Identification and regional distribution of the dopamine D_{1A} receptor in the gastrointestinal tract

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DOPAMINE (DA) is an important modulator of gastrointestinal exocrine secretary (60), fluid absorptive (10), motility (35), blood flow (28), and cytoprotective functions in mammalian species, including humans (19). Although physiological effects of DA in the gut can be mediated through α- or β-adrenoreceptor activation, a dopaminergic receptor-specific response also has been implicated. Saturable high-affinity membrane-bound DA binding sites are demonstrable within gastrointestinal tissues (44, 60). Dopamine-immunoreactive and tyrosine hydroxylase-positive cells are identifiable throughout the length of the gastrointestinal tract (GIT), and substantial quantities of DA are synthesized by mesenteric organs (8, 11, 17). It has been suggested that this large DA productive capacity is reflective of the release of the endogenous agonist for a local nonneuronal dopaminergic autocrine or paracrine system (11, 12). A rate differential synthesis of DA from its circulating precursor (3,4-dihydroxyphenyl)-L-alanine has been demonstrated in nonglandular and glandular stomach, duodenum, jejunum, ileum, and proximal and distal colon of the rat (58). In this manner the GIT may be functionally analogous to other peripheral organs, such as the kidney, where a similar endogenous DA production and autocrine/paracrine action has been documented (51).

The actions of DA were originally ascribed to the activation of two central nervous system DA receptor subtypes, D_1 and D_2, which had opposing actions on adenylyl cyclase activity (26). A number of selective ligands were developed for use in clinical therapeutics in the treatment of psychiatric, neurological, and endocrine disorders. Many of these drugs, such as metoclopramide, bromocriptine, and haloperidol, were shown to modify gastrointestinal function. DA receptors were subsequently localized in peripheral tissues, such as blood vessels, kidney, adrenal gland, and GIT, by ligand binding and autoradiographic studies. Because structural similarities between central and peripheral DA receptors were indeterminate, a separate nomenclature for peripheral DA receptors was adopted, D_{1A}-like and D_{2A}-like (20). However, with the advent of molecular biological cloning techniques, multiple subtypes of animal and human DA receptor subtypes have been identified along with the discovery of complex interactions with a variety of second messenger systems (45). At present, two members of the D_1 receptor family have been described, the D_{1A} and D_{1B} (D_{3a} human equivalent) receptors, along with three members of the D_2 family, the D_2, D_3, and D_4 receptors.

Our group recently demonstrated that the D_{1A} receptor, cloned from the rat brain, is expressed in rat kidney (39, 40), heart (42), and adrenal gland (1, 38). This was achieved using light microscopy immunohistochemistry with antipeptide antisera directed to

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epitopes on the D₁A receptor, as well as in situ amplification and hybridization using an oligonucleotide probe targeted to receptor-specific mRNA. This combined approach permits identification of receptor subtypes that remain indistinguishable when using agonist- and/or antagonist-based radioligand, biochemical, or functional studies. Apart from one other previous report, confined to the small intestine (34), the expression and localization of these recently cloned DA receptor subtypes has not been studied, to our knowledge, in the GIT. The present study determines the regional distribution of the D₁A receptor throughout the rat GIT. The localization of receptor in both epithelial and muscle cell layers throughout the esophagus, stomach, and small and large intestines strongly suggests that this subtype may play a role in the modulation of gastrointestinal function previously ascribed to DA.

METHODS

Antisera. Polyclonal antibodies were raised against three synthetic peptide sequences derived from the predicted rat D₁A receptor amino acid sequence, corresponding to epitopes located on extracellular and intracellular portions of the receptor (40). These antibodies were shown to be specific for the D₁A receptor (40).

Light microscopic immunohistochemistry. Male Wistar-Kyoto rats, weighing 250–500 g, were anesthetized with pentobarbital sodium (50 mg/kg ip). Frozen sections from various regions of the GIT and brain were cut (7–10 μm) and mounted on positively charged slides. For immunohistochemical studies, the sections were incubated with the D₁A receptor primary antiserum diluted 1:1,500 to 1:4,000. Immunoreactive D₁A receptor was detected with an avidin-biotin immunoperoxidase reaction (Vectastain ABC Kit, Vector Lab, Burlingame, CA). Slides were lightly counterstained with hematoxylin. Controls included 1) omission of primary antiserum, 2) replacement of primary antiserum with preimmune serum, 3) immunohistochemical analysis of brain striatal regions known to have the D₁A receptor. In all but the last, immunoreactivity was abolished.

Western blot analysis. Western blot analysis was performed in a manner previously described (42).

In situ amplification and hybridization histochemistry. A transcription-based amplification system (3SR in situ) was employed to amplify D₁A mRNA before detection of the mRNA by in situ hybridization. Specific details about this novel molecular amplification method have been described elsewhere (39). In addition to sense controls, we omitted the oligonucleotide probe or the anti-digoxigenin antibody from other slides, both of which serve to control for nonspecific hybridization and background. These controls resulted in loss of the hybridization signal.

RT-PCR. Total RNA was isolated from various tissues of the rat gastrointestinal tract, from the striatum as well as from a murine fibroblast LTK⁻ cell line that had been stably transfected with a full-length rat D₁A receptor cDNA as previously described (39). Nontransfected LTK⁻ cells were used as a control source of RNA. RNA extraction was carried out using a standard guanidium-thiocyanate protocol. Genomic contamination was removed by DNase I (Boehringer Mannheim, Lewes, UK) treatment. First-strand cDNA synthesis (reaction volume 30 μl) using 10–100 ng of RNA was performed in the presence of avian myeloblastosis virus (AMV) RT buffer (Promega, Southampton, UK) with 0.5 μg oligo(dT)₁₅ primer (Promega), 0.20 mM dNTPs (Promega), 20 units RNasin (Promega), and 100 units AMV RT (Promega). The reaction was carried out at 37°C for 90 min. A control in which all the components of the reaction were added except the reverse transcriptase was tested in parallel with each sample.

PCR amplification of the cDNA was performed using primers designed specifically to the rat D₁A receptor mRNA. The sense primer 5'AGATCTCTTGGTGCTGCTG-3', corresponding to nucleotides 263–281, and the antisense primer 5'-ATAATGCGTCGCGAGTG-3', corresponding to nucleotides 688–706, resulted in the amplification of a 425-bp product. The PCR was carried out (total volume of 50 μl) in the presence of 1 μl of the cDNA reaction, 25 pmol of each primer, 0.25 mM dNTPs, 5 μl of 10× Taq buffer (Promega), and 2 μl of 50 mM MgCl₂. The reaction contents were heated at 94°C for 1 min before the addition of 2.5 units of Taq polymerase (Promega). The 40 reaction cycles were set at 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min. Analysis of the amplified product was conducted by size fractionation of an aliquot of the RT-PCR sample, in conjunction with molecular weight markers of known size, in a 2% agarose gel and visualization with ethidium bromide staining. All of the RT-PCR products thus analyzed gave a band of the expected size.

RESULTS

Western blot analysis revealed a single 50-kDa band for the D₁A receptor transfected LTK⁻ fibroblasts (Fig. 1B, lane 11), but not in wild-type fibroblast cells (Fig. 1B, lane 10). An identical 50-kDa size band was obtained for the esophagus, stomach, duodenum, jejunum, colon (Fig. 1B, lanes 2–6), and striatum (Fig. 1B, lane 9), with a further 40-kDa band noted in the latter tissue lanes. The molecular mass of the D₁A receptor is ~50 kDa, and we have previously seen additional bands on Western blot analysis of the brain D₁A receptor that may be related to partially degraded receptor protein. In vitro amplification, by RT-PCR, of RNA isolated from similar tissue sites resulted in the generation of a predicted 425-bp product identical to that seen for RNA isolated from D₁A receptor transfected LTK⁻ fibroblasts (Fig. 1A). In the whole stomach, the 425-bp product was not detected, but this product was detected in low abundance in the muscle layers of the fundus (data not shown). In contrast, no amplified product was detectable for RNA isolated from nontransfected LTK⁻ cells or from nontemplated control.

Light microscopic immunohistochemistry demonstrated an extensive distribution of D₁A receptor protein within the GIT. In the gastroesophageal junction, immunoreactivity was present on the cytoplasmic membranes of muscle fibers in the lower esophageal sphincter (Fig. 2A). Control sections, in which either preimmune or preadsorbed serum was used, were distinguished by their complete absence of immunoreactive staining (Fig. 2B). In situ hybridization histochemistry for the D₁A receptor mRNA corroborated this muscular localization of the D₁A receptor (Fig. 2C). In Fig. 2C, the digoxigenin-labeled antisense probe clearly hybridized to the muscle fiber membranes, resulting in a clear demarcation of individual fibers of the gastroesophageal sphincter. In contrast, consecutive sections probed with the matching digoxigenin-labeled sense probe had no hybridization signal (Fig. 2D).
In the stomach, immunohistochemical staining for D_{1A} receptor protein was present in both the gastric epithelium and smooth muscle layers. Strong staining was present in gastric glands throughout the cardiac, fundal, and pyloric regions of the stomach (Fig. 3A). Immunoreactive receptor protein appeared to be visible in both peptic and parietal cells at the base of gastric glands (Fig. 3A and B). Blood vessels within the gastric mucosa and the submucosa demonstrated strong immunoreactivity for the D_{1A} receptor (Fig. 3B). A D_{1A} immunoreactive signal was also present in smooth muscle of both the muscularis mucosa and in the muscularis externa (Fig. 3B). Control sections of stomach were consistently negative (Fig. 3C). In situ hybridization histochemistry for the D_{1A} receptor revealed an equivalent distribution of D_{1A} receptor mRNA. Figure 3D is an example of the mRNA distribution pattern found within the fundal region of the stomach. It can be seen that mRNA signal was present within the gastric lining epithelium, the muscularis mucosa, vessels of the submucosa, as well as within the muscularis externa. Control sections probed with sense probe had no visible hybridization signal.

Small intestinal staining for the D_{1A} receptor protein and mRNA was found in the duodenum, jejunum, and ileum, with a strong signal present in both epithelial and muscle cell layers. D_{1A} receptor protein immunoreactivity was present throughout the epithelium in intestinal villi and crypts, with further signal in the muscularis mucosa and muscularis externa (Fig. 4, A and B). Immunoreactive receptor protein was also visible in lamina propria cells throughout the small intestine (Fig. 4C). A very strong signal was present in Auerbach’s plexus in the small intestinal wall (Fig. 4D), although control sections were consistently negative. D_{1A} receptor mRNA signal was detectable within similar tissue sites of the small intestine. Epithelial cells lining intestinal villi stained strongly for mRNA (Fig. 4E), and signal appeared to be predominantly within enterocytes rather than goblet cells. mRNA signal was also very evident within both the muscularis mucosa and muscularis externa (Fig. 4F). It appeared that signal was more intense within the inner circular region of the muscularis externa (Fig. 4F). As before, control sections probed with sense probe had no visible hybridization signal (Fig. 4G).

D_{1A} receptor immunoreactivity had a transmural distribution in the colon (Fig. 5A). Strong D_{1A} receptor immunoreactivity was detected in the cytoplasm of cells in both the apices and bases of colonic epithelial crypts. In colonic crypts, immunostaining was present in discrete cell populations, whereas other cells lining the crypts did not demonstrate any immunoreactivity. Strong immunostaining was also evident in myocytes.
throughout the muscularis mucosa. Immunostaining was absent in both the submucosa and submucosal blood vessels. The muscularis externa also exhibited strong immunoreactivity, with an increased signal in the longitudinal muscle layer relative to the circular muscle layer. Consecutive sections processed with either preimmune serum, omission of primary antibody, or omission of secondary antibody did not reveal any immunoreactivity (Fig. 5B). Sections probed for D1A receptor mRNA gave a positive in situ hybridization signal in the same colonic epithelium. This closely correlated with the signal obtained immunohistochemically. The smooth muscle of muscularis mucosa showed a very intense hybridization signal (Fig. 5C). A positive hybridization signal was present in cells of the muscularis externa, although the signal was weaker than in the muscularis mucosa. Control sections treated with a sense oligonucleotide probe resulted in the abolition of the hybridization signal (Fig. 5D). Likewise, sections from which the anti-digoxigenin antibody were omitted failed to develop a positive hybridization signal.

Overall, there was a very close correlation between the results obtained immunohistochemically for D1A receptor protein expression and those for D1A receptor message. Table 1 summarizes the gastrointestinal distribution of the D1A receptor found in this study.

DISCUSSION

The presence of DA receptors throughout the GIT has long been suspected on the basis of the known pharmacological effects of DA and dopaminergic agonists and antagonists on various regions of the gut. DA is a known enteric neurotransmitter (60) located in the
nerve terminals of the myenteric plexus. In addition, autoradiographic studies have revealed high-affinity binding sites for DA at various gastrointestinal sites, including the stomach, duodenum, and ileum (44, 60), and immunohistochemical studies have shown the presence of DA-immunoreactive and tyrosine hydroxylase-positive cells within the various nonneuronal tissue layers of the GIT (8, 11, 36). It is interesting to note that certain peptidergic cells of the amine precursor uptake and carboxylation series within the gut may have dopaminergic precursors (54). High quantities of both free and conjugated DA along with DA metabolites are present within mesenteric organs (8, 11, 12, 17). These findings have prompted the hypothesis that the GIT is an integral component of a peripheral catecholaminergic system, wherein DA acts as an autocrine or paracrine hormone being inactivated by conjugation (21).

In the gastroesophageal sphincter, the D_{1A} receptor was located on the cytoplasmic membrane of striated muscle fibers. DA promotes a relaxation or inhibition of lower esophageal pressure in several species (60). Concurrent use of DA antagonists such as metoclopramide and domperidone counteracts this effect, increasing lower esophageal sphincter (LES) tone when used clinically in humans (56).

Pharmacological characterization of the LES suggests that both D_{1}-like and D_{2}-like receptors are present with opposing actions on sphincter motility, the D_{1}-like receptor modulating contraction and the D_{2}-like receptor modulating relaxation (37, 47). On the basis of the above pharmacological profile, the expression of the D_{1A} receptor on fibers of the LES as demonstrated in the present study implies an integral role in the modulation of the described D_{1}-like receptor increase in LES tone. Such receptors also may have an etiological role in certain pathophysiological LES conditions, as suggested by a recent study wherein an imbalance in D_{1}-like/D_{2}-like receptor function was demonstrated in esophageal achalasia (46).
A number of physiological actions have been ascribed to DA within the stomach. Several investigators have demonstrated that DA can reduce intragastric pressure and antral motility in a number of species, including humans (3, 31, 57), and that such effects can be inhibited by the DA antagonists, such as domperidone, haloperidol, and sulpiride (31, 57). Such actions have been ascribed to peripheral DA receptors located in the enteric nervous system on the basis of the fact that neither DA nor domperidone crosses the blood-brain barrier and because tetrodotoxin can abrogate, at least in part, these DA inhibitory effects (50). However, further studies have demonstrated the presence of DA receptors directly on smooth muscle cells of the stomach (8) that also can modulate gastric smooth muscle responses. DA appears to elicit two major actions within gastric smooth muscle (30): 1) contraction of the circular smooth muscle layer through a direct action on smooth muscle at adrenoceptor and 2) relaxation of the longitudinal smooth muscle layer. The latter response is mediated by two separate pathways: a direct action on smooth muscle D₁-like receptors, which is maximal under basal conditions, and an additional, though lesser, neurally mediated component. The present study clearly demonstrates the presence of the D₁A receptor subtype on gastric longitudinal smooth muscle cells.

An expanding body of literature suggests that DA is involved in experimental gastric ulcer genesis. A cytoprotective role for DA and its agonists in stress-induced

Fig. 4. Light photomicrographs of small intestinal staining for the D₁A receptor protein and mRNA. D₁A receptor protein immunoreactivity was present throughout the epithelium and in the muscularis mucosa and muscularis externa (A; magnification ×100 and B; magnification ×250), with signal also visible in cells within the lamina propria (C; magnification ×400). A very strong signal was present in Auerbach’s plexus (AP) in the small intestinal wall (D; magnification ×400). mRNA was detectable at equivalent tissue sites throughout the small intestine. E: D₁A receptor mRNA in duodenal villi (magnification ×400). F: the mRNA signal in ileal smooth muscle layers. G: sense control of duodenum (magnification ×400).

Fig. 5. Light photomicrographs demonstrating D₁A receptor immunoreactivity in colon. A: positive staining found in colonic crypts, muscularis mucosa, and in the muscularis externa. No immunoreactivity was detectable within submucosal blood vessels (magnification ×250). Control sections probed with preabsorbed serum had complete absence of immune staining (B; magnification ×250). mRNA was detected at similar colonic sites (C; magnification ×250) with notable absence of signal within mucosal blood vessels, whereas consecutive sections probed with sense hybridization probes had complete absence of staining (D; magnification ×250).
gastric lesions is demonstrable, antagonized by the peripherally selective D₂-like receptor antagonist, domperidone, suggesting the involvement of peripheral DA receptors (23). D₁-like/D₂-like receptor interactions have been implicated in the regulation of gastric mucosal integrity during stress (43), with a key role being played by D₁-like receptors (18). DA is thought to mediate, in part, so-called gastric adaptive cryoprotection, a phenomenon wherein application of a mild irritant to the gastric mucosal surface confers protection against subsequent irritant exposures (33). Significant levels of DA are demonstrable within gastric mucosa (11, 17), and ligand-binding studies have revealed D₁-like receptors in both rat (8, 22) and human gastric mucosa (24). Immunohistochemical studies have revealed substantial reductions in gastric mucosal DA content with acute ulceration (25). An integral component of this anti-ulcerogenic effect of DA may be the resultant increased blood flow to gastric mucosa and submucosa, possibly mediated by DA receptors located in submucosal blood vessels (28). Blood vessels within the gastric epithelium as well as vessels within the submucosa stained strongly for the D₁A receptor in the present study. We previously described a widespread distribution of the D₁A receptor within the renal vasculature of the rat kidney, which is probably responsible for the well-described renal vasodilator effects of DA (39, 40). It is possible that this receptor also has a similar vasoactive role within gastric blood vessels. An additional action of D₁-like receptors that can enhance the DA induced anti-ulcerogenic effect is their reduction of gastric secretion. Thus various groups have shown that D₁-like receptor selective agonists reduce, whereas antagonists augment, gastric acid production (9, 18). The experiments presented here clearly demonstrate D₁A receptor protein and mRNA in gastric glands throughout the fundus and body and pyloric regions of the stomach, providing strong evidence that this DA receptor subtype mediates, at least in part, these gastric actions. These glands contain acid-secreting cells, as well as neuroendocrine cells that contain both DA and a variety of gastrointestinal hormones. The presence of DA receptors in close proximity to neuroendocrine cells raises the possibility that DA modulates their activity and hormone production. It is possible that DA also modulates gastric acid secretion through autocrine or paracrine mechanisms, as induction of acid secretion via activation of D₁-like receptors located on cholinergic neurons and on some nonneural cells has been demonstrated recently in the rat stomach (55).

Small intestinal staining for the D₁A receptor was detected in both the jejunum and ileum, with a strong signal present in both epithelium and muscle. D₁A receptor immunoreactivity was present throughout the epithelium, especially at the base of crypts, with a weaker signal in the muscularis mucosa and muscularis externa. A strong signal was also present in Auerbach’s plexus in the small intestinal wall. Several lines of investigation have provided other evidence of specific DA receptors throughout the small intestine. Studies similar to those cited for the stomach have implicated dopaminergic mechanisms in the pathogenesis of duodenal ulceration (52). D₁-like receptor binding sites are demonstrable in the mucosa and muscularis propria of rat stomach and duodenum (8, 52) along with the presence of a DA-sensitive adenyl cyclase. DA agonists such as bromocriptine and amantadine accelerate duodenal ulcer healing and reduce relapse rates (48). An integral part of this dopaminergic anti-ulcerogenic action in the duodenum is thought to be the stimulation of duodenal mucosal bicarbonate secretion (15, 27), which protects the epithelium from luminal acid. This effect is mediated through D₁-like receptors and is associated with an increase in the production of cAMP (14).

DA has a relaxing effect on isolated rat jejunum that is thought to be a direct effect mediated through specific DA receptors and is associated with increased cAMP levels (6, 32) in the manner usually evoked by D₁-like receptor agonists. There is evidence of dopaminergic receptors on intrinsic neural elements within the small intestine (7), which is supported by the localization of D₁A receptors in Auerbach’s plexi in the present studies. These DA receptors may play a role in modulating intrinsic enteric neural systems as evidenced by the ability of DA to alter stimulated endogenous opioid release in ileal longitudinal muscle (41). It is also possible that DA receptors modulate fluid and electrolyte absorption in the GIT, in both the ileum (2, 10) and jejunum (13). The previous description of the D₁A receptor revealed its presence on cells at the base of intestinal crypts (34). These findings are confirmed and extended in the present study, again supporting a role for this receptor subtype in modulating intestinal function.

Table 1. Distribution of dopamine D₁A receptors in the gastrointestinal tract

<table>
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<tr>
<th>Tissue</th>
<th>Cellular Distribution</th>
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<tbody>
<tr>
<td>Gastroesophageal junction</td>
<td>Myocytes of the lower esophageal sphincter</td>
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<tr>
<td>Stomach</td>
<td>Epithelial peptic and parietal cells: cardia, fundus, and pylorus</td>
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<td></td>
<td>Blood vessels of mucosa and submucosa</td>
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<td>Smooth muscle cells of muscularis mucosa and muscularis externa</td>
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<tr>
<td>Small intestine (duodenum, jejenum, ileum)</td>
<td>Enterocytes of villi and crypts</td>
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<td></td>
<td>Smooth muscle cells of muscularis mucosa and muscularis externa</td>
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<td></td>
<td>Lamina propria</td>
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<td>Auerbach’s plexus</td>
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<td>Colon</td>
<td>Epithelial cells of crypts</td>
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<td>Smooth muscle cells of muscularis mucosa and muscularis externa</td>
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A number of functional and pharmacological studies have demonstrated increases in colonic motility in response to DA and DA receptor agonists and decreases in colonic motility in response to DA antagonists, both in vivo and in vitro, in a variety of species (5, 16, 53, 59). Many of these studies, however, lack conclusiveness regarding the precise role of peripheral DA and DA receptors in the colon, because no precise pharmacological characterization was carried out, the ligands used were nonspecific, and the effects are likely to have been mediated by other receptor populations, such as adrenergic receptors, 5-hydroxytryptamine receptors, or by centrally mediated dopaminergic mechanisms. We have demonstrated positive immunostaining throughout the muscularis mucosa and in the muscularis externa for the D_{1A} receptor with an increased signal in the longitudinal layer of the colon relative to the circular layer. The presence of this receptor in these sites may suggest that this receptor can modulate colonic motility. Clinical studies support the importance of DA in the modulation of colonic motility. A study of patients with Parkinson’s disease and suffering from constipation showed that they have fewer colonic dopaminergic myenteric neurons as well as lower levels of DA in the muscularis externa than control subjects (49). Although the specific defect has not been elucidated in this enteropathy, the study highlights the functional importance of DA in the human colon.

DA has been shown to modulate blood flow in different vascular beds such as the renal (20) and coronary circulation (61). DA-induced intestinal vasodilation has been studied in a number of species, and there is general agreement that it is mediated through stimulation of specific DA receptors (29). Several studies have demonstrated that dopaminergic compounds have protective actions on the mucosa of the human stomach and duodenum. Although the mechanism is not well understood, it may be via improved mucosal blood flow. We have demonstrated prominent D_{1A} receptor immunoreactivity in both mucosal vessels and submucosal vessels of the stomach and small intestine, but not in blood vessels within the colon. This suggests that this DA receptor subtype modulates vasomotion in a regional manner in the GIT, supporting previous studies that revealed a regional variation in the intestinal vasodilation induced by DA (28). Many lamina propria cells within various gastrointestinal tissues were found to express the D_{1A} receptor. This is consistent with recent reports that demonstrated the presence of tyrosine hydroxylase in human lower digestive tract lamina propria cells (11) and immunocytes of gastric mucosa (36) and with other reports that demonstrated an ability of immune cells to synthesize catecholamines (4). The functional significance of dopamine receptors on such cells remains indeterminate.

**Perspectives**

The present study is the first to characterize the regional expression of the recently cloned central D_{1A} receptor gene in the GIT. The extensive distribution in different layers and different organs of the GIT supports previous studies that suggest that there is an important enteric dopaminergic autocrine or paracrine system that modulates a number of aspects of GIT function. Although precise functional studies demonstrating the physiological effects of this receptor subtype are lacking at present, the cellular distribution of this receptor subtype suggests diverse actions, including modulation of motility, fluid and electrolyte balance, and blood flow. In the future, the development of more specific ligands, as well as the use of gene deletion animal models, will allow the precise evaluation of the physiological importance of this receptor in the mammalian GIT.

**REFERENCES**


