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Investigation on the mechanisms involved in the central protective effect of amylin on gastric ulcers in rats

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1 The mechanisms involved in the protective effect of amylin (administered into the brain ventricle, i.c.v.) on gastric ulcers induced by the oral administration of ethanol 50% (EtOH, 1 ml/rat) or indomethacin (indomethacin, 20 mg kg^{-1}, at a dosing volume of 5 ml) were investigated in rats.

2 The possible involvement of endogenous nitric oxide (NO) in the beneficial effect of amylin against EtOH-induced ulcers was examined. The inhibitor of NO-synthesis, N^G-nitro-L-arginine methyl ester (L-NAME, 70 mg kg^{-1}, s.c.) was injected 30 min before amylin (2.2 μg/rat, i.c.v.) followed by EtOH after a further 30 min. Rats were sacrified 1 h after EtOH. L-NAME completely removed the protective effect of amylin.

3 The interaction between amylin and gastric nonprotein sulfhydryl groups was studied. The rats were treated with N-ethyl-maleimide (NEM, 25 mg kg^{-1}, s.c.) 30 min before amylin (2.2 μg/rat, i.c.v.) followed by EtOH 30 min after or by indomethacin 5 min after amylin. Rats were sacrified 1 h or 6 h respectively after EtOH or indomethacin. NEM counteracted the protective effect of amylin against EtOH-induced ulcers but not against those provoked by indomethacin.

4 To determine whether amylin was able to promote ulcer healing, the peptide was injected 5 min after EtOH or 1 h after indomethacin. In the case of EtOH, the beneficial effect of amylin was lost whereas it was still effective on indomethacin-induced ulcers.

5 The results indicate that: the mechanisms involved in the antiulcer effects of amylin are different in these two types of gastric lesions probably because of the different etiopathology of various types of ulcers. Endogenous NO and nonprotein sulfhydryl groups are involved in the mucosal protective effects of amylin on EtOH and not on indomethacin-induced ulcers. Furthermore the effectiveness of amylin against indomethacin-induced lesions when administered after the ulcerogenic process has started suggests that amylin is involved not only in the protection but also in the healing mechanisms in this type of ulcer.

Keywords: Amylin; gastric ulcers; nitric oxide; gastric mucosa sulfhydryls; ethanol; indomethacin

Introduction

Amylin is a 37-amino acid peptide that is mainly expressed in the islets of Langerhans in the pancreas (Cooper *et al*., 1987). As shown for other pancreatic hormones such as glucagon, somatostatin and pancreatic polypeptide, amylin-like immunoreactivity has also been detected in a variety of other tissues (Ferrier *et al*., 1989) including the mammalian gastro-intestinal tract (Toshimori *et al*., 1990; Miyazato *et al*., 1991) where the amylin message is expressed in the endocrine cells in the antrum and fundus of the rat stomach in a population of somatostatin-immunoreactive cells (Mulder *et al*., 1994; Pittner *et al*., 1994).

The peptide inhibits acid gastric secretion (Guidobono *et al*., 1994) and protects from gastric erosion produced in various experimental models in rats (Guidobono *et al*., 1997; Clementi *et al*., 1997). The activity of amylin on the stomach is remarkable and seems to be mediated by specific receptors for the peptide identified in several organs including the stomach and the central nervous system (Bhogal *et al*., 1992). Amylin binding sites are localized in the brain in areas involved in the control of gastro-intestinal functions such as the hypothalamus, the amygdala and the area postrema (Beaumont *et al*., 1993; Sexton *et al*., 1994; van Rossum *et al*., 1994). The gastroprotective effect in our experimental conditions, appears not to be dependent only on the inhibition of acid gastric secretion, which occurs when amylin is given by central and peripheral routes, whereas the protective effects of amylin on indomethacin- and ethanol-(EtOH)-induced lesions are detectable only when the peptide is injected directly into the brain (Guidobono *et al*., 1997). In a different experimental system, other authors found that amylin peripherally administered, reduces EtOH-induced gastritis in the short run (Gedulin *et al*., 1997). In this context it is important to outline that amylin behaves differently from the structurally related peptides calcitonin and CGRP which are not able to protect against EtOH-induced gastric lesions (Taché *et al*., 1988; Clementi *et al*., 1993).

Despite previous observations on the involvement of the prostaglandin system in the prevention of EtOH-induced ulcers by amylin (Guidobono *et al*., 1997), the possible mechanisms underlying the antiulcer properties of amylin remain to be clarified considering that the pathogenetic mechanisms vary with the model of ulcer used (Cho & Ogle, 1992). The fact that inhibition of nitric oxide (NO) synthesis did not remove the beneficial effect of amylin on indomethacin-induced lesions (Guidobono *et al*., 1997) does not exclude the participation of the NO-generating system in other types of ulcers. In this respect considering that changes in mucosal blood flow might play a key role in the pathogenesis of gastric ulceration induced by EtOH the possible involvement of NO, the major second messenger of endothelium relaxation (Moncada *et al*., 1991), in this type of ulcer was considered. Furthermore since gastric nonprotein sulfhydryl groups are one of the mucosal defence mechanisms against ulcerogenic

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agents (Szabo *et al*., 1981) we tested whether or not a sulfhydryl depletor drug N-ethyl-maleimide (NEM) could remove the beneficial effects of amylin in both EtOH and indomethacin-induced ulcers. Another aim of our study was to examine the possibility that amylin could have an effect not only in protection but also in healing mucosal damage. For this purpose the peptide was administered after the ulcerogenic agents when the mucosal damage is already in process.

On the basis of the results obtained showing that the time of amylin administration is critical for the protective effects against EtOH-ulcers we investigated whether or not the ineffectiveness of calcitonin in this type of ulcer (Taché *et al*., 1988) was due to the different protocol used compared with that for amylin.

Methods

Animals

Male Sprague-Dawley rats, weight range 180–200 g (Charles River, Calco, Italy) were placed in single cages which had wire-net floors to prevent coprophagy. All experiments were performed in conscious animals that were deprived of food for 24 h but given free access to water until 30 min before the beginning of experiments. Animals for i.c.v. studies were implanted with a polyethylene cannula (PE10) in the left lateral ventricle, 5 days before the experiment, as previously described (Guidobono *et al*., 1994).

Drugs

Rat amylin or salmon calcitonin (Peptide Institute, Inc. Japan) were dissolved in saline and administered intracerebroventricularly (i.c.v.) at concentrations suitable to be administered in 5 μl/rat. Ethanol (EtOH, BDH) was diluted to 50% in double distilled water and was given orally in a volume of 1 ml/rat. Indomethacin (Indomethacin, Sigma) administered orally was suspended in arabic gum at a concentration of 20 mg kg^{-1}, at a dosing volume of 5 ml. N^G-intro-L-arginine methyl ester (L-NAME, Tocris Cookson) was dissolved in saline to a concentration of 70 mg kg^{-1}, at a dosing volume of 1 ml. The dose used is far higher than that needed to raise mean arterial blood pressure in rats (Holzer *et al*., 1993). N-ethyl-maleimide (NEM, Sigma) was dissolved in saline at a concentration of 25 mg kg^{-1}, at a dosing volume of 1 ml and was injected subcutaneously (s.c.). Rats acting as controls were given a similar volume treatment of saline or the excipient by the same route.

Experimental procedures

Ulcers were induced by intragastric instillation of Indomethacin (20 mg kg^{-1}) or EtOH 50%. In one group of experiments we used the inhibitor of nitric oxide-synthase activity, L-NAME, in order to investigate the role of endogenous nitric oxide (NO) on the protective effect of amylin on gastric lesions induced by EtOH. L-NAME was administered at the dose of 70 mg kg^{-1}, s.c. 30 min before amylin (2.2 μg/rat, i.c.v.) followed by EtOH 30 min thereafter. Animals were killed 1 h after EtOH administration. In order to study the role of gastric sulfhydryl groups in the beneficial effect of amylin on ulcers, another group of experiments was carried out in which rats were treated with the sulfhydryl alkylating compound, NEM (25 mg kg^{-1}, s.c.) 30 min before amylin (2.2 μg/rat, i.c.v.) followed by EtOH 30 min thereafter or by indomethacin (20 mg kg^{-1}, o.s.) 5 min after. Animals were killed 1 h after EtOH and 6 h after indomethacin treatment.

To see whether or not amylin (2.2 μg/rat, i.c.v.) was able to protect against ulcer development when the lesions were already in process, the peptide was administered 5 min after EtOH and 1 h after indomethacin. Rats were killed 1 h after EtOH and 6 h after indomethacin. On the basis of the results obtained an experiment was performed to test the effect of central administration of salmon calcitonin (10- 100- 1900 ng/rat, i.c.v.). The highest dose of calcitonin used is equimolar to the effective dose of amylin against EtOH-induced ulcers. Treatment was performed 30 min before EtOH and rats were killed 1 h thereafter.

At the end of the experimental period the rats were anaesthetized with ether and the stomachs dissected out and opened along the lesser curvature. Necrotizing lesions were examined macroscopically by two or three observers unaware of the treatment. The lesions were classified by arbitrary scales in which the severity rating and the number of lesions, according to a modified scoring system of Adami *et al*. (1964), was as follows; 0 = no lesions; 1 = haemorrhagic suffusion; 2 = from one to five small ulcers <3 mm; 3 = many ulcers, more than five, or one ulcer of marked size; 4 = many ulcers of marked size; 5 = perforated ulcers. For EtOH ulcers we used a modified scoring system of Martin et al. (1994); 0 = no lesions; 1 = less than five slight lesions; 2 = more than five slight lesions; 3 = from one to three haemorrhagic bands of length <0.5 mm and width >2 mm; 4 = from one to three haemorrhagic bands >5 mm in length; 6 = complete lesions of the mucosa with haemorrhage. Mean scores for each group were calculated and expressed as the ulcer index.

Statistical analysis

All results are expressed as means $\pm$ s.e. Statistical comparisons were performed by one way analysis of variance followed by Bonferroni test. The 5% level of statistical significance was used in all experiments.

Results

Pretreatment of rats with the inhibitor of NO synthase, L-NAME (70 mg kg^{-1}, s.c.) did not increase the gastric lesions induced by EtOH but completely abolished the protective effect of amylin (2.2 μg/rat, i.c.v., Figure 1). Following administration of a sulfhydryl blocker, NEM (25 mg kg^{-1}, s.c.), EtOH-induced gastric damage was greater (although not statistically significant) than after EtOH alone and the effect of amylin (2.2 μg/rat, i.c.v.) disappeared (Figure 2). In contrast to this, amylin (2.2 μg/rat, i.c.v.) was still able to protect against ulcers induced by indomethacin (20 mg kg^{-1}, o.s.) even in rats pretreated with NEM (25 mg kg^{-1}, s.c.), Figure 3. In this experimental condition NEM exibited a tendency to reduce indomethacin ulceration and to potentiate the effect of amylin (although not reaching statistical significance).

In addition amylin was also effective even if administered 1 h after indomethacin, when the complex mechanisms of gastric damage are already in process (Figure 4). The same was not true for the effect of amylin on EtOH-induced lesions. When animals were treated with amylin (2.2 μg/rat, i.c.v.) 5 min after EtOH the peptide lost its beneficial effect on EtOH-induced ulcers (data not shown).

Calcitonin administered i.c.v. 30 min before EtOH failed to protect against the mucosal injury of EtOH (Figure 5), confirming previous data of Taché *et al*. (1988) who

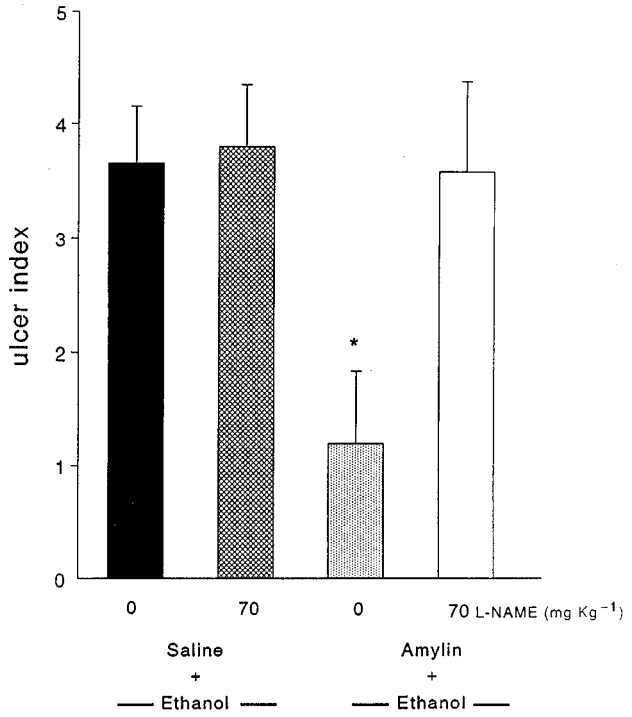

Figure 1 Effect of amylin (2.2 μg/rat, i.c.v.) given 30 min before inducing gastric ulcers by 50% ethanol (EtOH, 1 ml, orally) and in rats pretreated with L-NAME 30 min before amylin. Data are expressed as mean ± s.e.mean of five to six animals. Solid column, EtOH; cross-hatched column, EtOH + L-NAME; stippled column, amylin + EtOH; open column, L-NAME + amylin + EtOH. $^{*}P < 0.05$ vs EtOH.

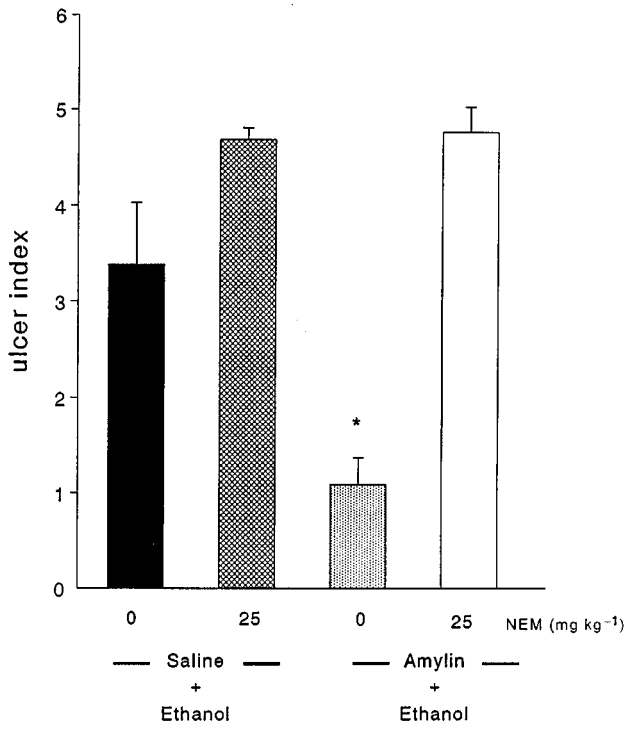

Figure 2 Effect of amylin (2.2 μg/rat, i.c.v.) given 30 min before inducing gastric ulcers by 50% ethanol (EtOH, 1 ml, orally) and in rats pretreated with NEM 30 min before amylin. Data are expressed as mean ± s.e.mean of eight to ten animals. Solid column, EtOH; cross-hatched column, EtOH + NEM; stippled column, amylin + EtOH; open column, NEM + amylin + EtOH. $^{*}P < 0.05$ vs EtOH.

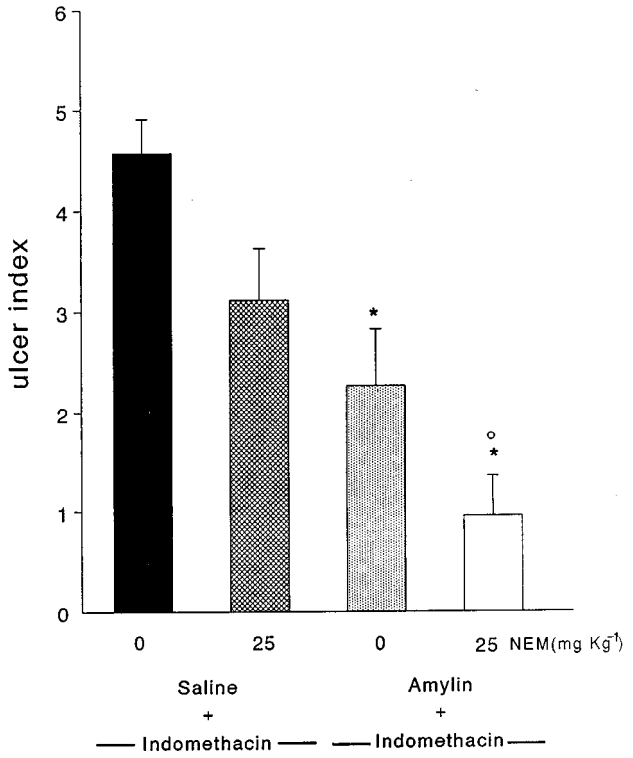

Figure 3 Effect of amylin (2.2 μg/rat, i.c.v.) given 5 min before inducing gastric ulcers with indomethacin (indomethacin, 20 mg kg^{-1}, orally) and in rats pretreated with NEM 30 min before amylin. Data are expressed as mean ± s.e.mean of 10–15 animals. Solid column, indomethacin; cross-hatched column, NEM + indomethacin; stippled column amylin + indomethacin; open column NEM + amylin + indomethacin. $^{*}P < 0.05$ vs indomethacin alone. $^{\circ}P < 0.05$ vs indomethacin + NEM.

administered calcitonin intracisternally only 5–7 min before exposure to EtOH.

Discussion

These results demonstrate that amylin administered i.c.v. prevents EtOH and indomethacin-induced gastric ulcers. In addition, amylin also promotes the healing of indomethacin-induced gastric lesions as shown by its effectiveness when the peptide was administered after indomethacin, i.e. during the development of ulcerations. However the lack of a repairing effect of amylin when administered after EtOH, shows that the mechanisms involved in the beneficial effects of amylin in these two types of experimental ulcers are different. This discrimination is probably due to the fact that the mucosal lesions induced by EtOH and indomethacin involve a different etiopathology not yet completely understood. EtOH disrupts the gastric mucosal barrier and causes profound microvascular changes with strong venocostriction accompanied by arteriolar dilatation responsible for engorgement of mucosal capillaries. Indomethacin, beside producing the well recognized prostaglandin cytoprotective deficiency, also enhances gastric motility which may contribute to the pathogenesis of gastric injuries (Cho & Ogle, 1992; Kapui *et al.*, 1993; Tacheuchi *et al.*, 1994). Our results showing that the blockade of NO synthase by L-NAME prevents the mucosal protective activity of amylin on EtOH-induced ulcers and not those of indomethacin (Guidobono *et al.*, 1997) is consistent with the importance of mucosal blood flow for the protection against EtOH-induced

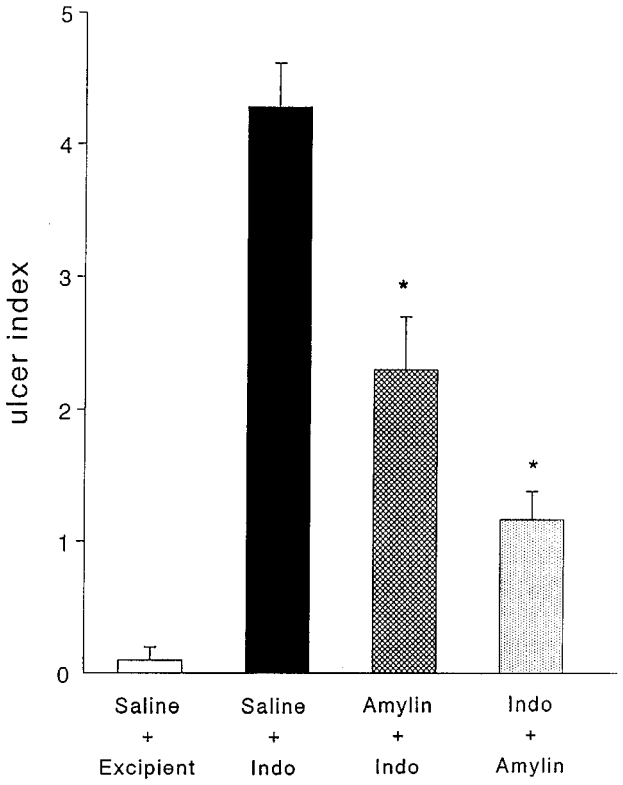

Figure 4 Effect of amylin (2.2 μg/rat, i.c.v.) on healing of gastric ulcers induced by indomethacin (indomethacin, 20 mg kg^{-1}, orally) in rats. Amylin was injected either 5 min before indomethacin (cross-hatched column) or 1 h after indomethacin (stippled column); open column, controls; solid column, indomethacin. Data are expressed as mean $\pm$ s.e.mean of six to eight animals. $^*P<0.05$ vs indomethacin.

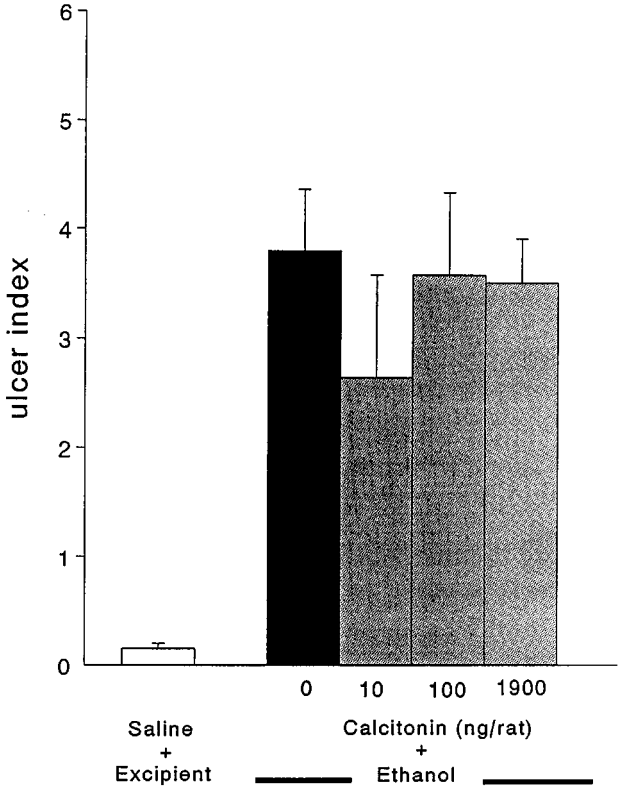

Figure 5 Effect of salmon calcitonin given i.c.v. 30 min before inducing gastric ulcers by 50% ethanol (EtOH, 1 ml, orally) in rats. Each value is the mean $\pm$ s.e.mean of six to eight animals.

ulcers. In addition the importance of the maintenance of mucosal integrity in preventing EtOH injuries comes from the evidence showing that the depletion of sulfhydryl groups by NEM is able to abolish the protective effect of amylin on EtOH-induced ulcers and not the protective effect of amylin against indomethacin-induced lesions.

There is evidence that the NO system beside playing a role in the regulation of the gastric mucosal blood flow which contributes to protection against injurious agents (Sorbye & Svanes, 1994), cooperates with the prostaglandin system in the maintenance of gastric mucosal integrity (Whittle *et al*., 1990). Furthermore these two mediators can regulate the synthesis of one another as NO has been shown to stimulate cyclooxygenase activity (Salvemini *et al*., 1993). Previous results have also shown that the inhibition of prostaglandin synthesis removes the protective effect of amylin on EtOH damage (Guidobono *et al*., 1997).

The mechanism by which amylin could activate the NO system is not known. Recently Morley *et al*. (1997) have shown that amylin, injected i.p. in mice, had no effect on NO synthase in the fundus of the stomach. However the effect of amylin administered peripherally or centrally in mice or rats could be totally different. The fact that amylin requires a latency time (30 min) to exert its gastroprotective effect against EtOH-induced lesions suggests that the effect of amylin on the NO system, if any, would be indirect and could be centrally mediated.

The current data, showing that depletion of nonprotein sulfhydryls by NEM removes amylin protection from EtOH induced gastric injuries, and worsens EtOH ulceration indicates that in addition to NO and prostaglandins, endogenous sulfhydryls also participate in the gastric beneficial effect of amylin. Although decreased sulfhydryl activity does not appear to play a role in the aetiology of mucosal ulcers induced by indomethacin, nevertheless, indomethacin-induced ulceration is reduced by sulfhydryl depletion and NEM enhances the protective effect of amylin although not significantly. The opposite effects of NEM in these two types of ulcers is in line with the data of Cho & Ogle (1992) that showed that NEM worsens the severity of EtOH-induced ulcers and protects against stress-induced gastric mucosal damage.

The possibility that amylin could act in the brain through calcitonin receptors in triggering the mechanisms subserving the protective effect on EtOH-induced ulcers, is ruled out as calcitonin despite being a potent amylin agonist (Beaumont *et al*., 1993; van Rossum *et al*., 1994) was ineffective against this type of lesions. An explanation for this evidence emerges from the data of van Rossum *et al*. (1994) showing that amylin has higher affinity than calcitonin for sites present in some brain areas.

The protective effect of amylin against indomethacin-induced ulcerations might involve the inhibitory effect of the peptide on gastric emptying. Amylin is known to be a potent inhibitor of gastric motility (Young *et al*., 1996a), an effect that is probably mediated by a central mechanism (Clementi *et al*., 1996) involving the vagus nerve since subdiaphragmatic vagotomy destroys the response to amylin (Jodka *et al*., 1996). Thus considering that enhanced gastric motility is an important factor in the pathogenesis of indomethacin and not in EtOH-induced gastric lesions (Takeuchi *et al*., 1994) and that NEM treatment was shown to decrease gastric motility (Takeuchi *et al*., 1991) such an effect could also account for the more pronounced protective effect of amylin on indomethacin-induced ulcers when the peptide was administered in rats treated with NEM.

The fact that amylin was effective even when administered one hour after indomethacin, when gastric damage has already started, suggests that amylin in this type of ulcer could not only act by stimulating the mucosal defence mechanisms but also by promoting the complex repair processes. In fact the epithelium can repair injuries quickly following disruption of its continuity, by rapid migration of healthy cells from the gastric pits over the denuded basement membrane (Wallace & Granger, 1996).

A decrease in parasympathetic input to the stomach consequent to the interaction of amylin with its own receptors located in the area postrema could contribute to the ulcer healing properties of amylin. This region regulates efferent vagal activity that appears necessary for the amylin inhibitory activity on gastric emptying (Young, 1997). Anterior unilateral vagotomy has, infact been reported to show a significant acceleration of the healing of gastric ulcers on the denervated side which appears to relate to the inhibition of both gastric acid secretion and gastric relaxation (Tsukimi & Okabe, 1994). The distension of the stomach could trigger the release of peptides from gastric endocrine cells. There is now evidence that the process of re-epithelialization and reconstruction of the mucosal architecture is under the control of growth factors produced locally by regenerating cells (Tarnawski *et al*., 1995). Trefoil growth factors, a family of protease-resistant peptides, that are released into the lumen of the gastro-intestinal tract could be the candidates in promoting ulcer healing as their expression is increased at the sites of gastro-intestinal ulceration (Diguass *et al*., 1994; Wright *et al*., 1993). Further evidence of the importance of growth factors in ulcer repair is the observation that when damage of the gastric mucosa occurs, a new lineage of cells that produce epidermal growth factors can be identified at the ulcer margin (Wright *et al*., 1990). The possibility that the final outcome of the ulcer healing activity of amylin could involve growth factors production and/or activity is based on the observation that growth factors can promote ulcer healing without the need to neutralize gastric acid secretion (Szabo *et al*., 1995) as it is the case for amylin antiulcer effects (Guidobono *et al*., 1997). Instead it can be excluded that the stimulating activity of amylin on glucose production (Cooper, 1994) could contribute to its antiulcer effects (Takeuchi *et al*., 1994); because in our experimental procedure we did not find hyperglycemia after i.c.v. administration of the peptide. Furthermore the gastroprotective effect of peripherally administered amylin (Gedulin *et al*., 1997) has been observed at doses 300 times lower than those required to elevate glucose in rats (Young *et al*., 1996b).

In conclusion our results show that different mechanisms are involved in the protective effects of amylin in different experimental gastric ulcers. The antiulcer effect of centrally administered amylin is probably mediated by its own specific receptors in the brain, in fact the structurally related peptide, calcitonin and CGRP, which are effective against indomethacin induced ulcers are ineffective against EtOH gastric lesions. The results suggest that amylin is a new candidate for modulation of gastric functions.

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