Minireview

# A novel system of peptidergic regulation

Andrei A. Karelin, Elena Yu. Blishchenko, Vadim T. Ivanov*

*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya, 16/10, 117871 Moscow, GSP-7, Russia*

Received 7 April 1998

**Abstract Systematic analysis of structure and biological activity of peptide components of tissue extracts and biological fluids allows us to formulate a novel concept of a peptidergic regulatory system, complementary to the conventional regulatory systems (i.e. nervous, endocrine and paracrine systems). According to that concept, the proteolytic degradation of tissue proteins carried out by a specific and regulated system of tissue-specific enzymes and protein substrates gives rise to a large group of peptides, which we define as tissue-specific peptide pool. As a result, functional proteins provide their proteolytically derived fragments for maintaining tissue homeostasis.**



*Key words:* Biologically active peptide; Proteolysis; Tissue-specific peptide pool; Tissue homeostasis

## 1. Introduction

It is well established that biologically active peptides are mediators of a large number of regulatory systems. Several thousands of the substances involved in signal transduction in nervous, immune, endocrine and paraendocrine and other systems have been identified and characterized. Most of these peptides are cleaved from specific precursors by trypsin-like processing enzymes and released from cells. No other function than giving birth to the peptides is known for these precursors. The secreted peptides further interact with specific receptors localized on the target cell surface and trigger the signal transduction into the cells. In this way the activity of the majority of peptide hormones, parahormones and neurotransmitters, i.e. 'classical' peptide bioregulators, is realized.

The biological function of this group of substances has been examined in detail and described in a number of reviews and monographs (e.g. [1–3]). At the same time, there are many biologically active peptides that do not correspond to the above mentioned features of 'classical' peptide bioregulators. The present minireview deals with that group of peptides.

## 2. Proteolysis of functional proteins in vitro leads to formation of biologically active peptides

For several decades it has been known that in vitro treatment of proteins by various proteinases results in generation of peptides exhibiting pronounced biological effects. The first observation of the activity of proteolytic protein fragments was made in 1941, when treatment of blood plasma proteins with pepsin gave rise to a mixture of substances inducing histamine release from mast cells [4]. At that time there was no experimental means to interpret the phenomenon in structural terms. Further work along similar lines has been continued in a number of laboratories confirming the conclusion that in vitro enzymatic digestion of biological preparations, including both tissue homogenates and individual proteins, leads to formation of biologically active peptides. More than 300 protein fragments have been identified and sequenced [5]. In contrast to 'classical' peptide bioregulators, this group of peptides is formed upon proteolysis of proteins with well established function in vivo.

At present bioactive fragments of functional proteins include peptides derived from β-casein (so called β-casomorphins), hemoglobin, myelin basic protein, gluten, serum albumin, cytochrome *c*, lactoferrin and some other proteins [5,6].

Several peptides originally obtained by proteolysis in vitro, i.e. opioid-like hemoglobin fragments (VV-hemorphin-7 and LVV-hemorphin-7 [7]), neurotensin-like fragment of albumin (HRP-1 [8]) and β-casomorphin-8 [9], were later identified in biological fluids or tissues in vivo. The presence of myelin basic protein fragments in biological preparations was also shown by means of immunochemical methods, however the sequences of peptide-like immunoreactive material have not been established [10].

At the same time, only a small fraction of peptides derived from in vitro proteolysis of functional proteins was identical to peptides derived in vivo from the same precursors. This discrepancy might be partially due to the technical aspects of isolation. Screening of compounds formed upon in vitro proteolysis, as a rule, is aimed at isolation of most active components, independently of their content in the mixture obtained, while screening of endogenous protein fragments in biological sources commonly results in isolation of the most abundant species.

## 3. Tissue-specific peptide pool – the result of endogenous proteolytic degradation of functional proteins

In spite of the fact that molecular fractions of tissue extracts have been routinely used as a source of 'classical' peptide bioregulators, the components of these fractions were not subjected to systematic structural studies until the early 1990s.

It is generally accepted that proteolytic enzymes non-specifically digest the proteins normally present in the tissue after fulfilling their function. In this case, the tissue extracts should contain a 'random', physiologically inert set of protein fragments. The content of these peptides should correspond to the level of protein precursors and the stability of these proteins to proteolytic degradation. Based on this concept the frag-

*Corresponding author. Fax: (7) (095) 3107007.
E-mail: ivavt@ibch.siobc.ras.ru


*PII* S0014-5793(98)00486-4

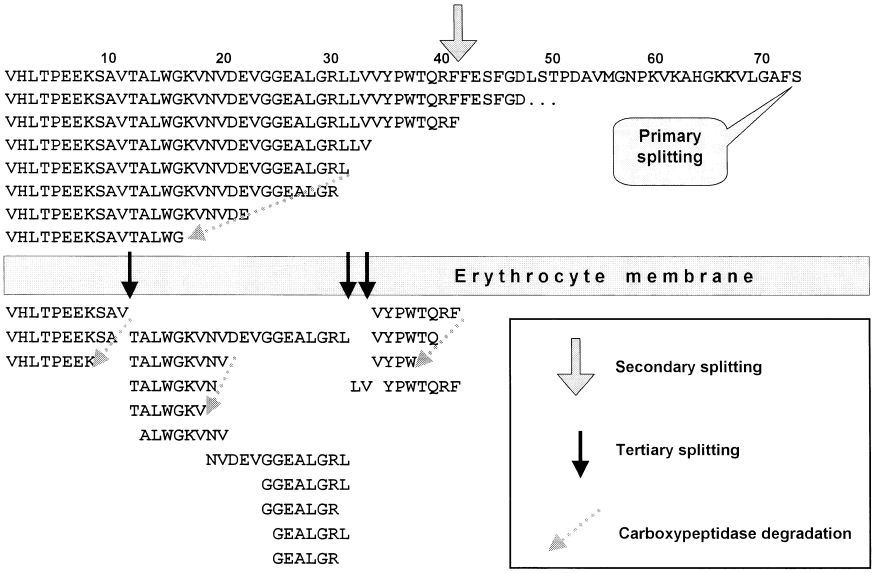


Fig. 1. Peptides derived from the N-terminus of human β-globin. Erythrocyte membrane separates the peptides found inside the erythrocyte and in the supernatant of the erythrocyte primary culture.

ments of hemoglobin identified in the course of isolation of LH-RH were considered artifacts, in spite of their biological activity [11,12]. Analogous views led to the search for a specific precursor of neokyotorphin, a pentapeptide identical to the C-terminal segment of the α-chain of hemoglobin isolated from bovine brain [13]. The broad spectrum of neuromodulatory effects shown for this peptide [6] did not allow us to consider it an artifact, on the one hand, or as a hemoglobin-derived fragment, on the other.

Since 1993 a large number of endogenous fragments of functional proteins have been isolated and the first results of systematic structural studies of components of low molecular fractions of tissue extracts have been obtained. Chromatographic analysis of the components of extracts of bovine [14,15] and rat [16,17] brain, human cerebellum [17,18], bovine bone marrow [14] as well as the lysate and the supernatant of primary culture of human erythrocytes [6,19] demonstrated that these sources contain a panel of compounds. The number of substances corresponding to the chromatographic peaks, distinguishable by means of computer analysis of the elution profiles, varies within the 200–800 range, depending on the biological source. Both the content and the composition of components characteristic for each tissue was highly reproducible and strongly tissue-dependent. Most of the components were identified as peptides. The overall content of peptides per 1 g of tissue comprises 0.02–0.5% of the total preparation weight [15,20]. Judging from their sequences these peptides are derived from a limited group of functional proteins or from non-defined protein precursors. Some of them are present at levels of 50–80 nmol/g of tissue [6,15,21,22], which is much higher than the typical content of 'classical' peptides in most tissues, i.e. 1–50 pmol/g of tissue [23,24].

Most of the components of tissue extracts were identified as hemoglobin fragments. In spite of the fact that hemoglobin, as a major blood protein, is widely distributed in the organism, the content and the composition of hemoglobin-derived peptides is also tissue-specific. For instance, the overall levels of hemorphins, i.e. the peptides belonging to the 32–40 site of β-globin, vary significantly in different biological tissues and fluids. The total level of hemorphins ranges from 100–500 nM/g of tissue in the rat heart and lung extracts [22] to 0.1–1 nM/g of tissue or less in bovine bone marrow [14], rat spleen

Table 1
Distinctive features of peptidergic regulatory systems

| Property | Peptidergic regulatory system | | |
|---|---|---|---|
| | Nervous | Endocrine and paracrine | Tissue-specific peptide pool |
| Family | Neurotransmitters | Hormones | Fragments of functional proteins |
| Precursor | Specific protein precursor | Specific protein precursor | Functional protein |
| Type of processing | Discrete site-specific processing | Discrete site-specific processing | Action of tissue proteinases |
| Content (nM/g tissue) | 0.001–1.0 | 0.001–1.0 | 0.1–100 |
| Type of regulation | Synaptic secretion | Extracellular secretion | Alteration of the level in the tissue |
| Mechanism of action | Binding to receptors in synaptic membranes | Binding to receptors in cellular membranes | Binding to receptors of homologous hormones |
| Receptor binding constants ($K_d$, nM) | 1–1000 | 0.1–10 | 100–10000 |
| Time range of action | seconds–minutes | minutes–hours | hours–days |
| Biological role | Transmission of nerve impulse | Regulation of physiological processes in the tissue or the whole organism | Maintenance of tissue homeostasis |

[22] or blood plasma [25]. Analogously, the content of neokyotorphin in rat lung tissue is 10–15-fold higher than in the rat brain [21]. Brain extracts also contain fragments of neurospecific proteins [15,16], the spleen extract is enriched with fragments of β-actin [26].

A number of peptides from tissue extracts were discovered earlier and found to be active in a variety of biological test systems. For instance, hemorphins are active in basic opioid tests: inhibition of binding of opioid ligands to brain membranes, induction of naloxone-dependent analgesia in rats in vivo and contractile activity at the guinea pig ileum [7]. Neokyotorphin was shown to terminate the hibernation of ground squirrels and to enhance the inward potential-dependent $Ca^{2+}$ current in cardiac myocytes of frog [27]. A number of hemoglobin fragments display hemopoietic activity [14]. All these data provided a critical mass for formulating the concept considering two patterns of peptide-mediated regulation. The first is mediated by the 'classical' peptide bioregulators formed from specific precursors by means of specific proteolytic enzymes and produced by specialized cells. The other implies the action of biologically active fragments of functional proteins. According to that concept each tissue contains a specific set of proteins, which are cleaved after completion of their function by tissue-specific proteolytic enzymes giving rise to a large group of peptides which can be defined as a 'tissue-specific peptide pool'. The composition and the content of components in the pool are characteristic for the given organ or tissue.

At the same time, significant changes in composition and content of components of tissue-specific peptide pools were shown to accompany several pathologies. For instance, the content of some intraerythrocyte hemoglobin fragments increased more than 10-fold in the cells of patients with Hodgkin's disease [28]. Alterations in content of long (more than 25 amino acid residues in length) hemoglobin fragments were detected in cerebellum of patients with Alzheimer's disease [17,18] and hippocampus of rats subjected to induced brain ischemia [17]. Alterations of the level of myelin basic protein fragments were shown to accompany several CNS pathologies of newborn children [14]. The composition and the content of components of tissue-specific peptide pools should be considered an important characteristic of the state of the tissue.

## 4. Formation of the components of tissue-specific peptide pools

The identified components of tissue-specific peptide pools can be divided into two groups: (i) the fragments of intracellular proteins; (ii) the fragments of extracellular and membrane-associated proteins. Some fragments of intracellular functional proteins were shown to act inside the cell. In particular, the fragment (24–62) of neurogranin (a calmodulin-binding protein kinase C (pkC) substrate [29]), exhibited calmodulin-binding activity. This fragment is also a good substrate of pkC [30]. Neurogranin belongs to a large group of B-50 proteins, that includes a number of calmodulin-binding substrates of pkC (F-1, GAP-43, neuromodulin). The N-terminal fragment of GAP-43-PIP inhibits phosphorylation of GAP-43 by pkC [31].

Since hemoglobin-derived peptides are the largest group of protein fragments found in all tissue extracts studied so far, comprising 30–90% of the total number of sequenced protein fragments [6,14–18], the patterns of endogenous hemoglobin fragmentation could be outlined in some detail. Hemoglobin degradation inside the erythrocytes seems to proceed in several consecutive steps: at the first stage α- and β-chains of hemoglobin are cleaved in the middle segment giving rise to peptides 60–80 amino acid residues in length. Study of myoglobin proteolysis has shown that removal of hemin makes the middle segment of that protein much more sensitive to the action of proteolytic enzymes with different specificity [32]. Bearing in mind the analogy of spatial structures of hemoglobin chains and myoglobin, in particular, of the site sensitive to the proteolytic attack in myoglobin with the putative primary splitting sites of α- and β-globin, we consider apoglobins the most probable substrates of erythrocyte proteinases.

The products of primary splitting are subjected to a secondary splitting stage at sites containing two hydrophobic residues, i.e. $Phe^{33}$–$Leu^{34}$ and $Leu^{105}$–$Leu^{106}$ in the case of the α-chain and at $Phe^{41}$–$Phe^{42}$ and $Leu^{110}$–$Val^{11}$ for the β-chain. The resultant fragments are further degraded, primarily by amino- and carboxypeptidases forming a set of stepwise N- and C-terminally shortened peptides 20–30 amino acid residues in length [6,19,20]. Formation of long peptides within erythrocytes is followed by tertiary splitting, accompanied by the release of smaller, 4–20-membered fragments into the surrounding medium.

The above described pattern is illustrated in Fig. 1. After secondary splitting of the β-chain at $Phe^{41}$–$Phe^{42}$ the resultant fragment is cleaved at the $Leu^{31}$..$Val^{33}$ site giving rise to the family of hemorphins, the dipeptide LV and the fragment (1–30). The former peptides are released from the erythrocytes, while the latter is subjected to C-terminal shortening inside the cells. The alternative tertiary splitting of the β-(1–30) peptide at $Val^{11}$–$Thr^{12}$ leads to peptide families derived from the segments (1–11) and (12–31). According to that description the peptides belonging to the β-(1–11) family might be formed by two complementary pathways: by means of C-terminal shortening of the fragment (1–30) inside the erythrocytes or by tertiary splitting at the erythrocyte membrane followed by excretion from the cells. In summary, proteolysis of hemoglobin within the erythrocytes takes place in three main stages. The intermediate products of these stages are subjected to N- and C-terminal shortening by amino- and carboxypeptidases giving rise to 'ladders' of structurally related peptides.

Altogether, amino acid sequences of 32 intraerythrocyte peptides and 36 peptides released from human erythrocytes have been established [6,19]. The structural set of the released peptides differs significantly from that observed in tissue (brain, bone marrow) extracts and inside the erythrocytes. In particular, a novel family corresponding to the (84–95) segment of the hemoglobin α-chain was discovered in the supernatant, as well as a novel pattern of formation of the peptides corresponding to β-chain segment (12–31). The related family found in bovine brain extract [15] is formed upon cleavage of β-globin (14–28) at the $Asp^{20}$–$Glu^{21}$ site giving rise to two groups of both N- and C-terminally shortened peptides. In contrast, in the erythrocytes the tertiary splitting takes place at $Val^{11}$–$Thr^{12}$ (instead of $Leu^{13}$–$Trp^{14}$) and the resultant fragments are in turn cleaved at two sites ($Val^{17}$–$Asn^{18}$ and $Asp^{20}$–$Glu^{21}$) forming two families of overlapping peptides. Moreover, erythrocytes excrete a novel subfamily of hemorphins, V-hemorphins, not found in other biological sources. Ten of the 36 peptides are known for their biological

effects, while most of the rest structurally overlap with other bioactive hemoglobin fragments.

By analogy with hemoglobin we suggest that other intracellular proteins are also subjected to stepwise degradation leading to formation of short peptides further excreted from the cells. Analysis of structures of peptides derived from cytochrome *c* oxidase, glutamate-ammonium ligase, glyceraldehyde-3-phosphate dehydrogenase and found in brain extract allows us to assume that the basic mechanisms of formation of these peptides are similar to that of hemoglobin fragments [14,15]. In particular, peptides are cleaved at sites containing two hydrophobic residues, analogous to the secondary splitting of hemoglobin and the resultant products are further trimmed by amino- and carboxypeptidases at N- and C-termini.

## 5. Biological activities of the components of tissue-specific pools

Of more than 250 sequenced components of tissue-specific pools, 60–65 peptides were shown to be biologically active [6,14]. The available data suggest that the activity of at least several groups of the components of the peptide pools (such as hemorphins [7] or HRP-1 [8], etc. [5]) might be realized through the same receptors as with neurotransmitters or hormones (such as opiate, neurotensin, bombesin). Typically, the binding affinities of the pool components are by several orders of magnitude lower than for 'classical' peptide bioregulators and their content in blood and other biological fluids is not normally sufficient for transduction of signals in the organism [19,25]. However, these peptides are present in higher amounts in tissues [21,22], in contrast to blood plasma and cerebrospinal fluid [25,33]. Therefore their potency is expected to be expressed mainly at the tissue level. Several structurally related families of fragments of functional proteins, for instance hemorphins [34,35] and casomorphins [36], were shown to induce cytolysis and to inhibit proliferation of tumor cells, i.e. to exhibit effects analogous to 'classical' ligands of opioid receptors [37,38]. In contrast to opioid activities of hemoglobin fragments, their oncolytic activity is comparable to that of 'classical' opioids. At the same time, the molecular targets of the majority of the tissue-specific peptides are not established. In spite of that, they exhibit pronounced effects at the cellular level. Neokyotorphin and, to a lesser degree, des-Arg-neokyotorphin were shown to stimulate proliferation of tumor cells. In contrast to neokyotorphin, its fragment induces cytolysis of tumor cells [21,39]. The presence of high amounts of neokyotorphin in lung carcinoma suggests its involvement in tumor development [40]. It was recently found that two groups of α-globin fragments, (1–32)-(1–30) and (106–141)-(110–141), induce a 30–60% increase of proliferative tumor cell growth [41]. The activity of hemoglobin fragments is concentration-dependent. For instance, peptides α-(12–24) and α-(12–23) cause a 35–45% decrease of live tumor cell number at $10^{-6}$–$10^{-7}$ M. At the same time, these peptides show no reliable effect on viability or proliferation of the same tumor cells in a $10^{-8}$–$10^{-9}$ M concentration range and stimulate proliferation at $10^{-10}$ M up to 60%. Similarly, long intraerythrocyte fragments of hemoglobin increase tumor cell growth in a $10^{-6}$–$10^{-8}$ M concentration range but show a slight growth-inhibitory effect at lower concentrations.

Since tumor cells are a rather simple and convenient model for evaluation of in vitro activity of different compounds, including proteins, peptide hormones, or neurotransmitters, we carried out a primary search for bioactive peptides derived from functional proteins using this particular test system. The available literature data speak of the involvement of such peptides in regulation of proliferation of normal cells. For example, VV-hemorphin-5 slightly inhibits proliferation of red bone marrow cells (20% growth inhibition, or 3 times less than that of tumor cells [34]). Neokyotorphin stimulates proliferation of proadipocytes [42], peptide α-(33–38) exhibits immunostimulatory activity [43] and a number of short hemoglobin fragments increase the proliferative rate of lymphocytes treated with radiation or cytostatic drugs [14]. The tetrapeptide AcSDKP, initially isolated from fetal bovine bone marrow extract, inhibits proliferation of hemopoietic progenitor cells [44]. LVV-hemorphin-3 was shown to restore PHA-induced proliferation of T-cells pretreated with immunosuppressive factors produced by HL-60 leukemia cells [45]. A neurotensin-related peptide, analogous to neurotensin, stimulates histamine release from mast cells [46].

In other words, the biological potency of the components of tissue-specific peptide pools is reliably documented at the cellular level, at least, in vitro. It is worth mentioning that components of the tissue-specific peptide pools have several features in common with the so called cytomedines, a term proposed in the early 1980s for the components from low molecular mass fractions of the total tissue extracts that are applied in clinical practice as tissue-restorative preparations [47,48]. Like cytomedines, the fragments of functional proteins might participate in maintenance or restoration of homeostasis of the given tissue in case of pathology.

## 6. Tissue-specific peptide pools as a novel system of peptidergic regulation

The above described data strongly suggest that the process of protein elimination should not be considered a random proteolysis leading to amino acids in turn employed for various metabolic purposes. Instead, it is a complex process carried out by a specific and regulated system of tissue-specific enzymes and protein substrates. As already mentioned, this process results in formation of a panel of peptides which we define as a tissue-specific peptide pool. In contrast to 'classical' peptide bioregulators, in particular, hormones or parahormones such as neurotensin or substance P, which are secreted by specified cells, the components of tissue-specific peptide pools are produced by all cells comprising the tissue. The composition and the content of each component in the pool are specific for the given organ or tissue.

Based on the studies of receptor properties of opioid-like fragments of functional proteins, i.e. hemorphins or casomorphins [7,49], it can be generalized that components of tissue-specific peptide pools modulate the availability of hormonal receptors to their 'true' ligands. In this case, the high content of fragments of functional proteins might compensate for their relatively low binding parameters. Tissue-specific pools contain peptides exhibiting a variety of effects, sometimes of opposite signs (see above) and might be considered polyfunctional and polyspecific 'buffers' providing the basic hormonal signals for tissue cells and regulating the availability of the signals to the given tissue. One can also envisage cases of inverse regulation, when changes in the efficiency of local

proteolytic processes take place as a result of hormonal signals.

At the same time, the molecular mechanisms of action of the majority of the components of tissue-specific pools are unknown. It might well be that they are not restricted to modulation of the activity of peptide hormones. Some of the peptides could be involved in regulation of cell adhesion [50,51] and the fragments of intracellular proteins, such as calmodulin [17] or neurogranin [15,16], might participate in the regulation of signal transduction at the intracellular level.

The following examples provide a clue to the possible endogenous role of the components of tissue-specific peptide pools. We have demonstrated recently that α-globin fragment α-(2–32) restores proliferation of red bone marrow cells pretreated with epirubicin, being inactive in the normally proliferating cells [41]. Similar data were obtained for the β-globin fragment (1–10), that was shown to restore proliferation of lymphocytes in mice treated with 5-fluorouracil [14]. It seems that the activity of such peptides is predominantly expressed in damaged cells or cells subjected to 'unfavorable' conditions. The data on the therapeutic effects of the preparations based on peptide components of tissues, Cerebrolysin or 'cytomedins' (see above), indicate their ability to restore, at least partially, the normal function of the tissue. For instance, the action of Cerebrolysin in the case of Alzheimer's disease is due to restoration of the connection between the neurons, and, as a result, to a notable improvement of brain activity [48]. It seems that the function of tissue-specific peptides can be essentially formulated as maintenance of the equilibrium between the growing, differentiating, normally functioning and dying cells of the given tissue and, consequently, as compensating for the effects caused either by exogenous damaging agents (such as cytostatic drugs) or by endogenous regulatory factors.

Both the composition and the content of tissue-specific peptides depend strongly on the content and the availability of protein substrates to proteolytic enzymes as well as on the activity of proteolytic enzymes in the given tissue. Accordingly, several pathologies are accompanied by changes in composition and content of the fragments of functional protein in tissues [14–18,28,40,41]. Notwithstanding the clinical and biochemical differences, these pathologies have a common principal feature – they involve alterations in tissue homeostasis or metabolic state of the cells, independently of the nature of the disease: cell transformation [40,41], tissue atrophy (Alzheimer's disease and ischemia [16–18]) or impaired lymphoproliferation (Hodgkin's disease [28]). Activity of the components of tissue-specific peptide pools in cell cultures in vitro shows that their biological role might be due to prevention of tumor cell growth or to control of viability, growth and differentiation of cells of the tissues, i.e. to maintenance of tissue homeostasis.

Summarizing the data described above we suggest that fragments of functional proteins form a novel system of peptidergic regulation. However, there is certainly no strict borderline between the peptides regulating the function of nervous, endocrine or paracrine systems and the peptides derived from functional proteins (Table 1). On the one hand, one cannot exclude that precursors of 'classical' peptide bioregulators exhibit certain not yet discovered in vivo functions. It is also known that their processing is not always trypsin-like and highly specific [52,53]. Some of the 'classical' peptide bioregulators are not secreted from the specified cells, for instance, 'tissue hormones' angiotensin II and bradykinin are formed in the tissues from extracellular proteins (T-kininogens) [54]. Moreover, as already described, a number of the components of tissue-specific peptide pools bind to the same receptors as peptide hormones and exhibit similar biological effects [7,8,49]. The above mentioned features are common to 'classical' peptide bioregulators and tissue-specific peptides.

On the other hand, any regulatory system should belong to a concrete systemic level, i.e. to the whole organism, to a given organ, tissue or isolated cells. Typically, the major role of a hormonal regulatory system is in ensuring the 'cooperative work' of different organs and tissues in order to maintain the integrity of the whole organism and in providing thereby an adequate reaction of the given organism to external signals. In contrast, the overall data on features of the components of tissue-specific peptide pools, i.e. (i) high level in tissues, as opposed to that in blood plasma, (ii) tissue specificity, and (iii) activity in cell cultures, suggest that the function of such peptides is realized mainly at the tissue and cellular levels. Stability of the composition and the content of such peptides in tissues at normal conditions suggests their participation in the maintenance of the appropriate ratio of dividing, differentiating and dying cells of the corresponding tissue.

On the other hand, the mediators of conventional regulatory systems (nervous, endocrine or immune system), as a rule, can be seen as inducing changes in the state of the tissue by transducing changes of the membrane potential, by stimulating or inhibiting secretion of bioactive compounds, etc., while the function of the components of tissue-specific pools might be due to bringing back the tissue to stationary condition. It can also be suggested that induction of the hormone-mediated effects takes place exclusively when the appropriate level of hormones in tissue is attained, i.e. tissue-specific peptides restrict the possibility of 'occasional' alterations of tissue state and consequently maintain the tissue homeostasis [6]. As a result, both systems maintain the equilibrium of biological processes at the level of the whole organism.

Since the change of the proteolytic activity within the tissue is a relatively slow process, compared with the action of hormones and neurotransmitters, we believe that the components of tissue-specific peptide pools should be predominantly involved in long-term regulation of tissue or organism state.

On the basis of the above discussed data it can also be assumed that the proteolysis-based peptidergic system is phylogenetically a more ancient regulatory system than the nervous and endocrine systems. This proposal follows from the fact that the peptide pool does not necessarily require the entire organism to express its activity. Most of its inherent potency could be employed at the cellular level. Moreover, some of the fragments of intracellular proteins might regulate a variety of responses within a single cell, i.e. such mechanism could be applied even to unicellular organisms.

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