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Immunohistochemical Localization of Endocrine Cells in the Alimentary Tracts of Six Frog Species

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A peroxidase-antiperoxidase method was used to detect the cells showing immunoreactivities to six hormone antibodies in the alimentary tracts of six frog species, *Rana nigromaculata, R. rugosa, R. amurensis coreana, R. catesbeiana, Bombina orientalis*, and *Hyla arborea japonica*, inhabiting Korea. The cells immunoreactive to gastrin and cholecystokinin-8 were observed in the pylorus of the stomachs and in the small intestines of all frog species examined. In contrast, these somatostatin-immunoreactive cells were identified in the esophagus and the whole gastrointestinal tracts, but were absent from the large intestines in *R. rugosa, R. catesbeiana, B. orientalis*, and *H. arborea japonica*. The pancreatic polypeptide (PP)-immunoreactive cells represented their distribution limited to the small intestines of *R. amurensis coreana* and *H. arborea japonica*, and they were additionally identified in the pylorus of the stomachs in the other four species. Serotonin- and glucagon- immunoreactive cells revealed different regional distributions in which the former were observed throughout the whole alimentary tracts in all frog species investigated, whereas the latter were not found in these regions at all. Endocrine cells were relatively abundant in the pyloric portion of the stomach compared to other organs. The present study showed that all endocrine cells except for PP had a similar distribution in the alimentary tracts of all frog species used.

Various endocrine cells have been identified in the mucosa epithelium of the alimentary tract in vertebrates, which secrete regulatory peptides or amines to stimulate digestive activity in the autonomic nervous system as well as motility of the alimentary tract (Henderson and Henderson, 1995). From lower vertebrates to mammals, various types of enteroendocrine cells are observed, with different ultrastructure and distribution between species (Holmgren et al., 1982; El-Salhy, 1984). Thus, immunohistochemical investigation on these cells is considered to be an important part of a phylogenic study (El-Salhy and Grimelius, 1981; Rajjo et al., 1988; Arena et al., 1990; Agungpiryono et al., 1994; D'Este et al., 1994).

Although many researches have been conducted on the distribution of endocrine cells in the alimentary tract of various vertebrates including frogs, understanding the overall features of these cells along the whole alimentary tract of frog species still remains poor. To date, most research on amphibian enteroendocrine cells have elucidate the function of the ultrastructure of these cells. Several immunohistochemical studies also have been carried out for these cells, but limited to specific organs which provide researchers with fragmentary information (Rajjo et al., 1988; Valverde et al., 1993; Trandaburu and Nürnberger, 1995; Bodegas et al., 1997). The present study aims to characterize these cells on the whole alimentary tracts of six frog species inhabiting Korea, some of which have never been studied for this purpose.

Materials and Methods

Six frog species used for this study were: *Rana nigromaculata, Rana rugosa, Rana amurensis coreana, Rana catesbeiana, Bombina orientalis*, and *Hyla arborea japonica*, which were collected in Kyungnam Province, South Korea. For quantitative immunohistochemical studies, the esophagus, stomach, small intestine, and large intestine were dissected out, fixed in Bouin's solution for 24 h, and embedded in paraffin. Serial sections of 5 μm were obtained.

After deparaffinizing, the sections were applied for 30 min by 0.25% methanolic hydrogen peroxide to remove peroxidase which remained in tissues, followed by washing with PBS (phosphate buffered saline, 0.01 M at pH 7.4). To prevent non-specific immunoreaction, tissues were treated with 3.3% normal serum albumin at room temperature for 30 min and then stained using the peroxidase-antiperoxidase technique (Sternberger,

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Table 1. The primary antisera and their working dilutions used

Antisera	Working dilution	Code no	Sources
Somatostatin	1:300	A566	DAKO Corp.
Gastrin	1:200	A568	DAKO Corp.
Cholecystokinin-8	1:500	20078	Incstar Corp.
Pancreatic polypeptide	1:600	A619	DAKO Corp.
Serotonin	1:500	20080	Incstar Corp.
Glucagon	1:300	A565	DAKO Corp.

*Raised in rabbit.

1979).

These sections were treated with 6 types of primary antisera diluted (Table 1) at 4℃ in the moisture chamber for 24 h and washed by PBS. These sections were then incubated in the secondary antisera, horseradish peroxidase conjugated anti-rabbit IgG (DAKO, P448), diluted to 1:200 for 60 min followed by washing with phosphate buffer (0.01 M, pH 7.4). Then sections were incubated in Tris buffer (0.05 M, pH 7.6) mixed with 3,3′-diaminobenzidine tetrahydrochloride and 0.003% H_2O_2 and were counterstained using Mayer's hematoxylin. For the controls, treatment with primary and secondary antibodies were omitted. The serial stained sections were observed under the light microscope to identify the localization and distribution of endocrine cells.

Results

The results of the present study on endocrine cells in the alimentary tract of six frog species are outlined in Tables 2-6.

Cells immunoreactive to somatostatin were identified in the esophagus and the whole gastrointestinal tracts of all frog species examined, but were absent from the large intestines in *R. rugosa, R. catesbeiana, B. orientalis,* and *H. arborea japonica* (Fig. 1).

Cells immunoreactive to gastrin and cholecystokinin-8 (CCK-8) represented a similar pattern of distribution in both the pylorus and small intestine. All species except *R. nigromaculata* represented the distribution of these cells in the pylorus and anterior part of the small intestine. On the other hand in the *R. nigromaculata,* these cells were distributed in the pylorus and throughout the small intestine (Figs. 2 and 3).

Table 2. The frequency and distribution of somatostatin-immunoreactive cells in various parts of the alimentary tract of the frog

Species	ES	CS	BS	PS	PSI	MSI	DSI	LI
Rana nigromaculata	++	++	++	++	++	+	+	+
R. rugosa	+	+	+	+++	++	+	+	-
R. amurensis coreana	+	+	+	+++	+	+	+	+
R. catesbeiana	++	++	+	+++	++	+	+	-
Bombina orientalis	+	+	+	++	+	+	+	-
Hyla arborea japonica	+	+	+	++	+	+	+	-

ES, esophagus; CS, cardiac part of the stomach; BS, body of the stomach; PS, pyloric part of the stomach; PSI, proximal part of the small intestine; MSI, middle part of the small intestine; DSI, distal part of the small intestine; LI, large intestine; -, not observed; +, few; ++, moderate; +++, numerous.

Table 3. The frequency and distribution of gastrin-immunoreactive cells in various parts of the alimentary tract of the frog

Species	ES	CS	BS	PS	PSI	MSI	DSI	LI
Rana nigromaculata	-	-	-	++++	+	++	++	-
R. rugosa	-	-	-	++++	+	+	-	-
R. amurensis coreana	-	-	-	+++	+	+	-	-
R. catesbeiana	-	-	-	++++	+	-	-	-
Bombina orientalis	-	-	-	+++	+	+	-	-
Hyla arborea japonica	-	-	-	+++	+	+	-	-

Abbreviations are given in Table 2. ++++, very numerous.

Table 4. The frequency and distribution of cholecystokinin-8-immunoreactive cells in various parts of the alimentary tract of the frog

Species	ES	CS	BS	PS	PSI	MSI	DSI	LI
Rana nigromaculata	-	-	-	+++	+	+	+	-
R. rugosa	-	-	-	+++	+	+	-	-
R. amurensis coreana	-	-	-	++	+	-	-	-
R. catesbeiana	-	-	-	+++	+	-	-	-
Bombina orientalis	-	-	-	++	+	+	-	-
Hyla arborea japonica	-	-	-	++	+	+	-	-

Abbreviations are given in Table 2.

Unlike other endocrine cells, pancreatic polypeptide (PP)-immunoreactive cells revealed different regional distribution between species. That is, in *R. nigromaculata* and *B. orientalis,* PP-immunoreactive cells were identified in the pylorus and whole part of the small

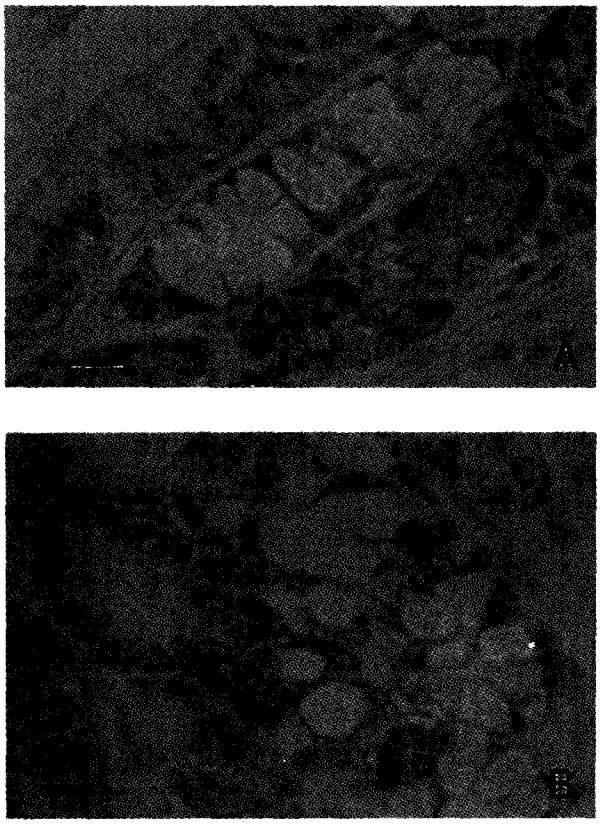

Fig. 1. Somatostatin-immunoreactive cells in the esophagus (A) and pyloric region of the stomach (B) of *R. rugosa.* Scale bars=30 μm.

Table 5. The frequency and distribution of pancreatic polypeptides-immunoreactive cells in various parts of the alimentary tract of the frog

Species	ES	CS	BS	PS	PSI	MSI	DSI	LI
Rana nigromaculata	-	-	-	++	+	+	+	-
R. rugosa	-	-	-	+	+	-	-	-
R. amurensis coreana	-	-	-	-	+	-	-	-
R. catesbeiana	-	-	-	++	+	-	-	-
Bombina orientalis	-	-	-	++	+	+	+	-
Hyla arborea japonica	-	-	-	-	+	+	+	-

Abbreviations are given in Table 2.

Table 6. The frequency and distribution of serotonin-immunoreactive cells in various parts of the alimentary tract of the frog

Species	ES	CS	BS	PS	PSI	MSI	DSI	LI
Rana nigromaculata	+	+	++	++++	+++	++	++	++
R. rugosa	++	++	++	+++	++	++	++	++
R. amurensis coreana	+	+	++	+++	++	++	++	++
R. catesbeiana	+	++	++	+++	+++	+	++	++
Bombina orientalis	++	++	++	++++	++	++	++	++
Hyla arborea japonica	+	+	++	+++	+++	++	++	++

Abbreviations are given in Table 2.

intestine, whereas *R. rugosa* and *R. catesbeiana* found in the pylorus and proximal part of the small intestine, *R. amurensis coreana* in the proximal part of the small intestine, and *H. arborea japonica* all over the small intestine (Fig. 4). While cells immunoreactive to serotonin were observed in the esophagus and whole gastrointestinal tract, those to glucagon were not found in these regions at all (Fig. 5). Immunoreactive cells were relatively abundant in the pyloric part of the stomach compared to other organs.

It is notable that each type of endocrine cells have a different distribution pattern within the same organ. In the esophagus, somatostatin-immunoreactive cells were abundantly found within esophageal glands, whereas serotonin-immunoreactive cells were found in the surface epithelium. In the stomach, immunoreactive cells were mainly distributed in the glands, especially in the upper portion of fundic and pyloric glands, and in the intestine, immunoreactive cells were detected over the intestinal epithelium.

Discussion

Our results on somatostatin-immunoreactive cells showing their distribution throughout all alimentary tracts except the large intestine were consistent with the previous results from *Xenopus laevis* by Hacker et al. (1983), *R. temporaria* by Valverde et al. (1993), *R. esculenta* by Trandaburu and Nürnberger (1995), and *Bufo regularis* by El-Salhy et al. (1981).

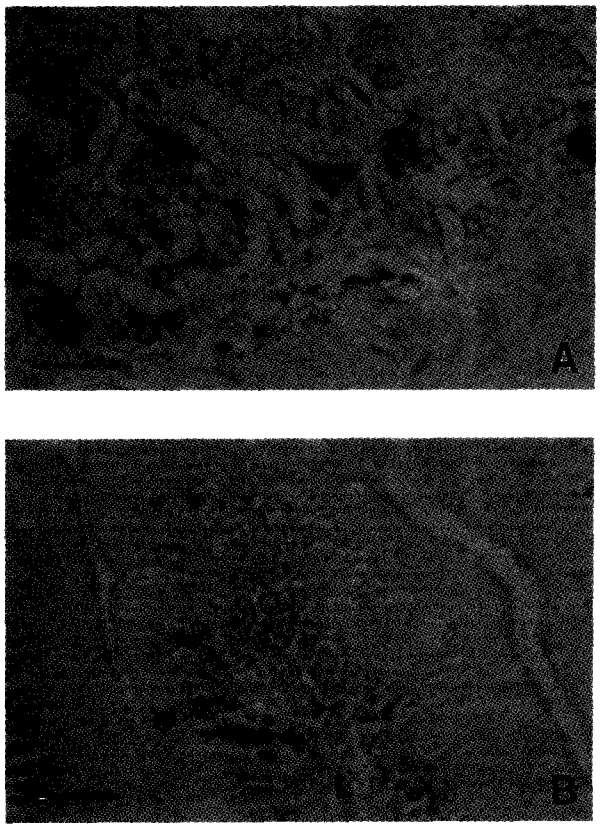

Fig. 2. Gastrin-immunoreactive cells in the pyloric region of the stomach (A) and middle region of the small intestine (B) of *B. orientalis*. Scale bars=30 μm.

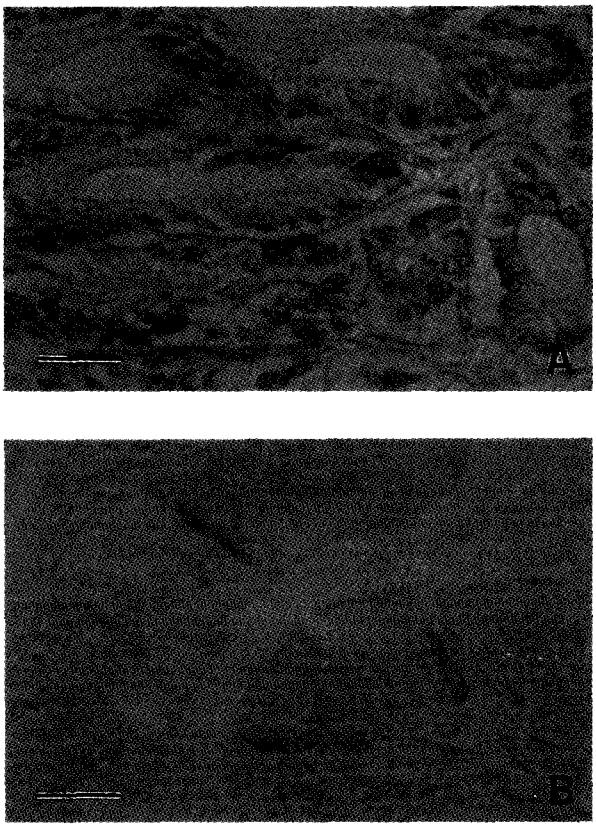

Fig. 3. CCK-8-immunoreactive cells in the pyloric region of the stomach (A) and distal region of the small intestine (B) of *R. nigromaculata*. Scale bars=30 μm.

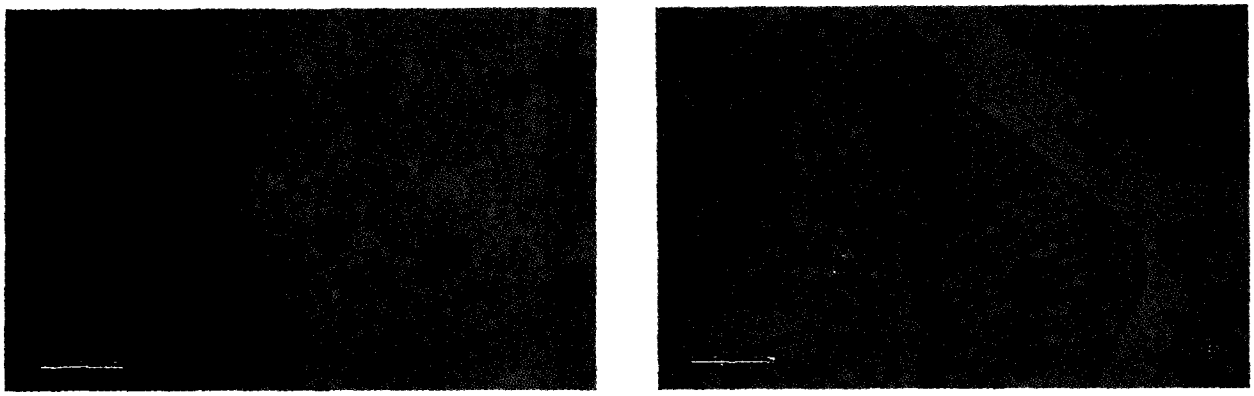

Fig. 4. PP-immunoreactive cells in the pyloric region of the stomach (A) and middle region of the small intestine (B) of *R. nigromaculata*. Scale bars= 30 μm.

Buchan (1986) also identified somatostatin-immunoreactive cells in the stomach and small intestine of *B. bombina*, *Alytes obstetricans*, and *Atelopus oxyrhynchus* and in the stomach and whole intestinal tracts of *R. temporaria*, *Bufo bufo*, and *Acris crepitans*, but indicated that they were observed only in the stomach and small intestine of *R. esculenta* and *H. arborea*, respectively.

Amphibian somatostatin-immunoreactive cells appeared to have a similar distribution pattern as shown in other vertebrates from fishes (Noaillac-Depeyre and Holland, 1981; Groff and Youson, 1997) to mammals (Kitamura et al., 1984, 1985, 1990; Krause et al., 1985; Agungpiryono et al., 1994), which showed a dense distribution of them in the stomach and the proximal part of the small intestine and loose distribution in the lower part of the alimentary tracts.

As for gastrin-immunoreactive cells, research with other amphibian species (Gibson et al., 1976; El-Salhy et al., 1981) showed results similar to ours. As shown in somatostatin, the distribution pattern of these cells seemed to be very conservative throughout the whole taxa of animals (El-Salhy, 1984; Krause et al., 1985; Hashimoto et al., 1993).

Research on CCK-immunoreactive cells have mainly focused on CCK-33 known as pancreozymin in the alimentary tracts. In amphibian research, the cells were identified in the stomach as well as in the duodenum of *R. catesbeiana* (Rajjo et al., 1988) and CCK-8 were especially observed in the intestine of larvae of the *R. temporaria* (Bodegas et al., 1997).

The presence of CCK-immunoreactivity in the digestive tracts is common among vertebrates, but toward the higher vertebrates it was detected in the stomach or proximal part of the intestine, which is related to the compact pancreas (Rajjo et al., 1988). It can be considered that both gastrin and CCK-8 originated from the same ancestor since they show similar C-terminal structures, which were detected through immunoreactivity using the gastrin/CCK antiserum recognizing both gastrin and CCK-8 responded gastrin/CCK family (Larsson and Rehfeld, 1977).

This combined type of endocrine cells was reported to exist in the stomach, and small intestines of *B. bombina* and *A. obstetricans*, the intestinal tracts of *R. temporalis*, and the antrum of *R. esculenta* (Buchan, 1986). The distribution of gastrin/CCK family in other vertebrates revealed that the C-terminal antiserum was observed in the stomach and intestines. However,

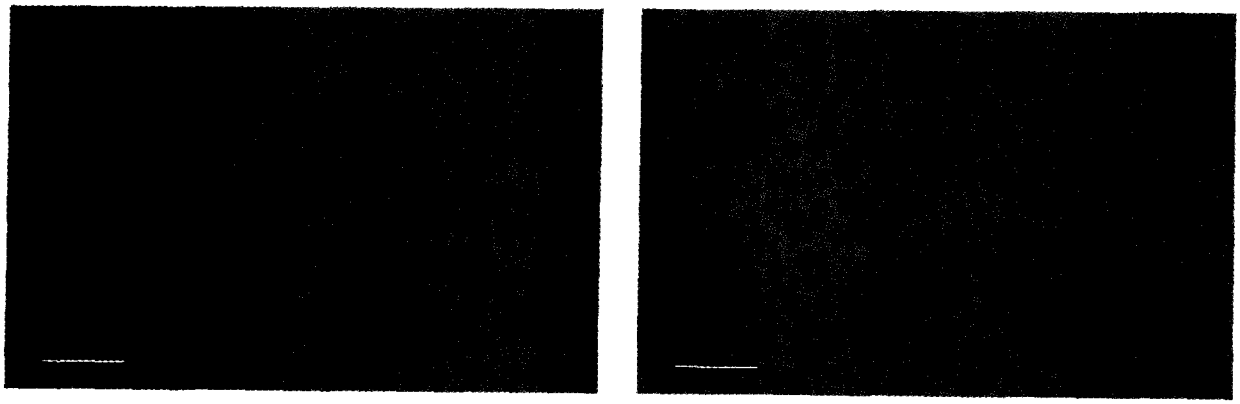

Fig. 5. Serotonin-immunoreactive cells in the esophagus (A) and pyloric region of the stomach (B) of *R. catesbeiana*. Scale bars=30 μm.

Buchan (1986) reported that the N-terminal 17 gastrin antibody or antiserum to CCK9-20 in two anuran and eight anomuran species were not immunoreactive. Considering that the gastrin antiserum used in this study also responded to CCK-8 simultaneously, more specific antiserum to gastrin or CCK should be available for better results.

The distribution of PP-immunoreactive cells, called pancreatic endocrine cells varied by species as known in previous research (El-Salhy et al., 1981; Valverde et al., 1993). Buchan (1986), however, presented different results from two anurans and eight anomuran species, which showed no PP reactivity. In other vertebrates, these cells were in general detected either in the stomach or in the small intestine or both (Krause et al., 1985; Arena et al., 1990; Agungpiryono et al., 1994; Groff and Youson, 1997).

Among the endocrine cells tested in this study, serotonin-immunoreactive cells were observed as the most widely distributed among whole alimentary tracts of the six frog species. This would be true for all other vertebrates from fish to mammals (El-Salhy, 1984; Krause et al., 1985; Arena et al., 1990; Agungpiryono et al., 1994). Especially those reported in the esophagus of King's skink (Arena et al., 1990) and *R. catesbeiana* (Nada et al., 1984) were also found in all frog species used in the present study. The function of these cells is not known but regarded as a new cell type which are related to paraneuron or the amine precursor uptake and decarboxylation (APUD) system.

In conclusion, this result clearly demonstrated that the distribution of endocrine cells except for PP was similar among the six frog species examined. To obtain a clearer understanding of the distribution pattern of these cells in the alimentary tract of the frog species, further research is needed in relation to the function of each cell type to the respective organ. Especially, high priority should be given to serotonin- and somatostatin-immunoreactive cells which show their presence in the esophagus.

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