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Analogs of Neurokinin A(4–10) Afford Protection Against Gastroduodenal Ulcers in Rats

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EVANGELISTA, S., C. A. MAGGI, P. ROVERO, R. PATACCHINI, S. GIULIANI AND A. GIACHETTI. *Analogs of neurokinin A(4–10) afford protection against gastroduodenal ulcers in rats*. PEPTIDES **11**(2) 293–297, 1990.—We have developed a novel series of peptides, related to the NKA(4–10) sequence, in which substitution of selected amino acids determined variations in the affinity for the TK receptor subtypes. Subcutaneous pretreatment of rats with some peptides of this series reduced gastric ulcers induced by ethanol, indomethacin or acetylsalicylic acid (ASA) as well as duodenal ulcers induced by dulcerozine. In particular [Ala^5]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10) possess a broad spectrum of antiulcer activity which is long lasting and stronger than the precursor NKA(4–10). The observation that the prevention of ethanol-induced gastric lesions could be reversed by pretreatment with indomethacin favors the possible involvement of prostaglandins in the observed gastroprotection by TK analogs.

Neurokinin A (NKA) NK-2 receptors Gastric and duodenal ulcers Tachykinins (TKs) Peptides Indomethacin Ethanol lesions Acetylsalicylic acid (ASA) Dulcerozine

TACHYKININS (TKs) are a family of peptides which share the common C-terminal sequence (Phe-X-Gly-Leu-Met-NH_2). In mammals, at least three peptide molecules have been proposed to act as neurotransmitters, namely substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). Each peptide displays a high affinity for one of the TK receptor subtypes termed NK-1, NK-2 and NK-3, respectively (1).

TKs are widely distributed in neurons and nerve fibers of the gastrointestinal tract (2,7). In this tissue, neuronal TK-like immunoreactivity originates either from intrinsic neurons (3) or sensory nerve fibers of extrinsic origin, which are sensitive to the neurotoxin capsaicin (9). Although the physiological role of these peptides in the gastrointestinal tract has not been delineated, there are indications that they may participate in the capsaicin-sensitive "gastrointestinal defense mechanisms" (5, 8, 18). In fact, depletion of gastrointestinal content of these peptides by neonatal treatment with capsaicin renders the animals more susceptible to gastric and duodenal injuries caused by noxious stimuli [see (12) for review].

Recently, a number of TKs were shown to exert a gastroprotective action toward the lesions induced by ethanol in rats (4). The order of potency exhibited by TKs in providing protection suggested that this effect might be associated with activation of NK-2 receptors. To substantiate this conclusion we have examined a novel series of peptides, related to the NKA(4–10) sequence in which substitution of selected amino acids determined variations in the affinity for the TK receptors subtypes, in some experimentally induced gastroduodenal ulcers.

METHOD

Sprague-Dawley rats of both sexes, weighing 180–210 g, were housed at constant room temperature (21 ± 1°), relative humidity (60%) and with 12 hr light-dark cycle (light on 6:00 a.m.).

In Vitro Preparations

All peptides under study were tested on in vitro systems designed to detect the interaction with one of the three receptors: a) NK-1 receptor: contractile response of the isolated guinea pig ileal longitudinal muscle (GPI) (16) in the presence of atropine (3 μM) and chlorpheniramine (1 μM); b) NK-2 receptor: potentiation of the nerve-mediated contractions of the rat isolated vas deferens (RVD) (14,19); c) NK-3 receptor: contraction of the rat isolated portal vein (RPV) (13,16).

All experiments were performed in Krebs solution of the following composition (in mM): NaCl 119; $NaHCO_3$ 25; KCl 4.7; $MgSO_4$ 1.5; KH_2PO_4 1.2; $CaCl_2$ 2.5 and glucose 11. The solution was gassed with 96% O_2 and 4% CO_2. Initial load was 0.5 g. Concentration-response curves were performed in a cumulative (for RVD) or noncumulative manner (20–30 min between doses).

Induction of Gastric Ulcers

Male rats were housed 24 hr in plastic cages with wire bottom to minimize coprophagy and deprived of food but not water. The peptides to be tested were administered subcutaneously at a dose (0.5 mg kg^{-1}) selected in previous investigations (4), and 30 min

TABLE 1

BIOLOGICAL ACTIVITY OF NKA, NKA(4–10) AND ANALOGUES ON THE GUINEA PIG ILEUM (GPI), RAT VAS DEFERENS (RVD) AND RAT PORTAL VEIN (RPV)

	GPI			RVD			RPV		
Peptide	pD_2*	RA†	a‡	pD_2*	RA†	a‡	pD_2*	RA†	a‡
NKA	7.48 ± 0.19	100	1.0	6.52			6.45		
NKA(4–10)	7.43 ± 0.11	100	1.0	5.98 ± 0.15	100	1.0	6.79 ± 0.05	100	1.0
[βAla^8]NKA(4–10)	6.61 ± 0.24	15	0.8	6.91 ± 0.05	851	1.4	6.01 ± 0.09	17	1.0
[Ala^5,βAla^8]NKA(4–10)	7.14 ± 0.19	51	0.8	6.72 ± 0.08	549	1.5	6.72 ± 0.12	85	1.2
[Ala^5]NKA(4–10)	8.16 ± 0.12	537	0.9	6.40 ± 0.10	263	1.0	6.05 ± 0.23	18	1.8
[βAla^5]NKA(4–10)	7.23 ± 0.15	63	1.0	5.64 ± 0.24	46	1.0	6.19 ± 0.10	25	1.0
[βAla^6]NKA(4–10)	inactive			inactive			inactive		

*pD_2: − log of molar concentration of agonist producing 50% of maximal response. Each value is mean ± S.E. of at least 4 experiments.

†RA: relative affinity expressed as % of the affinity of NKA(4–10).

‡a: intrinsic activity expressed as a fraction of the maximal effect of NKA(4–10).

later the rats received the ulcerogens: 96% ethanol (5 ml kg^{-1} rat), indomethacin (20 mg kg^{-1}) or acetylsalicylic acid (ASA, 150 mg kg^{-1}) by gavage.

Dose-effect and time-dependence studies of some peptides were performed as detailed in Figs. 1 and 2.

The rats were killed 1 hr after the ethanol or ASA challenge and 5 hr after indomethacin challenge and their stomachs removed, opened along the greater curvature and examined for the presence of gastric lesions. The degree of gastric ulcers was scored according to an arbitrary scale (0 to 4 in relation to size and intensity) by an observer unaware of the treatment (5) and their length measured by means of a stereomicroscope (× 10).

In other experiments rats were given indomethacin (5 mg kg^{-1} PO) 1 hr before the peptides, followed by the ethanol challenge as described above. Indomethacin pretreatment has been reported to decrease PGI_2 and PGE_2 gastric mucosal content (10).

Induction of Duodenal Ulcers

Fasted female rats received the peptides (0.5 mg kg^{-1} SC) 1 hr before and 4 and 23 hr after the ulcerogen dulcerozine [300 mg kg^{-1} PO; see (11)]. Duodenal ulcers were evaluated following an arbitrary scale: 0 = no ulcers, 0.5 = redness, 1 or 1.5 = superficial mucosal erosions covering a small or a large duodenal area, 2 or 2.5 = deep ulcer usually with a small or large transmural necrosis, 3 or 3.5 = small or large perforated ulcer, 4 = death (6).

Drugs

The peptides were synthetized in our peptide synthesis laboratory by a conventional solid-phase method and purified by gel filtration and preparative high pressure liquid chromatography as described previously (17). They were dissolved in a few drops of ethanol (maximum 2% of the final solution), diluted in saline and administered in a volume of 5 ml kg^{-1}. Indomethacin (Sigma) and dulcerozine (Daiichi, Japan) were suspended in a vehicle containing 0.9% NaCl, 0.5% carboxymethylcellulose and 0.5% Tween 80 while ASA (Sigma) was suspended in 0.1 M HCl and Tween 80 (1 drop per 20 ml).

Statistics

Data relative to degree and length of gastric lesions were analyzed by means of Smirnov's test for nonparametric samples and ANOVA followed by a multiple comparison test, respectively.

RESULTS

In Vitro Studies

The C-terminal heptapeptide NKA(4–10) represents the minimal sequence of NKA maintaining full biological activity at the TK receptors (16). Substitution of the natural glycine in position 8 with β-alanine gives rise to a potent and selective NK-2 receptor agonist {[βAla^8]NKA(4–10)} more potent than NKA(4–10) on RVD (Table 1). Similar substitution in position 5, e.g., β-alanine in place of serine {[βAla^5]NKA(4–10)}, results in a limited loss of potency without alteration of the spectrum of activity at the three receptors (Table 1). On the contrary, introduction of alanine in position 5 {[Ala^5]NKA(4–10)} considerably enhances the apparent affinity of the peptide for the NK-1 and NK-2 receptors (Table 1). A further increase in affinity for the NK-2 receptor, but not for the NK-1, is obtained by introducing β-alanine in position 8 {[Ala^5,βAla^8]NKA(4–10)}. Finally, introduction of β-alanine in position 6 in place of the natural phenylalanine {[βAla^6]NKA(4–10)} gives rise to a completely inactive peptide (Table 1).

Ulcer Studies

NKA, NKA(4–10), [Ala^5]NKA(4–10) and [Ala^5,βAla^8] NKA (4–10), but not [βAla^8]NKA(4–10), [βAla^5]NKA(4–10) and [βAla^6]NKA(4–10), reduced degree of gastric lesions induced by ethanol (Table 2).

NKA(4–10) and [Ala^5,βAla^8]NKA(4–10) were the more effective lesion-reducing peptides in this test since they were also able to reduce the length of gastric lesions (Table 2).

Gastric ulcers induced by indomethacin were reduced in score and length only by [Ala^5]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10) (Table 2). These latter peptides and NKA(4–10) were able to reduce the parameters examined (score and length) of ASA-induced gastric ulcers (Table 2).

Duodenal ulcers induced by dulcerozine were prevented only by the administration of [Ala^5]NKA(4–10) and [Ala^5,βAla^8] NKA(4–10), the former being the more effective antiulcer peptide in this model (Table 2).

Protective effect exerted by peptides toward gastric and duodenal lesions can be summarized as follows (see Table 2): 1)

TABLE 2

EFFECT OF NKA, NKA(4–10) AND ANALOGS (0.5 mg kg^{-1} SC) ON EXPERIMENTALLY INDUCED GASTRODUODENAL ULCERS

	Gastric Ulcers						Duodenal Ulcers
Ulcerogen:	Ethanol		Indomethacin		ASA		Dulcerozine
Treatments	Score	Length	Score	Length	Score	Length	Score
Controls	2.40	28.2 ± 8.5	2.38	16.4 ± 3.3	2.15	13.6 ± 2.8	2.35
NKA	1.33*	13.3 ± 7.0	2.30	14.8 ± 4.2	2.02	11.7 ± 3.1	2.63
NKA(4–10)	1.28†	9.2 ± 5.8*	2.38	15.6 ± 4.1	1.10*	4.5 ± 1.3*	2.33
[Ala^5]NKA(4–10)	1.30*	13.0 ± 8.1	1.18†	2.4 ± 1.4†	0.50*	1.8 ± 1.1†	0.77†
[βAla^8]NKA(4–10)	2.20	17.6 ± 6.0	1.64	5.4 ± 1.6*	2.10	12.0 ± 4	2.78
[Ala^5,βAla^8]NKA(4–10)	0.91†	4.0 ± 2.3*	0.70†	3.4 ± 2.4†	0.80*	3.0 ± 2.5*	1.22*
[βAla^5]NKA(4–10)	1.76	15.8 ± 6.1	2.65	20.9 ± 7.9	1.52	8.4 ± 3.5	1.52
[βAla^6]NKA(4–10)	2.50	32.5 ± 9.9	2.47	21.8 ± 8	1.83	9.2 ± 3.5	2.29

*$p<0.05$ and †$p<0.01$ as compared to controls. n = 5–8 for each group.

Peptides providing significant protection in all experimental models examined {[Ala^5]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10)}. 2) Peptides marginally active toward gastric lesions {NKA, NKA(4–10) and [βAla^8]NKA(4–10)}. 3) Peptides showing no protective effect at the dose tested {[βAla^5]NKA(4–10) and [βAla^6]NKA(4–10)}.

Figures 1 and 2 depict dose- and time-dependency studies performed with the two peptides exerting the broadest pattern of protective activity, namely [Ala^5]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10). The model employed was the gastric ulcers produced by ASA administration. Figure 1 shows that [Ala^5,βAla^8]NKA(4–10) and [Ala^5]NKA(4–10) exerted dose-related protective effect in the range of doses examined (50–500 μg kg^{-1} SC).

When tested at the highest active dose, both peptides exhibited considerable gastroprotection up to 8 hr after administration with a maximum effect between 0.5 and 2 hr (Fig. 2).

Other experiments studied the influence of indomethacin pretreatment upon the protection from ethanol lesions afforded by NKA(4–10), [Ala^5]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10). It appears from results reported in Fig. 3 that indomethacin curtails the activity of the latter two peptides whereas that of NKA(4–10) was unaffected.

DISCUSSION

The previous observations from this laboratory indicated that the TKs displaying high affinity for the NK-2 subtype such as NKA, NKA(4–10) and the amphibian peptide kassinin also possessed gastroprotective effects toward ethanol-induced lesions (4), raising the possibility of a causal relationship between antiulcer effect (at least against ethanol lesions) and NK-2 receptor agonistic properties.

We have attempted to verify this assumption by investigating a series of NKA(4–10) analogs in which replacement of selected

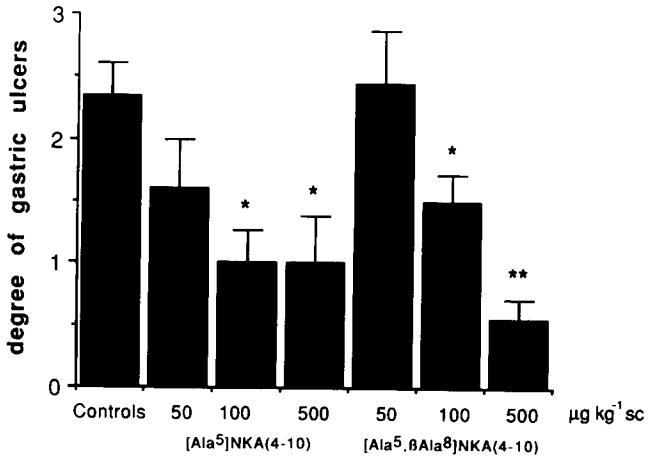

FIG. 1. ASA ulcers. Effect of different doses of [Ala^5]NKA(4–10) or [Ala^5,βAla^8]NKA(4–10), administered 30 min before ASA challenge, on degree of gastric ulcers induced by acetylsalicylic acid (ASA; 150 mg kg^{-1} PO). Mean ± S.E., n = 12, *$p<0.05$ and **$p<0.01$ as compared to controls.

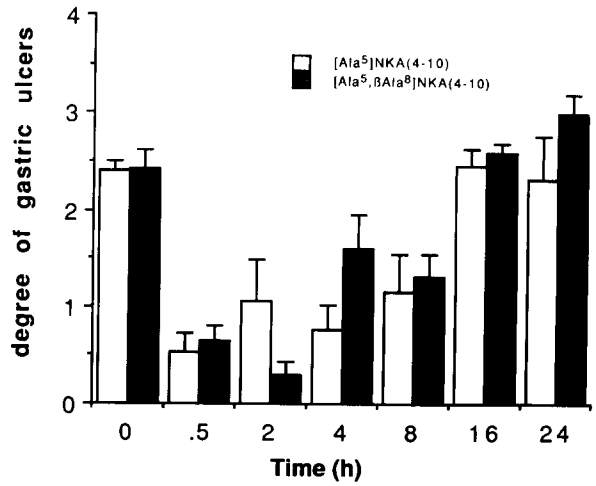

FIG. 2. ASA ulcers. Effect of pretreatment at different times before ASA challenge with 0.5 mg kg^{-1} of [Ala^5]NKA(4–10) or [Ala^5,βAla^8]NKA(4–10) on degree of gastric ulcers induced by acetylsalicylic acid (ASA; 150 mg kg^{-1} PO). Mean ± S.E., n = 12.

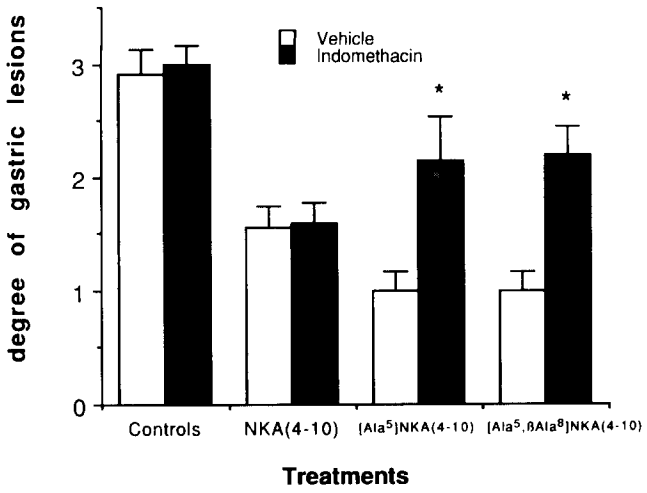

FIG. 3. Effect of indomethacin (5 mg kg^{-1} PO, 90 min before ethanol) pretreatment on 0.5 mg kg^{-1} SC (30 min before ethanol) of NKA(4–10)-, [Ala^5]NKA(4–10)- or [Ala^5,βAla^8]NKA(4–10)-induced inhibition of degree of gastric lesions induced by 96% ethanol. Mean ± S.E., n = 12, $*p<0.05$ as compared to respective group treated with vehicle.

amino acids in the peptide sequence yields compounds with various degrees of selectivity for the three TK receptor subtypes. Reasoning that TKs might influence a more extensive gastrointestinal protective mechanism rather than opposing specific noxious stimuli (e.g., ethanol), we extended previous investigations to include gastric and duodenal lesions induced by a variety of agents.

Analysis of the receptor interaction studies shows that, in the limited series of peptides synthesized, three compounds demonstrated considerable improvement over the heptaptide NKA(4–10) of agonist affinity and/or selectivity or both. Introduction of a β-alanine in position 8, as exemplified by compounds [βAla^8]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10), appears to confer to the peptides increased affinity for the NK-2 receptor accompanied by a reduced activity at both NK-1 and NK-3 receptors. Thus, both compounds resulted more selective for the NK-2 subtype. The introduction of alanine residue in position 5 enhances the agonist activity, particularly at the NK-1 receptor with a moderate effect on NK-2-mediated activity. The results obtained with the peptide in which β-alanine was introduced in position 6, showing complete loss of agonistic and antagonistic properties (data not reported), are of great interest since they demonstrate that the natural amino acid phenylalanine is crucial for receptor interaction.

As for the protective effects exerted by the peptides, it is difficult to draw any firm conclusion from the observations made in this study about the involvement of NK-2 receptor in mediating this action. However, some of the results summarized in Table 2 clearly favor a relationship between NK-2 receptor activation and gastrointestinal protection. For example, the peptides containing the alanine residue in position 5 exert potent protective effects on all ulcer models and have a distinctly higher affinity for the NK-2 sites than the parent compound NKA(4–10). Supporting the receptor hypothesis, although indirectly, is the observed inactivity of [βAla^6]NKA(4–10) in ulcer models and in stimulating NK receptors. On the other hand, several discrepancies can be noted. As an example, [βAla^8]NKA(4–10), which is a potent and selective NK-2 agonist, was distinctly less active as antiulcer than [Ala^5]NKA(4–10) or [Ala^5,βAla^8]NKA(4–10). Thus, an alanine residue in position 5 seems to improve the antiulcer activity of the NKA(4–10) derivatives, an effect not shared by β-alanine in the same position. In terms of affinity for the three receptors, the only major change produced by alanine in position 5 is to increase potency at NK-1 receptors. However, it seems unlikely that this effect can be related to the antiulcer activity of these peptides, because [Ala^5,βAla^8]NKA(4–10), ten times less potent than [Ala^5]NKA(4–10) at NK-1 sites, exhibited the same broad spectrum of antiulcer activity.

Apart from changes in affinity for the various TK receptors, substitutions in position 5 and 8 of the NKA(4–10) backbone might have improved the antiulcer activity of peptides also by confering resistance to metabolism. Relatively little information is available about inactivation of NKA, however, the Ser^5-Phe^6 and Gly^8-Leu^9 bonds of NKA and NKA(4–10) are sites for enzymatic cleavage of these peptides by endopeptidase 24.11 (20). Indeed, we have preliminary data showing that replacement of Gly^8 with β-alanine confers partial resistance to endopeptidase metabolism (15).

An indirect effect, possibly mediated by prostaglandins, is indicated by the experiments with indomethacin. However, this may not apply to all peptides since gastroprotective properties of NKA(4–10) are unaffected by indomethacin.

At this stage the possibility cannot be ruled out that the antiulcer activity of tested peptides might involve systemic and/or local cardiovascular changes. A systemic cardiovascular effect seems unlikely for [βAla^8NKA(4–10), which is virtually devoid of significant effects on blood pressure or heart rate up to a dose of 30 nmol kg^{-1} IV (Maggi *et al.*, submitted). Indeed, preliminary data indicate that [Ala^5]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10) have little if any gastric antisecretory activity in rats.

Clearly, further studies are needed to firmly assess the mechanism and site of action of the antiulcer effect described in this study, but the present results indicate that the NK-2 agonists [Ala^5]NKA(4–10) and [Ala^5,βAla^8]NKA(4–10) possess a broad spectrum of antiulcer activity which is stronger and longer lasting than the precursor NKA(4–10).

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