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Cellular localization of dopamine receptors in the gastric mucosa of rats

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ABSTRACT

Dopamine (DA) plays a critical role in the protection of gastric mucosa and is mediated through corresponding receptors. However, the details of the expression of DA receptors (D1–D5) in the gastric mucosa are lacking. The present study investigated the expression and cellular localization of DA receptors in rat gastric mucosa by means of real-time PCR and immunofluorescent techniques. The results indicated that the mRNA expressions of all five subtypes of DA receptors were found in the gastric mucosa, among which the D2 level was the highest. The immunopositive cells of D1–D3 and D5 were primarily localized to the basilar gland of the epithelial layer in gastric corpus, but D4 immunoreactivity (IR) was only observed in the enteric nerve plexus. The D1, D2, and D5 IR were found in pepsin C-IR cells except D3. No IR of any DA receptor was detected in the H^+/K^+-ATPase- or mucin 6-IR cells. In conclusion, for the first time, this study demonstrates the predominant distribution of DA receptors in the chief cells, not the parietal and mucous neck cells, in rat gastric mucosa, thus suggesting that DA may not directly regulate the function of parietal cells or mucous neck cells, but it may modulate the function of chief cells through the D1, D2, and D5 receptors.

1. Introduction

DA as an important neurotransmitter, plays an important role in the regulation of gastrointestinal (GI) motility [1], GI epithelial ion transport [2,3] and the protection of gastroduodenal mucosa. A high incidence of gastroduodenal ulcer has been noted in patients with Parkinson disease, a well-known dopaminergic neuronal degenerative disease, whereas a low incidence has been noted in patients with schizophrenia, who have excess DA secretion in the brain [4]. Anti-dopaminergic drugs have been reported to induce ulcers, and DA agonists are able to protect against gastroduodenal mucosa ulcer formation [5]. A gastric ulcer animal model has been induced with the intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [6]; in this model, the dopaminergic markers and DA content in the GI tract are significantly decreased [7]. However, little is currently known about which DA receptors are involved in the protection of DA in the gastric mucosa.

DA signaling is mediated by five subtypes of DA receptors. These subtypes have been grouped into two families on the basis of structure and pharmacology: D1-like (D1 and D5), which can activate adenylyl cyclase; and D2-like (D2, D3, and D4), which can inhibit adenylyl cyclase [8]. In addition to the central nervous system, DA receptors have also been detected in peripheral tissues, such as blood vessels, kidneys, and adrenal glands, by ligand binding and autoradiographic studies [9]. The gastric epithelium consists of several distinct cell populations that are essential to digestive processes. These cells include acid-secreting parietal cells, pepsinogen-producing chief cells and mucus-producing mucous neck cells. Parietal cells in the stomach are able to produce a substantial amount of DA [10] in addition to neuronal DA coming from the autonomic and enteric nervous systems [11]. A great majority of studies in vivo have reported that DA or dopaminergic compounds inhibit the secretion of gastric acid or pepsin [12], stimulate the secretion of mucus or bicarbonate [13,14], and regulate mucosal blood flow [15]. Recently, the IRs of DA receptors have been found in the enteric neurons of mouse ileum and mRNA expressions were detected in the gastric mucosa of rats, respectively [16]. However, very little information is available regarding the cellular localization of DA receptors in the gastric mucosa [17]. The aim of the present study was to investigate the

Abbreviations: DA, dopamine; IR, immunoreactivity; GI, gastrointestinal; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; real-time RT-PCR, real-time reverse-transcription polymerase chain reaction; SST, somatostatin; PGE2, prostaglandin E2; CP, control peptide; DAPI, 4′,6-diamidino-2-phenylindole.

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Table 1
Sequences of primers.

Primer	GenBank accession number	Primer sequence	Primer location in the sequence
β-Actin	NM031144	Forward: 5′-TTC AAC ACC CCA GCC ATG T-3′	460–478
		Reverse: 5′-GTG GTA CGA CCA GAG GCA TAC A-3′	506–527
D1	NM012546	Forward: 5′-GGA TGA CAA CTG TGA CAC AAG GTT G-3′	1297–1321
		Reverse: 5′-AAG CTG ATG AGG GAC GAT GAA-3′	1339–1359
D2	NM012547	Forward: 5′-CAC CAC GGC CTA CAT AGC AA-3′	1280–1299
		Reverse, 5′-GGC GTG CCC ATT CTT CTC T-3′	1321–1339
D3	NM017140	Forward: 5′-ACG GCA CCG GGC AGA GC-3′	500–516
		Reverse: 5′-GAG GGC AGG ACA CAG CAA AAG-3′	563–583
D4	NM012944	Forward: 5′-GGC GTG TGG CTG CTG AG-3′	410–426
		Reverse: 5′-TTG AAG ATG GAG GCG GTG C-3′	471–489
D5	NM012768	Forward: 5′-CCA CTG CCT CCA TCC TGA ATC-3′	365–385
		Reverse: 5′-GGC TAC TCG CTG GGT CAT CTT G-3′	444–465

Table 2
Primary antibodies used in the study.

Antigen	Immunizing antigen	Host species	Dilution	Source/catalog no.
D_1	Peptide SSHHEPRGSISKDC corresponding to amino acid residues 372–385 of rat D1 Dopamine receptor	Rabbit	1:200	Alomone/ADR-001
D_1	13 Amino acid peptide sequence (amino acids 403–415) from rat D1A	Rabbit	1:50	Chemicon/LV1486607
D_2	Synthetic peptide: AARRAQELE, corresponding to amino acids 272–282 of Human Dopamine D2 Receptor	Rabbit	1:1000	abcam/ab21218
D_2	Synthetic peptide conjugated to KLH derived from within residues 1–100 of Human Dopamine D2 Receptor	Goat	1:100	abcam/ab32349
D_3	Peptide CGAENSTGVNRARPH corresponding to amino acid residues 15–29 of rat D3 dopamine receptor	Rabbit	1:200	Alomone/ADR-003
D_4	D4 (Ac-176–185) covalently attached to a carrier protein	Rabbit	1:1000	abcam/ab20424
D_5	Peptide EEGWELEGRTENC corresponding to amino acid residues 199–211 of rat D5 dopamine receptor	Rabbit	1:200	Alomone/ADR-005
D_5	Recombinant rat dopamine receptor D5, carboxy terminus	Mouse	1:1000	abcam/ab78511
H^+–K^+-ATPase	Purified 34 kDa core peptide from deglycosylated hog gastric microsomes	Mouse	1:4000	abcam/ab2866
Pepsin C	Peptide mapping within an internal region of pepsin C of mouse origin	Goat	1:400	SANT CRUZ/sc-51188
Mucin	Full length native protein (purified) (Human)	Mouse	1:1000	Novus biologicals/NB120–11335

expression of DA receptors in the gastric mucosa and the cellular localization of DA receptors in the gastric epithelia by real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) and double-labeling immunofluorescence. This study may help us to better understand the mechanism underlying the protection of DA in the gastric mucosa.

2. Materials and methods

2.1. Animals and tissue preparation

Experimental protocols were approved by the Beijing Administration Office of Laboratory Animals, following the Administration Regulations on Laboratory Animals of Beijing Municipality. Adult male Sprague–Dawley rats (Laboratory Animal Services Center, Capital Medical University, Beijing, China) ranging in weight from 220 to 250 g were housed in an animal care facility at a temperature of 23 °C on a 12-h light/dark cycle and had free access to standard rodent laboratory food and water.

The rats were sacrificed by cervical dislocation. The stomach was removed and quickly immersed in ice-cold Kreb's solution. The stomach was split open along the greater curvature. The gastric corpus was reserved and washed free of luminal contents. Some gastric corpuses were cut into 5–7 pieces, fixed by immersion in ice-cold 4% paraformaldehyde in 0.1 M PBS, refrigerated overnight, and processed for paraffin sectioning by standard methods. Paraffin sections (4 μm) were mounted on glass slides and kept refrigerated until immunohistochemical processing. Other gastric corpuses were pinned mucosal side down in a Sylgard-lined Petri dish containing ice-cold oxygenated solution. The serosa, muscularis, and submucosa were stripped away with fine forceps to obtain the mucosa preparation (including mucosa and a small amount of adherent submucosal plexus and connective tissue) for RT-PCR.

2.2. RNA extraction and preparation of cDNA

Total RNA was extracted from the mucosal layer of gastric corpus using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The first strand cDNA was synthesized following the protocol of the Superscript first-strand synthesis system for RT-PCR (Invitrogen).

2.3. Real-time PCR

Real-time PCR was used to quantify mRNA encoding DA receptors in the samples from the rats. The expression of β-actin was used as an internal control for normalization. Pairs of oligonucleotide primers for amplification of the cDNA encoding DA receptors and β-actin were designed according to published cDNA sequences of rat (Table 1). Transcripts encoding DA receptors in samples of the rats were measured with the Brilliant SYBR Green QPCR Master Mix kit (Stratagene Company, La Jolla, CA, USA) using a Light Cycler instrument (Stratagene Company). Data were analyzed with computer assistance using the MxPro QPCR software (version 3.0, Mx3000P system, Stratagene Company). The relative expressions of DA receptor mRNA were analyzed using Pfaffl's method.

2.4. Immunofluorescence staining for confocal microscopy

Information on the primary antibodies used in this study is summarized in Table 2. Double labeling of DA receptors with H^+/K^+-ATPase, pepsin C or mucin 6 was used to identify the cell

Table 3
Secondary antibodies used in the study.

Secondary antibody	Conjugation	Company/catalog no.	Dilution
IHC			
Goat anti-rabbit IgG	DyLight™488	Zhongshan company/ZF-0411	1:400
Donkey anti-goat IgG	Texas red	Proteintech group/00005-3	1:100
Goat anti-mouse IgG	TRITC	Zhongshan company/ZF-0313	1:200
Donkey anti-rabbit IgG	FITC	Jackson/711–095–152	1:150
Donkey anti-goat IgG	TRITC	Jackson/705–025–147	1:150

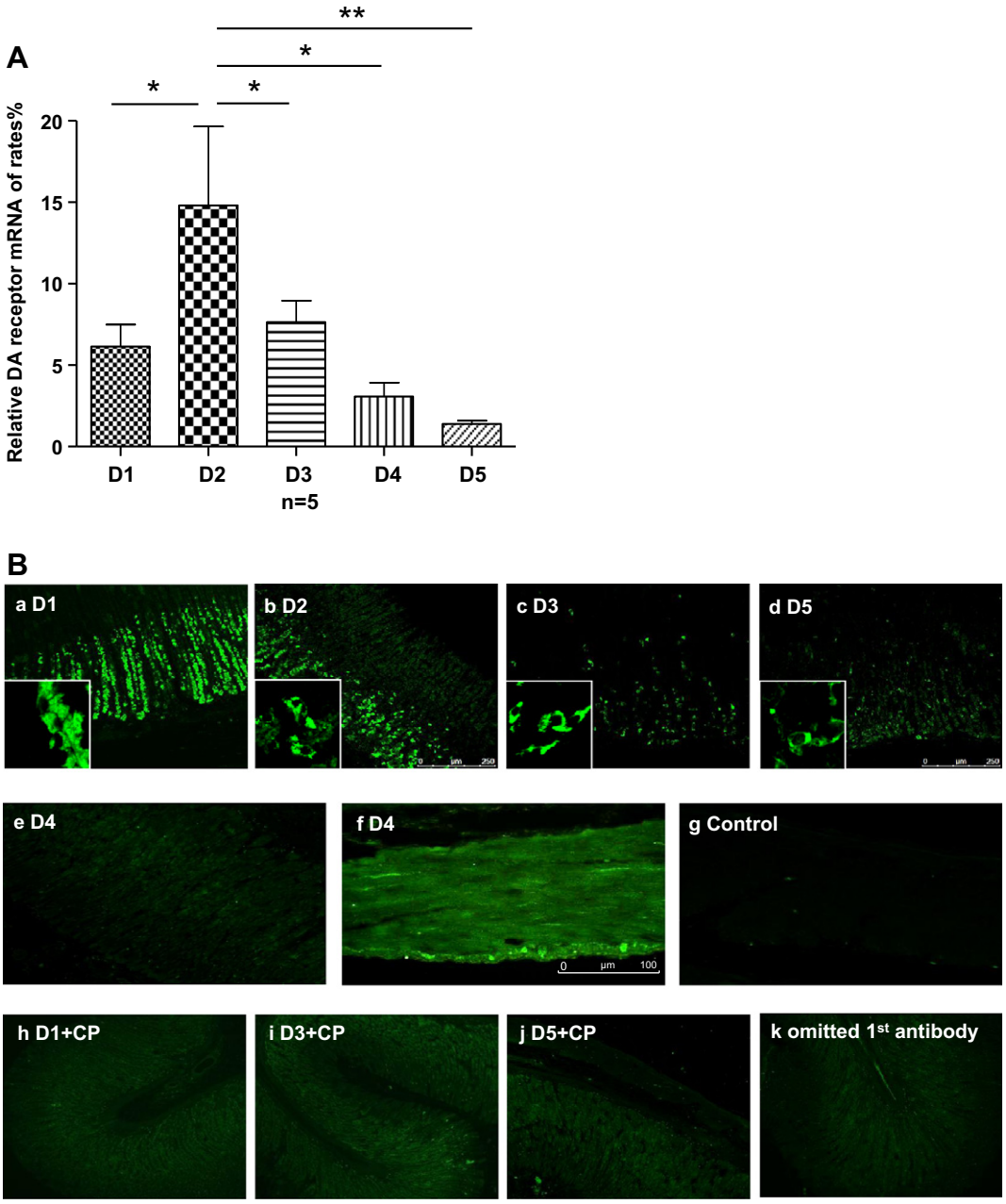

Fig. 1. Expression of 5 subtypes of DA receptors in gastric mucosa of rats. (A) The mRNA expression of the D2 receptor in gastric corpus mucosa was significantly higher than that of other DA receptor subtypes as analyzed by real time-PCR. β-Actin was used as an internal control. Data were presented as means (±SD). $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Rat gastric mucosa stained with antibody against D1–D5. D1, D2, D3, and D5 IR were expressed in the epithelia of the stomach (a–d). High-power magnifications are shown in the insets. D4 IR was not found in the gastric mucosa (e) but was only observed in the gastric enteric plexus (f). The primary antibodies to DA receptors were omitted, and no immunofluorescence was observed (g, k). Preabsorption of D1, D3, and D5 antibodies with their corresponding control peptides (CP) resulted in no immunostaining (h–j). Bars 250 μm (a–e, h–k); Bars 100 μm (f and g).

types that expressed DA receptors. For immunofluorescent staining, paraffin sections were first baked at 60 °C for 2 h and then deparaffinized in xylene 20 min. Then slides were rehydrated in descending concentrations of alcohol and transferred from 70%

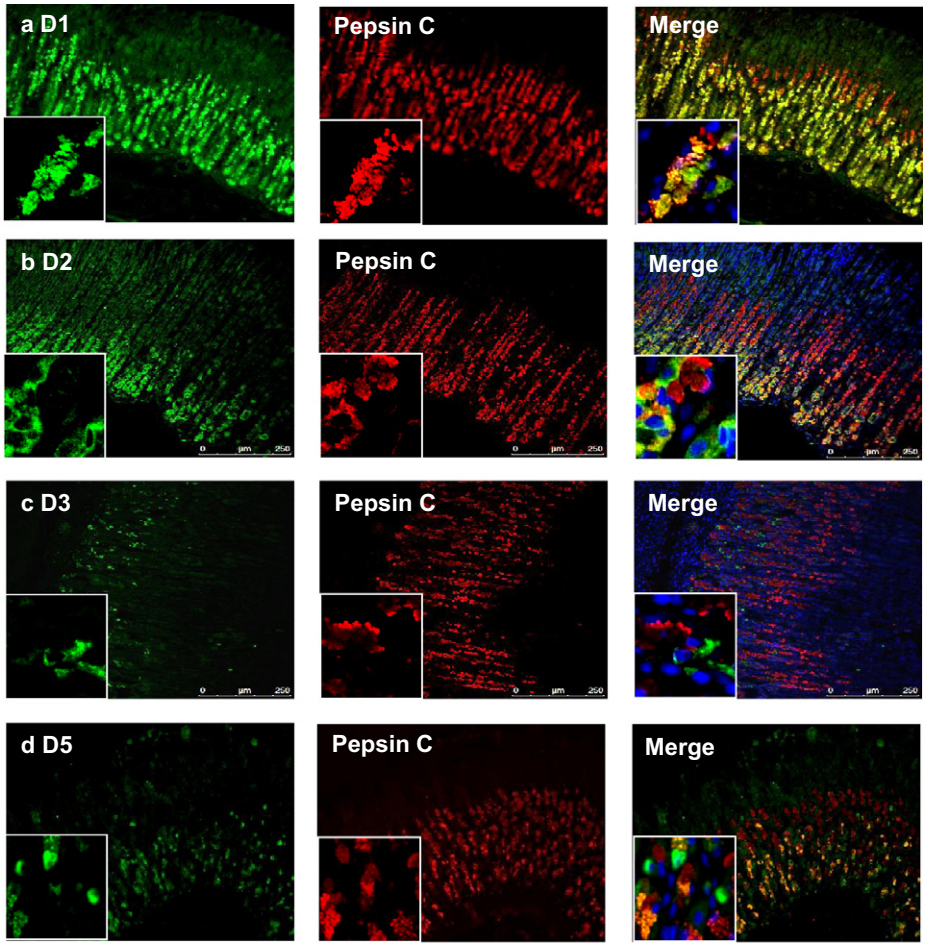

Fig. 2. Double-label immunofluorescence of DA receptors with pepsin C in rat gastric mucosa. Paraffin sections were double-labeled with DA receptor-specific antibodies and the chief cell marker pepsin C. Pepsin C-positive chief cells were localized to the deeper region of the gastric glands. D1, D2, and D5 IR were expressed in some pepsin C-positive cells in the gastric glands (a, b and d). No colocalization of D3 IR with pepsin C IR was found in gastric epithelial cells (c). Each figure is magnified underneath. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Bars 250 μm (a–d). (For interpretation of the references to color in this figure legend the reader is referred to the web version of this article.)

alcohol solution to distilled water for rinsing. Before staining, slides were pretreated with 10 mM citric acid (pH 6.0) at 92–98 °C for 10 min and incubated in 10% normal serum and 0.1% Triton X-100 for 30 min. The sections were then incubated with the primary antibody for the DA receptors, followed by the appropriate secondary antibody (Table 3). Finally, the sections were washed in PBS and mounted in 90% glycerin PBS. The analysis of double-label immunofluorescence was performed by Leica confocal microscopy and Leica processing software. Counts of double-labeled cells were assessed in three animals for each chemical marker. At least 10 sections in every animal were counted.

To ensure the specificity of the staining, two or three antibodies from different companies for each DA receptor were used. Preabsorption of DA receptor antibodies with their corresponding immunizing peptides was performed to determine if this would abolish the immunostaining. Immunostaining was also performed with omission of the primary antibody. Control experiments were conducted to test the cross-reactivity between antibody pairs in double labeling immunofluorescence. No cross-reactivity was found between these antibody pairs.

2.5. Statistical analysis

The values were presented as means ± SEM; *n* refers to the number of rats or the number of pairs. Statistical analyses were performed by one-way analysis of variance followed by the Newman–Keuls test or the Student's paired or unpaired *t*-test. Statistics and graphs were generated using GraphPad Prism version 4.0. *P*-values less than 0.05 were assumed to denote a significant difference.

3. Results

3.1. The mRNA expression of D1–D5 in gastric mucosa

The mRNA transcripts encoding all five DA receptor subtypes were found in the gastric mucosa (Fig. 1A). The relative mRNA quantity was normalized to β-actin in each preparation. The rank order for the expression of DA receptors was D2 > D3 > D1 > D4 > D5. The mRNA level of the D2 receptor was the highest among all five types of DA receptors ($P < 0.05$).

3.2. Distribution of D1–D5 in gastric mucosa

The IRs of all other DA receptors subtypes, except D4, were found in the gastric mucosa, especially in the basal gastric gland (Fig. 1Ba–e). D4 IR was only observed in the gastric enteric plexus (Fig. 1Bf). Furthermore, at higher magnification, DA receptors were expressed clearly at the cell membrane and sometimes in the cytoplasm. Granular DA receptor staining was not uniformly distributed within the cytoplasm. In all cases, no nuclear staining was observed. DA receptor-IR cells were diversiform; i.e., they

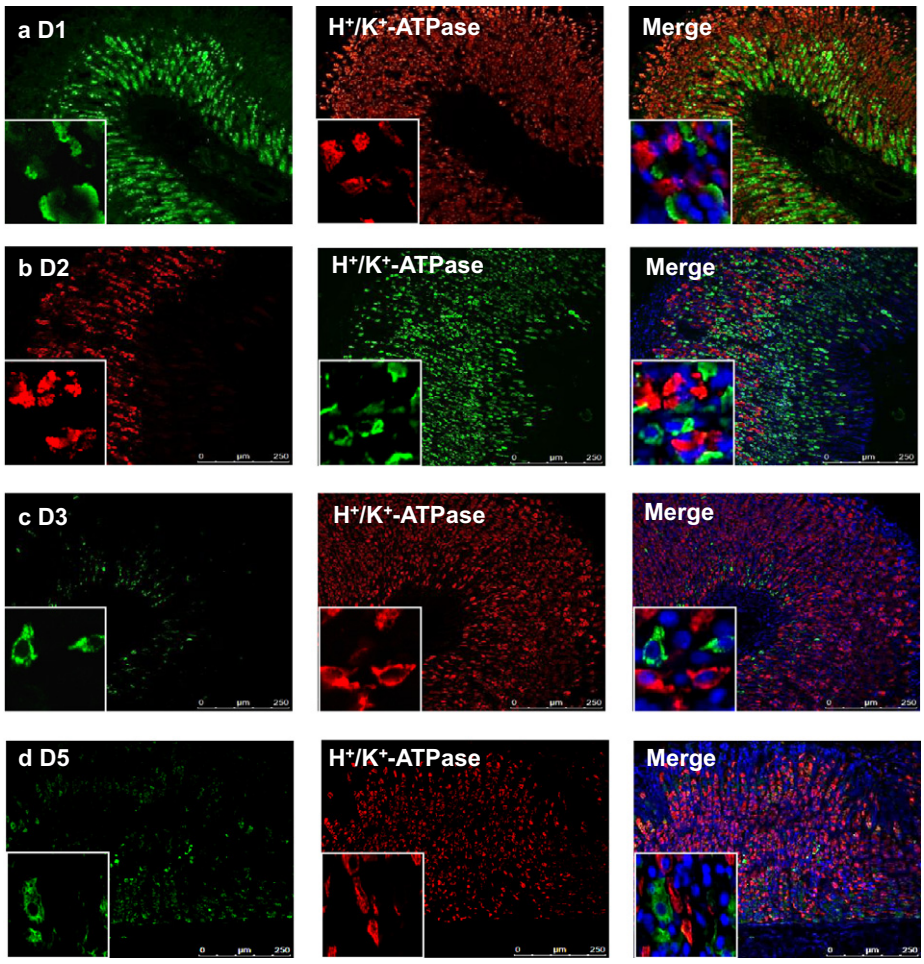

Fig. 3. Double-label immunofluorescence of DA receptors with H^+/K^+-ATPase in rat gastric mucosa. Paraffin sections double-labeled with DA receptor-specific antibodies and the parietal cell marker H^+/K^+-ATPase. No DA receptor subtype colocalized with H^+/K^+-ATPase in the gastric mucosa (a–d). Each figure is magnified underneath. Nuclei were stained with DAPI (blue). Bars 250 μm (a–d). (For interpretation of the references to color in this figure legend the reader is referred to the web version of this article.)

were oval, basiconic or spindle-shaped. D1 IR and D2 IR showed a prominent distribution in the gastric mucosa. D1 IR was more abundant at the basilar half of the gland than at the superficial region, with a very sharp transition (Fig. 1Ba). However, D2 IR was prominent at the extreme base of the gland (Fig. 1Bb). D3-IR cells were scatted in the entire gastric gland, and their immunostaining near the muscularis mucosae was the most intense (Fig. 1Bc). The total number of D3-IR cells in the gastric mucosa was much lower than the number of D1- or D2-IR cells. D5-IR cells were mostly distributed in the basilar one-third of the gland; the immunostaining was lighter, and the number was lower compared with D1- and D2-IR cells (Fig. 1Bd). A similar labeling pattern was obtained with another antibody against DA receptor (data not shown). When the primary antibodies to D1–D5 were omitted, no immunofluorescence was observed (Fig. 1Bg and k). Similar results were also achieved by preabsorption of D1, D3, and D5 antibodies with their corresponding immunizing peptides (Fig. 1Bh–j).

3.3. Cellular localization of DA receptors in gastric mucosa

For a more accurate cellular localization of D1, D2, D3, and D5 in the gastric mucosa, double-labeling immunofluorescence for three cell lineage markers, pepsin C (chief cells), H^+/K^+-ATPase (parietal cells) and mucin 6 (mucous neck cells), were performed.

3.3.1. Localization of DA receptors in chief cells

As expected, strong pepsin C IR was mainly found in the deeper region of the gastric glands and showed up as diffuse granular cytoplasmic staining pattern. D1, D2, and D5 IR were found in the pepsin C-IR chief cells. However, the expression of DA receptors did not fully colocalize with pepsin C. Immunostaining of DA receptors was observed throughout the membrane and cytoplasm, whereas pepsin C IR was restricted to the perinuclear area of the cytoplasm. A majority of the D1-IR cells (84%; $n = 3$) also expressed pepsin C IR. Conversely, approximately 66% ($n = 3$) of pepsin C-IR cells were D1 IR (Fig. 2a). Approximately 40% ($n = 3$) of pepsin C-IR cells manifested D2 IR. As shown from higher magnification, a small amount of D2-IR cells (23%; $n = 3$) were pepsin C-negative (Fig. 2b). Approximately 20% ($n = 3$) of pepsin C-positive cells expressed D5 IR, which was 50% ($n = 3$) of the total number of D5-IR cells (Fig. 2d). No colocalization of D3 IR with pepsin C IR was found in gastric epithelial cells (Fig. 2c).

3.3.2. Localization of DA receptors in parietal cells

Parietal cells are another major cell type in the gastric gland. To investigate the distribution of DA receptors in parietal cells, gastric mucosa was co-stained with anti-DA receptor and anti-H^+/K^+-ATPase antibodies. As shown in Fig. 3, H^+/K^+-ATPase-IR cells were distributed along most of the length of the gastric gland. None of H^+/K^+-ATPase-positive cells manifested D1, D2, D3 or D5 IR (Fig. 3a–d).

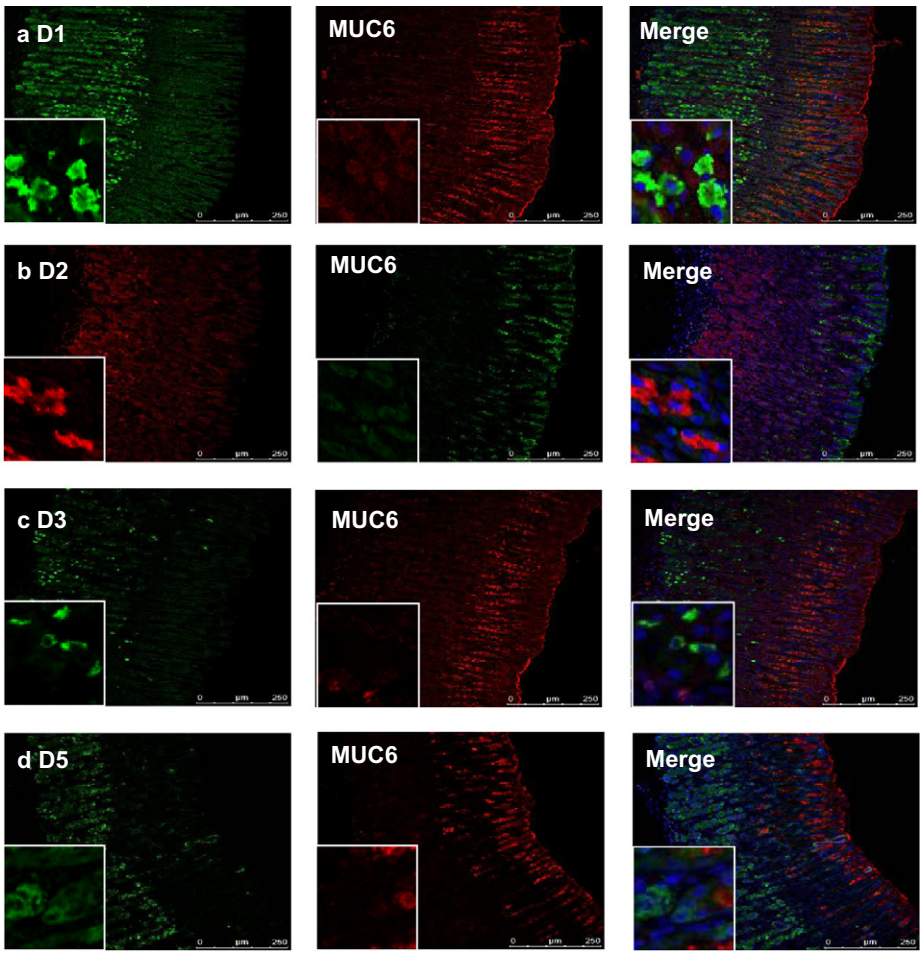

Fig. 4. Double-label immunofluorescence of DA receptors with mucin 6 in rat gastric mucosa. Paraffin sections double-labeled with DA receptor-specific antibodies and the mucous neck cell marker mucin 6. Mucin 6-positive cells were localized to the superficial one-third of the gastric gland. No DA receptor subtype colocalized with mucin 6 in the gastric mucosa (a–d). Each figure is magnified underneath. Nuclei were stained with DAPI (blue). Bars 250 μm (a–d). (For interpretation of the references to color in this figure legend the reader is referred to the web version of this article.)

3.3.3. Localization of DA receptors in mucous neck cells

To investigate the colocalization of DA receptors with mucous neck cells in the gastric gland, a well-established marker for mucous neck cells, mucin 6, was used. As expected, mucin 6 heavily stained the neck region of the gastric glands. Similar to the results from parietal cells, no double labeled-cells of mucin 6 IR and D1, D2, D3 or D5 IR were detected in the gastric mucosa (Fig. 4a–d). Being different from the distribution of DA receptors, the IR of mucin 6 was mainly located at the superficial, not basilar, region of the gastric gland.

4. Discussion

The present study demonstrates, for the first time, the distribution of DA receptors D1, D2, D3, and D5 in the gastric mucosa of rats; only pepsin C-positive chief cells manifest D1, D2, and D5 IR, and no subtype of DR was detected in the H^+/K^+-ATPase-positive parietal cells or mucin 6-positive mucous neck cells.

According to the in vivo functional studies, DA and DA receptors are involved in the regulation of pepsin output [18]. Activating D1 with fenoldopam and inhibiting D2 with sulpiride can significantly decrease pepsin output in the gastric juice and lessen gastric mucosal injury in the restraint plus water-immersion stress or pylorus-ligated models. Conversely, inhibiting D1 with SCH 39166 and activating D2 with quipirole led to a significant increase in pepsin secretion. A similar result has been reported by Glavin and Hall [19]. However, the functional studies lack the support of morphologic data. Our present finding, the localization of D1, D2, and D5 in the chief cells, provides morphological evidence for the regulation of pepsin secretion by DA, acting through D1, D2, and D5 in the chief cells. The specific regulatory mechanisms need to be further investigated.

In the present study, no DA receptor IR was detected in the H^+/K^+-ATPase- or mucin 6-IR cells, thus indicating that the parietal cells and mucous neck cells may not express any subtype of DA receptor. However, a large number of studies in vivo have reported the inhibitory effect of DA on gastric acid secretion [20] and the stimulatory effect on mucus and bicarbonate secretion. In our understanding of the effects of DA on gastric acid, mucus and bicarbonate secretion may not be a direct consequence of DA on the parietal cells and/or mucous neck cells. It has been reported that DA is able to promote the release of somatostatin (SST) and prostaglandin E2 (PGE2) [21], both of which are recognized to inhibit gastric acid secretion [22,23]. Whether the stimulatory effect of DA on the release of SST and PGE2 is directly mediated by DA receptors needs to be further investigated.

Mezey et al. [24] have reported the expressions of D1–D5 mRNA in the gastric mucosa of rats with the highest expression of D5 based on the in-situ hybridization histochemistry technique. A high expression of D2 mRNA in the enteric nerve plexus, but not in the gastric mucosa, has been reported in [16] using the

RT-PCR technique. In the present study, the mRNA transcripts encoding all of five subtypes of DA receptors, from D1 to D5, have been detected in the gastric mucosa, among which the level of the D2 mRNA is the highest. This different result may be due to the varying detection or tissue preparation methods. In our study, real-time PCR and strips of gastric corpus mucosa with a small amount of remnant submucosal tissue were employed, while in the study of Mezey, the results were based on the in-situ hybridization histochemistry technique and did not distinguish between the different parts of the rat stomach.

We speculate that DA is synthesized in the parietal cells. It may act on D1, D2 and/or D5 receptors in the chief cells in a paracrine manner to regulate pepsin secretion. As these receptor subtypes belong to two families, the direct regulatory role of DA on pepsin release is bidirectional. DA may not regulate gastric acid in an autocrine manner because no DA receptor was detected in the parietal cells. In other words, the regulation of gastric acid secretion by DA might not occur directly through the parietal cells; DA might indirectly inhibit gastric acid secretion by regulating the release of hormones from other endocrine cells. D3 and some D5 receptors were scattered in the cells (most likely endocrine cells) of the gastric corpus mucosa. They could be related to the regulation of gastric acid, mucus–bicarbonate secretion, mucosal blood flow and/or the function of mobile immune cells in the GI tract. D1/D5 receptor agonists have been shown to increase bicarbonate secretion in both rats and humans [25,26].

In conclusion, the present study demonstrates, for the first time, the distribution of D1, D2, and D5 in the majority of chief cells. No DA receptor subtype is present in parietal or mucous neck cells. This study provides important morphological evidence to better understand the possible role of DA on gastric mucosa protection. Functional studies should be undertaken to investigate the mechanisms underlying the DA-regulated release of pepsin, gastric acid and mucus and, therefore, the protection of the gastric mucosa.

Acknowledgments

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