) was superfused repetitively in a calcium-free buffer plus 1 mM EGTA. Each ATP exposure was separated by 300 s of calcium-free buffer perfusion. Following the ATP superfusions, thapsigargin (10 μM) was superfused for 300 s. These tracings are typical of 89 (A) and 44 (B) glia.

ATP may release D-*myo*-inositol 1,4,5-trisphosphate-sensitive calcium stores.

ATP activates PLC in enteric glia

The aminosteroid U-73122 has been shown to inhibit PLC-coupled responses in a wide variety of cells (Bleasdale and Fisher, 1993). Pretreatment of enteric glia with U-73122 (10 μM, 60 s) inhibited ATP-evoked calcium transients by 92 ± 8% (n = 29; $p < 0.05$ vs. second ATP exposure alone; Fig. 9). U-73343 (10 μM), an inactive analogue of U-73122, had no effect on ATP-evoked responses (n = 59, data not shown), suggesting the effect of U-73122 was not due to nonspecific cell toxicity.

Phorbol ester inhibits ATP-evoked calcium transients

Phorbol ester inhibition of both nucleotide-evoked calcium transients and D-*myo*-inositol 1,4,5-trisphosphate accumulation have been reported in rat glioma cells (Munshi et al., 1993) and a neuronal cell line (Iredale et al., 1992). Brief preincubation of enteric glia with PMA (100 nM, 300 s) inhibited ATP-evoked

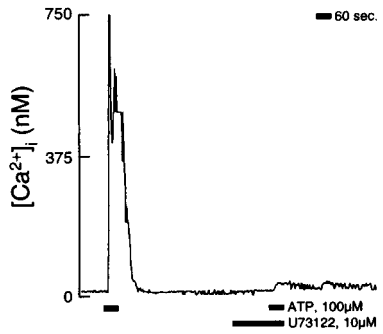

FIG. 9. Enteric glia were initially exposed to ATP (100 μM, 60 s), followed by 300 s of buffer, 60 s of U-73122 alone (10 μM), and then U-73122 plus ATP (100 μM) for 60 s. Percent inhibition of ATP-evoked calcium flux calculated as per Materials and Methods. This tracing is typical of 29 glia.

calcium fluxes by 93 ± 7% (n = 68; $p < 0.05$ vs. second ATP exposure alone; Fig. 10). Preincubation of enteric glia with 4αPMA (100 nM, 300 s), a negative control (Duuren et al., 1979), had no effect (n = 8; data not shown).

Effect of pertussis toxin

P_{2U} receptors couple to PLC either partially through pertussis toxin-sensitive G proteins (Erb et al., 1993) or exclusively through toxin-insensitive G proteins (Munshi et al., 1993). Enteric glia were preincubated with pertussis toxin at 100 ng/ml for 24 h. No significant difference in peak $[Ca^{2+}]_i$ was seen on ATP superfusion (100 μM) between pertussis toxin-treated glia (peak $[Ca^{2+}]_i$ = 621 ± 76 nM, n = 43) vs. control glia (peak $[Ca^{2+}]_i$ = 577 ± 99 nM, n = 37).

DISCUSSION

This study suggests that (a) cultured enteric glia, like glia of the CNS and PNS, exhibit neuroligand signal transduction systems linked to increases in cytosolic calcium levels; (b) enteric glia in vitro are phar-

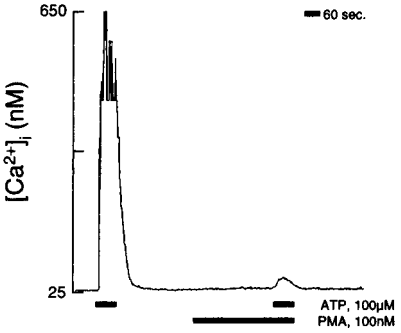

FIG. 10. Enteric glia were initially exposed to ATP (100 μM, 60 s), followed by 200 s of buffer, 300 s of PMA alone (100 nM), and then PMA plus ATP (100 μM) for 60 s. Percent inhibition of ATP-evoked calcium flux was calculated as per Materials and Methods. This tracing is typical of 48 glia.

macologically heterogeneous; (c) enteric glia possess at least a P_{2U} receptor for ATP; and (d) ATP-evoked Ca^{2+} fluxes in enteric glia are coupled by a pertussis toxin-insensitive mechanism to activation of PLC and release of D-*myo*-inositol 1,4,5-trisphosphate-sensitive calcium stores.

Enteric glia, originating from the neural crest, have been characterized using ultrastructural, immunocytochemical, and molecular techniques. Studies with thin-section electron microscopy have revealed an intimate association between enteric glial and neuronal membranes, with numerous specialized contacts of unknown significance between the two cell types (Gabella, 1981). In addition to staining strongly for GFAP, enteric glia, like astrocytes, express the enzyme glutamine synthetase, the antigen Ran-2 (Jessen and Mirsky, 1983), and the Ca^{2+} binding protein S-100 (Ferri et al., 1982).

This work extends prior characterization of cultured enteric glia and raises new possibilities for their function. Although glia of the CNS and PNS respond to various neurotransmitters, responses of enteric glia have not previously been examined. Fura-2-loaded type 1 and type 2 astroglia exhibit calcium signaling in response to 2-methylthio-ATP, ATP, carbachol, serotonin, glutamate, and histamine (Dave et al., 1991; McCarthy and Salm, 1991; Kastritsis et al., 1992). Likewise, oligodendroglia express neuroligand receptors linked to calcium mobilization; responses to ATP, glutamate, norepinephrine, histamine, bradykinin, carbachol, and substance P have been reported (Kastritsis and McCarthy, 1993). In contrast to CNS glia, peripheral glia such as Schwann cells have displayed negligible or only partial responses to neuroligands such as bradykinin, histamine, and glutamate, whereas nearly all responded to ATP (Lyons et al., 1994). Regarding responses to neurotransmitters, enteric glia appear more like peripheral than central glia, despite established similarities between enteric glia and astrocytes in expression of cytoskeletal and other proteins. Enteric glia did not respond to glutamate, norepinephrine, carbachol, or substance P. Minor subpopulations of enteric glia responded to bradykinin, histamine, and serotonin, whereas all cells examined responded to ATP. Whether enteric glia in vivo demonstrate differing neurotransmitter responsiveness is unknown. Differences in membrane properties of glia in vitro versus in situ have been reported: Optic nerve type 1 astrocytes express fewer types of ion channels in culture than in situ (Barres et al., 1990).

This report suggests that enteric glia are pharmacologically heterogeneous, supporting the few previous observations of enteric glial variability. For example, a minor subgroup of cultured enteric glia stain positively for Leu 7, a monoclonal antibody that recognizes specific adhesion cell molecules (Bannerman et al., 1987). Likewise, the monoclonal antibody anti-GFAP3, which recognizes GFAP-containing intermediate filaments in astrocytes, binds selectively to a subgroup of enteric glia (Jessen and Mirsky, 1985). In this study, discrete subpopulations of enteric glia responded with calcium signaling to serotonin (4%), bradykinin (11%), histamine (31%), and ATP (100%). It is likely that differences in receptor expression account for these variations in responsiveness, although differences in signaling pathways distal to the receptor could also be a factor. It is possible that cultured enteric glia isolated from elsewhere along the gastrointestinal tract would demonstrate different responsiveness, as astroglial properties and functions vary depending on their location within the brain (Murphy and Pearce, 1987).

Based on the actions of purinoceptor agonists, enteric glia possess at least a P_{2U} receptor subtype for ATP. Purinergic receptors were first classified in 1978 (Burnstock) as P_1 (adenosine) and P_2 (ATP/ADP), with many new subclasses being defined since that time. Abbracchio and Burnstock (1994) have recently put forth a new framework for P_2 receptors, suggesting that the variously named P_2 purinoceptors that are G protein coupled should be incorporated into numbered subclasses of the P_{2Y} family. Thus, the recently cloned P_{2U} receptor (Erb et al., 1993; Lustig et al., 1993) would be renamed P_{2Y2}. This receptor, which will continue to be referred to as P_{2U} for the purposes of this article, has been expressed in a human cell line where its actions are independent of extracellular calcium and partially pertussis toxin sensitive (Erb et al., 1993; Lustig et al., 1993). It has been localized to cells as diverse as human neutrophils, human skin fibroblasts, aortic smooth muscle cells, pulmonary artery endothelium, and renal cortical collecting tubule (for review, see O'Connor et al., 1991). Some cells, such as the C6 glioma line (Lin and Chuang, 1994) have a P_{2U} receptor plus a second purinoceptor, with more than one signal transduction mechanism activated on ATP binding. P_{2U} receptor activation is linked to varied processes, including prostaglandin production in adrenal medulla endothelial cells, neutrophil superoxide release, and relaxation of rat aorta (O'Connor et al., 1991).

The enteric glial P_{2U} receptor and its calcium signaling pathway display both similarities and differences compared with other described P_{2U} receptors. As in enteric glia, the C6 glioma P_{2U} receptor is linked to PLC by a pertussis toxin-insensitive mechanism (Lin and Chuang, 1994). However, suramin was a competitive antagonist for the C6 glioma receptor, whereas it had no effect in enteric glia. Like enteric glia, increases in levels of cytosolic calcium and D-*myo*-inositol 1,4,5-trisphosphate are stimulated by a P_{2U} receptor in bovine glomerular endothelial cells (Briner and Kern, 1994). The P_{2U} receptor in NG108-15 cells, a neuroblastoma/glioma hybrid line, has been linked not only to PLC activation, but also to a plasma membrane calcium channel, because chelation of extracellular calcium by EGTA reduced ATP-evoked $[Ca^{2+}]_i$ increases by 40% (Lin et al., 1993).

Experiments using inhibitors, e.g., U-73122 and

PMA, were performed by comparing the peak Ca^{2+} response of the second ATP exposure in the presence of the inhibitor to the peak Ca^{2+} response to ATP expected in the absence of the inhibitor. Based on multiple observations, there was a 41% decrease in peak $[Ca^{2+}]_i$ between the first and second ATP exposures ($n = 68$; Fig. 4). Decreases in peak $[Ca^{2+}]_i$ observed in the presence of the inhibitors, e.g., $92 \pm 8\%$ decrease with U-73122, were thus compared with this expected decrement. Decreases in responsiveness on repetitive stimulation have been seen with several receptors linked to phosphoinositide hydrolysis (Sugiya et al., 1987; Munshi et al., 1993). One potential complication in data interpretation using this experimental paradigm is that the inhibitors may be affecting calcium influx rather than the D-*myo*-inositol 1,4,5-trisphosphate-mediated release of calcium from intracellular stores. The compound U-73122 has been extensively characterized (Bleasdale and Fisher, 1993) as an inhibitor of phosphoinositide-specific PLC, with little or no direct inhibition of phospholipases A_2 and D. Experiments by other investigators have demonstrated rapid inhibition of Ca^{2+} oscillations with U-73122 in the presence of lanthanum, indicating that U-73122 was unlikely to inhibit Ca^{2+} oscillations by interfering with Ca^{2+} influx (Yule and Williams, 1992). Furthermore, experiments using thapsigargin ruled out the possibility that U-73122 functioned to interfere with the storage or release of Ca^{2+} in pancreatic acini (Yule and Williams, 1992). Thus, although we cannot rule out potential effects of inhibitors on Ca^{2+} influx pathways, our results using inhibitors are consistent with previously published actions of these compounds on the phosphoinositol pathway.

Reported functions of neuroligand-responsive glia from the CNS and PNS include regulation of energy metabolism, synthesis and release of neurotransmitters, and buffering of electrolytes. Neuroligands such as serotonin, histamine, and vasoactive intestinal peptide activate enzymatic breakdown of [^{3}H]glycogen stores in astrocytes (Pentreath et al., 1986). It has been suggested that neighboring neurons use the products of astroglial glycogen turnover as energy sources (Shank and Campbell, 1984). Furthermore, because astrocytes have ion channels, they are believed to participate in electrolyte homeostasis, thus controlling the neuronal microenvironment (Cserr and Bundgaard, 1986). Specifically, astrocytes regulate extracellular K^+ levels by taking up excess potassium, redistributing it through extensive gap junctions, and releasing it into the bloodstream (Newman, 1986). Thus, CNS glia could be involved in information processing by modulating local amine and ion concentrations. Whether enteric glia possess such neuromodulatory functions remains to be determined.

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