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Presence of functionally active protease-activated receptors 1 and 2 in myenteric glia

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Abstract

Protease-activated receptors (PARs) belong to the family of membrane receptors coupled to G-proteins; their presence is reported in a wide variety of cells. The object of this study was to demonstrate the presence of PAR-1 and PAR-2 in myenteric glia of the guinea pig, and to elucidate the cellular mechanisms that are triggered upon receptor activation. Thrombin and PAR-1 agonist peptide (PARP-1) activate PAR-1 with a maximum mean ± SEM change in intracellular calcium concentration with respect to basal level ($\Delta[Ca^{2+}]_i$) of 183 ± 18 nM and 169 ± 6 nM, respectively. Trypsin and PAR-2 agonist peptide (PARP-2) activate PAR-2 with a maximum $\Delta[Ca^{2+}]_i$ of 364 ± 28 nM and 239 ± 19 nM, respectively. Inhibition of phospholipase C by U73312 (1 μM) decreased the $\Delta[Ca^{2+}]_i$ due to PAR-1 activation from 167 ± 10 nM to 87 ± 6 nM. The PAR-2-mediated $\Delta[Ca^{2+}]_i$ decreased from 193 ± 10 nM to 124 ± 8 nM when phospholipase C activity was inhibited. Blockade of sphingosine kinase with dimethylsphingosine (1 μM) decreased the $\Delta[Ca^{2+}]_i$ due to PAR-2 activation from 149 ± 19 nM to 67 ± 1 nM, but did not influence the PAR-1-mediated $\Delta[Ca^{2+}]_i$. PAR-1 and PAR-2 were localized in myenteric glia by immunolabeling. Our results indicate that PAR-1 and PAR-2 are present in myenteric glia of the guinea pig, and their activation leads to increases in intracellular calcium via different signal transduction mechanisms that involve activation of phospholipase C and sphingosine kinase.

Keywords: calcium, enteric glia, protease-activated receptors, signal transduction.

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The enteric nervous system (ENS) regulates secretion, absorption, motility and vascular reactivity of the gastrointestinal tract. The ENS diverges functionally and structurally from other divisions of the PNS, maintaining integrated function in the absence of input from the CNS, and containing non-neuronal cell populations that resemble CNS astrocytes (Jessen and Mirsky 1983; Gershon and Sherman 1987). The two major cell types that constitute the ENS, neurons and glia, are present in a ratio of 1 : 2 (Gabella 1982). Recent evidence suggests that enteric glia are functionally active in information transfer in the ENS.

Protease-activated receptors (PARs) belong to a G-protein-coupled family of receptors. Most studies of PARs have been related to cellular responses to injury and inflammation (Vergnolle *et al*. 2001). PARs become activated through a unique mechanism that involves proteolytic cleavage of the extracellular N-terminal domain, exposing a new N-terminus that acts as a tethered ligand, binding and activating the receptor itself. To date, four different PARs have been cloned. PAR-1 and PAR-3 are activated by thrombin; PAR-2 is activated by trypsin and mast cell tryptase; and PAR-4 is activated by both trypsin and thrombin (Vu *et al*. 1991; Nystedt *et al*. 1995; Ishihara *et al*. 1997; Xu *et al*. 1998). PARs have been localized to a variety of tissues and cell types, including the CNS. In the ENS, the presence of PARs has been reported in submucosal and myenteric neurons (Corvera *et al*. 1999; Green *et al*. 2000). The presence of PARs in enteroglial cells has not yet been established.

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Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium concentration; CCE, capacitative calcium entry; $\Delta[Ca^{2+}]_i$, change in $[Ca^{2+}]_i$ with respect to basal level; DAPI, 4′,6-diamidino-2-phenylindole; DMS, dimethylsphingosine; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; IP3, inositol triphosphate; PAR, protease-activated receptor; PARP, protease-activated receptor peptide; PLC, phospholipase C; S1P, sphingosine-1-phosphate.

The object of this study was to demonstrate the presence of PAR-1 and PAR-2 in myenteric glial cells, and to elucidate the cellular mechanisms that are triggered upon receptor activation. We report that PAR-1 and PAR-2 are expressed and are functionally active in myenteric glial cells of the guinea pig. The current study demonstrates the existence of receptor-specific signal transduction mechanisms that involve activation of phospholipase C (PLC) and sphingosine kinase following PAR occupation in enteric glia.

Materials and methods

Materials

Trypsin was from Worthington Biochemicals (Lakewood, NJ, USA). Dimethylsphingosine (DMS), penicillin–streptomycin solution, tetrodotoxin (TTX), thrombin and collagenase type V were obtained from Sigma (St Louis, MO, USA). U-73343 and U-73122 were purchased from RBI (Natick, MA, USA). Fura-2 acetoxymethyl ester and fura-2 free acid were from Molecular Probes (Eugene, OR, USA). Recombinant human tryptase was from Promega (Madison, WI, USA). PAR peptide (PARP) receptor antagonists (PARP-1: TFLLR-NH2; PARP-2: tc-LIGRO-NH2) were a kind gift from Dr Nigel Bunnet (Department of Surgery and Physiology, University of California, San Francisco, CA, USA). The primary antibodies for PAR-1, PAR-2, c-*fos* and glial fibrillary acidic protein (GFAP) were from Santa Cruz (La Joya, CA, USA). Hank's balanced salt solution, M199 culture media and L-glutamine were from GibcoBRL (Grand Island, NJ, USA).

Cell culture

Primary cultures of guinea pig myenteric plexus were obtained as described previously (Kimball and Mulholland 1996). Briefly, tenia coli from 1-day-old male Duncan–Hartley guinea pigs (Simonsen, Gilroy, CA, USA) were removed and placed in collagenase for 18–20 h at 4°C, followed by 30 min incubation at 37°C. The layers of longitudinal and circular muscle were separated from the myenteric plexus using a dissecting microscope. The plexus was treated with a trypsin–EDTA solution for 30 min at 37°C to allow enzymatic digestion of the tissue. After a brief centrifugation (2000 *g* for 5 min), the cell pellet was mechanically disaggregated, and then plated in collagen-coated glass coverslips. The cultures were maintained in M199 complete medium supplemented with 5% BD™ Nu-Serum, and 0.001% trypsin inhibitor (type I-S from soybean). For the first 48 h, penicillin–streptomycin solution was added to the cultures at 2% dilution. The cultures were incubated at 37°C and 5% CO_2 for 6–8 days. Under a phase-contrast microscope, neurons can be distinguished as presenting with phase bright and compact bodies with very few ramifications, relative to a larger cytoplasm and denser nucleus characteristic of glial cells. Under these conditions, cultures contained approximately 90% glial cells, as additionally confirmed by GFAP immunostaining.

Calcium imaging

Myenteric plexus cultures were incubated in fresh, warm medium containing the fluorescent, cell permeable calcium dye fura-2 acetoxymethyl ester (final concentration 2 μM) for 45 min at 37°C. Cultures were washed with control buffer (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_3, 0.8 mM $MgSO_4$, pH 7.4) and were then placed in a superfusion chamber. During all experiments, the superfusion conditions were 1 mL/min at 37°C. In experiments in which calcium-free solutions were used, $CaCl_2$ was removed from the control buffer, and 1 mM EGTA was added. To determine intracellular calcium concentration ($[Ca^{2+}]_i$), a Zeiss (Thornwood, NY, USA) Axiovert inverted microscope and Attofluor digital imaging system (Rockville, MD, USA) were used. The intracellular calcium concentration was obtained from the ratio of fluorescence intensity of fura-2 at 334 and 380 nm, with an emission wavelength of 540 nm. Calibration of the system was conducted with the following two-point standardization equation using fura-2 free acid:

$$[\mathrm{Ca}] = K_\mathrm{d}[(R - R_\mathrm{Lo})(R_\mathrm{Hi} - R)]B,$$

where K_d is the dissociation constant of the Ca–fura-2 complex (225 nM), R is F_{334}/F_{380} (F, fluorescence intensity), R_{Lo} is the ratio at [Ca] = 0 (EGTA 1 mM), R_{Hi} is the ratio at high [Ca] (1 mM $CaCl_2$), and B corresponds to F_{380}(zero [Ca])/F_{380}(saturating [Ca]). Frames were not averaged, and a ratio pair was taken every 2 s. To assure cell viability, cellular responses to ATP were tested, and only cells that showed increased intracellular calcium after the treatment were analyzed.

Immunofluorescence

Primary glial cultures grown on glass coverslips were fixed with 4% paraformaldehyde for 30 min at 4°C, and then permeabilized with 0.1% saponin in phosphate-buffered saline for 20 min at room temperature (22°C). To block non-specific binding, cultures were treated with 10% serum for 30 min at room temperature. Fixed glial cells were incubated with antibodies against PAR-1, PAR-2, c-*fos* or the glial marker GFAP for 1 h at room temperature, followed by incubation with a biotinylated secondary antibody and streptavidin–FITC (for PAR-1, PAR-2 and c-*fos*) or streptavidin–Alexa Fluor 594 (Molecular Probes) (for GFAP) for 40 min at room temperature. Cultures were visualized using a LSM 510 confocal laser scanning microscope (Zeiss).

Data analysis

Each experiment was repeated at least three times with different cell preparations, and only one microscope field per coverslip was studied. For calcium imaging experiments, each microscope field contained at least 50 cells. Only cells with a basal calcium concentration of < 100 nM were considered for the data analysis. Increase in intracellular calcium concentration was obtained by subtracting basal calcium levels from the maximum calcium concentration achieved after the treatment. Only cells that demonstrated a minimum 50 nM calcium increase were considered responsive. Peak intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) was measured as the highest Ca^{2+} concentration achieved during exposure to the agonist. No differences in the results obtained were observed when neuronal activity was blocked by incubation with 1 μM tetrodotoxin, and therefore the possibility of neuronally mediated effects on glia was discarded. Tracings shown are representative of experimental observations. Results are presented as mean ± SEM. Data were analyzed by Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

PAR-1 and PAR-2 activation increases $[Ca^{2+}]_i$

To functionally characterize the presence of PARs, we examined the effects of PAR-1 and PAR-2 activation on the intracellular calcium levels in enteric glial cells. To determine working concentrations, dose–response curves were generated using both the physiological agonist (thrombin or trypsin) and the corresponding specific synthetic peptide (PARP-1 or PARP-2), and the increase in intracellular calcium was quantified with respect to basal levels ($\Delta[Ca]_i$).

Thrombin in a range from 7.3 nM to 7.3 μM (0.001–1.0 U/mL) produced dose-dependent increments in $[Ca^{2+}]_i$ (Fig. 1). $[Ca^{2+}]_i$ increased rapidly on exposure to thrombin, with calcium levels returning gradually to baseline upon withdrawal of agonist. Thrombin (7.3 nM) elicited a threshold increment in $[Ca^{2+}]_i$. A maximum increment in $[Ca^{2+}]_i$ of 183 ± 18 nM was elicited in 61 ± 17% of glial cells at 7.3 μM thrombin. Approximately 60% of cells were responsive to thrombin (Fig. 1c).

Exposure of cultured enteric glial cells to PARP-1 also caused dose-dependent increases in $[Ca^{2+}]_i$ over a concentration range from 10 nM to 2 μM (Fig. 2). Threshold responses were noted at 10 nM PARP-1. PARP-1 (1 μM) caused increases in $[Ca^{2+}]_i$ of 169 ± 6 nM relative to basal levels, with a maximum of 70 ± 11% of cells responding.

Exposure of glial cells to trypsin caused an increase in $[Ca^{2+}]_i$, with a maximum $[Ca^{2+}]_i$ of 364 ± 28 nM at 1 μM concentration (Fig. 3). At 1 μM trypsin, 90 ± 8% of cells responded with increases in $[Ca^{2+}]_i$.

Similar to trypsin, exposure of enteric glia to PARP-2 resulted in a maximum of 89 ± 7% of cells demonstrating $[Ca^{2+}]_i$ increments (Fig. 4). The time course of $[Ca^{2+}]_i$ transients was similar to that observed for thrombin. PARP-2 increased intracellular calcium by 239 ± 19 nM over baseline at a concentration of 1 μM. Enteric glial cells also responded to tryptase, a PAR-2 agonist, with increments in $[Ca^{2+}]_i$. For 30 nM tryptase, 79 ± 3% of cells responded; the $\Delta[Ca^{2+}]_i$ was 118 ± 17 nM.

Based on dose–response studies, concentrations of agonists that exerted a submaximal calcium increase were chosen for subsequent experiments. The concentrations of thrombin and PARP-1 used were 0.73 μM (0.1 U/mL) and 100 nM, respectively. To study PAR-2 activation, trypsin and PARP-2 concentrations of 100 nM were used.

Receptor desensitization

To characterize responsiveness after trypsin and thrombin exposure, glial cells were repeatedly exposed to agonist peptides. As shown in Fig. 5, pretreatment of cells with PARP-1 (100 nM) decreased, but did not eliminate, the response to thrombin. In contrast, exposure of enteric glial cells to thrombin eliminated subsequent responsiveness to a second thrombin application.

Similarly, exposure of glial cells to trypsin (100 nM) eliminated subsequent increases in $[Ca^{2+}]_i$ upon secondary exposure to trypsin (Fig. 6). Pretreatment with PARP-2

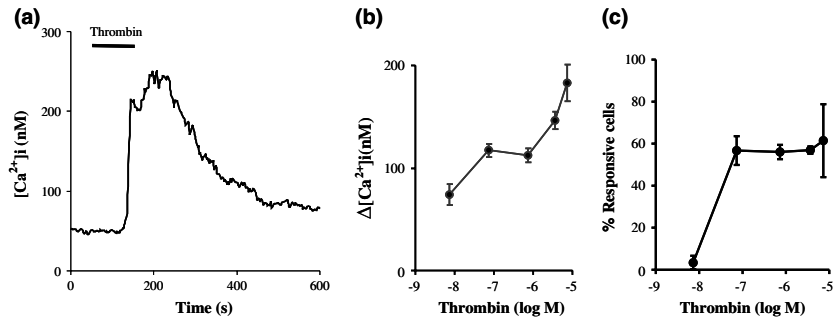

Fig. 1 (a) Effect of thrombin (0.73 μM) on $[Ca^{2+}]_i$ in myenteric glia. Tracing representative of 124 cells. (b) Effect of increasing concentrations of thrombin on the $\Delta[Ca^{2+}]_i$ in myenteric glia. Values are mean ± SEM. (c) Dose-dependent increases in percentage of cells responding to the treatment. Values are mean ± SEM.

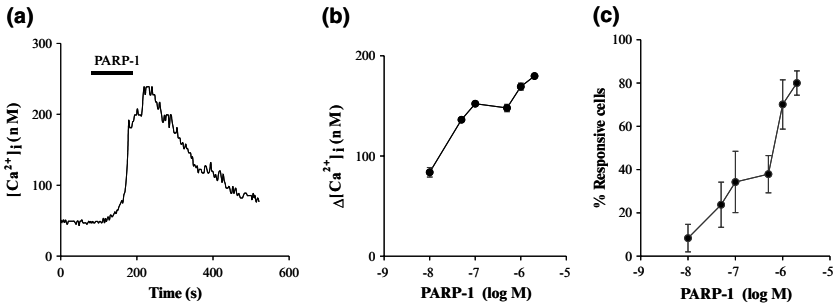

Fig. 2 (a) Effect of PARP-1 (100 nM) on $[Ca^{2+}]_i$ in myenteric glia. Tracing representative of 152 cells. (b) Effect of increasing concentrations of PARP-1 on $\Delta[Ca^{2+}]_i$ in myenteric glia. Values are mean ± SEM. (c) Dose-dependent increases in percentage of cells responding to the treatment. Values are mean ± SEM.

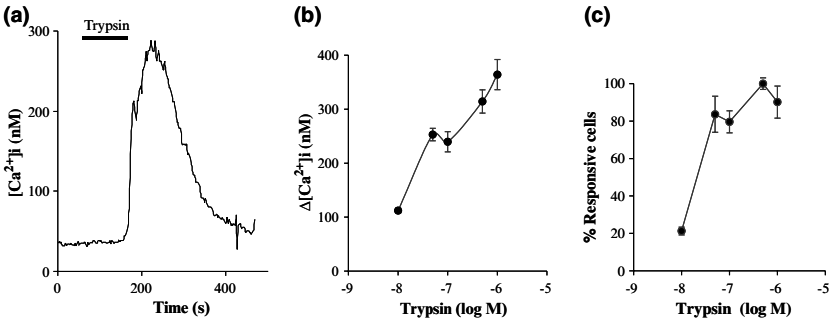

Fig. 3 (a) Effect of trypsin (100 nM) on $[Ca^{2+}]_i$ in myenteric glia. Tracing representative of 126 cells. (b) Effect of increasing concentrations of trypsin on $\Delta[Ca^{2+}]_i$ in myenteric glia. Values are mean ± SEM. (c) Dose-dependent increases in percentage of cells responding to the treatment. Values are mean ± SEM.

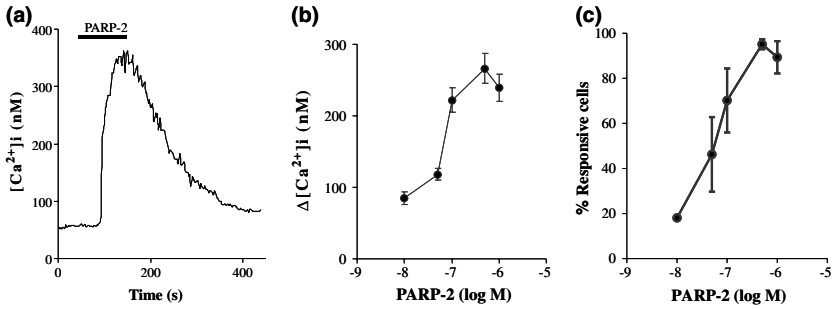

Fig. 4 (a) Effect of PARP-2 (100 nM) on $[Ca^{2+}]_i$ in myenteric glia. Tracing representative of 111 cells. (b) Effect of increasing concentration of PARP-2 on $\Delta[Ca^{2+}]_i$ in myenteric glia. Values are mean ± SEM. (c) Dose-dependent increases in percentage of cells responding to the treatment. Values are mean ± SEM.

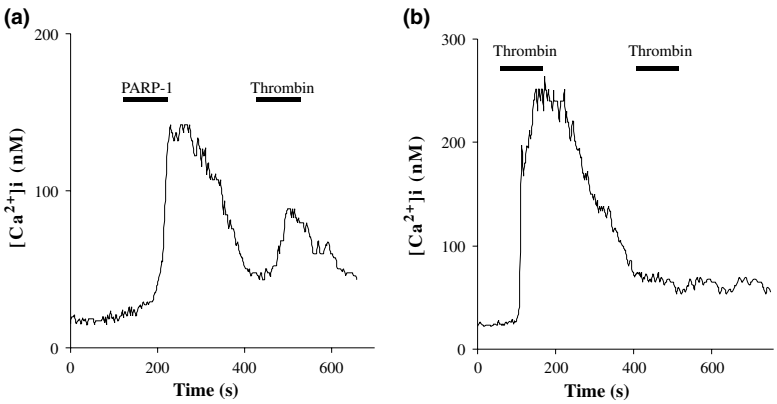

Fig. 5 PARP-1 receptor desensitization caused by initial activation with PARP-1 (100 nM) (a) or thrombin (0.73 μM) (b), and subsequent exposure to thrombin (0.73 μM). Tracings were representative of 98 cells.

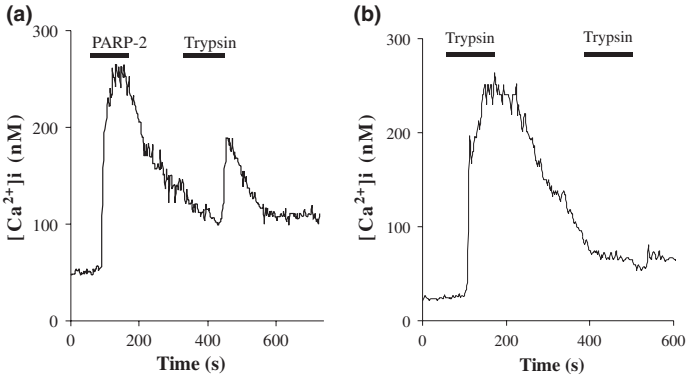

Fig. 6 PARP-2 receptor desensitization caused by initial activation with PARP-2 (100 nM) (a) or trypsin (100 nM) (b), followed by exposure to trypsin (100 nM). Tracings are representative of 104 cells.

(100 nM) decreased, but did not eliminate, the $[Ca^{2+}]_i$ response to secondary trypsin exposure.

To eliminate the possibility of decreased responsiveness due to depletion of intracellular calcium stores, cells were exposed to ATP 100 μM at the end of each experiment (data not shown). All cells responded with $\Delta[Ca]_i$ characteristic of this agonist (Kimball and Mulholland 1996). These results are in accordance with previous observations in which primary exposure to the enzyme (thrombin or trypsin) renders the receptor non-responsive to successive agonist exposure.

PAR receptor activation induced capacitative calcium entry

Capacitative calcium entry (CCE) refers to a process of extracellular calcium influx across the cellular membrane induced by intracellular calcium depletion. CCE is postulated to replenish intracellular stores. To investigate the occurence of CCE, a protocol was followed in which the cells were exposed to agonist in calcium-free buffer, resulting in increased $[Ca^{2+}]_i$ secondary to calcium release from intracellular compartments. When $[Ca^{2+}]_i$ returned to baseline, calcium was reintroduced in the perfusion buffer, allowing for observation of subsequent CCE. Cells were exposed to PARP-1 or PARP-2 in calcium-free conditions. As shown in Fig. 7, PARP-1 and PARP-2 increased intracellular calcium levels in absence of extracellular calcium, consistent with release from internal stores. When calcium was returned to the superfusate, subsequent CCE was observed as a secondary increase in $[Ca^{2+}]_i$.

PAR signal transduction

To determine the possible mechanisms through which PAR-1 or PAR-2 activation increase intracellular calcium, the effects of PLC and sphingosine kinase inhibition were investigated. To inhibit PLC activity, the aminosteroid U73122 (1 μM) was added to the perfusion buffer before addition of the corresponding agonist peptide. This compound has been shown to inhibit PLC activity in a variety of cell types (Jin *et al.* 1994). As a negative control, U73343 (1 μM), an inactive analog of U73122, was used (data not shown). U73343 was without effect on $[Ca^{2+}]_i$.

Dimethylsphingosine (DMS) was used to inhibit sphingosine kinase activity (Edsall *et al.* 1998). To demonstrate the specificity of DMS, experiments were performed in which the effects of DMS on ATP-mediated increases in $[Ca^{2+}]_i$ were examined. It has been documented previously that ATP-mediated increases in $[Ca^{2+}]_i$ in enteric glia are solely dependent on PLC activation (Kimball and Mulholland 1996), and therefore would be expected to be independent of sphingosine kinase activity. Exposure of myenteric glia to DMS 1 μM for 5 min did not affect baseline $[Ca^{2+}]_i$. In addition, pretreatment with DMS did not inhibit increases in $[Ca^{2+}]_i$ due to exposure to ATP.

PARP-1-mediated increases in $[Ca^{2+}]_i$ were significantly attenuated by pretreatment with U73122 (87 ± 6 nM vs. 167 ± 10 nM for controls) (Fig. 8a). The percentage of cells responsive to the peptide was also decreased by inhibition of PLC (18 ± 7% compared with 56 ± 2%) (Fig. 8b). Exposure of glial cells to 1 μM DMS did not alter $[Ca^{2+}]_i$ increments after PAR-1 exposure.

PARP-2-induced $[Ca^{2+}]_i$ increments were decreased from 193 ± 10 to 124 ± 8 nM when glial cells were exposed to U73122 before receptor activation (Fig. 9a). Pre-exposure of enteroglial cells to 1 μM DMS significantly inhibited responsiveness to PARP-2 ($\Delta[Ca^{2+}]_i$ 77 ± 13 mM; 11 ± 1% of cells responding; Fig. 9b). Inhibitory effects of DMS were dose dependent (data not shown).

Detection of PAR by immunofluorescence

To localize PAR-1 and PAR-2 in our primary cultures, cells were incubated with the relevant primary and secondary antibodies and visualized by confocal laser scanning microscopy (Figs 10a and 10b, respectively). To verify the glial nature of the cultures, cells obtained identically from the same cultures were stained for the glial marker GFAP. As seen in Fig. 10c and previously reported (Kimball and Mulholland 1996), the vast majority of the cells in the culture were glial cells. As a negative control, primary antibodies were replaced with antibody solvent (2% serum in

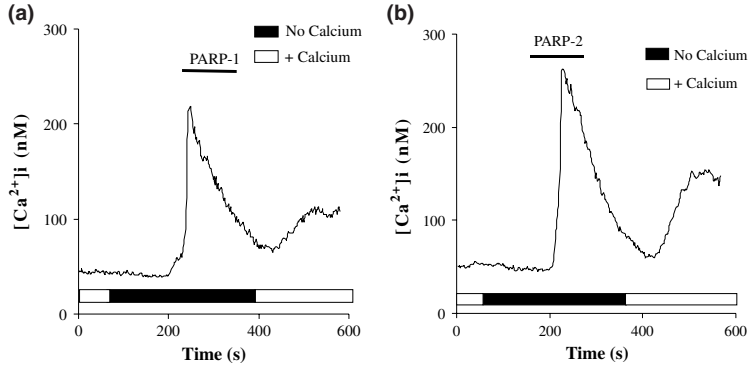

Fig. 7 PARP-1 (a) and PARP-2 (b) caused CCE in enteric glia. Application of the corresponding agonist peptide (100 nM) in calcium-free buffer induces release of $[Ca^{2+}]_i$ and influx from the extracellular space once calcium is returned to the perfusion medium.

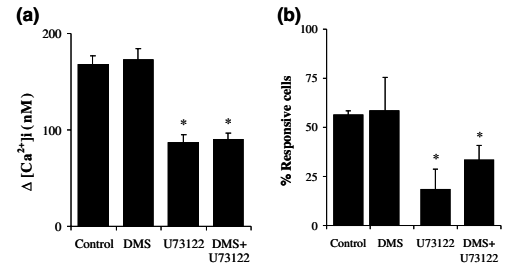

Fig. 8 Signal transduction pathways involved in PARP-1-induced $\Delta[Ca]_i$ in myenteric glia. (a) Glial cells were exposed to U73122 (1 μM), DMS (1 μM), or a combination of DMS and U73122, and changes in $[Ca^{2+}]_i$ in response to PARP-1 (100 nM) were measured. (b) Percentage of glial cells that responded to PARP-1 in the presence of inhibitors. Values are mean ± SEM. $^*p < 0.05$ versus control group (Student's *t*-test).

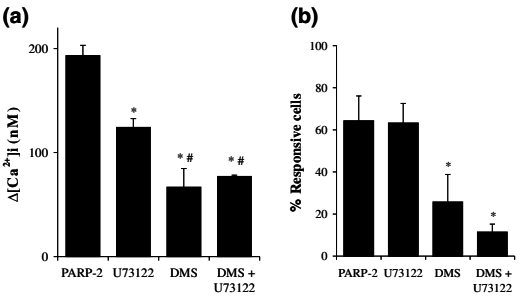

Fig. 9 PARP-2-induced $\Delta[Ca]_i$ in myenteric glia. Glial cells were exposed to the PLC inhibitor U73122 (1 μM), DMS (1 μM), or a combination of DMS and U73122, and changes in $[Ca^{2+}]_i$ (a) and percentage of responsive cells (b) in response to PARP-2 (100 nM) were measured. Responsiveness to ATP (100 μM) was not altered by DMS 15 μM. Values are mean ± SEM. $^*p < 0.05$ versus PARP-2, $\#p < 0.05$ versus U73122 alone (Student's *t*-test).

phosphate-buffered saline). No staining was observed in the control cultures (Fig. 10d).

Expression of c-*fos* in response to PARP-2

To determine if protease exposure increased c-*fos* expression in enteric glia, primary cultures were exposed to 1 mM PARP-2 for varying lengths of time. Significant and time-dependent increase in c-*fos* immunoreactivity were noted, reaching peak values with 30 min of incubation (Fig. 11).

Discussion

The present study demonstrates the presence of functionally active PAR-1 and PAR-2 in myenteric glial cells. Five distinct observations support this conclusion.

(i) Glial cells respond dose dependently with increments in $[Ca^{2+}]_i$ to both thrombin and trypsin.

(ii) Dose-dependent increases in $[Ca^{2+}]_i$ and in the percentage of cells responding were observed with exposure to synthetic PARPs.

(iii) Receptor densensitization typical of PARs was noted with repetitive exposure.

(iv) Enteroglial cells were immunocytochemically positive for PAR-1 and PAR-2.

(v) Exposure to PARP-2 induced significant and time-dependent increases in c-*fos* immunoreactivity in glial cells.

Enteric glial cells also responded to the PAR-2 agonist, tryptase.

Activation of glial PAR-1 or PAR-2 releases Ca^{2+} from intracellular stores, with subsequent CCE. The signaling pathway for PAR-1-induced Ca^{2+} release involves PLC. In contrast, in enteroglial cells, PAR-2-stimulated Ca^{2+} release depends upon both PLC and sphingosine kinase. These observations constitute the first report of PAR expression in enteric glial cells.

The presence of PARs has been demonstrated in a variety of cell systems. Most initial studies of PAR-1 focused on the role of thrombin in wound healing and vessel repair. PAR-1 has been demonstrated in platelets, endothelium, neutrophils and leukocytes, with functional activities linked to cellular aggregation and mucosal barrier function (Bizios *et al*. 1986; Malik *et al*. 1986; Andersen *et al*. 1999). Recently, PAR-1 expression has been reported in neurons of the ENS (Corvera *et al*. 1999). PAR-2 is also widely expressed; it has been demonstrated in vascular tissue, the digestive system and epidermis (Al-Ani *et al*. 1995; Santulli *et al*. 1995; Derian and Eckardt 1997; D'Andrea *et al*. 1998; Corvera *et al*. 1999). Within the gastrointestinal tract, PAR-2 has been reported in enterocytes, smooth muscle cells and myenteric neurons. Our observations indicate that both PAR-1 and PAR-2 are present in myenteric glia.

Activation of both PAR-1 and PAR-2 has been associated with increases in $[Ca^{2+}]_i$ in different cell types, through G-protein-mediated activation of different calcium signaling cascades (Wang *et al*. 1996; Brass *et al*. 1993; Corvera *et al*. 1999). The intracellular origin of the initial calcium increase has been demonstrated in epithelial cell and myenteric neurons, where activation of these receptors induces increased $[Ca^{2+}]_i$ in absence of extracellular calcium (Bohm *et al*. 1996; Corvera *et al*. 1999).

The specificity of agonist peptides PARP-1 and PARP-2 has been demonstrated previously in different models, mimicking thrombin and trypsin receptor action, respectively (Chao *et al*. 1992; Hollenberg *et al*. 1997; Vergnolle *et al*. 1998; Corvera *et al*. 1999). PARs demonstrate a unique mechanism of receptor activation through cleavage at the N-terminus. This receptor cleavage is believed to contribute to receptor desensitization, making the modified receptor molecule non-responsive to a second exposure to protease.

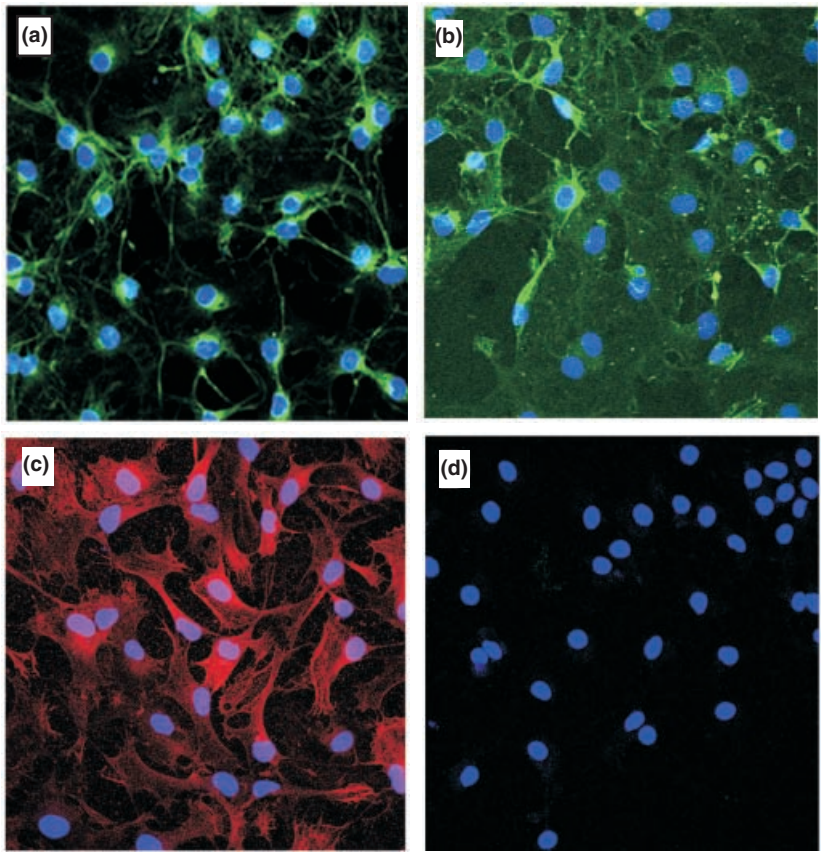

Fig. 10 PAR immunolocalization in glial primary cultures. PAR-1 (a) and PAR-2 (b) were detected by immunofluorescence in glial cultures. 4′,6-Diamidino-2-phenylindole (DAPI) was used as nuclear counterstain. Additionally, identical cultures were characterized using GFAP as a glial marker (c). Control cultures lacking primary antibody incubation showed no significant staining (d).

This mechanism of desensitization has been noted in previous reports in which primary exposure to trypsin or thrombin abolished subsequent responsiveness to the proteases. In contrast, primary exposure to PARP-1 or PARP-2 results in diminished, but still present, responsiveness to repeated exposure to the synthetic peptides (Hammes and Coughlin 1999; Corvera *et al*. 1999).

Detailed study of PAR-1 and PAR-2 signaling in enteroglial cells has indicated that receptor activation leads to rapid release of calcium from intracellular stores, followed by secondary influx of calcium from extracellular sources. This secondary process has been termed CCE. CCE is typical for calcium signaling in a variety of non-excitable cells, and has been demonstrated previously in enteroglial cells (Zhang *et al.* 1997; Sarosi *et al*. 1998). CCE appears to modulate a wide range of biological pathways, such as proliferation, regulation of adenylate cyclase and hormonal regulation. It may serve to replenish internal stores previously depleted by calcium-mobilizing agonists such as inositol trisphosphate (IP3) (Berridge 1995).

The current investigation indicates that the cellular intermediates that participate in PAR-1 and PAR-2 calcium signaling in enteroglia differ. PAR-1-induced Ca^{2+} transients were significantly diminished by PLC inhibition, but were unaffected by DMS, an inhibitor of sphingosine kinase. PAR-1 has been shown previously to couple to PLC, stimulating IP3 production, and release of calcium from IP3-sensitive intracellular stores (Babich *et al*. 1990; Garcia 1992; Hung *et al*. 1992).

In contrast, calcium signaling in response to PAR-2 activation was decreased by inhibition of both PLC and sphingosine kinase. There are previous reports that suggest that PAR-2 signaling occurs through PLC activation in enterocytes and smooth muscle cells, among others (Kong *et al*. 1997; Berger *et al*. 2001). Sphingosine kinase catalyzes the intracellular phosphorylation of sphingosine to produce sphingosine-1-phosphate (S1P). Production of S1P and subsequent calcium release has been observed after activation of several membrane receptors (Choi *et al*. 1996). These effects are blocked by sphingosine kinase inhibitors, demonstrating the involvement of S1P production in the reported calcium transient. DMS is a newly described sphingosine kinase inhibitor (Edsall *et al*. 1998) that shows higher specificity than previously used inhibitory compounds. The current investigation is the first report of the involvement of S1P in PAR-2 signaling.

S1P is a versatile molecule that can act extracellularly by binding to G-protein-coupled membrane receptors, as well as intracellularly, as a second messenger stimulating calcium release from the endoplasmic reticulum (Ghosh *et al*. 1994;

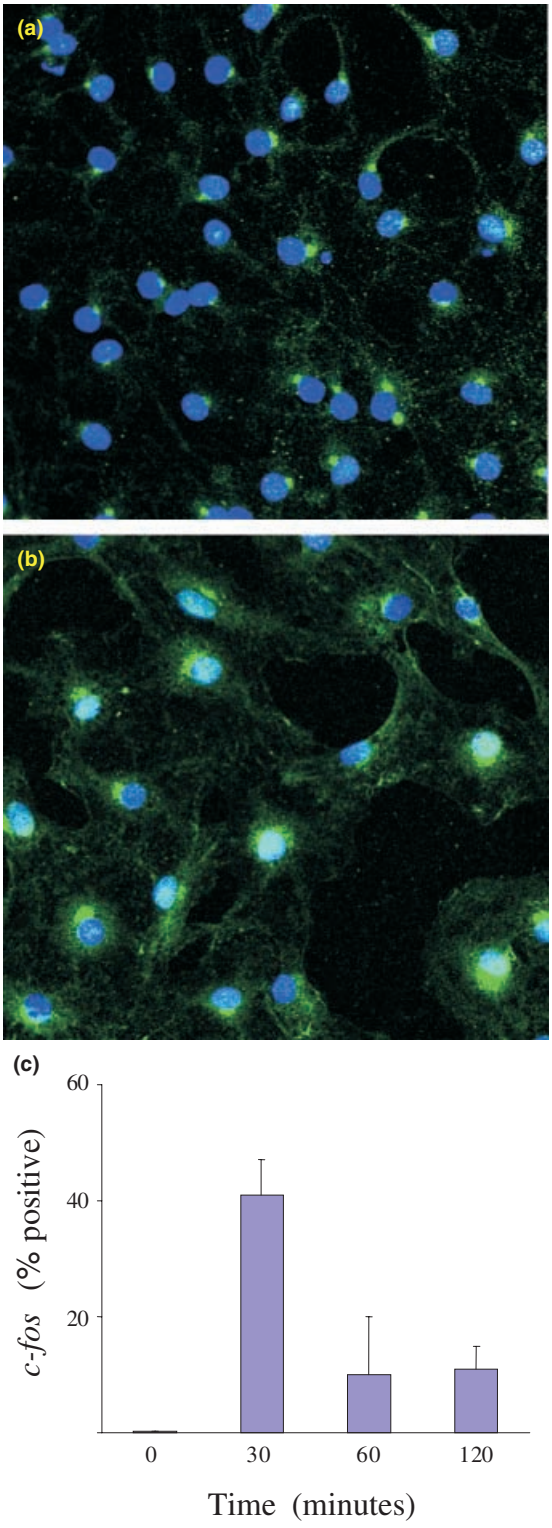

Fig. 11 Immunoreactive c-*fos* in primary glial cultures. Glial cells were exposed to PARP-2 (1 μM) for 30, 60 or 120 min. DAPI was used as nuclear counterstain. Basal expression (a); time interval 30 min (b). (c) Mean ± SEM percentage of cells that stained positively. Time intervals of 30, 60 and 120 min were significantly different from basal.

Postma *et al*. 1996). The effects of S1P in other systems have been related to control of cell proliferation and migration (Zhang *et al*. 1990). It has recently been reported that intracellular S1P is involved in thrombin-stimulated histamine release from mast cells (Gordon *et al*. 2000). The mechanisms governing PAR signaling may vary, depending upon the system studied (Grand *et al*. 1996).

The physiologic role of PAR signaling in enteric glial cells is unknown. The recent finding of a thrombin-generating system in the brain and the presence of PAR-1 in neurons and glia suggest that PAR-1 may have functions in addition to revascularization (Ubl *et al*. 1998). PAR-2 activation has been related to release of proteinases in inflammatory and allergic conditions, vasodilatation of cerebral arteries, release of prostaglandins, and regulation of cytokine production in enterocytes (Wakita *et al*. 1997; Sobey *et al*. 1999). In the gastrointestinal tract, PAR-2 may be activated by trypsin or tryptase, presumably released by mast cells within the gut wall (Corvera *et al*. 1999). The widespread localization of PAR-1 and PAR-2 within the digestive tract makes them an attractive target for functional investigation.

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