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Signal Transduction Pathways Involved in the Mechanical Responses to Protease-Activated Receptors in Rat Colon

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ABSTRACT

Recording simultaneously in vitro the changes of endoluminal pressure (index of circular muscle activity) and isometric tension (index of longitudinal muscle activity), we examined the mechanisms responsible for the apamin-sensitive relaxant and contractile responses induced by protease-activated receptor (PAR)-1 and PAR-2 activating peptides, SFLLRN-NH_2 and SLIGRL-NH_2, respectively, in rat colon. In the circular muscle, the inhibitory effects of SFLLRN-NH_2 and SLIGRL-NH_2 were significantly reduced by ryanodine, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum, but unaffected by 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), a phospholipase C (PLC) inhibitor, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride (GF109203X), a protein kinase C (PKC) inhibitor, or genistein, a tyrosine kinase inhibitor. In the longitudinal muscle, the contractile responses to SFLLRN-NH_2 and SLIGRL-NH_2 were significantly reduced by nifedipine, an L-type calcium channel blocker, ryanodine, GF109203X, genistein, and abolished by U73122. The effects of genistein were additive with GF109203X but not with nifedipine. In the longitudinal muscle, the relaxant responses to the highest concentrations of SFLLRN-NH_2 and SLIGRL-NH_2 were abolished by nifedipine, reduced by genistein, and unaffected by ryanodine or GF109203X. In conclusion, influx of extracellular Ca^{2+} through L-type voltage-dependent channels or release of Ca^{2+} from intracellular stores are determining for the opening of the apamin-sensitive K^+ channels responsible for longitudinal muscle relaxation or circular muscle inhibitory response, respectively, in rat colon. The longitudinal muscle contraction is mediated by activation of PLC; PKC and tyrosine kinase are involved in the cascade process, playing a parallel role. Indeed, tyrosine kinase and L-type Ca^{2+} channels would act sequentially. The influx of Ca^{2+} in turn would cause release of Ca^{2+} from sarcoplasmic reticulum.

Proteinase-activated receptors (PARs) are a recently described novel family of seven-transmembrane G-protein-coupled receptors, which are involved in several pathophysiological processes, including inflammation (Dery et al., 1998; Schmidlin and Bunnett, 2001; Vergnolle et al., 2001). Rather than being stimulated through ligand receptor occupancy, activation is initiated by cleavage of their extracellular N-terminal domain by a serine protease resulting in the generation of a new "tethered ligand" binding and activating the receptor itself (Dery et al., 1998; Macfarlane et al., 2001). To date, four PARs have been identified: PAR-1 and PAR-3 both preferentially activated by thrombin (Vu et al., 1991; Ishihara et al., 1997), PAR-2, which selectively activated by trypsin or mast-cell tryptase (Nystedt et al., 1994; Molino et al., 1997), and PAR-4, activated by thrombin or trypsin (Xu et al., 1998). Short synthetic peptides based on the proteolytically revealed receptor sequences (PAR-activating peptides) can selectively activate PAR-1, PAR-2, or PAR-4, but thus far, activating peptides for PAR-3 have not been identified.

Numerous studies have been performed to clarify the role of PARs in the physiology and pathophysiology of the gastrointestinal tract because these tissues, more than others, are exposed to proteinases and PARs are highly expressed throughout the gastrointestinal tract (Vergnolle, 2000). PAR-2 appears to be involved in exocrine secretion (Nguyen et al., 1999; Kawabata et al., 2000c) and intestinal ion transport (Vergnolle et al., 1998). PAR-1 and PAR-2 modulate smooth muscle motility (Kawabata et al., 2001), and their activation can induce relaxant, contractile, or biphasic responses (Al-Ani et al., 1995; Saifeddine et al., 1996; Corvera et al., 1997; Hollenberg et al., 1997, 1999; Zheng et al., 1998; Cocks et al., 1999; Kawabata et al., 1999, 2000a; Tognetto et al., 2000; Mulè et al., 2002).

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ABBREVIATIONS: PAR, protease-activated receptor; PLC, phospholipase C; PKC, protein kinase C; U73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; GF109203X, bisindolylmaleimide I, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride.

The signal transduction mechanisms coupled with PAR activation do not necessarily appear to be the same in distinct tissues. Extracellular Ca^{2+} influx, activation of tyrosine kinase, activation of phospholipase C (PLC) with consequent increase of inositol 1,4,5-trisphosphate and diacylglycerol, formation of metabolites of arachidonic acid, and activation of apamin-sensitive K^+ channels are the main mechanisms reported to be involved in the biochemical transduction (Hollenberg et al., 1993, 1997, 1999; Zheng et al., 1998; Cocks et al., 1999; Kawabata et al., 1999, 2000a,b; Mulè et al., 2002). To date, however, it is unclear if these mechanisms represent different steps of a single cascade or are parallel signal transduction pathways.

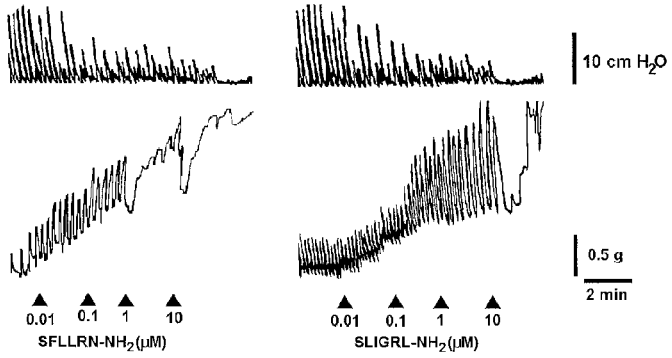

Fig. 1. Representative recordings of the effects induced by SFLLRN-NH_2, a PAR-1 agonist, and SLIGRL-NH2, a PAR-2 agonist, on the circular muscle (upper tracings) and on longitudinal muscle (lower tracings) of an isolated segment of rat colon. The arrow indicates the application of the agonist.

In our previous study, a role of PAR-1 and PAR-2 receptors in the stimulation of the intestinal transit was hypothesized in rat colon because PAR activation induced different mechanical responses on the two muscular layers: an inhibitory effect on the circular muscle and a contractile effect, which became biphasic at the highest concentration used, in the longitudinal muscle (Mulè et al., 2002). In rat colon, an involvement of products of cyclooxygenase in the action of PAR-1 and PAR-2 activating peptides was ruled out. The inhibitory responses to PAR-1 and PAR-2 agonists mainly occurred via activation of apamin-sensitive K^+ channels, but the source of Ca^{2+} necessary to activate the K^+ channels was not investigated.

The present study was undertaken in the attempt to characterize the signal transduction mechanisms for the rat colonic relaxant and/or contractile responses due to PAR-1 and PAR-2 activation. Specifically, we examined 1) the relative contribution of intracellular and extracellular Ca^{2+}, 2) the possible involvement of PLC, protein kinase C (PKC), and tyrosine kinase in the inhibitory and contractile responses to PAR-1 and PAR-2 activation, and 3) the sequence of events leading to the contraction.

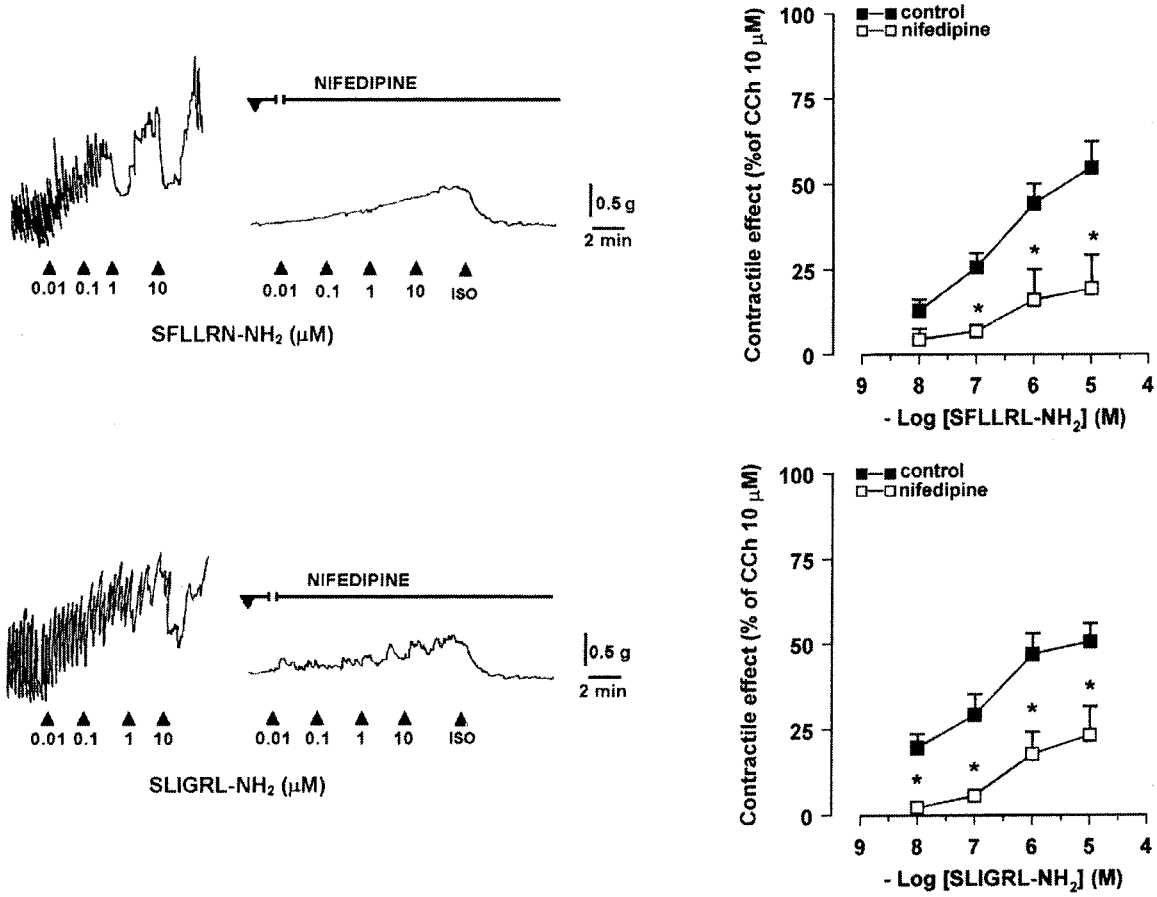

Fig. 2. Effects of nifedipine on the responses of the longitudinal muscle of rat colon to SFLLRN-NH_2, a PAR-1 activating peptide, and SLIGRL-NH2, a PAR-2 activating peptide. Left panel, typical recordings of the effects induced by the peptides on the tension oscillations before and after nifedipine (1 μM). The arrow indicates the application of the agonist. ISO, isoproterenol (1 μM). Right panel, concentration-response curves to PAR-1 and PAR-2 activating peptides in the absence or in the presence of nifedipine (1 μM). The contractile response of longitudinal muscle is expressed as a percentage of the maximal contraction to carbachol (10 μM). Each value is mean ± S.E. of five experiments. ⋆, $P < 0.05$ compared with the control value.

Materials and Methods

Tissue Preparation and Mechanical Recording. Colonic segments were obtained from male Wistar rats weighing 250 to 400 g. Animals were cared for in accordance with the Declaration of Helsinki and were killed by rapid cervical dislocation. The abdomen was immediately open, and the colon was removed. The colonic lumen was cleaned with Krebs' solution, and three segments of about 2 cm in length were cut proximally to caecum. The preparation was then placed in a continuously perfused horizontal organ bath containing 5 ml of gassed (95% O_2 and 5% CO_2) Krebs' solution with the following composition: 119 mM NaCl, 4.5 mM KCl, 2.5 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, and 11.1 mM glucose. The solution was maintained at 37°C. As previously described (Mulè et al., 2002), the distal end of the intestinal segment was tied around the mouth of a J-tube, which was connected via a T catheter to a standard pressure transducer (Ugo Basile, Biological Research Apparatus, Varese, Italy) and to a syringe for filling the preparation with Krebs' solution. The ligated proximal end was secured with a silk thread to an isometric force transducer (DY2; Ugo Basile). Preparations, filled with 0.1 ml of Krebs' solution, were subjected to an initial tension of 1 g and were allowed to equilibrate for at least 30 min before starting the experiment. Colonic contractions were monitored as changes in intraluminal pressure and isometric tension, which are mainly generated by circular and longitudinal muscle activity, respectively. Mechanical activities were recorded on an ink writer recorder (Gemini, Ugo Basile). The correspondence of the intraluminal pressure and tension recordings to the mechanical activity of circular and longitudinal muscle, respectively, has been already demonstrated in our previous studies (Mulè et al., 1992; 1999).

Experimental Protocol. After the equilibration period, the preparation was challenged with carbachol at 10 μM, which in preliminary experiments was demonstrated to induce maximal effect. Then, the responses of the preparations to cumulative concentrations of PAR-activating peptides were examined in the absence and presence of different inhibitors/antagonists. In some experiments, we confirmed that the noncumulative addition of the peptides caused reproducible concentration-response curves. The peptides tested were SFLLRN-NH_2 (0.01–10 μM), a PAR-1 agonist that is reported to have weak agonistic activity toward PAR-2 (Hollenberg et al., 1997), and SLIGRL-NH_2 (0.01–10 μM), a murine PAR-2-specific agonist (Nystedt et al., 1994). We previously demonstrated that in our preparation, SFLLRN-NH_2 mimicked the effects of TFLLR-NH_2, a highly specific PAR-1 analog (Hollenberg et al., 1997), and that the response to SFLLRN-NH_2 was not affected by prior desensitization to SLIGRL-NH_2 (Mulè et al., 2002). Therefore, the responses to SFLLRN-NH_2 in the present study have to be considered to result from activation of PAR-1. The peptides were added into the bath in volumes of 50 μl after switching off the perfusion. Each concentration was left in contact with the tissue for 2 min. The inhibitors/antagonists were the following: nifedipine, an L-type Ca^{2+} channel inhibitor (1 μM), ryanodine, an inhibitor of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (10 μM), U73122, a PLC inhibitor (0.5–5 μM), GF109203X, a protein kinase C inhibitor (1 μM), and genistein, a tyrosine kinase inhibitor (1–10 μM). For the most of the drugs, the concentration used was known to be effective in our and other systems (Mulè and Serio, 1997; Cocks et al., 1999; Kawabata et al., 2000b). Some antagonists were coadministered to clarify if the effectors represent different steps of the same pathway. These agents were added to the perfusing solution at least 30 min before testing the PAR-activating peptides. Sometimes to verify the specificity of the effect observed, the responses to carbachol (1 μM) or isoproterenol (1 μM) at concentrations shown to be submaximal were evaluated in the absence and in the presence of the inhibitors.

Data Analysis and Statistics. The inhibitory response of the circular muscle to PAR-1 and PAR-2 activation was taken as the percent change from the resting spontaneous activity (e.g., −100% corresponds to the abolition of spontaneous activity). In this view, the mean amplitude of the pressure waves was determined for 10 min before and after administration of agonists. The contractile response of the longitudinal muscle was defined as a change in the resting tone (the bottom level of the tension oscillations) and was expressed as a percentage of the contraction caused by 10 μM carbachol. All data obtained are expressed as mean ± S.E. n indicates the number of animals from which an intestinal segment was taken. Statistical analyses were performed by a Student's paired t test for comparison between two-group data (responses in the absence and in the presence of inhibitors) or by analysis of variance, followed by a Bonferroni test for multiple treatment comparison. $P < 0.05$ was regarded as significant.

TABLE 1

Effects of different inhibitors on the longitudinal muscle relaxation evoked by PAR-1 and PAR-2 activating peptides in rat colon

The relaxation is expressed in grams. Values are means ± S.E. for the number of experiments shown in parentheses.

	SFLLRN-NH_2 (1 μM)	SFLLRN-NH_2 (10 μM)	SLIGRL-NH_2 (10 μM)
Control	0.86 ± 0.3	1.1 ± 0.3	1.2 ± 0.1
Nifedipine (1 μM)	0* (5)	0* (5)	0* (5)
Control	1.3 ± 0.3	1.6 ± 0.3	1.3 ± 0.3
Ryanodine (10 μM)	1.6 ± 0.4 (7)	2.3 ± 0.4 (7)	1.7 ± 0.4 (7)
Control	0.92 ± 0.3	1.1 ± 0.3	0.9 ± 0.3
GF109203X (1 μM)	0.6 ± 0.2 (6)	0.7 ± 0.2 (6)	0.7 ± 0.2 (6)
Control	0.8 ± 0.2	1.4 ± 0.3	0.9 ± 0.2
Genistein (7.5 μM)	0.2 ± 0.1* (6)	0.5 ± 0.2* (6)	0.4 ± 0.2* (6)

* Significantly different from the control, $P < 0.05$.

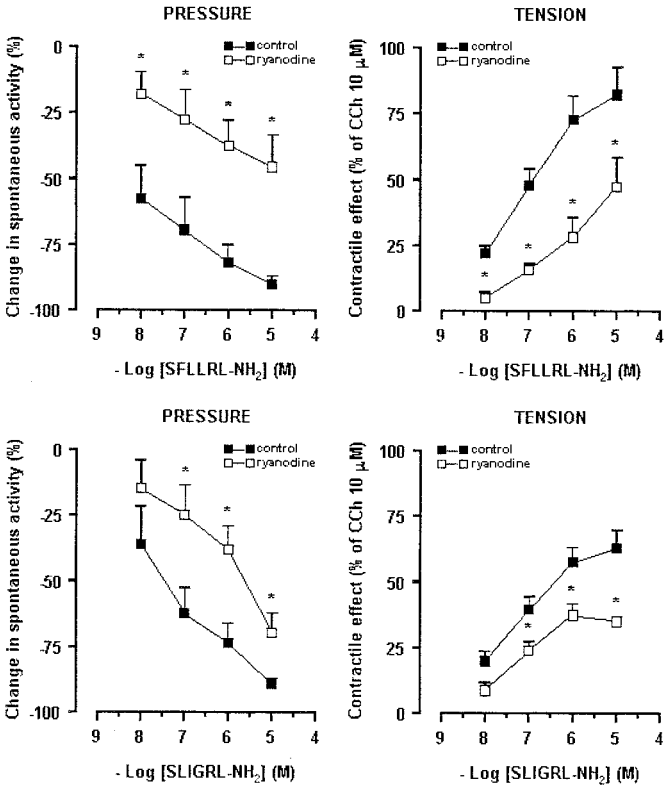

Fig. 3. Concentration-response curves for effects evoked by PAR-1 and PAR-2 agonists on the pressure waves and on the tension oscillations of the rat colon in the absence and in the presence of ryanodine (10 μM). The inhibitory responses of circular muscle are expressed as the percent change from the resting spontaneous activity (−100% corresponds to the abolition of spontaneous activity). The contractile effects of longitudinal muscle are expressed as a percentage of the maximal contraction to carbachol (10 μM). Each value is mean ± S.E. of seven experiments. ⋆, $P < 0.05$ compared with the control value.

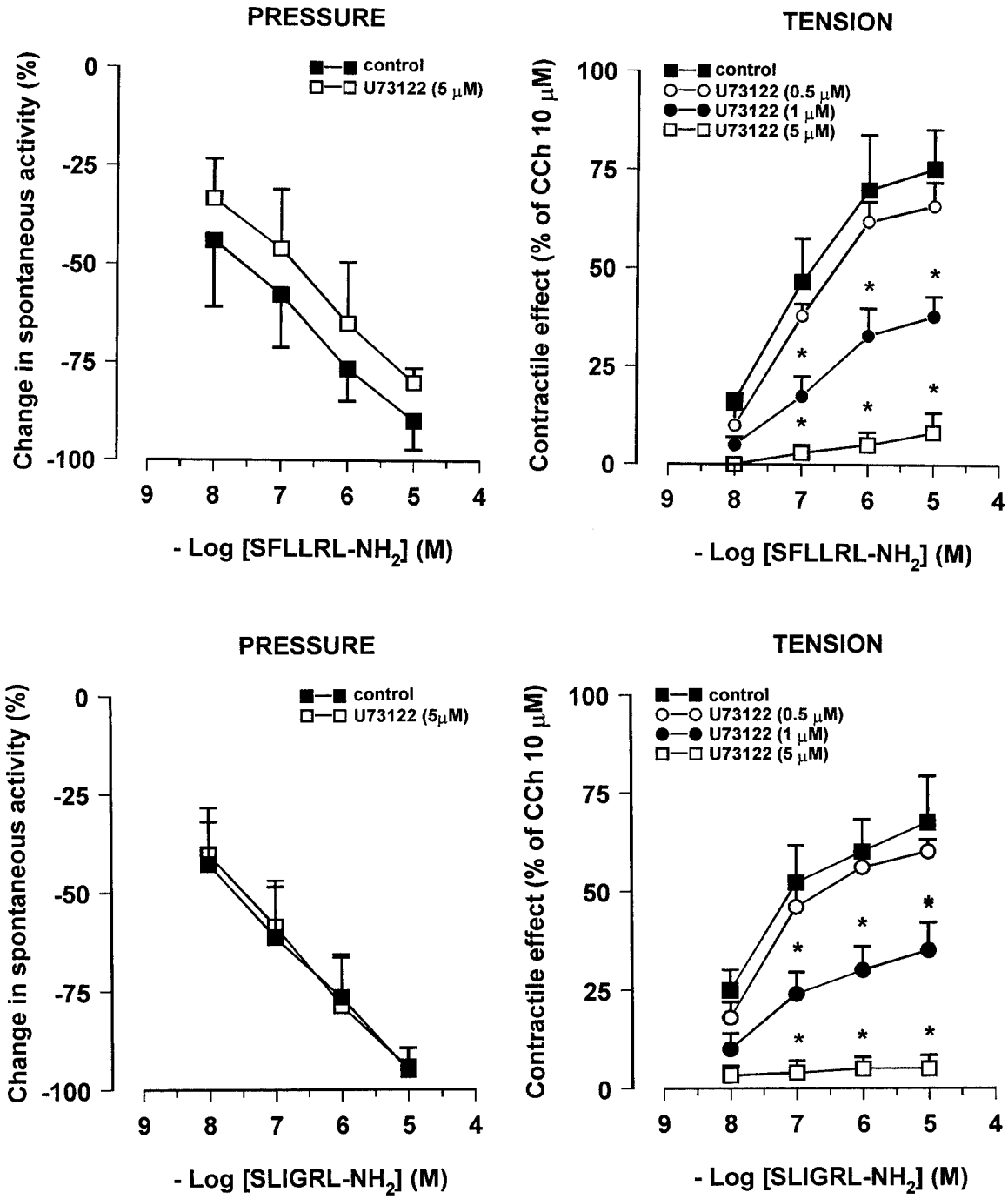

Fig. 4. Concentration-response curves for effects evoked by PAR-1 and PAR-2 agonists on the pressure waves and on the tension oscillations of the rat colon in the absence and in the presence of U73122, a PLC inhibitor. The inhibitory responses of circular muscle are expressed as the percent change from the resting spontaneous activity (−100% corresponds to the abolition of spontaneous activity). The contractile effects of longitudinal muscle are expressed as a percentage of the maximal contraction to carbachol (10 μM). Each value is mean ± S.E. of five experiments. ⋆, $P < 0.05$ compared with the control value.

Drugs. The following drugs were used: carbamylcholine chloride (carbachol), isoprenaline hydrochloride, ryanodine (Sigma-Aldrich, St. Louis, MO), nifedipine, bisindolylmaleimide I (GF109203X), genistein, U73122, and phorbol-12,13-dibutyrate (Calbiochem, Darmstadt, Germany). SFLLRN-NH_2 was obtained from Bachem (Bubendorf, Switzerland), and SLIGRL-NH_2 was supplied by Dr. D. McMaster of the Peptide Synthesis Core Facility at the University of Calgary (Calgary, AB, Canada). PAR-related peptides were prepared by standard solid-phase synthesis procedures. The concentration, purity, and composition of the peptides were determined by high-performance liquid chromatography, mass spectrometry, and quantitative amino acid analysis. Ryanodine was dissolved in ethanol. Nifedipine, U73122, GF109203X, and genistein were dissolved in dimethyl sulfoxide. All other chemicals were dissolved in distilled water. Control tests showed that the solvents alone had no effect on the preparation. To avoid photodecomposition, experiments with nifedipine and genistein were performed with no external lighting.

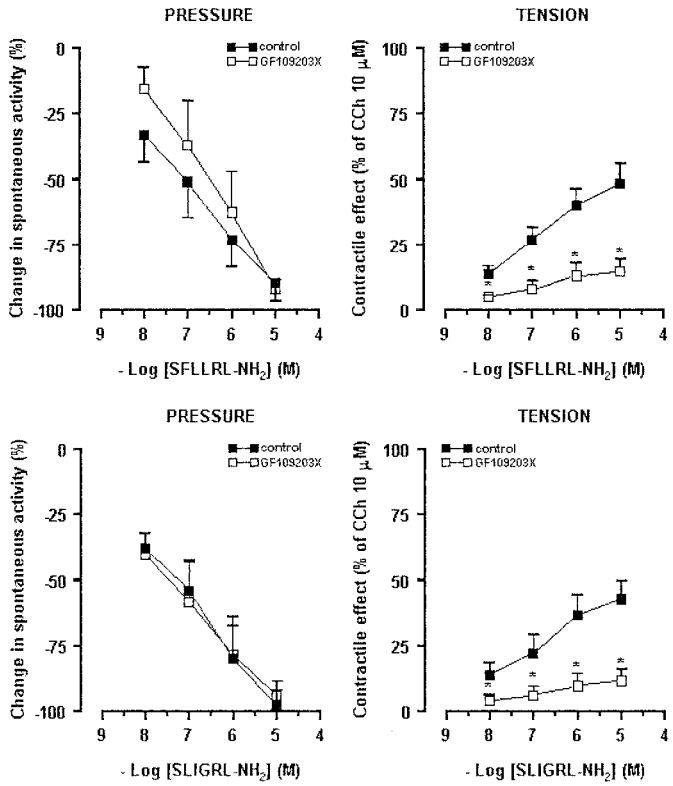

Fig. 5. Concentration-response curves for effects evoked by PAR-1 and PAR-2 agonists on the pressure waves and on the tension oscillations of the rat colon in the absence and in the presence of GF109203X, a PKC inhibitor (1 μM) and a PLC inhibitor. The inhibitory responses of circular muscle are expressed as the percent change from the resting spontaneous activity (−100% corresponds to the abolition of spontaneous activity). The contractile effects of longitudinal muscle are expressed as a percentage of the maximal contraction to carbachol (10 μM). Each value is mean ± S.E. of six experiments. ⋆, $P < 0.05$ compared with the control value.

Results

As previously described (Mulè et al., 2002), the PAR-1 and PAR-2 peptide activators SFLLRN-NH_2 (10 nM–10 μM) and SLIGRL-NH_2 (10 nM–10 μM), respectively, which are mimetic of each tethered ligand sequence, caused different effects on the two muscular layers. They produced an inhibitory response consisting in a concentration-dependent reduction in the amplitude of the spontaneous rhythmic contractions of the circular muscle and a contractile response in the longitudinal muscle, at least at the lowest concentrations tested. In fact, in the longitudinal muscle, a biphasic effect, relaxation followed by contraction, in response to the PAR-1 and PAR-2 activating peptides was observed (Fig. 1) at concentrations over 1 μM.

Role of Extracellular and Intracellular Calcium. To investigate the involvement of Ca^{2+} in the responses due to PAR-1 and PAR-2 activation and the relative contribution of the extracellular and intracellular source, we used nifedipine, an L-type voltage-dependent calcium channel blocker, and ryanodine, an inhibitor of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum. Nifedipine at 1 μM greatly reduced the spontaneous phasic contractions of the colonic segments; therefore, its effects on the inhibitory response in the circular muscle could not to be evaluated. In the longitudinal muscle, nifedipine significantly reduced the contractile effects induced by the activation of PAR-1 and PAR-2, and it abolished the relaxation in response to the highest concentrations of the PAR-1 and PAR-2 activating peptides without affecting the relaxation to isoproterenol (1 μM) (Fig. 2; Table 1). Moreover, the contraction to carbachol (1 μM) was not significantly modified in the presence of nifedipine (2.5 ± 0.2 g before and 2.3 ± 0.3 g after nifedipine, $n = 5$, $P > 0.05$). Ryanodine (10 μM) by itself did not modify the mechanical spontaneous activity or the contraction to carbachol (1 μM) (2.8 ± 0.4 g before and 2.6 ± 0.5 g after ryanodine, $n = 7$, $P > 0.05$). It significantly reduced both the inhibitory effects on the circular muscle and the contractile effects on the longitudinal muscle induced by activation of PAR-1 and PAR-2 (Fig. 3). It failed to affect the longitudinal muscle relaxation in response to SFLLRN-NH_2 (1–10 μM) or SLIGRL-NH_2 (10 μM) (Table 1).

Effects of Inhibitors of PLC, PKC, and Tyrosine Kinase. In the rat duodenal smooth muscle, the PAR-1-induced effect depends on the activation of PLC and PKC (Kawabata et al., 1999; 2000b); therefore, we verified the involvement of these enzymes in the signal transduction responsible for the PAR-1- and PAR-2-induced effects in rat colon. U73122 (0.5–1 μM), a PLC inhibitor, significantly reduced the PAR-1- and PAR-2-induced contraction in a concentration-dependent manner (Fig. 4). U73122 at a concentration of 5 μM, which slightly reduced the spontaneous contractions, abolished the contractile response of the longitudinal muscle to SFLLRN-NH_2 and SLIGRL-NH_2 but did not modify the inhibitory response on the circular muscle (Fig. 4). In the presence of U73122, the longitudinal muscle relaxation in response to high concentrations of the PAR-1 and PAR-2 peptides could not be observed, probably due to lacking of mechanical tone. GF109203X (1 μM), a PKC inhibitor, which per se failed to affect the spontaneous activity, significantly reduced the PAR-1- and PAR-2-induced longitudinal muscle contractile effect without affecting the relaxation (Fig. 5 and Table 1). In the presence of GF109203X, the inhibitory response to peptides of the circular muscle persisted unaltered (Fig. 5). Moreover, GF109203X at 1 μM was found to completely block contractions elicited in the preparation by the PKC activator phorbol dibutyrate.

Since tyrosine kinase has been reported to be involved in apamin-sensitive relaxation of longitudinal colonic muscle (Takeuchi et al., 1999) as well as in the PAR-evoked gastric contraction in rats (Saifeddine et al., 1996), we also tested the effects of PAR-1 and PAR-2 activating peptides in the presence of genistein, a tyrosine kinase inhibitor. Genistein (1–10 μM) decreased the amplitude of the spontaneous contractions and significantly reduced in a concentration-dependent manner the contractile and the relaxant effects on the longitudinal muscle in response to PAR-1 activating peptide. It did not affect, however, the inhibitory effects on the circular muscle. Maximal inhibition for SFLLRN-NH_2-induced contractions was observed at 7.5 μM. Similar results were obtained with PAR-2 activating peptide (Fig. 6; Table 1). The suppressive effects of the two kinase inhibitors genistein (7.5 μM) and GF109203X (1 μM) when coadministered on the PAR-1- and PAR-2-evoked longitudinal contraction were additive (Fig. 7). The combined pretreatment of the colonic segment with genistein (7.5 μM) and GF109203X (1 μM)

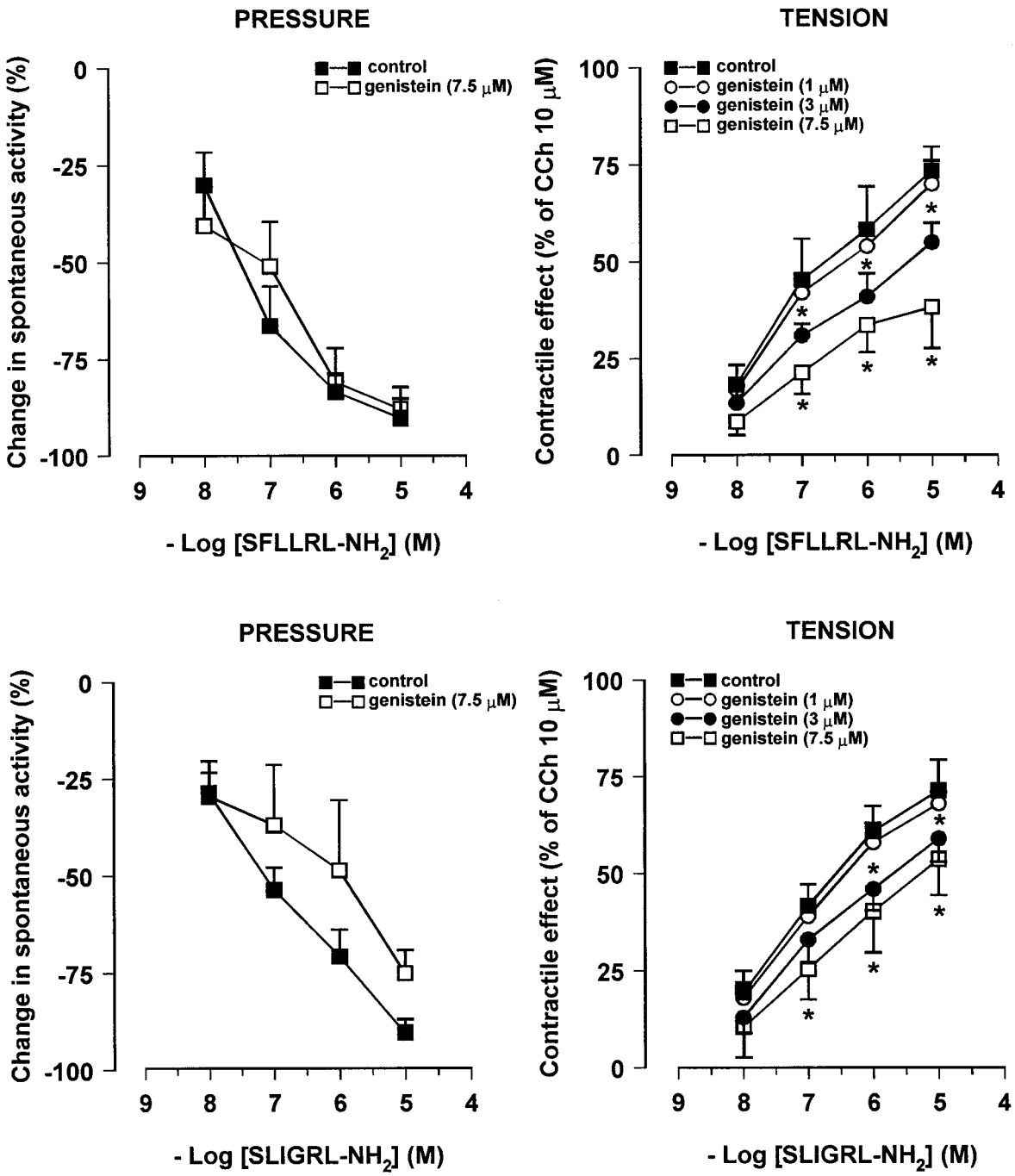

Fig. 6. Concentration-response curves for effects evoked by PAR-1 and PAR-2 agonists on the pressure waves and on the tension oscillations of the rat colon in the absence and in the presence of genistein, a tyrosine kinase inhibitor. The inhibitory responses of circular muscle are expressed as the percent change from the resting spontaneous activity (−100% corresponds to the abolition of spontaneous activity). The contractile effects of longitudinal muscle are expressed as a percentage of the maximal contraction to carbachol (10 μM). Each value is mean ± S.E. of six experiments. ⋆, $P < 0.05$ compared with the control value.

abolished the spontaneous contractions; therefore, possible additive effects on the inhibitory response of the circular muscle could not be examined. Lastly, genistein (7.5 μM) failed to further reduce the PAR-1 or PAR-2-induced longitudinal muscle contraction residual to nifedipine (1 μM) (Fig. 7). The contractile effects to PAR-1 and PAR-2 agonist peptides, however, were completely abolished by the coadministration of nifedipine (1 μM), genistein (7.5 μM), and GF109203X (1 μM) (Fig. 7).

Discussion

The present results suggest that the signal pathways activated by PAR-1 and PAR-2 receptors to cause contractile or inhibitory responses are the same in rat colon. Activation of PLC, PKC, tyrosine kinase, and intracellular and extracellular calcium are involved in the contractile effects of the longitudinal muscle. Influx of extracellular Ca^{2+} through L-type voltage-dependent channels or release of Ca^{2+} from intracel-

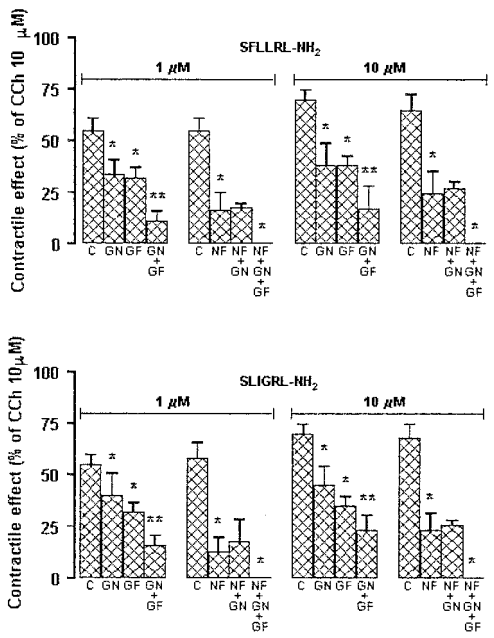

Fig. 7. Histograms showing the contractile effects on longitudinal muscle exerted by the PAR-1 agonist SFLLRN-NH_2 (1–10 μM) or by the PAR-2 agonist SLIGRL-NH_2 (1–10 μM) in the absence or in the presence of genistein (GN; 7.5 μM), GF109203X (GF; 1 μM), and nifedipine (NF; 1 μM), alone or in combination. The contractile responses are expressed as a percentage of the maximal contraction to carbachol (10 μM). Each value is the mean ± S.E. of at least five experiments. Data refer to two different sets of experiments. ⋆, $P < 0.05$ compared with the control value. ⋆⋆, $P < 0.05$ compared with genistein or GF109203X alone.

lular stores are determining for the opening of the apamin-sensitive K^+ channels responsible for longitudinal muscle relaxation or circular muscle inhibitory response, respectively.

Based on our own recent data (Mulè et al., 2002) and on work of others (Hamilton and Cocks, 2000), we are confident that our results, using the receptor-activating peptide SFLLRN-NH_2, reflect the selective activation of PAR-1 and not of PAR-2 because SFLLRN-NH_2 mimicked the effects of TFLLR-NH_2, a highly specific PAR-1 analog (Hollenberg et al., 1997), and the response to SFLLRN-NH_2 was not affected by prior desensitization to SLIGRL-NH_2.

The intestinal smooth muscle responses and the signal transduction mechanisms coupled with PAR activation do not appear to be the same in the intestinal smooth muscle and depend on the tissues and the animal species. Moreover, the signal transduction pathways triggered by PAR-1 and PAR-2 are complex and the "cascade" processes are not completely understood. In fact, although activation of L-type calcium channels, cyclooxygenase, protein kinase C, and tyrosine kinase for contractile effects (Hollenberg et al., 1992; Zheng et al., 1998; Kawabata et al., 1999; Kawabata et al., 2000b) and activation of apamin-sensitive K^+ channels for relaxant effects (Cocks et al., 1999; Kawabata et al., 1999, 200b; Mulè et al., 2002) appear to be involved, the sequence of events leading to intestinal smooth muscle contraction or relaxation has not been yet clarified. Moreover, indomethacin-sensitive cyclooxygenase products might act in parallel to drive the effects. In our preparation, however, no role for cyclooxygenase products in PAR-mediated responses was previously pointed out (Mulè et al., 2002).

In our preparation, the contractile responses to PAR-1 and PAR-2 activating peptides were greatly reduced by nifedipine suggesting that contractile effects to PAR-1 and PAR-2 are mediated through the activation of L-type Ca^{2+} channels. Moreover, nifedipine also abolished the relaxation of the longitudinal muscle evoked by PAR-1 and PAR-2 peptide agonists, which we previously demonstrated to be apamin-sensitive. This finding suggests that the activation of the apamin-sensitive, Ca^{2+}-activated, small conductance K^+ channels is also mediated by calcium influx through L-type channels. A similar result was reported in mouse gastric fundus (Cocks et al., 1999). It is likely that the calcium concentration enhances in close proximity to the sarcolemma, evoking outward hyperpolarizing currents due to opening of apamin-sensitive K^+ channels and subsequent closure of the voltage-dependent Ca^{2+} channels responsible for the relaxation in response to the high concentrations of SFLLRN-NH_2 and SLIGRL-NH_2. The specificity of the effect of nifedipine was confirmed by the findings that the inhibitor of the Ca^{2+} channels did not alter the contraction to carbachol or the relaxation induced by isoproterenol. The finding that both the longitudinal muscle contraction and the circular muscle inhibitory effects induced by PAR-1 and PAR-2 activating peptides were also inhibited by ryanodine, an inhibitor of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum, suggests that Ca^{2+} release from the sarcoplasmic reticulum triggered by PAR activation contributes to the contraction and it is somehow linked to the circular muscle inhibitory effect in this tissue. Therefore, in the circular muscle, Ca^{2+} derived from sarcoplasmic reticulum is involved in the activation of the apamin-sensitive K^+ channels, which are responsible for the inhibitory effects. PAR-1- and PAR-2-mediated relaxation via ryanodine-sensitive and -insensitive activation of small-conductance, Ca^{2+}-activated K^+ channels has been previously reported in mouse gastric fundus (Cocks et al., 1999).

Moreover, our data imply that the contraction of the rat colonic longitudinal muscle evoked by the activation of PAR-1 and PAR-2 is mediated by PLC and, in addition, the activation of PKC and tyrosine kinase participate in the signaling transduction. In fact, the inhibitor of PLC, U73122, antagonized the contractile responses in a concentration-dependent manner to PAR-1 and PAR-2 agonists in the longitudinal muscle, up to blocking it, without affecting the inhibitory response in the circular muscle, indicating that the contraction is entirely dependent by activation of PLC. Partial involvement of PLC in the PAR-1-mediated rat duodenal muscle has been already reported (Kawabata et al., 2000b). Moreover, we tested the possible involvement of PKC and tyrosine kinase because it is well known that PLC produces diacylglycerol that in turn activates PKC and recent data indicate that tyrosine kinase is also involved in the signal transduction associated with rat intestinal contractions (Ohta et al., 2000) or relaxation (Takeuchi et al., 1999) through the modulation of L-type calcium channels or apamin-sensitive K^+ channels, respectively. In our preparation, the protein kinase C inhibitor GF109203X at a concentration sufficient to block contraction induced by a PKC activator, phorbol dibutyrate, and the tyrosine kinase inhibitor genistein concentration dependently significantly reduced, but not abolished, the contractile effects, indicating an involvement of both enzymes in the mechanism triggered by PAR-1 or PAR-2 activation. The observation that the com-

bined inhibitory effect of the two-protein kinase inhibitors was greater than that of each one suggests that the activation of PKC and tyrosine kinase are two distinct steps of different pathways acting in parallel. On the contrary, the suppressive effects induced by genistein were not additive with those induced by nifedipine suggesting that tyrosine kinase and Ca^{2+} channels probably represent two steps of the same sequential pathway leading to contraction. One suggested hypothesis is that genistein inhibits the activation of the Ca^{2+} channels. In fact, tyrosine phosphorylation has been reported to modulate the L-type calcium channels (Liu and Sperelakis, 1997; Evans and Pocock, 1999). Our pharmacological data, however, do not allow us to establish whether phosphorylation of the Ca^{2+} channels occurs in rat colon or to clarify which type of tyrosine kinase is involved. In fact, genistein is reported to be a tyrosine kinase broadly specific inhibitor at the ATP-binding site (Akiyama et al., 1987). Further experiments using biomolecular techniques will need to clarify this point.

It is interesting to note that the PAR-induced relaxation of the longitudinal muscle was significantly also reduced by genistein, indicating that tyrosine kinase is involved in the regulation of the apamin-sensitive K^+ channels in this tissue, although it was not possible establish whether the action of phosphorylation is direct or via other kinases. Tyrosine kinase and PKC, however, do not participate in the mechanism responsible for the inhibitory effects observed in circular muscle in response to PAR-1 and PAR-2 activating peptides.

In conclusion, PAR-1 and PAR-2 mediate inhibitory effects in the circular muscle via apamin-sensitive K^+ channels in which activation depends on Ca^{2+} released from intracellular store. The contraction of the longitudinal muscle in response to PAR-1 and PAR-2 activating peptides is mediated by activation of PLC. In the cascade process, tyrosine kinase and PKC play a parallel role, whereas tyrosine kinase and L-type Ca^{2+} channels would act sequentially. The influx of Ca^{2+} in turn would cause release of Ca^{2+} from sarcoplasmic reticulum. The longitudinal muscle relaxation in response to high concentrations of PARs depends on apamin-sensitive K^+ channels, opened by influx of Ca^{2+} through L-type channels, and modulated by tyrosine kinase.

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