

# Matrix metalloproteinase-1 activates a pertussis toxin-sensitive signaling pathway that stimulates the release of matrix metalloproteinase-9

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## Abstract

The matrix metalloproteinases (MMPs) are a family of structurally related metalloendopeptidases so named due to their propensity to target extracellular matrix (ECM) proteins. Accumulating evidence, however, suggests that these proteases cleave numerous non-ECM substrates including enzymes and cell surface receptors. MMPs may also bind to cell surface receptors, though such binding has typically been thought to mediate internalization and degradation of the bound protease. More recently, it has been shown that MMP-1 coimmunoprecipitates with the  $\alpha_2\beta_1$  integrin, a receptor for collagen. This association may serve to localize the enzymatic activity of MMP-1 so that collagen is cleaved and cell migration is facilitated. In other studies, however, it has been shown that integrin engagement may be linked to the activation of signaling cascades including those mediated by  $G_{i\alpha}$  containing heterotrimers. As an example,  $\alpha_2\beta_1$  can form a complex

with CD47 that may associate with  $G_{i\alpha}$ . In the present study we have therefore investigated the possibility that MMP-1 may affect intracellular changes that are linked to the activation of a  $G_i$  protein-coupled receptor. We show that treatment of neural cells with MMP-1 is followed by a rapid reduction in cytosolic levels of cAMP. Moreover, MMP-1 potentiates proteinase activated receptor-1 (PAR-1) agonist-linked increases in intracellular calcium, an effect which is often observed when an agonist of a  $G_i$  protein-coupled receptor is administered in association with an agonist of a  $G_q$  coupled receptor. In addition, MMP-1 stimulates pertussis toxin sensitive release of MMP-9 both from cultured neural cells and monocyte/macrophages. Together, these results suggest that MMP-1 signals through a pertussis toxin-sensitive  $G$  protein-coupled receptor.

**Keywords:** CD47,  $G$  protein-coupled receptor, integrins, MMP-1, MMP-9.

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Matrix metalloproteinases (MMPs) belong to a family of structurally similar, zinc-dependent endopeptidases, which degrade specific components of the extracellular matrix (ECM) including collagen, laminin, and entactin (Yong *et al.* 1998). MMPs play an important role in diverse processes including the inflammatory response. The release of MMPs from numerous cell types is typically increased by pro-inflammatory stimuli such as interleukin (IL)-1 $\beta$ , and these enzymes may contribute to both inflammatory cell migration and effector functions (Woessner and Nagase 2000). With respect to the central nervous system (CNS) in particular, MMPs released by infiltrating leukocytes and/or resident cells may contribute both to pathological events such as destruction of the blood–brain barrier (Mun-Bryce and

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**Abbreviations used:** BBB, blood–brain barrier; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; IBMX, 3-isobutyl-1-methyl xanthine; LRP, low density lipoprotein-receptor related protein; MP, metalloproteinase; MMP, matrix metalloproteinase; PAR-1, proteinase-activated receptor-1; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PKA, protein kinase A; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; PTX, pertussis toxin; SDS, sodium dodecyl sulfate; TGF- $\beta$ , tumor growth factor- $\beta$ .

Rosenberg 1998), and physiological events such as neurite outgrowth (Szkларczyk *et al.* 2002).

While MMPs were named for their ability to target matrix proteins, it is becoming increasingly evident that these proteases target numerous non-matrix substrates including cytokines such as tumor growth factor- $\beta$  (TGF- $\beta$ ), cell surface proteins such as the low density lipoprotein-receptor related protein (LRP) and FGF-R1, and chemokines such as MCP-3 (Quinn *et al.* 1997; McCawley and Matrisian 2001). Of further interest, MMPs may cleave cell surface Fas ligand (Powell *et al.* 1999), and it has also been shown that an MMP inhibitor blocks cleavage of the mannose receptor (Martinez-Pomares *et al.* 1998), which may be involved in antigen presentation by microglia. Previous studies have also shown that cleavage events mediated by MMPs may generate ligands which may in turn activate the EGF receptor (Prenzel *et al.* 1999).

In addition to their ability to cleave non-matrix substrates, MMPs have been shown to bind to specific cell surface receptors including LRP (Barmina *et al.* 1999; Yang *et al.* 2001) and CD44 (Yu and Stamenkovic 2000). While binding of an MMP to cell surface receptors may mediate internalization and degradation of the protease, or appropriate localization of enzymatic activity, binding might also lead to receptor activation. With respect to MMP-1 in particular, a recent study has shown that this protease will coimmunoprecipitate with the  $\alpha_2\beta_1$  integrin (Dumin *et al.* 2001). Select integrins, including  $\alpha_2\beta_1$ , form complexes with neighboring non-integrin cell surface proteins, and these integrin/integrin associated protein complexes may in turn be linked to  $G_i\alpha$  signaling (Brown and Frazier 2001). Examples of such signaling include that mediated by the urokinase receptor and  $\alpha_3\beta_1$  (Wei *et al.* 2001), as well as the thrombospondin receptor (CD47) and integrins including  $\alpha_2\beta_1$  (Brown and Frazier 2001). It has also been shown that integrin/CD47/ $G_i\alpha$  complexes are detergent stable and can be recovered by immunoprecipitation (Frazier *et al.* 1999). Of further interest, it has been shown that the activation of CD47 and/or its integrin partner may be linked to processes including memory formation, cell spreading, cell migration, and cell death (Chang *et al.* 1999; Reinhold *et al.* 1999; Wang *et al.* 1999; Pettersen 2000; Liu *et al.* 2001).

In the present study, we investigated the possibility that MMP-1 stimulates signaling events mediated by the activation of a  $G_i\alpha$  protein-coupled receptor (GiPCR). We therefore examined the effects of MMP-1 on intracellular cAMP and calcium. Moreover, we determined whether MMP-1 stimulates the release of MMP-9 through a pertussis toxin sensitive mechanism. The release of MMP-9 is linked to the activation of  $G_i\alpha$  protein-coupled receptors in other systems (Vliagoftis *et al.* 2000), and may also contribute to changes in cell or cell process migratory ability. If such migratory ability is significantly affected, then signaling by MMP-1 could have important consequences in plasticity and immunopathology of the CNS.

## Experimental procedures

### Cells and cell culture

Neuronal cultures were prepared from 19-day-old embryonic Sprague–Dawley rats as previously described (Haughey *et al.* 1999). Briefly, tissue was dissociated by gentle trituration in a calcium-free Hank's balanced salt solution and was centrifuged at 1000 g. Cells were resuspended in minimal essential media containing 10% fetal bovine serum (FBS) and 1% antibiotic solution ( $10^4$  U of penicillin G/mL, 10 mg streptomycin/mL and 25  $\mu$ g amphotericin B/mL; Sigma, St Louis, MO, USA). Neurons were then plated at a density of 100 000 cells/mL on poly-D-lysine coated glass coverslips. Three hours after plating the media was replaced with serum-free Neurobasal media containing 1 X B-27 supplement (Gibco, Rockville, MD, USA). Cultures were used between 10 and 14 days *in vitro*. Immunofluorescent staining for MAP-2 (neurons) and glial fibrillary acidic protein (GFAP) (astrocytes) showed that cultures were > 98% neurons; the remainder of cells were predominantly astrocytes.

Slice cultures from rat spinal cord were prepared from lumbar spinal cords of 8-day-old rat pups as previously described (Drachman and Rothstein 2000). Three hundred and fifty micrometer slices were prepared with a McIlwain tissue chopper. Slices were cultured in millicell CM semipermeable culture inserts at a density of five slices/well. Under these conditions, a stable population of neurons persisted in excess of three months. Culture medium (serum-free neurobasal media containing 1 X B-27 supplement from Gibco) was changed twice weekly. Of note, culture inserts were not coated with extracellular matrix proteins.

Human fetal neuronal cultures were prepared from human fetal brain specimens of 12–17 weeks gestation obtained in accordance with NIH guidelines, and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) with 10% heat-inactivated FBS (Sigma) and 1% antibiotic–antimycotic solution (penicillin G sodium, streptomycin sulfate, and amphotericin B in 0.85% saline; Gibco BRL). Culture methods and characterization were performed as previously described (Vos *et al.* 2000a). These cells were cultured at a density of approximately  $10^5$  cells/mL.

Cultured human monocytes were obtained through adherence-purification following isolation of peripheral blood mononuclear cells (PBMCs) by Ficoll gradient as previously described (Vos *et al.* 2000b), and cultured at approximately  $10^6$  cells/mL. Because serum contains MMPs, prior to treatment of neuronal or monocyte cultures, medium was replaced with a comparable serum-free preparation.

### Purified MMPs and inhibitors

Purified MMPs were purchased from R & D systems (Minneapolis, MN, USA), Oncogene Research Products (San Diego, CA, USA) and Chemicon International (Temecula, CA, USA). MMPs were aliquoted and stored at  $-70^\circ\text{C}$  upon their arrival. MMP-1 from R & D Systems (Minneapolis, MN, USA) was human recombinant, expressed in a mouse myeloma cell line, MMP-1 from Oncogene Research Products (San Diego, CA, USA) was purified from human dermal fibroblasts by affinity chromatography, ion exchange chromatography and gel filtration, and MMP-1 from Chemicon (Temecula, CA, USA) was purified from transfected P2AH2A cells also by ion exchange and affinity chromatography. Except where indicated, MMP-1 from R & D Systems was used in the experiments shown. MMP-1 preparations from R & D Systems and Chemicon

contained both pro- and activated MMP-1 while that from Oncogene contained only the pro-enzyme. MMP-1 preparations used in experiments were tested by western blot. Occasional preparations showed degradation products and were not used. Recombinant active MMP-7, which was used as a control in experiments which measured intracellular calcium, was purchased from Chemicon. Note, 50–100 ng/mL MMP-1 was used in experiments since previous studies showed that IL-1 $\beta$  stimulated astrocyte supernatants contained amounts in this range (Vos *et al.* 2000a).

GM-6001 was obtained from Chemicon, pertussis toxin (PTX) was from Calbiochem (San Diego, CA, USA), and anti- $\beta$ 1 integrin chain antibody was purchased from Cymbus Biotechnology Limited (CBL 481, Chandlers Ford, UK). Activity of GM-6001 against MMP-1 was confirmed using a casein substrate zymogram (Bio-Rad Laboratories, Hercules, CA, USA). Toxicity controls included trypan blue staining.

#### Measurements of cAMP

To raise cAMP levels to detectable limits, phosphodiesterase was inhibited by the addition of 3-isobutyl-1-methyl xanthine (IBMX; 50  $\mu$ M) to cultures for 30 min. MMP-1 (100 ng/mL) was added to cultures for 5, 10, 15, 20, 25 and 30 min before lysis of cells. Forskolin (10  $\mu$ M) or pertussis toxin (100 ng/mL) was added to cultures for 30 min prior to MMP-1. Direct cAMP measurements were carried out using the non-acetylated version of a commercial assay kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's protocol. Cyclic AMP concentrations were standardized to protein content using the Pierce BCA kit.

#### Calcium studies

Calcium influx was assessed in various conditions as follows. Cytosolic calcium concentrations ( $[Ca^{2+}]_c$ ) were determined using the  $Ca^{2+}$ -specific fluorescent probe Fura-2/AM. Cells were incubated 25 min at 37°C in Locke's buffer (154 mM NaCl, 3.6 mM NaHCO<sub>3</sub>, 5.6 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, 2.3 mM CaCl<sub>2</sub>, 10 mM glucose) with 2 mM Fura-2/AM; pH 7.4. Cells were washed with Locke's to remove extracellular Fura-2 and incubated 10 min at 37°C to allow for complete de-esterification of the probe. Fura-2 loaded cells were excited at 340 and 380 nm and emission recorded at 510 nm. Random fields were imaged using a 40x oil immersion objective and calcium levels were measured in all cells of the field. The  $R_{max}/R_{min}$  ratios were converted to nM  $[Ca^{2+}]_c$  with reference standards (Molecular Probes, Eugene, OR, USA) as described elsewhere (Haughey *et al.* 1999). During calcium imaging, MMP-1 or MMP-7 (100 ng/mL) was added to cultures for 2, 5, 15 and 30 min prior to the application of a peptide agonist for protease activated receptor 1 (TFLLRNPNDK-NH<sub>2</sub>) which had been prepared by solid phase synthesis and purified by RP-HPLC. Forskolin (10  $\mu$ M), IBMX (50  $\mu$ M) or pertussis toxin (100 ng/mL) was added to cultures 30 min prior to imaging.

#### Western blot for MMP-9

Western blot was performed for MMP-9 using 30  $\mu$ L of cell culture supernatant which was mixed with 2  $\times$  Laemmli sample buffer containing 5% 14.3 M  $\beta$ -mercaptoethanol. Samples were run on a 15% Tris-glycine polyacrylamide gel. Following protein transfer to a polyvinylidene difluoride (PVDF) membrane, the blot was probed with a polyclonal antibody to MMP-9 (AB19047, Chemicon). Immunoreactive bands were visualized by electrochemilumines-

cence (Amersham). Recombinant MMP-9 (Chemicon) was used as a positive control.

#### ELISA

ELISA for MMP-9 was performed using a commercially available kit (R & D Systems) in accordance with the manufacturer's instructions. This ELISA detects total (pro- and active) MMP-9.

#### Zymography

Samples (30  $\mu$ L supernatant) were mixed 1 : 2 with sample buffer [62.5 mM Tris-CL, pH 6.8, 4% sodium dodecyl sulfate (SDS), 25% glycerol, and 0.01% bromophenol blue], and run at 100 V on a 10% polyacrylamide (w/v), 0.1% SDS, 0.1% protein (gelatin) containing gel. The gel was then placed in 2.5% Triton X-100 to allow for renaturation of the embedded proteins. Subsequently, the gel was placed in buffer (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 5 mM CaCl<sub>2</sub>, and 0.02% Brij-35) at 37°C for 16 h to optimize metallo-proteinase activity. The gel was stained for 1 h in 40% methanol/10% acetic acid/0.5% (w/v) Coomassie brilliant blue G-250 and then destained in the same buffer without Coomassie brilliant blue. Proteinase activity was subsequently inferred by the presence of clear bands which appeared against a blue background. Molecular weights of the proteinases were determined by comparison to protein molecular weight standards (Bio-Rad).

#### Immunoabsorption

Immunoabsorption of MMP-1 was performed as previously described (Conant *et al.* 1996) using protein sepharose (Pharmacia) and an antibody to human MMP-1 (Chemicon, AB8105). This antibody reacts with both pro- and active MMP-1 as determined by western blot.

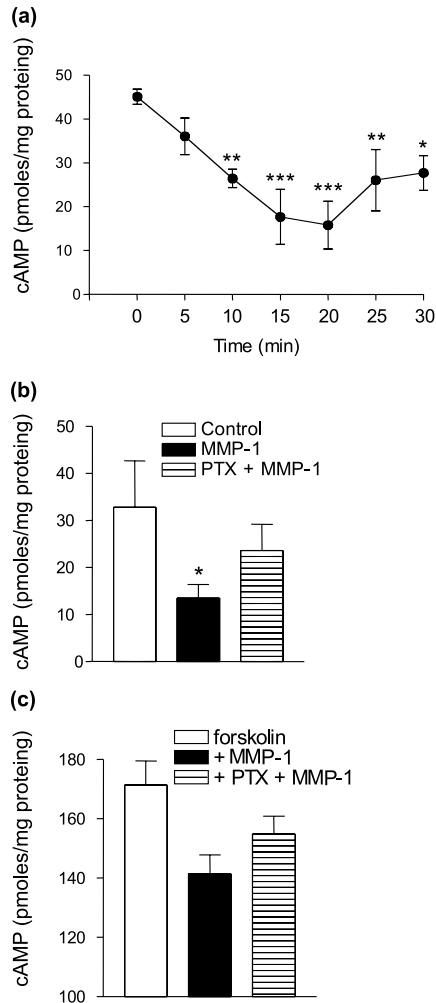
#### Immunoprecipitates

Following incubation with MMP-1 (R & D Systems) for 1 h in serum free medium except where otherwise indicated, monocytes were washed twice in phosphate-buffered saline (PBS) and then lysed in a Triton-X containing buffer [50 mM Tris, 150 mM NaCl, 1% Triton X, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Lysates were spun and supernatant was incubated 1 : 1 with pre-swollen protein A sepharose (Pharmacia) for 2 h at 4°C, a step taken to eliminate proteins in the lysate which may bind non-specifically to the Protein A. The mix was subsequently spun and the supernatant was incubated at 4°C overnight with anti-MMP-1 (Chemicon, AB8105) or an isotype-matched control antibody (Chemicon, AB5320). This mix was next incubated for 2 h with protein A sepharose and, following three washes, the precipitate was analyzed by western blot using a primary antibody to Gi $\alpha$ -1 and -2 (Signal Transduction International, San Clemente, CA, USA; A0705). All incubations (antibody and protein A) were performed on a rotary table at 4°C, and all spins were performed using a desktop Eppendorf centrifuge at 4°C for 5 min at maximum speed (9000 g).

## Results

### MMP-1 decreases cytosolic levels of cAMP

Activation of Gi $\alpha$  coupled receptors typically results in decreased levels of cAMP by inhibiting adenylate cyclase



**Fig. 1** Matrix metalloproteinase (MMP)-1 decreases cytosolic levels of cAMP in dissociated rat neuronal cultures by a PTX sensitive mechanism. (a) MMP-1 (100 ng/mL) significantly decreased cytosolic levels of cAMP within 15 min of treatment. (b) Inhibition of  $G_{i\alpha}$  with pertussis toxin (PTX; 100 ng/mL) prevented significant decreases in cAMP levels following a 15-min treatment with MMP-1. (c) Increased levels of cAMP following the direct activation of adenylate cyclase with forskolin (10  $\mu$ M) were significantly decreased by coincubation with MMP-1. Inhibition of  $G_{i\alpha}$  signaling with pertussis toxin partially reversed the ability of MMP-1 to decrease forskolin-induced increases in cAMP ( $n = 3$  experiments conducted in duplicate. Data are the mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to time 0; ANOVA with Tukey's *post-hoc* comparisons). Note, 50  $\mu$ M IBMX was added to all cultures, 30 min prior to experiments, in order to raise cAMP levels to detectable limits.

activity. Consistent with the activation of  $G_{i\alpha}$ , cells exposed to MMP-1 exhibited transient decreases in cytosolic levels of cAMP that were significantly depressed between 15 and 25 min poststimulation (Fig. 1a). Direct activation of adenylate cyclase with forskolin increased cytosolic levels of cAMP, and MMP-1 reduced forskolin-induced increases in cAMP (Fig. 1c). Pre-treatment of cells with pertussis toxin,

which leads to the ADP-ribosylation of  $G_{i\alpha}$ , inhibited the ability of MMP-1 to reduce cAMP in the presence and absence of forskolin (Figs 1b and c). Thus, MMP-1-induced decreases in cytosolic levels of cAMP require  $G_{i\alpha}$ -associated signaling.

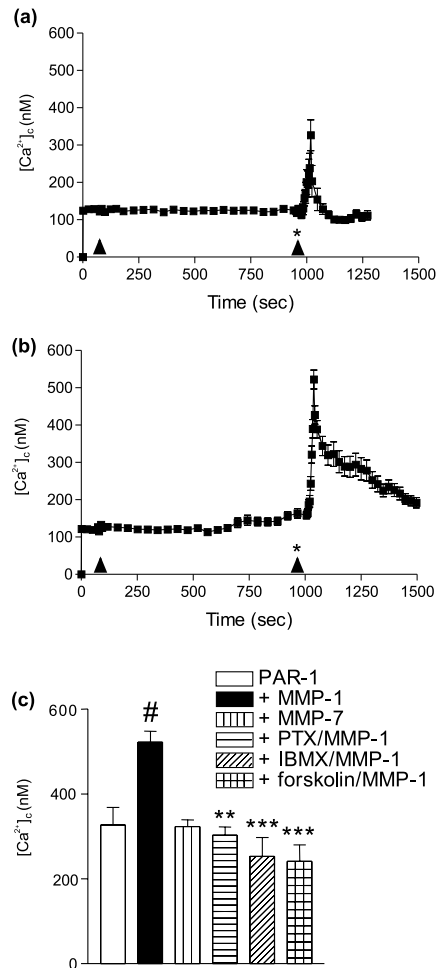
### MMP-1 potentiates protease activated receptor-linked calcium responses

Previous studies have shown that  $G_i$  protein-coupled receptor ( $G_i$ PCR) activation can potentiate intracellular calcium release mediated by activation of  $G_q$  PCRs (Selbie and Hill 1998). Several mechanisms may contribute to this effect including reduced phosphorylation/deactivation of IP<sub>3</sub> receptors by protein kinase A (PKA) (Bugrim 1999), and phospholipase C (PLC) activation by both the  $\beta\gamma$  subunits of the  $G_i$ PCR and the  $\alpha$  subunit of the  $G_q$  (Zhu and Birnbaumer 1996). We therefore determined whether MMP-1 could potentiate increases in intracellular calcium mediated by activation of protease activated receptor-1 (PAR-1), which can couple to  $G_q$ . Following a 15-min exposure to MMP-1, rat neurons showed a significantly increased peak calcium response to the PAR-1 agonist (Figs 2a–c), an effect which was not observed with MMP-7 (Fig. 2c). Consistent with the mechanism of PAR inactivation, which involves receptor internalization, second applications of the PAR-1 agonist resulted in minimal calcium increases ( $59 \pm 15$  nM, mean  $\pm$  SE 24 cells from four experiments) over baseline. To test the requirement of  $G_{i\alpha}$  signaling in this effect, we also used pertussis toxin in these experiments. As shown in Fig. 2(c), pertussis toxin did prevent facilitation of the PAR-1 agonist calcium response by MMP-1. Forskolin, an activator of adenylate cyclase, and IBMX, an inhibitor of phosphodiesterase, also reduced the ability of MMP-1 to potentiate PAR-1 associated changes in  $[Ca^{2+}]_c$ , suggesting that increased levels of cAMP inhibit PAR-1 and/or MMP-1 effects on  $[Ca^{2+}]_c$ .

In separate experiments, we observed that when MMP-1 and the PAR-1 agonist were applied simultaneously, potentiation by MMP-1 was not observed ( $334 \pm 24$  nM versus  $344 \pm 38$  nM, mean  $\pm$  SE of three experiments using 24–56 cells in each). A 1-min pre-treatment with MMP-1 was also ineffective ( $367 \pm 49$  versus  $336 \pm 42$ , mean  $\pm$  SE of three experiments using 24–56 cells). Potentiation effects shown in Fig. 2 therefore seem to occur through mechanisms which take minutes to evolve.

### MMP-1 stimulates MMP-9 release

MMP release has been observed following activation of  $G_{i\alpha}$  protein coupled receptors (Vliagoftis *et al.* 2000). Moreover, increased expression of MMP-9 has been observed following ligation of CD47 by thrombospondin (Qian *et al.* 1997). Ligation of integrins including  $\alpha_2\beta_1$  has also been linked to MMP-9 release as well as to processes which may be MMP dependent such as neurite outgrowth (Werb *et al.* 1989; Toyota *et al.* 1990; Sefror *et al.* 1992; Hou *et al.* 2000).



**Fig. 2** MMP-1 potentiates increases in intracellular calcium in mediated by a PAR-1 agonist. (a) Vehicle was applied to cells (arrow) 15 min before intracellular calcium increases were triggered by the application of a PAR-1 receptor agonist (arrowhead + asterisk). (b) Pre-treatment of cells with MMP-1 (100 ng/mL; arrowhead + asterisk) potentiated calcium increases triggered by a PAR-1 agonist (arrowhead + asterisk). (c) Peak levels of cytosolic calcium were significantly increased by pre-treatment of cells with MMP-1 (100 ng/mL) but not by MMP-7 (100 ng/mL). Inhibition of  $G_{i\alpha}$  with pertussis toxin (PTX; 100 ng/mL), inhibition of phosphodiesterase with IBMX, or direct activation of adenylate cyclase with forskolin, significantly decreased MMP-1 potentiation of PAR-1 triggered calcium release ( $n = 26$ – $47$  cells from three to four experiments. Data are mean  $\pm$  SEM. # $p < 0.01$ , compared with PAR-1; \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared with MMP-1; ANOVA with Tukey's *post-hoc* comparisons).

To determine if MMP-1 stimulates the release of MMP-9, we treated neural cultures and monocyte/macrophages with MMP-1 and then measured MMP-9 levels in the supernatant by ELISA and western blot. Organotypic spinal cord cultures were initially used in these experiments due to their high cell density, which allowed secreted proteins to be more easily detected by western blot. As shown in Fig. 3(a), MMP-1 stimulated the release of MMP-9 from these cultures. Since

these cultures are fairly heterogenous, however, we also examined dissociated human neuronal cultures, from which MMP-9 release can be evaluated by ELISA. As shown in Fig. 3(b), MMP-1 also stimulated MMP-9 release from these cultures ( $p = 0.05$ , Student's *t*-test). Moreover, as shown in Fig. 3(c), MMP-1 stimulated the release of MMP-9 from human monocytes which, like neurons, express both CD47 and the  $\alpha_2\beta_1$  integrin (Brown and Frazier 2001; Pacific *et al.* 1991; Colognato *et al.* 1997; Mi *et al.* 2000). MMP-1 stimulated release of MMP-9 from monocytes was confirmed by both zymography (Fig. 3d) and western blot (Fig. 3e). Of note, MMP-1 obtained from both Chemicon and R & D Systems stimulated MMP-9 release with similar efficacy and immunoabsorption of MMP-1 blocked this effect (not shown).

Since both pro- and active MMP-1 can bind to the  $\alpha_2\beta_1$  integrin (Dumin *et al.* 2001; Stricker *et al.* 2001), we also used a preparation (Oncogene Science) containing only pro-MMP-1 in some experiments. This preparation also stimulated MMP-9 release, though we cannot rule out autocatalysis or activation of pro-MMP-1 at the cell surface following its addition to our cultures. Therefore, experiments using an inhibitor of MMP-1 activity, GM-6001, were also performed in some of the studies above (see Fig. 3c). The results of these experiments suggested that, at least in monocytes, enzymatic activity was not required for increased release of MMP-9.

#### MMP-1 stimulated release of MMP-9 is PTX sensitive

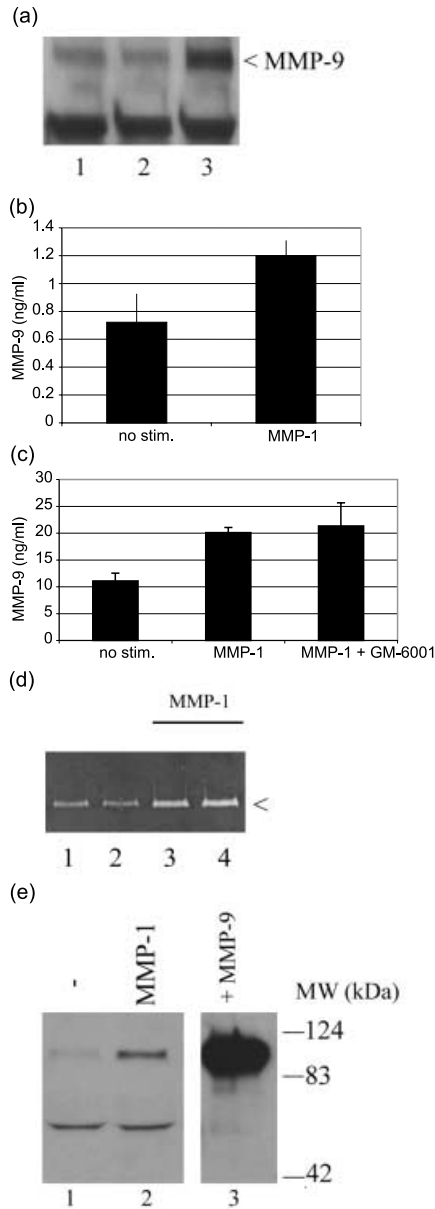
To further and more specifically address the question of whether a GPCR may be involved in MMP-1-stimulated release of MMP-9 we tested the ability of PTX to block this effect. As shown in Fig. 4(a and b), pre-treatment of organotypic spinal cord cultures with 250 ng/mL PTX inhibited the ability of 50 ng/mL MMP-1 to stimulate the release of MMP-9. As shown in Fig. 4(c), a similar effect was observed with cultured monocytes. As used in these studies, PTX was not associated with cytotoxicity as determined by LDH release or trypan blue uptake.

#### MMP-1 coimmunoprecipitates with a protein recognized by an antibody to $G_{i\alpha}$

We next examined whether MMP-1 might coimmunoprecipitate with  $G_{i\alpha}$ . As shown in Fig. 5, MMP-1 coimmunoprecipitated with a with a 41-kDa protein recognized by anti- $G_{i\alpha}$  (Fig. 5a, lane 2). Moreover, as shown in Fig. 5(b) (lane 2), immunoprecipitates made using an isotype-matched control antibody did not contain this 41 kDa band, demonstrating specificity of the effect. We also were unable to coimmunoprecipitate  $G_{i\alpha}$  from cells which had been washed but then not treated for 1 h with exogenous MMP-1 (Fig. 5a, lane 1).

#### An antibody to the $\beta_1$ integrin chain blocks MMP-1 stimulated release of MMP-9

Since MMP-1 has been shown to bind to the  $\alpha_2\beta_1$  integrin (Dumin *et al.* 2001), and this integrin may form a complex



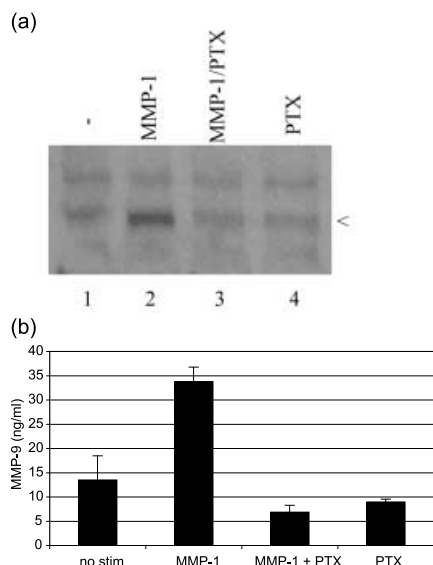
**Fig. 3** MMP-1 is associated with increased MMP-9 release from organotypic spinal cord, human neuronal and human monocyte/macrophage cultures. (a) MMP-1 stimulates the release of MMP-9 from organotypic spinal cord cultures. Organotypic spinal cord cultures were stimulated with serum free medium (SFM) (lane 1), or SFM containing 10 ng/mL MMP-1 (lane 2) or 100 ng/mL MMP-1 (Fig. 2a, lane 3). 24 h later, supernatants were sampled and MMP-9 assessed by western blot. As can be appreciated at left, MMP-9 immunoreactivity (arrowhead) is increased in association with 100 ng/mL (approximately 2 nM) MMP-1. Based on molecular weight, the predominant faster migrating band is likely to represent MMP-2, which is also recognized by this antibody. (b) MMP-1 stimulates release of MMP-9 from dissociated human neuronal cells, as detected by ELISA. In this experiment, supernatants were examined by ELISA following 16 h stimulation with serum free medium with or without 50 ng/mL MMP-1 as indicated. Data represent the mean  $\pm$  SE of a single experiment done in triplicate. (c) MMP-1 stimulates the release of MMP-9 from monocytes as determined by ELISA, and the MMP-1 inhibitor GM-6001 does not block this effect. Monocytes were stimulated with SFM in the presence or absence of 50 ng/mL MMP-1 and/or 50 nM GM-6001 as indicated. Supernatants were taken 16 h later and analyzed by ELISA. Results represent the mean  $\pm$  SE of an experiment done in quadruplicate. Differences between no stimulation and MMP-1 were significant at  $p = 0.002$ , and differences between no stimulation and GM-6001 were significant at 0.041. There was no significant difference between MMP-1 and MMP-1 plus GM-6001 (Student's *t*-test). (d and e) MMP-1 stimulates increased release of MMP-9 from human monocytes as detected by gelatin substrate zymography and western blot. These experiments were similar to that shown in (c), except that monocytes were obtained from different normal donors and supernatants were analyzed by gelatin substrate zymography (d) and western blot (e). In (d), pro-MMP-9 activity, as inferred by molecular weight, is indicated by the arrowhead. Pro-MMP-9 activity is relatively elevated in those lanes in which MMP-1-treated cell supernatants (lanes 3 and 4) versus untreated cell supernatants (lanes 1 and 2) were run. In (e), supernatants from untreated cells were run in lane 1, and those from cells which were treated with MMP-1 were run in lane 2. The immunoreactive band between 83 and 124 kDa represents MMP-9, while the faster migrating band is likely to represent MMP-2. Purified MMP-9 was run in lane 3 as a control.

with CD47 that is in turn linked to the activation of  $G_{i\alpha}$  signaling, we tested an antibody to the  $\beta_1$  integrin chain in terms of its ability to inhibit MMP-1 stimulated release of MMP-9. As shown in Fig. 6, treatment of monocytes with 4% anti- $\beta_1$  inhibited this effect. Antibody was added to cultures 30 min prior to their stimulation with MMP-1 and supernatants were sampled 16 h later. On its own, anti- $\beta_1$  did not significantly diminish MMP-9 release from that observed in unstimulated cultures, and cells treated with anti- $\beta_1$  did not show evidence of toxicity as determined by trypan blue uptake. Anti- $\beta_1$  did, however, significantly reduce MMP-1 stimulated MMP-9 release ( $p = 0.018$ , Student's *t*-test). These results are consistent with activation of a  $\beta_1$  containing receptor complex on monocytes by MMP-1.

## Discussion

The metalloproteinases (MPs), which include angiotensin-converting enzyme, insulin-degrading enzyme, tumor necrosis factor- $\alpha$  converting enzyme and the potential  $\beta$ -amyloid degrading enzyme neprilysin, are a family of structurally related metalloendopeptidases (Woessner and Nagase 2000; Yong *et al.* 2001). The MMPs represent a subset of MPs which are so named due to their propensity to target ECM proteins including collagens and laminin.

MMPs are thought to play an important role in numerous pathological and physiological processes including wound healing, emphysematous tissue destruction, angiogenesis, and cancer metastasis (Hautamaki *et al.* 1997; Woessner and Nagase 2000). With respect to the central nervous system, MMPs have been particularly well-studied in terms of their

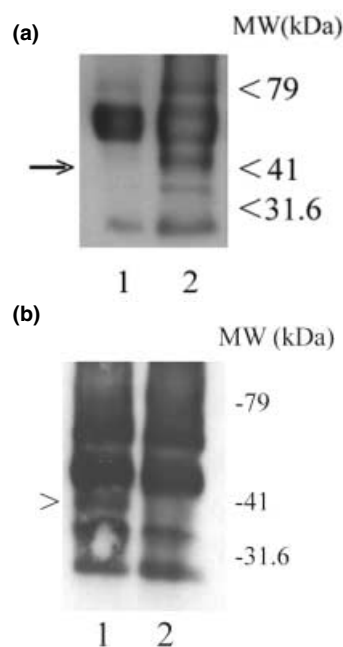


**Fig. 4** PTX inhibits the ability of MMP-1 to stimulate increased release of MMP-9. (a) Western blot analysis of supernatants from organotypic spinal cord cultures. Supernatants from organotypic spinal cord cultures which were treated for 16 h with medium alone were run in lane 1, with 100 ng/mL MMP-1 in lane 2, with 100 ng/mL MMP-1 plus 250 ng/mL PTX in lane 3, and with 250 ng/mL PTX alone in lane 4. The MMP-9 specific band, which runs between the 124 and 83 kDa M.W. markers (arrowhead), is reduced in association with PTX. (b) Analysis of supernatants from human monocyte/macrophage cultures by ELISA. Cultures were treated for 16 h with SFM, SFM with 50 ng/mL MMP-1 or SFM with 250 ng/mL PTX and 50 ng/mL MMP-1 as indicated. Results shown represent the mean  $\pm$  SE of an experiment that was done in triplicate. The difference between MMP-1 and MMP-1 plus PTX was significant at  $p = 0.015$  (Student's  $t$ -test).

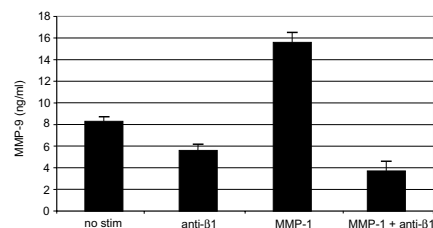
effects on the blood–brain barrier (BBB). MMPs are made by leukocytes so as to facilitate their passage across this barrier. In addition, MMPs are made by parenchymal cells of the CNS in response to inflammatory mediators (Gottschall 1995). Such MMPs may in turn affect the BBB from the parenchymal side thereby also contributing to leukocyte ingress and possibly, to the CNS ingress of toxic serum components such as thrombin.

While MMPs have been extensively studied in terms of their effects on ECM proteins including those that form the BBB, it is becoming increasingly evident that these proteases have significant biological effects that are relatively ECM independent. These effects include cleavage of cytokines and cell surface receptors (McCawley and Matrisian 2001).

In previous studies, we have observed that MMP-1 is produced by astrocytes, the most numerous cells in the CNS, and that such production is increased in association with inflammatory stimuli including IL-1 $\beta$  (Vos *et al.* 2000a). MMP release may also be increased following stimulation of cells with amyloid- $\beta$  (Deb and Gottschall 1996) and, of particular interest, it has recently been demonstrated that



**Fig. 5** MMP-1 coimmunoprecipitates with a with a 41-kDa protein recognized by anti-Gi $\alpha$ . A 41-kDa band can be appreciated in MMP-1 immunoprecipitates (a, lane 2; b, lane 1). This is the expected molecular weight for Gi. Moreover, as shown in Fig. 5 (b) (lane 2), immunoprecipitates made using an isotype matched control antibody did not contain this 41 kDa band, demonstrating specificity of the effect. We also were unable to coimmunoprecipitate Gi $\alpha$  from cells which had been washed but not subsequently treated for 1 h with exogenous MMP-1 (Fig. 5a, lane 1), consistent with low levels of MMP-1 in the monocyte cell cultures/supernatants that were used. Of note, the large non-specific band just above that thought to represent Gi is likely to be protein A (as determined by experiments not shown).



**Fig. 6** MMP-1 stimulated release of MMP-9 is inhibited by anti- $\beta_1$ . Monocyte/macrophages were treated with 40  $\mu$ L/mL anti- $\beta_1$ , 50 ng/mL MMP-1, or 40  $\mu$ L/mL anti- $\beta_1$  plus 50 ng/mL MMP-1 as indicated. Anti- $\beta_1$  was associated with inhibition of MMP-1 stimulated MMP-9 release, consistent with the possibility that MMP-1 signals through a  $\beta_1$  integrin containing receptor complex. Data represent the mean  $\pm$  SE of an experiment performed in duplicate.

MMP-1 levels are increased in the CNS in association with Alzheimer's type dementia (Leake *et al.* 2000).

Recently, it has been demonstrated that MMP-1 coimmunoprecipitates with  $\alpha_2\beta_1$  (Dumin *et al.* 2001). In other studies, it has been shown that  $\alpha_2\beta_1$  can associate with CD47

and that this complex may in turn be linked to the activation of Gi signaling (Brown and Frazier 2001). We therefore tested the possibility that MMP-1 may stimulate Gi signaling, an attractive possibility because activation of Gi could conceivably lead to downstream changes including changes in cell shape and/or the increased release of additional ECM-degrading MMPs.

Our observations were consistent with stimulation of a GiPCR by MMP-1. This protease decreased intracellular cAMP, and potentiated intracellular calcium changes mediated by a Gq coupled receptor agonist. The latter effect required pre-treatment with MMP-1 on the order of minutes and may therefore involve mechanisms including those that follow reductions in intracellular levels of cAMP, such as decreased phosphorylation of the IP<sub>3</sub> receptor by PKA. Phosphorylation of the IP<sub>3</sub> receptor can significantly diminish its calcium release properties (Bugrim 1999).

We next examined a potential downstream effect of GiPCR activation by MMP-1. In these experiments, we measured the release of MMP-9. In other studies, MMP-9 release has been linked both to GPCR activation and to thrombospondin, an agonist of CD47 (Qian *et al.* 1997). MMP-9 is also known to affect BBB permeability (Mun-Bryce and Rosenberg 1998), and has been associated not only with the breakdown of myelin basic protein (Chandler *et al.* 1995) but with the activation of IL-1 $\beta$  (Schonbeck *et al.* 1998). It is produced by monocytes and microglia, and also by neurons. Recent studies suggest that MMP-9 may also be involved in axonal transection (Newman *et al.* 2001) and a second gelatinase, MMP-2, has been associated with cytotoxicity (Johnston *et al.* 2001). In terms of cell or cell process migration, MMP-9 may be involved in neuronal process outgrowth following injury to the adult hippocampus (Szklaarczyk *et al.* 2002).

Our studies showed that MMP-1 did increase the release of MMP-9 and that this effect was pertussis toxin sensitive. In combination with the results of cAMP experiments, these results suggest that MMP-1 signals through a GPCR. Additional experiments will be necessary to determine which forms of MMP-1 may best activate Gi signaling, as both pro- and active forms of MMP-1 have been shown to bind the  $\alpha_2$  integrin (Dumin *et al.* 2001; Stricker *et al.* 2001), and our results suggest that enzymatic activity is not required for this effect. Additional experiments, including functional studies and sequencing of proteins recovered by immunoprecipitation, will also be necessary to determine whether MMP-1 activates Gi signaling through direct effects on an integrin/integrin associated protein complex. Results of experiments using an antibody to the  $\beta_1$  integrin chain suggest that this may be the case. It is possible that MMP-1 may also cleave a ligand which can in turn signal through a GPCR. Regardless of the mechanism(s) involved, however, the ability of MMP-1 to activate GPCR signaling could have important biological consequences. Such activation may contribute

both to normal physiological processes such as neurite outgrowth (Szklaarczyk *et al.* 2002), and to pathological conditions such as inflammatory disease associated cell migration (Liu *et al.* 2001).

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