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Pepsinogen and H,K-ATPase mediate acid secretion in gastric glands of *Triturus carnifex* (Amphibia, Caudata)

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Summary

The gastric glands of *Triturus carnifex* (Amphibia, Caudata) have been examined by histochemical and immunohistochemical methods with particular regard to hydrochloric acid and pepsinogen secretion. Fundic glands consist of mucous neck cells, endocrine cells and oxynticopeptic cells producing both pepsinogen and hydrochloric acid.

The neck cells showed an unexpected distribution pattern which was only observed in the oral fundus, and produced neutral mucins with glycosidic residues of GalNAc and Gal β1,3GalNAc, and in this respect they differ from the neck cells of anuran amphibians.

The secretion of pepsinogen and hydrochloric acid as demonstrated by immunolabelling with anti-H,K-ATPase and with anti-pepsinogen, respectively, seems not to vary significantly along the longitudinal axis of the stomach. The mechanism of gastric acid secretion seems to be mediated by an ATPase, having similar features to the mammalian gastric H,K-ATPase, and is localised in the luminal membrane and in the subapical cytoplasm of the oxynticopeptic cells. Unusually, the same cytoplasmic areas revealed binding specificity for the winged pea lectin (WPA) from *Lotus tetragonolobus*, even after β elimination, indicating the presence of fucosyl residues in *N*-linked oligosaccharidic chains in glycoproteins of β-H,K-ATPase subunits.

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Introduction

Gastric juice in vertebrates consists of two main components, namely pepsinogen and hydrochloric acid. Generally, in non-mammalian vertebrates, both of these components are secreted by one type of cells, the oxynticopeptic cells, which are clustered in glands in the gastric mucosa (Smit, 1968; Helander, 1981). Some Anura deviate from this pattern, because pepsinogen and hydrochloric acid are not produced by the same cell type in the same region. Specifically, pepsinogen is mainly produced by peptic cells clustered in oesophageal glands, whereas hydrochloric acid is mainly produced by oxyntic cells arranged in gastric glands. The genera sharing this condition are found in Ranoidea (Norris, 1959; Suganuma et al., 1981; Hirji, 1982; Hirji and Nikundiwe, 1982; Bani et al., 1992; Gallego-Huidobro and Pastor, 1996; Ferri et al., 2001). In non-ranoidean frogs, the general non-mammalian pattern seems to occur.

In the green toad, *Bufo viridis*, a functional gradient was observed in oxynticopeptic cells from the oral to the aboral fundus, with a decrease in pepsinogen secretion and an increase in HCl secretion towards the aboral fundus (Liquori et al., 2002).

These functional variations of the oxynticopeptic cells from the oral to the aboral fundus in *B. viridis* are very similar to those observed in the river stingray *Potamotrigon* sp., a cartilaginous fish (Gabrowsky et al., 1995), and in some squamates such as the seps, *Chalcides chalcides* (Liquori et al., 2000), and the ruin lizard, *Podarcis sicula campestris* (Ferri et al., 1999), and can be probably extended to other non-mammalian vertebrates with oxynticopeptic cells in their gastric glands.

However, only a few non-mammalian species have been examined regarding this phenomenon. In particular, little is known about the histochemistry of the secretory cells producing gastric juice in Caudata.

We have investigated the oesophagogastric tract of *Triturus carnifex* where we have defined the histological and histochemical features of the gastric glands and verified the existence of an oro-aboral gradient in the production of pepsinogen and hydrochloric acid.

We tested the latter by (i) staining with the modified Bowie's method for zimogen granules (Bonucci, 1981), (ii) immunolabelling with anti-pepsinogen for pepsinogen identification, (iii) labelling with *Dolichos biflorus* lectin, DBA, which specifically binds to α-GalNAc residues on the intracellular canalicular membranes that in mammalian parietal cells produce hydrochloric acid (Peschke et al., 1983; Ito et al., 1985; Kessimian et al., 1986) and thus it has been considered as a marker for this cellular type, and (iv) immunolabelling with H,K-ATPase, which in mammals is an integral protein of tubulovesicular and secretory canalicular membranes of the gastric parietal cells and is responsible for gastric acidification. These latter two tests provide indirect information on hydrochloric acid secretion by oxynticopeptic cells.

Binding with six FITC or peroxidase labelled lectins was also assessed to characterise glycosidic residues contained in oligosaccharidic chains of glycoproteins in mucous neck cells and in β-subunit of H,K-ATPase of oxynticopeptic cells.

Material and methods

Four adult *T. carnifex* were collected from areas around Altamura, Bari (Italy). The animals were sacrificed under ether anaesthesia and their digestive tracts were quickly removed. Samples of the digestive tracts were fixed in 10% v/v formalin in distilled water, dehydrated through a graded ethanol series, and embedded in paraffin wax. Five-micrometer-thick serial sections were cut by a Reichert Jung 2030 microtome.

Histochemistry

Zymogen granules were identified with a modified Bowie's staining method according to Bonucci (1981). Furthermore, some sections were stained with the Masson–Fontana silver method to detect argentaffin cells (Pearse, 1972).

Glycohistochemistry

Deparaffinised and rehydrated sections were stained with the periodic acid—Schiff (PAS) method (Mowry and Winkler, 1956) and with Alcian blue (AB) at pH 2.5 (Lev and Spicer, 1964). Binding with six FITC or peroxidase conjugated lectins (Sigma, St. Louis MO, USA) was also performed to characterise glycosidic residues contained in oligosaccharidic chains of glycoproteins in mucous neck cells and in β-subunit of H,K-ATPase of oxinticopeptic cells. The lectins tested are the same employed in our previous investigations in other amphibian and reptilian species (e.g., Ferri and Liquori, 1994; Ferri et al., 1999; Liquori et al., 2002).

For FITC-conjugated lectins (PNA, PWM, SBA, WPA) deparaffinised and rehydrated sections were incubated for 1 h at room temp with the FITC-lectin solutions, then rinsed in PBS and mounted in PBS

glycerin. For peroxidase labelled lectins (Con A and DBA) rehydrated sections were exposed to 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity and then incubated for 30 min at room temperature with solutions of peroxidase-labelled lectins. The horseradish peroxidase label was then visualised histochemically with 3,3′-diaminobenzidine (DAB)–hydrogen peroxide medium (Graham and Karnowsky, 1966) for 10 min. Finally, the sections were dehydrated, cleared and mounted with DPX.

The lectins utilised, their concentration and sugar specificities are detailed in Table 1.

Lectin binding was also performed after β-elimination with 0.2 M KOH in dimethylsulphoxide–H_2O–ethanol (50:40:10) for 1 h at 45 °C and subsequent neutralisation with 10 mM HCl and washing in PBS (Downs et al., 1973). Only the O-linked glycans are removed from glycoproteins by this method.

Controls for the lectin labelling procedures included (i) substitution of the lectin with PBS; (ii) incubation with the lectin with addition of the appropriate inhibitory sugar to confirm specificity of lectin labelling, as detailed in Table 1.

A variant of the Con A peroxidase method, the paradoxical Con A staining (PCS method: periodate oxidation, borohydride reduction and Con A labelling; Katsuyama and Spicer, 1978), was also carried out to identify mucous neck cells that in other amphibians, in reptiles and in mammals strongly react with this method (Katsuyama and Spicer, 1978; Suganuma et al., 1981; Ferri et al., 2001; Liquori et al., 2002).

Immunoistochemistry

For immunohistochemical assay, deparaffinised and rehydrated sections were treated with blocking buffer (1% normal goat serum, supplied by Sigma, in PBS) for 30 min at room temperature. Then they were incubated overnight with the primary antibody against the α-subunit of porcine H,K-ATPase (Chemicon, International, Temecula, CA, USA) diluted 1:5000 in blocking buffer at 4 °C. After several rinses in PBS, sections were then incubated in anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) diluted 1:500 in PBS for 5 h at room temperature. After rinses in PBS, sections were sequentially incubated overnight, at 4 °C, in a monoclonal antibody to human pepsinogen I (DPC Biermann, Bad Nauheim, Germany) diluted 1:500 in PBS and in anti-mouse Alexa Fluor 568 (Molecular Probes) for 5 h at room temperature. Finally, all sections were washed in PBS and mounted in PBS glycerin.

Controls were performed by omitting the primary antibodies or by using a peptide block (co-incubation with the immunising peptides).

The images were captured using an epifluorescence E 600 photomicroscope equipped with a DMX 1200 digital camera (Nikon, Kawasaki, Japan). Figures illustrating double immunofluorescence labelling (Figs. 2C and D) were created by mixing separate images using Adobe Photoshop 6.0 software.

Results

Oesophagus

The oesophagus of the *T. carnifex* was lined by a columnar ciliated epithelium with widespread mucous goblet cells. The mucosa appeared to be folded and no oesophageal glands were observed. Goblet cells contained large secretory vesicles that were PAS-positive and reacted with AB at pH 2.5 (Fig. 1A).

Table 1. Characteristics of the plant lectins utilised

Lectin	Source	Binding specificity	Lectin concentration (mg/ml)	Inhibitory sugar
Con A	*Canavalia ensiformis*	D-mannose D-glucose	0.05	0.1 M MαM
PWM	*Phytolacca americana*	(GlcNAc β1,4)$_3$	0.02	0.2 M GlcNAc
SBA	*Glycine max*	GalNAc	0.02	0.2 M GalNAc
PNA	*Arachis hypogaea*	Galβ1,3GalNAc	0.06	0.2 M Gal
WPA	*Lotus tetragonolobus*	L-Fucose	0.10	0.2 M L-Fucose
DBA	*Dolichos biflorus*	α-GalNAc	0.02	0.2 M GalNAc

Abbreviations: Con A, concanavalin A; PWM, pokeweed mitogen; SBA, soybean agglutinin; PNA, peanut agglutinin; WPA, winged pea agglutinin; DBA, *Dolichus biflorus* agglutinin; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; MαM, methyl-α-mannopyranoside.

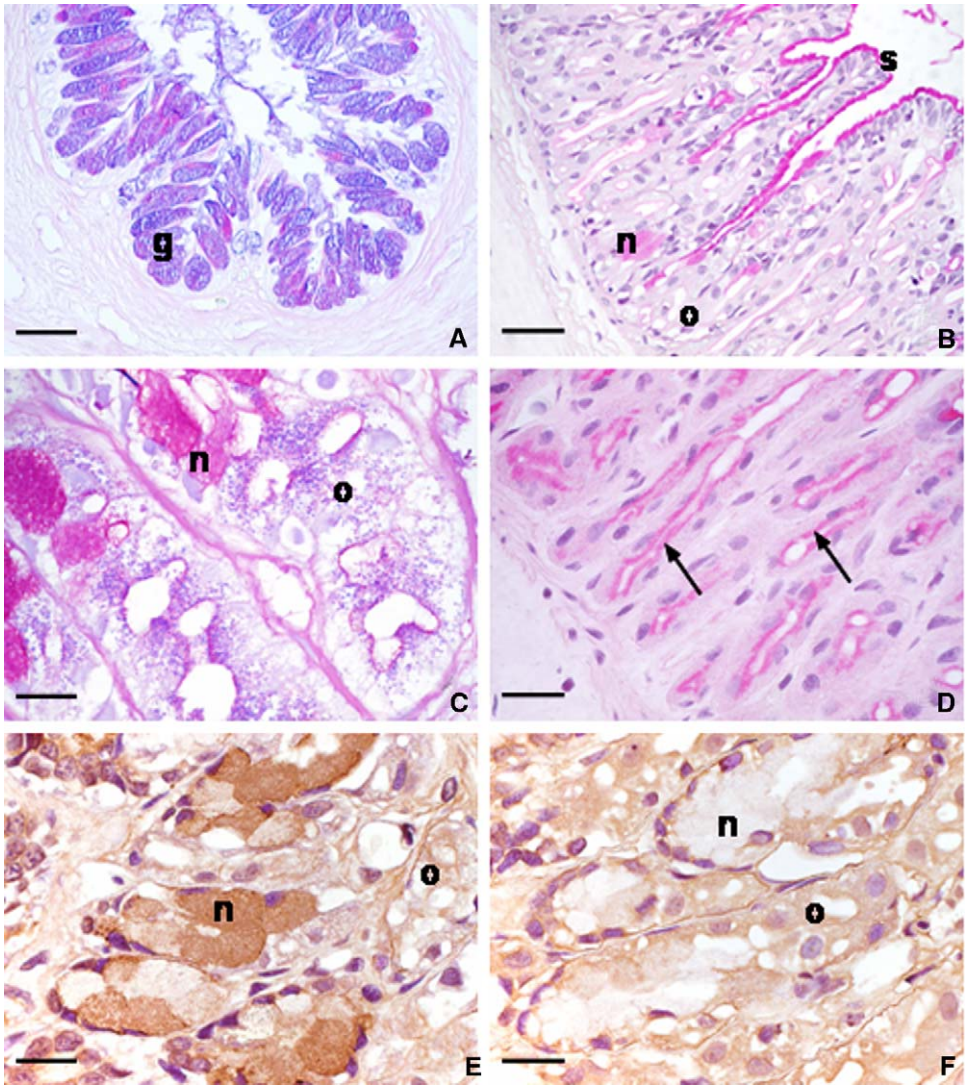

Fig. 1. Gastro-oesophageal mucosa of the *T. carnifex* stained using different histochemical methods. (A) Oesophagus; (B–F) Fundus. (A) Oesophageal goblet cells (g) are PAS positive and react with Alcian blue. The mucosa does not contain oesophageal glands. Alcian-PAS staining. (B) Surface cells (s) of the gastric mucosa stain intensely with the PAS reaction. Fundic glands are of the simple or ramified type and mainly consist of mucous neck cells (n) and oxynticopeptic cells (o). PAS-hemalum staining. (C) Oxynticopeptic cells (o) are filled with blue stained zymogen granules. Mucous neck cells (n) are PAS positive. PAS-Bowie staining. (D) The apical cytoplasm of the oxynticopeptic cells (arrows) is positive with the PAS reaction. PAS-haemalum staining. (E) More neck cells (n) show binding sites for DBA lectin. o, oxynticopeptic cells. DBA-labelling. (F) Mucous neck cells (n) do not label with Con A lectin using the paradoxical Con A method. o, oxynticopeptic cells. paradoxical Con A staining. Bars: (A,B), 65 μm; (C) 20 μm; (D) 30 μm; and (E,F) 25 μm.

Stomach

The stomach of *T. carnifex* appeared to be histologically subdivided into a corpus, or fundus, and a wide pars pylorica. These are characterised by fundic and pyloric glands, respectively, both emptying into gastric pits. The stomach lumen was lined with a single layer of mucus-secreting cells. The mucosa was arranged in a few longitudinal folds.

(I) *Surface epithelial cells*: These cells were PAS-positive, but they did not react with AB (Fig. 1B).

(II) *Fundic glands*: These glands were of the simple or ramified tubular type and mainly consisted of mucous neck cells and oxynticopeptic cells (Figs. 1B and C). Scattered argentaffin cells were also present.

Oxynticopeptic cells were filled with zymogen granules which stained blue with the Bowie method (Fig. 1C). Glycoconjugate histochemistry revealed the presence of neutral glycoproteins in the apical cytoplasm of the oxynticopeptic cells, by positive staining with the PAS reaction (Fig. 1D). No significant morphological or histochemical differences

were found between oxynticopeptic cells from oral and aboral areas of the stomach.

The mucous neck cells were observed only in the oral fundus and were PAS-positive, but they did not react with AB at pH 2.5 (Figs. 1B and C). Most neck cells showed binding sites for DBA (Fig. 1E) and they did not label with WPA, PWM or Con A using the PCS method (Fig. 1F). These cells labelled intensely with SBA lectin (Fig. 2A) and less intensely with Con A (Fig. 2B) and PNA (Fig. 2C). In control sections, no specific labelling was seen. The Masson–Fontana silver reaction demonstrated the presence of numerous endocrine cells in the basal third of the glandular tubules (data not shown). Beta elimination did not significantly affect the lectin binding.

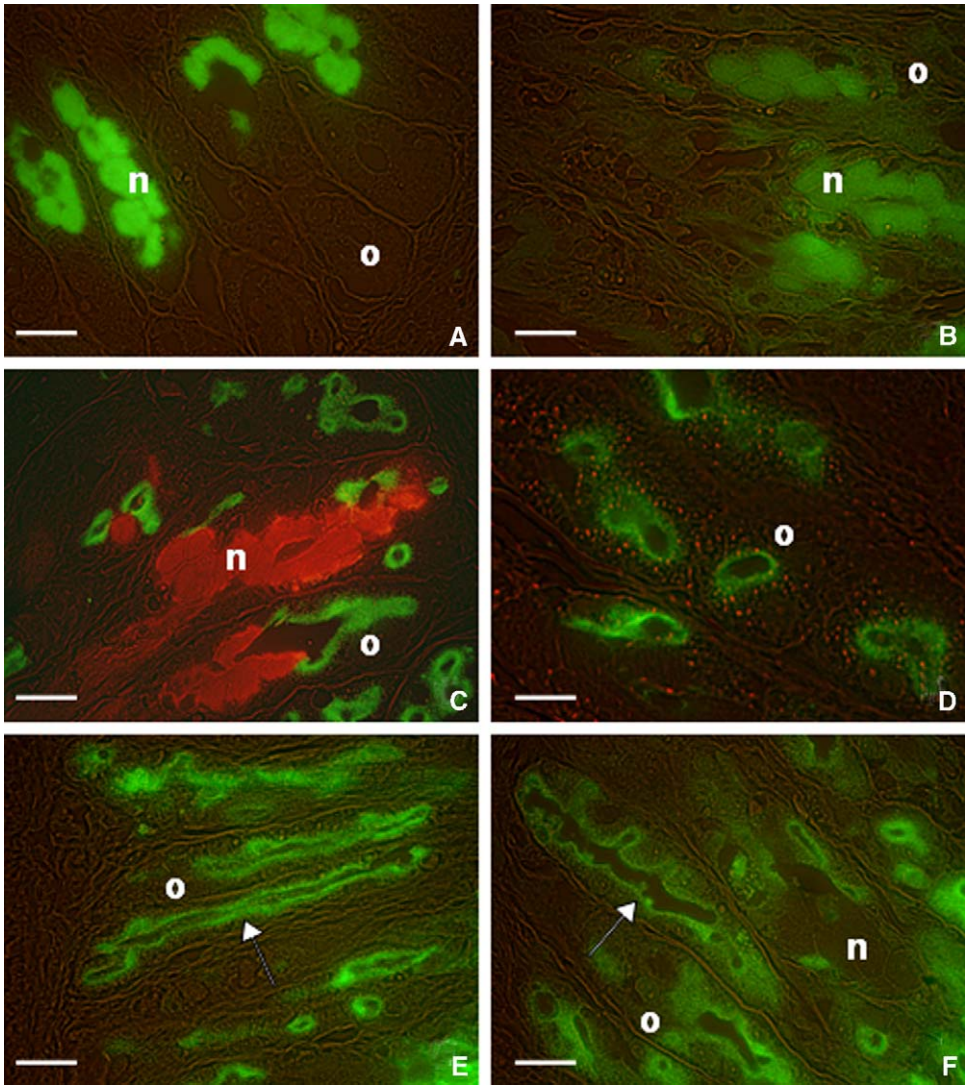

Fig. 2. Immunohistochemistry and lectin histochemistry of fundic mucosa of T. *carnifex*. (A) Soybean lectin (SBA) strongly labels neck cells (n) oxynticopeptic cells (o) are unreactive. SBA-FITC lectin labelling. (B) Con A lectin labelling is positive only in mucous neck cells (n). o, oxynticopeptic cells. Con A-FITC lectin labelling. (C) Double labelling with anti-H,K-ATPase (green labelling), for oxynticopeptic cells (o), and PNA-TRITC (red labelling) for mucous neck cells (n). Peanut lectin (PNA) strongly labels mucous neck cells. Anti-H,K-ATPase-Alexa Fluor 488 and PNA-TRITC lectin labelling. (D) Secretory granules of oxynticopeptic cells (o) immunoreacts (red labelling) with anti-pepsinogen and are mixed with smooth vesicles immunoreactive with anti-H,K-ATPase (green labelling). Anti-pepsinogen-Alexa Fluor 568 and anti-H,K-ATPase-Alexa Fluor 488. (E) Also in the aboral fundus the luminal membrane and the apical cytoplasm of the oxynticopeptic cells (o) labelled with anti-H,K-ATPase primary antibody. Anti-H,K-ATPase-Alexa Fluor 488. (F) The same cytoplasmic areas bound LTA lectin. (o) oxynticopeptic cells; (n) mucous neck cells. LTA-FITC labelled lectin. Bars, 30 μm.

Immunolocalisation of pepsinogen and H,K-ATPase

Immunolabelling with anti-pepsinogen antibody confirmed the presence of abundant proteolytic enzyme in the oxynticopeptic cells of *T. carnifex* (Fig. 2D). Secretory granules were mainly distributed in the supranuclear cytoplasm. In the apical cytoplasm, pepsinogen granules appeared mixed with vesicles of the smooth endoplasmic reticulum immunolabelled with the antibody directed against the C-terminus of the α-subunit of H,K-ATPase (Fig. 2D). This proton pump was found in the luminal membrane and in the apical cytoplasm of oxynticopeptic cells both in oral (Figs. 2C) and in aboral (Fig. 2E) gastric glands. No labelling was seen in control sections in which the primary antibodies were substituted with PBS or co-incubated with the immunising peptides.

The areas of the oxynticopeptic cells labelled with the anti-H,K-ATPase antibody were also PAS positive (Fig. 1D), bound WPA lectin (Fig. 2F), but did not react with DBA, Con A, SBA, PNA, PWM or with the PCS method. Beta elimination did not significantly affect the lectin binding with WPA.

(III) *Pyloric glands*. Pyloric glands were shorter than the fundic ones, and consisted of mucous cells and enteroendocrine cells. Mucous cells contained a few PAS positive secretory granules in their apical cytoplasm (Fig. 3A). No staining was seen using the Bowie method. Pyloric glands were unreactive with anti-H,K-ATPase and with anti-pepsinogen primary antibodies. Staining with the Masson–Fontana silver method revealed numerous endocrine cells in pyloric glands (Fig. 3B).

Discussion

This study reveals that the oesophagogastric tract of *T. carnifex* has some unusual histological and histochemical features. This is consistent with the morphofunctional heterogeneity that seems to exist among the digestive tracts of amphibians.

The oesophageal epithelium of *T. carnifex* is of the columnar ciliated type with widespread goblet cells that produce acidic mucins.

Oesophageal glands are lacking, as reported for non-ranoidean frogs (Loo and Wong, 1975; Hirji and Nikundiwe, 1982; Liquori et al., 2002). Thus, the oesophageal mucosa of *T. carnifex* differs from those of ranoidean frogs in which serous oesophageal glands produce most of the pepsinogen in gastric juice, while gastric glands mainly produce hydrochloric acid (Shirakawa and Hirschowitz, 1986; Bani et al., 1992; Gallego-Huidobro et al., 1992).

Epithelial surface cells of the stomach of *T. carnifex* mainly produce neutral glycoproteins as seen in the green toad *B. viridis* (Liquori et al., 2002). This is different to the situation in the red-legged frog *Rana aurora* where these cells secrete mainly sulpho-sialo glycoproteins (Ferri et al., 2001). Fundic glands in *T. carnifex* consist of mucous neck cells, endocrine cells and oxynticopeptic cells producing both pepsinogen and hydrochloric acid, as seen in *B. viridis* (Liquori et al., 2002) and *Bombina variegata* (Bani et al., 1992).

Interestingly, the neck cells in *T. carnifex* show an unusual distribution pattern and histochemical features as they are observed only in the oral fundus and have been found to produce neutral mucins with glycosidic residues of GalNAc and Gal β1,3GalNAc in *N*-linked glycoproteins. Various treatments carried out prior to the Con A method

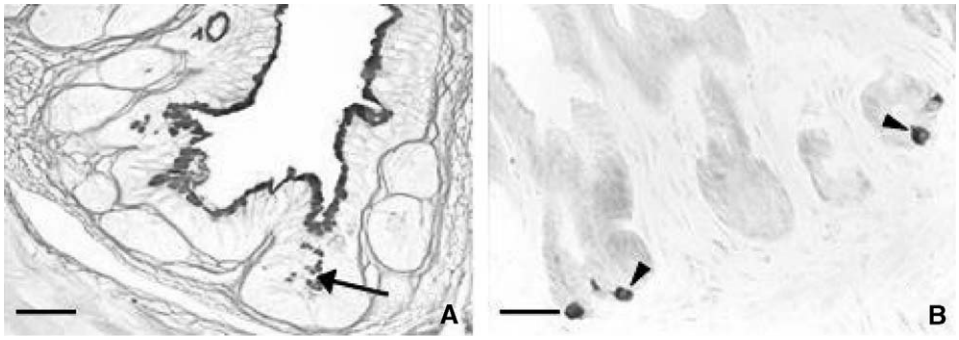

Fig. 3. Pyloric mucosa of T. *carnifex*. (A) Pyloric glands are shorter than the fundic ones, and mainly consist of mucous cells containing a few PAS positive secretory granules in their apical cytoplasm (arrow). PAS staining. (B) The Masson–Fontana silver reaction demonstrates the presence of numerous endocrine cells (arrows) in the basal third of the glandular tubules. Masson–Fontana staining method. Bars, 30 μm.

were found to affect labelling and allowed differentiation of three main classes of complex carbohydrates in the mammalian alimentary tract (Katsuyama and Spicer, 1978). Class I mucosubstances lose Con A reactivity while classes II and III gain Con A reactivity after periodate oxidation. Class II mucosubstances lose reactivity whereas class III gain or increase their reactivity with a reduction step interposed between oxidation and Con A staining (paradoxical Con A staining). In *T. carnifex*, the neck cells did not label with the paradoxical Con A method and are thus distinct from the neck cells of some anuran amphibians, such as *R. aurora* (Ferri et al., 2001) and *B. viridis* (Liquori et al., 2002), various reptiles and mammals (Suganuma et al., 1981) in which these cells contain paradoxical Con A positive class-III mucosubstances.

It is conceivable that *T. carnifex* retains a primitive feature, since neck cells first appeared phylogenetically in amphibians. Numerous mitotic figures were observed in the deeper tract of glandular tubules in the aboral fundus, which lacks neck cells that might act as a precursor of oxynticopeptic cells or mucous cells, as hypothesised by Oinuma et al. (1991) in the clawed toad *Xenopus laevis*.

Pyloric glandular cells, like those of other amphibians such as *Rana perezi* (Gallego-Huidobro and Pastor, 1996), *R. aurora* (Ferri et al., 2001) and *B. viridis* (Liquori et al., 2002), are mainly of the mucus-secreting type and secrete neutral glycoproteins. Numerous endocrine cells have been also found in pyloric glands.

In *T. carnifex,* as in most non-mammalian vertebrates, the fundic gland oxynticopeptic cells are able to synthesise both pepsinogen and hydrochloric acid. In the toad *Bufo marinus* the secretion of both these components of the gastric juice can be stimulated by the same agents, histamine or carbachol (Ruiz et al., 1993). Our results suggest that also in Caudata the mechanism of gastric acid secretion could be mediated by an ATPase having similar features to the mammalian gastric H,K-ATPase.

This is supported by the fact that both the luminal membrane and the apical cytoplasm of oxynticopeptic cells show clear immunoreactivity to α-H,K-ATPase, a gastric proton pump that in other vertebrates has been demonstrated to be responsible for gastric acidification. Significantly, in the toad *B. marinus* acid secretion is inhibited by omeprazole (Ruiz et al., 1993), an agent that is known to block the gastric H,K-ATPase pump (Lorentzon et al., 1987).

The anti α-H,K-ATPase immunoreactive cytoplasmic areas are also PAS positive, a pattern shared with the oxyntic or oxynticopeptic cells of other vertebrates (Sedar, 1968; Smolka et al., 1994). This is most likely due to the presence of heavily glycosylated β-subunits of H, K-ATPase.

In mammals, it has been hypothesised that the β-H,K-ATPase is involved in the structural and functional maturation of the holoenzyme and the intracellular transport of ATPase (Chow et al., 1992), as well as possibly playing a role in protecting the holoenzyme from acidic and peptic insults (Forte and Forte, 1970; Chow and Forte, 1995). The mammalian β-subunit of H,K-ATPase has six or seven *N*-linked sites of glycosylation (Tyagarajan et al., 1996, 1997) which are conserved among different species, although differences in the nature of the oligosaccharidic chains may occur between species (Appelmelk et al., 1996; Crothers et al., 1996).

In *T. carnifex*, the oxynticopeptic cells did not label with the DBA lectin, which has been reported to bind specifically to the α-GalNAc residues in the intracellular canalicular and vesicular membranes of mammalian parietal cells (Peschke et al., 1983; Ito et al., 1985; Kessimian et al., 1986), which are probably rich in the β-subunits of H,K-ATPase. Labelling with the DBA lectin has also been observed in oxynticopeptic cells in some non-mammalian vertebrates, such as the green toad *B. viridis* (Liquori et al., 2002) and the ruin lizard *P. sicula campestris* (Liquori et al., 2000). Unusually, the apical cytoplasm of the oxynticopeptic cells in *T. carnifex* revealed labelling with the winged pea lectin (WPA), also after β-elimination, indicating the presence of fucosyl residues in *N*-linked oligosaccharidic chains in glycoproteins of β-H,K-ATPase subunits.

Our findings suggest that differences in the nature of the oligosaccharidic chains may occur between species of vertebrates.

In active oxynticopetic cells of *T. carnifex*, pepsinogen granules are mainly distributed in the supranuclear cytoplasm where they are mixed with vesicles of the smooth endoplasmic reticulum immunolabelled with the antibody directed against the C-terminus of the α-subunit of H,K-ATPase.

Hydrochloric acid and pepsinogen secretion, as demonstrated by immunolabelling with anti-H,K-ATPase and with anti-pepsinogen antibodies, respectively, seem not to vary significantly along the longitudinal axis of the stomach. The morpho-functional pattern of the gastric cells secreting the two main components of the gastric juice clearly show differences when compared to those described in anuran amphibians.

In fact, in the anuran *B. viridis*, as in other non-ranoidean frogs, in which pluricellular oesophageal

glands are lacking, oxynticopeptic cells of the oral fundic glands mainly produce pepsinogen, while in the aboral fundus they mainly synthesise hydrochloric acid (Liquori et al., 2002).

A similar pepsinogen and hydrochloric acid gradient has also been found in a cartilagineous fish (Gabrowsky et al., 1995) and in some squamates (Giraud et al., 1979; Ferri and Liquori, 1994; Ferri et al., 1999; Liquori et al., 2000).

In the anurans of the superfamily Ranoidea, peptic cells have been found clustered in oesophageal glands with the gastric glands producing mainly hydrochloric acid from oxynticopeptic cells (Hirji, 1982; Hirji and Nikundiwe, 1982; Gallego Huidobro et al., 1992; Gallego Huidobro and Pastor, 1996; Ferri et al., 2001).

In both *B. viridis* (Liquori et al., 2002) and *R. aurora* (Ferri et al., 2001), the production of pepsinogen along the oesophagogastric mucosa precedes that of HCl, even if this is accomplished in different ways. In both cases, food is first surrounded by pepsinogen in the oesophagus (*R. aurora*) or in the oral fundus (*B. viridis*), and pepsinogen is converted to pepsin in the acid environment of the whole fundus (*R. aurora*) or in its aboral part (*B. viridis*) and then proteolytic activity begins.

Pyloric glands are shorter than the fundic ones and consist of mucous cells and enteroendocrine cells.

In conclusion, the results reported in this study support the hypothesis that the mechanism and the molecules involved in gastric acidification are substantially similar in different vertebrate species. Nevertheless, some differences could exist between species related to different habitat and diet. Hydrochloric acid and pepsinogen may be secreted from different cellular types localised in different areas of the oesophagogastric tract. Our study also contributes to demonstrating that there is morphological and functional variability between the gastroesophageal tracts of amphibians. However, we cannot as yet say if the histomorphological features observed in *T. carnifex* are shared among Caudata. Future investigations will be necessary to address this possibility.

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