PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2016, № 2, p. 33–42

Biology

NATURAL SUBSTRATES OF DIPEPTIDYL PEPTIDASE IV

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A widely expressed multifunctional, membrane-anchored cell surface or in a soluble form in the plasma and other body fluids serine protease, dipeptidyl peptidase IV (DPPIV), cleaves dipeptide from peptides containing proline or alanine in the N-terminal penultimate position. Several important regulatory peptides have been identified as DPPIV substrates, including neuropeptides, chemokines, and the incretin hormones, which share this conserved sequence at their N-termini. Natural substrates of DPPIV are involved in immunomodulation, psycho/neuronal modulation and physiological processes in general and the cleavage by DPPIV of these peptides resulted in their inactivation or degradation. Therefore, targeting of DPPIV and especially its proteolytic activity has much therapeutic potential. Some known and new natural substrates were discussed in this review.

***Keywords*:** dipeptidyl peptidase IV, natural substrates of DPPIV, proteolytic activity of DPPIV, regulatory peptides, proline rich peptides, amyloid beta peptides, modification of bioactive peptides.

Introduction. The increasing number of biological processes appear controlled by proline-containing peptides. The unique amino acid residue proline forms special conformation of peptide chain protecting it against degradation by usual peptidases even with broad specificity. Proteolysis and breakdown of proline-containing neuropeptides and hormones are realized by proteases specific for proline bonds [1, 2]. Dipeptidyl peptidases (DPP) are unique serine proteases removing N-terminal dipeptides from the polypeptides and proteins, containing proline or alanine on the penultimate position [3].

Dipeptidyl Peptidase IV as a Member of Dipeptidyl Peptidases Family. 766 amino acids containing, type II transmembrane protein Dipeptidyl Peptidase IV (DPPIV, EC 3.4.14.5) is a member of the family of DPPs. It is a multifunctional, ubiquitous enzyme, firstly described by Hopsu-Havu and Glenner in 1966 [4]. In 1992 DNA of human DPPIV was sequenced [5].

DPPIV exists in two forms: membrane-bound, located on the luminal surface of endothelial cells in numerous tissues, and blood circulating soluble. The membrane-located DPPIV has an extracellular domain possessing the enzymatic activity, a transmembrane part and a short cytoplasmic domain. It is expressed in the brush borders of the gut and the kidney (where it participates in digestion of

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proteins and peptides), in the placenta, lung, liver, pancreas, at low levels in skeletal muscle, heart, brain. DPPIV is expressed on T-, B- and natural killer (NK) cells, hematopoietic progenitor and stem cells, subsets of macrophages, epithelial, endothelial and acinar cells of a variety of tissues [6].

Blood circulating soluble DPPIV missed the intracellular and transmembrane regions. Being released from the appropriate cell types (lymphocytes, hepatocytes, adipocytes, etc.), it is detected in the blood serum/plasma, urine, bile, semen, cerebrospinal, pleural and synovial fluids [7]. The identity of DPPIV and delivering costimulatory signal to T-cells activation antigen CD26 has been manifested [8]. In the hematopoietic system DPPIV/CD26 is found on CD4+ T-memory cells, CD8+ effector/T-memory cells. In the blood, DPPIV is functioning as an immune enhancing protein in antigen-presenting cells.

The functioning of DPPIV as a proteolytic enzyme, cell surface receptor, costimulatory protein, signal transduction mediator, in adhesion and apoptosis, depends on both the cell type and the intracellular/extracellular environment [7].

Besides cell surface and circulating forms, nuclear localization of DPPIV has been reported in malignant mesothelioma and malignant T-cell lines and in human thyroid carcinomas [9]. The importance of DPPIV/CD26 in immunology, autoimmunity, HIV, diabetes and cancer is discussed widely.

Interaction of DPPIV with Different Proteins and Enzymes. Along with the enzymatic function, DPPIV interacts with various proteins, peptides and enzymes: it forms heterodimer with fibroblast activating protein α (FAP α), associates with fibronectin, collagen, mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR), C-X-C chemokine receptor type 4 (CXCR4), C-C chemokine receptor type 5 (CCR5), tyrosine phosphatase (CD45), plasminogen 2 and adenosine deaminase (ADA) [10, 11].

The binding of highly sialylaled glycoforms of plasminogen 2 to DPPIV and its activation by urokinase type plasminogen activator induce a significant increase in the cytosolic free Ca^{2+} concentration [12]. DPPIV colocalization with the stromal cell-derived factor 1α (SDF-lα) receptor CXCR4 induces chemotaxis and antiviral activity of Th2-cells. CXCR4–CD26/DPPIV complex is likely a functional unit, in which CD26/DPPIV directly modulates SDF-lα induced chemotaxis and antiviral activity of lymphocytes [13]. T-cell activation resulted in enhanced mannose 6 phosphorylation of DPPIV/CD26. The cross-linking of CD26 with antibody induced internalization and colocalization of CD26 with M6P/IGFIIR plays a role in CD26-mediated T-cell costimulation [14]. Induced by anti-CD26 modulation of DPPIV/CD26 from T-cell surface enhances the phosphorylation of receptor signaling molecules (c-Cbl, p56lck, ZAP-70, Erk l/2 and CD3ζ) [15]. It was shown that CD26 colocalizes on the T-cell surface with CD45. This interaction enhances tyrosine kinase activity and stimulates TCR signaling. The enzymatic activity of DPPIV/CD26 enhances the cellular responses of immune cells to CD26-mediated external stimuli and/or CD3/T-cell receptor complex, increasing IL-2 production. IL-2 has to be processed by CD26/DPPIV to become fully activated [16].

ADA is one of the clue enzymes in purine metabolism, responsible for degradation of (deoxy)adenosine. The enzyme is presented in all of mammalian cells and its primary function in humans is development, differentiation and maturation of the lymphoid system. Association of ADA with epithelial cell differentiation,

neurotransmission and gestation maintenance has been demonstrated [17]. Besides of low-molecular intracellular form of ADA, its high-molecular, extracellular form has been descried as a complex of the former with the ADA-binding protein. In 1993 this protein has been identified as DPPIV/CD26 [18].

Truncation of Different Peptides by DPPIV. DPPIV forms a homodimer on the cell surface membrane. Each of its subunits consists of α/β-hydrolase and 8-bladed β-propeller domains. The catalytic site is located in a large cavity, called a central tunnel, formed between the α/β-hydrolase and β-propeller domains [19]. The 22 residues anchor the enzyme in the membrane. The substrate recognition by DPPIV is defined by the size and amino acid sequence of the substrate. The central tunnel is a part of the inner cavity surface, in which catalysis occurs [20]. The propeller domain protects the large peptides and proteins from proteolysis limiting their occupying the catalytic pocket. The molecular environment of the catalytic triad (Ser630-Asp708-His740) in the active site of DPPIV is mainly responsible for its substrate specificity [21].

A number of natural substrates of DPPIV have been identified so far, including incretin hormones, neuropeptides and other physiologically important peptides and proteins. Interacting directly with various cell surface and intracellular molecules, DPPIV regulates the function of various chemokines and cytokines, the specificity of receptors, etc. via its enzymatic activity [22, 23]. The proteolysis and modulation of target substrates by DPPIV activate/inactivate the bioactive peptides, facilitate degradation of macromolecules by other peptidases [24].

Although peptides with proline and alanine in the penultimate position are preferential substrates of DPPIV, those with several other residues in this position also are cleaved at lower rates [25]. The *in vitro* K_m values of purified human DPPIV for natural substrates are in the micromolar range, while *in vivo* they act in the pico-/nano-molar concentrations [24]. The k_{cat}/K_m is used for comparing the potency of DPPIV towards substrates at physiological concentrations. Some identified substrates of DPPIV are presented in the Table.

DPPIV substrates and their functions

Substrate	Localization	Function	Refs
1	2	3	4
RANTES	T-cells, monocytes, T-helper cells and eosinophils	Chemoattractant for blood monocytes, memory T-helper cells and eosinophils; releases histamine from basophils and activates eosinophils; binds to CCR1, CCR3, CCR4 and CR5 receptors	[26]
Monocyte chemoattractant protein 2, (MCP-2)	Immune cells	Chemotactic for and activates mast cells, eosinophils, basophils, (implicated in allergic responses), monocytes, T- and NK-cells; binds to CCR1, CCR2B, CCR5 receptors; potent inhibitor of HIV-1	[27]
Monokine induced by gamma interferon (MIG)	Monocytes and T-lymphocytes	Affects the growth, movement, activation state of cells involved in immune and inflammatory responses; chemotactic for activated T-cells, monocytes, T-lymphocytes; binds to CXCR3	[23]
Interferon gamma-induced protein 10 (IP-10)	Monocytes, endothelial cells and fibroblasts	Chemoattractant for monocytes/macrophages, T-, NK- and dendritic cells; promotes T-cell adhesion to endothelial cells, inhibition of bone marrow colony formation and angiogenesis; binds to CXCR3	[23]

1	2	3	4
Interferon-inducible T-cell alpha chemoattractant (I-TAC)	Leukocytes, liver, pancreas, thymus, spleen, lung, small intestine, placenta and prostate	Chemotactic for interleukin-activated T-cells; induces calcium release in activated T-cells; is important in CNS diseases involving T-cells, in response to the allergens, promotes the accumulation of eosinophils; binds to CCR3 and CXCR3	[23]
Stromal cell-derived factor 1α (SDF-1α)	Different cells and tissues, in the area of inflammatory bone destruction	Chemoattractant for T-lymphocytes, monocytes. Activates the chemokine receptor CXCR4; binds to ACKR3, a scavenger receptor for SDF-1α; regulates monocyte migration and adhesion; stimulates migration of monocytes and T-lymphocytes through receptors, CXCR4 and ACKR3	[28]
Macrophage-derived chemokine (MDC)	Secreted by dendritic cells and macrophages	Trafficking of activated/effector T-lymphocytes to inflammatory sites; chemotactic for monocytes, dendritic and NK-cells; mild chemoattractant for primary activated T-lymphocytes, a potent chemoattractant for chronically activated T-lymphocytes; binds to CCR4	[23]
Neuropeptide Y (NPY)	Neurons of the sympathetic nervous system	Strong vasoconstrictor, controls epileptic seizures, feeding and secretion of gonadotropin-release hormone, increasing food intake and storage of energy as fat, reducing anxiety, stress, pain, alcohol intake, blood pressure, affecting the circadian rhythm	[29]
Peptide YY (PYY)	Released by cells in the ileum and colon in response to feeding	Suppresses pancreatic secretion, decreases appetite (anorexigenic), inhibits gastric motility and emptying, increases water and electrolyte absorption in the colon, digestion and absorption of nutrient; it exerts the action through NPY receptors	[30]
Substance P	Brain and spinal cord	Neurotransmitter, neuromodulator, vasodilator, associates with inflammatory processes, regulates mood disorders, neurogenesis, stress, anxiety, reinforcement, respiratory rhythm, neurotoxicity, nociception, pain	[31]
Glucagon-like peptide 1 (GLP-1)	Intestinal L-cells	Stimulates glucose-dependent insulin release, inhibits gastric motility and glucagon secretion, has growth promoting activities on intestinal epithelium, regulates the hypothalamic pituitary axis via effects on LH, TSH, CRH, oxytocin and vasopressin secretion, stimulates pancreatic β-cell proliferation, inhibits their apoptosis	[32]
Gastric inhibi-tory polypep-tide (GIP)	Intestinal K-cells	Potent stimulator of insulin secretion and relatively poor inhibitor of gastric acid secretion	[32]
Vasoactive intestinal peptide (VIP)	Suprachiasmatic nuclei of gut, pancreas, hypothalamus	Stimulates contractility in heart, causes vasodilation, increa-ses glycogenolysis, lowers arterial blood pressure, relaxes the smooth muscle of trachea, stomach and gall bladder	[25]
Growth hormone-releasing factor (GHRF)	Arcuate ucleus of the hypothalamus	Released in a pulsatile manner, stimulates growth hormone secretion by binding to the GHRH receptor; promotes slow-wave sleep; its expression has been demonstrated in the pancreas, epithelial mucosa of gastrointestinal tract, in tumor cells	[33]
TNF-a	Activated macrop hages CD4+, NK and mast cells, neurons, lymphocytes, eosinophils, neutrophils	Regulats the immune cells; induces fever, cell apoptosis, cachexia, inflammation, inhibits tumorigenesis, viral replication, responds to sepsis via IL1 and IL6 producing cells; deregulation of its production implicates Alzheimer's desaese, cancer, major depression, psoriasis, inflammatory bowel disease, etc.	[34]

1	2	3	4
Endomorphin-1 and 2 (EM-1, EM-2)	EM-1 is more prevalent in the brain, EM-2 in the spinal cord	High affinity, selective agonists of the μ-opioid receptor, its endogenous ligands, produce analgesia equal with morphine; EM-2 induces the release of dynorphin A and Metenkephalin in the spinal cord and brain	[35]
Calcitonin gene-related peptide 1 (CGRP)	Peripheral and central neurons	Induces vasodilation of coronary, cerebral and systemic vasculature vessels, neurotransmitter and neuromodulator in the CNS, elevates platelet cAMP, participates in cardiovascular homeostasis and nociception	[36]
Bradikinin	Blood plasma	Inflammatory mediator, vesodilatator (via the release of nitric oxide, prostacyclin and EDHF), lowering blood pressure; protected by angiotensin converting enzyme inhibitors	[36]
Procalcitonin	Thyroid, lung and intestine	Precursor of calcitonin hormone involved in calcium homeostasis	[37]
Eotaxin	Eosinophils	Promotes the accumulation of eosinophils in response to allergens; binds to CCR3	[38]

Several members of CXC and CC chemokine subfamilies contain penultimate proline or alanine at their N-termini verifying the DPPIV substrate specificity [23].

The modification or proteolytic removal of a few N-terminal amino acids leads to the significant changes in the functional activity of chemokines. The *in vivo* experiments have shown that DPPIV is an important regulator for chemotactic responses and inflammation. After DPPIV-mediated modification, CCL3, CCL5, CCL11, CCL22 and CXCL12 exhibit altered chemotactic activity [23, 25]. DPPIV is preferentially expressed by Th1-cells and DPPIV-mediated chemokine cleavage potentially contributes to the down regulation of Th2 responses by Th1-cells and increases the chemoattraction of monocytes via hydrolysis of CXCL12 and CCL3.

In normal conditions, the incretin hormones GLP-1 and GIP are released from intestinal L- and K-cells in response to the ingestion of nutrients. They are responsible for normal glucose tolerance after meal intake [42]. GLP-1 (7-36) is characterized as the most potent insulinotropic hormone, being a therapeutic agent for treatment of T2D patients.The continuous infusion of this peptide decreases plasma glucose and improves β-cell function. However, active GLP-1 (7-36) is intensively converted into inactive GLP-1 (9-36) by DPPIV [43].

NPY is a neuropeptide in the central and peripheral nervous system, involved in the control of feeding, energy homeostasis, and blood pressure [44]. PYY (1-36) is released in proportion to nutrient intake along the gut. Both NPY and PYY are potent endogenous agonists of the Y1 receptor (stimulating food intake, promoting vasoconstriction and cell proliferation, involved in the regulation of heart rate, anxiety, and bone homeostasis). The enzymatically formed PYY (3-36) and NPY (3-36) are inactive at Y1, but active at the receptors Y2 (inhibiting the release of NPY and noradrenaline) and Y5 (involved in feeding behaviour) [45]. PYY (3-36) is anorexigenic, it decreases appetite.

In the list of DPPIV substrates, NPY (1-36) and PYY (1-36) are of particular interest, because k_{cat}/K_m for them are much higher compared with those for GLP-1 and GIP. Higher rate constant has been reported also for the growth hormone-releasing hormone (GRH).

The New Substrates of DPPIV.

Proline Rich Peptides (PRP). New type cytokines of the neurosecretory granules of neurohypophysis, have been discovered by academician A.A. Galoyan

and colleagues. The precursor protein of PRPs, neurophysin-, vasopressin-associated glycoprotein is synthesized in the neurosecretory cells of hypothalamus by genetically determined mechanisms. During axonal transport, this hypothalamic neuropeptides are released from the precursor by partial proteolysis. One of PRPs, the best studied 15 amino acids containing PRP-1 (AGAPEPAEPAQPGVY) has been found in immunocompetent blood cells, neurosecretory cells of hypothalamus, bone marrow granulocytes, etc. It possessed the cytokine activity, stimulated the antigen-presenting function of macrophages, the expression and release of human growth factor by transformed BALB/c mice fibroblasts, etc. The regulation of both humoral and cellular immunity, differentiation of thymocytes, and myelopoesis by PRP-1 were shown. It possessed antibacterial, antitumor and antiviral activity, was recommended for treating of infectious diseases, immunodeficiency, etc. [46]. PRP-1 is a mediator of hypothalamus-neurohypophysis-bone marrow-thymus axis and participates in the regulation of differentiation and proliferation of bone marrow stem and progenitor cells [47]. Several important functions of the 10 amino acids containing PRP-4 (APEPAEPAQP) have been manifested also [48].

We demonstrated the breakdown of two PRP cytokines of the neurohypophysis PRP-1 and PRP-4 by DPPIV purified from bovine kidney [49] The observed increase of the optical absorbance at 334 *nm* due to complex formation of primary amines (NH_2) with o-phtalaldehyde (OPA) dye evidenced the increase of NH_2 amount confirming the scission of peptide links in PRPs. To confirm the responsibility of DPPIV for this scission, we used the known inhibitor of the enzyme, Diprotin A. However, this inhibitor (Ile-Pro-Ile) contains proline at N-penultimate position and actually undergoes to truncation by DPPIV. The usage of the grape leaf and clove extracts, inhibiting DPPIV with high efficiency [50], has been more successful [51]. Increasing the amount of primary amines in the assay mixture has been prevented by the inhibition of PRP-1 truncation by DPPIV. The breakdown of PRP-1 in the presence of DPPIV has been confirmed by the gel-filtration analysis of the assay mixture on Sephadex G-25 column [51]. The obtained diagrams clearly demonstrated the appearing of many small peptide peaks along with the decrease of initial PRP-1 peak. The same result has been fixed at paper electrophoresis. DPPII (E.C. 3.4.14.2), another member of DPP enzymes family, discovered in lysosomes of different tissues [52], failed to truncate PRP-1 in the identical experiment.

Using graphical analysis, the catalytic parameters of catalyzed by DPPIV enzymatic reactions have been determined as: V_{max}= 1.27 ± 0.11 *nmol*/*min* and K_m= 0.38 ± 0.1 *mM* for PRP-1 truncation; and V_{max}= 3.1 ± 0.2 *nmol*/*min* and K_m= 0.114 ± 0.01 *mM* for PRP-4 truncation. These parameters indicate PRP-4 being a better substrate for DPPIV than PRP-1. This finding corroborates the fact that a proline residue is beneficial for DPPIV penultimate N-termini of PRP-4.

Hence, the new cytokines from neurosecretory granules of bovine neurohypophysis, Proline Rich Neuropeptides PRP-1 and PRP-4, possessing a lot of physiological functions, are degraded by the multifunctional DPPIV and represent new natural substrates of the enzyme.

The Aggregates of Amyloid Beta Peptides (Aβs) are considered as one of the main pathological hallmarks of Alzheimer's disease. The neurotoxicity of Aβs

correlated with their aggregative ability [53]. It was expected that proteolysis of Aβs could decrease their steady-state concentration and deposition in brain.

As alanine is a N-terminal penultimate residue in Aβs: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG GVVIA, we presume that DPPIV can modify them. Using the above mentioned NH_2–OPA complex formation, we have shown that DPPIV (but not DPPII) truncated Aβ16, Aβ40 and Aβ42 peptides *in vitro*. The parameters of the enzymatic breakdown by DPPIV were determined for Aβ40 (K_m=37.5 μM; k_{cat}/K_m=1.7·10^3 $M^{-1}s^{-1}$) and Aβ42 (K_m=138.4 μM; k_{cat}/K_m=1.90·10^2 $M^{-1}s^{-1}$) [54]. The *in vitro* scission of N-terminal dipeptides from the synthetic Aβ(1-40/42) peptides by DPPIV has been confirmed in our experiments using MALDI-TOF mass spectrometry [unpublished]. Earlier, it was suggested that N-terminal truncation of the dipeptide from Aβs might be followed by Glutaminyl cyclase (QC, EC 2.3.2.5) catalyzed pGlu-modification, enhancing Aβs pathogenesis and toxicity [55]. Indeed, in our experiments, the *in vitro* conversion of Aβ(1-40/42) into the more toxic species pE-Aβ(3-40/42) at the simultaneous presence of DPPIV and QC has been observed for the first time.

We have controlled the aggregation-disaggregation of Aβs by visualization of transmitter electron microscope and by Thioflavin-T fluorescence on spectro-fluorimeter and fluorescent microscope. The results of our study indicated that the aggregation/fibrillation of Aβ(1-40/42) peptides was hindered by DPPIV [54], but accelerated in the simultaneous presence of DPPIV and QC.

We suggest that the inhibition of DPPIV and QC can be considered as a new treatment strategy to prevent Aβs toxicity and Alzheimer's disease development.

The Modifying Bioactive Peptides DPPIV as a Therapeutic Target. DPPIV activity processes and modulates biologically active peptides and their metabolism. Often it impacts their activity, and the inhibition of the enzyme seems necessary. For example, DPPIV degrades GHRF and administration of DPPIV inhibitor together with GHRF appeared useful in treatment of children with the hormone deficiency via prolonging its availability [33]. DPPIV is involved in the degradation of GIP and GLP-1 incretin hormones. The scission of N-terminal dipeptides by DPPIV abolishes their insulinotropic activity. The simultaneous injection of the enzyme inhibitor enhances the insulin secretion and improves glucose tolerance by the peptide hormones in type 2 diabetes (T2D) patients [56]. The DPPIV inhibitors increase circulating SDF-1α and endothelial progenitor cell levels at T2D [57]. The binding of plasminogen to DPPIV initiates a signal transduction mechanism, regulates expression of MMP-9 by prostate cancer cells [12], and the enzyme inhibition has been shown blocking the metastasis. The enzyme inhibitors in a dose-dependent manner suppress inflammation and tissue destruction. They may be useful as immunosuppressants in treatment of autoimmune diseases (e.g. rheumatoid arthritis), as well as in preventing the rejection of transplants [57]. The DPPIV inhibition after acute myocardial infarction improves cardiac homing of stem cells and enhances heart function [58].

In conclusion, DPPIV inhibitors prolong the availability of bioactive molecules and can be used as therapeutic agents in cases when the bioactive peptides are processed. However, their target, DPPIV, has a wide range of biologic functions and it has to be realized that processing of regulatory peptides is not

accomplished by CD26/DPPIV alone. Like many other biological systems, the alternative routes may limit the use of DPPIV inhibitors.

Received 10.03.2016

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