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Release of dynorphin, somatostatin and substance P from the vascularly perfused small intestine of the guinea-pig during peristalsis

Josef Donnerer, Peter Holzer & Fred Lembeck

Department of Experimental and Clinical Pharmacology of the University of Graz, Universitätsplatz 4, A-8010 Graz, Austria

1 The release of dynorphin-(1–17), somatostatin and substance P into the venous effluate of the isolated and vascularly perfused guinea-pig small intestine was measured during rest and peristaltic activity. The peptides were determined by specific radioimmunoassays.

2 Increasing the intraluminal pressure by 5 mbar increased the release of dynorphin-(1–17), somatostatin and substance P. A substantial increase in the release of substance P was only seen in the presence of naloxone (1.5 μM) indicating an inhibitory influence of opioid peptide-containing neurones on the release of substance P. The pressure-induced release of substance P and dynorphin-(1–17) was completely prevented by tetrodotoxin (1.3 μM), which suggests a neural origin of these two peptides. The pressure-induced release of somatostatin was only partially inhibited by tetrodotoxin (1.3 μM) suggesting that somatostatin may also be released from non-neuronal sources, i.e. endocrine mucosal cells.

3 Dimethylphenylpiperazinium (32 μM) increased the release of somatostatin and substance P and this effect was inhibited by tetrodotoxin (1.3 μM). Cholecystokinin-octapeptide (38 nM) induced a large increase in the release of somatostatin but only a minute increase in the release of substance P; these effects of cholecystokinin-octapeptide were not blocked by tetrodotoxin (1.3 μM).

4 Noradrenaline (59 μM) inhibited the pressure-induced release of substance P but not that induced by dimethylphenylpiperazinium (32 μM). Neither the pressure-induced nor the dimethylphenylpiperazinium-evoked release of somatostatin was significantly diminished by noradrenaline.

5 These results indicate that dynorphin-(1–17), somatostatin and substance P may be transmitters involved in the coordination of the peristaltic reflex. Part of the inhibitory effects of opioid peptides and noradrenaline on intestinal motility may be brought about by inhibition of the release of substance P.

Introduction

Somatostatin and substance P have been localized by immunocytochemistry in neurones of the myenteric and submucous plexus as well as in cells of the mucosa (Schultzberg *et al.*, 1980; Furness *et al.*, 1980). Substance P serves as excitatory transmitter in the peristaltic reflex (Barthó *et al.*, 1982 b,c). Somatostatin exerts inhibitory effects on intestinal motility which can be differentiated from those of opiates and adrenoceptor agonists (Cohen *et al.*, 1979; Jhamandas & Elliott, 1980). Somatostatin was shown to inhibit the release of acetylcholine from myenteric neurones (Guillemin, 1976; Furness & Costa, 1979; Yau *et al.*, 1983a) and to activate inhibitory neurones of the enteric plexuses (Furness & Costa, 1979). The release of gastrointestinal hormones is also inhibited by somatostatin (McQuillan, 1980).

Dynorphin, an endogenous opioid peptide, has been determined in the duodenum of the pig (Tachibana *et al.*, 1982) and guinea-pig ileum (Kromer *et al.*, 1981). Endogenous opioid peptides depress the motility of the guinea-pig small intestine (Barthó *et al.*, 1982b,c; Kromer & Schmidt, 1982), and they may do so by inhibiting the neuronal release of both acetylcholine and substance P (Yau *et al.*, 1983b; Holzer, 1984).

The release of substance P from intrinsic neurones in response to electrical field stimulation (Holzer, 1984) and during peristaltic activity (Donnerer *et al.*, 1984) has recently been demonstrated. The purpose

of the present study was to compare the release of dynorphin, somatostatin and substance P into the vascular bed of the guinea-pig isolated small intestine during peristaltic activity, to elucidate the sources from which the peptides are released, and to unravel further the complex interactions between the different neurones of the enteric nervous system. To this end, drugs known either to stimulate enteric neurones (cholecystokinin-octapeptide, CCK-8, 1,1-dimethyl-4-phenyl-piperazinium, DMPP) or to inhibit their activity (tetrodotoxin, noradrenaline) were used to investigate the release of dynorphin, somatostatin and substance P from intramural neurones.

Methods

Experimental protocol

Guinea-pigs (350–500 g) were deprived of solid food overnight but had access to water. Under pentobarbitone anaesthesia (50 mg kg^{-1} i.p.) the mesenteric artery was cannulated and perfused with Tyrode solution (1.5 ml min^{-1}). After cannulation of the portal vein the whole small intestine was removed from the body and placed in a 200 ml organ bath containing Tyrode solution at 37°C. The oral and aboral ends of the intestine were connected to inflow and outflow cannulae, respectively. The lumen of the intestine communicated, via the inflow cannula, with a Mariotte bottle containing Tyrode solution to allow adjustment of the intraluminal pressure. The set-up for eliciting peristalsis has been described in detail by Holzer & Lembeck (1979) and Donnerer *et al.* (1984). The Tyrode solution used for vascular perfusion as well as that contained in the organ bath were gassed with a mixture of 95% O_2 and 5% CO_2. The perfusion solution also contained bovine serum albumine 10 mg ml^{-1} to protect the peptides from adsorption to surfaces, bacitracin 20 μM to avoid enzymatic degradation of peptides (Holzer, 1984) and, if not stated otherwise, naloxone 1.5 μM to block the inhibition of transmitter release by endogenous opioid peptides. Drugs were infused into the arterial supply in volumes of 15–60 μl min^{-1}.

The experiments began with a 20 min period of equilibration followed by a 5 min period during which the intraluminal pressure was increased to 5 mbar. After a recovery period of 20 min, CCK-8 or DMPP was infused for 3 min and, after a second recovery period of 20 min, the K^+-concentration of the arterial perfusate was raised to 108 mM by isoosmotic replacement of Na^+ for 5 min. At the end of the experiment, the small intestine was removed from the bath, freed of the mesentery, and weighed (3.5–4.5 g).

Radioimmunoassay

For the determination of somatostatin and substance P, 1 min samples were collected in tubes containing acetic acid to give a final concentration of 2 M acetic acid. The perfusion samples were freeze-dried and redissolved in assay buffer. Somatostatin was measured by a specific radioimmunoassay according to Lee *et al.* (1981) using antiserum R–143. This antiserum is apparently specific for the tetradecapeptide somatostatin (Lee *et al.*, 1981). Substance P was measured by radioimmunoassay according to Mroz & Leeman (1979) using antiserum Rd_2. This antibody is directed to the C-terminal part of substance P, including the C-terminal hexapeptide, and shows full cross-reactivity with substance P sulphoxide. For the determination of dynorphin-(1–17) immunoreactivity, the guinea-pig small intestine was perfused with Tyrode solution containing bovine serum albumin (10 mg ml^{-1}) but no bacitracin and naloxone. Fractions were collected at 2.5 min intervals in tubes containing hydrochloric acid to give a final concentration of 0.1 M HCl. The samples were freeze-dried and redissolved in assay buffer. Dynorphin-(1–17) was determined by a radioimmunoassay using synthetic dynorphin-(1–17) as standard, [^{125}I]-dynorphin-(1–17) as tracer and antiserum 'Myriam' at a final dilution of 1:20 000. In this assay, dynorphin-(1–13) has less than 1% the activity of dynorphin-(1–17) whereas Leu enkephalin (60 nM) does not cross-react with the antiserum. None of the drugs used interferred with the radioimmunoassays. The substance P antibody was a gift of Dr S.E. Leeman, the somatostatin antibody a gift of Dr P.C. Emson, and the dynorphin-(1–17) antibody a gift of Dr V. Höllt.

The limit of detection was 7 pg g^{-1} min^{-1} for somatostatin, 4 pg g^{-1} min^{-1} for substance P, and 3 pg g^{-1} min^{-1} for dynorphin-(1–17). The 'total evoked release' of the peptides means the release during the period of stimulation minus the mean release in the 2 periods preceding the stimulus. All results are presented as means ± s.e.mean. The statistical significance of differences was tested using the *t* test for unpaired samples.

Gel chromatography

To determine the nature of the immunoreactivities of the vascularly released peptides, 5–10 samples (pressure or K^+ stimulation) were pooled, freeze-dried and subjected to gel exclusion chromatography. Separation of somatostatin- and substance P-like immunoreactivity was accomplished on a column of Sephadex G-25 fine (1,2 × 60 cm) and dynorphin-(1–17) immunoreactivity was chromatographed on a Bio-Gel P-6 column (1.8 × 25 cm). Somatostatin and

Substance P were eluted with radioimmunoassay buffer, dynorphin-1(1–17) with acetic acid 0.1 M at 4°C at a flow rate of 12 ml h^{-1}. Fractions of 2 ml were collected and 200 µl aliquots assayed for immunoreactivity.

Drugs

Cholecystokinin-octapeptide (Squibb, Princeton, U.S.A.), 1,1dimethyl-4-phenylpiperazinium iodide (Ega, Steinheim, F.R.G.), hexamethonium chloride, (±)-noradrenaline tartrate (Fluka, Buchs, Switzerland), somatostatin, substance P, dynorphin-(1–17) (Peninsula, San Carlos, U.S.A.) and tetrodotoxin (Sankyo, Tokio, Japan) were used.

Results

The basal release of somatostatin was about 18 pg g^{-1} min^{-1} and that of dynorphin about 12 pg g^{-1} min^{-1}. The basal release of substance P was sometimes below, sometimes just above the detection limit of the assay (4 pg g^{-1} min^{-1}). It has to be considered that the amounts of the peptides found in the portal vein effluent reflect only a part of the whole amount released within the intestinal wall. When 10 ng min^{-1} somatostatin or substance P was added to the arterial perfusion only 18 ± 6% of somatostatin (n = 6) and 12 ± 5% of substance P (n = 5) was recovered in the venous outflow.

Donnerer *et al.* (1984) have shown that, in the presence of naloxone, increasing the intraluminal pressure to 5 mbar increased the release of substance P. The present results show that the release of somatostatin (Figure 1) and dynorphin-(1–17) (Figure 2) is also increased by raising the intraluminal pressure. It was also found that naloxone (1.5 µM) enhanced severalfold the pressure-induced release of substance P (Table 1). Likewise, the peristalsis evoked by increasing the intraluminal pressure was also more vigorous in the presence of naloxone than in its absence. In the absence of naloxone, pressure-induced peristalsis faded away after 2–3 min, whereas it continued throughout the whole period of increased pressure when naloxone was present. The pressure-induced increase in the release of somatostatin was also enhanced by naloxone, but only by

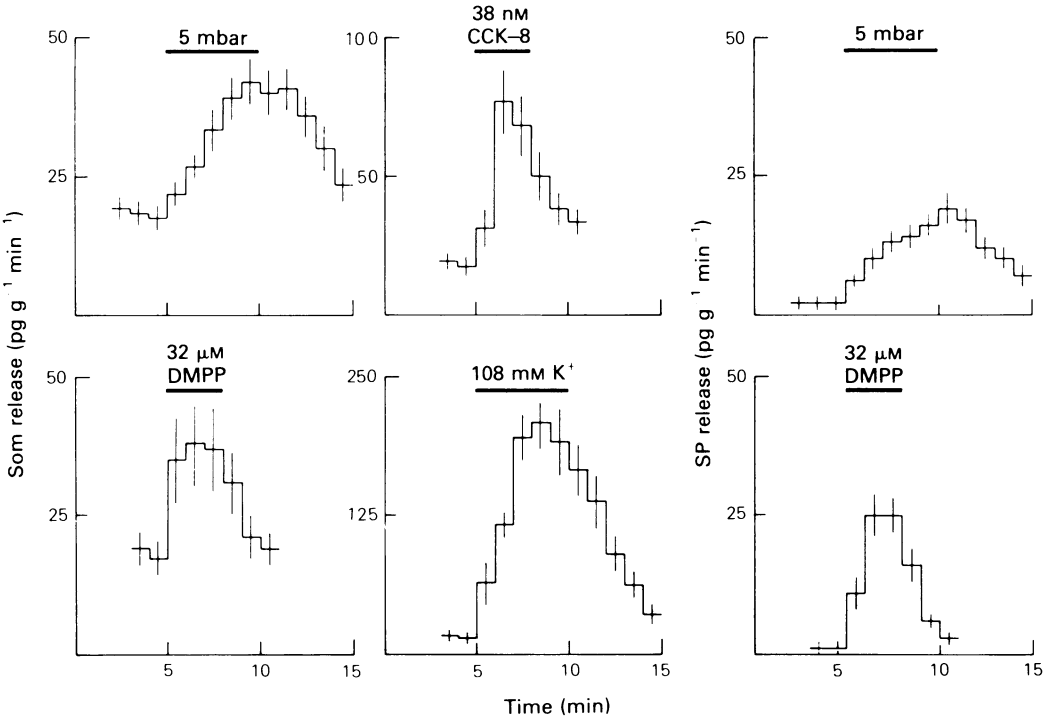

Figure 1 Somatostatin (Som) and substance P (SP) release into the vascular system of the guinea-pig isolated small intestine in the presence of naloxone (1.5 µM). Release was evoked by 5 mbar intraluminal pressure, by dimethylphenylpiperazinium (DMPP), cholecystokinin-octapeptide (CCK-8) or by 108 mM K^+ (40 times the normal K^+ concentration). Means ± s.e.mean, n = 6–13.

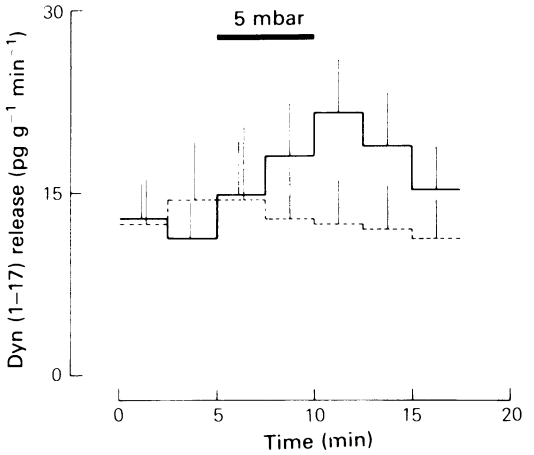

Figure 2 Dynorphin (Dyn-(1–17)) release into the vascular system of the guinea-pig isolated small intestine in the absence of naloxone. The dotted lines indicate the dynorphin release during the infusion of tetrodotoxin (TTX, 1.3 μM). Means with vertical lines showing s.e.mean; $n = 6$.

52% (Table 1). However, neither the basal release of substance P nor that of somatostatin was influenced by naloxone.

The pressure-induced release of somatostatin was reduced by about 50% in the presence of tetrodotoxin (1.3 μM), whereas that of substance P (Table 1) and dynorphin-(1–17) (Figure 2) was completely suppressed by tetrodotoxin. Raising the intraluminal pressure completely failed to evoke peristalsis in the presence of tetrodotoxin. Also noradrenaline (59 μM) inhibited the pressure-induced release of substance P by 89% whereas that of somatostatin was reduced by only 44% (Table 1). Noradrenaline also caused a complete relaxation of the gut muscle so that peristalsis was no longer evoked by increasing the intraluminal pressure.

The infusion of CCK-8 (38 nM) induced contractions of the intestine and a marked release of somatostatin whereas the release of substance P increased only slightly, but significantly ($P < 0.05$) (Figure 1, Table 1). These effects of CCK-8 were not inhibited by tetrodotoxin.

DMPP (32 μM) caused an increase in the release of somatostatin and substance P (Figure 1) which was completely blocked by tetrodotoxin (Table 1). However, neither the DMPP-induced release of somatostatin nor that of substance P was influenced by noradrenaline.

Raising the K^+-concentration of the perfusion solution by a factor of 40 induced the most pronounced release of somatostatin and substance P (Figure 1, Table 1).

Gel chromatography of pooled samples, which had been collected during pressure or K^+ stimulation, showed that 92% of the somatostatin and dynorphin-(1–17) immunoreactivity and all the substance P-like immunoreactivity was eluted at the position of the synthetic substances (Figure 3). About 8% of the somatostatin- and dynorphin-immunoreactivity was eluted at positions of a molecular weight higher than that of the authentic peptides. The recovery of the immunoreactivities applied to the columns amounted to 80–95%.

Table 1 Total release of substance P and somatostatin from the vascularly perfused, isolated small intestine of the guinea-pig

	Substance P (pg g^{-1})	n	*Somatostatin* (pg g^{-1})	n
5 mbar intraluminal pressure				
Without naloxone	17 ± 8*	8	103 ± 20	6
Naloxone (1.5 μM)	112 ± 16	13	157 ± 21	13
Naloxone (1.5 μM), noradrenaline (59 μM)	12 ± 6*	6	88 ± 12	5
Naloxone (1.5 μM), tetrodotoxin (1.3 μM)	1 ± 1*	5	73 ± 16*	5
Cholecystokinin-octapeptide infusion (38 nM)				
Naloxone (1.5 μM)	12 ± 5	6	187 ± 39	7
Naloxone (1.5 μM), tetrodotoxin (1.3 μM)	12 ± 3	4	141 ± 33	5
Dimethylphenylpiperazinium infusion (32 μM)				
Naloxone (1.5 μM)	85 ± 11	12	82 ± 16	7
Naloxone (1.5 μM), noradrenaline (59 μM)	91 ± 17	8	62 ± 17	5
Naloxone (1.5 μM), tetrodotoxin (1.3 μM)	22 ± 8*	6	4 ± 2*	4
108 mM K^+ infusion				
Naloxone (1.5 μM)	348 ± 40	13	1086 ± 168	6

Means ± s.e.mean. *$P < 0.05$ compared with naloxone-group, two sample t test. The amount of peptides released is expressed as pg g^{-1} released during the period of stimulation minus the mean release in the 2 periods preceding the stimulus.

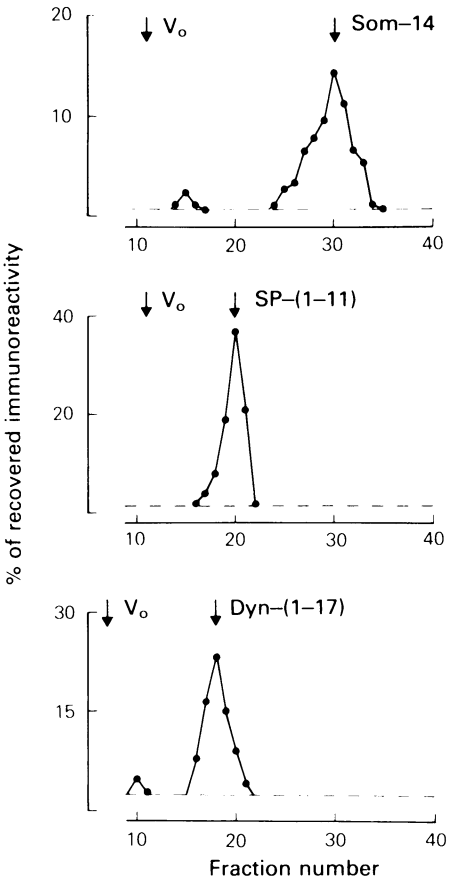

Figure 3 Gel chromatography of somatostatin, substance P and dynorphin-(1–17) immunoreactivity released from the vascularly perfused small intestine of the guinea-pig by pressure and K^+ stimulation. Release samples of several experiments were pooled and chromatographed. The columns were calibrated in separate runs. V_0, void volume; Som–14, authentic somatostatin; SP-(1–11), authentic substance P; Dyn-(1–17), dynorphin-(1–17). The interrupted horizontal lines indicate the detection limit of the assays.

Discussion

Release by pressure stimulation

Taken together, the present results indicate that pressure stimulation activates enteric neurones containing opioid peptides, somatostatin and substance P. The released opioid peptides inhibit the release of substance P, for which there is already both indirect (Barthó *et al.*, 1982 a,b,c; Gintzler & Scalisi, 1982; Kromer & Schmidt, 1982) and direct evidence (Holzer, 1984). The present finding of a pressure-induced release of dynorphin into the venous effluent does not necessarily contradict the reports that the release of Met enkephalin (Clark & Smith, 1981) and dynorphin (Kromer *et al.*, 1981) into the bath medium is decreased during peristaltic activity. It is likely that the different experimental methods account for this discrepancy.

The inhibitory effects of tetrodotoxin indicate that the release of both substance P and dynorphin-(1–17) involves propagated action potentials and is not due to an unspecific effect of the raised intraluminal pressure *per se*. It further demonstrates that the released substance P and dynorphin-(1–17) are of neural origin. The tetrodotoxin-insensitive part of the released somatostatin may stem from endocrine cells in the mucosa.

The results obtained with noradrenaline are in line with the findings that noradrenaline or efferent stimulation of sympathetic nerves can inhibit the release of substance P from the myenteric plexus (Barthó *et al.*, 1983).

Release by DMPP and CCK-8

There is evidence that cholinergic neurones synapse on enteric substance P neurones (Franco *et al.*, 1979), and both acetylcholine (Holzer, 1984) and the ganglion stimulant dimethylphenylpiperazinium (DMPP) (Donnerer *et al.*, 1984) enhance the release of substance P from enteric neurones. The present findings show that the peptides released by DMPP are of neural origin, because their release was inhibited by tetrodotoxin. Since noradrenaline did not inhibit the DMPP-induced release of substance P, the substance P neurones activated by DMPP may not be completely identical to those activated during peristaltic activity. A previous study suggested that during pressure-induced peristalsis, substance P is released from both pre- and postganglionic substance P neurones (Donnerer *et al.*, 1984).

The excitatory effects of CCK-8 in the gut are due not only to release of acetylcholine but also of substance P from enteric neurones (Hutchinson & Dockray, 1981; Barthó *et al.*, 1983; Holzer, 1984). The present results confirm this and show in addition that CCK-8 also releases somatostatin. Since these effects were tetrodotoxin-insensitive, it is not yet clear whether the peptides are released from non-neuronal sources or whether they are released only from dendrites or somata of neurones (see North, 1982).

Significance of the findings

This study has shown that the release of substance P and somatostatin, and in addition of dynorphin-(1–17), from the isolated small intestine is pharmacologically discernible. Of particular importance

is the fact that the pressure-induced release of substance P is inhibited by both endogenous opioid peptides and by noradrenaline whereas the release of substance P induced by DMPP is unaffected by noradrenaline. Since there is now good evidence to regard substance P as an excitatory transmitter involved in the peristaltic reflex (Barthó *et al.*, 1982 b,c; Yokoyama & North, 1983), it seems reasonable to argue that opioid peptides and noradrenaline inhibit intestinal motility, at least in part, by inhibiting the release of substance P. The fact that the release of substance P caused by different stimuli, i.e. by pressure stimulation, DMPP and CCK-8, is influenced by different drugs to a different extent may suggest that there are different populations of enteric substance P neurones which may have different physiological roles.

Endogenous opioid peptides seem also to be involved in controlling intestinal motility (see Kromer & Schmidt, 1982). They not only inhibit the release of acetylcholine but, as shown in this study, also inhibit the release of enteric substance P. Little is known about the significance of somatostatin in intestinal peristalsis. Somatostatin has been shown to inhibit the release of acetylcholine in the gut and to activate inhibitory neurones of the enteric nervous system (see Introduction). It is thus conceivable that both opioid peptides and somatostatin are inhibitory transmitters involved in the peristaltic reflex.

It is now well established that intestinal peristalsis is coordinated by a complex array of excitatory and inhibitory neurones within the enteric nerve plexuses. The integrated activity of these neurones enables the gut to propel its contents in an aboral direction. The complexity of this system makes it difficult to identify the many types of nerve cells which mediate the various components of the peristaltic reflex and the interaction between these neurones. One approach is to record the release of transmitters during peristalsis as executed in the present study. This approach will have to be extended to all substances which are believed to be transmitters of the enteric nervous system (see Furness & Costa, 1982). The demonstration that a substance is released during peristalsis is certainly an argument in favour of a physiological role of this substance in peristalsis but does not prove it. There are some further limitations of release studies like this, which must not be overlooked. It is impossible to identify exactly the source from which a substance is released and, as has been shown in this study for substance P and somatostatin, only a small portion of the substances released within the gut wall appears in the venous effluent.

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