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ORIGINAL ARTICLE

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Localization and characterization of neuropeptide receptors in human colon

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Abstract Information about the expression of neuropeptide receptors is limited in human peripheral tissues, such as the gastrointestinal tract, as compared to the brain. A detailed evaluation of binding sites for gastrin-releasing peptide (GRP), neuropeptide Y, vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating polypeptide (PACAP), gastrin/cholecystokinin, neurotensin, substance P and somatostatin was therefore undertaken in human colon using in vitro receptor autoradiography and subtype characterization with receptor-selective ligands. GRP receptors, Y_2 receptors, PACAP type$_1$-receptors, cholecystokinin-A receptors, neurotensin1 and sst$_2$ receptors were abundantly expressed in the myenteric plexus. Y_2, neurotensin1 and sst$_2$ receptors were also strongly expressed in the submucosal plexus. Furthermore, expression of GRP receptors, neurokinin (NK)$_1$ receptors, VIP type$_2$-receptors and sst$_2$ receptors was found in the mucosa-directed margin of the circular smooth muscle where the interstitial cells of Cajal are located. A variable and complementary expression of GRP receptors, VIP/PACAP receptors, Y_2, neurotensin1, NK_1 and somatostatin receptors was found in the circular and longitudinal smooth muscle. NK_1 and Y_1 receptors were often detected in arteries and veins, while VIP/PACAP and sst$_2$ receptors were found in lymphoid follicles. Y_2, VIP type$_1$ and sst$_2$ receptors were present in the colonic mucosa. Y_2 was strongly expressed in the muscularis mucosae.

This study shows that neuropeptide receptors are expressed in high amounts and in highly specific patterns in distinct targets in the human colon, suggesting a major physiological role for these peptides. The data represent the molecular basis to investigate the regulation by neuropeptides of colonic functions and to develop neuropeptide drugs aimed at interacting with these receptors in colonic diseases, such as Hirschsprung's and Crohn's diseases.

Keywords Peptide receptors · Human colon · Myenteric and submucosal plexus · Interstitial cells of Cajal · Smooth muscle · Mucosa · Lymphatic tissue · Vascular system

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Introduction

The autonomic innervation of the lower gastrointestinal tract is very complex. The enteric nervous system contains about as many neurons as the spinal cord and is capable of functioning independently of the central nervous system (Goyal and Hirano 1996). Despite its independence, this system is connected with the central autonomic network through sympathetic and parasympathetic afferent and efferent neurons (Surprenant 1994). Cell bodies of the intrinsic neurons are grouped into ganglions which were found, in the myenteric and submucosal plexus, to be widely connected among themselves (Gershon et al. 1994; Ibba-Manneschi et al. 1995). Neurotransmission is mediated not only by the "classical" neurotransmitters but also by neuropeptide transmitters like somatostatin (SRIF), substance P (SP), neurotensin, gastrin, cholecystokinin (CCK), neuropeptide Y (NPY), gastrin-releasing peptide (GRP), vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) (Furness et al. 1992; McConalogue and Furness 1994; Schultzberg et al. 1980). These neuropeptides have been identified with immunohistochemical methods in the enteric nervous system in rats and guinea-pigs (Ekblad et al. 1988; Hökfelt et al. 2001; Larsson and Sundler 1990; Shen et al. 1992), and shown to interact with many specific targets like the smooth muscles, the vessels, the mucosa and the lymphoid tissues. They act on the regulation of gut motility, blood flow, secretion and nutrient absorption as well as on the modulation of the immune response (McConalogue and Furness 1994). The action of these

neuropeptides is mediated by specific G protein-coupled receptors (Jacobson and Bunnett 1997; Johnson 1994; McConalogue and Bunnett 1998; Sanders 1998). Although neuropeptide receptors were shown to be located in specific target cells of the gastrointestinal tract in various animals (Jackerott and Larsson 1997; Peaire et al. 1997; Sternini et al. 1997), little is known about the distribution of these receptors and their subtypes in the human colon, in particular in the colonic nerve plexus, an important local relay station, and in the other targets of the human colon innervated by the peripheral nervous system.

Peptide receptor expression and localization in the healthy and diseased gastrointestinal tract has increasing diagnostic and therapeutic implications (Fischman et al. 1993; Lamberts et al. 1991; Reubi 1995). In vitro observations of peptide receptors over-expressed in tumors have led to the development of diagnostic and radiotherapeutic applications of radiolabeled peptides (octreotide, VIP, gastrin and CCK) for in vivo receptor scintigraphy or peptide radiotherapy in tumor patients (Behr et al. 1998; Krenning et al. 1989, 1995; Reubi 1995; Virgolini et al. 1994). The evaluation of peptide receptors in nondiseased tissues is, however, equally important, not only for the interpretation of in vivo receptor scintigraphy data in nondiseased areas (Krenning et al. 1995), but also for predicting potential side effects of peptide therapy. Moreover, such a study should provide basic information relevant to future investigations of colonic diseases, such as Hirschsprung's disease, neuronal intestinal dysplasia, Crohn's disease or idiopathic chronic constipation, where alterations of neuropeptide contents have previously been reported (Basbaum and Levine 1991; Kawana et al. 1990; Kimura et al. 1994; Koch et al. 1988; Sjolund et al. 1997).

Our aim was to evaluate and compare the expression of receptors for somatostatin, SP, neurotensin, gastrin/CCK, NPY, GRP and VIP/PACAP in the myenteric and submucosal plexus, the smooth muscle layers, the vessels, the mucosa and the lymphoid tissue in normal human colonic samples gained 5–10 cm away from surgically resected tumors. Receptor subtypes were characterized with subtype-selective analogs, where available. The receptors were measured with receptor autoradiography, a method that permits the evaluation of receptors in complex human tissues since it preserves the morphological integrity of the tissues and allows in most instances to localize the receptors in the specific target tissues. Although immunohistochemical detection of these receptors would be more precise at the cellular level (Reubi et al. 1998b), the lack of availability of adequate antibodies to target the majority of these human peptide receptors determined the choice of the receptor autoradiographic method.

Materials and methods

Normal human colonic tissue was obtained from nine patients. It was resected 5–10 cm away from surgically operated tumors. A total of 27 tissue samples (3 samples per patient) were used for autoradiographic evaluation of each investigated ligand binding site (Table 1). All tissues were frozen on dry ice immediately after surgical resection and stored at –80°C. In vitro receptor autoradiography was performed on 20-µm-thick cryostat (Microm HM 500 OM or Leitz 1720; Rockleigh, N.J., USA) sections of the tissue samples, mounted on microscope slides and stored at –20°C for at least 3 days to improve adhesion of the tissue to the slide, as previously described (Reubi et al. 1990). Corresponding sections stained by H&E and immunohistochemically stained for synaptophysin or CD-56 (neural cell adhesion molecule, NCAM; Dako, Glostrup, Denmark) were used to identify the cells expressing the corresponding receptor.

GRP receptor autoradiography. [^{125}I]Tyr^4-bombesin was used as radioligand, known to specifically label GRP receptors. As reported previously (Markwalder and Reubi 1999), tissue sections were first preincubated in 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (HEPES) buffer, pH 7.4, for 5 min at room temperature. They were then incubated in 10 mM HEPES, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM ethyleneglycol-bis (β-aminoethylether)-*N*,*N*'-tetraacetic acid, 0.1% bovine serum albumin, 100 µg/ml bacitracin (pH 7.4), and approximately 100 pM [^{125}I]Tyr^4-bombesin (2000 Ci/mmol; Anawa, Wangen, Switzerland) in the presence or absence of 1 µM bombesin for 1 h at room temperature. Additional adjacent sections of selected samples were incubated in the presence of increasing amounts of nonradioactive bombesin, GRP, neuromedin B or somatostatin to generate competitive inhibition curves. After incubation, the sections were washed four times for 2 min each in 10 mM HEPES with 0.1% bovine serum albumin (pH 7.4) at 4°C. Finally, the slides were rinsed twice for 5 s each at 4°C in distilled water. The slides were then dried at 4°C under a stream of cold air. The slides were apposed to ^{3}H-Hyperfilms (Amersham, Aylesbury, UK) and exposed for 7 days in X-ray cassettes.

NPY receptor autoradiography. ^{125}I-labeled peptide YY (PYY, 2000 Ci/mmol; Anawa, Wangen, Switzerland) was used as radioligand. As reported previously (Dumont et al. 1993; Reubi et al. 2001), the slide-mounted tissue sections were preincubated in 119 mM NaCl, 3.2 mM KCl, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 2.53 mM $CaCl_2 \times 2H_2O$ and 10 mM D-glucose, pH 7.4 (preincubation solution), for 60 min at room temperature. The slides were then incubated in a solution containing the same medium as the preincubation solution to which the following compounds were added: 0.1% bovine serum albumin, 0.05% bacitracin, pH 7.4, and the radioligand, at approximate concentration of 22 pM [^{125}I]PYY. The slides were incubated at room temperature with the radioligand for 120 min. In all cases, increasing amounts of nonradioactive [Leu^{31},Pro^{34}]-NPY, an Y_1-selective ligand, and PYY(3–36), an Y_2-selective ligand, were added to the incubation medium to generate competitive inhibition curves. On completion of the incubation, the slides were washed four times for 5 min each in ice-cold preincubation solution, pH 7.4. They were rinsed twice in ice-cold distilled water, and then dried under a stream of cold air at 4°C, apposed to ^{3}H-Hyperfilms and exposed for 7 days in X-ray cassettes.

VIP/PACAP receptor autoradiography. ^{125}I-labeled VIP (2000 Ci/mmol; Anawa, Wangen, Switzerland) was used as radioligand as described previously (Reubi et al. 1999b). The slide-mounted tissue sections were allowed to reach room temperature and were then incubated for 90 min in a solution of 50 mM Tris-HCl, pH 7.4, containing bovine serum albumin (2%), EGTA (2 mM), bacitracin (0.1 mM) and $MgCl_2$ (5 mM) to inhibit endogenous proteases, in the presence of approximately 30 pM [^{125}I]VIP, at room temperature. To estimate nonspecific binding, paired sections were incubated as described above, except that 1 µM VIP (Bachem, Bubendorf, Switzerland) was added to the incubation medium. After this incubation, the slides were washed twice in ice-cold 50 mM Tris-HCl, pH 7.4, containing 0.25% bovine serum albumin, then in buffer alone, and quickly dried under a stream of cold air. The sections were subsequently exposed to ^{3}H-Hyperfilms for 1 week. In selected cases, displacement experiments using successive serial sections of human colon were performed with increas-

ing concentrations of unlabeled VIP, PACAP-27 as well as somatostatin 14. In samples with VIP receptor-positive tissue, the $VPAC_1$-selective analog [Lys^{15},Arg^{16},Leu^{27}]-VIP(1–7)/GRF(8–27) and the $VPAC_2$-selective Ro 25–1553 were used to differentiate $VPAC_1$ from $VPAC_2$ receptor subtypes.

Adjacent sections were also evaluated with [^{125}I][Ac-His^1]-PACAP-27 (2000 Ci/mmol; Anawa) for further characterization of receptor subtype specificity (Reubi et al. 1999b); displacement experiments under the same conditions as for VIP receptor autoradiography using increasing concentrations of unlabeled VIP and PACAP-27 were performed to differentiate PAC_1 from $VPAC_1$/$VPAC_2$ binding sites. In separate experiments, [^{125}I]Ro 25–1553 (2000 Ci/mmol; Anawa) was used to identify $VPAC_2$-selective sites, as reported previously (Reubi et al. 2000a).

CCK-A and -B receptor autoradiography. The CCK analog [^{125}I]D-Tyr-Gly-Asp-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-amide ([^{125}I]CCK) was used as radioligand (Reubi et al. 1997). The sections were preincubated in 50 mM Tris-HCl, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM ethylene glycol-*bis*[β-aminoethyl ether] *N*,*N*,*N*',*N*'-tetraacetic acid, and 0.5% bovine serum albumin, pH 7.4 (preincubation solution), for 30 min at 25°C. The slides were then incubated in a solution containing the same medium as the preincubation solution except that the bovine serum albumin was omitted, and the following compounds were added: 0.025% bacitracin, 1 mM dithiothreitol, 2 μg/ml chymostatin, 4 μg/ml leupeptin, pH 6.5, and the radioligand, 45 pM [^{125}I]CCK (2000 Ci/mmol; Anawa). The slides were incubated at room temperature with the radioligand for 150 min. Increasing amounts of nonradioactive sulfated CCK-8 and gastrin (as a CCK-B receptor-selective ligand) were added to the incubation medium to generate competitive inhibition curves. In these experiments, CCK-B receptors were distinguished from CCK-A receptors by their high affinity for gastrin. On completion of the incubation, the slides were washed six times for 15 min each in ice-cold preincubation solution, pH 7.4. They were rinsed twice in ice-cold distilled water for 5 s each, and then dried under a stream of cold air at 4°C, apposed to ^{3}H-Hyperfilms and exposed for 1–7 days in X-ray cassettes.

Neurotensin receptor autoradiography. [^{125}I][Tyr^3]-neurotensin was used as a radioligand. Neurotensin binding was performed as shown previously (Reubi et al. 1998c) by incubating the sections with 90 pM monoiodo [^{125}I][Tyr^3]-neurotensin (2000 Ci/mmol; Anawa, Wangen, Switzerland) in 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM $MgCl_2$, 0.2% bovine serum albumin and 5×10^{-5} M bacitracin at 4°C for 1 h. Additional sections were incubated in the presence of increasing amounts of nonradioactive neurotensin, acetyl-neurotensin(8–13), neurotensin(1–11) and the neurotensin-2-receptor-selective levocabastine (Janssen, Beerse, Belgium), to generate competitive inhibition curves. After incubation, the sections were washed for 8 min at 4°C in four consecutive baths containing 50 mM Tris-HCl buffer, pH 7.6. The sections were then dried under a stream of cold air at 4°C, apposed to ^{3}H-Hyperfilms, and exposed for 7 days to X-ray cassettes.

Neurokinin (NK) receptor autoradiography. The radioligand used was [^{125}I]Bolton-Hunter-SP ([^{125}I]BHSP, 2000 Ci/mmol; Anawa, Wangen, Switzerland) as reported previously (Hennig et al. 1995). The sections were incubated for 2 h in a solution of 50 mM Tris-HCl (pH 7.4) containing bovine serum albumin (0.02%), chymostatin (2 mg/l), leupeptin (4 mg/l), bacitracin (40 mg/l), $MgCl_2$ (5 mM), and 50 pM [^{125}I]BHSP, at room temperature. To estimate nonspecific binding, paired serial sections were incubated as described above, except that 1 μM SP (Bachem, Bubendorf, Switzerland) was added to the incubation medium. After this incubation, the slides were rinsed with four washes of 30 s each in ice-cold 50 mM Tris-HCl (pH 7.4), dipped in ice-cold distilled water, and then quickly dried in a refrigerator under a stream of cold air. The sections were subsequently exposed to a ^{3}H-Hyperfilm for 1 week. In addition to SP, the NK_1-selective agonist [Sar^9,$Met(O_2)^{11}$]-SP, as well as the NK_2-selective agonist [Nle^{10}]-neurokinin $A_{4–10}$ and the NK_3-selective agonist senktide (Bachem, Bubendorf, Switzerland), were used in order to assess the NK receptor subtype involved.

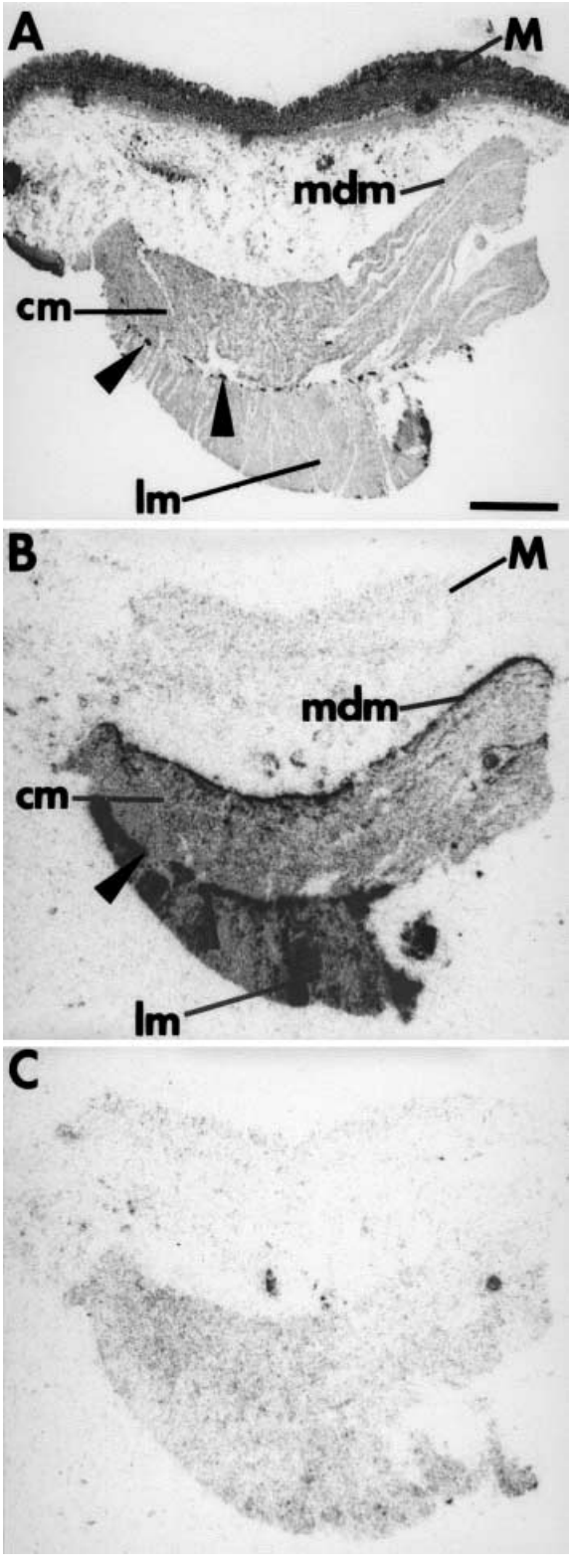
Somatostatin receptor autoradiography. The radioligands used were the somatostatin analogs [^{125}I][Tyr^3]-octreotide and [^{125}I][Leu^8, D-Trp^{22},Tyr^{25}]-SRIF-28 ([^{125}I][LTT]-SRIF-28) known to specifically label somatostatin receptors. Both ligands were iodinated, purified by high pressure liquid chromatography column and characterized in standard binding assays. Sections were then incubated for 2 h at ambient temperature in the presence of 65 pM iodinated ligand (Reubi et al. 1990). The incubation solution was 170 mM Tris-HCl buffer (pH 8.2) containing 1% bovine serum albumin, bacitracin (40 μg/ml) and $MgCl_2$ (10 mM) to inhibit endogenous proteases. Nonspecific binding was determined by adding 1 mM solution of unlabeled [Tyr^3]-octreotide (Novartis, Basel, Switzerland) or SRIF-28 (Bachem, Bubendorf, Switzerland). Incubated sections were washed twice for 5 min in cold incubation buffer containing 0.25% bovine serum albumin, then in buffer alone, and dried quickly. Finally, the sections were apposed to ^{3}H-Hyperfilms and exposed for 1 week in X-ray cassettes. In selected cases, dis-

Table 1 Neuropeptide receptor distribution in human colon [*mdm circular* mucosa-directed margin of the circular smooth muscle (interstitial cells of Cajal), +++ strongly positive in all cases, ++ moderately positive in all cases, + weakly positive in all cases, – no receptors identified, –/+ negative in most cases, weakly positive in a few, –/++ negative in some cases but moderately positive in all others, *n.a.* not assessable]

Targets	GRP-R	Y_1	Y_2	VIP/ PACAP-R	CCK-A	CCK-B	NT1	NK_1	SRIF-R
Myenteric plexus	+++	–	+++	+[a]	++	–	+++	n.a.	+++[d]
Submucosal plexus	n.a.	–	+++	n.a.	n.a.	n.a.	+++	n.a.	+++[d]
Smooth muscle									
mdm circular (Cajal cells)	++	–	n.a.	+[b]	–	–/+	n.a.	++	+[d]
Circular	+	–	++	+	–	–	++	++	–/+
Longitudinal	++/+++	–	+	+	–/++	–	+/++	–	–/+
Blood vessels	–	++	–	–/+	–	–	–	+++	+[d]
Lymphoid follicles	–	–	–	+	–	–	–	–	++[d]
Mucosa	–	–	++/+++	++[c]	–	–	–	–	+/++[d]
Muscularis mucosae	–/+	–	++	–	–	–	–	–	–

[a]Identified as PAC1
[b]Identified as VPAC2
[c]Identified as VPAC1
[d]Identified as sst_2

placement experiments with [^{125}I][Tyr^3]-octreotide were performed in successive tissue sections using increasing concentrations of the sst_2-selective L-779,976 (Reubi et al. 2000b), the sst_3-selective sst_3-ODN-8 (Reubi et al. 2000c) and the sst_5-selective L-817,818 (Reubi et al. 2000b), in order to identify the receptor subtype labeled by [^{125}I][Tyr^3]-octreotide.



In all the displacement experiments, the autoradiograms corresponding to successive sections incubated with increasing concentrations of cold peptides were quantified with a computer-assisted image-processing system, as previously described (Reubi et al. 1990). Radiolabeled tissue sections were exposed to ^{3}H-Hyperfilms together with standards (Autoradiographic [^{125}I]microscales; Amersham, Aylesbury, UK) that contained known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentration. The image analyzer was calibrated to the standards; it performed interpolation to read values that lay between those of the film standards. Each point in the curves represents the optical density of binding measured in the area of interest, with nonspecific binding subtracted from all values.

Results

An overview of the data showing the distribution of the nine investigated peptide receptors in various targets of the normal human colon is given in Table 1.

GRP receptors

The GRP receptor status in normal colonic tissue is summarized in Table 1. Figure 1 illustrates these data and shows a representative example of receptor autoradiography from a colon sample with GRP receptors heavily expressed in the myenteric plexus. The labeling is often difficult to identify because of the high density of GRP receptors in the smooth muscle close to the myenteric plexus. Furthermore, a high density of GRP receptors is identified in the longitudinal smooth muscle and a lower density in the circular smooth muscle (Fig. 1); however, there is an individual variability of the receptor density in both layers. Moreover, the circular smooth muscle shows a marked and topical expression of GRP receptors in its mucosa-directed margin (sometimes with interruptions), corresponding to the site where the interstitial cells of Cajal are localized. The muscularis mucosae shows in a few samples a measurable amount of GRP receptors. Other targets such as vessels, lymphoid follicles and mucosa do not express GRP receptors (Table 1; Fig. 1). The characterization of GRP receptors in the smooth muscle is shown in the displacement curve in Fig. 2, with a high affinity binding for GRP and bombesin and lower affinity for neuromedin B. Unrelated peptides like somatostatin are inactive.

Fig. 1A–C GRP receptors in normal human colon sample. **A** Section immunohistochemically stained for CD 56 showing the myenteric plexus (*large arrowheads*), the smooth muscle layers (*cm*, *lm*), the mucosa (*M*) and the mucosa-directed margin (*mdm*). *Bar:* 1 mm. **B** Autoradiogram showing total binding of [^{125}I][Tyr^4]-bombesin. The myenteric plexus (*large arrowheads*) is strongly labeled. The longitudinal smooth muscle (*lm*) is strongly but not homogeneously labeled. The circular smooth muscle (*cm*) is labeled in particular in the margin (*mdm*) on the mucosa-directed side of the muscle. **C** Autoradiogram showing non-specific binding of [^{125}I][Tyr^4]-bombesin (in the presence of 10^{-6} M unlabeled bombesin)

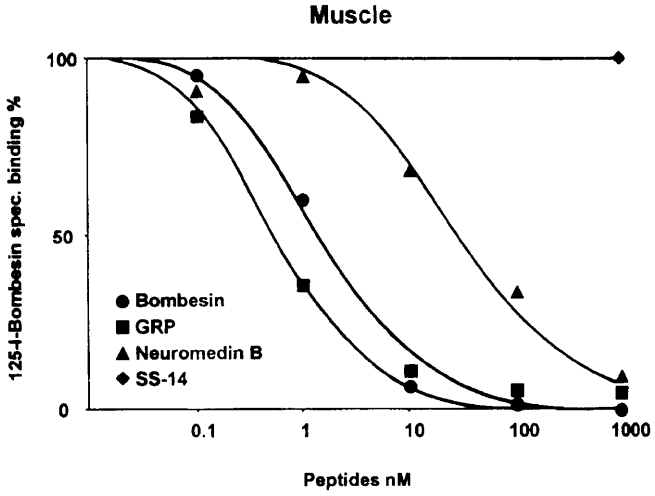


Fig. 2 GRP receptors in normal human colon. High affinity and specificity of the [^{125}I][Tyr^4]-bombesin binding in displacement experiments. Sections containing longitudinal smooth muscle were incubated with [^{125}I][Tyr^4]-bombesin and increasing concentrations of unlabeled bombesin (●), GRP (■), neuromedin B (▲) and somatostatin (◆). High-affinity displacement of the radioligand is found with bombesin and GRP, whereas neuromedin B shows lower affinity. Somatostatin has no effect. Non-specific binding is subtracted from all of the values. The observed rank order of potencies of these analogues is characteristic for the GRP receptor subtype

NPY receptors

In human colon, NPY receptors are distributed in the myenteric and submucosal plexus, smooth muscle, vessels, mucosa and muscularis mucosae, but not in lymphoid follicles. As seen in Table 1, the receptor expression for Y_1 and Y_2 subtype is different depending on the investigated target. A high density of Y_2 receptors, characterized by a [^{125}I]PYY labeling fully displaced by PYY(3–36) but not by [Leu^{31},Pro^{34}]-NPY, is identified in myenteric and submucosal plexus (Fig. 3). Y_1 receptors are not detectable in these targets. Light microscopic examination of successive sections, immunostained for CD-56 to visualize the peripheral nerves, reveals a staining of the myenteric plexus that closely corresponds to the distribution of the NPY receptors. There is a moderate density of Y_2 recep-

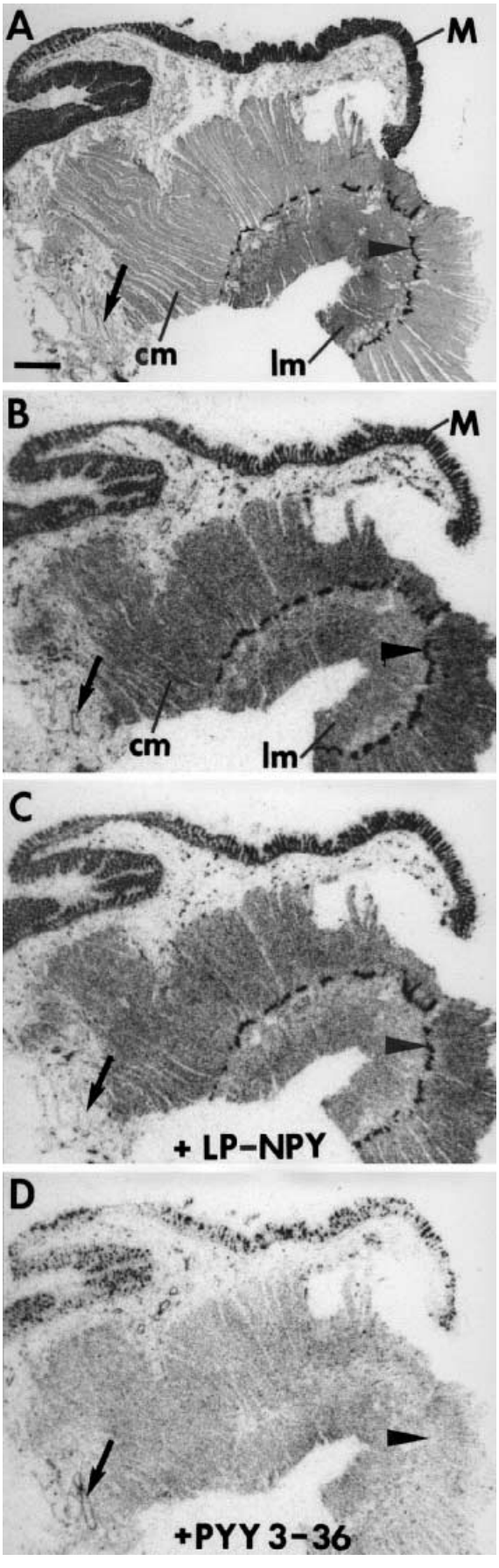


Fig. 3A–D NPY receptors in normal human colon sample. **A** Section immunohistochemically stained for CD 56 showing the myenteric plexus (*large arrowhead*), the smooth muscle layers (*cm*, *lm*), the mucosa (*M*) and the vessels (*arrow*). *Bar:* 1 mm. **B** Autoradiogram showing total binding of [^{125}I]PYY. **C** Autoradiogram showing binding of [^{125}I]PYY in presence of 10^{-6} M [Leu^{31},Pro^{34}]-NPY (= LP-NPY). **D** Autoradiogram showing binding of [^{125}I]PYY in presence of 10^{-6} M PYY(3–36). Y_1 receptors are present only in vessels (*arrow*) where [^{125}I]PYY is displaced by [Leu^{31},Pro^{34}]-NPY (C) but not by PYY(3–36) (D). The myenteric plexus (*large arrowhead*) and the mucosa (*M*) are strongly labeled for Y_2 receptors since [^{125}I]PYY is displaced by PYY(3–36) (D) but not by [Leu^{31},Pro^{34}]-NPY (C). The longitudinal and circular smooth muscle (*lm*, *cm*) are also labeled for Y_2 receptors showing displacement by PYY(3–36) (D) but not by [Leu^{31},Pro^{34}]-NPY (C). Residual labeling of muscle and mucosa in D represent non-specific binding

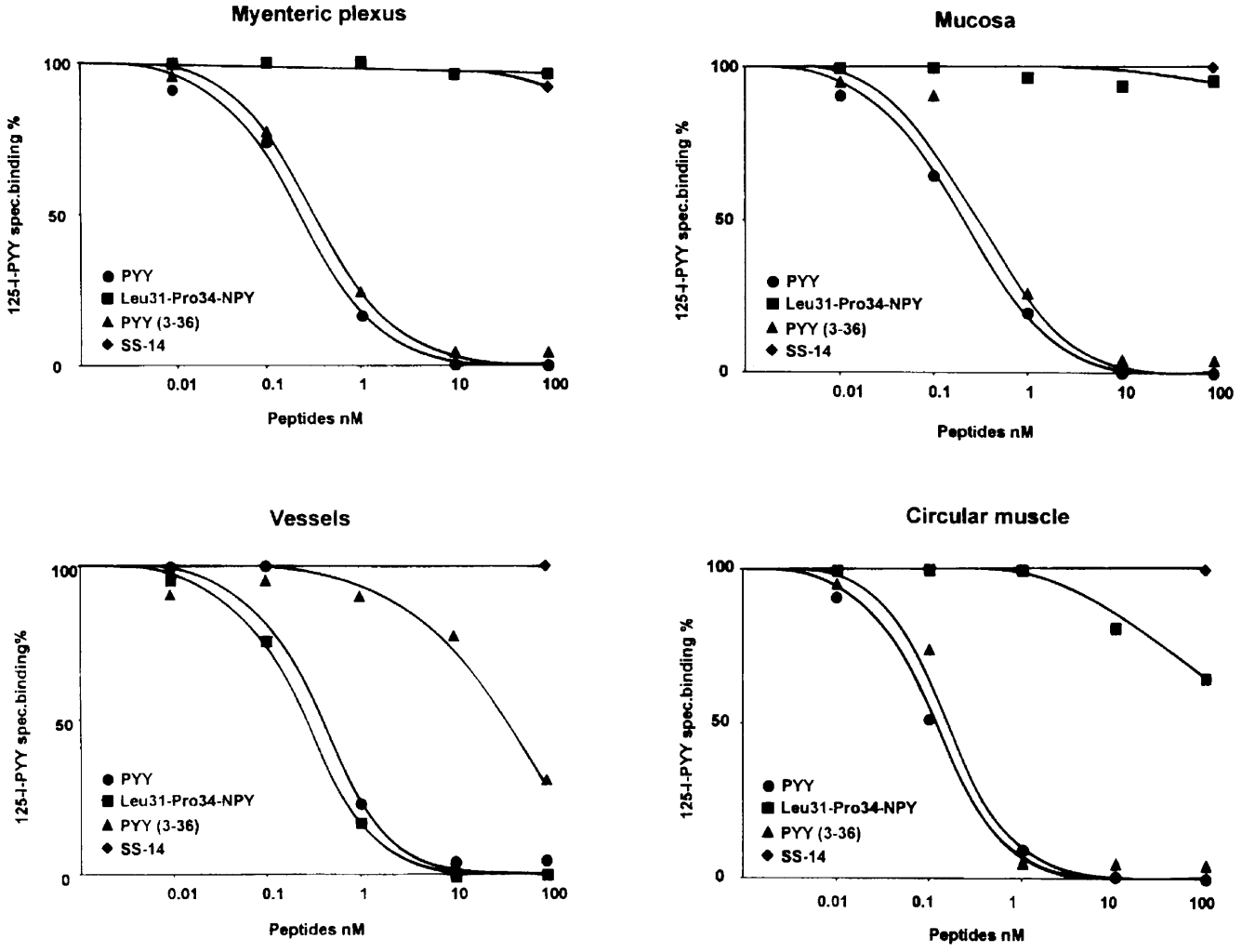


Fig. 4 NPY receptors of the Y_1 and Y_2 subtype in normal human colon. Characteristics of [^{125}I]PYY binding in displacement experiments using sections containing myenteric plexus, vessels, mucosa and smooth muscle. Sections were incubated with [^{125}I]PYY and increasing concentrations of unlabeled PYY (●), [Leu^{31}, Pro^{34}]-NPY (■), PYY(3–36) (▲) and 1000 nM SRIF-14 (◆). Each *point* represents the optical density of binding measured in the area of interest. Non-specific binding was subtracted from all values. The high affinity displacement by PYY(3–36) and the low affinity displacement by [Leu^{31},Pro^{34}]-NPY in the myenteric plexus, the mucosa and the smooth muscle indicate Y_2 receptors. The high affinity displacement by [Leu^{31},Pro^{34}]-NPY and the low affinity displacement by PYY(3–36) in the vessels indicate Y_1 receptors

tors in the circular smooth muscle and a lower density in the longitudinal smooth muscle. No measurable levels of Y_1 receptors are identified in the smooth muscle, while vessels express a moderate density of the Y_1 subtype. In lymphoid follicles there is no measurable expression of Y_1 or Y_2 receptors. Conversely, we identify a high density of the Y_2 subtype in the mucosa and a lower density in the muscularis mucosae. The displacement experiments (Fig. 4) show Y_1 sites in the vessels, where [^{125}I]PYY is displaced by nanomolar concentrations of the analog [Leu^{31},Pro^{34}]-NPY, and Y_2 sites in the myenteric plexus, the mucosa and the smooth muscle, where [^{125}I]PYY is displaced by nanomolar amounts of the analog PYY(3–36).

VIP/PACAP receptors

As shown in Table 1, the human colon tissue expresses VIP/PACAP receptors. The superficial mucosa contains VIP/PACAP receptors having a high affinity for PACAP and VIP. Receptor subtyping identifies $VPAC_1$ receptors, based on the high affinity for the $VPAC_1$-selective analog [Lys^{15},Arg^{16},Leu^{27}]-VIP(1–7)/GRF(8–27) (Reubi et al. 2000a). Figure 5 shows that the myenteric plexus (and, to a lesser extent, the surrounding muscle) express a moderate density of receptors labeled with [^{125}I][Ac-His^{1}]-PACAP-27, representing predominantly the PAC_1 subtype, with a high affinity for PACAP but not for VIP. Conversely, the mucosa displays a high density of VIP/PACAP receptors having high affinity for PACAP and VIP. Moreover, in most samples, a marked VIP/PACAP receptor expression is identified in the mucosa-directed margin of the circular smooth muscle, which corresponds to the localization of the interstitial cells of Cajal; the la-

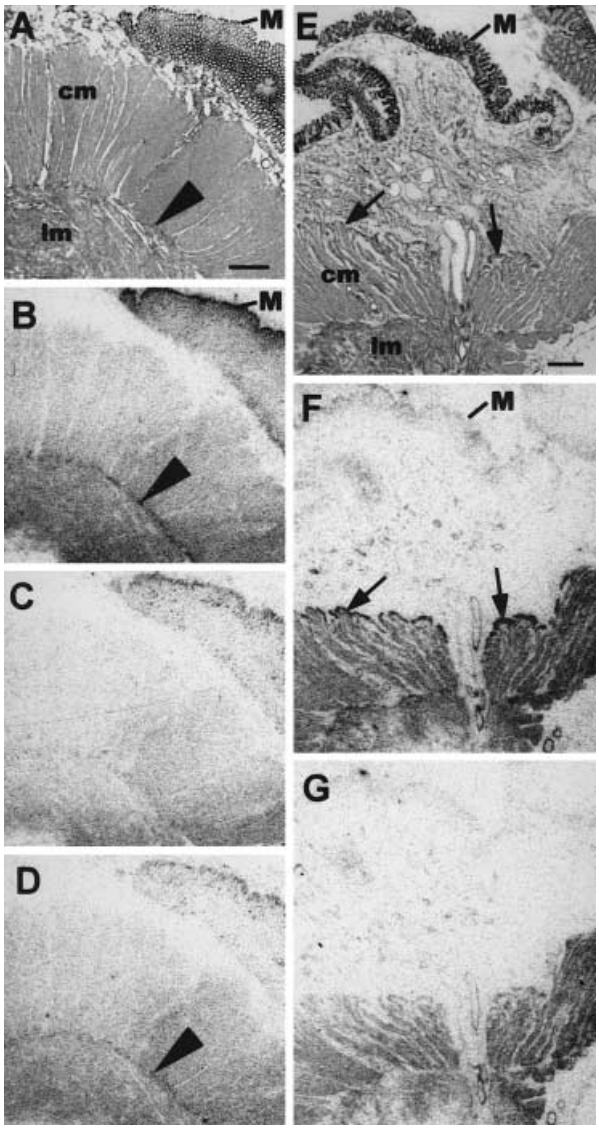


Fig. 5 VIP/PACAP receptors in normal human colon labeled with **A–D** [^{125}I][Ac-His^{1}]-PACAP-27 or **E–G** [^{125}I]Ro 25–1553. **A** Section stained by H&E showing the myenteric plexus (*arrowhead*), the smooth muscle layers (*cm, lm*) and the mucosa (*M*). *Bar:* 1 mm. **B** Autoradiogram showing total binding of [^{125}I][Ac-His^{1}]-PACAP-27. **C** Autoradiogram showing binding of [^{125}I][Ac-His^{1}]-PACAP-27 in presence of 20 nM unlabeled PACAP. **D** Autoradiogram showing binding of [^{125}I][Ac-His^{1}]-PACAP-27 in presence of 20 nM unlabeled VIP. The myenteric plexus (*arrowhead*) and, to a lesser extent, the circular (*cm*) and longitudinal smooth muscle (*lm*) contain PAC_1 receptors, since the [^{125}I][Ac-His^{1}]-PACAP-27 is displaced by PACAP (C), but only partly by VIP (D). Conversely, the mucosa (*M*) contains $VPAC_1$ receptors, since the [^{125}I][Ac-His^{1}]-PACAP-27 binding is displaced by both PACAP (C) and VIP (D), as well as by [K^{15},R^{16},L^{27}]-VIP(1–7)/GRF(8–27) (not shown). **E** H&E-stained section showing the smooth muscle (*cm, lm*) with the mucosa-directed margin (*arrow*) of the circular muscle (*cm*) and the mucosa (*M*). *Bar:* 1 mm. **F** Autoradiogram showing total binding of the $VPAC_2$-selective [^{125}I]Ro 25–1553. **G** Autoradiogram showing nonspecific binding (in presence of 100 nM Ro 25–1553). The $VPAC_1$-expressing mucosa M is not labeled with this $VPAC_2$-selective ligand. The mucosa-directed margin of the circular muscle (*arrow*) is strongly labeled, while the remaining muscle as well as some vessels are weakly labeled, indicating a general expression of $VPAC_2$ in smooth muscle elements

beling of this area with the iodinated $VPAC_2$-selective Ro 25–1553 (Reubi et al. 2000a) indicates a predominance of $VPAC_2$, while the remaining muscle expresses $VPAC_2$ only weakly. Vessels expressing VIP/PACAP receptors, preferentially of the $VPAC_2$ subtype, i.e. with low affinity for [Lys^{15},Arg^{16},Leu^{27}]-VIP(1–7)/GRF(8–27) and high affinity for Ro 25–1553 can be seen in some samples and can be specifically labeled by [^{125}I]Ro 25–1553 (Fig. 5). No expression of VIP/PACAP receptors is identified in the muscularis mucosae. The lymphoid follicles usually have VIP/PACAP receptors, as reported previously (Reubi et al. 1998a). VIP/PACAP receptors cannot be assessed in the submucosal plexus.

CCK receptors

Table 1 summarizes the results of the CCK receptor distribution in various targets of normal human colon. The main CCK target is the myenteric plexus, which has predominantly CCK-A receptors (Fig. 6), as identified by the low affinity for gastrin. Four of nine patients show a moderate density of CCK-A receptors in the longitudinal smooth muscle whereas the other patients have a low density or absence of CCK-A receptors in this tissue. A case with a lack of CCK-A receptors in the longitudinal smooth muscle was chosen in Fig. 6, in order to optimally identify the receptor-positive myenteric plexus. While the circular smooth muscle is negative for CCK-A and CCK-B receptors, its mucosa-directed margin is CCK-B receptor-positive in a few samples. Neither CCK-A nor CCK-B receptors are expressed in the vessels, the lymphoid tissue, the mucosa or the muscularis mucosae (Table 1; Fig. 6). CCK receptors in nerve cells of the myenteric plexus are shown in displacement curves to have a high affinity for CCK-8 but not for gastrin, which is characteristic for CCK-A receptors (data not shown).

Neurotensin receptors

Figure 7 illustrates the high density of neurotensin receptors in the myenteric and submucosal plexus as well as in the circular and longitudinal smooth muscles of the human colon. The density of neurotensin receptors in longitudinal and circular smooth muscle is variable throughout the evaluated samples but usually a higher density is found in the circular than in the longitudinal smooth muscle. Moreover, the whole circular smooth muscle is usually labeled whereas the longitudinal one is only partly labeled. No receptors are identified in the lymphoid tissue, the vessels, the mucosa or the muscularis mucosae (Table 1; Fig. 7). Neurotensin receptors are of high affinity and specific for biologically active neurotensin, such as the natural neurotensin or acetyl-neurotensin(8–13), but not for the biologically inactive neurotensin(1–11). They do not have any affinity (up to 1000 nM) for the NT2-selective ligand levocabastine, suggesting a predominant expression of NT1 receptor subtype (data not shown).

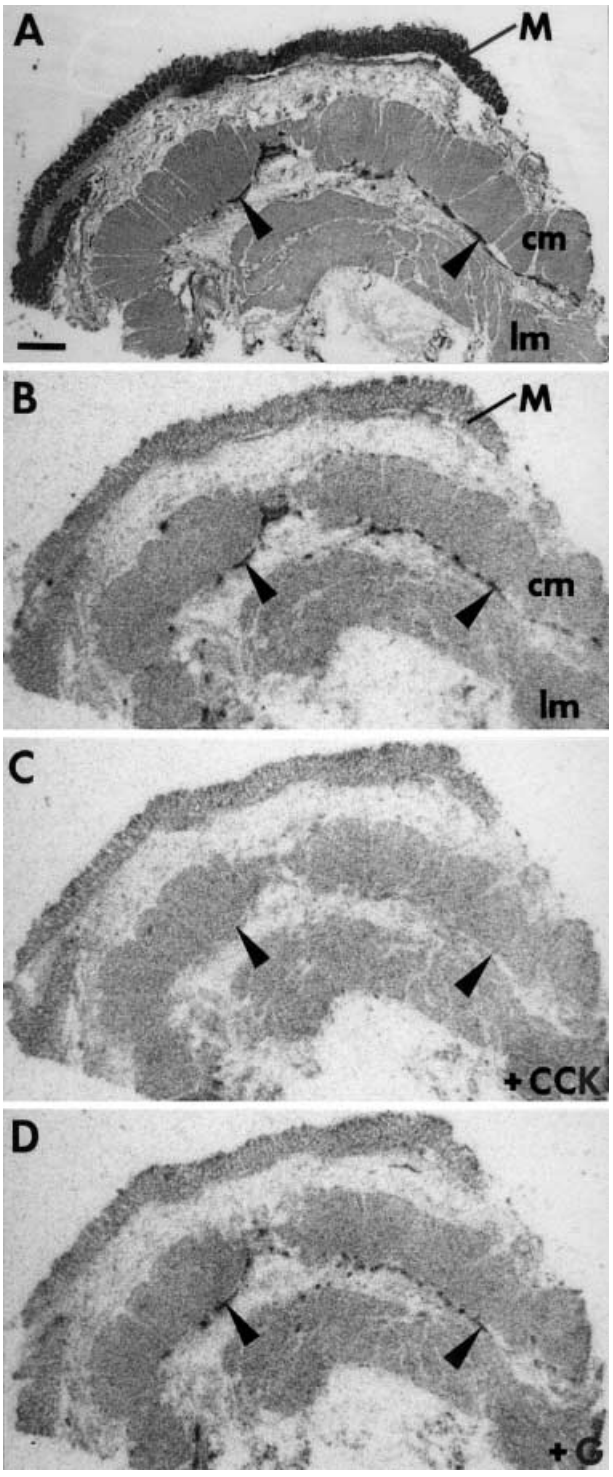


Fig. 6A–D CCK-A receptors in normal human colon. **A** Section immunohistochemically stained for synaptophysin showing the myenteric plexus (*large arrowheads*), the smooth muscle layers (*cm*, *lm*) and the mucosa (*M*). *Bar:* 1 mm. **B** Autoradiogram showing total binding of [^{125}I]CCK representing CCK-A and/or CCK-B receptors. **C** Autoradiogram showing binding of [^{125}I]CCK in presence of 50 nM sulfated CCK-8. **D** Autoradiogram showing binding of [^{125}I]CCK in presence of 50 nM gastrin. CCK-A receptors are present in the myenteric plexus (*large arrowheads*), since [^{125}I]CCK is displaced by CCK (C) but not by gastrin (D). Vessels, mucosa (*M*), the circular smooth muscle (*cm*) and, in this patient, the longitudinal smooth muscle (*lm*) have only non-specific labeling

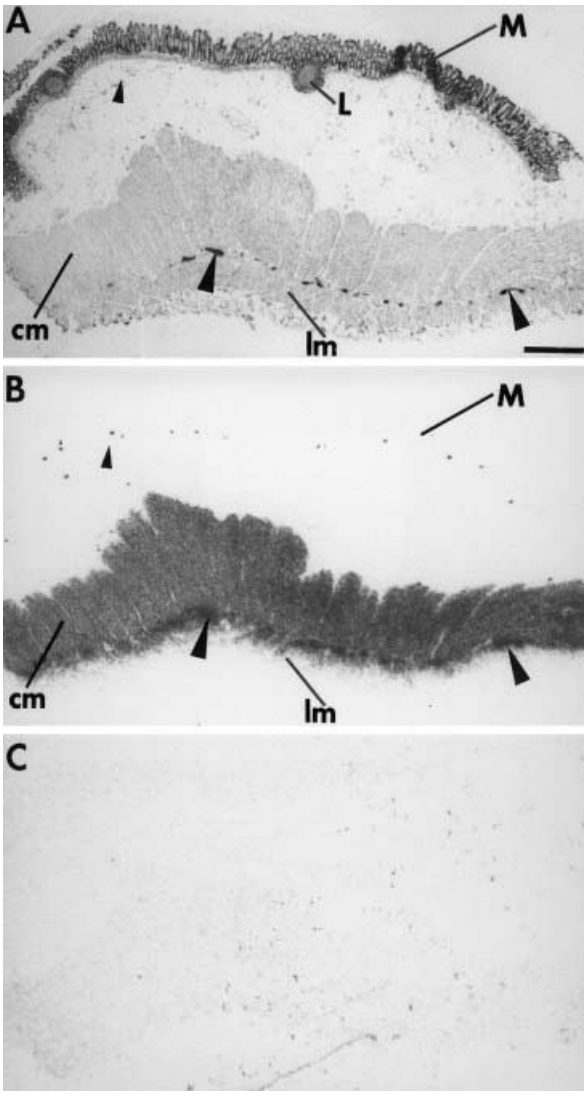


Fig. 7A–C Neurotensin receptors in normal human colon. **A** Section immunohistochemically stained for synaptophysin showing the myenteric plexus (*large arrowheads*), the submucosal plexus (*small arrowhead*), the smooth muscle layers (*cm*, *lm*), the mucosa (*M*) and the lymphoid follicle (*L*). *Bar:* 1 mm. **B** Autoradiogram showing total binding of [^{125}I][Tyr^3]-neurotensin. The myenteric (*large arrowheads*) and submucosal (*small arrowhead*) plexus, the circular (*cm*) and part of the longitudinal smooth muscle (*lm*) layers are strongly labeled, but not the mucosa (*M*) and the lymphoid follicle (*L*). **C** Autoradiogram showing non-specific binding of [^{125}I][Tyr^3]-neurotensin (in the presence of 10^{-6} M unlabeled neurotensin)

NK receptors

In normal human colonic tissue NK receptors are expressed in large quantities in vessels (arteries and veins) and in the outer layer of the circular smooth muscle. Moreover, a very strong and topical expression of NK receptors is seen in the mucosa-directed margin of the circular muscle (Fig. 8). The longitudinal smooth muscle does not express NK receptors, except in one sample. Unfortunately, the presence of NK receptors in nerve cells of

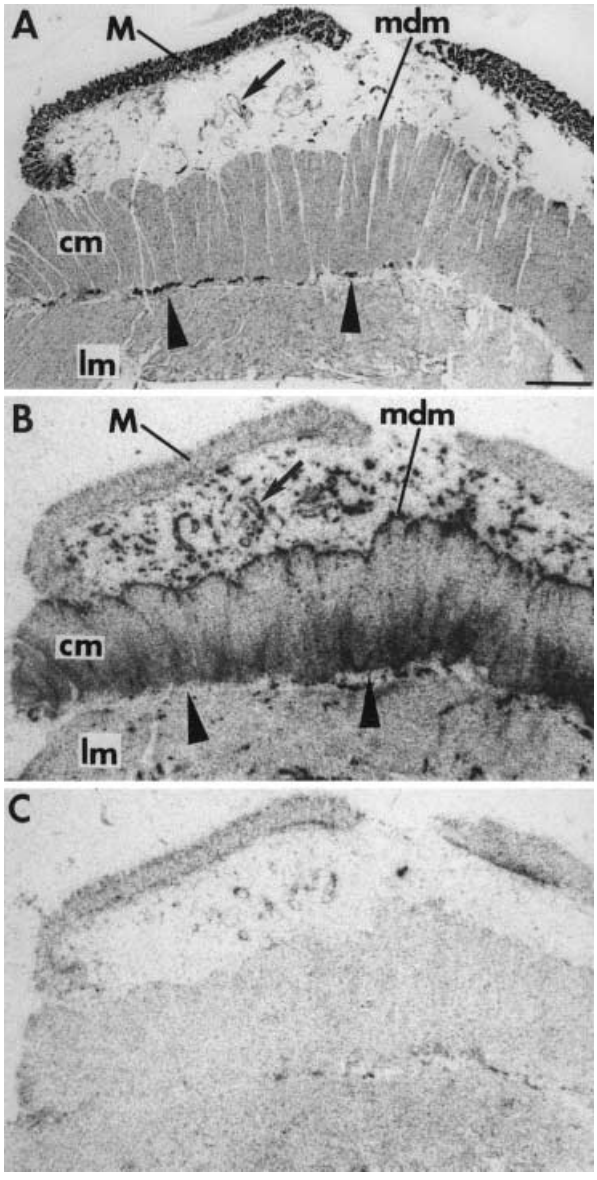


Fig. 8A–C NK receptors in normal human colon sample. **A** Section immunohistochemically stained for synaptophysin showing the submucosa with vessels (*arrow*), the myenteric plexus (*large arrowheads*), the smooth muscle layers (*cm*, *lm*), the mucosa-directed margin (*mdm*) and the mucosa (*M*). *Bar:* 1 mm. **B** Autoradiogram showing total binding of [^{125}I]BHSP. **B,C** Vessels (*arrow*) and the circular smooth muscle layer (*cm*) are labeled, especially the part directed towards the myenteric plexus, and the mucosa-directed margin (*mdm*) of the smooth muscle. The longitudinal smooth muscle layer (*lm*) and the mucosa (*M*) are not labeled. The labeling status of the myenteric plexus cannot be assessed due to the very strong labeling of the adjacent muscle (*b*). **C** Autoradiogram showing non-specific binding of [^{125}I]BHSP (in the presence of 10^{-6} M unlabeled SP)

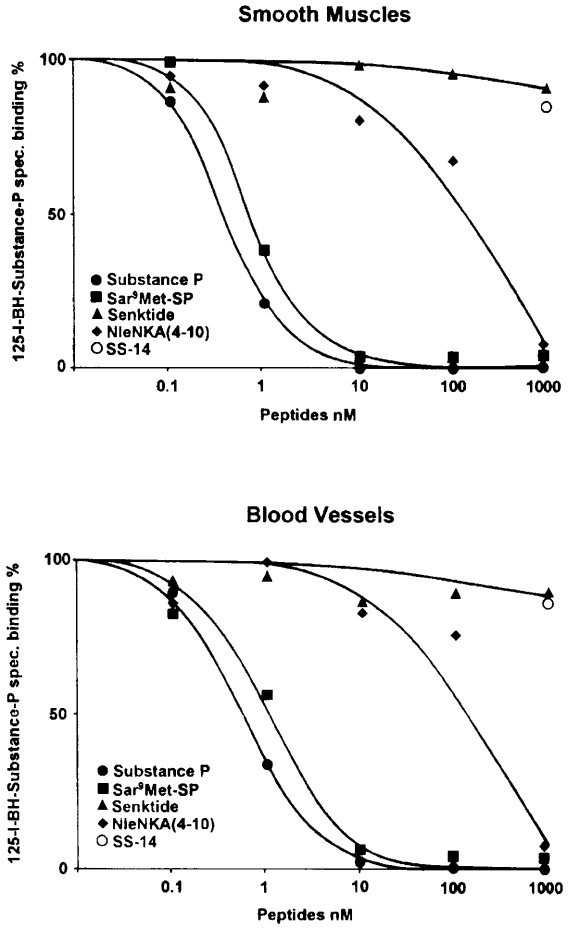


Fig. 9 NK receptors in normal human colon. Characteristics of [^{125}I]BHSP binding in displacement experiments using sections containing circular smooth muscle and vessels. Sections were incubated with [^{125}I]BHSP and increasing concentrations of unlabeled SP (●), [Sar^{9},Met(O$_2$)^{11}]-SP (■), [Nle^{10}]-neurokinin A$_{4–10}$ (◆), senktide (▲) and SRIF-14 (○). High affinity displacement by [Sar^{9},Met(O$_2$)^{11}]-SP indicates NK$_1$ subtype

myenteric and submucosal plexus cannot be assessed unequivocally due to the high density of receptors in vessels and muscles. The lymphoid follicles, the mucosa and the muscularis mucosae do not show measurable levels of NK receptors (Table 1; Fig. 8). The displacement curves (Fig. 9) illustrate the presence of NK$_1$ receptors in smooth muscle and blood vessels, as shown by the high affinity displacement of [^{125}I]BHSP by the NK$_1$-selective analog [Sar^{9},Met(O$_2$)^{11}]-substance P and low affinity displacement by the NK$_2$-selective [Nle^{10}]-neurokinin A$_{4–10}$ and the NK$_3$-selective senktide.

Somatostatin receptors

Table 1 summarizes the distribution of somatostatin receptors in human colon. The myenteric and submucosal plexus show a high density, the germinal center of lymphoid follicles a moderate density of somatostatin receptors. The circular and longitudinal muscles are usually receptor-negative but may, in a few cases, diffusely express a very low density of somatostatin receptors. However, the circular smooth muscle shows a topical expression of somatostatin receptors in its mucosa-directed margin. Blood vessels (veins) and the mucosa show low densities

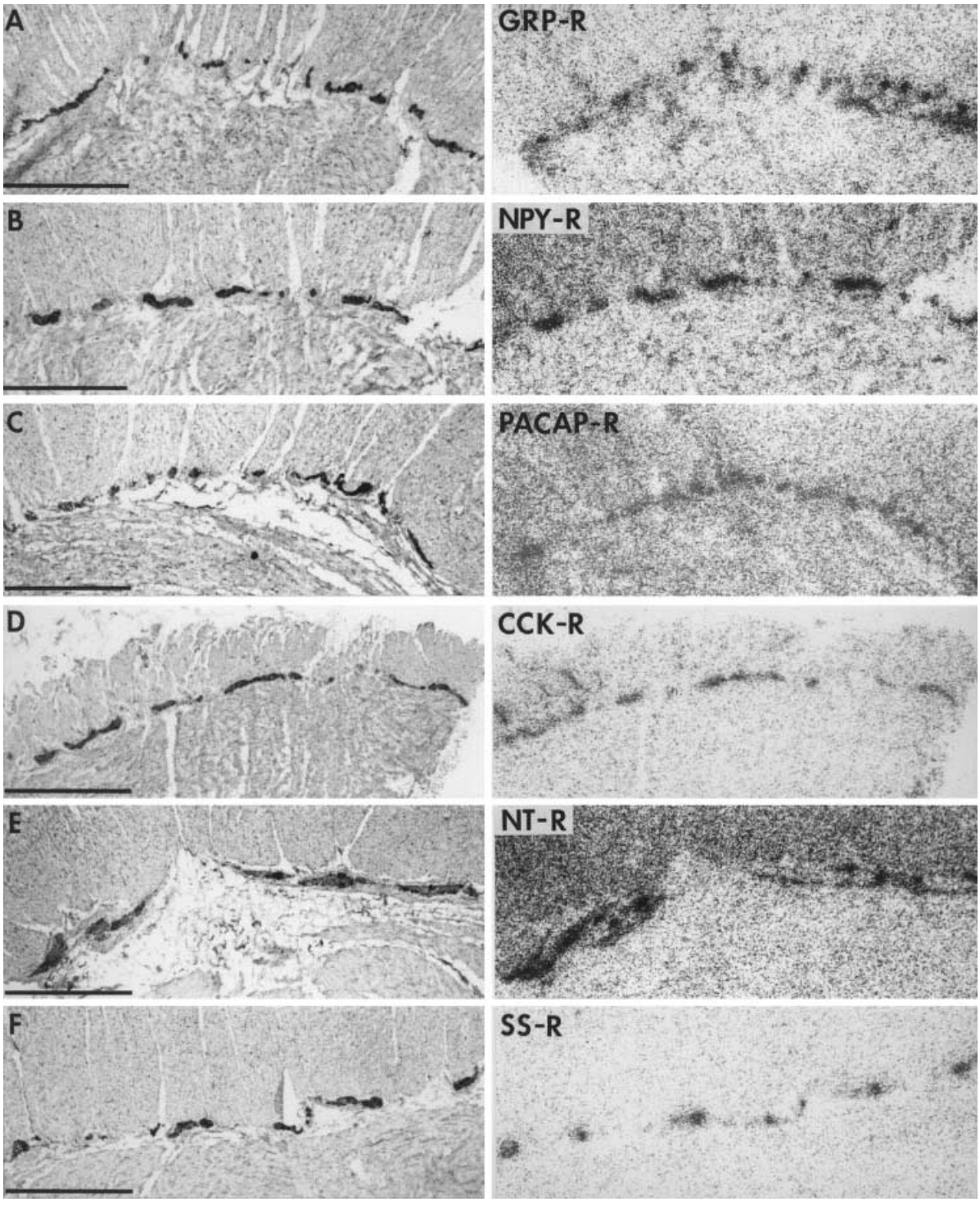


Fig. 10A–F Autoradiographic identification of different peptide receptors in the myenteric plexus compared with a corresponding section showing the immunohistochemical staining (synaptophysin or CD56) of the myenteric plexus at high magnification. Immunohistochemically stained sections are on the *left side*, autoradiograms are on the *right side*. In all sections the circular part of the smooth muscle is located above the myenteric plexus, the longitudinal part, below the plexus. **A** Total binding of [^{125}I][Tyr^{4}]-bombesin. **B** Total binding of [^{125}I]PYY. **C** Binding of [^{125}I]-*N*-acetyl-PACAP 27 in presence of 10^{-6} M unlabeled VIP. **D** Total binding of [^{125}I]CCK. **E** Total binding of [^{125}I][Tyr^{3}]-neurotensin. **F** Total binding of [^{125}I][LTT]-SRIF-28. In all cases, non-specific binding is negligible

of somatostatin receptors. The muscularis mucosae expresses no somatostatin receptors.

The above-mentioned targets were more strongly labeled with [^{125}I][Tyr^3]-octreotide than with [^{125}I][Leu^8, D-Trp^{22},Tyr^{25}]-SRIF-28, suggesting a predominant expression of sst_2, sst_5 or sst_3. For further identification of subtypes, [^{125}I][Tyr^3]-octreotide was used in displacement experiments with subtype-selective analogs: in the plexus, the germinal centers, the vessels, the mucosa and the margin of the circular muscles, 50 nM of the sst_2-selective L-779,976 completely displaced the radioligand, whereas the sst_3-selective analog sst_3-ODN-8 or the sst_5-selective L-817,818 were inactive, suggesting a predominant expression of sst_2 in these tissues.

Comparative analysis of receptor distribution in the various targets

Figure 10 compares the density and localization of the investigated peptide receptors, such as GRP receptor, Y_2 receptor, PAC_1 receptor, CCK-A receptor, NT1 receptor and sst_2 receptor in the myenteric plexus and the adjacent smooth muscle at high magnification. It also compares the distribution of the different peptide receptors with the corresponding positive immunostaining of the myenteric plexus for synaptophysin. For a better GRP receptor identification in the myenteric plexus, a sample has been chosen with a longitudinal smooth muscle expressing only a low receptor density.

While Fig. 10 is a comparative illustration of the complex distribution of several neuropeptide receptors and indicates the importance of the myenteric plexus as a relay station for neuropeptides in the human enteric nervous system, Table 1 allows to compare the distribution of the neuropeptide receptors in the other colonic targets. The smooth muscle is a very important neuropeptide target as well, as it expresses GRP receptors, Y_2 receptors, VIP/PACAP receptors, NT1 receptors, NK_1 receptors and somatostatin receptors. Moreover, a complementary distribution of these receptors is seen in the longitudinal (predominantly GRP and NT1 receptor-positive) and circular (predominantly NK_1, NT1 and Y_2 receptor-positive) smooth muscle. Interestingly, GRP receptors, NK_1 receptors, $VPAC_2$ receptors and sst_2 receptors are all identified in the mucosa-directed part of the circular smooth muscle, an observation that may suggest a special role for this muscle area which corresponds to the localization of the interstitial cells of Cajal (Vannucchi 1999). It appears, furthermore, that vascular receptors include predominantly Y_1 receptors and NK_1 receptors and to a lesser extent $VPAC_2$ and sst_2 receptors. The lymphoid follicles are complementarily labeled, expressing VIP/PACAP receptors in the peripheral zone and sst_2 receptors in the germinal centers. In the mucosa, a strong $VPAC_1$ labeling in the superficial layers as well as a Y_2 labeling of the whole mucosa is observed.

Discussion

This study is a detailed and systematic report on the comparative evaluation of neuropeptide receptor expression in the human colon; this was achieved by using morphological methods such as receptor autoradiography. This method visualizes the specific targets for these neuropeptides in this complex organ and identifies the tissues characterized by a co-localization of several receptor types, as a basis for possible functional interactions among these neuropeptides. It also gives basic information on the status of neuropeptide receptors in the normal human colon. This will permit future studies in diseased conditions of the colon, i.e. Hirschsprung's disease or inflammatory bowel disease. It should be noticed that the methodology used, in particular the characterization of receptor subtypes in displacement experiments with selective analogs, allows to identify the receptor subtype predominantly expressed in a tissue, but may not necessarily detect other subtypes concomitantly expressed in only low amounts, in the same tissue.

The wide distribution of neuropeptidic targets and the pattern specificity of neuropeptide receptor expression in human colon suggest an important physiological role for these peptides in this part of the gastrointestinal tract. The myenteric plexus, for example, as the most important neuronal relay station of this area, contains high densities of GRP receptors, Y_2 receptors, NT1 receptors and sst_2 receptors and lower densities of PAC_1 receptors and CCK-A receptors. They are usually homogeneously distributed in the whole plexus. The present results provide a molecular basis of the neuropeptide regulation of many colonic functions through specific G protein-coupled receptors (Furness et al. 1992; Jacobson and Bunnett 1997; McConalogue and Bunnett 1998; Sanders 1998). Regulation of water- and ion transport, for example, is a well-established VIP action (McCabe and Dharmsathaphorn 1988) that is likely to be mediated by the $VPAC_1$ receptors located in the superficial layers of the mucosa (Broyart et al. 1981; McConalogue and Furness 1994; Zimmerman et al. 1989). Other receptors located in the mucosa such as Y_2 receptors and sst_2 receptors may mediate peptide effects related to the regulation of fluid and electrolyte transports as well (Jackerott and Larsson 1997; Saria and Beubler 1985; Walsh et al. 1993). Regulation of immune processes by VIP and somatostatin are likely to be mediated by VIP/PACAP receptors and sst_2 receptors, respectively, located either in the peripheral zone or in the germinal center of the lymphoid follicles (ODorisio 1988; Reubi 1992; Reubi et al. 1992). The example of the lymphoid follicle is a good illustration of the potential complementary role of these receptors for the regulation of immunological processes (Kimata et al. 1992; ODorisio 1988; Stanisz et al. 1987). The regulation of blood flow in the colon may represent a very subtle process involving the interaction of at least four different peptides, SP, NPY, VIP and SRIF; based on the amount of receptors, it may be primarily mediated by NK_1 receptors and Y_1 receptors, to a lesser ex-

tent by VPAC1 and sst_2 receptors located in arteries and/or veins (Nandha et al. 1991; Peaire et al. 1997; Walsh et al. 1993). The regulation of the motility of the gastrointestinal tract (Furness et al. 1992) may also be mediated concomitantly by several peptide receptors including GRP receptors, NT1 receptors, Y_2 receptors and NK_1 receptors located in high density in longitudinal and/or circular smooth muscle. The differential expression of these receptors in the various muscle compartments, including longitudinal and circular muscle as well as muscularis mucosae, illustrates the complexity of the regulation of motility and suggests an extensive interaction and complementary role among neuropeptides. The predominant expression of most neuropeptide receptors in the nerve plexus confirms the crucial neurotransmitter role of neuropeptides previously suggested on the basis of immunohistochemical studies in this tissue. This may indicate that the neuropeptide receptors in most colon targets evaluated in this study may primarily be involved with a neurotransmitter function.

The high expression of GRP and NK_1 receptors, but also of $VPAC_2$ and sst_2 receptors in the mucosa-directed margin of the circular smooth muscle strongly suggest that this area may deserve particular interest, as a site for a major neuropeptidergic role. In laboratory animals, this margin is known to contain numerous interstitial cells of Cajal that express NK and sst_2 receptors (Sternini et al. 1997; Vannucchi 1999). These cells are known to play a predominant role as pacemakers for the gut rhythmic activity and as a relay station for neuronal inputs to the muscle (Thomsen et al. 1998; Vannucchi 1999). Therefore, the expression, in this area, of NK_1 and sst_2 receptors, but also of GRP and $VPAC_2$ receptors and, in some cases, CCK-B receptors, is probably related to the function of these interstitial cells of Cajal. However, the limited spatial resolution of the autoradiographic techniques does not allow a precise identification of the receptor-positive elements.

The evaluation of the expression of several neuropeptide receptors had been reported previously for the human colon. Compared to former studies, however, the present one characterizes the subtypes of the peptide receptors. Moreover, we could find in several cases more sites of receptor expression than previously described. For instance, we identified CCK-A in the myenteric plexus while this had not been seen before (Mantyh et al. 1991) except for an incidental report in a colon cancer study (Reubi et al. 1999a). While VIP receptors had been reported already in the human colonic mucosa and smooth muscles (Korman et al. 1989; Mantyh et al. 1991; Zimmerman et al. 1989), we could identify PAC_1 expressed in the myenteric plexus and confirm therefore previous findings in rat and mice (Ekblad et al. 1988). Moreover, we observed $VPAC_2$ expressed in the circular smooth muscle at the site where the interstitial cells of Cajal are known to be localized. New for human tissue is also the observation of GRP receptors, NK_1 and sst_2 receptors at the site of location of Cajal cells, in the mucosa-directed margin of the circular muscle. While we could confirm Y_1 expression in vessels (Peaire et al. 1997), we could not detect measurable amounts of Y_1 in the myenteric plexus and in the muscles; conversely, high amounts of Y_2 were found in the myenteric and submucosal plexus, in the smooth muscles and in the mucosa. Whereas our finding of Y_2 in the plexus are in agreement with previous autoradiography data by Walsh et al. (1993), they differ from mRNA studies reporting Y_1 in the plexus and the mucosa (Peaire et al. 1997; Wharton et al. 1993); these studies, however, only evaluated Y_1 and did not investigate Y_2. While our results suggest a high expression of Y_2 in the plexus, we cannot exclude that the method missed small amounts of Y_1 that would have been masked by the high amount of Y_2. Furthermore, we can confirm the previously reported high expression of bombesin receptors in the plexus and smooth muscle cells (Mantyh et al. 1991), which we characterized here as the GRP subtype. We can also confirm the high expression of NK receptors in smooth cells and blood vessels (Korman et al. 1989), characterized as NK_1 subtype in the present study.

This study is based exclusively (1) on human tissue; as the peptide receptor expression among species varies greatly, our results may differ from results obtained in studies investigating receptors in other species (Jackerott and Larsson 1997; Sternini et al. 1997; Walsh et al. 1993); (2) on tissue taken from samples resected 5 cm or 10 cm from surgically operated tumors. Although the investigated colonic tissue has been taken at distance from the diseased site and no histopathological abnormalities have been noticed, it may not necessarily represent normal human colon tissue in all cases. The good concordance in the receptor data among the tissues of the nine patients and the little individual variability of receptor expression suggest a rather homogeneous and representative sample population in this series. Nevertheless, the smooth muscle can show an individual variability of expression for somatostatin receptors (circular and longitudinal layers) and CCK-A receptors (longitudinal layer). The mucosa-directed margin can show a variability for CCK-B receptors, the muscularis mucosae for GRP receptor, and the vessels for VIP/PACAP receptor expression. Since an internal control for adequate tissue processing has been provided by the strong expression of other peptide receptors in these same cases, these variable results are likely due to a real receptor inhomogeneity in a given tissue and/or to individual variabilities in receptor density among the patients.

A sufficiently high local level of endogenous peptides is necessary for a receptor to be functional. Unfortunately, a comparison between the distribution of receptors and the presence of the corresponding endogenous ligands cannot be completed at present time because human data on endogenous peptide contents in the colon are lacking for several of the peptides. Nevertheless, the peptides somatostatin, SP, NPY and VIP have been found to be present in human colonic nerves (Domoto et al. 1990; Koch et al. 1987; Larsson and Sundler 1990), suggesting that the endogenous ligand is present in the area of the receptor-expressing tissues. VIP is known to have a rich distribution in the nerve fibers of several targets in the colon, including the myenteric and submucosal plexus, the

smooth muscle and the mucosa (Domoto et al. 1990; Koch et al. 1987; Larsson and Sundler 1990); receptor expression has been found in all these VIP-innervated tissues, the highest amounts being observed in the mucosa (Broyart et al. 1981; Korman et al. 1989). SP has been shown to be moderately distributed in nerve fibers located in the colon, for instance in the myenteric and submucosal plexus, the smooth muscles and the mucosa (Kimura et al. 1994; Koch et al. 1987; Larsson and Sundler 1990); interestingly, in some of these targets, i.e. the mucosa, there is virtually no expression of NK receptors (Korman et al. 1989; and the present study).

Our morphological study on the localization of neuropeptide receptors in the human colon has potential clinical implications: (1) it will allow a comparison with prospective in vitro peptide receptor analysis of diseased human colonic tissues including tumors, Hirschsprung's disease, degenerative and autoimmune pathologies, where neuropeptides may play a prominent pathophysiological role (Basbaum and Levine 1991; Kimura et al. 1994; Koch et al. 1988); (2) it may represent basic information for the development of neuropeptide drugs aimed at interacting with the various targets in the colon in clinical settings, but it may also be useful for the understanding and prediction of potential side effects occurring in human colon by the use of synthetic peptides for systemic therapy (Lamberts et al. 1991).

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