Enzyme-ultracytochemical study of adenylate and guanylate cyclases in normal and pathologic human nasal mucosa

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The ultracytochemical localization of adenylate cyclase (AC) and guanylate cyclase B (GC-B) and C (GC-C) activity was studied after stimulation with pituitary adenylate cyclase activating peptide, C-type natriuretic peptide and guanylin, respectively, in normal human respiratory nasal mucosa and mucosa of nasal polyps. To demonstrate these enzymatic activities, we employed enzyme-ultracytochemical methods for electron microscopy. Both normal and pathologic nasal mucosa contained AC, GC-B and GC-C activity. In the upper portion of respiratory epithelium, the enzymes were detected on ciliary and microvillar membranes. In ciliary membranes, GC-B was the predominant form expressed. In goblet cells and in glands of the lamina propria, enzymatic activities were localized mainly on plasma membranes and on membranes lining secretory granules. The results did not reveal any evident differences between the enzymatic activities in normal and pathological nasal mucosa and suggest complementary activities for these enzymes and their stimulators in the regulation of mucociliary transport and glandular secretion.

Key words: adenylate cyclase, guanylate cyclase, PACAP, guanylin, C-type natriuretic peptide, human nasal mucosa, nasal polyps

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Pituitary adenylate cyclase activating peptide (PACAP) is a 38-amino acid peptide originally isolated from bovine hypothalamus (Miyata et al., 1989). It is a neurotransmitter, neuromodulator and neutrophic factor in the central nervous system, though PACAP-like immunoreactivity has been detected also in peripheral organs such as exocrine and endocrine glands (Arimura and Shioda, 1995; Przywara et al., 1996; Simonneaux et al., 1998). PACAP is a member of the family of vasoactive intestinal peptide (VIP) and the N-terminal portion of PACAP has 68% homology with porcine VIP (Miyata et al., 1989). The biological effect of PACAP and VIP in target tissues is the activation of adenylate cyclase (AC) with cAMP production. C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family which cooperates in regulating fluid volume and electrolyte homeostasis in target tissues (Drewett and Garbers, 1994).

Guanylin is a peptide that plays a role in decreasing fluid absorption and in stimulating the secretion of Cl^- in the intestinal mucosa (Forte and Currie, 1995).This peptide, originally isolated from the intestine, has also been detected in other organs (Fan et al., 1997). In the target tissues, CNP and guanylin elicit their physiological effects by activating two isoforms of membrane-bound guanylate cyclase (GC) with cGMP production. The isoform GC-B is the receptor for CNP, and the isoform GC-C is the receptor for guanylin (Drewett and Garbers, 1994). GC-C is also a receptor for *Escherichia coli* heat-stable enterotoxin (Sta toxin), which has structural homology with guanylin (Field et al., 1978).

Electrolyte transport by nasal epithelium is important for controlling the quantity and composition of the nasal fluid and may play an important role in the development of nasal polyps (Lee et al., 2002). Mucus hypersecretion is a normal feature of nasal polyposis (Kim et al., 2000).

Previously, in biochemical studies, the presence

and activity of VIP and PACAP were demonstrated in the airways of various animals and in human nasal mucosa (Uddman et al., 1991; Hauser-Kronberger et al., 1996; Khun and Arnold, 1996; Wagner et al., 1998). In human and murine airway epithelial cells, an increase in cGMP levels was found in response to CNP, but not to other natriuretic peptides or Sta toxin (Geary et al., 1993, 1995; Kelley et al., 1997). In bovine tracheal epithelial cells, Sta toxin produced increases in cGMP (Range et al., 1997).

While biochemical analyses are essential to understand how enzyme activities are regulated, it is also important to determine where these enzyme activities are distributed in a tissue or cell type. Enzyme-ultracytochemical methods may be a valid means to localize an enzyme within a cell and to analyze changes in distribution of enzymatic activity under different experimental or pathological conditions. Thus, the aim of the present study was to survey in detail the ultrastructural localization of AC, GC-B and GC-C activity by enzyme-ultracytochemical methods after stimulation by their activators in normal nasal respiratory mucosa and in mucosa of nasal polyps, which are consistently associated with chronic inflammation, in order to examine eventual different enzymatic activities.

Materials and Methods

Samples of normal nasal respiratory mucosa from 6 patients and samples of mucosa of nasal polyps from 6 patients were used. The samples were obtained during surgery under local anaesthesia and were fixed immediately after the excision in cold (4°C) 0.25% or 1% glutaraldehyde in 0.1 M cacodylate buffer containing 8% glucose, pH 7.4, for AC localization and in cold (4°C) 0.25% glutaraldehyde - 0.25% paraformaldehyde or 1% glutaraldehyde - 1% paraformaldehyde in the same buffer for GC-B and GC-C localization. After 20 min the samples were washed several times in the same buffer to remove the fixative. Thin sections were cut from each sample under the stereomicroscope and stored at 4°C overnight before further processing.

Ultracytochemical localization of AC activity

The method was based on a previous enzyme-ultracytochemical procedure for the detection of AC activity (Yamamoto et al., 1998, Rambotti et al., 2002a). A freshly prepared medium consisted of 80 mM Tris-maleate buffer pH 7.4, 8% glucose, 2 mM theophylline, 4 mM MgSO4, 0.5 mM adenylyl-imidodiphosphate sodium salt (App(NH)p), 2 mM $Pb(NO_3)_2$, 1 mM GTPγS, 10 mM ouabain (basal medium).

Samples were incubated in (i) basal medium, (ii) basal medium plus 0.01 μM PACAP.

Ultracytochemical localization of GC-B and GC-C activity

The method was based on previous enzyme-ultracytochemical procedures for the detection of GC activity (Schulze and Krause, 1983; Rambotti et al., 1997, 2000). The incubation medium for the detection of GC-B activity consisted of 80 mM Tris-maleate buffer, 2 mM $Pb(NO_3)_2$, 3 mM $MgCl_2$, 1 mM guanylyl-imidodiphosphate sodium salt (Gpp(NH)p), 0.8 mM ATPγS, 2 mM theophylline, 0.175 M glucose and 1 mM levamisole (basal medium). The basal incubation medium for the detection of GC-C contained the same components but the concentrations of $MgCl_2$ and ATPγS were 6 mM and 1 mM, respectively.

Samples were incubated in: (i) basal medium at pH 7.4 or pH 8 for the detection of GC-B or GC-C activity, respectively, (ii) basal medium plus 1 μM CNP at pH 7.4 for the detection of GC-B activity, (3) basal medium plus 0.1 μM guanylin at pH 8 for the detection of GC-C activity.

In control experiments, samples were incubated in media containing all the components but without App(NH)p or Gpp(NH)p, or they were preheated at 60°C for 20 min prior to incubation in media containing all components.

After incubation for 45 min at 37°C, the incubation media were aspirated and the samples rinsed several times in 0.1 M cacodylate buffer, and post-fixed in 1% osmium tetroxide for 45 min. The samples were dehydrated in a graded ethanol series and embedded in an Epon-Araldite mixture. After a brief staining in a saturated solution of uranyl acetate in 50% ethanol, ultrathin sections were examined in a Philips TEM 400 electron microscope.

For enzyme-ultracytochemical demonstration, the method used allows the detection of a precipitate of enzymatically released imidodiphosphate and lead ions at the site of AC or GC activity (reaction product).

App(NH)p, ATPγS, Gpp(NH)p, GTPγS (non-hydrolyzable analogs of ATP and GTP), were used. GTP and ATP are necessary to increase AC and GC

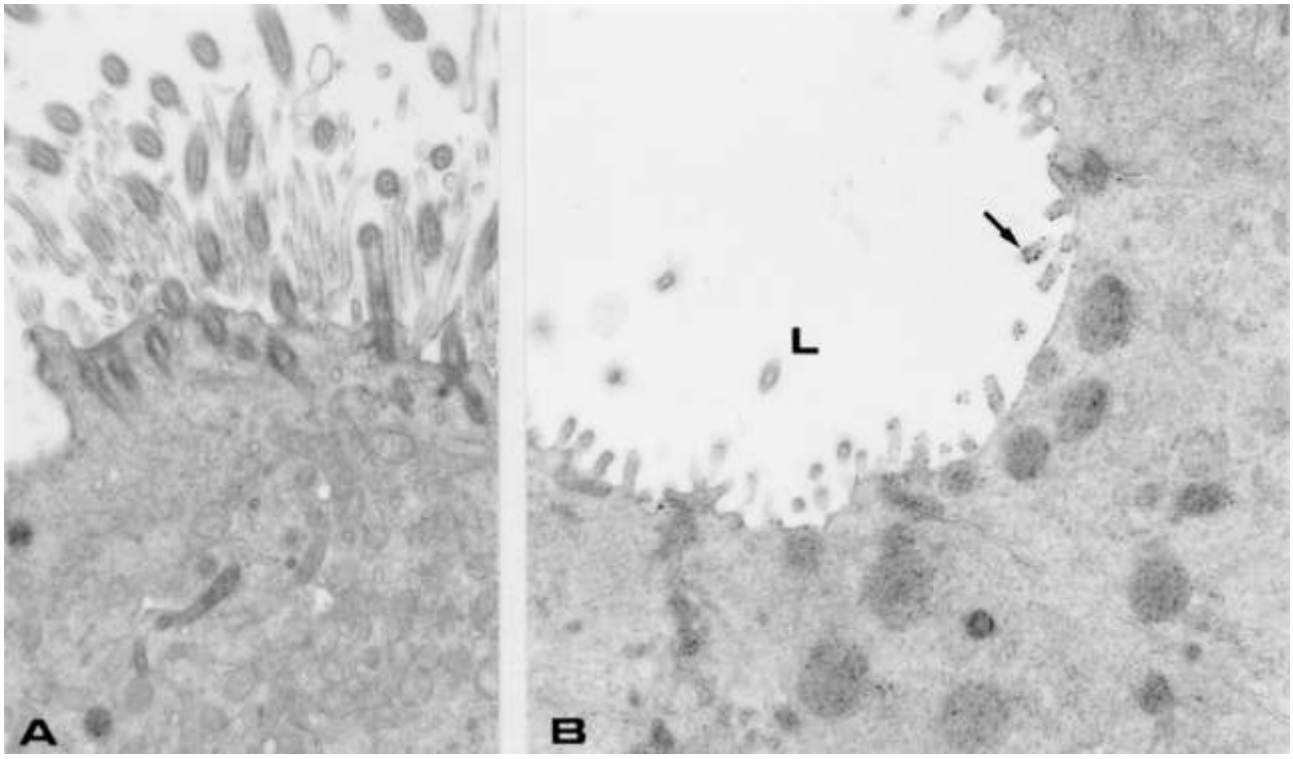

Figure 1. Normal respiratory nasal mucosa: epithelium. Samples fixed in 0.25% fixative and incubated in basal medium. Enzyme-ultracytochemical localization of GC (A) and AC (B) activity. No reaction products of basal GC activity are localized on the upper portion of the epithelium. In glands of the lamina propria, a few reaction products of AC basal activity are localized on apical plasma membranes (arrow). L: lumen of the gland. A,B: x14000.

activity, respectively. Theophylline was used as an inhibitor of phosphodiesterases, ouabain was included to inhibit Na^+,K^+ATPase, levamisole was included in the medium to inhibit non-specific alkaline phosphatase.

App(NH)p, Gpp(NH)p, CNP, were obtained from Sigma, Milan, Italy, ATPγS and GTPγS from Boehringer Mannheim, Germany, guanylin from Alexis Corporation, San Diego, CA, USA.

Results

At the electron microscope, control samples from normal and pathological mucosa incubated in basal media plus activators, but in absence of the substrates App(NH)p or Gpp(NH)p, or heat inactivated tissue, did not reveal AC, GC-B, GC-C reaction products (*data not shown*).

In samples from normal and pathological mucosa fixed in 1% fixative and incubated in basal media, no AC or GC activity could be detected (data not shown). In samples from normal and pathological mucosa fixed in 0.25% fixative and incubated in basal media, no AC or GC activity was found in the epithelium, but some reaction products of AC or GC activity were present on the plasma membrane of glandular cells. An example of these results is showed in Figures 1A and 1B.

Samples, fixed in 0.25% or 1% fixative and incubated in basal media plus activators showed enzymatic activity by the presence of reaction products.

Since it was found that the localizations of enzymatic activities were similar in samples fixed in 0.25% or 1% fixative, the figures showing only the enzymatic activity of samples fixed in 1% fixative are given.

Normal mucosa

In samples exposed to PACAP, guanylin or CNP, the reaction products of AC (Figure 2A), GC-C (Figure 2B), GC-B (Figure 2C,D) activity were found on ciliary and microvillar membranes of the apical portion of the epithelium. Only in samples exposed to guanylin (Figure 2B) or CNP (Figures 2 C, D), were the reaction products evident on basal bodies of ciliated cells. In the goblet cells, enzymatic reaction products of AC (Figure 3A), GC-C (Figure 3B), GC-B (Figure 3C) were present on the plasma

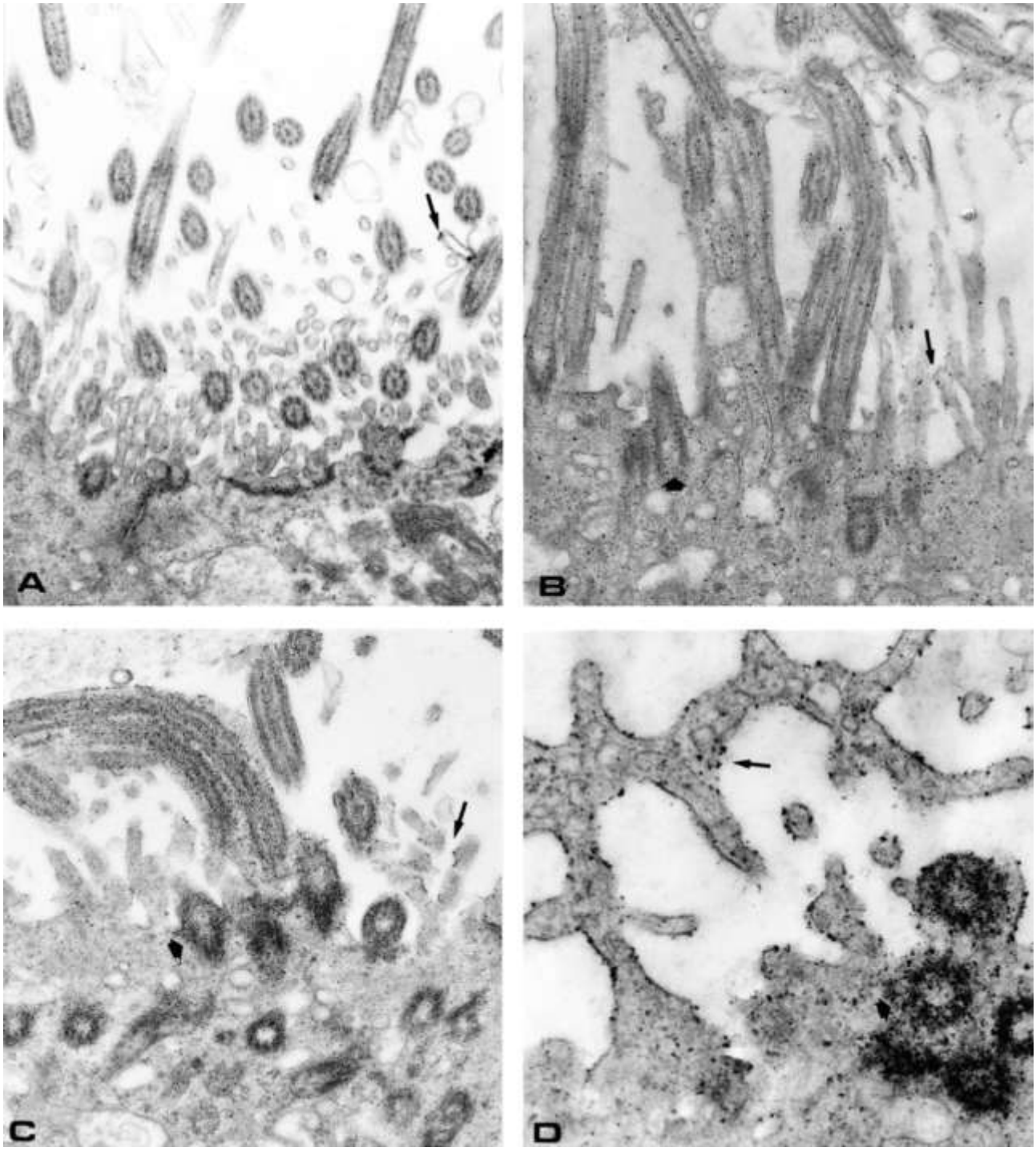

Figure 2. Normal respiratory nasal mucosa: epithelium. Enzyme-ultracytochemical localization of AC (A), GC-C (B) and GC-B (C,D) activity. Incubation in media containing activators. Reaction products are localized on ciliary and microvillar (arrow) membranes and on basal bodies (arrowhead). A: x20000; B: x24000; C: x30000; D: x50000.

membrane and on the membranes surrounding mucus globules. In the lamina propria, enzymatic positivities were localized at the level of the glands. The reaction products of AC (Figure 4A), GC-C (Figure 4B), GC-B (Figure 4C) activity were found on apical and lateral plasma membranes of glandular cells. Very occasionally, reaction products could be seen on membranes lining secretory granules.

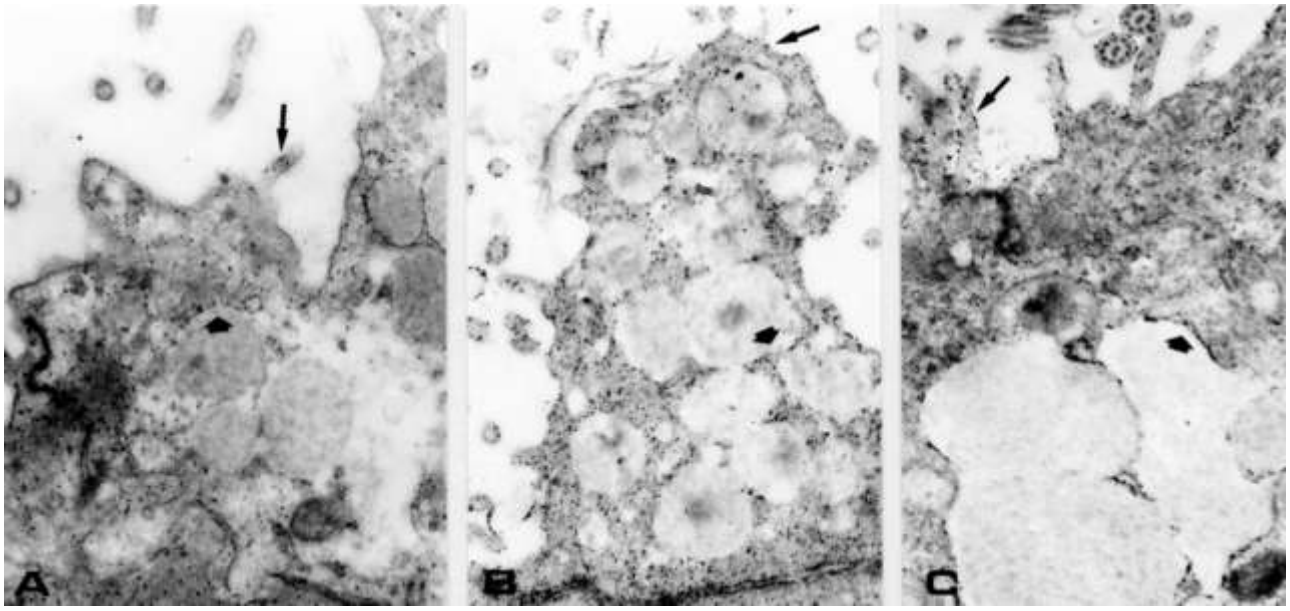

Figure 3. Normal respiratory nasal mucosa: epithelium. Enzyme-ultracytochemical localization of AC (A), GC-C (B) and GC-B (C) activity. Incubation in media containing activators. In goblet cells, reaction products are localized on plasma membrane (arrow) and on membranes lining secretory granules (arrowhead). A,C: x20000; B: x24000

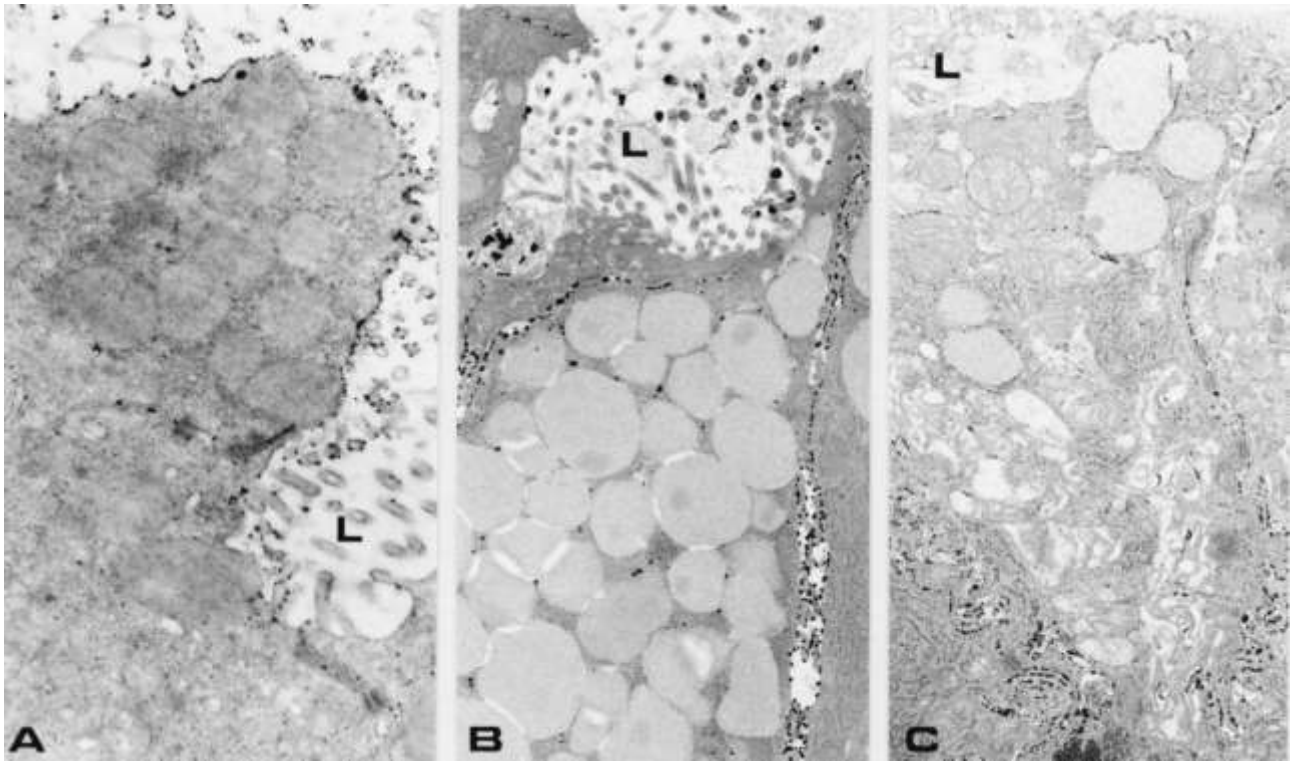

Figure 4. Normal respiratory nasal mucosa: glands of the lamina propria. Enzyme-ultracytochemical localization of AC (A), GC-C (B) and GC-B (C) activity. Incubation in media containing activators. Reaction products are localized on plasma membrane and on membranes lining secretory granules. L: lumen of gland. A: x18000; B: x15000; C: x12000.

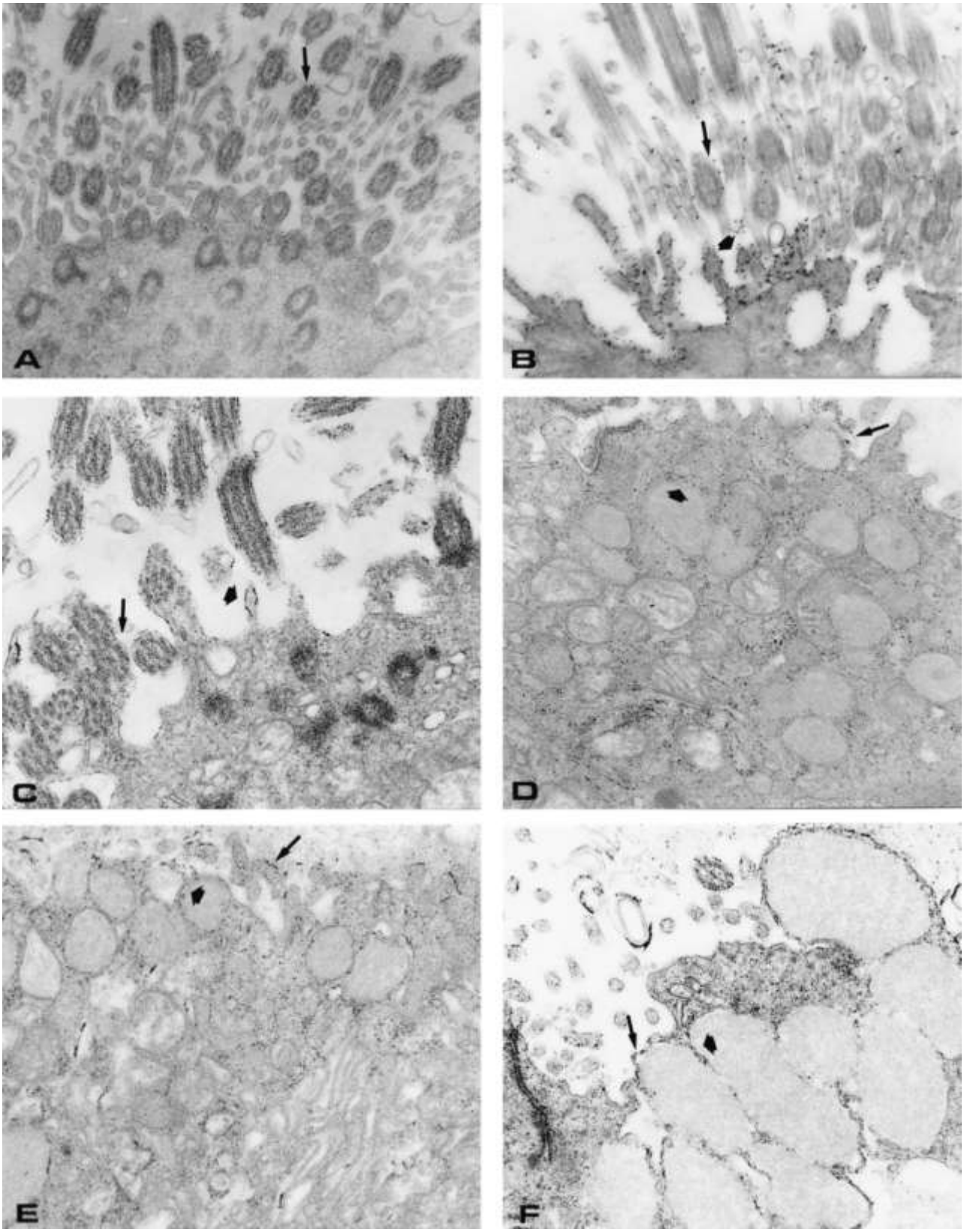

Figure 5. Nasal polyps: epithelium. Enzyme-ultracytochemical localization of AC (A, D), GC-C (B, E), and GC-B (C, F). Incubation in media containing activators. Reaction products are localized on ciliary (A-C) (arrow) and microvillar (B,C) (arrowhead) membranes. In goblet cells (D-F), reaction products are localized on plasma membrane (arrow) and on membranes lining secretory granules (arrowhead). A: x18000; B: x20000; C-F: x 25000.

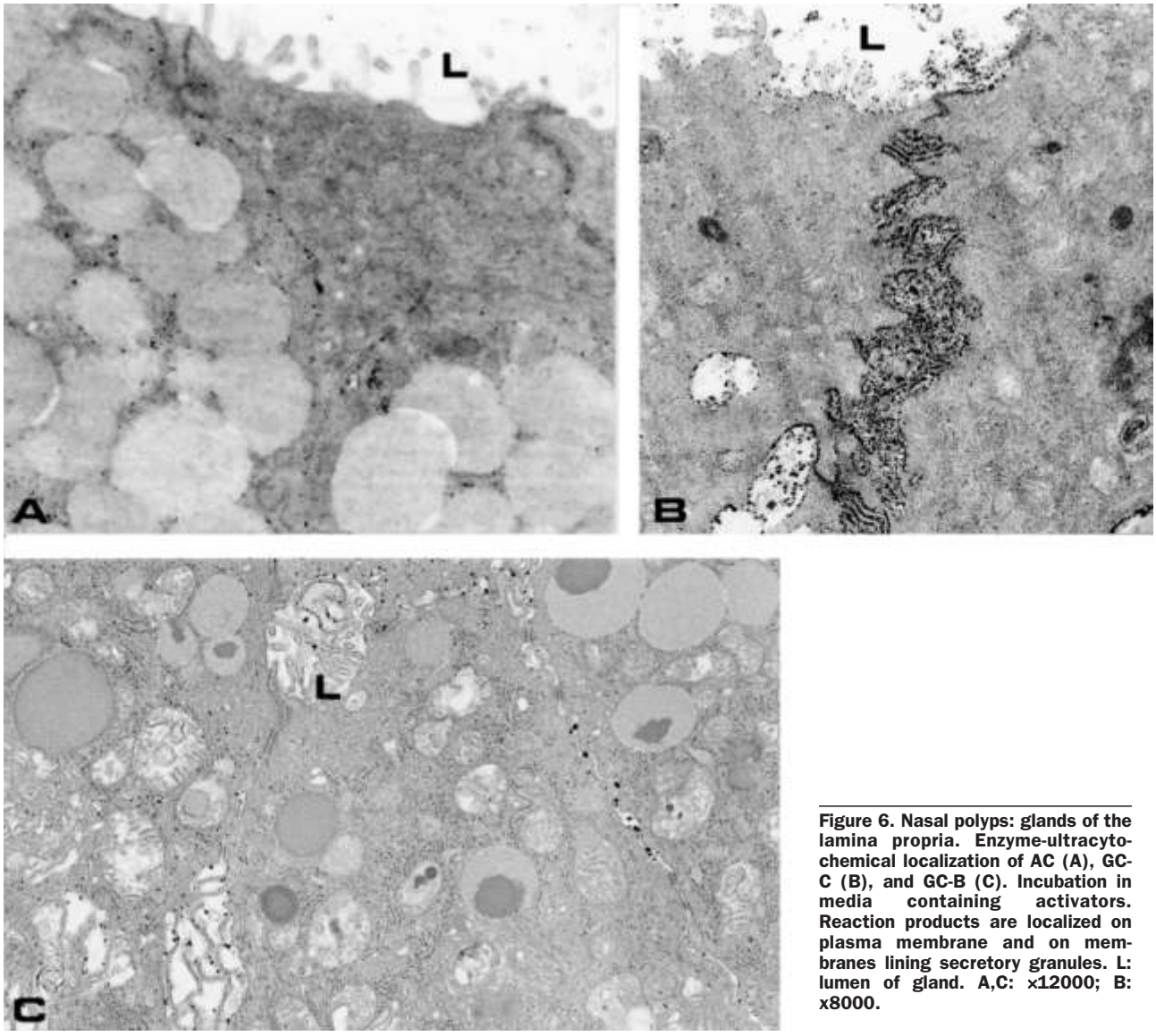

Figure 6. Nasal polyps: glands of the lamina propria. Enzyme-ultracytochemical localization of AC (A), GC-C (B), and GC-B (C). Incubation in media containing activators. Reaction products are localized on plasma membrane and on membranes lining secretory granules. L: lumen of gland. A,C: x12000; B: x8000.

Mucosa of polyps

The epithelium of nasal polyps is typically a pseudostratified respiratory epithelium with ciliary cells and goblet cells, although in some areas it is subject to morphological changes such as hyperplasia and squamous metaplasia (Larsen and Tos, 1989; Coste et al., 1996a, 1996b).

In the epithelium of samples exposed to stimulation by PACAP, guanylin and CNP, AC (Fig.5A), GC-C (Figure 5B), GC-B (Figure 5C) activity was localized on ciliary membranes but only guanylin (Figure 5B) and CNP (Figure 5C) activated their enzymes also on microvillar membranes. In the goblet cells (Figures 5D-F), the localization of the enzymatic activities was similar to that seen in samples of normal mucosa. In the glands of the lamina propria, reaction products of AC (Figure 6A), GC-C (Figure 6B) and GC-B (Figure 6C) activity were detected on apical and lateral plasma membranes of glandular cells; some reaction products were also detected on membranes lining secretory granules.

Discussion

In previous studies, we investigated the ultrastructural localization of natriuretic peptide- and guanylin-activated GC in various organs. The enzyme-ultracytochemical method we used throughout allowed the detection of variations in the activation state of membrane-bound GC in tissues and cells under different functional conditions, during tissue growth and cellular proliferation and in

pathologic conditions (for a review see Rambotti et al., 2002b). We also investigated the enzyme-ultrastructural localization of AC activity under different functional and pathologic conditions (Rambotti et al., 1988a,b; Spreca et al., 1988; Spreca et al., 1991; Rambotti et al., 2002a).

Using the same techniques as in our previous studies, we herein present morphological evidence that normal human respiratory nasal mucosa and mucosa of nasal polyps contain active AC, GC-B and GC-C in the presence of their stimulators in samples incubated in 0.25% or 1% fixative. The absence or presence of few reaction products in samples fixed in 1% or 0.25% fixative and incubated in basal medium suggests that the basal activity of the enzymes was at the detection limit of the technique used. On the other hand, it is known that fixation and exposure to lead significantly decrease these enzymatic activities to various extents, but that a certain amount of activity still remains, and appropriate stimulators can be used to restore such activity (Cutler, 1983; Schulze and Krause, 1983; Fukushima and Gay, 1991).

We used CNP at a concentration of 1 μM. It has been observed that this concentration produces a good stimulatory effect in other situations (Sorci et al., 1995; Suda et al., 1996; Spreca et al., 1999). Previous biochemical studies on the stimulation of GC-C activity showed that guanylin at a concentration of 0.1 μM produced the maximal effect for stimulating intracellular cGMP accumulation at an alkaline pH (Khare et al., 1994; Hamra et al., 1997) and GC-C activity was markedly accentuated by ATPγS in the presence of Mg^{2+} (Khare et al., 1994). Furthermore, the results we previously obtained for the ultrastructural localization of GC-C stimulated by guanylin in the alimentary tract and associated glands suggest that the use of ATPγS in the presence of Mg^{2+} in the incubation medium at pH 8 seems to be essential for the complete ultrastructural detection of GC-C activity (Rambotti et al., 2000). From the biochemical assays, the maximal stimulatory effect of PACAP was produced at a concentration of 0.01 μM (Watanabe et al., 1992), and in our previous study, this concentration produced a good stimulatory effect on AC activity in human sweat glands (Rambotti et al., 2002a).

We found that the distribution of AC, GC-B and GC-C activity was similar in both normal mucosa and in mucosa of polyps. Only very few differences were detected. Enzymatic activities in epithelial layer cells and in glands were detected.

In ciliated cells, the present morphological observations indicate a different density of reaction products in both normal mucosa and mucosa of polyps.

CNP produced the highest amount of GC-B reaction products on ciliary membranes and basal bodies in both normal mucosa and mucosa of polyps. This result is in agreement with previous biochemical studies showing that CNP was the most potent of the natriuretic peptides in airway epithelial cells (Geary et al., 1993, 1995; Range et al., 1997). In primary ciliated airway epithelial cells, CNP induced increases in ciliary beat frequency via a cGMP-dependent mechanism (Geary et al., 1995). The presence of many reaction products of GC-B activity also in ciliary membranes of polyps seems to be in agreement with previous studies which found an increase in ciliary beat frequency in patients with nasal polyps compared to controls (Slater et al., 1996; Braverman et al., 1998).

Previous biochemical studies showed conflicting results over the presence of GC-C in ciliated cells (Geary et al., 1993; Range et al., 1997). We demonstrated that guanylin activated GC-C in ciliary membranes and basal bodies in normal mucosa. Very few reaction products of GC-C activity were present on ciliary membranes of polyps. This result may suggest a reduced activity of the ciliostimulatory effect in pathological mucosa.

Remarkable positivity of GC-B and GC-C activity was also detected on microvillar membranes in both normal mucosa and mucosa of polyps. This result may demonstrate that CNP and guanylin regulate other physiological processes, such as electrolyte transport. The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel protein that plays an important role in electrolyte and water transport through the respiratory epithelial cells, where it has a typical apical distribution. The ability of CNP and guanylin to stimulate Cl^- transport by CFTR activity has been detected in murine nasal epithelium and in normal human bronchial epithelial cells (Kelley et al., 1997; Zhang et al., 1998).

In normal and pathological mucosa, small quantities of AC reaction products were present on ciliated and microvillar membranes; AC activity was detected mainly in glands.

In the respiratory system of various animals, PACAP was found in close association with glandular structures where it stimulated mucus secretion

(Wagner et al., 1998; Uldman et al., 1991; Hauser-Kronberger et al., 1996). PACAP had no significant effect on the airway ionic currents in normal human bronchial epithelial cells (Zhang et al., 1998).

We demonstrated that PACAP, CNP and guanylin could stimulate enzymatic activity in goblet cells and in glands, suggesting a significant role in the secretion function. The reaction products were present on plasma membranes; but mainly in goblet cells do they appear to be localized also on the intracellular membranes which participate directly in the formation and discharge of secretory products. In some previous studies, we detected enzymatic activity on plasma and intracellular membranes in exocrine and endocrine cells. (Rambotti et al., 2002a,b). The subcellular distribution of AC activity is in agreement with previous enzyme-ultracytochemical studies (Poeggel and Luppa, 1988; Yamamoto et al., 1998). The mechanism whereby natriuretic peptides activate GC on intracellular membranes remains to be elucidated.

In conclusion, the results obtained by our methodological approach, comparing localization and amount of reaction products of AC, GC-B, GC-C activity, indicated no evident differences in the enzymatic activities of normal and pathological nasal mucosa.

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