INTESTINE INFLAMMATION

Evidence for the presence of functional protease activated receptor 4 (PAR4) in the rat colon

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**Background and aims:** Protease activated receptors (PARs) have been postulated to play a role during intestinal inflammation. The presence and role played by PAR4 in gastrointestinal functions have not been fully clarified. The aims of this study were: (i) to examine expression of PAR4 in rat proximal colon; (ii) to determine the mechanical effects induced by PAR4 activation in longitudinal muscle; and (iii) to characterise the underlying mechanisms.

**Methods:** PAR4 expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. Mechanical activity was recorded as changes in isometric tension.

**Results:** A PCR product corresponding to the predicted size of the PAR4 signal was amplified from RNA prepared from the colon of rats, showing the presence of PAR4 in those tissues. Immunohistochemistry revealed that PAR4 protein was expressed on epithelial surfaces and submucosa. PAR4 activating peptides, GYGPKF-NH2 and AYPGKG-NH2, produced concentration dependant contractile effects on longitudinal muscle. Tetrodotoxin (TTX) or atropine significantly reduced the contractile responses to AYPGKG-NH2, and atropine after TTX did not cause any further reduction. NK1 receptor antagonist, SR140333, or NK2 receptor antagonist, SR48968, alone or in combination, produced a reduction in PAR4 induced contractile effect, and when coadministered with TTX abolished it. Capsaicin markedly reduced the contractions evoked by AYPGKG-NH2.

**Conclusions:** The present results suggest that PAR4 is functionally expressed in rat colon and its activation induces contraction of the longitudinal muscle both through TTX sensitive release of acetylcholine and release of tachykinins, probably from sensory nerves. These actions may contribute to motility disturbances during intestinal trauma and inflammation.

**METHODS**

**Animals**
Male Wistar rats weighing 250–300 g were obtained from Morini (Italy) and from Charles River Laboratories (Quebec, Canada). All animals were housed in temperature controlled rooms and had free access to food and water. Local animal care committees approved all experimental protocols. Rats were sacrificed by cervical dislocation, and tissues were immediately removed for analysis.

**Abbreviations:** PARs, protease activated receptors; PBS, phosphate buffer saline; TTX, tetrodotoxin; GADPH, glyceraldehyde-3-phosphate dehydrogenase; EC_{50}, half maximal contractile concentration; RT-PCR, reverse transcription-polymerase chain reaction.
RT-PCR
For PAR4 reverse transcription-polymerase chain reaction (RT-PCR), total RNA from rat colonic tissues was isolated using the Trizol method (Gibco, Canada). RNA (2 μg) was reverse transcribed and DNA was amplified according to the following cycle conditions: dissociation of nucleic strands at 94°C for one minute, annealing at 55°C for 30 seconds, and extension for one minute at 72°C. The primer sequences for rat PAR4 were 5'-GGAGTGTTGAGAGAACGCA A and 3'-GACAAGGAGGGTACACCATT C, and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-GGACATCAAAGGTATTGGTCG T and 3'-AGGCTCTCTCATGAGT GGT GATAAC. Twenty seven cycles were performed for PAR4 and 23 cycles for GAPDH. PCR products were then separated on a 1% agarose gel with ethidium bromide and the gel was scanned under UV light.

Immunohistochemistry
Rat colon tissues were harvested and fixed in Zamboni's buffer overnight. Tissues were then washed three times in phosphate buffer saline (PBS) and placed in 20% sucrose with PBS. Tissues were embedded in OCT and frozen sections (10 μm) were placed on “superfrost plus” slides. Tissue sections were washed with PBS alone and then incubated for one hour at room temperature with PBS containing 10% donkey serum. Sections were then incubated with primary antibody (affinity purified goat polyclonal anti-PAR4 antibody, Santa Cruz Biotechnology, California, USA; 1:100 dilution) in PBS with 10% donkey serum overnight at 4°C. In control experiments, the anti-PAR4 antibody was preincubated (for 24–48 hours at 4°C with 1 or 10 μM of) with the blocking peptide used for immunisation (peptide mapping the C terminal domain of mouse PAR4; Santa Cruz Biotechnology). Slides were washed in PBS and incubated with secondary antibody (Cy3 conjugated AffiniPure donkey antigoat, 1/500 dilution in PBS plus 1.5% donkey serum) for one hour at room temperature. Tissue sections were then covered with Fluorsave reagent and examined using a Olympus IX50/FRA fluorescent microscope equipped with a coolsnap-fx camera.

Mechanical recording
Rat colon was removed distally to the caecum. The colonic lumen was cleaned with Krebs solution and segments of 2 cm in length were cut. The preparation was then placed in a continuously perfused organ bath containing 5 ml of gassed (95% O2 and 5% CO2) and heated (37°C) Krebs solution with the following composition (mM): NaCl 119; KCl 4.5; MgSO4 2.5; NaHCO3 25; KH2PO4 1.2; CaCl2 2.5; and glucose 11.1. Preparations were then tied with silk thread to an isometric force transducer (DY2: Ugo Basile, Comerio, Varese, Italy) for recording of isometric tension of the longitudinal muscle on an ink writer recorder (Gemini; Ugo Basile). Segments were allowed to equilibrate for at least 30 minutes under a 1 g load before starting the experiment.

Experimental protocol
After the equilibration period, the preparation was challenged with carbachol at 10 μM which, in preliminary experiments, was demonstrated to induce a maximal effect. Then, responses of the preparations to cumulative concentrations of GYPGKF-NH2, the murine synthetic PAR4 activating peptide, AYPGKG-NH2, which is reported to be a stronger agonist for PAR4, or YAPGKG-NH2, the inactive control peptide, were examined. The peptides were added into the bath in volumes of 50 μl after switching off the perfusion to give the concentrations indicated. Each concentration was left in contact with the tissue for two minutes. In preliminary experiments, in order to assess the reproducibility of the responses, we found that concentration related responses obtained with the first concentration-response curve were not significantly different from the second curve.

The response to PAR4 activation with AYPGKG-NH2 was assessed in the absence and presence of the following inhibitors/antagonists: tetrodotoxin (TTX 1 μM), a Na+ channel blocker; atropine (1 μM), an antagonist of cholinergic muscarinic receptors; SR140333, a selective antagonist of NK1 receptors (1 μM); SR48968, a selective antagonist of NK2 receptors (1 μM); and capsaicin at a concentration of 10 μM, which is reported to cause depolarisation of tachykinins from sensory nerve fibres. Some antagonists were coadministered to clarify whether the effectors represented different steps of the same pathway. For each drug, the concentration used was known to be effective in our preparation. The antagonists were added to the perfusing solution at least 30 minutes before testing the PAR activating peptides, except capsaicin. To prove that TTX treatment was effective, the preparation was stimulated through a pair of platinum ring electrodes.

Data analysis and statistics
Because of spontaneous phasic contractions of the tissues, the contractile response of longitudinal muscle was defined as change in resting tone (the bottom level of the tension oscillations), and was expressed as a percentage of contraction caused by 10 μM carbachol. All data are expressed as means (SEM) where n indicates the number of animals from which intestinal segments were taken. The half maximal contractile concentration (EC50) was calculated from individual experiments by non-linear regression. Differences in peptide induced responses in the absence (control) and presence of inhibitors were analysed by the Student’s t test or analysis of variance (ANOVA) and Bonferroni’s correction, when required. A probability value of less than 0.05 was regarded as significant.

Drugs
The following drugs were used: carbachol chloride (carbachol), capsaicin, tetrodotoxin (TTX) (Sigma, Chemical Corp, St Louis, Missouri, USA), ((S)-N-methyl-N-[4-(4-acetyl-amino-4-phenylpiperi-dino)-2-(3, 4-dichloro-phenyl)-butyl]benzamide (SR48968), (S)-[1-2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxy-phenylacetyl) piperidin-3yl] ethyl]-4-phenyl-1 azaniciobicyclic 2.2.2 octane chloride (SR140333) (a kind gift from Sanofi Recherche, Montpellier Cédex, France), [Sa2+], Met(O2)11-SP, [β-Ala8]-NKA(4–10) (Calbiochem-Novabiochem AG, Laufelfingen, Switzerland). All drugs were dissolved in distilled water, except for the following: SR48968 and SR140333 dissolved in DMSO, capsaicin dissolved in absolute ethanol, [Sa2+], Met(O2)11-SP dissolved in diluted acetic acid, and [β-Ala8]-NKA(4–10) dissolved in diluted ammonia. Working solutions were prepared fresh on the day of the experiment by diluting the stock solutions. Control experiments using the different solvents alone showed that none had effects on the tissue responses studied.

GYPGKF-NH2, AYPGKG-NH2 and the inactive control peptide YAPGKF-NH2 were purchased from the Peptide Synthesis Core Facility at the University of Calgary (Canada), and prepared by standard solid phase synthesis procedures.

RESULTS

PAR4 expression
A PCR product of a predicted size of 463 bp was amplified from RNA prepared from the colon of rats (lanes 1 and 2; fig 1), showing PAR4 presence in those tissues. In the absence of PAR4 primer, only GAPDH amplified product (306 bp) was detected in those tissues (lane 3; fig 1). PAR4 protein
immunoreactivity was also observed in rat colon (fig 2). PAR4 expression was prominently localised to the mucosal surface (colonocytes; arrowheads in fig 2A), but staining was also observed in the submucosa (arrows in fig 2A). Staining was abolished by preabsorption of antibody with the receptor fragment used to raise the anti-PAR4 antibody (fig 2B).

Functional studies

Under the present experimental conditions, rat colon smooth muscle showed spontaneous mechanical activity, consisting in rhythmic phasic changes of isometric tension (from 1 to 2.5 g). The PAR4 activating peptides, GYPGKF-NH2 (0.01–10 μM) and AYPGQV-NH2 (0.01–10 μM), caused a concentration dependent contractile response in the longitudinal muscle of rat colon, consisting of an increase in basal tension and maintenance of the phasic contraction (fig 3). AYPGQV-NH2 (EC50 = 0.08 (0.06) μM; n = 6) was more potent than GYPGKF-NH2 (EC50 = 0.3 (0.1) μM; n = 8) but efficacy was similar, both peptides being able to reach approximately 90% of the contraction induced by carbachol (10 μM) at the maximal concentration tested (fig 4). In order to verify the specificity of the response, we applied to the preparations the inactive control peptide YAPGQV-NH2 (0.01–10 μM) and this had no effect (figs 3, 4).

To clarify the mechanism underlying the contractile effect produced by PAR4 activation, we evaluated the responses evoked by AYPGQV-NH2 in the presence of TTX. TTX (1 μM), which per se did not modify spontaneous contractions, significantly reduced the responses induced by the PAR4 activating peptide, but did not abolish them, indicating partial involvement of neural mechanism (fig 5A).

As tachykinins and acetylcholine are among the major excitatory neurotransmitters to intestinal smooth muscle, we evaluated if these mediators could be involved in the PAR4 agonist induced contractile response. Therefore, we tested the effects of their antagonists on responses evoked by PAR4 activation both in the absence and presence of TTX. Pretreatment of tissues with the muscarinic antagonist atropine (1 μM) significantly decreased the contractile responses induced by AYPGKF-NH2 (fig 5A). Contractile responses to 10 μM carbachol were absent in these tissues (data not shown). It is interesting that the reduction in the PAR4 contractile effect induced by atropine was of the same degree as that induced by TTX; furthermore, subsequent addition of atropine (1 μM) after TTX caused no further reduction of the PAR4 induced contractile effect (fig 5A).

Both NK1 and NK2 receptor antagonists, SR140333 (1 μM) and SR48968 (1 μM), respectively, reduced independently the contractile effects induced by PAR4 activating peptide (fig 5B). The concentration of the NK1 receptor selective antagonist SR140333 that we used (1 μM) antagonised the contractile response to the NK1 receptor agonist [SAR9, Met(O2)11]-SP but not to the NK2 receptor agonist [β-alal]-neurokinin A (4–10). The concentration of the NK2 receptor selective antagonist SR48968 that we used (1 μM) antagonised the contractile response to the NK2 receptor agonist [β-alal]-neurokinin A (4–10) but not to the NK1 receptor agonist [SAR9, Met(O2)11]-SP (data not shown). The suppressive effects of the NK1 receptor selective antagonist SR140333 (1 μM) were additive to those induced by the NK2 receptor selective antagonist SR48968 (1 μM) (fig 5B). When both antagonists were coadministered with TTX (1 μM), the contractile effects evoked by PAR4 activating peptide were abolished (fig 5B).

Lastly, pretreatment of the preparation with capsaicin (10 μM) for 90 minutes significantly reduced the contractile effects induced by PAR4 activating peptide (fig 6).

DISCUSSION

Possible physiological/pathophysiological roles for PAR4 in tissues or cells other than platelets are poorly understood, in spite of their wide distribution in varying tissues.12–21 30–32 Considering the effects of PAR4 agonists on smooth muscles, some evidence indicates the PAR4 is not involved in modulation of the motility of rat duodenum16 or guinea pig gall bladder.19 However, Hollenberg and colleagues12 have reported that PAR4 agonists induce contraction of gastric longitudinal smooth muscle and nitric oxide dependent

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**Figure 1** Detection of protease activated receptor 4 (PAR4) in rat colon by reverse transcription-polymerase chain reaction. Lanes 1 and 2, colon tissues where PAR4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were amplified; a specific 463 bp fragment corresponds to PAR4 while GAPDH showed an expected amplified fragment of 306 bp. Lane 3 shows omission of PAR4 primer.

**Figure 2** Immunolocalisation of protease activated receptor 4 (PAR4) in rat colon. In (A), tissues were incubated with an anti-PAR4 primary antibody and then with a Cy3 conjugated secondary antibody. The control (B) shows incubation of primary anti-PAR4 antibody with blocking peptide, followed by Cy3 conjugated secondary antibody. m, mucosa, sm, submucosa, cm, circular muscle. Arrowheads shows colonocyte staining, arrows shows submucosa staining. Scale bar, 10 μm.
relaxation of vascular smooth muscle. It is not yet clear whether PAR4 activation plays a role similar or opposite to PAR1. In fact, PAR4 activation appears to mimic the effects of PAR1 activation in gastric and airway smooth muscle, whereas in the rat oesophageal muscularis mucosae, PAR4 appears to mediate relaxation, in contrast to PAR1.22

The present study demonstrates that PAR4 is present in rat colon and its activation evokes contraction of intestinal longitudinal smooth muscle. Both GYPGKF-NH2, corresponding to the murine PAR4 tethered ligand, and AYPGKF-NH2, reported to be a more potent peptide analogue for PAR4,25 induced a concentration dependent contractile response. The control inactive peptide YAPGKF-NH2 had no contractile effect on longitudinal smooth muscle preparations, thus confirming the specificity of action of the two PAR4 peptide agonists used. AYPGQV-NH2 was more potent than GYPGKF-NH2, which is in accordance with data from the literature.25 The effective concentration range of GYPGKF-NH2 and AYPGQV-NH2 for induction of the contractile responses was lower than that required for production of contraction in other preparations.2 One possible explanation for this observation is that in our preparation the PAR4 activating peptides were not susceptible to rapid protease degradation, or alternatively the high potency of agonist may reflect greater expression of this type

![Figure 3](image-url)

**Figure 3** Representative recordings of the contractile effects induced by the protease activated receptor 4 (PAR4) activating peptides, GYPGKF-NH2 and AYPGKF-NH2, by the inactive control peptide, YAPGKF-NH2, and by carbachol (CCh 10 μM) on the longitudinal muscle of rat colon. Arrows indicate application of the agonist, W, washout.

![Figure 4](image-url)

**Figure 4** Concentration-response curves for the effects evoked by the protease activated receptor 4 (PAR4) activating peptides, GYPGKF-NH2, and AYPGKF-NH2, and the inactive control peptide, YAPGKF-NH2, on the longitudinal muscle of rat colon. The contractile effects are expressed as a percentage of the contraction to carbachol (CCh 10 μM). Each value is the mean (SEM) of 4–8 experiments.

![Figure 5](image-url)

**Figure 5** (A) Effects of tetrodotoxin (TTX) and atropine, alone or in combination, on the contractile responses evoked by protease activated receptor 4 (PAR4) activating peptide, AYPGKF-NH2 on the longitudinal muscle of rat colon. The contractile responses to AYPGKF-NH2 were significantly reduced by TTX (1 μM) or atropine (1 μM). The combination of atropine (1 μM) and TTX (1 μM) did not cause any further reduction. (B) Effects of SR140333 and SR48968, alone, in combination, or after treatment with TTX, on the contractile responses evoked by PAR4 activating peptide, AYPGKF-NH2, on the longitudinal muscle of rat colon. The contractile responses to AYPGKF-NH2 were significantly reduced by SR140333 (1 μM), an antagonist of NK2 receptors, by SR48968 (1 μM), an antagonist of NK1 receptors, and even more by a combination of SR48968 (1 μM) and SR140333 (1 μM). AYPGKF-NH2 did not induce any response in the presence of a combination of SR48968 (1 μM), SR140333 (1 μM), and TTX (1 μM). The contractile effects are expressed as a percentage of the contraction to carbachol (CCh 10 μM). Each value is mean (SEM) of five experiments. *p<0.05 compared with control value; †p<0.05 compared with TTX or atropine alone; ‡p<0.05 compared with SR140333 and SR48968 alone; §p<0.05 compared with the combination of SR140333 and SR48968.
of receptor in the colon than in the stomach, as reported by Xu and colleagues.\textsuperscript{21}

However, the contractile effects we demonstrated for PAR\textsubscript{4} agonists in rat colonic smooth muscle preparations suggest a role for this receptor in motility functions of the gut. Whether or not this role is associated with pathophysiological circumstances may depend on the availability of endogenous proteases in the vicinity of the receptor. Which endogenous protease activates PAR\textsubscript{4} in intestinal tissues remains to be established. Both thrombin and trypsin have been reported to activate PAR\textsubscript{4}.\textsuperscript{21} In a previous study, we demonstrated that thrombin and trypsin induced contractile effects in rat colonic longitudinal muscle preparations, showing biphasic responses: relaxation followed by contraction for the highest concentrations.\textsuperscript{19} These effects were mimicked by PAR\textsubscript{1} and PAR\textsubscript{2} activating peptides, suggesting that thrombin and trypsin could be responsible for endogenous activation of PAR\textsubscript{1} and PAR\textsubscript{2}, respectively. However, the present results highlight PAR\textsubscript{4} as another potential effector of both thrombin and trypsin. The similar effects of PAR\textsubscript{1}, PAR\textsubscript{2}, and PAR\textsubscript{4} could make redundant the effects of thrombin and trypsin in rat intestinal longitudinal muscle as was suggested for platelets and smooth muscles from gastric, vascular, and airway tissues.\textsuperscript{12, 24, 30, 31} Recently, the neutrophil granule protease cathepsin G\textsuperscript{18} has been found to stimulate PAR\textsubscript{4}. This adds cathepsin G to the list of potential endogenous activators of PAR\textsubscript{4} in intestinal tissues. Moreover, it links PAR\textsubscript{4} activation in those tissues to the presence of granulocytes, suggesting that inflammatory pathophysiological circumstances, such as inflammatory bowel diseases, may be associated with PAR\textsubscript{4} activation.

We examined the mechanism by which PAR\textsubscript{4} activation induces contractile effects in rat colonic longitudinal muscle by treating the preparation with TTX, a neuronal conduction blocker, to assess the role of neurotransmission in the evoked effect. The finding that the contractile responses to PAR\textsubscript{4} activating peptides were antagonised, although not completely, by TTX, suggests that a neurogenic component is involved in the PAR\textsubscript{4} agonist evoked effect. Previous studies have reported that PAR\textsubscript{1} and PAR\textsubscript{2} are expressed by a large proportion of neurons of the myenteric plexus of the guinea pig intestine\textsuperscript{34} and PAR\textsubscript{2} receptors are expressed on cholinergic nerves in porcine ileum.\textsuperscript{35} However, information on the cellular localisation of PAR\textsubscript{4} in intestinal tissue is lacking. In mouse, PAR\textsubscript{4} expression on peripheral nerves of bladder has been shown.\textsuperscript{36} We observed immunostaining for PAR\textsubscript{4} in submucosal structures which could be submucosal neurones (fig 2A), but double labelling with a neuronal marker would be necessary to ascertain that PAR\textsubscript{4} is expressed on neurones in the rat colon. We have evaluated if acetylcholine or tachykinins could be responsible for the PAR\textsubscript{4} neurally mediated effect because these chemical mediators are considered the major excitatory neurotransmitters of intestinal smooth muscle. Atropine reduced the contractile response to PAR\textsubscript{4} activation to the same extent as TTX but failed to decrease it further after TTX treatment. This indicates that PAR\textsubscript{4} activation can induce acetylcholine release through an action potential dependent neural mechanism. In addition, NK\textsubscript{1} and NK\textsubscript{2} receptor antagonists, when administered separately or in combination, reduced contraction due to PAR\textsubscript{4} activation, suggesting involvement of these receptors in the effects evoked by AYPGKF-NH\textsubscript{2}. The presence of these receptors, which mediate contraction in the rat colon, has been demonstrated previously by histochemical and functional studies.\textsuperscript{37, 38} On the other hand, the action of both SR140333 and SR48696 has to be considered as specifically linked to occupancy of NK\textsubscript{1} or NK\textsubscript{2} receptors, respectively, as these drugs antagonised exclusively contraction induced by selective agonists of the respective receptors. Because the suppressive effects of NK\textsubscript{1} and NK\textsubscript{2} receptor antagonists were additive to the effect of TTX, it could be hypothesised that PAR\textsubscript{4} activation can induce tachykinin release without the contribution of propagated action potential (TTX resistant release). In fact, TTX may not inhibit release of neurotransmitters following direct stimulation of receptors present in nerve terminals. Therefore, we hypothesise that prejunctional PAR\textsubscript{4} may directly induce the release of tachykinins, which are mostly released by neurones in the gut.\textsuperscript{26}

In addition, capsaicin significantly reduced the response induced by PAR\textsubscript{4} activating peptide. Although capsaicin can cause different effects, such as VR1 receptor desensitisation or blockade of voltage gated calcium channels, it is likely that the effect of 10 \textmu M capsaicin for 90 minutes can be attributed to an inhibitory effect on the release of biologically active substances, such as tachykinins, by sensory nerve fibres.\textsuperscript{39} Depletion of tachykinins from sensory nerves could account for the reduction in contractile effect to PAR\textsubscript{4} activation. It has been reported that PAR\textsubscript{2} activation stimulates the release of substance P from terminals of capsaicin sensitive primary sensory neurones, thus causing neurogenic inflammatory responses in peripheral tissue.\textsuperscript{39} Interestingly, a similar system has also been described for trypsin induced contraction of the guinea pig bronchus, with release of tachykinins from sensory nerves being responsible for the final contractile response.\textsuperscript{40, 41} In addition, our recent data have suggested that PAR\textsubscript{1} and PAR\textsubscript{2} activation can induce neural tachykinin release.\textsuperscript{29}

In conclusion, this study has demonstrated for the first time that PAR\textsubscript{4} is functionally expressed in rat colon. It can mediate a contractile response of longitudinal muscle through TTX sensitive release of acetylcholine and through action potential independent release of tachykinins, probably from sensory nerves. This mechanism of action may support a proinflammatory role for PAR\textsubscript{4}, which could contribute to motility disturbances observed during intestinal trauma and inflammation.

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