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NEUROPEPTIDE K: A MAJOR TACHYKININ IN PLASMA AND TUMOR TISSUES FROM CARCINOID PATIENTS

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Summary: Evidence is presented for the presence of an entire family of tachykinin-immunoreactive peptides in plasma and tumor tissues from patients with carcinoid tumors. The peptides include in addition to substance P and neurokinin A; neurokinin B, an eledoisin like peptide and neuropeptide K - a 36 amino acid long tachykinin which contains neurokinin A at its C-terminus. Neuropeptide K seems to be the tachykinin which is present in highest concentrations in plasma as well as in acetic acid extracts of tumor tissues. It is highly biologically active, and may therefore contribute to the clinical symptoms of carcinoid tumors.

Carcinoid tumors contain and secrete several biologically active substances such as 5 hydroxy tryptamine (1), histamine (2), kallikrein (3), prostaglandins (4) and tachykinins such as substance P (5) and neurokinin A (6). Tachykinins occur both in mammals, e.g. substance P (SP) (7), neurokinin A (NKA) (8,9, 10,11), neurokinin B (NKB) (12), neuropeptide K (13) as well as in nonmammalian species e.g. eledoisin (ELE) and kassinin (KAS) and physalemin (PHY) (14,15). The various tachykinins have C-terminal amino acid homologies and share characteristic biological effects such as smooth muscle contraction and lowering of the blood pressure. Recently the structure of two types of bovine brain SP precursors (α and β preprotachykinins; α-PPT and β-PPT

Abbreviations: NPK, neuropeptide K; NKA, neurokinin A (neurokinin α, neuromedin L); NKB, neurokinin B (neurokinin β, neuromedin K); SP, substance P; ELE, eledoisin; KAS kassinin; PHY, physalemin; rpHPLC, reversed phase high performance liquid chromatography.

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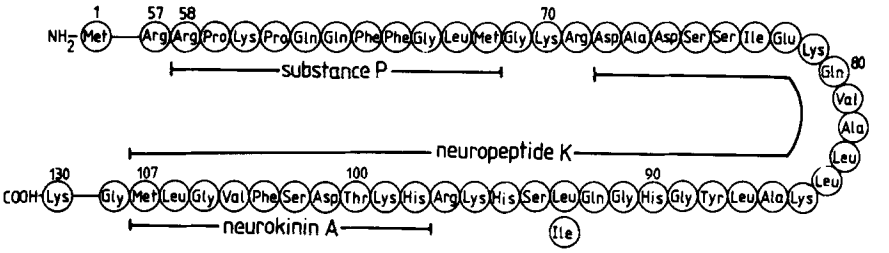


Figure 1. Primary structure of the part of the β-preprotachykinin contanining the sequence for SP, NPK and NKA.

respectively) was elucidated by determining their cloned cDNA sequences (8,9) (Fig 1). β-PPT contains not only the SP sequence, but also the sequence for NKA and the elongated form of NKA, neuropeptide K (NPK) (13), whereas α-PPT contains only the sequence for SP, thus supporting the hypothesis that a number of tachykinins may be present in mammalian tissues.

Using antisera raised against ELE and KAS we recently found elevated levels of tachykinin immunoreactivity in plasma and tumor tissues from patients with carcinoid tumors (6). Chromatographic analysis indicated the presence of several immunoreactive components, of which one was immunochemically and chromatographically similar to NKA. However, a major immunoreactive component occurring both in plasma and in acetic acid extracts of tumor tissues did not coelute with any of the tachykinins known at that time. We now present data strongly indicating that this component is identical with NPK.

MATERIALS AND METHODS

Plasma samples from 7 patients and tumor tissues from 5 patients (liver metastases or primary tumors obtained either during operative treatment, or at liver biopsy) were investigated. The tumor tissues were all positive according to the Masson (16) and Grimelius (17) technique. All patients investigated had liver metastases, and all had daily flush attacks and watery diarrhoea.

Blood samples were collected in chilled tubes containing heparin (Kabi, Stockholm) and aprotinin (Trasylol^{R}, Bayer, Leverkusen) to give a final concentration of 20 IU/ml blood and 400 KIU/ml blood respectively. The blood samples were kept on ice for not more than 15 min before centrifugation at 4°C. The supernatants were sucked off and kept at - 20°C. Plasma samples were thawed and extracted in acid ethanol. 1.5 ml of concentrated (min 37%) hydrochloric acid (Merck) was added to 1000 ml of absolute ethanol and 1.0 ml of the acid ethanol added to 0.5 ml of plasma. After vortexing for 30 sec, the samples were centrifugated at 2500 x g for 15 min. The supernatants were decanted into other labelled tubes and evaporated to dryness at 45°C under nitrogen

gas. The samples were redissolved in the starting eluent before chromatographic analysis.

Tissue samples were rapidly frozen and stored not more than 3 weeks at - 20°C before extraction. They were weighed in the frozen state, cut in small pieces, and boiled for 10 min in neutral water before homogenization with a polytron and centrifugation. The supernatants were collected and stored at - 20°C until chromatography. The pellets were then reextracted in 10 vol of 1 mol/l acetic acid in the same manner, and the supernatants frozen and lyophilized.

Cation-exchange chromatography was performed using CM Sephadex C-25 (Pharmacia Fine Chemicals) column 2.6x20 cm. It was eluted at 4°C with a gradient made by having 250 ml of 0.025 M ammonium bicarbonate in the mixing chamber and 250 ml 0.25 M ammonium bicarbonate in the reservoire. Both eluents contained 0.02% sodium azide. Two ml fractions were collected at an elution rate of 0.6 ml/min, lyophilized, reddissolved in 100 µl of distilled water, and assayed for immunoreactivity in the tubes used for collection of fractions.

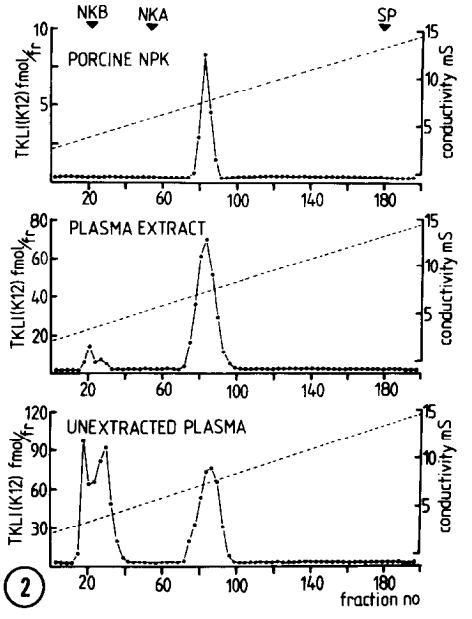
Reversed phase high performance liquid chromatography (rpHPLC) was performed using nucleosil C18, 5µ (Merck),4.6x300 mm column eluted with a 40 min linear gradient of 20% acetonitrile (Rathburn Chemicals, HPLC grade S) in water containing 0.1% trifluoroacetic acid (TFA) to 40% acetonitrile in water containing 0.1% TFA. Two LDC/Milton Roy Constametric pumps were controlled by Sinclair Spectrum/Bercol microcomputer. Samples were passed through Millipore GS filters (0.22 µm) to remove particulate matter before chromatography. Samples of 100 µl were injected using Rheodyne injector (7l5S). 0.5 ml fractions were collected at an elution rate of 1.0 ml/min. Each fraction was lyophilized and redissolved in 100 µl of distilled water. The fractions were assayed for immunoreactivity in the tubes used for collection of fractions.

A competitive immunoassay was based on antiserum K12 (11) raised in a rabbit against a kassinin conjugate. NKA was labelled to a specific activity of 69 Bq/fmol according to Bolton and Hunter (18) and used as radioligand and synthetic NKA was used as standard. The IC_{50} value of antiserum K12 (the amount of unlabelled peptide in the assay tube inhibiting the maximal binding by 50%) was 21.8 fmol of NKA (19) and the detection limit for NKA was 0.6 fmol/tube (20). Using the crossreactivity to NKA as the 100% reference, the crossreactivity of antiserum K12 to other tachykinins was KAS 84%, ELE 30%, NKB 26% and NPK 18%. The crossreactivity for SP, PHY, neuromedin B, neuromedin C and bombesin was less than 0.01%. NPK diluted in paralell with the NKA standard curve.

SP was determined using a competitive radioimmunoassay based on antiserum SP2 (21) which was very specific for SP (less than 0.05% crossreactivity for NKA, NKB, NPK, KAS, ELE, PHY and bombesin).

RESULTS AND DISCUSSION

The immunoreactive material detected with antiserum K12 (subsequently termed TKLI(K12)) in unextracted plasma eluted as two major components at cation-exchange chromatography (Fig. 2). The first component, which eluted in the void volume, was removed by ethanol extraction of the plasma samples prior to chromatography. The second component, which constituted the major portion



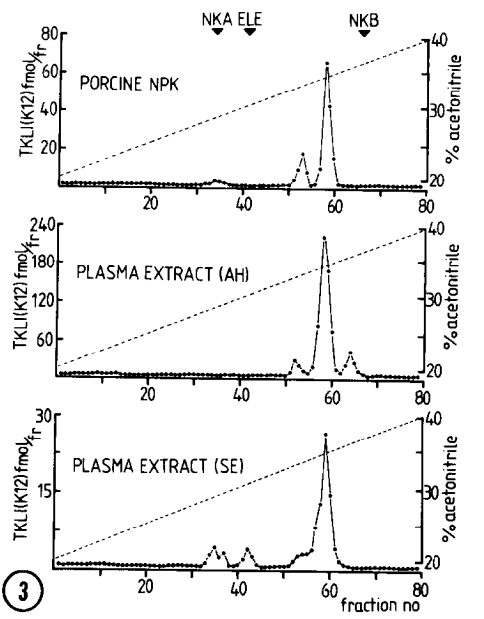


Figure 2. Chromatographic analysis of purified porcine NPK, extracted and unextracted plasma from a carcinoid patient (AH) on CM Sephadex C-25. The column was calibrated with NKB, NKA and SP.

Figure 3. Reversed phase high performance liquid chromatography of plasma from carcinoid patients. The immunoreactivity was analysed with antiserum K12. The column was calibrated with NKA, ELE and NKB.

of TKLI(K12) in plasma extracts, eluted in the position of porcine NPK. Also at rpHPLC a component with the chromatographic characteristics of NPK was found in extracts of plasma samples from all seven patients at rpHPLC (Fig. 3). This was the major TKLI(K12) component in plasma from all patients, but smaller immunoreactive peaks corresponding to NKA, ELE and NKB were found in five, two and one out of seven plasma samples respectively.

Reversed phase HPLC of neutral extracts of tumor tissue from all five patients separated the TKLI(K12) into two major immunoreactive components eluting in the position of NKA and ELE respectively (Fig. 4). The same two components were also found in the acetic acid extracts, but the latter component was in that case considerably smaller (Fig. 4). The TKLI(K12) in a neutral water extract applied to cation-exchange column (same conditions as in Fig. 2) eluted also as two components, one in the void volume and the other in the position of NKA. When the first component from the cation-exchange chromatography was applied to the rpHPLC column, it eluted in the position of ELE, while the second component eluted in the position of NKA (results not

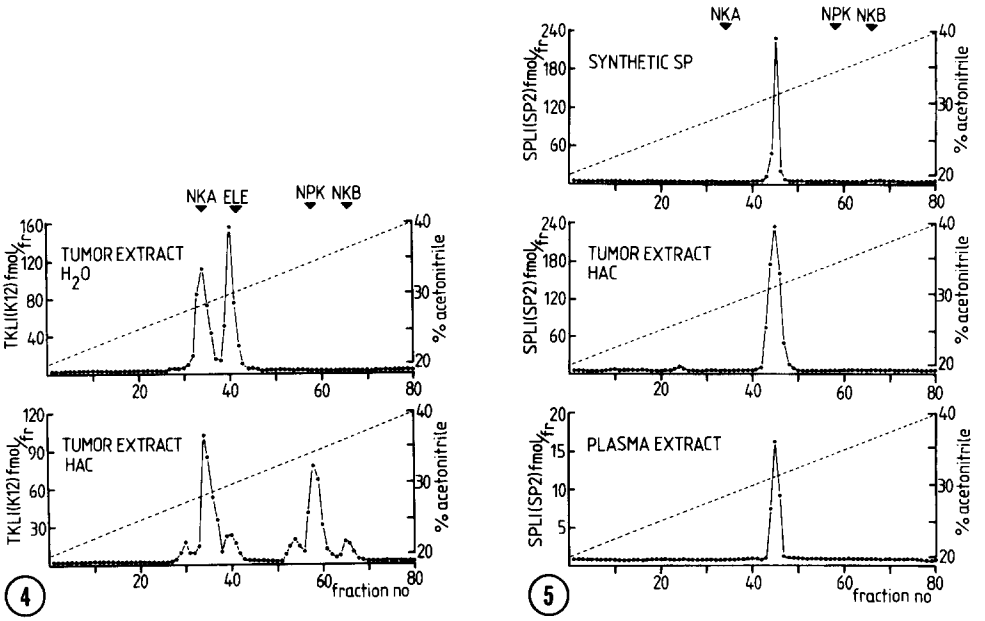


Figure 4. Reversed phase high performance liquid chromatography of tumor extracts from carcinoid patients. The immunoreactivity was analysed with antiserum K12. The column was calibrated with NKA, ELE, NPK and NKB.

Figure 5. Reversed phase high performance liquid chromatography of plasma and tumor extract from a patient with carcinoid tumor. The immunoreactivity was analysed with antiserum SP2. The column was calibrated with NKA, NPK and NKB.

shown). Acetic acid extracts revealed the presence of two additional components in the position of NPK and NKB respectively (Fig. 4). The component corresponding to NPK was present in acetic acid extracts of all five tumor tissues, but the component corresponding to NKB was only present in one out of five extracts.

SP-like immunoreactivity eluting in the position of synthetic SP was detected in three out of seven plasma samples at rpHPLC (Fig. 5). SP immunoreactivity was similarily found in four out of five in tumor extracts eluting as a single peak in the position of synthetic SP (Fig. 5).

The present results show the presence of an entire family of immunoreactive tachykinins in tumor tissues and plasma from carcinoid patients. Our data support earlier findings of SP (22) and NKA (6) in plasma and tumor extracts from carcinoid patients.

ELE and NKB eluted both in the void volume at ion-exchange chromatography. The identity of the immunoreactive material eluting in the void volume could therefore not be determined using this chromatographic system. At rpHPLC, immunoreactive NKB

was only found in acetic acid extract of tumor tissues from one patient, and in plasma from another patient. This may not necessarily mean that NKB is of rare occurrence in carcinoid tumors since antiserum K12 has rather low immunoreactivity for NKB (26% in relation to NKA). Furthermore, the presently used extraction methods may not have favored recovery of NKB since it has poor solubility in aquous solutions. On the other hand, an immunoreactive component eluting in the position of ELE at rpHPLC was found in neutral water extracts of tumor tissues from all five patients. Furthermore, when the void volume component from the cation-exchange column was applied to the rpHPLC, it eluted at the position of ELE, indicating the presence of an eledoisin-like component.

The presence of NPK was established in plasma and tumor tissues from all patients investigated. Thus an immunoreactive component with the chromatographic characteristics of NPK both at cation-exchange chromatography and at rpHPLC was abundant in plasma and in acetic acid extracts of tumor tissues from all patients. NPK was on the other hand not found in the neutral water extracts. NPK was the most abundant tachykinin measured with antiserum K12 in all seven plasma samples even though antiserum K12 only crossreacts to 18% with NPK in comparison with NKA which was used as standard due to the scarcity of NPK. The present radioimmunoassay is therefore likely to grossly underestimate the concentration of NPK both in plasma and in the tumor extracts. However, elevated plasma levels of tachykinin-like immunoreactivity as measured with antiserum K12 have been found in 75% of patients with advanced carcinoid tumors (6).

NPK is very potent in inducing long lasting bronchoconstriction and hypotension due to vasodilation (13). Since pulmonary and circulatory phenomena are major symptoms of the carcinoid tumor, NPK may be one of the substances which mediate carcinoid symptoms.

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REFERENCES

1. Lembeck, F. (1953) Nature 172, 910-911.
2. Waldenström, J., Pernow, B. and Silwer, H. (1956) Acta Med. Scand. 156, 73-83.
3. Oates, J.A., Pettinger, W.A. and Doctor, R.B. (1966) J. Clin. Invest. 45, 173-178.
4. Sandler, M. (1968) Lancet 1, 104.
5. Håkansson, R., Bengmark, S., Brodin, E., Ingemarsson, S., Larsson, L.-I., Nilsson, G. and Sundler, F. (1977) Substance P pp. 55-58, Raven Press, New York.
6. Norheim, I., Theodorsson-Norheim, E., Brodin, E., Öberg, K., Lundquist, G. and Rosell, S. (1984) Regulatory Peptides 9, 245-257.
7. Pernow, B. Pharmacol. Rev. (1983) 35, 86-141.
8. Nawa, H., Hirose, T., Takashima, H., Inayama, S. and Nakanishi, S. (1983) Nature 306, 32-36.
9. Nawa, H., Kotani, H. and Nakanishi, S. (1984) Nature 312, 729-734.
10. Kimura, S., Okada, M., Sugita, Y., Kanazawa, I. and Munekata, E.(1983) Proc. Jap. Akad. Sci. 59, 101-104.
11. Theodorsson-Norheim, E., Brodin, E., Norheim, I. and Rosell, S.(1984) Regulatory Peptides 9, 229-244.
12. Kanagawa, K, Minamino, N., Fukuda, A.and Matsou, H. (1983) Biochem. Biophys. Res. Commun. 114, 533-540.
13. Tatemoto, K., Lundberg, J.M., Jörnvall, H. and Mutt, V. (1985) Biochem. Biophys. Res. Comm. 128, 947-953.
14. Erspamer, V. (1981) Trends. Neurosci. 4, 267-269.
15. Lazarus, L.H., Linnoila, R.I., Hernandez, O. and Di Augustine, R.P. (1980) Nature 287,555-558.
16. Masson, P. (1914) C.R. Acad. Sci. Paris 158, 59-61.
17. Grimelius, L. (1968) Acta Soc. Med. Uppsala, 73,243-278.
18. Bolton, A.E. and Hunter, W.M. (1973) Biochem J. 133, 529-539.
19. DeLean, A.P., Munson, P.J. and Rodbard, D. (1978) Am J. Physiol. 235, E97-E102.
20. Rodbard, D., Hutt, D., Faden, V.B. and Huston, J.R. (1974) RIA and related procedures in medicine. pp. 165-193, International Atomic Energy Agency, Vienna.
21. Brodin, E., Dalsgaard, C.-J., Lindefors, N., Theodorsson-Norheim, E. and Rosell, S. (1985) Manuscript in preparation.
22. Gamse, R., Saria, A., Busics, A. and Lembeck, F. (1981) Peptides 2, Suppl. 2, 275-280.