Modulation by opioid peptides of mechanosensory pathways supplying the guinea-pig inferior mesenteric ganglion

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1. Radioimmunological techniques were used in isolated guinea-pig inferior mesenteric ganglion (IMG)–colon preparations to determine whether opioid peptides and neurotensin$^{8-13}$ (NT^{8-13}), the *C*-terminal region of NT^{1-13} recognized by neurotensin receptors, modulate distension-induced release of substance P (SP)- and vasoactive intestinal polypeptide (VIP)-like immunoreactive (LI) material.

2. Colonic distension significantly increased the amount of SP- and VIP-LI material released in the ganglionic superfusate. A low-Ca^{2+} (0·1 mM), high-Mg^{2+} (15 mM) solution blocked their release.

3. *In vivo* capsaicin pretreatment abolished release of SP-LI material during colonic distension but had no significant effect on distension-induced release of VIP-LI material.

4. The addition of [Leu^5]enkephalin, [Met^5]enkephalin, PL017 (a μ-receptor agonist) and DPDPE (a δ-receptor agonist) to the ganglion side of a two-compartment chamber blocked distension-induced release of SP-LI material. The addition of naloxone and ICI-174,864 (a δ-receptor antagonist) to the ganglion compartment reversed the inhibitory effect of the μ- and δ-receptor agonists.

5. Addition of [Leu^5]enkephalin and [Met^5]enkephalin to the ganglion compartment had no significant effect on release of VIP-LI material during colonic distension.

6. Addition of NT^{8-13} to the ganglion compartment significantly increased in the amount of SP-LI material released during colonic distension but had no affect on distension-induced release of VIP-LI material.

7. The results suggest the hypothesis that under *in vivo* conditions, enkephalinergic nerves decrease and neurotensinergic nerves increase the release of SP from peripheral branches of primary afferent sensory nerves.

A non-cholinergic late slow excitatory postsynaptic potential (LS-EPSP) was first described in bullfrog sympathetic neurones (Nishi & Koketsu, 1968) and subsequently in neurones of the guinea-pig inferior mesenteric ganglion (IMG) (Crowcroft & Szurszewski, 1971) and myenteric ganglia of the small intestine (Katayma & North, 1978). A number of electrophysiological studies suggest that LS-EPSPs in the guinea-pig IMG may be due to a direct postsynaptic action of substance P (SP) (Dun & Jiang, 1982; Tsunoo, Konishi & Otsuka, 1982; Dun & Kiraly, 1984), vasoactive intestinal polypeptide (VIP) (Love & Szurszewski, 1987), cholecystokinin (CCK) (Mo & Dun, 1986; Schumann & Kreulen, 1986), neurokinin A (NKA) (Saria, Ma & Dun, 1985), and vasopressin (Peters & Kreulen, 1985). Nerve fibres and terminals containing SP-like immunoreactive (LI) material surround principal ganglion cells of the guinea-pig IMG (Hökfelt, Elfvin, Schultzberg, Goldstein & Nilsson, 1977; Gamse, Wax, Zigmond & Leeman, 1981; Matthews & Cuello, 1982). These nerve fibres arise from cell bodies of the dorsal root ganglia (Matthews & Cuello, 1982, 1984). Nerve fibres and terminals containing VIP-LI material are also found in the guinea-pig IMG (Dalsgaard *et al.* 1983). These nerve fibres arise from enteric ganglion cells of the distal colon (Dalsgaard *et al.* 1983). Recent studies have shown that radial distension of the distal colon releases SP- and VIP-LI material in the IMG (Parkman, Ma, Stapelfeldt & Szurszewski, 1993).

Methionine^5 ([Met^5])-enkephalin-LI (Schultzberg *et al.* 1979; Dalsgaard, Vincent, Hökfelt, Christensson & Terenius, 1983; Heym, Reinecke, Weihe & Forssmann, 1984) and neurotensin (NT)-LI material (Lundberg,

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Rökaeus, Hökfelt, Rosell, Brown & Goldstein, 1982; Reinecke, Forssmann, Thiekötter & Triepel, 1983; Heym *et al.* 1984) are two other neuropeptides found in prevertebral ganglia. Both peptides are located in nerve fibres derived from different sets of spinal preganglionic neurones (Reinecke *et al.* 1983; Kondo, Kuramoto, Wainer & Yanaihara, 1985). Results from electrophysiological experiments show that the enkephalinergic pathway decreases the amplitude and duration of the LS-EPSP probably by inhibiting release of SP from axon collaterals of primary afferent sensory nerves (Konishi, Tsunoo & Otsuka, 1979; Konishi, Tsunoo, Yanaihara & Otsuka, 1980), and that the neurotensinergic pathway increases the amplitude and duration of the LS-EPSP probably by facilitating release of SP (Stapelfeldt & Szurszewski, 1989*a*, *b*). To date, there is no direct evidence to support the hypothesis that the enkephalinergic and neurotensinergic nerves in the IMG mediate or modulate release of SP. The present study was designed to test directly the hypothesis that the enkephalins and NT modulate the release of SP. Release of VIP was also studied because it too has been implicated as a neuromodulator/neurotransmitter of the LS-EPSP (Baron, Jänig & McLachlan, 1985), and it is released in the IMG during colonic distension (Kuo, Krauthamer & Yamasaki, 1981).

Some of the results have been previously communicated in abstract form (Ma & Szurszewski, 1990).

METHODS

Neuropeptide release experiments

The adult male guinea-pigs used in this study were killed by a blow to the head and bled (approved by the Mayo Clinic Animal Care and Use Committee). For each experiment, IMGs with attached segments of distal colon were dissected from four guinea-pigs and placed in a two-compartment chamber. After cleaning off the adherent adipose and connective tissue, the ganglia were securely pinned down to a Sylgard base of the central chamber (200 μl) and superfused at 35–37 °C with normal Krebs solution of the following composition (mM): Na^+, 137·4; K^+, 5·9; Ca^{2+}, 2·5; Mg^{2+}, 1·2; Cl^-, 134; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; glucose, 11·5; equilibrated with 97% O_2 and 3% CO_2. The segments of colon were covered with moist strips of tissue paper and placed in a surrounding chamber which was separately perfused with normal Krebs solution (35–37 °C). The four segments of colon were joined together by polyethylene catheters. One of the catheters was a T-fitting catheter. It was connected to a 10 ml syringe so as to gain access to the colonic lumens for simultaneous distension of all four segments. Unless otherwise stated, the colonic segments were distended by injection of air (3 ml per segment) to a colonic intraluminal pressure of 20–25 cmH_2O. In a few experiments, prewarmed normal Krebs solution with bradykinin was used to fill and distend the colonic segments. In these experiments, prewarmed normal Krebs solution was used during control periods. In some experiments, colonic intraluminal pressure was measured using a procedure previously described (Stapelfeldt & Szurszewski, 1989*a*). The ganglia were superfused for 20 min with a Krebs solution containing 0·01% collagenase (Sigma) to facilitate recovery of released neuropeptides followed by a Krebs solution containing bovine serum albumin (BSA, 0·2% w/v) (Sigma) and Trasylol (aprotinin, 500 units ml^{-1}) (FBA Pharmaceuticals, West Haven, CT, USA) to diminish enzymatic breakdown of released neuropeptides. The ganglia were superfused with the latter solution for the remainder of the experiment. The concentration of BSA and Trasylol used in these experiments did not interfere with the radioimmunoassays employed as determined by recovery of >94% of neuropeptides added to blank superfusion media.

The experimental protocol consisted of stopped-flow periods (each 2 min long) separated by free-flow periods (each 5 min long) (Fig. 1). During the first and third stopped-flow periods, the colonic segments were not distended. These periods served as controls and are referred to as control and recovery/control, respectively. During the intervening second period of stopped-flow, all four colonic segments were distended simultaneously. At the end of each stopped-flow period, the superfusate (200 μl) was aspirated from the ganglion chamber and immediately frozen on dry ice for subsequent determination of the amount of SP- and VIP-LI material by radioimmunoassay (RIA), as previously described (Stapelfeldt & Szurszewski, 1989*a*). This sequence of three 2 min stopped-flow periods separated by free-flow periods was repeated three times and the three samples of aspirated superfusates from the three identical periods were combined and frozen for RIA (Fig. 1). Experiments consisted of two or more sets of stopped-flow periods (Fig. 1). In the first set, normal Krebs solution was used to perfuse the ganglion chamber. In the second set, a test substance (either tetrodotoxin (TTX), leucine ([Leu^5])-enkephalin, [Met^5]enkephalin, dynorphin$^{1-13}$, neurotensin$^{8-13}$ [NT^{8-13}], or an opioid receptor antagonist, denoted test substance 1; see Fig. 1) was added to the Krebs solution perfusing only the ganglion compartment, or a Krebs solution containing bradykinin was used to fill the colonic lumens and to distend them. In a third set, an opioid receptor antagonist (test substance 2; Fig. 1) was added to the Krebs solution perfusing only the ganglion compartment. This was followed by a fourth set in which the opioid receptor agonist and antagonist were added to the ganglion compartment. In some experiments, the ganglia were superfused with a high-K^+ (80 mM) Krebs solution (Fig. 1). Thus, in summary, each sample of ganglionic superfusate used for RIA was 600 μl and consisted of normal Krebs solution (control), Krebs solution containing either a test substance(s) or TTX, or high-K^+ Krebs solution (Fig. 1). Following some experiments, the IMGs were processed to determine their content of SP- and VIP-LI material.

[Leu^5]enkephalin, [Met^5]enkephalin, dynorphin$^{1-13}$ (10^{-5} M) and NT^{8-13} (5×10^{-6} M) (Sigma) did not cross-react with the SP antiserum or VIP antiserum used in this study, and did not interfere with the binding characteristics of our assay when added to the SP standard or VIP standard.

Radioimmunoassay procedures

The samples of superfusate were lyophilized until dry, reconstituted with 200 μl of assay buffer, and run as duplicate 100 μl samples for radioimmunoassay of SP-LI and VIP-LI materials. Specimens of IMGs from normal, vehicle-treated, and capsaicin-pretreated guinea-pigs were individually weighed, dissolved (1 : 20) in 0·1 M HCl, boiled for 20 min and homogenized with a Polytron® homogenizer (Brinkmann Instrument Co., Westbury, NY, USA). After centrifugation for at least 20 min, the supernatant of each specimen was decanted and stored at −20 °C for subsequent neuropeptide analysis. Previous studies have shown this extraction procedure to yield reliable recoveries of SP and VIP

(Yaksh, Abay & Go, 1982) for specific radioimmunoassays. The antisera used in this study were developed in the Radioimmunoassay Research Core Facility of our institution.

Radioimmunoassay for SP. The assay method employed was similar to one previously described (Micevych, Yaksh & Go, 1982). Synthetic (Try[8])-SP (Peninsula Labs, Belmont, CA, USA) was ^{125}I-radiolabelled by a modified Greenwood–Hunter method. Purification steps included absorption to cellulose powder, washing with 0·2 M glycine (pH 8·8), elution with acidified plasma (10% human plasma, 0·2 M acetic acid), and gel chromatography over a Bio-Gel P10 column (Bio Rad Labs, Richmond, CA, USA). The assay buffer (pH 6·5) consisted of 0·04 M KH_2PO_4, 1% BSA, 0·01 M EDTA, 2% polyethylene glycol (PEG) (8000), 0·25% Trasylol and 0·2% thimerosal. Sample volumes of 0·1 ml diluted SP standard, samples of superfusate or neutralized tissue extract were incubated with 0·1 ml rabbit SP antiserum (No. 4892, 1:15 000) and 0·7 ml assay buffer for 16 h of pre-incubation. ^{125}I-SP diluted in assay buffer containing 4% normal rabbit serum at a specific activity of 8000 c.p.m. per 0·15 ml was added and the mixture was incubated for an additional 24 h. The bound complex was separated by adding a titred amount of goat anti-rabbit serum (Calbiochem, San Diego, CA, USA), incubated for another 30 min and centrifuged at 2500 r.p.m. for 15 min (double-antibody method). After discarding the supernatant, the activity of the bound antibody was counted in a gamma counter for 5 min. The detection threshold (assay sensitivity) was 1·0 pg tube^{-1} and the inter- and intra-assay variations were 11 and 5%, respectively. The antiserum showed 100% cross-reactivity with the oxidized molecular form of SP and less than 1% cross-reactivity with other tachykinins such as kassinin, neurokinin A and substance K.

Radioimmunoassay for VIP. Rabbit VIP antiserum (No. 4823) was raised against natural VIP coupled to BSA, as previously described (Yaksh *et al.* 1982). The VIP antiserum was pre-incubated in a 1:100 000 dilution with 200 µl standard or tissue extract samples in 0·01 M phosphate buffer (pH 7·6) containing 0·05 M EDTA, 0·2 M benzamidine and 0·1% (w/v) BSA at a total volume of 0·8 ml for 15–18 h. ^{125}I-VIP, prepared by the chloramine-T method, was added at 1500 c.p.m. per 0·1 ml and incubated for another 24 h. In a similar separation procedure to that described above, 0·1 ml of goat anti-rabbit serum, 0·2 ml of 2% normal rabbit serum and 4% PEG (8000) were added, the mixture was incubated for another 6 h, and the bound activity was counted after centrifugation. This procedure yielded an assay sensitivity of 4 pg tube^{-1} at inter- and intra-assay variations of 10 and 4%, respectively.

In vivo capsaicin treatment

Two groups of guinea-pigs were used. In one group, the animals were pretreated with capsaicin *in vivo*. The procedure for *in vivo* capsaicin treatment was previously described in detail (Stapelfeldt & Szurszewski, 1989*a*). A second group of guinea-pigs was pretreated with only the vehicle solution following the procedure used for administering capsaicin. These animals served as controls. At 5–8 days after the second injection, animals in both groups were killed by a sharp blow to the head and bled.

Drugs

Atropine sulphate, bradykinin, hexamethonium bromide, leucine-enkephalin ([Leu[5]]enkephaline: Tyr-Gly-Gly-Phe-Leu), methionine-enkephalin ([Met[5]]enkephaline: Tyr-Gly-Gly-Phe-Met), dynorphin$^{1-13}$naloxone, neurotensin$^{8-13}$ (NT$^{8-13}$), and

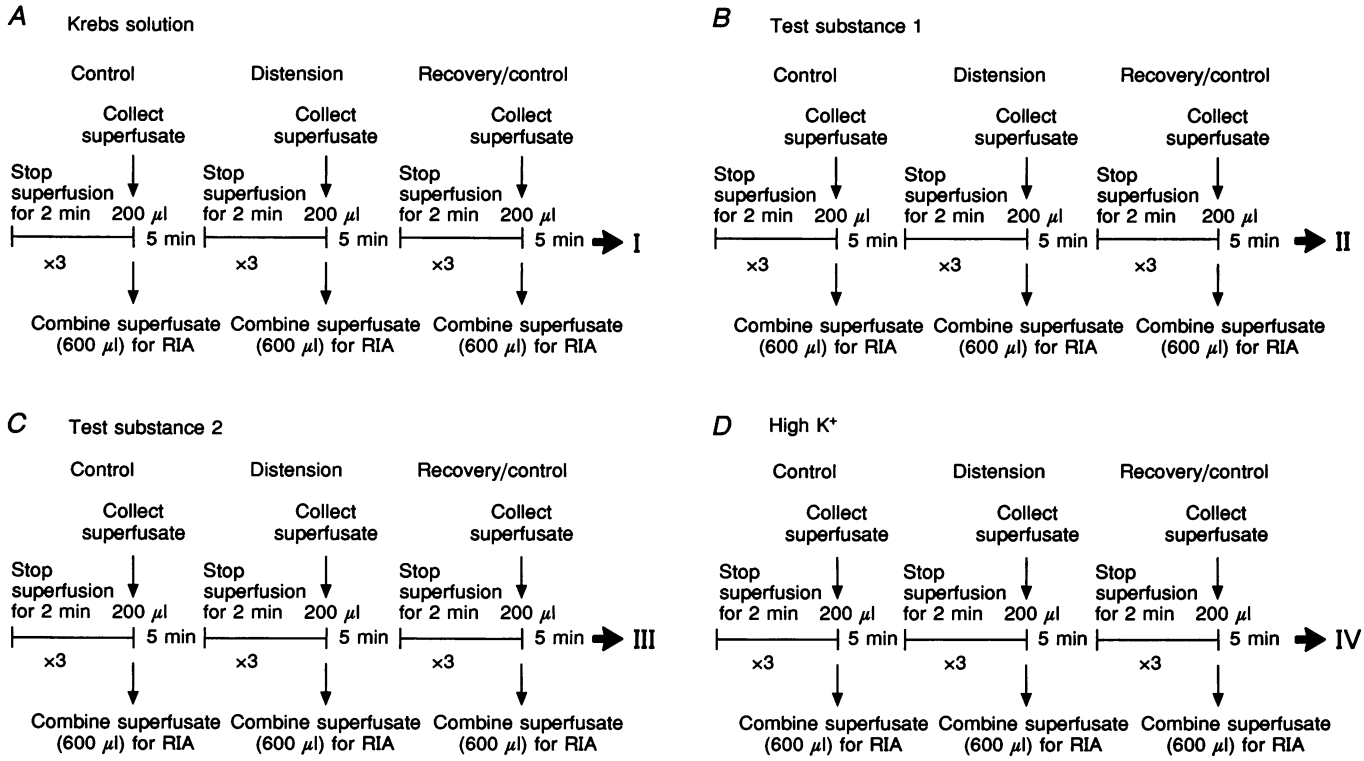

Figure 1. Schematic outline of the experimental protocol used in this study

I, to *B* or *D*, or repeat *A* using different pressures. II, end of experiment, or to *C*. III, end of experiment, or repeat *B* and *C*. IV, end of experiment, or repeat *A* in low Ca^{2+} and high Mg^{2+}. For details, see text.

tetrodotoxin were purchased from Sigma. The μ-, δ- and κ-receptor agonists, PL017 (Try-Pro-*N*-MePhe, D-Pro-NH_2; Peninsula Labs, Belmont, CA, USA), DPDPE ([D-Pen^2, D-Pen^5] enkephalin; Sigma), and U-50,488H ([*trans*-3,4-dichloro-*N*-methyl-*N*-(2-91 pyrolidinyl)-cychlohexyl]-benzeneacetamide methanesulphonate; Upjohn, Kalamazoo, MI, USA), respectively, and the δ- and κ-receptor antagonists ICI-174,864 (Cambridge Research Biochemicals, Valley Stream, NY, USA) and nor-BNI (nor-binaltomorphine; Research Biochemicals Incorporated), respectively, were also used. The drug concentrations cited in the text and figures are the final concentrations added to the chamber. In some experiments, a solution low in calcium (0·1 mM) and high in magnesium (15 mM) was used to block neurotransmitter release. A high-K^+ solution (80 mM) was prepared by replacing NaCl with an isosmotic equivalent of KCl.

Statistical analysis

The results are expressed as means ± S.E.M., in picograms per millilitre for SP-LI material and in nanograms per millilitre for VIP-LI material. Individual experiments were designed to allow repeated measurements of release of SP-LI and VIP-LI material from the same ganglia. Each period of colonic distension was preceded and followed by a control period (Fig. 1). During both periods, the ganglia were superfused with the same solution. In none of the experiments was there a significant difference within an experiment between either the amount of SP-LI or VIP-LI released during the two control periods. Thus, for convenience and sake of clarity, the amounts of SP- and VIP-LI material released during distension was compared with the amount released during the control period immediately preceding distension. Under these circumstances, differences in release of SP-LI were tested for significance using the Wilcoxon ranked sum test. This test was used because the amount of SP-LI released during control periods was below detection threshold (assay sensitivity). Differences in release of VIP-LI were tested for significance using Student's *t* test. In both instances significance was accepted at $P < 0.05$. Some of the results also were used for making multiple comparisons, for example, comparing the amount of SP-LI and VIP-LI released during colonic distension to the amounts released during treatment of the ganglia with 80 mM K^+. Under this and other circumstances when multiple comparisons were made between unrelated samples, differences were tested for significance using a one-way analysis of variance (ANOVA) followed by the Bartlett multiple comparison test and by the Tukey–Kramer multiple comparison test. The $P < 0.05$ level of probability was considered significant. Unless stated in the text, *F* values are reported in the figure legends. ANOVA also was used to test the significance of the content of SP-LI and VIP-LI material in IMG tissue among vehicle-pretreated (control) and capsaicin-pretreated groups. Values of $P < 0.05$ were considered significant. All statistical analyses were made using the Instat Computer program (GraphPAD Software Inc., San Diego, CA, USA).

RESULTS

Release of SP and VIP

In the absence of colonic distension, SP-LI material in the superfusate was below detection threshold (assay sensitivity). Colonic distension significantly increased the amount of SP-LI material in the superfusate to 26·6 ± 4·8 pg ml^{-1} ($n = 40$, $P < 0.05$) (Fig. 2*Aa*). Treatment of the ganglia for 2 min with high K^+ also significantly increased the amount of SP-LI material in the superfusate ($P < 0.05$, $n = 33$; Fig. 2*Aa*). The amount of SP released by high K^+ was not significantly different from the amount released during colonic distension (Fig. 2*Aa*). Colonic distension and treatment of the ganglia with high K^+ also significantly increased the amount of VIP-LI material in the superfusate (Fig. 2*Ab*). The amount of VIP-LI material released by high K^+ was not significantly different from the amount released during colonic distension (Fig. 2*Ab*).

The amount of SP- and VIP-LI material in the superfusate was measured when colonic intraluminal pressure was increased to three different levels. In these experiments, atropine (2×10^{-6} M) and hexamethonium (2×10^{-4} M) were added only to the ganglion chamber to interrupt cholinergic transmission during colonic distension, thereby preventing cholinergic-dependent reflex sympathetic activity from returning to the colonic segments while they were distended. Three ranges of increased colonic intraluminal pressure were studied: 7–5, 15–10 and 25–20 cmH_2O. After the initial increase in pressure (first value), intraluminal pressure steadily declined in 10–15 s to a lower pressure (second value) which was maintained throughout the remainder of the distension period. SP-LI material was not detected during the control period when the colonic segments were not distended, nor was any detected when colonic intraluminal pressure was increased to 7–5 cmH_2O. The first detectable and significant (compared with control) increase occurred at pressures ranging from 15–10 cmH_2O (Fig. 2*Ba*). A further increase (25–20 cmH_2O) in intraluminal pressure resulted in an increase in the amount of SP-LI material in the ganglionic superfusate. In contrast, the amount of VIP-LI material in the ganglionic superfusate significantly ($P < 0.05$, compared with control) increased with each step-increase in colonic intraluminal pressure (Fig. 2*Bb*).

The release of SP- and VIP-LI evoked by colonic distension was blocked when tetrodotoxin (10^{-5} M) was present in the chamber which contained the four colonic segments (Fig. 3*A*). In these experiments, tetrodotoxin was added 10 min prior to colonic distension. Note that tetrodotoxin also reduced release of VIP-LI material during the control period (Fig. 3*Ab*).

Effect of low Ca^{2+} and high Mg^{2+} on release of SP and VIP

This series of experiments was designed to determine whether the release of SP-LI and VIP-LI material was calcium dependent. After a 20 min superfusion of the ganglia with a low-Ca^{2+} (0·1 mM), high-Mg^{2+} (15 mM) Krebs solution, SP- and VIP-LI material was not detected in the ganglionic superfusate during the control period (Fig. 3*B*). The low-Ca^{2+}, high-Mg^{2+} solution also blocked the increase in SP- and VIP-LI material normally seen during colonic distension and high K^+ (Fig. 3*B*). Thus, release of both neuropeptides was a calcium-dependent process.

Effect of bradykinin on release of SP and VIP

In these experiments, a bradykinin-containing (5 μM) Krebs solution was used to distend simultaneously the four segments of colon. Mucosal treatment with bradykinin increased the amount of SP-LI material detected during colonic distension (Fig. 4*A*), and SP-LI material was detected during the control period when the segments were not distended. The amount of SP-LI detected during distension when bradykinin was present was significantly greater ($P < 0{\cdot}05$) when compared with the amount released by distension when only Krebs solution was used. The increase in SP-LI material during the control period was also significantly ($P < 0{\cdot}05$) greater when compared with the amount released during the immediately preceding control period. There was no significant difference between the amount of VIP-LI material released during distension in the presence of bradykinin compared with the amount released when normal Krebs solution was used to distend the segments of colon (Fig. 4*B*).

Effect of *in vivo* capsaicin pretreatment on IMG content and release of SP and VIP

In this series of experiments, guinea-pigs were divided into a control (vehicle-pretreated) group and a capsaicin-pretreated group. *In vivo* capsaicin pretreatment depleted SP-LI material in the IMG and lumbar dorsal root ganglia, and abolished release of SP-LI during colonic distension and during exposure of the IMGs to high K^+ (data not shown). *In vivo* capsaicin pretreatment, however, had no significant ($F[2,90] = 0{\cdot}004$, $P > 0{\cdot}05$) effect on the content of VIP-LI material in the IMG, no significant effect on the release of VIP-LI material before ($F[7,40] = 0{\cdot}61$, $P > 0{\cdot}05$) and during ($F[7,40] = 1{\cdot}51$, $P > 0{\cdot}05$) colonic distension, and no significant

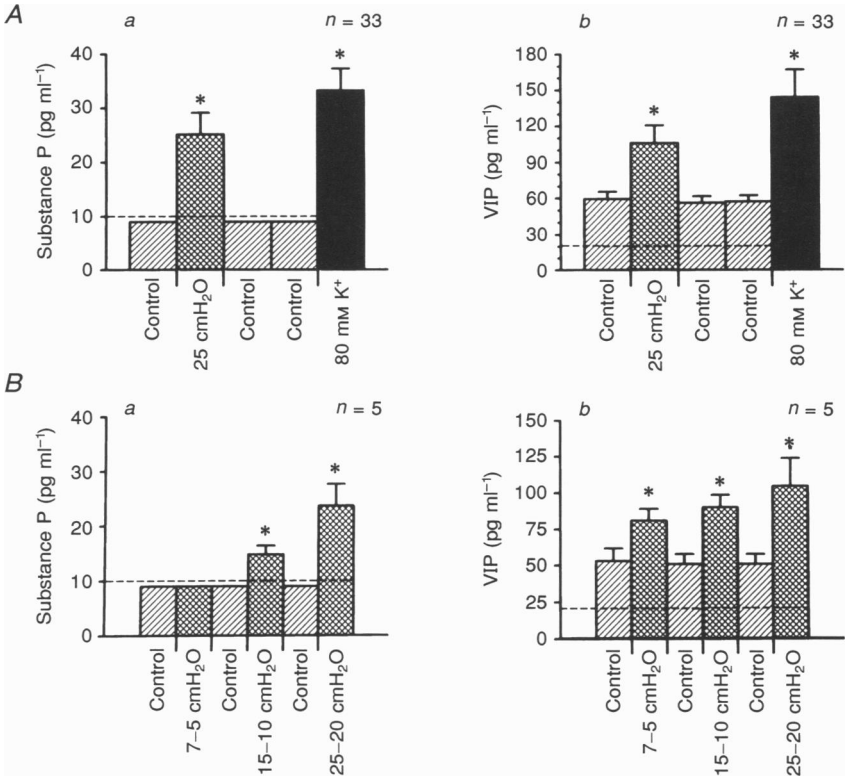

Figure 2. Increase in the amount of SP- (*a*) and VIP-LI (*b*) material released during colonic distension using high pressure and during exposure of the ganglia to high K^+

A, before colonic distension, the amount of SP-LI in the ganglionic superfusate was not different from the detection threshold (assay sensitivity). During colonic distension (25 cmH_2O) and during exposure of the IMGs to high K^+ (80 mM), the amount of SP-LI material and the amount of VIP-LI material in the ganglionic superfusate increased significantly ($P < 0{\cdot}05$) compared with control. The amount of SP- and VIP-LI material released by high K^+ was not significantly different from the amounts released during colonic distension (for SP: ($F[4,160] = 3{\cdot}16$, $P > 0{\cdot}05$; for VIP: $F[4,160] = 3{\cdot}45$, $P > 0{\cdot}05$). *B* shows the effect of different distension pressures on release of SP- and VIP-LI. Note that a distension pressure of 15–10 cmH_2O was required for detection of SP-LI whereas VIP-LI was detected during control conditions and at a distension pressure of 7–5 cmH_2O. In this and subsequent figures, the detection thresholds (assay sensitivity) for SP- and VIP-LI material are indicated by a dashed line. * $P < 0{\cdot}05$ compared with control.

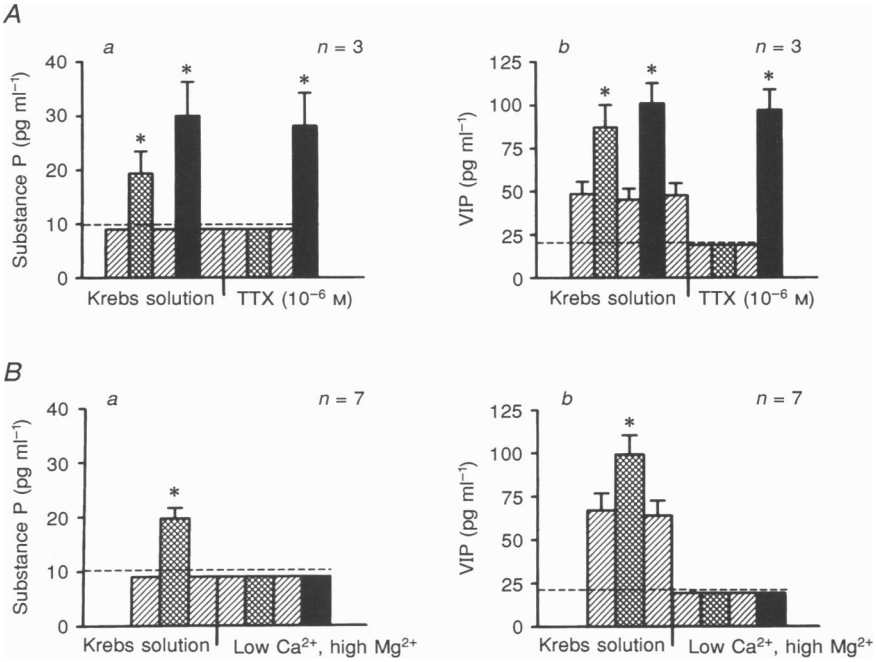

Figure 3. Effect of tetrodotoxin added to the colon chamber (*A*) and of a low-Ca^{2+}, high-Mg^{2+} solution in the ganglion chamber (*B*) on release of SP- (*a*) and VIP-LI (*b*) material during colonic distension and during exposure of the IMGs to high K^+

Note that TTX (10^{-5} M) blocked distension-evoked release of SP- and VIP-LI material. The release of both neuropeptides by the high K^+ (80 mM) solution when TTX was present most probably was due to a direct depolarization by K^+ of the synaptic terminals. During superfusion of the IMGs with the low-Ca^{2+} (0·1 mM), high-Mg^{2+} (15 mM) solution, the amounts of SP- and VIP-LI material before and during colonic distension, and during exposure of IMGs to high K^+ were below the detection threshold. * $P < 0·05$ compared with control. ▨, control; ▩, distension; ■, 80 mM K^+.

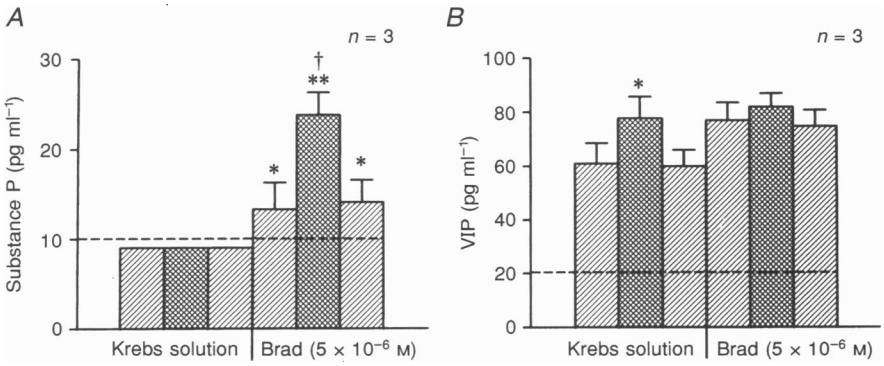

Figure 4. Effect of adding bradykinin to the colonic lumen on release of SP- (*A*) and VIP-LI (*B*) in the IMGs

Note that the amount of SP-LI detected in the ganglionic superfusate was: (a) greater ($P < 0·05$) during the control period with bradykinin compared with the amount detected during the immediately preceding Krebs solution control period; (b) greater during distension with bradykinin compared with the amount detected during distension with normal Krebs solution ($F[5,12] = 5·86$, $P < 0·05$). Bradykinin (Brad, 5×10^{-6} M) had no significant ($F[5,12] = 1·94$, $P > 0·05$) effect on distension-induced release of VIP-LI material. * $P < 0·05$ compared with Krebs solution; ** $P < 0·05$ compared with bradykinin; † $P < 0·05$ compared with distension in Krebs solution. ▨, control; ▩, distension.

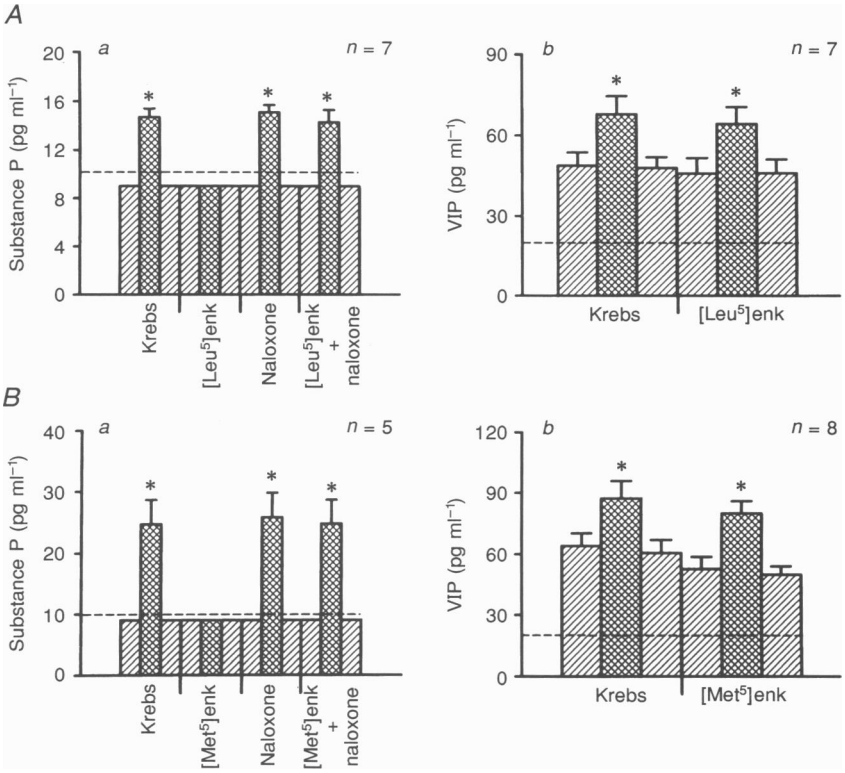

Figure 5. Effect of adding leucine-enkephalin (*A*) and methionine-enkephalin (*B*) to the ganglion chamber on control and distension-induced release of SP- (*a*) and VIP-LI (*b*) material

[Leu⁵]-enkephalin ([Leu⁵]enk, 10^{-5} M) and [Met⁵]enkephalin ([Met⁵]enk, 10^{-5} M) blocked distension-induced release of SP-LI in the IMGs. The inhibitory effect of the enkephalins was reversed by naloxone (10^{-6} M). Release of VIP-LI was not significantly affected by either of the enkephalins (*Ab* and *Bb*). * $P < 0.05$ compared with control. ▨, control; ▩, distension.

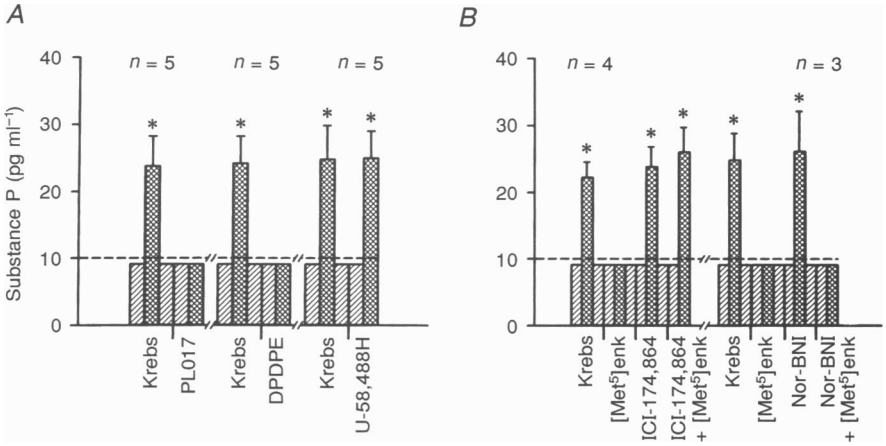

Figure 6. Effect of adding opioid receptor agonists (*A*) and antagonists (*B*) to the ganglion chamber on release of SP-LI in the IMGs

The amount of SP-LI in the ganglionic superfusate during colonic distension was not different from the detection threshold of the assay when PL017 (μ-receptor agonist) and DPDPE (δ-receptor agonist) were added to the ganglion chamber. The amount of SP-LI material detected during colonic distension when U-50,488H (κ-receptor agonist) was present was not significantly ($F[10,64] = 0.08$, $P > 0.05$) different compared with the amount released during distension in normal Krebs solution. ICI-174,864 (δ-receptor antagonist) antagonized the inhibitory effect of [Met⁵]enkephalin (10^{-5} M) whereas nor-BNI (κ-receptor antagonist) did not. * $P < 0.05$ compared with control. ▨, control; ▩, distension.

($F[7,40] = 1{\cdot}00$, $P > 0{\cdot}05$) effect on release of VIP-LI during exposure of the IMGs to high K^+ (data not shown).

Effect of opioid receptor agonists and antagonists on the release of SP and VIP

Addition of [Leu^5]enkephalin (10^{-5} M) or [Met^5]enkephalin (10^{-5} M) to the ganglion chamber blocked distension-induced release of SP-LI material, an effect reversed when the ganglia were pretreated for 10 min with naloxone (10^{-6} M) (Fig. 5*Aa* and *Ba*). In sharp contrast, [Leu^5]enkephalin (10^{-5} M) and [Met^5]enkephalin (10^{-5} M) had no significant effect on distension-induced release of VIP-LI material (Fig. 5*Ab* and *Bb*). Dynorphin, another member of the opioid peptide family, had no significant effect on distension-evoked release of either SP-LI or VIP-LI (data not shown).

The subtype of opioid receptor which mediated the inhibitory effect of the enkephalins was determined by studying the effect of μ-, δ- and κ-receptor agonists on distension-evoked release of SP-LI material. PL017 (10^{-5} M) and DPDE (10^{-5} M) added separately to the ganglion chamber blocked the distension-induced increase in SP-LI material (Fig. 6*A*). However, U-50,488H (10^{-5} M) had no significant effect on distension-evoked release of SP-LI material (Fig. 6*A*). Further evidence supporting the idea that μ- and/or δ-receptors mediated the inhibitory effect of the enkephalins was obtained by studying the effect of specific enkephalinergic receptor antagonists. ICI-174,864 antagonized the inhibitory effect of [Met^5]enkephalin. When nor-BNI was used, [Met^5]enkephalin was still able to block distension-induced release of SP-LI material (Fig. 6*B*).

Effect of NT$^{8-13}$ on the release of SP and VIP

The amount of SP-LI material in the ganglionic superfusate during high-pressure (25–20 cmH_2O) colonic distension was significantly ($P < 0{\cdot}05$) greater when NT$^{8-13}$ was present in the ganglion chamber compared with the amount released when normal Krebs solution was used to perfuse the ganglion chamber (Fig. 7*A*). NT$^{8-13}$ had no effect on control release of SP-LI material (Fig. 7*A*). NT$^{8-13}$ had no effect on control or distension-induced increase in VIP-LI material in the IMG (Fig. 7*B*). These data indicate that NT$^{8-13}$ specifically facilitated release of SP but not of VIP in the IMGs during colonic distension.

DISCUSSION

The results of this study show that the enkephalins and neurotensin, neuropeptides known to be present in sympathetic preganglionic terminals surrounding principal ganglion cells of the guinea-pig IMG (Schultzberg *et al.* 1979; Dalsgaard *et al.* 1983; Heym *et al.* 1984), modulate colonic distension-induced release of SP-LI material in the guinea-pig IMG. Neither neuropeptide affected release of VIP-LI material. Thus, the data support the hypothesis that under *in vivo* conditions, preganglionic enkephalinergic and neurotensinergic nerves ending in the IMG modulate colonic distension-induced release of SP.

The vast majority of SP-containing primary afferent nerve fibres which pass through the prevertebral ganglia give off axon collaterals to form synapses in the ganglion neuropil (Elfvin & Dalsgaard, 1977; Matthews & Cuello, 1982, 1984). The depletion of SP-LI material in the IMG and lumbar DRG after *in vivo* capsaicin treatment confirms previous observations (Matthews & Cuello, 1984; Stapelfeldt & Szurszewski, 1989*b*) which show that the SP-containing nerve fibres arise from cell bodies of the lumbar DRG. Thus, the *en passant* synapses between the axon collaterals of the SP-containing fibres and the principal ganglion neurones of the IMG constitute a sensory axon peripheral reflex.

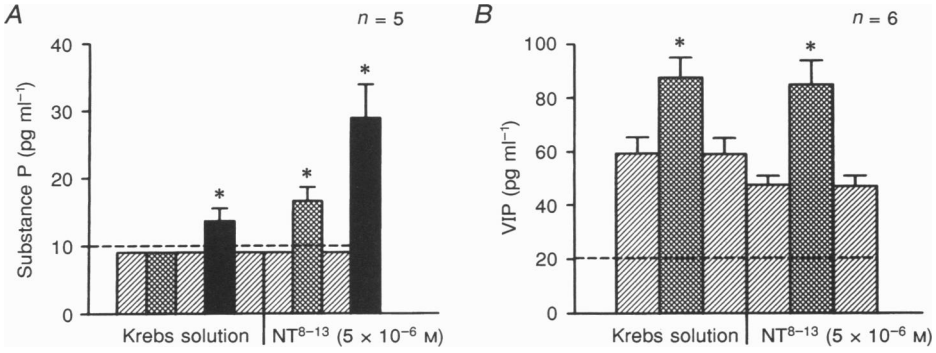

Figure 7. Effect of adding NT$^{8-13}$ to the ganglion chamber on release of SP- (*A*) and VIP-LI (*B*) material during colonic distension

When NT$^{8-13}$ was present in the ganglion bath, the amount of SP-LI material released during the high-pressure (25–20 cmH_2O) distension was significantly ($F[8,36] = 8{\cdot}04$, $P < 0{\cdot}05$) greater compared with the amount released during distension when only Krebs solution was present in the ganglion chamber. NT$^{8-13}$ had no significant effect on the release of VIP-LI during the control period (Krebs solution) or during colonic distension ($F[5,30] = 0{\cdot}43$, $P > 0{\cdot}05$). * $P < 0{\cdot}05$ compared with control. ▨, control; ▩, distension, 7–5 cmH_2O; ■, distension, 25–20 cmH_2O.

The present study confirms a previous one which showed that colonic distension releases SP-LI in the guinea-pig IMG (Parkman *et al.* 1993), and it extends it in two ways. Firstly, the results of the present study suggest the possibility that the SP pathway was a high threshold pathway activated by intraluminal colonic pressures well above those required to release VIP (this study) and acetylcholine in the IMG (Parkman *et al.* 1993). However, since release of SP-LI material during control periods was below the detection threshold (assay sensitivity), the possibility that there was a real but undetectable increase in release by the smallest distension cannot be excluded. Secondly, the enkephalins and NT^{8-13} modulated release of SP in the IMG when the SP pathway was activated by colonic distension. Previous studies have shown that NT^{8-13} potentiates release of SP in the IMG during electrical stimulation of the lumbar colonic nerves (Stapelfeldt & Szurszewski, 1989*a*). The present study extends these observations by showing that both neuropeptides modulate release of SP-LI material during colonic distension.

The inhibitory effect of the enkephalins on the release of SP-LI material was mediated by δ- and/or μ-receptors but not by κ-receptors. The significant decrease in the amount of SP-LI material in the ganglionic superfusate when PL017 and DPDPE, agonists with greater affinity for μ- (Chang, Wei, Killian & Chang, 1983) and δ-receptors (Mosberg *et al.* 1983), respectively, were present in the ganglionic superfusate supports this suggestion. Furthermore, ICI-174,864, a selective δ-receptor antagonist (Kosterlitz, 1985), blocked the effect of [Met^5]enkephalin further supporting a role for δ-receptors. The lack of effect of U-50,488H, an agonist for κ-receptors (Vonvoigtlander, Lahti & Ludens, 1983), of $dynorphin^{1-13}$, which acts mainly on the κ-receptor (Chavkin, James & Goldstein, 1982) and of nor-BNI (Takemori, Ho, Naeseth & Portoghese, 1988), a selective κ-opioid receptor antagonist, excludes a role for κ-receptors. Although dynorphin contains the [Leu^5]enkephalin amino acid sequence at its N-terminal, both biochemical and immunohistochemical studies in the brain and periphery have suggested that dynorphin and enkephalin immunoreactive systems are independent (Bornstein, Furness, Messenger & Stebbing, 1992). Dense networks of both dynorphin- and enkephalin-positive fibres are present in the prevertebral ganglia, including the IMG. It has been shown that dynorphin-immunoreactive fibres in the IMG originate from the gastrointestinal wall and reach the ganglion predominantly via the colonic nerves and to a lesser extent via the hypogastric and intermesenteric nerves, whereas the enkephalin-positive fibres in the IMG originate from the spinal cord and reach the ganglia via the lumbar splanchnic nerves (Dalsgaard *et al.* 1983). The results obtained in the present study show that addition of $dynorphin^{1-13}$ to the ganglion chamber had no significant effect on the release of either SP-LI or VIP-LI material in IMG during colonic distension, suggesting that dynorphin had a different effect from that of enkephalins in the guinea-pig IMG. The functional importance of dynorphin in IMG remains for future study.

The function and modulation of the peripheral afferent VIP pathway contrasts markedly with the SP pathway. The ongoing, TTX-sensitive release of VIP during periods when the colonic segments were not distended indicates the existence of ongoing activity in viscerofugal VIP-containing enteric neurones. There are no data from the present study indicating whether these neurones were the same population that responded to distension or whether they respond to mucosal stimulation and chemical stimulation. However, the increase in VIP-LI material in the ganglionic superfusate when the colon was distended with pressures less than 10 cmH_2O suggests that there was a population of enteric neurones that had a low threshold for activation. Neither the enkephalins nor NT^{8-13} had any significant effect on the amount of VIP-LI material released during control periods when the colonic segments were not distended. Thus, unlike the SP pathway, synaptic events mediated by VIP would not be expected to be modulated by preganglionic enkephalinergic or neurotensinergic nerves.

In summary, this study provides direct evidence supporting the hypothesis that under *in vivo* conditions, preganglionic enkephalinergic and neurotensinergic nerves ending in the IMG modulate colonic distension-induced release of SP from *en passant* synapses of polymodal, primary afferent visceral sensory nerves. Thus, mechanosensory information arriving in the IMG via the peripheral extension of primary afferent sensory fibres of DRG cells containing SP can be separately modulated in the IMG without alteration of the signal referred centrally via the central extension of the same DRG cell.

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