International Journal of Biochemistry Research & Review
3(4): 380-400, 2013

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Peptides Corresponding to Intracellular Regions of GPCR as a New Generation of Selective Drugs

Alexander O. Shpakov[1]

[1]*Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, Thorez av. 44, 194223 St. Petersburg, Russia.*

Author's contribution

This whole work was carried out by the author AOS.

Research Article

Received 27th June 2013
Accepted 13th August 2013
Published 21st August 2013

ABSTRACT

In the G protein-coupled receptors (GPCRs) relatively short regions of their intracellular loops and cytoplasmic C-terminal domain are responsible for specific interaction with G-proteins. GPCR-derived peptides corresponding to these regions are able to influence the activity of signal pathways involving the cognate receptors. Modified by hydrophobic radicals, these peptides turn into their cell-penetrating analogs, pepducins, possessing the activity both *in vitro* and *in vivo*. This review is devoted to the analysis of the available data and the prospects for GPCR-peptides and their lipophilic derivatives to be used in experimental and clinical medicine in the treatment of vascular diseases, cancers, inflammation, and endocrine dysfunctions.

Keywords: Angiogenesis; cancer; G protein-coupled receptor; heterotrimeric G protein; inflammation; intracellular loop; palmitate; pepducin; peptide.

ABBREVIATIONS

AC, adenylyl cyclase; GPCR, G protein-coupled receptor; 5-HTR, 5-hydroxytryptamine receptor; ICL, intracellular loop; LH, luteinizing hormone; MMP1, matrix metalloprotease-1;

**Corresponding author: Email: alex_shpakov@list.ru;*

PAR, protease-activated receptor; PLCβ, phospholipase Cβ; S1PR, sphingosine 1-phosphate receptor; TM, transmembrane domain; TSH, thyroid-stimulating hormone.

1. INTRODUCTION

The interaction between G protein-coupled receptors (GPCRs), seven times penetrating the plasma membrane, and the downstream signal proteins, such as heterotrimeric G-proteins, β-arrestins and others, is realized via relatively short regions of the intracellular loops (ICLs) and their interfaces including the segments of transmembrane domains (TMs) proximal to cytoplasm. In a majority of GPCRs the second and third intracellular loops (ICL2 and ICL3) are responsible for their specific interaction with G-proteins, and the ICL3 and the cytoplasmic C-terminal domain for interaction with β-arrestins. Ligand binding is followed by changes in the conformation and localization of intracellular regions, which makes these regions accessible for effective and selective interaction with G-proteins, β-arrestins and other regulators of signal transduction and induces activation of intracellular signaling cascades, including the enzymes generating the second messengers and G protein-gated ionic channels.

In the early 1990s it was found that synthetic peptides corresponding to functionally important intracellular regions of GPCR can affect *in vitro* the activity of receptors and signaling pathways regulated by them [1-4]. The biological activity of GPCR-peptide derived from receptor ICL was shown to depend on the integrity of the G-protein-activating site in its primary structure and on the similarity of 3D-structures of the peptide and homologous region in the cognate receptor. As the G protein-binding sites of receptor are located primarily in the membrane-proximal regions of the ICLs, the conformation of sites in the full-size receptor is stabilized by an adjacent hydrophobic helix of TM. Due to this, it would be natural to expect that the modification of GPCR-peptide by TM segments or hydrophobic radicals simulating TM will increase its effectiveness. This supposition is in excellent agreement with the results of investigations of GPCR-peptides modified by TM segments with the length of about 2/3 of the entire TM or by fatty acid radicals which are similar in physicochemical properties to these segments [5-14].

The lypophilic derivatives of GPCR-peptides were discovered by Athan Kuliopulos et al. in 2002 and designated as pepducins [5,6]. The cellular distribution of pepducins showed that they are capable of penetrating through the lipid bilayer of the plasma membrane to intracellular target proteins, which allows them to be used *in vivo* [15,16]. The action of pepducins is carried out at lower concentrations compared to unmodified GPCR-peptides. This is due to the fact that their lipophilic portion is anchored in the membrane, which significantly increases the concentration of pepducins near target protein and stabilizes their biologically active conformation, providing effective and selective interaction with it.

The most significant achievement in the study of GPCR-peptides, which in many respects predetermined the modern approaches to their development and practical application, is the new model based on the specific interaction of GPCR-peptides with the complementary regions of homologous receptor, after penetration of peptides into the cell [5,7,15]. Furthermore, one can not exclude the interaction of GPCR-peptides with complementary regions located in intracellular domains of non-homologous receptor which forms a heterodimeric complex with receptor homologous to the peptides [17]. Thus, the action of GPCR-peptides can be implemented only in the presence of a homologous receptor and its oligomeric complexes, and, as a result, has the receptor and tissue specificity

[5,6,8,11,12,14,18-22]. The specificity and selectivity of regulatory action of GPCR-peptides derived from receptor ICL on the intracellular signaling cascades and their activity *in vivo* open new ways to create GPCR-based regulators of hormonal signaling systems. Note that the peptides derived from receptor TM also demonstrate high biological activity *in vitro* and *in vivo*. They are able to be embedded into the lipid matrix of the membrane and specifically interact with complementary TM of homologous receptor [23-25]. This review describes recent advances in the development of GPCR-peptides derived from receptor intracellular domains, and the prospects of their use in clinical practice.

2. MECHANISM OF ACTION OF GPCR-PEPTIDES

Nowadays two main molecular mechanisms of action of GPCR-peptides and their lypophilic derivatives, pepducins, are a focus of attention (Fig. 1).

According to one mechanism of the action of GPCR-peptides developed by the Kuliopulos group, GPCR-peptide mimicking the intracellular region of receptor homologous to it is able to act as an agonist, it binds to the high-affinity site localized within the receptor ICLs and stabilizes or induces the active state of ligand-free GPCR, making it possible for the latter to interact effectively with G proteins and trigger signaling cascade [5,6]. Pepducin can also act as an antagonist, it binds to the lower-affinity, inhibitory site and blocks the effective interaction between G protein and cognate GPCR and to prevent the transduction of hormonal signal to G protein. According to this two-site mechanism, GPCR-peptide specifically interacts with its cognate receptor complementary regions which in full-size receptor have contact with the region homologous to the peptide. The GPCR-peptides can function as allosteric regulators of the agonist binding with cognate receptor, causing changes in conformation of the ligand-binding site. They influence the di- and oligomerization of GPCRs, which in many cases accounts for their functional activity, acting either as positive allosteric regulators inducing the appropriate cellular response to agonist, even at a very low concentration, or as negative allosteric regulators blocking partially or completely this response [15,26].

The other mechanism of action of GPCR-peptides is based on their ability to interact directly with the $G\alpha$ subunit and to influence the functional activity and signal properties of G protein and its complex with GPCR (Fig. 1). The main targets of GPCR-peptides are the C-terminal region of $G\alpha$ subunit responsible for selective interaction of different types of G proteins with ligand-activated GPCRs, as well as the $\alpha4$ helix and the $\alpha4/\beta6$ loop located closely to the C terminus of $G\alpha$ subunit, that are involved in the interaction between GPCR and G protein and determine the specificity of their interaction in the case of $G\alpha_i$ subunit [27]. The synthetic peptides corresponding to the C-terminal region of $G\alpha$ subunits selectively decrease the regulatory effects of GPCR-peptides on the activity of G proteins and signaling cascades dependent of them [9,28]. This gives the evidence for a direct interaction between GPCR-peptide and $G\alpha$ subunit C terminus. As is shown in the case of peptides corresponding to ICL3 of m_2- and m_3-muscarinic cholinergic receptors, GPCR-peptides can also interact with $G\beta$ subunits [29]. The key role in the interaction between GPCR-peptides and G protein subunits is ascribed to ionic contacts between basic amino acids of GPCR-peptide usually having a net positive charge and negatively charged $G\alpha$ and $G\beta$ subunits, as it is observed in a full-size GPCR–G protein interaction [30,31]. The role of positively charged amino acids located in the membrane-proximal intracellular regions of GPCRs coupling to G_s proteins is less significant than that in GPCR coupling to $G_{i/o}$ and $G_{q/11}$ proteins. A direct interaction of GPCR-peptides and G proteins was demonstrated by the specific binding of $G_{i/o}$ proteins to

chemisorbed positively charged GPCR-peptides corresponding to ICL2 and ICL3 of α_2-adrenergic receptor [32,33]. This view concerning the direct interaction between GPCR-peptides and G proteins found support in the activation and modulation of Gα subunits by positively charged peptides, such as peptide toxins of insect, some peptide hormones of vertebrates and artificial cationic peptides, non-homologous to receptors but similar to their G protein-interacting and -activating regions at the level of secondary structure and in the distribution of positively charged amino acids [34-41]. However, the action of non-GPCR-peptides is not specific, and they can affect a wide spectrum of G protein-dependent signaling systems.

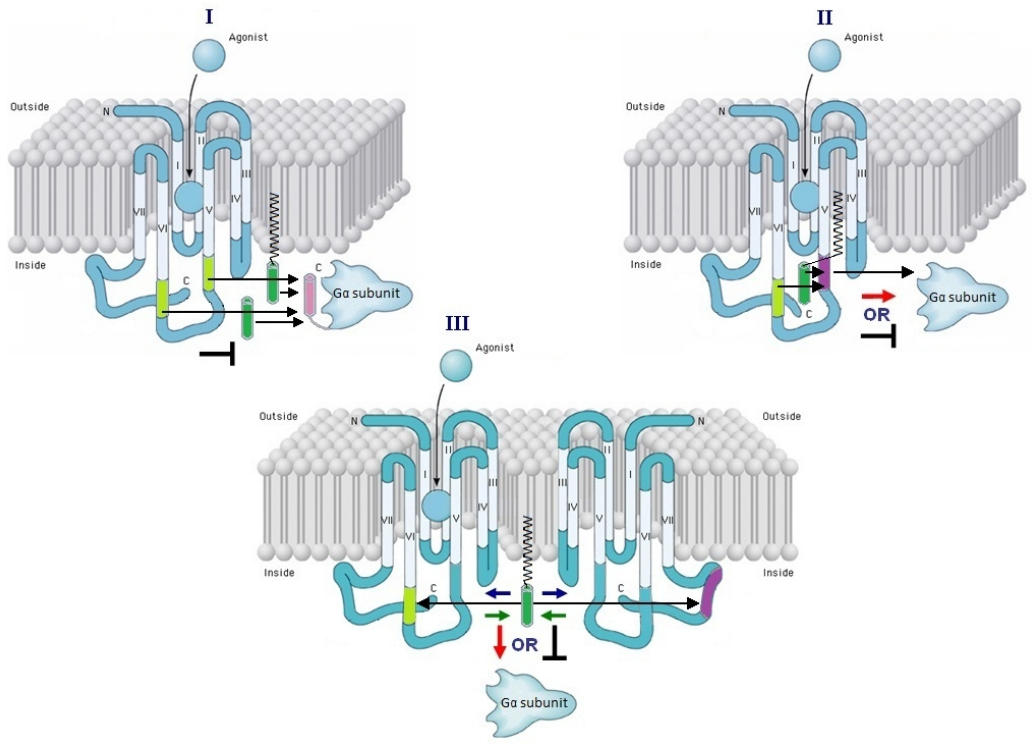

Fig. 1. The molecular mechanisms of action of GPCR-peptides and their lypophilic derivatives

I – Direct interaction of positively charged GPCR-peptides and their hydrophobic radical-modified derivatives with negatively charged C-terminus of Gα subunit which leads to blocking of functional interaction between agonist-activated receptor and G protein; II – Specific interaction of GPCR-peptides with complementary regions of the cognate GPCR which induces the activation of G protein or prevents the signal transduction from the ligand-activated receptor; III – The influence of GPCR-peptides on the di- and oligomerization of GPCRs due to their specific interaction with the intracellular regions of the cognate receptor responsible for the formation of GPCR complexes

Finally, the mechanism of action of GPCR-peptides can be a combination of the above mechanisms, depending on the structure and modifications of GPCR-peptide, its concentration, the location of region homologous to it in full-size receptor, the ability of the cognate receptor to form a functionally active homo- or heterodimeric complex, and other things, as was shown for cyclic peptide mimicking ICL3 of V_2-vasopressin receptor and for GPCR-peptides corresponding to ICL3 of somatostatin receptors of the types 1 and 2

[17,42,43]. Even minor structural changes in GPCR-peptide led to significant alterations in its biological activity. Thus, as a rule, the non-palmitoylated analogs of GPCR-peptides were less active and less specific compared with the palmitoylated counterparts [5,9,11,13,14].

3. GPCR-PEPTIDES AS SELECTIVE REGULATORS OF SIGNAL TRANSDUCTION

Thirty years ago, the data was obtained showing that peptides corresponding to the ICLs of G_s protein-coupled adrenergic and dopamine receptors are capable of activating the adenylyl cyclase (AC) and affect the signal transduction via a receptor homologous to them [1,2,4]. Later it was shown that peptides derived from different intracellular regions of many GPCR, including the receptors of the biogenic amines, polypeptide hormones, prostaglandins, fatty acids and cannabinoids also possess specific biological activity and regulate and modulate intracellular signaling cascades [15,26,44-47].

The action of GPCR-peptides was characterized by receptor specificity and G-protein selectivity [11,14,17,48-54]. Peptide KHSRKALKASL$^{258–268}$ and its C-palmitoylated analog corresponding to the ICL3 of 5-hydroxytryptamine receptor of the type 6 (5-HT_6R), inhibited the stimulatory effect of selective 5-HT_6R-agonist EMD-386088 on AC activity and GTP-binding of G_s proteins, but did not influence regulatory effects of agonists of other 5-HTR types [11]. Peptides 306–316 and 300–316 derived from HT_{1B}R ICL3 reduced both the inhibitory effect of serotonin and 5-HT_1R agonists, especially selective 5-HT_{1B}R agonist 5-nonyloxytryptamine, on AC activity and their stimulating effect on the GTP binding of G_i proteins, but were ineffective in the case of other receptors [53]. Peptide corresponding to the ICL2 of sphingosine 1-phosphate receptor (S1PR) of the type 3 induced the internalization of the cognate receptor, unlike the type 1 S1PR possessing structural and functional similarity to the type 3 S1PR [51]. Lipophilic derivatives of peptides corresponding to the intracellular loops of the protease-activated receptors (PAR), chemokine receptors, relaxin receptor RXFP1, and the receptors of luteinizing (LH) and thyroid-stimulating hormones (TSH) also demonstrated high receptor specificity [5,6,9,12-14].

GPCR-peptides selectively activated G-proteins coupled with the cognate receptor. Peptides 301–317 and 329–344 corresponding to the ICL3 of CB_1-cannabinoid receptor specifically blocked the functional interaction of the receptor with G_{i1} and G_{i2} proteins, but did not change the activity of structurally and functionally related G_{i3} protein not coupled with CB_1-cannabinoid receptor [49]. GPCR-peptides corresponding to the ICL3 of G_s-coupled prostacyclin receptor, LH and TSH receptors, relaxin receptor RXFP1 and glucagon-like peptide-1 receptor selectively activated G_s proteins [9,11,12,14,50,51], whereas the peptides corresponding to the same loop of G_i-coupled δ- and μ-opioid receptors, 5-HT_{1B}R and somatostatin receptors selectively stimulated the GTP-binding capacity and GTPase activity of G_i proteins [17,48].

The regulatory effects of GPCR-peptides were tissue specific and observed in the tissues where the cognate receptors are generally expressed [11,12,14,54]. Peptide NKDTKIAKK-*Nle*-A$^{562–572}$-K(Pal)A, the derivative of the ICL3 of LH receptor, activated AC primarily in the testes, peptide KHSRKALKASL$^{258–268}$-K(Pal)A derived from 5-HT_6R ICL3 in the brain, and peptide QYNPRDKDTKIAKR-Nle-A$^{612–627}$-K(Pal)-A derived from TSH receptor ICL3 preferably stimulated the enzyme activity in the thyroid [14,54]. In the tissues where the cognate receptors are expressed weakly, if at all, the changes of AC activity were not significant. These observations coincide very well with the molecular model of GPCR-

peptides action that is based on the complementary interaction between the peptide and appropriate regions of cognate receptor.

The fact that the GPCR-peptides were not active in the cell cultures which lack cognate receptor is another indication of their tissue specificity. This is illustrated by the data obtained on the peptides derived from the intracellular regions of PAR of the type 1 (PAR1). In the cells where the cognate receptor was absent or where mutant PAR1 lacking the C-terminal domain was expressed, the peptide corresponding to the N-terminal portion of the ICL3 had no effect on the activity of G-proteins and phospholipase Cβ (PLCβ) [6]. Palmitoylated peptide 374–386 corresponding to membrane-proximal region of the C-terminal domain of PAR1 stimulated phosphoinositide turnover only in the cell cultures with expressed PAR1 [55].

The ability of the GPCR-peptides to regulate the functional activity of hormonal signaling systems in a specific manner and to influence the fundamental cellular processes, such as growth, metabolism, differentiation, and apoptosis, allows them to be used as functional sensors in the study of structural and functional organization of these systems, the molecular mechanisms of signal transduction, and the intracellular cascades and transcriptional factors, the targets of hormonal action. Of particular interest is the ability of GPCR-peptides to control the physiological and biochemical processes, as this opens prospects for creation of GPCR-peptide-based drugs.

The study of GPCR-peptides, derivatives of the intracellular regions, carried out in the last ten years, showed that modification of these peptides by hydrophobic radical gives their analogs, pepducins, possessing the activity both *in vitro* and *in vivo* [5,6,8,15,26,45-47]. It has been already mentioned that such high efficiency of pepducins as regulators of biochemical and physiological processes *in vivo* is due, on the one hand, to their cell-penetrating properties, the ability to attach to the membrane inner surface and to the hydrophobic core of the cognate receptor, and on the other to the similarity between the 3D-structure of pepducin and that of homologous region in intact receptor. At the same time, there are data showing that the unmodified peptides can also possess activity *in vivo*, for example, in the case of a short, 9-amino acid, peptide KRX-725 derived from the ICL2 of the type 3 S1PR, possessing pro-angiogenic activity [51]. This peptide mimicked the regulatory effects of sphingosine 1-phosphate, induced extensive and dense vascular sprouts in aortic ring assay, an *ex vivo* model of angiogenesis, and initiated neovascularization in a mouse corneal pocket assay *in vivo*, its pro-angiogenic effects being synergistic with those of basic fibroblast growth factor, stem cell factor, and vascular endothelial growth factor. These results indicate that KRX-725 can be used to treat peripheral vascular disease, myocardial ischemia, and diabetic wound healing [51].

4. PAR-DERIVED PEPDUCINS

4.1 The Influence on Platelet Aggregation

Thrombin-dependent activation of platelets increases in the case of rupture of atherosclerotic plaques, erosion, percutaneous coronary intervention, which leads to arterial thrombosis, one of the main causes of myocardial infarction and ischemic stroke. Platelets adhere to damaged blood vessels, aggregate and induce thrombin production. Thrombin activates platelets, and its regulatory effect is realized via PAR1 and PAR4, both located in the plasma membrane of platelets and functionally coupled with different types of G-proteins, such as $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ [56]. Regulatory effects of thrombin are exerted mainly via PARs

coupled with $G_{q/11}$-proteins and PLCβ. Thrombin-induced PLCβ stimulation leads to an increase of intracellular calcium concentration and the synthesis of diacylglycerol activating protein kinase C. This causes activation and surface recruitment of integrins αIIb/β3, which enhances the platelet aggregation, induces the release of ADP that binds to platelet purinergic P2Y12-receptor, and triggers the synthesis of thromboxane A_2, an important mediator of platelet aggregation and degranulation. The usual practice to prevent platelet aggregation likely to occur in acute coronary syndromes and percutaneous coronary intervention is to use aspirin lowering the production of thromboxane A_2, antagonists of $P2Y_{12}$-receptor, and inhibitors of the platelet glycoprotein complex GPIIb/IIIa involved in platelet adhesion and aggregation. However, these approaches are not very effective and prevent less than 17 % of deaths caused by arterial thrombosis [57]; this speaks in favor of an urgent task to develop new drugs that will effectively control PAR1- and PAR4-dependent signaling pathways.

The data obtained in the study of PAR-derived peptides indicate that the pepducins corresponding to the ICLs of PAR1 and PAR4 possess a high specific activity *in vitro* and function as modulators of PAR1/PAR4-mediated signal transduction *in vivo*.

It was shown that pepducin Pal-ATGAPRLPST (P4pal-i1) corresponding to the ICL1 of PAR4 selectively inhibited the transduction of thrombin signal via PAR4 [58]. This pepducin blocked the PAR4-mediated chemotactic response and prevented platelet aggregation induced by AYPGKF-amide, a selective PAR4-agonist, but had no influence on the same processes mediated via PAR1. The combined use of P4pal-i1 and selective PAR1-antagonist RWJ-56110 led to the disappearance of thrombin effects, which suggests the high efficiency of simultaneous blocking of PAR1 and PAR4 [58]. The combined use of P4pal-i1 and thrombolytic agent bivalirudin, whose action is based on its specific binding to thrombin, led to a significant increase in the efficacy of both agents and completely prevented platelet aggregation induced by submaximal concentration (10 nM) of α-thrombin [58,59]. In the *in vivo* experiments, using the model of the carotid artery occlusion in guinea pigs, it was shown that P4pal-i1 (0.13 mg/kg) prolonged the occlusion time and prevented arterial thrombosis. The combined administration of P4pal-i1 and bivalirudin caused a significant protection against acute arterial occlusion and gave much better results than bivalirudin alone [58].

Pepducin Pal-SGRRYGHALR (P4pal-10) that corresponds to the ICL-3 of PAR4 was less specific compared with P4pal-i1 and was found to be PAR4-antagonist and inverse PAR1-agonist [6,60]. P4pal-10 completely blocked the stimulating effect of PAR4-agonist AYPGKF-amide on PLCβ activity, and reduced by 36 % the same effect induced by PAR1-agonist SFLLRN-amide. The pepducin reduced by 45-70% platelet aggregation induced by AYPGKF-amide, but had a weak effect on SFLLRN-amide-induced aggregation and did not affect platelet aggregation that is mediated by activation of receptors that do not belong to PAR family [6]. *In vivo* P4pal-10 prevented thrombosis of the mouse carotid artery induced by $FeCl_3$ treatment [8].

Pepducin Pal-RCLSSSAVANRS (P1pal-12) and its truncated analog P1pal-7 corresponding to N-terminal portion of PAR1 ICL3 inhibited the stimulation of PLCβ and the increase of intracellular level of Ca^{2+} induced by PAR1-agonist [5,6,61]. At micromolar concentrations P1pal-12 decreased by 75-95% platelet aggregation caused by PAR1-agonist SFLLRN-amide [5]. This pepducin partially inhibited the vasorelaxation mediated by PAR1-agonist TFLLR-amide, but not PAR2-agonist SLIGRL-amide, in the rat aorta [7]. The drug PZ-128

developed on the basis of P1pal-7 had the secondary structure that was similar to that of homologous C-terminal region of ICL3 and significantly inhibited PAR1-mediated platelet aggregation and arterial thrombosis in guinea pigs and monkeys [62]. The therapeutic effect of the PZ-128 was enhanced in the presence of antiplatelet agent clopidogrel, which inhibits the binding of ADP with the platelet receptors and the activation of the glycoprotein complex GPIIb/IIIa and prevents platelet aggregation. The treatment with the PZ-128 resulted in full recovery of platelet function during the day. It should be noted that the PZ-128 had no side effects. Neither did it cause bleeding or coagulation in the blood from primates and from the patients with percutaneous coronary intervention. Based on these findings, Zhang et al. concluded that the PZ-128 is a potent inhibitor of PAR1 and can be a good alternative to the low-molecular PAR1-antagonists used to treat arterial thrombosis [62].

The elongated pepducin Pal-RCLSSSAVANRSKKSRALF (P1pal-19), unlike P1pal-12 and P1pal-7, functioned as intracellular PAR1-agonist. P1pal-19 mimicked ligand-activated receptor and stimulated G_q- and $G_{i/o}$-proteins and their downstream signal proteins. The regulatory effects of P1pal-19 were similar to those of PAR1-agonists, TFLLR-amide and SFLLRN-amide [**5-7**]. Thus, the modification of pepducin not only affected its efficiency and selectivity, but in some cases could, in addition, change the mode of this action.

4.2 The Influence on Angiogenesis, Cancer and Metastasis

PAR1-mediated intracellular signaling pathways have an important role in the regulation of tumor angiogenesis and, thus, are involved in the invasive and metastatic processes of a wide spectrum of cancers, such as melanoma, breast cancer, ovary cancer, lung cancer, colon cancer and prostate cancer. In the tumor cells the density of PAR1 increases significantly, and the activity of matrix metalloprotease-1 (MMP1) that functions as PAR1-agonist is up-regulated [63,64]. PAR1-agonists enhance tumorigenesis, invasion, and metastasis, whereas PAR1-antagonists, on the contrary, suppress them [63,65].

Quite recently it was found that pepducin P1pal-7 (PZ-128), which, as was mentioned above, is the PAR1-antagonist and possesses thrombolytic activity, is also capable of suppressing the tumor growth and metastasis, and reduces the cell viability in breast, ovarian and lung carcinoma cells [63,64,66]. It should be noted that PAR1 was not expressed in normal breast epithelia but was up-regulated in invasive breast carcinomas, which led to blocking apoptosis and increased survival of malignant cells. P1pal-7 inhibited Akt-kinase, an important anti-apoptotic protein, and significantly promoted apoptosis in breast tumor xenografts expressing PAR1 [66]. P1pal-7, like MMP1 inhibitors, alone and in combination with Taxotere, an anti-cancer chemotherapy drug, inhibited growth of breast tumor xenografts and their metastasis to the lung.

In vitro P1pal-7 and its elongated analog P1pal-12 almost completely inhibited PAR1-dependent migration of ovarian carcinoma cells OVCAR-4 toward ascites fluid obtained from patients with ovarian cancer [63]. P1pal-7 and P1pal-12 also significantly decreased migration of other ovarian carcinoma cells, IGROV-1 and SKOV-3, to malignant human ascites fluid as well as fibroblast media. The six-week treatment of mice having the ovarian carcinoma with P1pal-7 caused 84–96% decrease of blood vessel density both in the center and edge of the OVCAR-4 tumors, and 90 % decrease in these parameters in the SKOV-3 tumors, as compared with control animals. The use of P1pal-7 enhanced the inhibitory effect of Taxotere on the angiogenesis in peritoneal ovarian cancer. The dual therapy with P1pal-7 and Taxotere caused a further significant decrease of invasion and metastasis from the peritoneal cavity to the thoracic organs, compared to Taxotere alone [63]. These data

indicate a potent inhibitory influence of P1pal-7 and P1pal-12, antagonists of PAR1, on the ovarian carcinomas and their potentiating effect on the specific activity of other drugs used to treat this tumor.

Pepducins derived from the ICLs of PAR1 were effective in the treatment of lung cancer. The expression of PAR1, unlike the other types of PAR, is markedly increased in lung cancer cells isolated from patient with lung tumors. Pepducins with PAR1-antagonistic activity, corresponding to the ICL1 and ICL3 of PAR1, significantly reduced PAR-mediated cell migration both in primary and established cell lines [19]. Besides, ICL3-derived pepducin P1pal-7 effectively inhibited PAR1-mediated activation of extracellular regulated kinase 1/2 and decreased lung tumor growth by 75 %, whose effectiveness is comparable with that of monoclonal VEGF-directed antibody drug Bevacizumab, an antitumor agent and angiogenesis inhibitor. This peptide suppressed invasion stellate growth of Lewis lung carcinoma cells in 3D matrices [64]. Pepducins corresponding to PAR1 ICL1, unlike P1pal-7, had no affect on extracellular regulated kinase activity and lung tumor growth.

Pepducin Pal-AVANRSKKSRALF (P1pal-13), a selective PAR1-agonist, had a pronounced pro-angiogenic effect in mice with carotid artery ligations [67]. The treatment of animals with P1pal-13 for 21 days at a dose of 2.5 mg/kg caused striking hyperplasia in the injured carotid artery, and this effect of pepducin required the presence of both PAR1 and PAR2. Therefore, a suggestion can be made that PAR1 is likely to form a functionally active heterodimeric complex with PAR2.

4.3 The Influence on Inflammation

Apart from the regulation of blood coagulation, angiogenesis and tumor growth, PAR1-mediated signaling pathways are involved in etiology and pathogenesis of inflammatory diseases, including sepsis. Agonist-induced PAR1 activation causes potent anti-inflammatory effect, protects endothelial cells against destruction [68]. However, such prolonged and very pronounced activation of PAR1 by high concentrations of thrombin in sepsis has the opposite effect; it leads to the dysfunction of the endothelial cells, the deterioration of the sepsis and the increase of mortality of patients with this disease [69,70].

To study the effect of PAR1-derived pepducins on the development of sepsis, a model of bacterial peritonitis in mice initiated by cecal ligation and puncture was used. Pepducins were injected subcutaneously either immediately or 4 h after puncture and the mortality rate was evaluated seven days after peritonitis induction. Pepducin Pal-RSLSSSAVANRS (P1pal-12S), a full PAR1-antagonist, administered at a dose of 2.5 mg/kg parallel to the induction of peritonitis, increased survival of two mice lines, CF-1 and C57BL/6, to 50-60 %, compared to untreated animals, which had 90-100 % mortality rate, while the injection of P1pal-12S 4 h later had no effect on mortality. At the same time, pepducin Pal-AVANRSKKSRALF (P1pal-13) with agonistic activity taken at the same dose was not effective when administered immediately after puncture, but increased the survival of CF-1 mice to 80 % in the case of delayed (4 h) administration, when there was a pronounced sepsis. It follows that in *Par1*$^{-/-}$ mice lacking PAR1 neither of the two pepducins had influence on sepsis, indicating that the receptor mediates the effects of PAR1-derived pepducins on inflammation [68].

One of the most severe manifestations of sepsis is septic shock, a complex syndrome characterized by profound cardiovascular derangement, including increase in cardiac output, alterations of microcirculation, blood flow redistribution between organs, decrease in

peripheral resistance and loss of intravascular fluid [71,72]. Early treatment of mice with peritoneal sepsis with PAR1-antagonist P1pal-12S or late treatment (4 h delay) with PAR1-agonist P1pal-13 gave protection against septic shock and prevented lung vascular leakage [68]. The treatment of septic mice with P1pal-12S 8 h after puncture increased lung vascular permeability, while the injection of P1pal-13 8 h after puncture was as effective as after a 4 h delay. These data indicate that optimization of the use of PAR1-derived pepducins with agonistic and antagonistic activity at the early and late stages of sepsis may result in pronounced therapeutic effect on this disease.

Like most of the PAR1-mediated effects, the regulation of inflammatory processes through PAR1 requires PAR2 to form heterodimeric PAR1-PAR2 complex. Crucial evidence for this was obtained with pepducins derived from PAR2 ICL3. Pepducin Pal-RSSAMDENSEKKRKSAIK$^{270–287}$ (P2pal-18S) with the replacement of Arg^{284} by serine at concentration of 0.14-0.2 mM completely blocked the migration of human neutrophils towards gradients of trypsin and selective PAR2-agonist SLIGRL-amide and the migration of mouse neutrophils toward 30 nM trypsin [67]. In the experiments with neutrophils chemotaxis, P2pal-18S had the receptor specificity, which did not affect the closely related PAR1, PAR4 and chemokine receptor CXCR1/2. It was shown also that P2pal-18S inhibited the migration of primary cholangiocarcinoma cells across a collagen membrane barrier, induced by trypsin and selective PAR2-agonist furoyl-LIGRLO-amide [73].

In the *in vivo* experiments the systemic administration of P2pal-18S caused the decrease of λ-carrageenan/kaolin-induced edema by 50 % and of edema induced by PAR2-agonist SLIGRL-amide by 85 % [67]. Even small changes in the length of PAR-derived pepducin and the substitutions of individual amino acids influenced the efficiency and selectivity of the pepducins, and, in addition, determined their activity as agonists or antagonists and influenced the choice of intracellular signaling cascades, the targets of pepdicin.

It was shown that the addition of three amino acid residues to the N-terminus of the pepducin P2pal-18S gives the potent PAR2-antagonist [67]. The replacement of the C-terminal lysine by phenylalanine gave the inverse PAR2-agonist. P2pal-18S with Lys^{287}Phe replacement did not have antagonistic activity and was a weak agonist of PAR2. These data lay the foundation stone in creation of the pepducin-based regulators of inflammatory processes, which can increase as well as decrease the inflammatory response and the pathological changes induced by inflammation.

4.4 The Influence on Acute Pancreatitis

PAR2 expressed in the pancreas by a variety of cell types, including endothelial cells, nerve cells, cells of the immune system, duct cells, and acinar cells, have an important role in acute pancreatitis, a complex inflammatory disease of the pancreas. PAR2, as indicated by the following facts, exerts a protective effect on secretagogue-induced pancreatitis. The activation of PAR2 by selective agonists reduced the severity of secretagogue-induced pancreatitis in rats [74], and the severity of this model of pancreatitis was increased in mice with deletion of *PAR2* gene [75]. At the same time, PAR2 exerts a worsening effect in the bile salt-induced experimental pancreatitis that clinically is more relevant to this disease. It was shown that pharmacological activation of PAR2 promoted the pathological effects of bile salts on pancreatic acini *in vitro*, and the deletion of *PAR2* gene markedly reduced the severity of bile salt-induced pancreatitis [76].

Pepducin P2pal-18S corresponding to PAR2 ICL3, that in acinar cells has the properties of PAR2-antagonist, significantly reduced the severity of experimental biliary pancreatitis in mice when administered before or 2 h after bile acid infusion. The pepducine was ineffective when administered 5 h after the induction of disease. In the experiments *in vitro* P2pal-18S protected acinar cells against injury induced by bile acid [77]. It follows that PAR2-derived pepducins with antagonistic activity may be successfully used in the clinical management of patients at risk for developing acute biliary pancreatitis [77]. Contrary to this, P2pal-18S *in vivo* increased the severity of secretagogue-induced pancreatitis.

5. CHEMOKINE RECEPTOR-DERIVED PEPDUCINS

The activation of PAR1 by MMP1 was followed by secretion of angiogenic chemokines, especially of interleukin-8 and chemokine CXCL1/GRO-α, from ovarian carcinoma cells. This induced the activation of endothelial chemokine receptors CXCR1 and CXCR2 sensitive to these chemokines and resulted in proliferation of endothelial cells, tube formation, and migration [10]. CXCR1 and CXCR2 are known to be involved in the control of survival, proliferation, and angiogenesis of vascular endothelial cells. Pepducin Pal-RTLFKAHMGQKHRAMR (X1/2pal-i3), a selective antagonist of CXCR1/2, that corresponds to the ICL3 of these receptors at a concentration of 300 nM inhibited the proliferation of endothelial cells induced by interleukin-8 and CXCL1/GRO-α, and significantly reduced MMP1-induced proliferation of cultured human umbilical vein endothelial cells with expressed PAR1. Peptide YSRVGRSVTD (X1/2LCA-i1), the derivative of CXCR1/2 ICL1, modified by lithocholic acid at the N-terminus and N-palmitoylated peptide X1/2pal-i3 both inhibited tube formation induced by interleukin-8, CXCL1/GRO-α and the media from the MMP1-treated OVCAR-4 cells. The inhibitory effect of X1/2pal-i3 on blood vessel formation in the mouse models of angiogenesis was comparable with that of anti-cancer drug Bevacizumab. Three-week treatment of mice with X1/2pal-i3 induced 5-fold decrease in angiogenesis and a significant decrease in tumor progression in ovarian cancer xenografts [10]. The injection of the truncated X1/2pal-i3-pepducin analog, Pal-RTLFKAHMGQKHR, at a dose 2.5 mg/kg to mice $Apc^{Min/+}$ reduced spontaneous formation of benign intestinal adenomas [22].

Chemokine receptor CXCR4 and its ligand, stromal cell-derived factor-1 CXCL12/SDF-1, are involved in regulation of many cellular processes, including leukocyte trafficking, B-cell lymphopoiesis, bone marrow myelopoiesis, and survival and proliferation of hematopoietic stem cells [78,79]. The CXCR4 is expressed to a high extent in many tumors, such as malignant brain neoplasms, ovarian cancer, melanoma, colorectal cancer, and myeloid and lymphoid leukaemia, some of which are difficult to treat or even incurable [80-82]. The cells of the tumors and their microenvironment produce a large amount of CXCL12/SDF-1 that binds to CXCR4 and induce tumor progression, angiogenesis, metastasis, and survival [83].

As was shown in preclinical and clinical studies, selective CXCR4-antagonists, CXCR4-derived pepducins in particular, inhibiting the signal transduction via the cognate receptor and in combination with chemotherapy effectively suppressed the tumor growth, manifesting themselves as novel promising drugs in the therapy of CXCR4-associated neoplasia [20,84,85]. It was shown that N-palmitoylated peptides corresponding to the region 63–74 of ICL1 and to the region 224–239 of ICL3 of human CXCR4, designated as PZ-218 and PZ-210, specifically inhibited CXCL12-mediated responses in human and mouse neutrophils and blocked CXCL12-mediated cell migration of lymphoma and lymphocytic leukemia [13,86]. Neither non-palmitoylated analogs of the pepducins PZ-218 and PZ-210, nor ICL1-

derived pepducin PZ-218 with an additional C-terminal lysine inhibited chemotaxis of human leukemia and lymphoma cells, indicating the palmitate moiety and the specific peptide composition to be essential for antagonist activity of the CXCR4 pepducins [13]. The CXCR4-derived pepducins both in monotherapy and in combination with rituximab, the monoclonal antibody to B-cell-associated antigen CD20, effectively suppressed the survival and metastasis of disseminated lymphoma xenografts, which is likely to be the basis of a new treatment strategy for lymphoid malignancies. Note that the pro-apoptotic effect of rituximab was completely blocked by the treatment with chemokine CXCL12. But in the combined treatment of tumor cells with CXCR4-derived pepducins and rituximab the inhibitory effect of the chemokine on apopotosis was blocked. Therefore, the pepducins increased the susceptibility of lymphoma cells to rituximab-mediated cell death, which pointed to the synergism of CXCR4-derived pepducins and rituximab action [13].

Thus, pepducins derived from the intracellular regions of PAR1 and chemokine receptors CXCR1, CXCR2 and CXCR4, possess potent antitumor activity. They are comparable to many anti-cancer drugs used to treat tumors of the reproductive system and lung, lymphocytic leukemias and other malignancies, and in some cases surpass them. The antitumor effect of pepducins comprises the increase of apoptosis of malignant cells, the inhibition of platelet aggregation and chemotaxis, and the suppression of tumor angiogenesis, which leads to a decrease of tumor survival and growth. Using pepducins, the new molecular mechanisms of tumorigenesis and metastasis including PAR1 and some chemokine receptors were revealed. They open up new possibilities for the control and treatment of cancer.

6. GPCR-PEPTIDES DERIVED FROM TSH RECEPTOR

There are many works on the *in vitro* activity of GPCR-peptides corresponding to ICL of the receptors involved in the regulation of endocrine functions [9,12,14,17,87-91]. But no information is available now on the activity of these peptides *in vivo*. The exception is palmitate-modified peptide QYNPRDKDTKIAKR-Nle-A$^{612–627}$-KA (612–627-K(Pal)A) derived from the ICL3 of TSH receptor synthesized and studied by us both the *in vitro* and *in vivo* conditions [14,92]. This peptide at micromolar concentrations stimulated the basal activity of AC and GTP-binding capacity of G_s-proteins and reduced the regulatory effects of TSH in the thyroidal membranes [14]. The effects of pepducin 612–627-K(Pal)A on AC signaling system were receptor and tissue specific. This is so because this pepducin was most active in the thyroid, where TSH receptor is, as a rule, intensively expressed, and exerted a very strong influence on the TSH-induced signal transduction.

Intranasal administration of 612–627-K(Pal)A into rats for 5 days at a daily dose of 450 mg/kg resulted in a significant and sustained increase of the free T4 level. The subsequent administration of the peptide for 10 days (with an interval of five days) led to a decrease of the level of free T4. The level of total T3 was increased after 5-day treatment, but returned to normal values in the case of long-term treatment. The level of TSH was markedly decreased after 10-day treatment [92]. Unmodified peptide 612-627-KA was not very active in the experiments *in vitro* and *in vivo*. This picture is similar to the results obtained using TSH-releasing hormone which in the short-term treatment increased the free T4 level, while in the long-term treatment reduced it, and influenced, but not significantly, the level of total T3 [93]. Thus, pepducin 612-627-K(Pal)A that we synthesized influenced the functional activity of the thyroid by acting on the initial steps of transduction of TSH signal and, in the future, it can be used to develop the pepducin-based regulators of thyroid functions.

7. CONCLUSION

The data presented above suggest that a significant progress has been achieved in the recent years allowing creation of highly selective and highly effective GPCR-peptides corresponding to the intracellular regions of receptors. However, the potential and prospects of the use of GPCR-peptides in clinical medicine are not fully realized yet. There are a number of problems to be solved for a further progress in this area. These are, first, developing new approaches for the modification of GPCR-peptides, which would create their biologically active analogs with the desired properties, and second, expanding the range of GPCR-peptides under study by increasing the list of receptors. Now, a detailed study of GPCR-peptides and their analogs has been carried out for a very limited number of GPCRs, i.e. PARs, some types of chemokine receptors, 5-HTRs, and the receptors of glycoprotein hormones. Finally, to determine the therapeutic potential of GPCR-peptides, they must be systematically studied *in vivo*, using experimental animal in norm and under pathological conditions.

Signal transduction from the ligand-activated GPCR to the intracellular targets can be mediated by G-protein-dependent pathways involving heterotrimeric G proteins as well as by G-protein-independent pathways involving β-arrestins [94,95]. β-Arrestins mediate the effects of β-arrestins-biased agonists on the activity of mitogen-activated protein kinases, phosphatidylinositol 3-kinase, non-receptor tyrosine kinases and other intracellular effectors. A key role in the interaction of ligand-activated receptor with β-arrestins belongs to its ICL3 and C-terminal domain [96]. There are a number of works showing the ability of GPCR-peptides to regulate β-arrestin signaling pathways [89,97-99]. However, the biological activity of β-arrestin-biased GPCR-peptides *in vivo* has not been studied, and in the *in vitro* experiments the main focus was on the use of the peptides in searching the molecular determinants of receptor involved in its specific binding and interaction with β-arrestins. The study of the effect of GPCR-peptides on β-arrestins-regulated pathways is a reliable way to design β-arrestin-biased GPCR-peptides selectively regulating the β-arrestin, G protein-independent, signaling pathways. Such peptides will be of value in fundamental biology as functional probes to be used in the study of the β-arrestin signaling and the molecular mechanisms of receptor–β-arrestin coupling, and in medicine as selective β-arrestin-biased intracellular agonists and antagonists.

ACKNOWLEDGEMENTS

This work was supported by Grant No. 12-04-00351 from the Russian Foundation of Basic Research and by the Ministry of Education and Science of Russian Federation (project No 8486 to IEPhB RAS). We express our thanks to Inga Menina for linguistic assistance.

COMPETING INTERESTS

The author reports no conflict of interest in this research.

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