The Relative Frequency and Topographical Distribution of Somatostatin-, GRP-, APP-, Glucagon-, 5-HT-, and Neurotensin-Immunoreactive Cells in the Proventriculus of Seven Species of Birds*

Junzo Yamada, Nobuo Kitamura and Tadayuki Yamashita

Department of Veterinary Anatomy (Prof. T. Yamashita), Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan

Received April 22, 1985

Summary. The relative frequency and topographical distribution of proventricular endocrine cells were examined immunohistochemically in seven species of birds: common finch, pigeon, quail, chicken, duck, gull and kite. Gastrin releasing peptide (GRP), somatostatin-, avian pancreatic polypeptide (APP)-, glucagon-, 5-hydroxytryptamine (5-HT)- and neurotensin-immunoreactive cells were observed in this study. GRP- and somatostatin-immunoreactive cells were found in all species examined. All six kinds of immunoreactive cells were found with varying frequency in the pigeon, quail and gull, but not all immunoreactives were found in the other species examined. Species differences with regard to the relative frequency and topographical distribution of proventricular endocrine cells were observed.

The avian stomach consists of two anatomically distinct parts: the proventriculus or glandular stomach; and the gizzard or muscular stomach. The proventriculus secretes digestive enzymes and gastric acid, whereas the gizzard converts food into chyme by mechanical means. It is well established that the morphology of these two stomachs shows wide variation according to the species and its feeding habits (Pernkopf and Lehner, 1937). Generally, the proventriculus is best developed in carnivorous birds, whereas the gizzard is best developed in seed and grain eating birds, (Kent, 1983).

Several studies have examined the endocrine cells in the proventriculi histologically (Dawson and Moyer, 1948; Aiken, 1958; Toner, 1964; Gabe, 1972; Okamoto et al., 1976; Iwanaga and Yamada, 1980), ultrastructurally (Kataoka, 1974, Andrew, 1976; Iwanaga and Yamada, 1980; Okamoto and Yamada, 1981), and immunohistochemically (Polak et al., 1974; Alumets et al., 1977; 1978a; Vaillant et al., 1979). These studies suggested that the endocrine cells of the proventriculus consist of argyrophil cells only and that these can be subdivided into at least four types by their ultrastructural profiles. Recently, five kinds of immunoreactive cells: gastrin releasing peptide (GRP or bombesin)-, somatostatin-, avian pancreatic polypeptide (APP)- and glucagon-immunoreactive cells, were identified in this region. Most of the immunohistochemical studies have dealt with either a single peptide or with a single avian species.

*Supported by a Research Grant-in-Aid from the Ministry of Education, Japan (Grant No. 58560282).
Our previous study demonstrated that the distribution and frequency of proventricular endocrine cells varied in the five species of birds examined (YAMADA et al., 1979). The present study examines the proventriculus of seven avian species using immunohistochemical techniques.

MATERIALS AND METHODS

Three to five birds from seven avian species: the common finch (Uroloncha striata var domestica); pigeon (Columba libia var domestica); Japanese quail (Coturnix coturnix japonica); chicken (Gallus gallus var domestica); white Beijing duck (Anas platyrhynchos platyrhynchos var domestica); black-tailed gull (Larus crassirostris); and black-eared kite (Milvus migrans); were examined in this study. Tissues from the proventriculus were dissected free and fixed in Bouin's fluid, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Serial sections were cut at 2 or 4 μm in thickness and stained immunohistochemically using the peroxidase-antiperoxidase (PAP) method (STERNBERGER, 1979) and the bridge method (MAson et al., 1969). Details of the antisera used are shown in Table 1. The specificity of each immunohistochemical reaction was determined as recommended by STERNBERGER (1979), and included replacement of the specific antiserum with the antiserum preincubated with the corresponding antigen and related peptides, and incubation with some fragments of these peptides. After immunohistochemical staining, the sections were lightly counterstained with Mayer’s hematoxylin, dehydrated, cleared in xylene and mounted. The relative frequency of immunoreactive cells in each species was graded subjectively into five groups according to their frequencies as seen under the microscope.

RESULTS

Cells immunoreactive to antisera against GRP, somatostatin, APP, glucagon, 5-HT and neurotensin were demonstrated in the avian proventriculi. Each kind of immunoreactive cell never showed any other immunoreactivities against the antisera listed in the Table 1, although a coexistence of the peptide and amine or existence of two kinds of polypeptides was carefully examined in the serial sections. The relative frequency, topographical distribution and kinds of immunoreactive cell varied among the species examined as shown in Table 2. Generally, immunoreactive cells occurred more frequently in the proventricular glands than in the superficial epithelium.

Superficial epithelium: The superficial epithelium that lines the proventricular lumen consists of columnar cells which tend to decrease in height towards the base of formed sulci. Immunoreactive cells were observed primarily in the basal portions of the folds and sulci. A total of six kinds of immunoreactive cells were observed in this region, although not all types were detected in each of the avian species examined (Table 2, Fig. 1). In fact, no single species had all six kinds of immunoreactive cells in this region of the proventriculus (Table 2). The immunoreactive cells observed were found more frequently in the quail than in the other species examined. GRP-, somatostatin- and 5-HT-immunoreactive cells were the most frequently observed endocrine cells in this region, while other immunoreactive cells were observed only occasionally. Many immunoreactive cells in the surface epithelium were oval in shape and covered by epi-
Table 1. Antisera used

<table>
<thead>
<tr>
<th>Antisera raised to</th>
<th>Code</th>
<th>Specificity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-hydroxytryptamine</td>
<td>Lot. 16302</td>
<td>—</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Synthetic substance P</td>
<td>R-2404</td>
<td>No cross reaction with gastric releasing polypeptide</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Synthetic human cyclic somatostatin</td>
<td>—</td>
<td>—</td>
<td>1:3,000</td>
</tr>
<tr>
<td>Synthetic porcine glucagon</td>
<td>GL-5</td>
<td>Reacts with pancreatic glucagon</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Avian pancreatic polypeptide</td>
<td>Lance-10/5/81 Bleed</td>
<td>No cross reaction with glucagon</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Synthetic human gastrin</td>
<td>GP-1304</td>
<td>No cross reaction with cholecystokinin-8</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Leucine-enkephalin</td>
<td>I671</td>
<td>—</td>
<td>1:80,000</td>
</tr>
<tr>
<td>Gastric inhibitory polypeptide</td>
<td>G/R/34-IIID</td>
<td>No cross reaction with glucagon</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Natural porcine cholecystokinin-33</td>
<td>—</td>
<td>Reacts with cholecystokinin 11-20; no cross reaction with gastrin</td>
<td>1:3,000</td>
</tr>
<tr>
<td>Synthetic porcine secretin</td>
<td>R-801</td>
<td>Reacts with the C- and N-terminals</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Synthetic porcine motilin</td>
<td>R-1104</td>
<td>—</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Synthetic bovine neurotensin</td>
<td>R-3501</td>
<td>—</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Synthetic porcine vasoactive intestinal polypeptide</td>
<td>R-502</td>
<td>Reacts against entire molecules</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Synthetic porcine gastrin releasing polypeptide</td>
<td>R-6902</td>
<td>Reacts primarily with the C-terminal; no cross reaction with substance P</td>
<td>1:1,000</td>
</tr>
</tbody>
</table>

a: All antisera were raised in rabbit except that against gastrin which was raised in guinea pig.
b: f: g: These antisera were purchased from Immunonuclear Corp., Stillwater; UCB-Bioproducts, Brussels; Guildhay, Surrey, respectively.
c: d: e: h: These antisera were kindly donated by Dr. N. Yanaihara, Shizuoka; Dr. S. Ito, Niigata; Dr. J. R. Kimmel, Kansas City; Dr. D. Grube, Hannover, respectively.

Table 2. Distribution and frequency of the endocrine cells in the proventriculus

<table>
<thead>
<tr>
<th></th>
<th>Finch</th>
<th>Pigeon</th>
<th>Quail</th>
<th>Chicken</th>
<th>Duck</th>
<th>Kite</th>
<th>Gull</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>APP</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Glucagon</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5-HT</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Superficial epithelium

<table>
<thead>
<tr>
<th></th>
<th>Finch</th>
<th>Pigeon</th>
<th>Quail</th>
<th>Chicken</th>
<th>Duck</th>
<th>Kite</th>
<th>Gull</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP</td>
<td>+</td>
<td>#, C</td>
<td>#, C</td>
<td>#, C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>#</td>
<td>#, C &amp; P</td>
<td>#, M</td>
<td>#, M</td>
<td>#, M</td>
<td>#, M</td>
<td>#, M</td>
</tr>
<tr>
<td>APP</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>−</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Glucagon</td>
<td>±</td>
<td>+, M</td>
<td>+, M</td>
<td>+, M</td>
<td>−</td>
<td>−</td>
<td>+, M</td>
</tr>
<tr>
<td>5-HT</td>
<td>±</td>
<td>+, I</td>
<td>+</td>
<td>+, I</td>
<td>−</td>
<td>+</td>
<td>+, P</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Proventricular glands

### 1.000

= neumerous, = moderate, = a few, = rare, = absent (not detected). C = distributed in central portion of the glandular lobules, I = distributed in inner half zone, M = distributed in middle zone, P = distributed in peripheral zone.
J. Yamada, N. Kitamura and T. Yamashita:

Epithelial cells. Some exhibited long or short cytoplasmic processes that coursed along the basement membrane (Fig. 1). On rare occasions, GRP-, somatostatin- or glucagon-immunoreactive cells established luminal contact via an apical cytoplasmic process.

Proventricular glands: The proventricular glands are the principal component of the proventriculus and constitute the greater part of the proventricular wall. The glands are composed of numerous rounded and elongated lobules that are arranged in small clusters. Each cluster drains into the lumen through a mucosal papillae. The wall of each lobule is composed of numerous tubular glands radiating out from the central lumen of the lobule.

Six kinds of immunoreactive cells were observed in the proventricular glands (Table 2, Fig. 2–6). The immunoreactive cells were located between the oxynticopeptic cells and the basement membrane, and did not establish contact with the lumen of the gland (Fig. 6). GRP- and somatostatin-immunoreactive cells were the principal endocrine cells in this region, and were observed in all the avian species examined (Table 2). Somatostatin-immunoreactive cells were the most frequently observed endocrine cells. They were found primarily in the middle zone of the glandular lobules in the quail, chicken, duck and kite, peripherally in the gull, centrally and peripherally in the pigeon and were scattered randomly in the finch (Table 2, Fig. 3b, 4). These endocrine cells were characterized by long cytoplasmic processes that coursed parallel to the long axis of the tubular glands (Fig. 6b). The ends of the cytoplasmic processes were enlarged into synaptic button-like swellings prior to termination on the neighboring oxynticopeptic cells or other somatostatin-immunoreactive cells (Fig. 6b). GRP-immunoreactive cells were numerous in the pigeon, quail, chicken and kite, moderate in number in the duck and gull, and few in number in the finch (Table 2, Fig. 2, 3a). They were also found primarily in the central or inner half zone of the lobules in the pigeon, quail, kite and gull, in the middle zone in the duck and randomly scattered in the finch (Table 2, Fig. 2, 3a). APP-immunoreactive cells were seen but rarely in most of the avian species examined (Table 2, Fig. 6c). Although glucagon-immunoreactive cells were not seen in the duck and kite, a small number of these cells was found in the other species examined (Table 2, Fig. 5a, 6d). Glucagon-immunoreactive cells were scattered primarily in the middle zone of the lobules (Fig. 5a). A few 5-HT-
immunoreactive cells were found in the inner half zone of the lobules in the pigeon and chicken, in the peripheral zone in the gull (Fig. 5b) and scattered randomly in the quail and kite (Table 2). The 5-HT-immunoreactive cells were a rare observation in the finch, and absent in the duck. Neurotensin-immunoreactive cells were seen but rarely in the pigeon and quail (Table 2). Because APP- and neurotensin-immunoreactive cells occurred in such low numbers, their pattern of distribution could not be determined in this study. The GRP-, APP-, glucagon-, 5-HT- and neurotensin immunoreactive cells were oval in shape, sometimes showing long or short cytoplasmic processes that coursed along the basement membrane (Fig. 6). The GRP-immunoreactive cells that exhibited long cytoplasmic processes were observed frequently in this
region but were not as plentiful as in the somatostatin-immunoreactive cells. The cytoplasmic processes of the GRP-immunoreactive cells, like the somatostatin-immunoreactive cells, terminated in synaptic, button-like swelling on neighboring cells (Fig. 6a).

**DISCUSSION**

In their first immunohistochemical study on avian gastrointestinal endocrine cells, Polak et al. (1974) failed to demonstrate immunoreactive cells in the proventriculus of the quail and chicken. However, numerous argyrophil cells, but no argentaffin cells, had been reported in the proventriculus of several avian species (Dawson and Moyer,
Fig. 6. Photomicrographs demonstrating the shapes of five kinds of immunoreactive cells in the proventricular glands. Note the long cytoplasmic processes terminating in synaptic button-like swellings. a. GRP-immunoreactive cells in the pigeon. b. Somatostatin-immunoreactive cells in the pigeon. c. APP-immunoreactive cell in the quail. d. Glucagon-immunoreactive cell in the quail. e. 5-HT-immunoreactive cells in the pigeon. PAP method. × 600

1948; Aiken, 1958; Okamoto et al., 1976). Our previous studies also confirmed the occurrence of endocrine cells in this region (Yamada et al., 1979; Iwanaga and Yamada, 1980; Okamoto and Yamada, 1981). Immunohistochemical studies have identified bombesin- (Vailant et al., 1979, in the turkey; Timson et al., 1979, in the chicken), GRP- (Buffa et al., 1982, in the chicken), APP- (Alumets et al., 1978a, in the chicken) and glucagon- (Timson et al., 1979, in the chicken) immunoreactive cells in the proventriculus. Although neurotensin-immunoreactive cells were not observed in the proventriculus of the quail (Reinecke et al., 1980) and chicken (Alumets et al., 1977), they were found in the proventriculus of the newly hatched chick (Rawdon and Andrew, 1981). Bombesin- and GRP-immunoreactives have been known to occur in the same endocrine cell (Buffa et al., 1982), and therefore five different endocrine cells have been identified in the avian proventriculus.

In general, the present observations correspond well with earlier immunohistochemical studies. One interesting observation of this study was that 5-HT-immuno-
reactive cells were identified in all the avian species examined with the exception of
the duck. Since argentaffin cells are generally regarded as being absent in the avian
stomach, the identification of the 5-HT-immunoreactive cells was an unexpected ob-
servation. Recently, Inokuchi et al. (1983) indicated that the population of enterochromaffin (EC) cells identified by the PAP method using a specific antiserum against
5-HT was larger than that of EC cells identified by the conventional silver impregna-
tion techniques. They isolated a small number of EC cells stained immunohistochemi-
cally that were neither argentaffin nor argyrophil. Furthermore, a few immunohisto-
chemical EC cells were shown to be argyrophil but not argentaffin. They have
concluded that the variance in population between EC cells revealed by the PAP
method and those shown by silver impregnation techniques is due to a difference in
the sensitivities of the techniques used. However, it is not yet clear whether the dif-
ference in staining of the non-argentaffin proventricular 5-HT-immunoreactive cells
and the argentaffin intestinal 5-HT-immunoreactive cells is dependent upon the amount
of 5-HT stored in the cells or whether there is a difference in the chemical components
of the secretory granules. The possibility that EC cells might contain, in addition to
5-HT, substance P (NiLSON et al., 1975; PEARSE and POLAK, 1975) or enkephalin (Alumets
et al., 1978b; Nihei et al., 1983) was suggested immunohistochemically. Examination
in serial sections in the present study indicated, however, that no 5-HT-immunoreac-
tive cells showed substance P- or leucine-enkephalin-immunoreactivity, and substance
P- and leucine-enkephalin-immunoreactive cells were absent in this organ.

Species differences with regard to the relative frequency and distribution of the
proventricular endocrine cells was pointed out in five species of birds in a previou-
s study using conventional histological methods (Yamada et al., 1979). The present
study using immunohistochemical technique has expanded and clarified these species
differences. However, the differences observed can not be explained solely on the feed-
ing habits or the species examined (Yamada et al., 1979). The functional significance
of the difference in distribution of the immunoreactive cells in the glandular lobules is
unknown at present. Future studies concerned with the microcirculation of the pro-
ventriculus will be essential in clarifying this question.

Although numerous argyrophil cells were observed in the peripheral or outer half
zone of the glandular lobules in the chicken, quail and kite (Yamada et al., 1979), no
immunoreactive cells were observed in the same zone of these species in the present
studies. This discrepancy suggests that other kinds of endocrine cells could be present
in the avian proventriculus. Ultrastructurally, the argyrophil cells which were not
immunoreactive to any of the antisera used in this study appeared similar to mam-
malian ECL cells (Iwanaga and Yamada, 1980). Mammalian ECL cells have been im-
plicated as histamine-synthesizing endocrine cells (Rubin and Schwartz, 1979).

Many immunoreactive cells in the proventricular glands exhibited elongated cyto-
plasmic processes that coursed along the basement membrane to terminate in swollen
endings reminiscent of synaptic buttons. In addition, most immunoreactive cells ob-
served in the proventricular glands were of the closed type. These morphological
features support the concept that many of the proventricular endocrine cells may func-
tion as mechanoreceptors and respond to mechanical stimuli such as pressure or tension
caused by foods (Kusumoto et al., 1979; Larsson et al., 1979; Yamada et al., 1979).
Somatostatin-immunoreactive cells in particular may function as mechanoreceptors as
well as typical paracrine cells as reported in mammalian fundic glands (Alumets
et al., 1979; Kusumoto et al., 1979; Larsson et al., 1979). It is well established that
somatostatin suppresses the secretion of endocrine as well as exocrine cells (Patel et
al., 1981) and that GRP (or bombesin) stimulates both directly and indirectly (via gastrin cells) gastric acid secretion by oxyntic cells (VARNER et al., 1981). In addition to the influence of gastrin cells and histamine on proventricular secretion (HILL, 1971), secretory activity also may be regulated at least in part by somatostatin- and GRP-immunoreactive cells. Future physiological studies on the proventriculus utilizing these particular gut peptides will be needed for further study on the control of secretion in this particular organ.

Acknowledgements. The authors wish to thank Prof. W. J. KRAUSE, Department of Anatomy, School of Medicine, University of Missouri, Columbia, for critically reviewing the manuscript. The gifts of antisera listed in Table 1 are also gratefully acknowledged.

REFERENCES


