INTESTINE INFLAMMATION

Evidence for the presence of functional protease activated receptor 4 (PAR_4) 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 PAR_4 in gastrointestinal functions have not been fully clarified. The aims of this study were: (i) to examine expression of PAR_4 in rat proximal colon; (ii) to determine the mechanical effects induced by PAR_4 activation in longitudinal muscle; and (iii) to characterise the underlying mechanisms.

Methods: PAR_4 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 PAR_4 signal was amplified from RNA prepared from the colon of rats, showing the presence of PAR_4 in those tissues. Immunohistochemistry revealed that PAR_4 protein was expressed on epithelial surfaces and submucosa. PAR_4 activating peptides, GYPGKF-NH_2 and AYPGKG-NH_2, produced concentration dependent contractile effects on longitudinal muscle. Tetrodotoxin (TTX) or atropine significantly reduced the contractile responses to AYPGKG-NH_2, and atropine after TTX did not cause any further reduction. NK_1 receptor antagonist, SR140333, or NK_2 receptor antagonist, SR48968, alone or in combination, produced a reduction in PAR_4 induced contractile effect, and when coadministered with TTX abolished it. Capsaicin markedly reduced the contractions evoked by AYPGKG-NH_2.

Conclusions: The present results suggest that PAR_4 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.

In addition to their established proteolytic roles, proteases such as trypsin, thrombin, or cathepsin G may also act as cell signalling molecules via protease activated receptors (PARs). This family of seven transmembrane G protein coupled receptors currently includes four receptor subtypes (PAR_1, PAR_2, PAR_3, PAR_4). PAR_1, PAR_3, and PAR_4, but not PAR_2, are activated by thrombin, whereas trypsin can activate PAR_2 and PAR_4.[1 2] PAR_4 can also be activated by the neutrophil granule protease cathepsin G.[3] Activation of PARs is initiated by proteolytic cleavage of their extracellular N terminal domain resulting in the unmasking of an extracellular N terminal sequence that acts as a tethered receptor activating ligand. PAR_1, PAR_2, and PAR_4 can also be activated by exogenously applied short synthetic peptides, based on the sequences of their tethered ligands.

Numerous studies have been performed to clarify the role of PARs in the physiology and pathophysiology of the gastrointestinal tract as these tissues, more than others, are exposed to proteinases and PARs are highly expressed throughout the gastrointestinal tract.[4] However, most of the studies concerned the functions of PAR_1 and PAR_2. PAR_1 and PAR_2 most likely play a role during inflammation, allergy, and/or haemorrhage where thrombin and/or mast cell tryptase become available as endogenous agonists. PAR_2 appears to be involved in exocrine secretion[5 6] and in intestinal ion transport.[7] PAR_1 and PAR_2 modulate smooth muscle motility[8] and their activation can induce relaxant, contractile, or biphasic responses.[9–20] Although northern blot analysis has shown that mRNA for PAR_4 is present in human small and large intestine,[21] the cell types that express PAR_4 are still unknown and the physiological role played by PAR_4 in the gastrointestinal tract has to be yet fully elucidated. Hollenberg and colleagues[12] have reported contractile effects on the longitudinal muscle of rat stomach due to activation of PAR_4 by the receptor activating peptide, GYPGQV-NH_2, and lack of cross reactivity with PAR_1 and PAR_2. In addition, in rat oesophageal muscularis mucosae[22] and isolated airways,[23] PAR_4 induces relaxation, a function opposed to PAR_1. In contrast, PAR_4 appears to mimic the actions of PAR_1 in the murine airway smooth muscle.[24]

The present study was designed to investigate whether PAR_4 is present in rat colon and if it affects gut motor functions. The purposes were to: (i) examine expression of PAR_4 in rat proximal colon; (ii) determine the mechanical effects induced by PAR_4 activation in longitudinal smooth muscle; and (iii) characterise the underlying mechanisms. Specifically, the possible involvement of neurogenic mechanisms and the relative contribution of different neurotransmitters were analysed.

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; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EC_{50}, half maximal contractile concentration; RT-PCR, reverse transcription-polymerase chain reaction

RT-PCR

For PAR_4 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℃ for one minute, annealing at 55℃ for 30 seconds, and extension for one minute at 72℃. The primer sequences for rat PAR_4 were 5′-GGA AGT CTT GAG AGA AAG GCA A and 3′-GAA CCA AGA GGC ATC ACC TAT C, and for glyceraldehyde-3-phosphate dehydrogenase (GADPH) 5′-CGG AGT CAA CGG ATT TGG TCG TAT and 3′-AGC CTT CTC CAT GGT GGT GAA GAC. Twenty seven cycles were performed for PAR_4 and 23 cycles for GADPH. 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-PAR_4 antibody; Santa Cruz Biotechnology, California, USA; 1/100 dilution) in PBS with 10% donkey serum overnight at 4℃. In control experiments, the anti-PAR_4 antibody was preincubated (for 24–48 hours at 4℃ with 1 or 10 μM of) with the blocking peptide used for immunisation (peptide mapping the C terminal domain of mouse PAR_4; 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/FLA 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 approximately 2 cm in length were cut. The preparation was then placed in a continuously perfused organ bath containing 5 ml of gassed (95% O_2 and 5% CO_2) and heated (37℃) Krebs solution with the following composition (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; $CaCl_2$ 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-NH_2, the murine synthetic PAR_4 activating peptide, AYPGKG-NH_2, which is reported to be a stronger agonist for PAR_4,[25] or YAPGKG-NH_2, 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 PAR_4 activation with AYPGKG-NH_2 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 NK_1 receptors (1 μM); SR48968, a selective antagonist of NK_2 receptors (1 μM); and capsaicin at a concentration of 10 μM, which is reported to cause depletion of tachykinins from sensory nerve fibres.[26 27] 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.[28 29] 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 (EC_{50}) 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' correction, when required. A probability value of less than 0.05 was regarded as significant.

Drugs

The following drugs were used: carbamylcholine 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-dichlorphenyl)-1 (3-isopropoxy-phenylacetyl) piperidin-3yl] ethyl]-4-phenyl-1 azaniabicyclo [2.2.2] octane chloride (SR140333) (a kind gift from Sanofi Recherche, Montpellier Cédex, France), [Sar^9, $Met(O_2)^{11}$]-SP, [β-Ala^8]-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, [Sar^9, $Met(O_2)^{11}$]-SP dissolved in diluted acetic acid, and [β-Ala^8]-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-NH_2, AYPGKG-NH_2, and the inactive control peptide YAPGKF-NH_2 were purchases from the Peptide Synthesis Core Facility at the University of Calgary (Canada), and prepared by standard solid phase synthesis procedures.

RESULTS

PAR_4 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 PAR_4 presence in those tissues. In the absence of PAR_4 primer, only GAPDH amplified product (306 bp) was detected in those tissues (lane 3; fig 1). PAR_4 protein

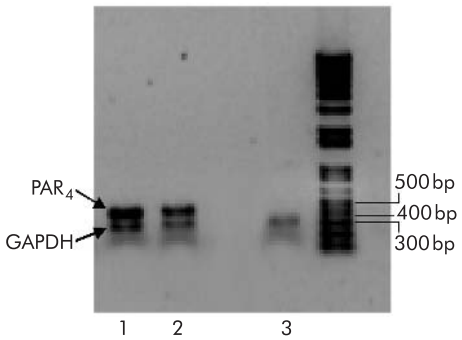

Figure 1 Detection of protease activated receptor 4 (PAR_4) in rat colon by reverse transcription-polymerase chain reaction. Lanes 1 and 2, colon tissues where PAR_4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were amplified; a specific 463 bp fragment corresponds to PAR_4 while GAPDH showed an expected amplified fragment of 306 bp. Lane 3 shows omission of PAR_4 primer.

immunoreactivity was also observed in rat colon (fig 2). PAR_4 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-PAR_4 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 PAR_4 activating peptides, GYPGKF-NH_2 (0.01–10 μM) and AYPGQV-NH_2 (0.01–10 μM), caused a concentration dependent contractile response in the longitudinal muscle of rat colon, consisting of an increase in basal tension with maintenance of the phasic contraction (fig 3). AYPGQV-NH_2 ($EC_{50} = 0.08$ (0.06) μM; $n = 6$) was more potent than GYPGKF-NH_2 ($EC_{50} = 0.3$ (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-NH_2 (0.01–10 μM) and this had no effect (figs 3, 4).

To clarify the mechanism underlying the contractile effect produced by PAR_4 activation, we evaluated the responses evoked by AYPGQV-NH_2 in the presence of TTX. TTX (1 μM), which per se did not modify spontaneous contractions, significantly reduced the responses induced by the PAR_4 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 PAR_4 agonist induced contractile response. Therefore, we tested the effects of their antagonists on responses evoked by PAR_4 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-NH_2 (fig 5A). Contractile responses to 10 μM carbachol were absent in these tissues (data not shown). It is interesting that the reduction in the PAR_4 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 PAR_4 induced contractile effect (fig 5A).

Both NK_1 and NK_2 receptor antagonists, SR140333 (1 μM) and SR48968 (1 μM), respectively, reduced independently the contractile effects induced by PAR_4 activating peptide (fig 5B). The concentration of the NK_1 receptor selective antagonist SR140333 that we used (1 μM) antagonised the contractile response to the NK_1 receptor agonist [SAR^9, $Met(O_2)^{11}$]-SP but not to the NK_2 receptor agonist [β-ala^8]-neurokinin A (4–10). The concentration of the NK_2 receptor selective antagonist SR48968 that we used (1 μM) antagonised the contractile response to the NK_2 receptor agonist [β-ala^8]-neurokinin A (4–10) but not to the NK_1 receptor agonist [SAR^9, $Met(O_2)^{11}$]-SP (data not shown). The suppressive effects of the NK_1 receptor selective antagonist SR140333 (1 μM) were additive to those induced by the NK_2 receptor selective antagonist SR48968 (1 μM) (fig 5B).When both antagonists were coadministered with TTX (1 μM), the contractile effects evoked by PAR_4 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 PAR_4 activating peptide (fig 6).

DISCUSSION

Possible physiological/pathophysiological roles for PAR_4 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 PAR_4 agonists on smooth muscles, some evidence indicates the PAR_4 is not involved in modulation of the motility of rat duodenum[16] or guinea pig gall bladder.[18] However, Hollenberg and colleagues[12] have reported that PAR_4 agonists induce contraction of gastric longitudinal smooth muscle and nitric oxide dependent

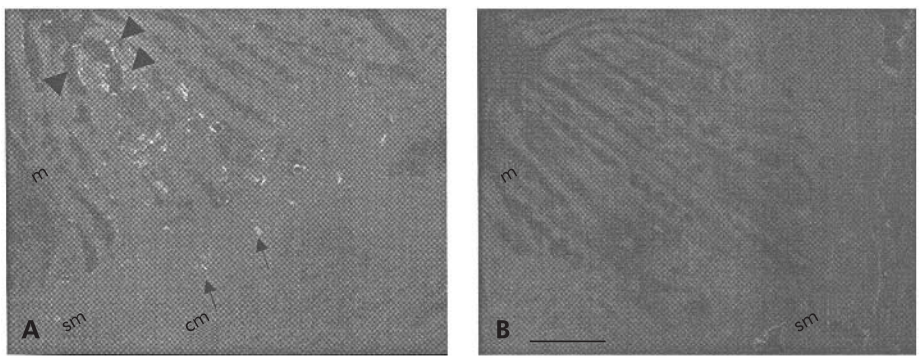

Figure 2 Immunolocalisation of protease activated receptor 4 (PAR_4) in rat colon. In (A), tissues were incubated with an anti-PAR_4 primary antibody and then with a Cy3 conjugated secondary antibody. The control (B) shows incubation of primary anti-PAR_4 antibody with blocking peptide, followed by Cy3 conjugated secondary antibody. m, mucosa, sm, submucosa, cm, circular muscle. Arrowheads shows colonocyte staining, arrows shows submucosa staining. Scale bar, 10 μm.

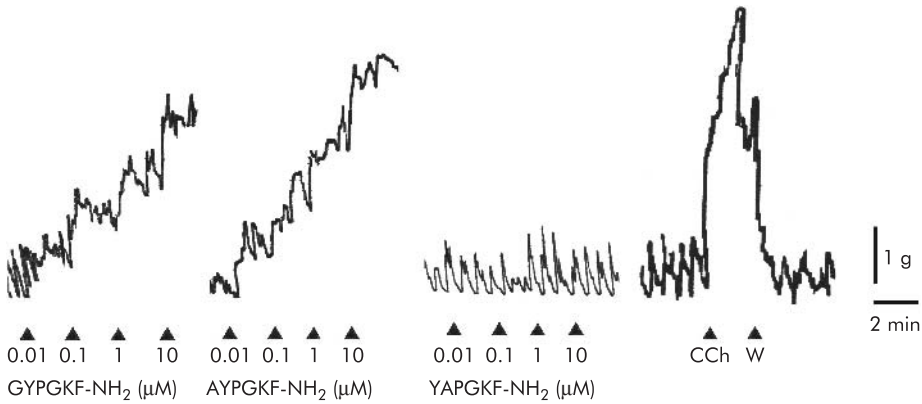

Figure 3 Representative recordings of the contractile effects induced by the protease activated receptor 4 (PAR_4) activating peptides, GYPGKF-NH_2 and AYPGKF-NH_2, by the inactive control peptide, YAPGKF-NH_2, and by carbachol (CCh 10 μM) on the longitudinal muscle of rat colon. Arrows indicate application of the agonist. W, washout.

relaxation of vascular smooth muscle. It is not yet clear whether PAR_4 activation plays a role similar or opposite to PAR_1. In fact, PAR_4 activation appears to mimic the effects of PAR_1 activation in gastric and airway smooth muscle,[12 24] whereas in the rat oesophageal muscularis mucosae, PAR_4 appears to mediate relaxation, in contrast to PAR_1.[22]

The present study demonstrates that PAR_4 is present in rat colon and its activation evokes contraction of intestinal longitudinal smooth muscle. Both GYPGKF-NH_2, corresponding to the murine PAR_4 tethered ligand, and AYPGKF-NH_2, reported to be a more potent peptide analogue for PAR_4,[25] induced a concentration dependent contractile response. The control inactive peptide YAPGKF-NH_2 had no contractile effect on longitudinal smooth muscle preparations, thus confirming the specificity of action of the two PAR_4 peptide agonists used. AYPGQV-NH_2 was more potent than GYPGKF-NH_2, which is in accordance with data from the literature.[25] The effective concentration range of GYPGKF-NH_2 and AYPGQV-NH_2 for induction of the contractile responses was lower than that required for production of contraction in other preparations.[12] One possible explanation for this observation is that in our preparation the PAR_4 activating peptides were not susceptible to rapid protease degradation, or alternatively the high potency of agonist may reflect greater expression of this type

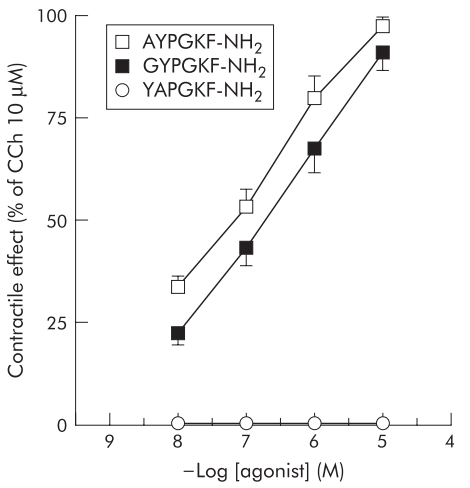

Figure 4 Concentration-response curves for the effects evoked by the protease activated receptor 4 (PAR_4) activating peptides, GYPGKF-NH_2 and AYPGKF-NH_2, and the inactive control peptide, YAPGKF-NH_2, on the longitudinal muscle of rat colon. The contractile effects are expressed as a percentage of the contraction to carbachol (CCh 10 μM). Each value is the mean (SEM) of 4–8 experiments.

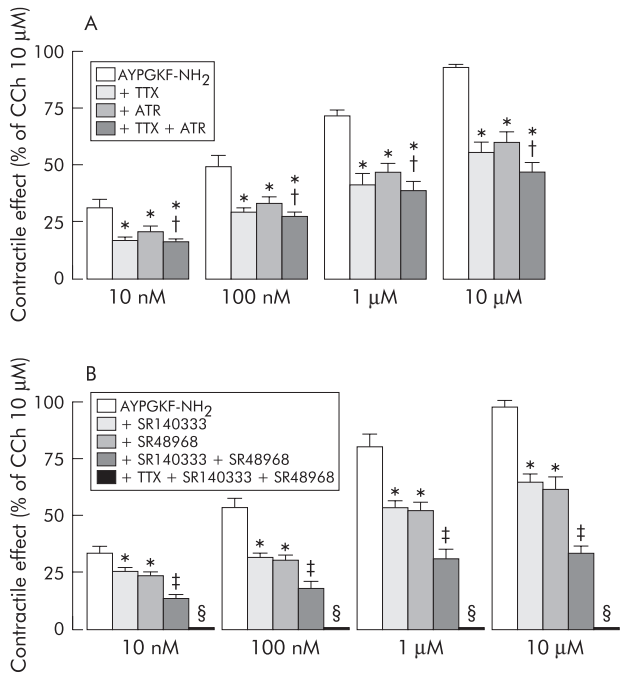

Figure 5 (A) Effects of tetrodotoxin (TTX) and atropine, alone or in combination, on the contractile responses evoked by protease activated receptor 4 (PAR_4) activating peptide, AYPGKF-NH_2, on the longitudinal muscle of rat colon. The contractile responses to AYPGKF-NH_2 were significantly reduced 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 PAR_4 activating peptide, AYPGKF-NH_2, on the longitudinal muscle of rat colon. The contractile responses to AYPGKF-NH_2 were significantly reduced by SR140333 (1 μM), an antagonist of NK_1 receptors, by SR48968 (1 μM), an antagonist of NK_2 receptors, and even more by a combination of SR48968 (1 μM) and SR140333 (1 μM). AYPGKF-NH_2 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.

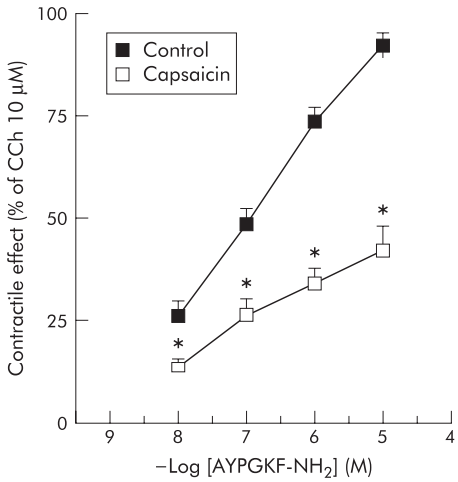

Figure 6 Concentration-response curves for the effects evoked by the protease activated receptor 4 (PAR_4) activating peptide AYPGKF-NH_2 on the longitudinal muscle of rat colon in the absence or presence of capsaicin. Capsaicin (10 μM) significantly antagonised the contractile response to the PAR_4 agonist. The contractile effects are expressed as a percentage of the contraction to carbachol (CCh 10 μM). Each value is the mean (SEM) of five experiments. *$p<0.05$ compared with control values.

of receptor in the colon than in the stomach, as reported by Xu and colleagues.[21]

However, the contractile effects we demonstrated for PAR_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_4 in intestinal tissues remains to be established. Both thrombin and trypsin have been reported to activate PAR_4.[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.[19] These effects were mimicked by PAR_1 and PAR_2 activating peptides, suggesting that thrombin and trypsin could be responsible for endogenous activation of PAR_1 and PAR_2, respectively. However, the present results highlight PAR_4 as another potential effector of both thrombin and trypsin. The similar effects of PAR_1, PAR_2, and PAR_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.[12 24 30 31 33] Recently, the neutrophil granule protease cathepsin G[3] has been found to stimulate PAR_4. This adds cathepsin G to the list of potential endogenous activators of PAR_4 in intestinal tissues. Moreover, it links PAR_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_4 activation.

We examined the mechanism by which PAR_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_4 activating peptides were antagonised, although not completely, by TTX, suggests that a neurogenic component is involved in the PAR_4 agonist evoked effect. Previous studies have reported that PAR_1 and PAR_2 are expressed by a large proportion of neurones of the myenteric plexus of the guinea pig intestine[34] and PAR_2 receptors are expressed on cholinergic nerves in porcine ileum.[35] However, information on the cellular localisation of PAR_4 in intestinal tissue is lacking. In mouse, PAR_4 expression on peripheral nerves of bladder has been shown.[36] We observed immunostainning for PAR_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_4 is expressed on neurones in the rat colon. We have evaluated if acetylcholine or tachykinins could be responsible for the PAR_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_4 activation to the same extent as TTX but failed to decrease it further after TTX treatment. This indicates that PAR_4 activation can induce acetylcholine release through an action potential dependent neural mechanism. In addition, NK_1 and NK_2 receptor antagonists, when administered separately or in combination, reduced contraction due to PAR_4 activation, suggesting involvement of these receptors in the effects evoked by AYPGKF-NH_2. The presence of these receptors, which mediate contraction in the rat colon, has been demonstrated previously by histochemical and functional studies.[26 37 38] On the other hand, the action of both SR140333 and SR48968 has to be considered as specifically linked to occupancy of NK_1 or NK_2 receptors, respectively, as these drugs antagonised exclusively contraction induced by selective agonists of the respective receptors. Because the suppressive effects of NK_1 and NK_2 receptor antagonists were additive to the effect of TTX, it could be hypothesised that PAR_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_4 may directly induce the release of tachykinins, which are mostly released by neurones in the gut.[26]

In addition, capsaicin significantly reduced the response induced by PAR_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 μM 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.[26 27] Depletion of tachykinins from sensory nerves could account for the reduction in contractile effect to PAR_4 activation. It has been reported that PAR_2 activation stimulates the release of substance P from terminals of capsaicin sensitive primary sensory neurones, thus causing neurogenic inflammatory responses in peripheral tissue.[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.[40 41] In addition, our recent data have suggested that PAR_1 and PAR_2 activation can induce neural tachykinin release.[29]

In conclusion, this study has demonstrated for the first time that PAR_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_4, which could contribute to motility disturbances observed during intestinal trauma and inflammation.

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