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In-vitro study of the enkephalinergic hypothesis for non-adrenergic, non-cholinergic innervation in the cat stomach

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It has been suggested that enkephalins are involved in the gastric relaxation induced by stimulation of the non-adrenergic, non-cholinergic vagal fibres in the cat stomach. Experiments were therefore performed on strips of cat stomach. With longitudinal and circular gastric fundus and corpus strips from reserpinized cats, non-adrenergic, non-cholinergic relaxatory responses could be elicited by transmural electrical stimulation in Tyrode solution containing atropine and 5-hydroxytryptamine. Morphine, leu-enkephalin and met-enkephalin did not influence the tone of the strips or the relaxation evoked by stimulation at 8 Hz, and neither did the opioid antagonist, naloxone. These results do not support the enkephalinergic hypothesis for the non-adrenergic, non-cholinergic vagal fibres in the cat stomach.

The presence of inhibitory non-adrenergic, non-cholinergic vagal fibres has been shown in the feline stomach (Martinson & Muren 1963; Martinson 1965) but the neurotransmitter involved is unknown. From data in the cat in-vivo, Edin et al (1980) suggested that enkephalinergic nerve fibres may control gastric motor function, producing pyloric contraction and gastric receptive relaxation upon stimulation. Few factors are known to influence the responses to non-adrenergic non-cholinergic nerve stimulation; in the guinea-pig taenia coli, morphine depresses the non-adrenergic non-cholinergic inhibitory response to transmural stimulation (Shimo & Ishii 1978), but this observation could not be repeated by Small & Yong (1983).

The experiments reported here were performed to study whether non-adrenergic, non-cholinergic relaxatory responses can be obtained in-vitro in cat gastric smooth muscle strips and whether enkephalins could be involved in this response. We studied the effect of transmural stimulation and of the opioid agonists, morphine, leu- and met-enkephalin on gastric smooth muscle strips of reserpinized cats, contracted with 5-HT in the presence of atropine. A non-adrenergic, non-cholinergic relaxatory response was obtained in gastric fundus and corpus strips, but no evidence was found for the involvement of enkephalins in this response. Preliminary results of these experiments have been presented (De Schaepdryver et al 1984).

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MATERIALS AND METHODS

Male or female cats (2·0–3·5 kg) were reserpinized (reserpine 3 mg kg^{-1} intraperitoneally; Trendelenburg & Weiner 1962) and were then fasted but had free access to water for 24 h, after which, they were killed by being anaesthetized with pentobarbitone (30 mg kg^{-1} i.p.). The abdomen was then opened by a midline incision and the stomach excised and put in Tyrode solution at room temperature (20 °C). From fundus, corpus and antrum, 2 longitudinal and 2 circular muscle strips (15 mm long, 2 mm broad), cut parallel or perpendicularly, respectively, to the greater curvature in the ventral part of the stomach, were obtained and the mucosal layer removed. All strips were used immediately.

The strips were mounted under a load of 1 g between 2 parallel platinum plate electrodes (48 × 8 mm) in organ baths containing 18 ml of Tyrode solution at 37 °C and bubbled with 95% O_2 and 5% CO_2. The composition of the Tyrode solution was (mM): NaCl 136·8, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·6, NaH_2PO_4 0·4; $NaHCO_3$ 11·9 and glucose 5·6. The strips were equilibrated for 60 min, the bathing medium being changed every 15 min. Contractions were recorded auxotonically (Harvard heart-smooth muscle transducer) and were registered on a Beckman Type R Dynograph recorder. Transmural electrical stimulation was applied via the two electrodes. A Grass stimulator (Model S88) supplied the current via a constant current unit (supramaximal voltage, 1 ms duration).

A *first series of experiments* was performed on fundus, corpus and antrum strips to see whether a

relaxatory response could be obtained upon electrical stimulation. The experiments were started in Tyrode solution containing eserine $7{\cdot}26 \times 10^{-8}$ M. After a 60 min equilibration period, a cumulative frequency-response (0·25–16 Hz) curve was performed; contractile responses were obtained and expressed in % of the maximum response. Twenty min later, atropine 10^{-6} M was added and 10 min later the frequency-response curve was repeated. The Tyrode solution containing eserine was then replaced by Tyrode containing atropine 10^{-6} M and 5-HT 3×10^{-6} M. This produced a marked and sustained increase in tone in the fundus and corpus strips but the basal tone of the antrum strips was not influenced. Thirty min after changing the Tyrode, a frequency-response curve (0·5–16 Hz) was obtained. In the fundus and corpus strips, marked relaxatory responses were induced and the frequency-response curve was repeated twice with intervals of 30 min. The first of these two curves was done in the presence of hexamethonium 5×10^{-4} M (10 min incubation), the second in the presence of tetrodotoxin 3×10^{-6} M (10 min incubation). The responses in the three curves were expressed in percent of the maximum response obtained in the first curve.

A *second series of experiments* was done on fundus and corpus strips, as in such strips marked relaxatory responses could be obtained upon transmural stimulation. The Tyrode solution contained atropine 10^{-6} M and 5-HT 3×10^{-6} M from the beginning of the experiment. After the equilibration period, electrical stimulation (8 Hz during 60 s) was performed eight times with an interval of at least 20 min between the stimulations. Two minutes before the 2nd, the 4th, the 6th and the 8th stimulation, morphine 10^{-5} M, leu-enkephalin 10^{-6} M, met-enkephalin 10^{-6} M (fundus strips) or 10^{-7} M (corpus strips), or naloxone 10^{-6} M was added. The response to electrical stimulation in the presence of an opiate agonist or antagonist was expressed in percent of the preceeding response.

Drugs used

The following drugs were used: atropine sulphate (Boehringer Ingelheim, FRG), eserine salicylate (Boehringer), hexamethonium chloride (Federa, Brussels, Belgium), leucine enkephalin (Sigma, St Louis, USA), methionine enkephalin (Sigma), morphine hydrochloride (Sterop, Brussels, Belgium), naloxone hydrochloride (Du Pont, Brussels, Belgium), reserpine (Aldrich Chemie, Brussels, Belgium), 5-hydroxytryptamine creatinine sulphate (Hoechst Belgium, Brussels, Belgium), tetrodotoxin (Sankyo Co, Tokyo, Japan). For morphine and naloxone, commercially available ampoules were used. Products were dissolved or diluted in isotonic NaCl solution, except tetrodotoxin which was dissolved in distilled water. For reserpine a stock solution of 10 mg ml^{-1} dissolved in 20% ascorbic acid was prepared. Drugs were added to the bath in a volume of 0·1 ml. Eserine, atropine and 5-HT were added to the Tyrode solution reservoir.

Values are expressed as means with their standard error (s.e.m.).

RESULTS

Fundus strips

The results obtained in longitudinal and circular fundus strips were similar. In eserinized Tyrode solution, transmural electrical stimulation induced frequency-dependent tonic contractions (n = 6 for both longitudinal and circular muscle strips); adding atropine 10^{-6} M to the bath did not influence the basal tension of the strips, but in the presence of atropine no response or a small relaxation (in 3 out of 6 strips of each type) were obtained upon transmural electrical stimulation. When the eserinized Tyrode solution was changed to Tyrode solution with atropine 10^{-6} M and 5-HT $3{\cdot}10^{-6}$ M, a sustained increase in tone was obtained; transmural stimulation now elicited consistently a marked, frequency-dependent relaxation of the strips. An example of the responses in a circular fundus strip is given in Fig. 1. The

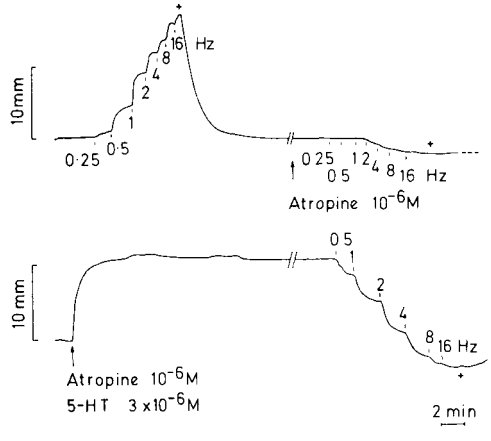

FIG. 1. Cat gastric fundus, circular muscle strip. Influence of transmural electrical stimulation at increasing frequencies (0·25–16 Hz, supramaximal voltage, duration 1 ms) on the tone of the strip. The upper panel shows the responses in Tyrode solution in the absence and in the presence of atropine 10^{-6} M. The lower panel shows the responses in Tyrode solution containing atropine 10^{-6} M and 5-HT 3×10^{-6} M. + Denotes the end of transmural electrical stimulation and rinsing.

frequency-response curve for relaxation was not changed by addition of hexamethonium 5×10^{-4} M (Fig. 2); in the presence of tetrodotoxin 3×10^{-6} M, the relaxatory responses were reduced (Fig. 2) and in one longitudinal fundus strip even abolished.

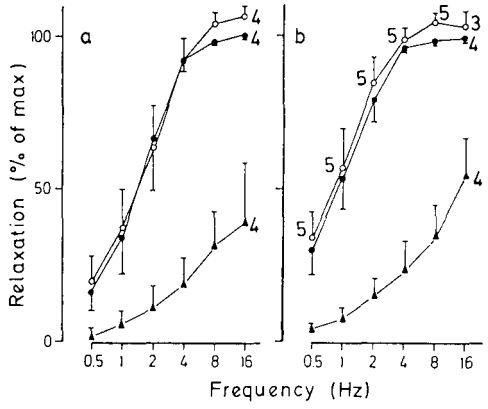

FIG. 2. Cat gastric fundus, longitudinal (left panel) and circular (right panel) muscle strips; relaxations in % of the maximal response. Influence of hexamethonium and tetrodotoxin on frequency-response curves to transmural stimulation (0·5–16 Hz, supramaximal voltage, duration 1 ms) in Tyrode solution containing atropine 10^{-6} M and 5-HT 3×10^{-6} M. Frequency-response curves are shown in control conditions (●), in the presence of hexamethonium 5×10^{-4} M (○) and in the presence of tetrodotoxin 3×10^{-6} M (▲). Each point represents the mean ± s.e.m. of 6 observations, except if otherwise indicated.

When fundus strips were stimulated repeatedly at 8 Hz in Tyrode solution containing atropine 10^{-6} M and 5-HT 3×10^{-6} M, reproducible relaxatory responses were obtained. The addition of morphine 10^{-5} M, leu-enkephalin, met-enkephalin or naloxone 10^{-6} M to the bath did not influence the basal tension of the strips nor the relaxation by transmural stimulation at 8 Hz, performed 2 min after their addition (n = 6 for each drug in both types of strips, except for met-enkephalin in the circular fundus strips, where n = 5).

Corpus strips

In both longitudinal (n = 6) and circular (n = 6) corpus strips, transmural electrical stimulation induced frequency-dependent tonic contractions in eserinized Tyrode solution, but in two strips of each type phasic contractions were superposed on the tonic contractions. The presence of atropine 10^{-6} M did not influence the basal tension of the strips but prevented completely the contractions upon electrical stimulation; in two of the longitudinal corpus strips, small relaxatory responses were obtained. When the bathing medium was changed to Tyrode solution with atropine 10^{-6} M and 5-HT 3×10^{-6} M, tone increased both in longitudinal and circular strips, but the increase was less pronounced in the longitudinal strips.

In the six circular corpus strips, transmural electrical stimulation elicited a frequency-dependent relaxation, which was not influenced by hexamethonium 5×10^{-4} M. The relaxation was abolished by tetrodotoxin 3×10^{-6} M in four strips; in two strips small relaxatory responses (at 16 Hz, 23 and 18% of the maximum response in the absence of tetrodotoxin) were still obtained. In Tyrode solution with atropine and 5-HT, transmural electrical stimulation elicited a frequency-dependent relaxation in two out of six longitudinal corpus strips. In these two strips, the relaxations were unaffected by hexamethonium but were abolished by tetrodotoxin.

Transmural stimulation at 8 Hz in Tyrode solution with atropine 10^{-6} M and 5-HT 3×10^{-6} M induced relaxation in four of six longitudinal strips and in the six circular strips investigated. In none of the strips did morphine 10^{-5} M, leu-enkephalin 10^{-6} M, met-enkephalin 10^{-7} M or naloxone 10^{-6} M influence the basal tension, nor the relaxation induced by transmural stimulation at 8 Hz, 2 min after addition of the substances (Fig. 3).

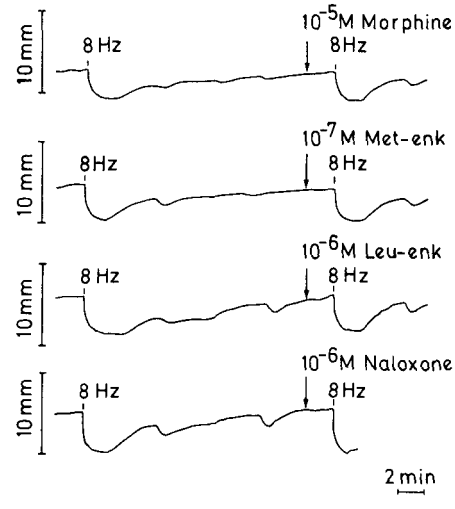

FIG. 3. Cat gastric corpus, longitudinal muscle strip. Influence of the opioid agonists morphine, met-enkephalin and leu-enkephalin and the opioid antagonist naloxone on the relaxation by transmural stimulation (8 Hz, supramaximal voltage, 1 ms, 60 s) in Tyrode solution containing atropine 10^{-6} M and 5-HT 3×10^{-6} M. Transmural stimulation was repeated 8 times with an interval of at least 20 min between the stimulations. Morphine 10^{-5} M was added 2 min before the 2nd, met-enkephalin 10^{-7} M 2 min before the 4th, leu-enkephalin 10^{-6} M 2 min before the 6th and naloxone 10^{-6} M 2 min before the 8th stimulation.

Antrum strips

In eserinized Tyrode solution, the longitudinal (n = 6) and circular (n = 6) muscle strips of the antrum showed a contractile response only at the higher frequencies of transmural stimulation. The response was a combined tonic and phasic one. In the presence of atropine 10^{-6} M, the contractile responses to transmural stimulation were abolished and no relaxatory responses were observed. Changing the eserinized Tyrode solution to one containing atropine 10^{-6} M and 5-HT 3×10^{-6} M did not influence the basal tension of the strips; no responses were obtained upon electrical stimulation in this medium.

DISCUSSION

The experiments were performed to study whether non-adrenergic, non-cholinergic relaxatory responses can be obtained in-vitro in cat gastric smooth muscle strips and whether enkephalins are involved in this response. The experiments were made on strips from reserpinized cats, to avoid noradrenergic components in the responses obtained; the dose of reserpine (3 mg kg^{-1} i.p. 24 h before death) has been shown to substantially reduce the noradrenaline content in different tissues (Trendelenburg & Weiner 1962). The noradrenaline content in pieces of fundus, corpus and antrum (mucosa removed) was determined by us using a HPLC method with electrochemical detection in two reserpinized and two non-treated cats: the noradrenaline content ranged between 120 and 392 ng g^{-1} tissue in the non-treated cats and between <2 and 13 ng g^{-1} tissue in reserpinized cats (unpublished results).

In the first series of experiments, the strips were bathed in Tyrode solution containing eserine $7{\cdot}26 \times 10^{-8}$ M. We have shown previously that with this concentration of eserine, contractions upon transmural electrical stimulation in rat and canine gastric fundus strips are more reproducible (Lefebvre et al 1983, 1984). In these conditions, transmural stimulation elicited contractions over the whole frequency range in fundus and corpus strips, but only at higher frequencies in antrum strips. In some corpus strips and in all antrum strips, the contractile response was tonic and phasic. In all strips, the contractions were abolished by atropine, illustrating their cholinergic nature. The different types of contractions probably reflect the stimulation by acetylcholine released upon electrical stimulation of the two different activation systems for smooth muscle activity, a P-system for phasic activity and a T-system for tonic activity (Boev et al 1976; Golenhofen 1976).

When 5-HT was added to the Tyrode solution together with atropine, an increase in tone was observed in the fundus and corpus strips and electrical stimulation induced marked frequency-dependent relaxatory responses. In antrum strips, 5-HT did not induce an increase in tone and no relaxatory responses were obtained upon electrical stimulation; an explanation for this discrepant behaviour is not apparent. As the cats were reserpinized and as atropine was present, the relaxatory responses in the fundus and corpus strips are non-adrenergic, non-cholinergic. We have no explanation why these responses were not seen in all longitudinal corpus strips. The relaxations were not influenced by hexamethonium, showing that postganglionic elements are involved. Surprisingly, the relaxatory responses were not completely abolished by tetrodotoxin 3×10^{-6} M, a concentration higher than or equal to that used to study the neurogenic origin of an electrically induced response in different gastrointestinal preparations (Anderson et al 1977; Ennis et al 1979; Ohga & Taneike 1977; Okwuasaba et al 1977). Relaxation due to direct smooth muscle cell stimulation is not likely, but it is possible that even in the presence of tetrodotoxin some depolarization occurs at the nerve endings, inducing some transmitter release not involving a conducted action potential in the nerves (Gershon 1967).

The opioid agonists were used in concentrations (morphine 10^{-5} M, leu-enkephalin 10^{-6} M, met-enkephalin 10^{-7} or 10^{-6} M) higher than those reported to clearly inhibit the electrically-induced contractions of longitudinal muscle in the guinea-pig ileum, an effect due to interaction with opioid receptor sites (Kosterlitz & Watt 1968; Hughes et al 1975). Morphine 10^{-5} M is also the concentration producing a maximal depression of the non-adrenergic non-cholinergic inhibitory responses in the guinea-pig taenia coli (Shimo & Ishii 1978). Naloxone 10^{-6} M is a concentration known to inhibit the effect of the enkephalins in the guinea-pig ileum (Hughes et al 1975). None of the opioid agonists influenced the 5-HT-induced tone of the fundus and corpus strips, while one would expect a relaxation if enkephalins were involved in the relaxation by the vagal inhibitory fibres. This result is in clear contrast with the influence of these opioid agonists on gastric tone when infused in-vivo via the splenic artery in cats: both leu-enkephalin and met-enkephalin induced a prompt gastric relaxation although morphine induced a slow increase of gastric tone (Edin et al 1980). The opioid antagonist, naloxone, did not influence the relaxation induced by transmural

stimulation at 8 Hz, again contrasting with naloxone's antagonizing effect on the atropine-resistant gastric relaxation by vagal nerve stimulation in cats in-vivo (Edin et al 1980). These in-vitro results thus do not confirm the hypothesis that enkephalins are involved in the relaxatory effect of the vagal inhibitory fibres.

The opioid agonists likewise did not influence the relaxation induced by transmural electrical stimulation at 8 Hz, in contrast to the depressant effect of morphine on the non-adrenergic, non-cholinergic inhibitory response to transmural stimulation reported in the guinea-pig taenia coli (Shimo & Ishii 1978) and to the inhibition of the non-adrenergic non-cholinergic inhibitory pathway by leu-enkephalin, met-enkephalin and morphine, suggested in the rabbit proximal colon by Blanquet et al (1982). However, the results of Shimo & Ishii (1978) could not be reproduced by other investigators (Small & Yong 1983).

It is concluded that non-adrenergic, non-cholinergic relaxatory responses can be obtained in cat gastric fundus and corpus strips but not in cat antrum strips; no evidence was found that enkephalins are involved in the relaxatory responses nor that enkephalins modulate them within the experimental conditions used.

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