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# Dynorphin expression, processing and receptors in the alveolar macrophages, cancer cells and bronchial epithelium of lung cancer patients

**Shaaban A. Mousa[1]*, Malgorzata Krajnik[2]*, Piotr Sobanski[3], Janusz Kowalewski[4], Elzbieta Bloch-Boguslawska[5], Zbigniew Zylicz[6] and Michael Schäfer[1]**

[1]Department of Anesthesiology and Intensive Care Medicine, Charité-Universitaetsmedizin Berlin, Campus Virchow-Klinikum and Campus Charite Mitte, Berlin, Germany, [2]Palliative Care Department, Collegium Medicum of the Nicolaus Copernicus University, Bydgoszcz, Poland, [3]Second Cardiology Department, Collegium Medicum of the Nicolaus Copernicus University, Bydgoszcz, Poland, [4]Department of Thoracic Surgery and Lung Disease, Collegium Medicum of the Nicolaus Copernicus University, Bydgoszcz, Poland, [5]Department of Forensic Medicine, Collegium Medicum of the Nicolaus Copernicus University, Bydgoszcz, Poland and [6]Dove House Hospice, Hull, United Kingdom

*These authors contributed equally to this work

**Summary.** Functional evidence suggests that opioid peptides such as dynorphin are involved in the regulation of airway macrophage functions and of human cancer growth. However, anatomical evidence for components of a putative dynorphin network within lung cancer patients is scarce. Tissue from lung cancer patients was examined immunohistochemically for all components of a local dynorphin (DYN) network. Double immunofluorescence microscopy analysis revealed colocalization of the opioid precursor PDYN with its end-product DYN, and key processing enzymes prohormone convertases 1 and 2 and carboxypeptidase E, as well as the kappa-opioid receptor (KOR) within alveolar macrophages and cancerous cells in varying degrees among patients. Moreover, chromograninA-immunoreactive pulmonary neuroendocrine cells expressing DYN were close to substance P- and KOR-immunoreactive sensory nerves. Our findings give a first hint of a neuroanatomical basis for a peripheral DYN network, conceivably regulating pulmonary, immune and cell-proliferative functions within the human lung, most likely in a paracrine/autocrine fashion.

**Key words:** Lung, Opioid peptides, Opioid receptors, Convertase, Carboxypeptidase

*Offprint requests to:* Malgorzata Krajnik, Palliative Care Department, Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Sklodowskiej-Curie 9, 85-094 Bydgoszcz, Poland. e-mail: malgorzata.krajnik@wp.pl

## Introduction

Dynorphin A, a 13 amino acid-long opioid peptide, was first isolated from the porcine pituitary (Chavkin et al., 1982) and has since been shown to be distributed widely throughout the central nervous system. Dynorphin (DYN) derives from processing of the precursor peptide prodynorphin (PDYN) and requires key enzymes, such as carboxypeptidase E (CPE) and prohormone convertases PC1 (also called PC1/3) and PC2, for post-translational processing into biologically active peptides (Dupuy et al., 1994; Berman et al., 2000, 2001; Boudarine et al., 2002). Evidence is accumulating that opioid peptides such as DYN, its precursor, and key processing enzymes are expressed by various cells of the immune system, e.g. lymphocytes and macrophages (Machelska et al., 2003; Mousa et al., 2004). These opioid peptides seem to play an important role in the regulation of both cellular and humoral immune responses and elicit receptor-mediated events such as cytokine production, phagocytosis and chemotaxis (reviewed by Carr and Blalock, 1989; Carr et al., 1988).

In addition to immune cells, PDYN has been identified in several different human tumour cell lines,

**Abbreviations.** ABC, Avidin-biotin complex; BSA, bovine serum albumin; CgA, chromogranin A; CPE, carboxypeptidase E; DAPI, 4'-6-diamidino-2-phenylindole; DAB, 3',3'-diaminobenzidine tetrahydrochloride; DYN, dynorphin; IR, immunoreactive; KOR, kappa-opioid receptor; PBS, phosphate-buffered saline; PC, prohormone convertase; PDYN, prodynorphin; PNECs, pulmonary neuroendocrine cells; SP, substance P.

e.g. small cell lung carcinoma (SCLC), large cell lung carcinoma (LCC), neuroblastoma and leukaemia (Geijer et al., 1991). Processing enzymes PC1, PC2 or CPE mRNA were detected in small cell carcinoma cells of the lung (Creemers et al., 1992; Mibikay et al., 1997; North and Du, 1998; Rounseville and Davis, 2000). Naltrexone, an opiate antagonist, had both stimulatory and inhibitory effects, depending on the dosage (Zagon and McLaughlin, 1983a). Interestingly, the blockade of endogenous opioids by naltrexone, a competitive antagonist at $\mu$- and κ-opioid receptors, inhibited cell proliferation in SCLC and non-SCLC cell lines (Zagon and McLaughlin, 1983b).

Although there is some evidence for DYN binding sites throughout the rat lung (Sato et al., 1990) and DYN influencing pulmonary function (Horii et al., 1990; Kamikawa and Shimo, 1990; Wang et al., 2003), as well as cell growth (Maneckjee et al., 1990), little is known regarding the exact localization and processing of DYN within human lung tissue.

Therefore, we systematically examined the presence of the endogenous opioid peptide DYN, its precursor, processing enzymes and corresponding opioid receptors throughout the anatomical structures of human lung tissue from lung cancer patients.

## Materials and methods

### *Patients and preparation of human lung tissue*

The study protocol adhered to the International Guidelines of the Declaration of Helsinki 2004 (World Medical Association: http://www.wma.net) and was approved by the Ethics Committee of the Nicolaus Copernicus University in Torun, Poland. Human lung tissue samples were obtained from 12 lung cancer patients undergoing a lobectomy or pneumonectomy at the Department of Thoracic Surgery and Lung Disease at the Oncology Centre in Bydgoszcz, Poland (Table 1). All patients were informed as to the purpose of the study and gave their written consent. The samples (1 $cm^3$ each) were taken from different areas of the lung tissue of these patients (e.g. margins of resected bronchi, lung parenchyma, non-cancerous and cancerous areas). Immediately after surgery, the tissue samples were fixed and tissue sections processed as described previously (Mousa et al., 2007a).

### *Single immunostaining for light microscopy*

The tissue sections were processed for DYN immunohistochemistry with a vectastain avidin-biotin peroxidase complex (ABC) kit as described previously (Mousa et al., 2007a,b). All incubations were carried out at room temperature and PBS was used for washing (three times for 10 min.) after each step. Sections were incubated for 45 min. in PBS with 0.6% $H_2O_2$ and 50% methanol to block endogenous peroxidase, and for 60 min. in PBS containing 0.3% Triton X-100, 1% BSA, 10% goat serum (Vector Laboratories, Burlingame, CA) (blocking solution) to prevent non-specific binding. The sections were then incubated overnight with polyclonal rabbit antibodies against DYN (1:1000, Peninsula Laboratories, Belmont, CA, USA), afterwards for 90 min. with a goat anti-rabbit biotinylated secondary antibody (Vector Laboratories) and for another 90 min. with ABC (Vector Laboratories). Finally, specific immunostaining was detected with 3',3'-diaminobenzidine tetrahydrochloride (Sigma, Taufkirchen, Germany). After the enzyme reaction, for the identification of cancerous cells by certain morphological criteria the slide-mounted tissue sections were counterstained with thionin, then dehydrated and cleared. Immunoreactive cancerous cells were identified by the following morphological criteria: cell division figures, nucleus enlargement and chromatin condensation. In addition, corresponding sections immunostained with hematoxylin-eosin were used for the histological characterization of the type of lung tumour.

**Table 1.** Patients' demographic characteristics.

| Nr | Sex | Age (years) | Histopathological diagnosis | TNM classification | Surgery |
|---|---|---|---|---|---|
| 1 | M | 65 | Adenocarcinoma | T2 N0 M0 | Lobectomy |
| 2 | M | 55 | Squamous cell carcinoma | T3 N1 M0 | Pneumonectomy |
| 3 | F | 57 | Adenocarcinoma | T2 N0 M0 | Lobectomy |
| 4 | M | 60 | Squamous cell carcinoma | T2 N0 M0 | Lobectomy |
| 5 | M | 51 | Leiomyosarcoma | T2 N0 M0 | Pneumonectomy |
| 6 | M | 52 | Adenocarcinoma | T1 N0 M0 | Lobectomy |
| 7 | M | 63 | Squamous cell carcinoma | T3 N1 M0 | Lobectomy |
| 8 | M | 55 | Small cell carcinoma | T2 N0 M0 | Pneumonectomy |
| 9 | M | 83 | Squamous cell carcinoma | T2 N2 M0 | Lobectomy |
| 10 | M | 64 | Squamous cell carcinoma | T2 N0 M0 | Lobectomy |
| 11 | F | 75 | Mesenchymoma | T2 N0 M0 | Lobectomy |
| 12 | M | 64 | Squamous cell carcinoma | T2 N0 M0 | Lobectomy |

M, male; F, female

### *Double immunofluorescence staining*

Double immunofluorescence staining was processed as described previously (Mousa et al., 2007b). Briefly, slide-mounted or floating tissue sections were incubated for 60 min. in a blocking solution. The sections were then incubated overnight with the following antibodies: 1) guinea pig anti-PDYN (Neuromics, MN, USA) in combination with rabbit anti-DYN (Peninsula Laboratories), anti-PC1, anti-PC2 or anti-CPE (all diluted to 1:1000); 2) rabbit anti-DYN in combination with mouse monoclonal anti-KOR (10 $\mu$g/ml, R&D Systems, Germany), anti-CD68 (clone KP1; Dako, Hamburg, Germany) or anti-chromogranin A (CgA; Abcam plc, Cambridge, UK); 3) rabbit anti-KOR (Dr. S.J. Watson, Ann Arbor, Michigan, USA) in combination with guinea pig antibody against substance P (SP; 1:1000; Peninsula Laboratories). After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with the appropriate secondary antibodies. Thereafter, sections were washed with PBS and the nuclei counterstained bright blue with 4'-6-Diamidino-2-phenylindole (DAPI) (0.1 $\mu$g/ml in PBS) (Sigma). Finally, the tissue sections were washed in PBS, mounted in vectashield (Vector Laboratories) and viewed under a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Göttingen, Germany).

### *Specificity controls*

To demonstrate specificity of staining, the following controls were included as described in detail elsewhere (Mousa et al., 2004): 1) pre-absorption of diluted antibody against DYN, KOR, CPE, PC1 or PC2 with 10 $\mu$g/ml purified DYN (Peninsula Laboratories), KOR, CPE, recombinant PC1 or PC2 antigens respectively; 2) omission of either the primary or secondary antibodies.

## Results

### *DYN immunoreactivity in different cells of human lung cancer tissue*

DYN immunoreactivity was identified in alveolar macrophages accumulating within the alveolar lumen (Fig. 1A), in numerous cancer cells infiltrating human lung tissue (Fig. 1B), in sparse solitary cells within bronchial epithelium (Fig. 1C) and nerve fibres arborizing within bronchial submucosa (Fig. 4Q). Pre-absorption of antibody against DYN with the respective antigenic peptide, as well as the omission of the primary antibody, revealed no significant immunoreactivity (data not shown).

### *Co-expression of PDYN, DYN and key processing enzymes in alveolar macrophages*

Double immunofluorescence confocal microscopy showed that most of the alveolar macrophages co-

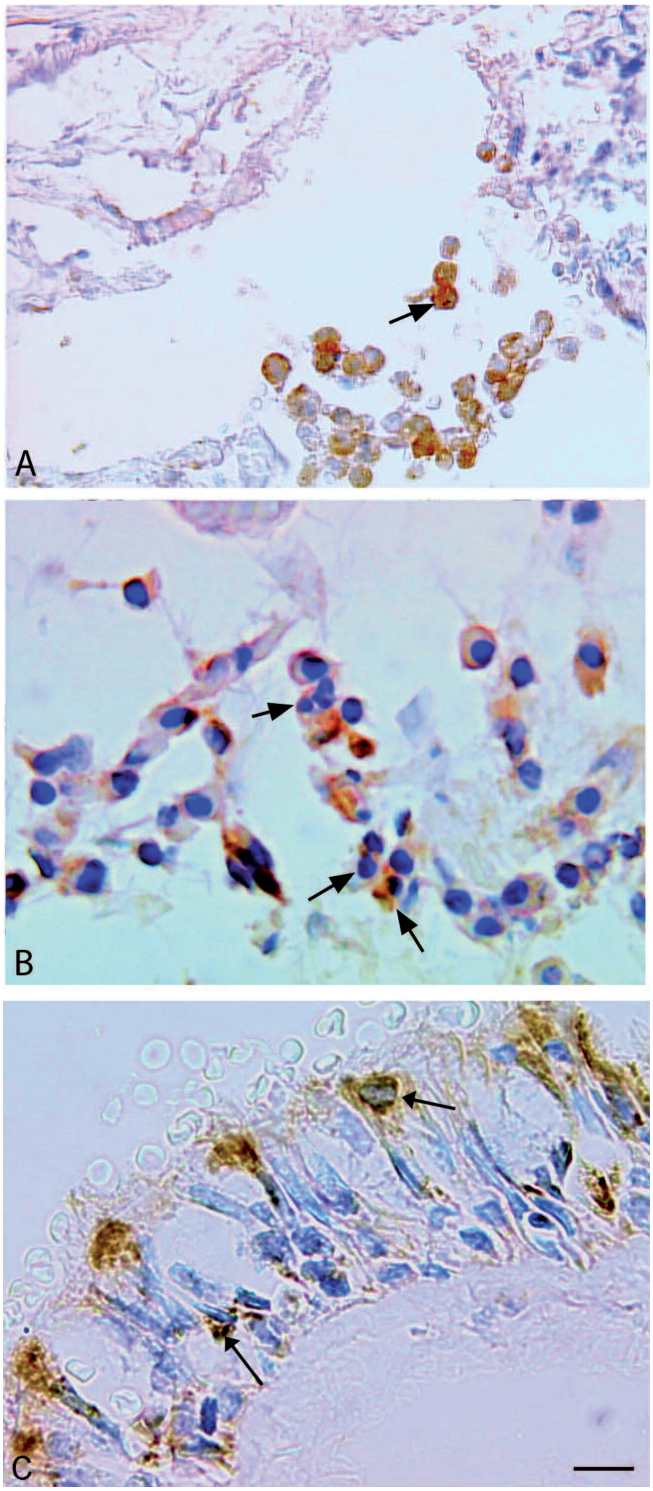


**Fig. 1.** Immunohistochemical localization of DYN in alveolar macrophages (arrow) within the alveolar lumen **(A)**, cancerous cells (arrows) **(B),** cells within epithelium (ep) **(C)** in lung cancer patients. **C** shows DYN immunoreactivity in sparse solitary cells (arrows) within the bronchial epithelium. **B** shows cell division figures (arrows), nucleus enlargement, and chromatin condensation of DYN-IR cancer cells. Bar: 20 $\mu$m.

expressed PDYN with DYN, PC1, PC2 or CPE. Only a few such cells were immunostained for PDYN, DYN, PC1, PC2 or CPE alone (Fig. 2A-P). We were able to demonstrate that the majority (≥80%) of the identified alveolar macrophages co-expressed the entire components required for DYN processing in all types of lung cancer. The identity of these cells as macrophages was confirmed by using CD68 as a cellular marker

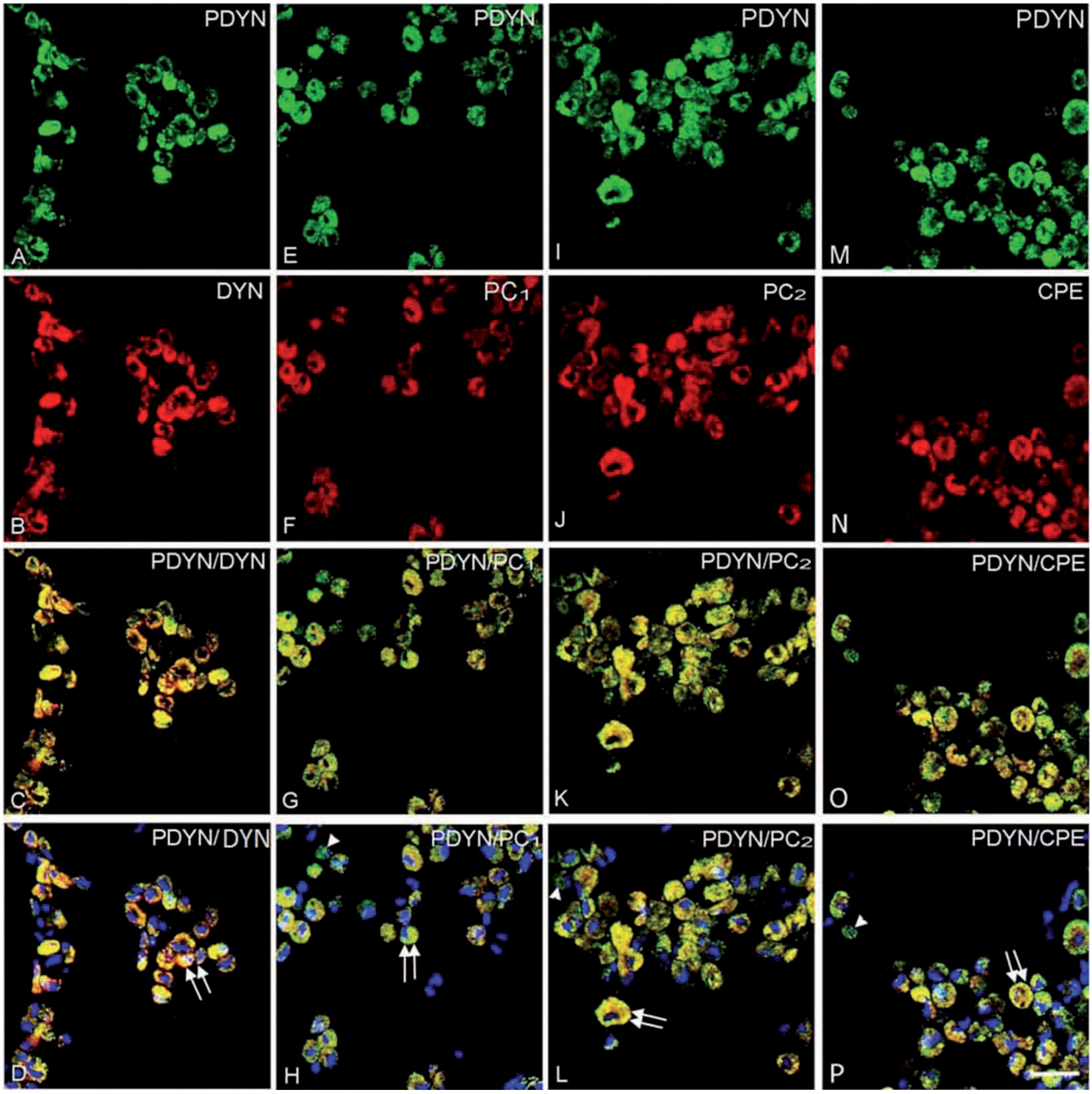


**Fig. 2.** Confocal microscopy of PDYN (**A, E, I, M)** (green fluorescence) with DYN (**B**), PC1 (**F**), PC2 (**J**) or CPE (**N**) (red fluorescence) double immunofluorescence in alveolar macrophages within tissue from lung cancer patients. Note that most of PDYN-IR alveolar macrophages express DYN (**C, D**), PC1 (**G, H**), PC2 (**K, L**) or CPE (**O, P**). **C, D, G, H, K, L, O and P** are combined images showing colocalization (double arrows) of PDYN with DYN (**C, D**), PC1 (**G, H**), PC2 (**K, L**) or CPE (**O, P**). Note: some PDYN-IR cancerous cells (arrowheads) do not express DYN (**D**), PC1 (**H**), PC2 (**L**) or CPE (**P**). **D, H, L and P** show DAPI nuclear staining (bright blue). Bar: 20 $\mu$m.

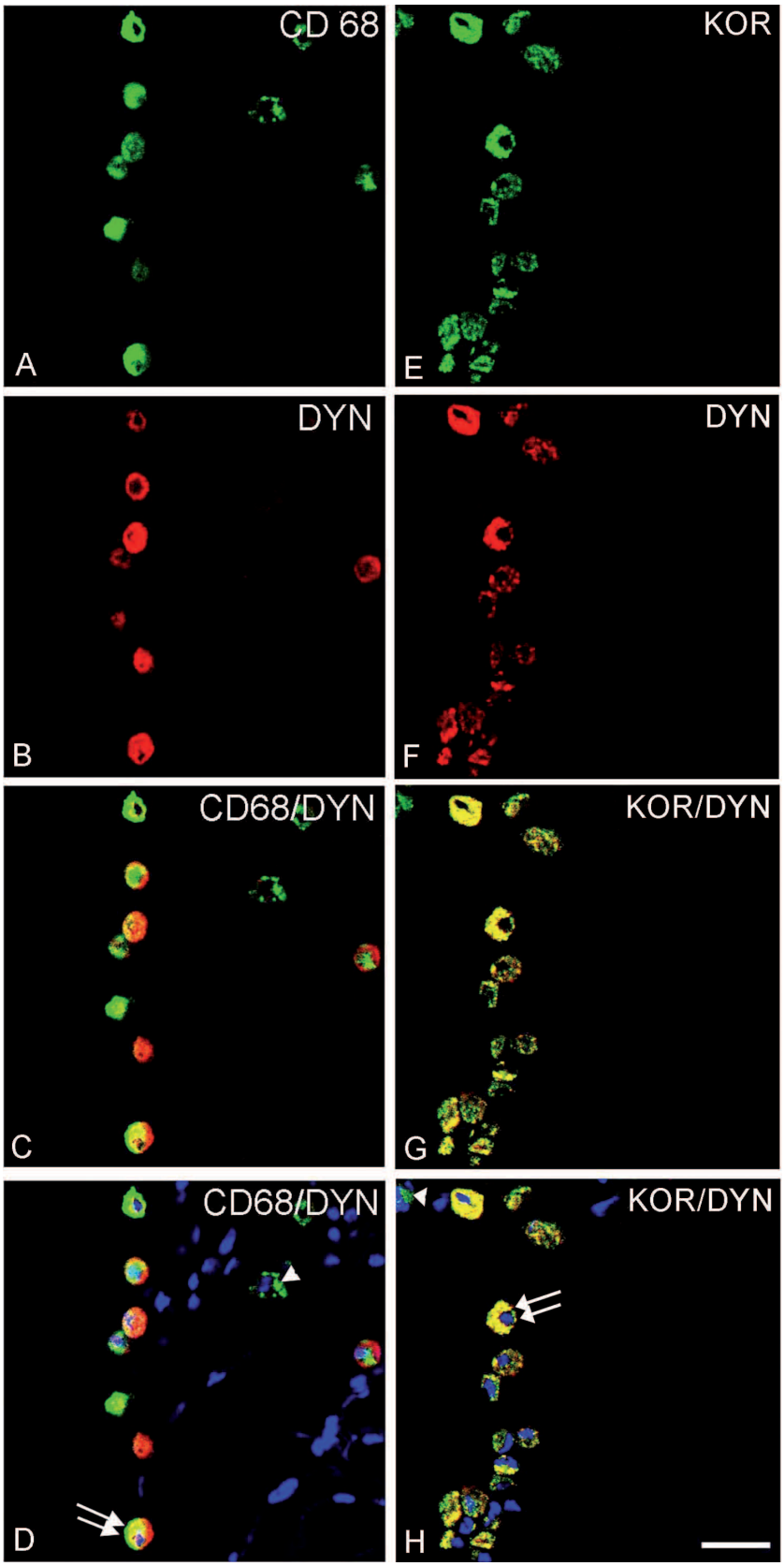


**Fig. 3.** Confocal microscopy of CD68 (**A**) or KOR (**E**) (green fluorescence) with DYN (**B, F**) (red fluorescence) double immunofluorescence in alveolar macrophages within tissue from lung cancer patients. Note that most of CD68- or KOR-IR alveolar macrophages express DYN (**B, F**). **C, D, G and H** are combined images showing colocalization of CD68 (**C, D**) or KOR (**G, H**) with DYN (double arrows) but some alveolar macrophages stained only with CD68 (arrowhead). **D and H** show DAPI nuclear staining (bright blue). Bar: 20 $\mu$m.

specific for macrophages (Fig. 3A-D). Moreover, DYN-immunoreactive (IR) alveolar macrophages expressed KOR (Fig. 3E-H).

### *Co-expression of PDYN, DYN and key processing enzymes in cancerous cells*

In areas of bronchial lesions infiltrated by numerous cancerous cells, double immunofluorescence staining showed colocalization of PDYN with DYN, PC1, PC2 or CPE. Only a few of the cancerous cells contained PDYN, DYN, PC1, PC2 or CPE alone (Fig. 4A-P). In the same cells, DYN immunoreactivity was detected with its corresponding receptor KOR (Fig. 4R-T). The active peptide DYN and its precursor were detected in cancer cells of all types of lung cancer. Apparently, the number of immunoreactive cancer cells infiltrating lung tissue varied among different types of cancer.

### *Co-expression of DYN and KOR in CgA-IR neuroendocrine cells and SP-IR sensory nerve fibres of bronchial epithelium*

Within bronchial epithelium, DYN immunoreactivity was observed in solitary pulmonary neuroendocrine cells (PNECs) identified by CgA, a

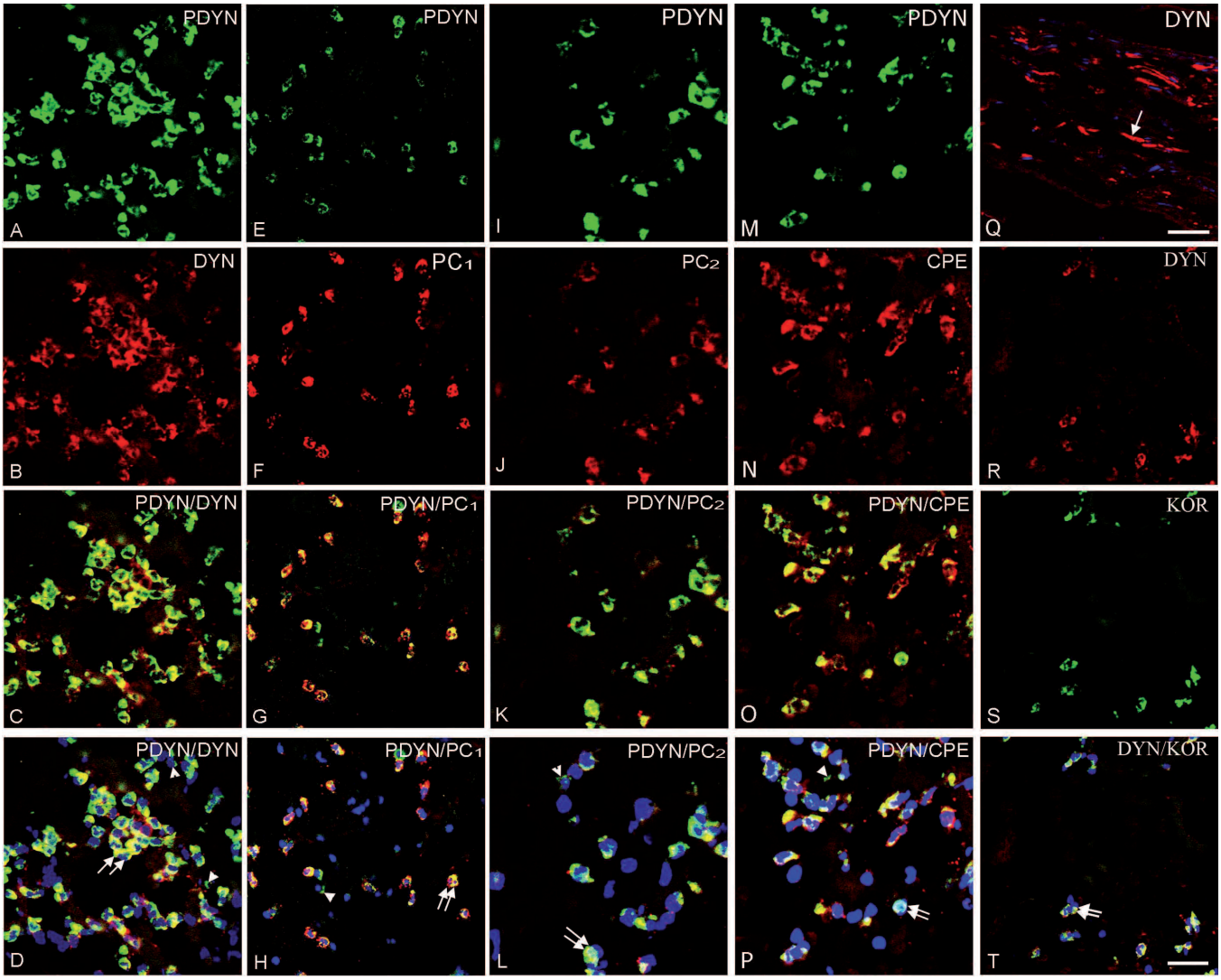


**Fig. 4.** Confocal microscopy of PDYN (**A, E, I, M**) (green fluorescence) with DYN (**B**), PC1 (**F**), PC2 (**J**) and CPE (**N**) (red fluorescence) and (**R**) DYN (red fluorescence) with KOR (**S**) (green fluorescence) double immunofluorescence within cancerous cells in tissue from lung cancer patients. **C, D, G, H, K, L, O, P, S and T** are combined images showing colocalization (double arrows) of PDYN with DYN (**C, D**), PC1 (**G, H**), PC2 (**K, L**) or CPE (**O, P**) and DYN with KOR (**T**). **D, H, L, P and T** show DAPI nuclear staining (bright blue). Note: some PDYN-IR cancerous cells (arrowheads) do not express DYN (**D**), PC1 (**H**), PC2 (**L**) or CPE (**P**). **Q** shows DYN immunoreactivity or nerve fibres in submucosa within tissue from lung cancer patients. Bar: 20 $\mu$m.

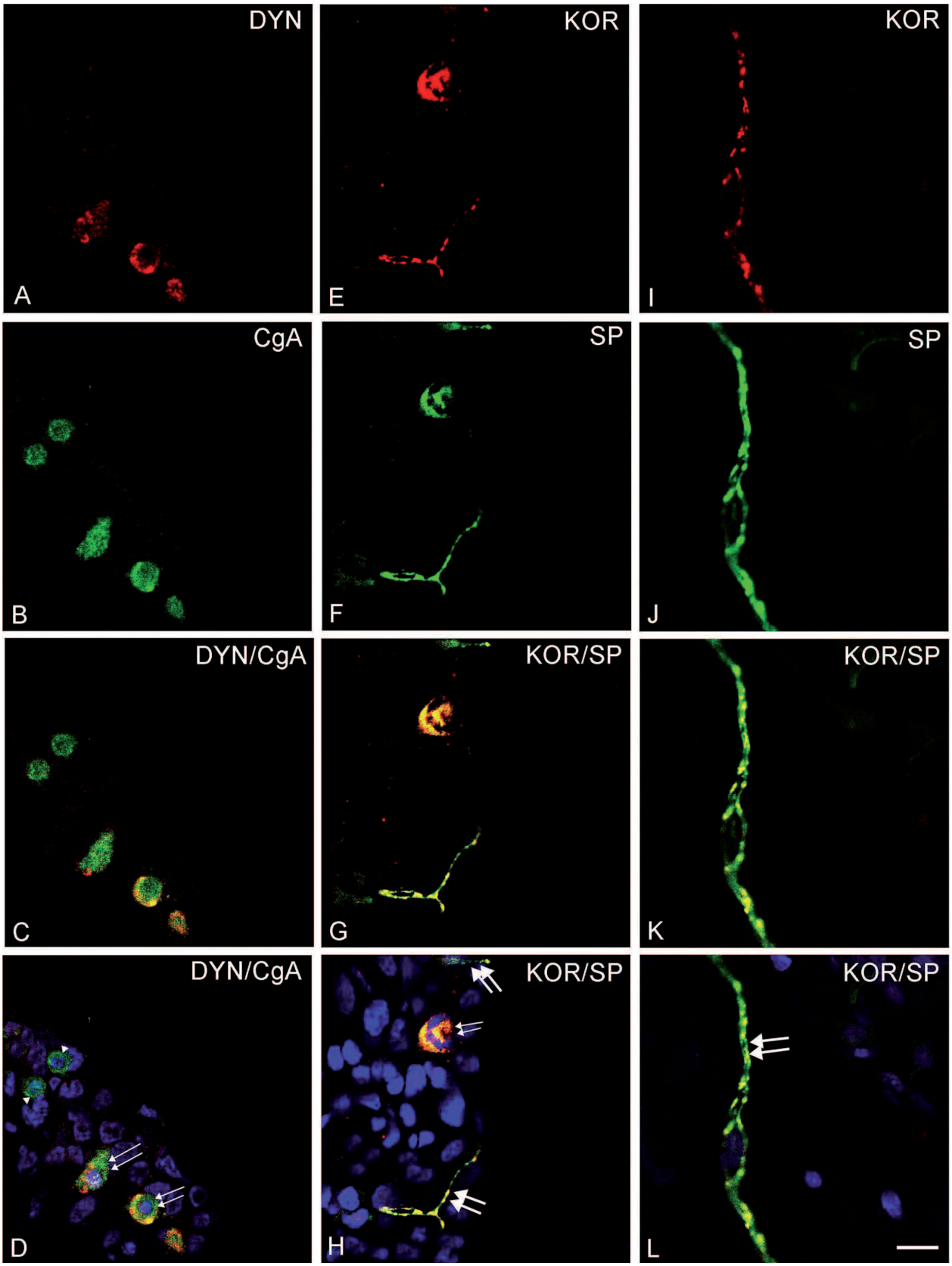


**Fig. 5.** Confocal microscopy of DYN (red fluorescence) with CgA **(B)** and DOR (**E, I)** (red fluorescence) with SP (**F, J**) (green fluorescence) double immunofluorescence in bronchial epithelium (**A-H**) or submucosa (**I-L**) in lung cancer patients. **C and D** are combined images showing colocalization of DYN with CgA-IR pulmonary neuroendocrine cells (PNECs) (**C, D**) (double arrow), but some CgA-IR PNECs cells do not express DYN (arrowheads). Within bronchial epithelium KOR-IR nerve fibres expressing sensory neuronal marker SP (double arrows) penetrate it up to its luminal surface (**G, H**). **K and L** show KOR-IR nerve fibres expressing sensory neuronal marker SP (double arrows) within submucosa. **D, H and L** show DAPI nuclear staining (bright blue). Bar: 20 $\mu$m.

marker for neuroendocrine structures (Cutz et al., 2007; Haworth et al., 2007) (Fig. 5A-D). Only a few PNECs did not express DYN (Fig. 5C, D). PNECs also co-expressed KOR with SP (Fig. 5E-H). Moreover, we were able to demonstrate nerve fibres co-expressing KOR with SP arborizing into the luminal part of the epithelium and forming a fine network of thin nerve fibres (Fig. 5E-H) and into bronchial submucosa (Fig. 5 I-L).

## Discussion

We identified to varying degrees essential components required for DYN synthesis, processing and its corresponding receptors within lung tissue of lung cancer patients by 1) the anatomical/histological localization of DYN-IR cells in different areas of human lung; 2) the colocalization of the opioid precursor PDYN with DYN, PC1, PC2, CPE and KOR in alveolar macrophages and cancerous cells; 3) the localization of DYN immunoreactivity in bronchioepithelial pulmonary neuroendocrine cells (PNECs); 4) the identification of KOR on alveolar macrophages, cancerous cells and SP-IR PNECs and sensory nerve fibres within bronchial epithelium. Taken together, our results provide the first evidence of the expression and processing of PDYN into functionally active peptides, such as DYN in alveolar macrophages, cancerous cells and bronchioepithelial PNECs, as well as the presence of its corresponding opioid receptors located – among others – on sensory nerve endings within bronchial epithelium and bronchial submucosa. These findings may offer a morphological basis for a putative immune and/or tumour modulatory role of DYN within the human lung, which may act in a paracrine/autocrine fashion (Maneckjee et al., 1990).

Using light microscopy, we provided new evidence for DYN immunoreactivity in macrophages accumulating within the alveolar lumen, in cancerous cells infiltrating the bronchial wall, and in sparse solitary cells within bronchial epithelium. Consistent with previous reports (Kurkowski et al., 1990; Kummer et al., 1992), we also found DYN immunoreactivity in a few nerve fibres within the bronchial wall.

In addition, double immunofluorescence confocal microscopy showed colocalization of the opioid peptide precursor PDYN with its end-product DYN and the key processing enzymes PC1, PC2 and CPE, as well as KOR in alveolar macrophages, clearly identified by the specific marker CD68. Although this is the first description of DYN within airway macrophages, PDYN mRNA has recently been detected in the human macrophage U-937 cell line (Sun et al., 2006). Previously, we also identified DYN-immunoreactivity in the mononuclear cells of inflamed subcutaneous tissue (Przewłocki et al., 1992; Machelska et al., 2003). Most interestingly, in our study DYN-IR alveolar macrophages colocalized with the corresponding opioid receptor KOR, suggesting an autocrine mechanism of regulation. These findings are in agreement with the identification of KOR on bone marrow macrophages (Maestroni et al., 1999). In functional studies, DYN seems to modulate macrophage functions such as chemiluminescence, cytokine release, chemokine production, chemotaxis and phagocytosis (Tosk et al., 1993; Ichinose et al., 1995; Sacerdote, 2003; Vujiç et al., 2004). Taken together, endogenous DYN and its receptors expressed in monocytes/macrophages of the lung may modulate airway (Belvisi et al., 1992), as well as immune (Bidlack et al., 2006) functions in an autocrine/paracrine way within the lungs of human lung cancer patients.

Several studies report that DYN and its receptor are potent inhibitors of cell growth in a variety of human and animal tumours, including lung tumours (Maneckjee and Minna, 1990; Hatzoglou et al., 1996; Kampa et al., 1996). However, nothing has been shown yet of PDYN, DYN and key processing enzyme expression within human lung cancer tissue. In this study, we were able to demonstrate colocalization of PDYN together with its key processing enzymes, its end-product DYN, and corresponding receptor KOR in cancerous cells of lung tissue. Our observations are in agreement with the identification of PDYN mRNA (Geijer et al., 1991) as well as immunoreactive opioid peptides (beta-endorphin, enkephalin or dynorphin) (Maneckjee and Minna, 1990) within cell lines of human small cell lung carcinoma. Our identification of KOR in lung cancer cells is in agreement with previous reports that α-casomorphins mediate their antiproliferative activity mainly via kappa-opioid binding sites in a breast carcinoma cell line which does not express mu-opioid receptors (Hatzoglou et al., 1996; Kampa et al., 1996). In the human breast carcinoma cell line MCF-7, mu-, delta- and kappa-opioid receptor-selective agonists were shown to suppress cell proliferation in a naloxone-reversible manner (Maneckjee et al., 1990). Importantly, the blockade of endogenous opioids by the receptor antagonist naltrexone inhibited (Zagon and McLaughlin, 1983a) or accelerated tumour proliferation (McLaughlin et al., 1999; Zagon and McLaughlin, 2005; Zagon et al., 2007) depending on the dosage. Taken together, our present findings provide morphological evidence for a possible endogenous opioid control of tumour cell proliferation within human lungs (Murgo, 1985; Scholar et al., 1987; Maneckjee et al., 1990; Geijer et al., 1991).

Another essential regulator of pulmonary functions, the PNECs within the bronchial epithelium, seems to be under the control of endogenous opioids. Our findings showed DYN immunoreactivity within PNECs. Through use of CgA, a specific marker for neuroendocrine cells, we could clearly differentiate PNECs from other cells of the bronchial epithelium (Cutz et al., 2007; Haworth et al., 2007). PNECs are part of the diffuse neuroendocrine system within the bronchial epithelium. They display endocrine and paracrine secretory mechanisms and are associated with a dense network of sensory nerve fibres (see reviews by Adriaensen et al., 2006; Linnoila, 2006). Consistently, we indentified KOR, not only directly on

PNECs but also on the numerous sensory SP-IR nerve fibres neighbouring them and arborizing from there up to the apical part of the bronchial epithelium and within bronchial submucosa. Consistently, previous studies have identified opioidergic nerves within airway mucosa and opioid binding sites within lung tissue (Cabot et al., 1994; Bhargava et al., 1997). Therefore, DYN may affect airway functions following its putative local release from either sensory nerve endings innervating bronchial epithelium (Shimosegawa et al., 1989; Barnes, 1991) or from pulmonary endocrine cells (Cutz et al., 1981).

In summary, our present study demonstrates the presence of PDYN as well as key processing enzymes (PC1, PC2 and CPE) required for PDYN processing into the biologically active peptide DYN within alveolar macrophages and cancerous cells of lung cancer patients. In addition, we identified a network of KOR on alveolar macrophages, cancerous cells, CgA -IR PNECs containing DYN, and on SP-IR sensory nerve fibres within bronchial epithelium and bronchial submucosa. This can be observed to varying degrees in all types of lung cancer. Taken together, we provide a first hint of a neuroanatomical basis for a peripheral DYN network within the human lung, conceivably regulating pulmonary, immune and cell proliferation functions, most likely in a paracrine/autocrine fashion.

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