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Protection by amylin of gastric erosions induced by indomethacin or ethanol in rats

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1 The effect of amylin on gastric ulcers induced by oral administration of indomethacin (Indo, 20 mg kg^{-1} at a dosing volume of 5 ml) or ethanol 50% (EtOH, 1 ml/rat) was investigated in conscious rats.

2 Amylin given intracerebroventricularly (0.22, 0.66 and 2.2 μg/rat, i.c.v.) demonstrated a dose-dependent cytoprotective effect against both Indo and EtOH-induced ulcers. In contrast, amylin, given subcutaneously at doses effective in inhibiting acid gastric secretion (2.5, 10 and 40 μg kg^{-1}, s.c.), did not show any cytoprotective effect.

3 The interaction between amylin and endogenous nitric oxide (NO) in the maintenance of gastric mucosal integrity was investigated by pretreating the rats with a selective inhibitor of NO-synthesis, N^G-nitro-L-arginine methyl ester (L-NAME, 25 and 70 mg kg^{-1}, s.c.). Administration of L-NAME to rats did not significantly increase the degree of the Indo-induced ulcer index and was not able to remove the protective effect of amylin on Indo-induced ulcers, thus excluding a role for endogenous NO in mediating the protective effect of this peptide.

4 To determine whether the cytoprotective effect of amylin was mediated by endogenous prostaglandins, we studied the effect of amylin (2.2 μg/rat, i.c.v.) on EtOH- induced ulcers in rats pretreated with Indo (10 mg kg^{-1}, s.c.) to inhibit prostanoid biosynthesis; Indo was injected 30 min before amylin and EtOH after a further 30 min. Pretreatment with Indo did not significantly increase the ulcer index induced by EtOH but counteracted the ability of amylin to prevent the ulcer formation.

5 These findings suggest that amylin exerts a gastroprotective activity that is not strictly related to inhibition of acid gastric secretion and can be partly explained through a prostaglandin-dependent mechanism mediated by receptors for the peptide in the brain. Amylin might be considered as a new brain-gut peptide.

Keywords: Amylin; brain; ulcers; ethanol; indomethacin

Introduction

Amylin is a 37-amino acid peptide isolated from pancreatic amyloid deposits in patients with non insulin-dependent diabetes mellitus (Cooper *et al*., 1987) as well as from human insulinoma (Westermark *et al*., 1987a). The peptide is stored in the pancreatic B-cells and is secreted in response to the same stimuli that induce insulin release (Westermark *et al*., 1987b; Mitsukawa *et al*., 1990). Unlike insulin, detectable amounts of amylin are found in extrapancreatic tissues including the lung, the central nervous system and the gut, from the stomach to the rectum (Toshimori *et al*., 1990; Miyazato *et al*., 1991). Amylin mRNA is present in the stomach, in the lung and in the dorsal root ganglion (Ferrier *et al*., 1989). The presence of amylin in the gastro-intestinal tract raises the possibility that the peptide has a functional role in this system. Very little is known so far concerning the role of amylin in gastric function. It has been found that the ingestion of food increases circulating levels of amylin (Butler *et al*., 1990); the administration of the peptide elicits anorectic effects (Morley *et al*., 1995) and inhibits acid gastric secretion (Guidobono *et al*., 1994).

Amylin shares a 46% amino acid homology with calcitonin gene-related peptide (CGRP) (Cooper *et al*., 1987) and a weaker homology with salmon calcitonin (CT) (Pittner *et al*., 1994). The three peptides have many activities in common and also show reciprocal cross-reactivity at their receptors, hence they are considered members of the same family of peptides (Poyner, 1995).

Amongst the biological actions shared by amylin, CT and CGRP there are hypocalcaemic effects, inhibition of glucose incorporation into muscle glycogen (Leighton & Cooper, 1988; Datta *et al*., 1989), inhibition of acid gastric secretion and food intake (Guidobono *et al*., 1990; Morley & Flood, 1991), although the three peptides act with a different rank order of potency (Cooper, 1994). Distinct receptors for amylin, CT and CGRP have been localized and characterized at both peripheral and central sites involved in the control of gastrointestinal functions (Skofitsch *et al*., 1995; van Rossum *et al*., 1995). However, it remains to be established whether or not amylin acts on its own receptors or through CT or CGRP receptors. Furthermore, it is not yet known whether amylin plays a role in the maintenance of gastric mucosal integrity and whether amylin antisecretory effect is relevant to this presumed activity. To characterize the potential gastroprotective activity of amylin, we evaluated the effect of central and peripheral administration of the peptide on gastric injury induced by indomethacin (Indo) or ethanol (EtOH), which are two types of ulcers differently influenced by CT and CGRP (Guidobono *et al*., 1991; Clementi *et al*., 1993).

Methods

Animals

Male Sprague-Dawley rats, weight range 180–200g (Charles River, Calco, Italy) were placed in single cages which had wire-net bottoms 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 the 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).

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Experimental procedures

Gastric mucosal damage Ulcers were induced by intragastric instillation of Indo (20 mg kg^{-1}) or EtOH 50%. Amylin or saline was administered 5 min before Indo and 30 min before EtOH and animals were killed 6 h after Indo and 1 h after EtOH exposure. The doses of amylin used for Indo-induced ulcers were 0.22, 0.66 and 2.2 μg/rat, i.c.v. and 2.5, 10 and 40 μg kg^{-1}, s.c. Rats treated with EtOH were injected with amylin at the doses of 0.66 and 2.2 μg/rat i.c.v. or 10 and 40 μg kg^{-1}, s.c. At the end of the experimental period the rats were anaesthetized with ether and the stomachs were dissected out and opened along the lesser curvature. Necrotizing lesions were examined macroscopically by 2 or 3 observers unaware of the treatment and lesions were classified with arbitrary scales in which the severity rating and number of lesions were considered according to a modified scoring system of Adami *et al.* (1964); 0 = no lesions; 1 = haemorrhagic suffusion; 2 = from 1 to 5 small ulcers <3 mm; 3 = many ulcers, more than 5, or 1 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 5 slight lesions; 2 = more than 5 slight lesions; 3 = from 1 to 3 haemorrhagic bands of length <5 mm and width >2 mm; 4 = from 1 to 3 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.

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 erosions. L-NAME was administered at doses of 25 and 70 mg kg^{-1}, s.c. 15 min before amylin (0.66 μg/rat, i.c.v.) followed by Indo (20 mg kg^{-1}, orally) 5 min thereafter.

In another group of experiments Indo was administered s.c. (10 mg kg^{-1}) to inhibit prostaglandin synthesis, amylin 2.2 μg/rat, i.c.v. was administered after 30 min followed by EtOH 30 min later.

Having found that amylin was able to protect from gastric ulcers at doses previously found not to inhibit acid gastric secretion (Guidobono *et al.*, 1994), we repeated previous experiments on acid gastric secretion with the current batch of amylin and a wider range of doses.

Acid secretion studies These were performed by the pylorus ligation method (modified from Shay *et al.*, 1945). Under light ether anaesthesia the pyloric sphincter was ligated through a small midline incision. The animals were killed 3 h later, stomachs were removed and the gastric content was collected. The volume of gastric juice was measured after centrifugation and the acid output was determined by titration with 0.01 N NaOH to pH 7.0. The experiments were repeated in order to have 7–9 animals per group. Results are expressed as acid concentration in μEq of acid in 3 h (total volume) and presented as percentage of the mean of the controls. Amylin (0.22, 0.66, 1.5, 2.7 and 5 μg/rat, i.c.v.) or saline was given at the time of pyloric ligation.

Drugs

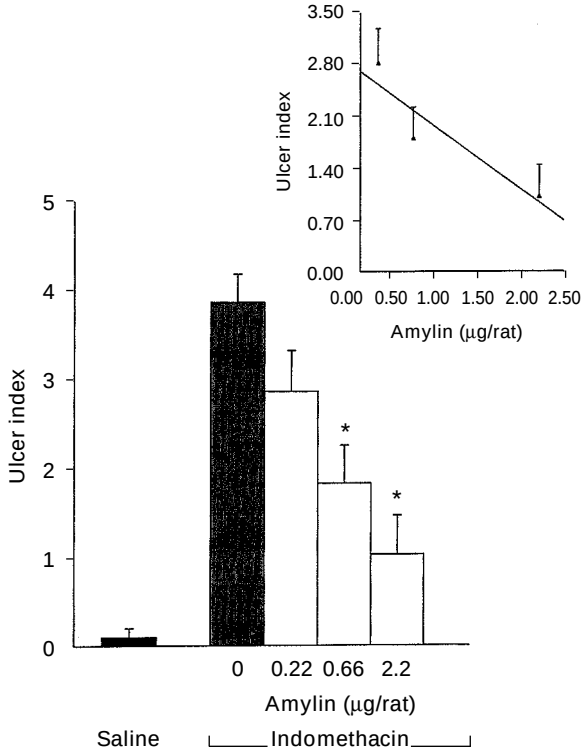
Rat amylin (Peptide Institute, Inc. Japan) for intracerebroventricular (i.c.v.) administration was dissolved in saline at concentrations suitable to be administered at 5 μl/rat; for subcutaneous (s.c.) injection amylin was dissolved in an appropriate diluent (1 g sodium acetate, 0.5 ml acetic acid, 0.1 g bovine serum albumin to 100 ml with double distilled water to give the final dosing volume of 1 ml kg^{-1}). Indomethacin (Indo, Sigma) for oral administration was suspended in arabic gum at a concentration of 20 mg kg^{-1} at a dosing volume of 5 ml; for s.c. injection. Indo was dissolved in $NaHCO_3$ 5% and diluted in double distilled water and administered at a final concentration of 10 mg kg^{-1} in a dosing volume of 1 ml. N^G-nitro-L-arginine methyl ester (L-NAME, Tocris Cookson) was dissolved in saline to a concentration of 25 and 70 mg kg^{-1}, at a dosing volume of 1 ml, which are the doses used by Clementi *et al.* (1994) to inhibit NO formation in rats in an experimental model of ulcer. The doses used were far higher than those needed to evoke a rise of mean arterial blood pressure in rats (Holzer *et al.*, 1993). Ethanol (EtOH, BDH) was diluted to 50% in double distilled water and was given in a volume of 1 ml/rat. In all the experiments groups consisted of 5–10 rats and control rats were treated with the vehicles used for the preparation of the drugs.

Statistical analysis

Data from pooled experiments were analysed by one way analysis of variance followed by Bonferroni tests. $P<0.05$ was considered significant. The dose- response curve and the linear regression analysis were performed by use of a statistic computer programme (Tallarida & Murray, 1981).

Results

The effects of amylin on gastric ulcers are shown in Figure 1. The peptide exhibited a protective effect in rats against Indo-induced ulcers in a dose-dependent manner ($r=-0.45$, $P<0.01$) with a range of doses between 0.22 and 2.2 μg/rat, i.c.v. Amylin was effective in protecting from Indo-induced ulcers at a dose of 0.66 μg/rat, i.c.v. (Figure 1). Under our experimental conditions, this was the minimal effective dose in inhibiting acid gastric secretion although gastric secretion volume and acidity were still substantial, being, respectively, 4.06 ± 0.29 ml and 113.3 ± 5.8 μEq ml^{-1}, compared to the values of controls that were 6.5 ± 0.4 and 118.3 ± 3.6 μEq ml^{-1} (Figure 2). The data showing a dose-related inhibition of acid gastric secretion confirm and widen previous results obtained



1Figure 1 Effect of different doses of amylin administered i.c.v. on gastric ulcers induced by indomethacin (Indo, 20 mg kg^{-1}, orally) Each value is the mean $\pm$ s.e.mean of 8–10 animals. $^*P<0.01$ vs Indo-treated group. Inset: linear regression analysis, $r=0.45$, $P<0.01$.

with a different batch of the peptide where amylin was effective only from 1.5 μg/rat, i.c.v. (Guidobono *et al*., 1994).

The s.c. administration of amylin at doses of 10 and 40 μg kg^{-1} was not able to prevent gastric erosions induced by Indo; no significant effects were seen with these doses (Figure 3). Higher doses were not used because data obtained previously on inhibition of acid gastric secretion showed that amylin 100 μg kg^{-1}, s.c., was less effective than 40 μg kg^{-1}, s.c. and the dose of 160 μg kg^{-1}, s.c. was completely ineffective; a typical bell-shaped curve was obtained, as often seen with peptides (Guidobono *et al*., 1994).

We considered the possibility that amylin might exert its protective effect on ulcers by increasing blood flow since it is well known that amylin, like CGRP, causes vasodilatation (Brain *et al*., 1990). As one of the possible endogenous factors responsible for such vasoactivity is NO, we examined the effect

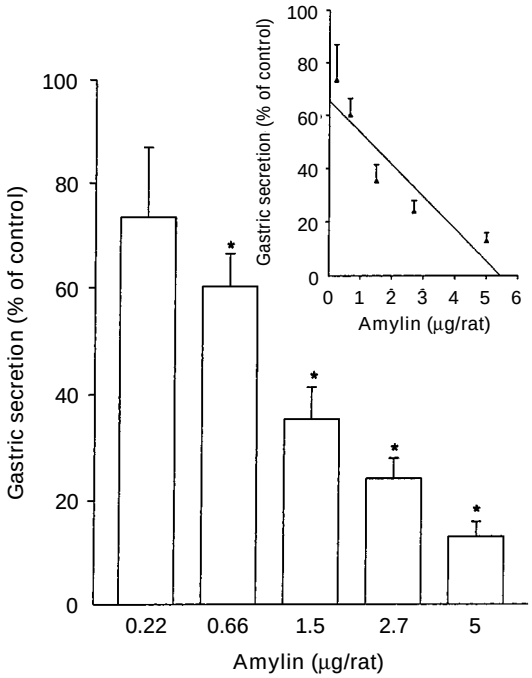


Figure 2 Inhibitory effect of different doses of amylin administered i.c.v. on gastric acid secretion in rats, 3 h after pylorus ligation. Data are expressed as % of controls (769.2 $\pm$ 41.4 μEq/total vol.). Each value is the mean $\pm$ s.e.mean of 7–9 animals, $^*P<0.05$. Inset: linear regression analysis, $r=-0.72$, $P<0.001$.

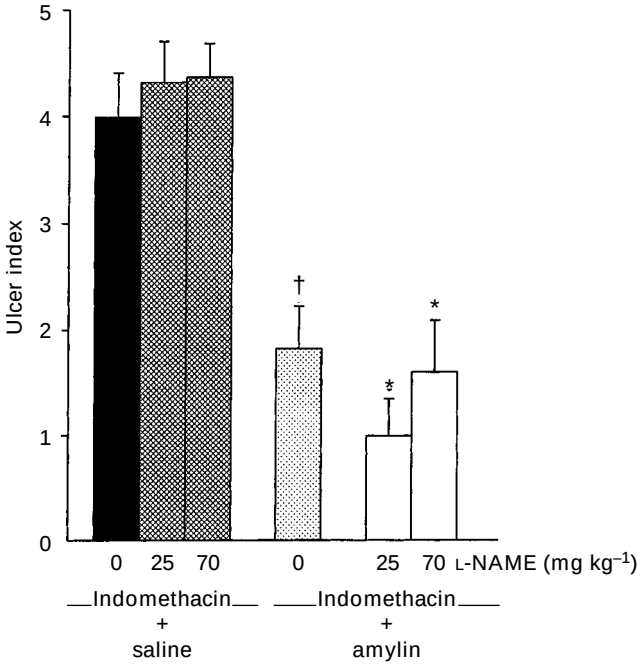


Figure 4 Effect of amylin (0.66 μg/rat, i.c.v.) on gastric ulcers induced by indomethacin (Indo, 20 mg kg^{-1}, orally) in rats pretreated with L-NAME 30 min before amylin. Data are expressed as mean $\pm$ s.e.mean of 6–8 animals. Solid columns, Indo; cross-hatched columns, Indo + L-NAME; stippled column, Amylin + Indo; open columns, L-NAME + Amylin + Indo. $\dagger P<0.05$ vs Indo, $^*P<0.05$ vs Indo + L-NAME treated group.

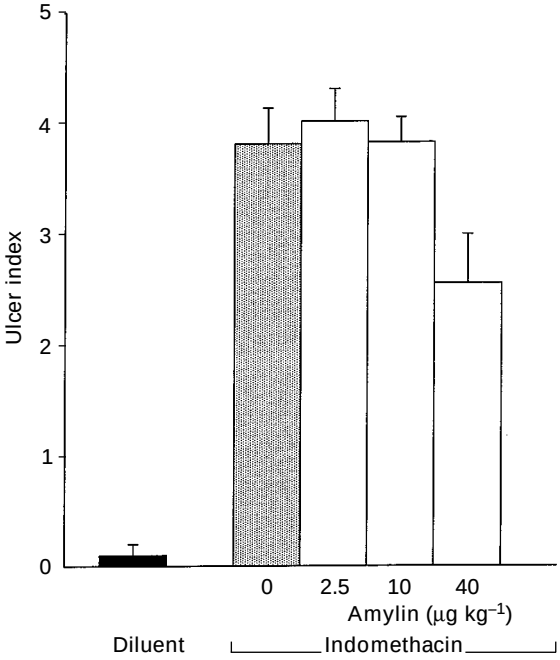


Figure 3 Effect of different doses of amylin given s.c. on gastric ulcers induced by indomethacin (Indo, 20 mg kg^{-1}, orally) in the rat. Data are expressed as mean $\pm$ s.e.mean of 5 animals.

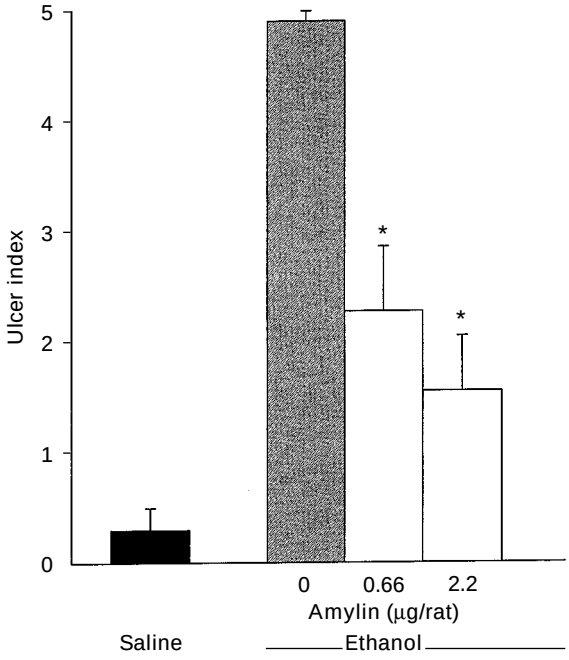


Figure 5 Effect of amylin given i.c.v. on gastric ulcers induced by 50% ethanol (EtOH, 1 ml, orally). Each value is the mean $\pm$ s.e.mean of 6–8 animals. $^*P<0.05$ vs EtOH-treated group.

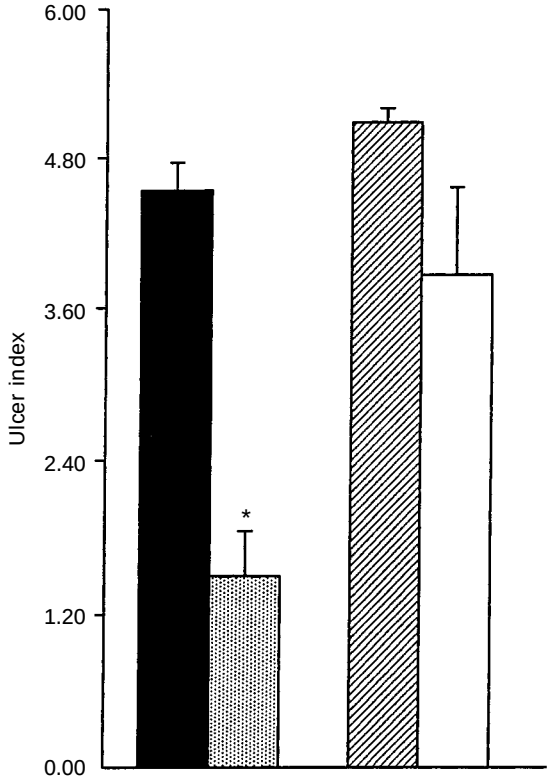


Figure 6 Effect of amylin (2.2 μg/rat, i.c.v.) on ethanol (EtOH)-induced ulcers in rats pretreated (30 min before amylin) with indomethacin (Indo, 10 mg kg^{-1}, s.c.) to inhibit synthesis of prostanoids. Each value is the mean ± s.e.mean of 6–7 animals. Solid column, EtOH; stippled column, Amylin + EtOH; hatched column, Indo + EtOH; open column, Indo + Amylin + EtOH. *$P < 0.05$ vs EtOH-treated group.

of amylin on Indo-induced ulcers in rats pretreated with L-NAME, an inhibitor of NO-synthase. As shown in Figure 4, L-NAME did not significantly increase the extent of Indo-induced gastric ulceration. In rats pretreated with L-NAME, 25 and 70 mg kg^{-1}, amylin 0.66 μg/rat, i.c.v., was still able to protect from Indo-induced ulcers, thus excluding the possibility that the antiulcer effect of amylin is mediated by NO production.

Amylin at doses of 0.66 and 2.2 μg/rat, i.c.v., was also effective in preventing gastric damage induced by EtOH (Figure 5). The s.c. administration of amylin, 10 and 40 μg kg^{-1}, did not protect rats from gastric lesions induced by EtOH (data not shown). Figure 6 presents the results of the experiments performed to examine whether or not endogenous prostaglandins are involved in the protective effect of amylin on EtOH-induced gastric ulcers. Pretreatment with Indo, 10 mg kg^{-1}, s.c. did not increase the ulcer index compared to that in EtOH-treated animals but the protective effect of amylin on EtOH-induced ulcers was significantly inhibited compared to the effect of amylin alone (ie amylin + EtOH group).

Discussion

We have demonstrated that amylin is able to protect against gastric ulcers induced by Indo or by EtOH. The antiulcer activity of i.c.v. administered amylin is evident when gastric acid secretion is significantly reduced by 40%, yet acid secretion is still substantial compared to controls. Amylin injected s.c., at doses previously shown to be effective in reducing gastric acid secretion by 66.5% (Guidobono *et al*., 1994), was not able to protect from gastric ulcers induced by Indo or by EtOH. These data thus emphasize that gastric protection by amylin involves mechanisms other than inhibition or neutralization of gastric acid secretion, in contrast with the classical antisecretory drugs, H^+- K^+- ATPase inhibitors, antimuscarinic and H_2 receptor antagonists whose anti-gastric ulcer and antisecretory effects go in parallel (Kromer *et al*., 1990). The present findings show that the administration of L-NAME did not prevent the protective effect of amylin on Indo-induced gastric erosion. However, pretreatment with Indo to block production of prostanoids, reduced the protective effect of amylin on EtOH-induced ulcers, indicating that prostanoids could, at least in part, be involved in this effect of amylin, as cytoprotection by prostaglandins is unrelated to the inhibition of gastric acid secretion (Robert *et al*., 1979).

The data obtained in this study rule out the possibility that the vasodilating effect of NO contributes to the antiulcer activity of amylin, in contrast with results obtained with CGRP whose antiulcer effect involving the gastric microcirculation, is inhibited by blockade of NO synthesis (Clementi *et al*., 1994; Holzer *et al*., 1995). Since the vascular effect of amylin is mediated by CGRP receptors (Beaumont *et al*., 1995) these findings suggest that the cellular mechanisms subserving the ulcer protective effects of amylin and CGRP are different.

The difference between amylin and CGRP is confirmed by the protective effect of amylin on EtOH-induced gastric lesions as neither CGRP (Evangelista *et al*., 1987; Evangelista & Maggi, 1991) nor CT (Guidobono *et al*., 1991) are effective against EtOH-induced ulcers. These results again imply that amylin acts through its own receptor and not through interaction with the receptors for CGRP or CT and that the three peptides have different functional activities. The mechanisms of mucosal protection by amylin remain to be explored. The present findings support the hypothesis that endogenous prostaglandins or prostacyclin contribute to the effect of amylin not only by exerting a local vasodilator action on the microcirculation but also by acting to enhance the gastric mucosal barrier.

Despite the fact that specific binding sites for amylin are present in the rat stomach (Bhogal *et al*., 1992), the peptide administered s.c. did not show a cytoprotective effect, in contrast to CT and CGRP which are able to prevent gastric ulceration when injected peripherally (Guidobono *et al*., 1991; Clementi *et al*., 1993). These differences support the concept of the existence of distinct receptors in the stomach linked to different functions for the homologous peptides CT, CGRP and amylin. It would appear that the cytoprotective effect of amylin is mediated by specific receptors for the peptide in the brain. Recently it has been shown that amylin and its binding sites are widely distributed in the central nervous system (Skofitsch *et al*., 1995; van Rossum *et al*., 1995). Amylin and its binding sites have been shown to be present in other brain regions, in areas involved in gastric functions like the amygdala and the hypothalamus (lateral, arcuate, dorsomedial and paraventricular nuclei), so it is possible that the peptide has a role in the central control of gastric function, as suggested by the results presented here. Studies on the hypothalamic control of gastric function have shown the presence of glucose-sensitive neurones that are believed to be implicated in feeding and other gastric related activities. These glucose responding neurones are supposed to receive information from the gastrointestinal tract through vagal afferents to the nucleus of the solitary tract that functions as a relay centre between the periphery, the medulla oblongata and the hypothalamus (Shiraishi, 1988).

Amylin has a well recognized role in glucose metabolism: the peptide activates glycogen phosphorylase and inhibits glycogen synthase (Young *et al*., 1990; Deems *et al*., 1991), stimulates glycogen breakdown to lactate and increases glucose production (Rink *et al*., 1993). In addition a marked reduction of blood glucose, as induced by insulin, can have a permissive role in the formation of gastric lesions even without significant changes in acid secretion (Takeuchi *et al*., 1994). Based on these facts, we propose that amylin could have a role

in the central glucoregulation as a metabolic partner to insulin and as such would oppose the effect of insulin hypoglycaemia on gastric functions. It is possible that amylin by acting in the CNS triggers mechanisms leading in the periphery to the stimulation of a local neuromodulator that regulates the mucosal resistance. Amylin by means of the vagus, might activate the release of neuropeptides contained in sensory fibres thus indirectly exerting a mucosal protective effect. In conclusion, although the role of this peptide in gastrointestinal function remains to be elucidated, it would seem that amylin should be included in the big family of brain-gut peptides.

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