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Intranuclear Signaling Cascades Triggered by Nuclear GPCRs

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Abstract

G protein-couped receptors (GPCRs) play a key role on cellular membranes, where they respond to a broad array of extracellular signals such as lipids, peptides, proteins and sensory agents. Intracellular biological responses triggered by these receptors include hormone secretion, muscle contraction, cellular metabolism a tyrosine kinase receptors transactivation. Recent results indicate that GPCRs localize to and signal also at nuclear level, thus regulating distinct signaling pathways which can also result from the integration of extracellular and intracellular stimuli. Nuclear GPCRs play a central role in many cellular processes, including regulation of gene transcription, cellular proliferation, neovascularization and RNA synthesis. On nuclear membranes and in nucleoplasm are present all the downstream signal transduction components of GPCRs, including G proteins, adenylyl cyclase, and second messengers such as Ca^{++}, ERKs, p38MAPK and other protein kinases. Nuclear GPCRs may be constitutively active or may be activated by ligands internalized from the extracellular space or synthesized within the cell. The translocation of membrane receptors to the nucleus could be attributed to the presence of a Nuclear Localization Signal, which is present in the eighth helix or in the third intracellular loop of a limited number of GPCRs. However, several sequence motifs that do not resemble classical Nuclear Localization Signals can promote import of GPCRs. In this review we discuss the most recent results on nuclear localization and signaling of several GPCRS.

Keywords: G protein-coupled receptors; Nuclear localization; Signal transduction

Introduction

G protein-coupled receptors comprise a large family of heptahelical transmembrane proteins involved in cellular signaling [1]. They represent the largest class of gene products targeted by therapeutic agents and recognized by extracellular stimulants of diverse nature [2]. GPCRs are viewed as cell surface functional entities and have been classified into seven families, based on phylogenic analysis of the seven transmembrane domain [3,4]. In conventional GPCR signaling, stimulation with a specific agonist triggers the dissociation of heterotrimeric G proteins. α and βγ subunits can stimulate downstream effector molecules associated with several cellular signaling pathways, thereby altering the generation of second messengers (e.g. cAMP, cGMP, inositol triphosphate, diacylglicerol, Calcium, nitric oxide). These signals are negatively regulated by intrinsic GTPase activity of Gα subunits, G protein-coupled receptor kinases (GRKs), β-arrestins, metabolism of second messengers, regulators of G proteins (RGS) signaling or internalization and down-regulation of the receptor [5].

Several independent studies have revealed that endocytosed GPCRs may regulate distinct signaling pathways, suggesting that the GPCR-mediated biological responses may also result from the integration of extracellular and intracellular signaling events [6-9]. Intracellular GPCRs may be constitutively active, or may be activated by agonists internalized from the extracellular space or synthesized within the cell. Furthermore, a number of GPCRs also localize to and signal at the nuclear membrane or within the nucleus in several cell types [4]. GPCRs may be inserted into nuclear membranes where they compose distinctive signaling units which respond to specific intracellular ligands by transducing nuclear transcriptional signals. They can regulate signaling cascades distinct from those activated by the same receptor on plasma membrane. Nuclear GPCRs may be constitutively active or activated by endogenous non-secreted ligands, thus regulating several physiological processes, such as cell proliferation, DNA synthesis, transcription and inflammatory responses [10-13]. A large number of downstream signal transduction components, normally associated with GPCRs at cell surface, are present in the nucleus and/or on the nuclear membranes including G proteins, adenylyl cyclases, phospholipases A2, Cβ and D, ion channels, β-arrestins and GRKs [14]. Furthermore, several signaling pathway components molecules, such as ERKs, p38MAPK, PKB, PKA and PKC are activated upon binding of cognate ligands to nuclear GPCRs [15-18]. The nuclear import of proteins into the nucleus involves the recognition of a nuclear localization signal (NLS), embedded in the protein, by importins. The most frequently encountered NLS consists of short stretches of mono- or bi-partite basic aminoacids residues (usually lysine-arginine or glycine-arginine repeats), even though several heterogenous sequences that do not resemble to classical basic NLS can promote nuclear import of several proteins [19]. A NLS motif has been identified in the eight helix or in the third intracellular loop of several GPCRs [20]. Here in we discuss the most recent results on nuclear localization and signaling of GPCRs.

Adrenergic Receptors

In mammalian cardiomyocytes three β-adrenergic receptors (β-ARs) are expressed and their nuclear localization is subtype specific. β1-AR and β3-AR, but not β2-AR, are expressed onto nuclear membrane and both receptors are differentially coupled to signaling pathways in isolated heart nuclei [21]. β1-AR stimulates Gs protein and activates adenylyl ciclase (AC), whereas β3-AR stimulates Gi and modulates transcriptional initiation in a PTX-sensitive manner [21]. In isolated cardiac nuclei, isoproterenol, a β3-AR selective agonist, stimulates PTX-sensitive de novo RNA synthesis and other signaling pathways in addition to Gi [22]. Stimulation of nuclear β-AR also induces up-regulation of 18S rRNA and a decrease in NF-κB mRNA, as well as mRNAs for genes involved in the NF-κB signaling pathway [5]. The transcriptional events mediated by nuclear β-AR are sensitive to p38MAPK, ERKs, Akt, and JNK inhibitors, suggesting that these pathways are involved in nuclear signaling [21,23]. The principal endogenous agonists for β-ARs are nor-adrenaline and adrenaline, which are not produced or secreted by β-AR-expressing cells in the heart. The interaction of nuclear β-ARs with the cognate ligands requires the transport of agonists to the nucleus. In neonatal and adult cardiomyocytes, nor-adrenaline is rapidly internalized and in neonatal rat ventricular cardiomyocites norepinehrine is transported to the nucleus [24]. Nuclear β-ARs can also regulate nitric oxide (NO) production, as demonstrated by the observation that isoproterenol increases NO production and that BRL 37344, a β3AR-selective agonist, increases NO synthesis in nuclei isolated from adult rat heart [25]. The orphan nuclear receptor Nur77 plays critical roles in cardiovascular diseases, and its expression is markedly induced in the heart after β-AR activation. In a time- and dose-dependent manner, isoproterenol and the overexpression of Nur77 increases the expression of this receptor in the nuclei of cardiomyocytes. Nur77 functionally interacts with NFATc3, a transcriptional factor involved in multiple cardiac hypertrophic signaling in the nuclei of cardiomyocytes, and GATA4 and inhibits their transcriptional activities, which are critical for the development of cardiac hypertrophy (Table 1) [26].

The three α1-adrenergic receptors (α1-ARs) subtypes (A, B, and D) are expressed in the heart, but cardiac myocites only express the α1A and the α1B receptor. In these cells, activation of endogenous nuclear α1-ARs induces Gαq, and PLCβ1 activation, which localize to the nucleus, as well as the accumulation of phosphorylated ERKs [27]. The α1A and β1B subtypes co-localize at the nucleus with the formation of receptor oligomers that affect receptor signaling [27], but no direct demonstration has been provided for interaction between β1-AR and β3-AR in the nucleus (Table 1) [21]. In the two α1-AR subtypes has been identified a bipartite NLS in α1A and a glycine-arginine repeat/arginine rich NLS in the α1B, in the carboxyl tail region of the respective receptors (Table 2) [28].

Endothelin Receptors

Endothelins act through two specific GPCR subtypes, ETAR and ETBR. ETAR is primarily expressed on surface membranes and transverse-tubules and to a lesser extent on nuclei isolated from the heart, while ETBR localizes primarily to the nuclei [29]. Nuclear endothelin receptors are capable of specific ligand binding and their stimulation induces a transient increase in nuclear cisternal Ca^{++} content, as well as the activation of endogenous nuclear protein kinases [29]. Furthermore, in human endocardial endothelial cells, in human aortic vascular smooth muscle cells and in adult rat heart, endothelin-1 (ET-1) induces an increase in nuclear Reactive Oxygen Species [30] and NO (Table 1) [25]. In cardiac nuclear membranes, the endothelin receptor is ETBR and the absence of N-glycosylation indicates that this receptor did not originate at the cell surface but traffics directly to the nuclear membrane after biosynthesis [31]. ETBR associated with the nuclear membranes regulates nuclear Ca^{++} signaling through an IP3-dependent pathway and exogenous endothelins are not ligands for for this receptor on nuclear membranes [31]. In fetal human left ventricular endocardial endothelial cells (EECLs), both plasma membrane ETAR and ETBR mediate ET-1-induced increase of intracellular calcium; this effect is mediated only by ETAR in right EECs (EECRs). On nuclear membrane of EECRs both ETAR and ETBR mediate the effect of cytosolic ET-1 on nuclear Ca^{++} increase. In contrast, in EECLs only nuclear membrane ETBR activation mediates the effect of cytosolic ET-1 [32]. A NLS motif has been identified in the eighth helix of ETAR and ETBR (Table 2) [20].

Vasoactive Intestinal Peptide Receptors

Vasoactive intestinal peptide (VIP) and its receptors (VPACs) are involved in proliferation, survival, and differentiation in several cell types. In human breast tissue samples VPAC1 is localized in cell nuclear fraction, whereas VPAC2 presents an extranuclear localization and its protein expression is lower than that of VPAC1 [33]. Both receptors are overexpressed in breast cancer and in estrogen-dependent and -independent human breast cancer cell lines the nuclear localization of VPAC1 is markedly enhanced. In these cells VPAC1 receptor is functional in nuclear membrane as demonstrated by cAMP production upon VIP stimulation. VIP seems to be involved in the regulation of VPAC1-receptor traffic from the plasma membrane to the nucleus [33]. VPACs are also involved in the regulation of glioma cell proliferation and migration (Table 1). In three human glioblastoma (GBM) cell lines, a strong nuclear staining for the VPAC1 receptor has been detected, which is correlated to glioma grades and to glioma progression [34].

GPR158

GPR158 belongs to the human GPCR family C, which contains 22 receptor subtypes [35], and is phylogenetically related to the GABA receptors. It is expressed in a variety of tissues, but it is expressed at highest levels in the brain. In cultured trabecular meshwork cells, glucocorticoid (GC) treatment and the transient overexpression of GPR158 stimulates levels of GPR158 mRNA and protein through transcriptional mechanisms [36]. Both endogenous and overexpressed GPR158 show an unusual subcellular localization pattern, being found almost entirely in the nucleus. The transient overexpression of GPR158 stimulates cell proliferation, up-regulates levels of the cell cycle regulator cyclin D1 and increases the levels of tight junction proteins ZO-1 (Table 1) [36]. Nuclear localization of endogenous and overexpressed GPR158 is prevented by inhibitors of clathrin-mediated endocytosis. This suggests that GPR158 traffics to the plasma membrane, where it rapidly undergoes endocytosis and translocation to the nucleus. A bipartite NLS has been identified in the eighth helix, at the proximal end of the C-terminal cytoplasmic tail of the receptor (Table 2) [36].

Chemokine Receptor Cysteine 4

Chemokine Receptor Cysteine 4 (CXCR4) is associated with the nucleus of malignant prostate cancer tissues. Likewise, expression of CXCR4 is detected in nuclear fractions among several prostate cancer

cell lines, compared to normal prostate epithelial cells [37]. A putative NLS (RPRK) between aminoacids 146-149 within CXCR4 that contributes to nuclear localization has been identified (Table 2). Nuclear CXCR4 interacts with Transportin β1 promoting CXCR4 nuclear translocation and its participation in nuclear G-protein signaling, as demonstrated by Ca^{++} mobilization and Gαi immunoprecipitation (Table 1) [37]. Emerging evidence demonstrates that CXCR4 translocates into the nucleus to facilitate cell migration and, therefore, determine the prognosis of several types of malignancies. However, the biological mechanism of nuclear location of CXCR4 remains unclear. Non-muscle myosin heavy chain-IIA (NMMHC-IIA) has been identified as a nuclear CXCR4-interacting protein and pharmaceutical inhibition of NMMHC-IIA dampenes the nuclear translocation of CXCR4, as well as the metastatic capacity of renal carcinoma cells [38].

Nuclear GPCR	Signalling	Ref.
-ARs	Gs stimulation, AC activation, Gi stimulation, RNA synthesis, Up-regulation 18S RNA, Decrease NFkB mRNA, p38MAPK, ERKs, Akt, JNK activation, NO production, nuclear Nur77 expression	[21,23,25,26]
-Ars	Gaq stimulation, PLCb1 activation, ERKs activation,Formation of receptor oligomers	[27]
Endothelin receptors	Increase in [Ca++], Activation protein kinases, Increase Reactive Oxygen Species, Increase NO	[25,29,30]
VPAC1	cAMP production, Cell proliferation and migration	[33,34]
GPR158	Cell proliferation, Up-regulation cyclin D1, Increases tight junction proteins ZO-1	[36]
CXCR4	[Ca++] mobilization, Gai stimulation,	[37]
B2R	[Ca++] mobilization, Akt activation,Acetylation histone H3, iNOS gene induction	[39]
OTR	Osteoblast differentiation genes, ERKs phosphorylation	[44]
UT	RNA synthesis, IP3 activation, [Ca++] mobilization, CREB and DREAM activation	[45,47]
AT1R/AT2R	p62 phosphorylation, RNA synthesis, NFkB expression, [Ca++] mobilization	[48,49]
F2rl1	Recruitment of Sp1, Vegfa expression, neovascularization	[50]
PAF receptor	Transcriptional regulation of cyclooxygenase-2 and iNOS, decrease in cAMP, [Ca++] mobilization, ERKs phosphorylation, NFkB activation	[52,53,54]
LPA1R	[Ca++] mobilization, iNOS and cyclooxygenase-2 expression, proteins phosphorylation	[55]
EP receptors	[Ca++] mobilization, gene transcription, iNOS expression, ERKs and Akt phosphorylation	[58,59,60]
GnRHRs	acetylation and phosphorylation of histone H3	[62]
Nuclear Opioid Receptors	increased opioid peptide gene transcription,PKC activation	[64]
CysLT1	Proliferative ERKs signaling, [Ca++] mobilization,up-regulation PAI-2 mRNA,	[65,66]
mGlu5	[Ca++] mobilization, CREB phosphorylation	[72,73]
FPR2	Gii stimulation, p42MAPK phosphorylation, c-Jun and c-Myc phosphorylation	[74]

Table 1: Signalling pathways triggered by nuclear GPCRs β-ARs: β-Adrenergic receptors; α-ARs: α-Adrenergic receptors; VPAC1: Vasoactive intestinal peptide receptor 1; CXCR4: Chmokine Receptor Cysteine 4; B2R: Bradikinin B2 receptor; OTR: Oxitocyn receptor; UT: Urotensin II receptor; AT1R/AT2R: Angiotensin II receptors; F2rl1: Coagulation Factor II Receptor-like 1; PAF receptor: Platelet-activating factor Receptor; LPA1R: Lysophosphatidic acid receptor-1; EP receptors: Prostaglandin E receptors; GnRHRs: Gonadotropin-Releasing Hormone Receptors; CysLT1: Cysteinyl Leukotriene 1 receptor; mGlu5: Metabotropic Glutamate receptor; FPR2: Formyl-peptide Receptor 2; AC: Adenylyl Cyclase; NO: Nitric Oxide; PLCβ1: phospholipase Cβ1; IP3: Inositol triphosphate; PKC: Protein Kinase C.

Bradykinin B2 Receptors

The mechanism of action of Bradykinin (BK) is primarily mediated by specific cell surface membrane B2 receptors (B2Rs). Some evidence has suggested, however, the existence of an intracellular/nuclear B2R population. Whether these receptors are functional and contribute to BK signaling remains to be determined. Nevertheless, highly purified nuclei from isolated rat hepatocytes contain a specific B2R that bind BK, inducing concentration-dependent transitory mobilization of nucleoplasmic calcium [39]. This response is prevented by B2R but not B1R antagonists. Furthermore, in isolated nuclei BK triggers Akt activation, acetylation of histone H3, which binds to promoter region of iNOS gene and ensuing pro-inflammatory iNOS gene induction [39]. This suggests that nuclear-localized B2R responsive to BK binding functions is a transcriptional regulator of specific genes (Table 1).

The transfer of B2R membrane to the nucleus can be attributed to the presence of a NLS motif (310-KRFRK-314), positioned just downstream of the seventh trans membrane domain within the eighth helix (Table 2) [40]. Mutation of the NLS sequence does not change nuclear distribution of B2R [40]. Nuclear B2R forms heterodimers with nuclear membrane protein lamin C and the sequence 303-320 in B2R has been identified as lamin C protein binding motif [41]. Interestingly, BK addition does not reduce the binding to lamin C or changes the distribution of B2R. The formation of this heterodimer seems to be essential to nuclear localization of B2R and plays an important role in cell signaling and function [41]. Agonist-independent nuclear localization of B2R has been demonstrated [20]. Nuclear B2R has been also detected in extravillous trophoblasts, where its expression is greater in the normal than in the preeclamptic placentas [42].

Oxytocin receptor

Oxytocin receptor (OTR) is a membrane GPCR known to mediate oxytocin (OT) effects, in both normal and neoplastic cells. However, human osteosarcoma (U2OS, MG63, OS15 and SaOS2), breast cancer (MCF7), and primary human fibroblastic cells (HFF) all exhibit OTR not only on the cell membrane, but also in the various nuclear compartments including the nucleolus [43]. OT induces internalization of OTR. The resulting vesicles accumulate in the

proximity of the nucleus and some of the perinuclear OTR enters the nucleus. OTR is first visible in the nucleoli and subsequently disperses within the nucleus, while other OTR diffuses throughout the nucleoplasm. Some cells require a ligand for transfer of OTR in nuclei. A constitutive internalization of OTR is observed only in osteosarcoma cells, while the nuclear localization in all other cells types is dependent on ligand binding. The amount of OTR within and in the proximity of the nucleus increases upon the treatment with OT in both constitutive and ligand-dependent type of cells [43]. In osteoblasts OT-dependent nuclear OTR translocation plays a key role in osteoblast maturation [44]. The passage of OTR into the nucleus is facilitated by successive interactions with β-arrestins, the small GTPase Rab5, importin-β, and transportin-1. siRNA-mediated knockdown of β-arrestins or transportin-1 inhibits OT-induced expression of the osteoblast differentiation genes without affecting ERKs phosphorylation (Table 1) [44]. Putative simple and bipartite NLSs are located within the cytoplasmic segments of OTR and include the following three clusters of positively charged amino acids: RTTRQKHSR (1st intracellular loop, aa 65-73), RSLRRRTDR (2nd intracellular loop, aa 146-154) and KGRR-7aa-KK (C-terminus, aa 353-365) (Table 2) [43].

Urotensin II receptor

In rat and monkey heart and central nervous system, Urotensin-II receptor (UT) is expressed at nuclear/perinuclear level. Both endogenous ligands of UT, urotensin-II and urotensin-II-related peptide (U-II and URP) are able to cross the plasma membrane in a receptor-independent manner and to activate the nuclear receptor. The activation by U-II or URP results in different modulatory effects on transcription [45]. Interestingly, the presence of different post-translational modifications between membrane and nuclear UT in brain extracts has been observed, and membrane and nuclear UT are differentially modulated in regard to subcellular localization [46]. U-II and URP induce de novo RNA synthesis which is prevented by an UT antagonist. Nuclear UT could induce transcription through a pathway involving the production of IP3 that would trigger IP3 receptor at the inner nuclear envelope, releasing calcium in the nucleoplasm. This latter event is crucial for the activation of transcription factors CREB and DREAM (Table 1) [47]. A NLS (Lys-Arg-Ala-Arg-Arg) is present in the third intracellular loop of human and monkey UT isoforms, while a Lys-Gln-Thr-Arg-Arg segment is observed in rat and mouse UT (Table 2) [47]. However, it is important to note that many NLS signals are still unknown and that the presence of an obvious NLS motif may mask the existence of still uncharacterized NLS sequences.

Nuclear GPCR	NLS	Ref.
α1-AR (subtype A)	Bipartite NLS in the carboxyl tail region	[28]
α1-AR (subtype B)	GR repeat/R rich in the carboxyl tail region	[28]
ETAR	KKFK in the eighth helix	[20]
ETBR	KRFK in the eighth helix	[20]
GPR158	Bipartite NLS in the eighth helix	[36]
CXCR4	RPRK (amino acids 146-149)	[37]
B2R	KRFRK (amino acids 310-314) in the eighth helix	[20,40]
OTR	Three clusters of positively charged amino acids: RTTRQKHSR (1st intracellular loop, aa 65-73), RSLRRRTDR (2nd intracellular loop, aa 146-154) KGRR-7aa-KK (C-terminus, aa 353-365).	[43]
UT	KRARR (human/monkey); KQTRR (rat/mouse)in the third intracellular loop	[47]
AT1R	KKFKR in the eighth helix	[20,48]
Apelin receptor	RKRRR in the third intracellular loop	[20]
PAF receptor	RSSRKCSRATTDTVTEVVVP (amino acids 311-320) in the carboxyl tail region	[54]
GnRHs	KKEKGKK (amino acids 66-72) in the first intracellular loop	[62]
CysLT1	Bipartite NLS: RKHSLSSVTYVPRKK (amino acids 310-324) in carboxyl tail region	[65]
PTH1R	Bipartite NLS: RKSWSRWTLALDFKRKARIN (amino acids 471-478) in carboxyl tail region	[71]
FPR2	KIHKK (amino acids 227-231) in the third cytoplasmic loop	[74]

Table 2: Nuclear localization signals of GPCRs

Angiotensin II Receptor 1

Incubation of neuronal cultures with Angiotensin II (Ang II) induces a time- and dose-dependent increase in the levels of Angiotensin II Receptor 1 (AT1R) immunoreactivity in the nucleus, which is prevented by losartan, an AT1 receptor-specific antagonist [48]. Ang II-induced nuclear localization is specific for AT1 receptor, because Ang II fails to produce a similar effect on neuronal AT2R. A putative NLS sequence (KKFKR) is present in the eighth helix of the cytoplasmic tail of the AT1 but not of AT2R receptor (Table 2) [20,48]. Ang II induces a time- and dose-dependent stimulation of p62 phosphorylation, a glycoprotein of the nuclear pore complex, which is completely prevented by losartan and not by AT2 receptor specific antagonists [48]. However, isolated cardiomyocytes indicate the presence of both AT1R and AT2R proteins that copurify with the nuclear membrane marker nucleoporin-62 and histone-3, but not markers of plasma (calpactin-I), Golgi (GRP-78), or endoplasmic reticulum (GM130) membranes [49]. In isolated nuclei from cardiomyocytes AT1R and AT2R ligands enhance de novo RNA synthesis and NFκB mRNA expression, a response that is prevented by co-administration of AT1R and/or AT2R antagonists. AT1R, but not AT2R, stimulation increases of Ca^{2+} concentration in isolated cardiomyocyte nuclei, and IP3 receptor blockade prevents AT1R-mediated Ca^{2+} release and attenuates AT1R-mediated transcription initiation responses (Table 1) [49].

Apelin Receptor

Nuclear localization of apelin receptor has been demonstrated in brain and cerebellum-derived D283 Med cells, as well as in human cerebellum and hypothalamus [20]. The predominant nuclear localization of the apelin receptor is particularly evident in the molecular and granular layers of the cerebellum, as well as in the

Paraventricular nucleus of the hypothalamus. In the cerebellum there are three different subcellular distributions for this receptor. The majority of neurons show expression of the apelin receptor exclusively in the nucleus, whereas in Purkinje cells it is expressed in both the nucleus and cytoplasm. In the Paraventricular nucleus of the hypothalamus, two different subcellular distribution patterns are observed: smaller parvocellular neurons show apelin receptor expression in the nucleus, whereas in magnocellular neurons apelin receptor is expressed in nuclei and cytoplasm [20]. A putative NLS motif (RKRRR) has been identified in the apelin receptor at the carboxyl-terminal region of the third intracellular loop (Table 2) [20]. Mutation of Lys242 with Gln prevents nuclear localization of the apelin receptor, suggesting that RKRRR is a functional NLS motif and that Lys residue is critical for the nuclear localization [20].

Coagulation Factor II Receptor-like 1

Coagulation Factor II Receptor-like 1 (F2rl1, previously known as Par2) is expressed to high levels in retinal ganglion cells and is associated with new blood vessel formation during retinal development, as well as in ischemic retinopathy. In stimulated retinal ganglion cells, F2rl1 translocates from the plasma membrane to the cell nucleus, using a microtubule-dependent shuttle that requires sorting nexin 11, which contains a NLS motif [50]. Plasma membrane activation of F2rl1 induces the expression of several genes, including Ang1, that are involved in vessel maturation. In contrast, nuclear F2rl1 facilitates recruitment of the transcription factor Sp1 to trigger Vegfa expression and, in turn, neovascularization (Table 1). Mutant constructs of F2rl1 that inhibit nuclear localization, but not plasma membrane activation, interfere with Vegfa expression [50].

Platelet-activating Factor Receptor

In nuclei isolated from rat liver, Platelet-activating factor (PAF) exerts its action through specific nuclear PAF binding sites [51]. PAF receptor also localizes at the cell nucleus of cerebral microvascular endothelial cells of newborn pig and cells overexpressing the receptor. Stimulation of isolated nuclei from porcine cerebral microvascular endothelial cells (pCMVECs) induces the production of PAF-molecular species in response to H_2O_2 and triggers several biological functions, including transcriptional regulation of cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) [52,53]. In PCECs, brain endothelial cells and Chinese hamster ovary cells stably overexpressing PAF stimulation of nuclei with methylcarbamate-PAF triggers a decrease in cAMP production and a pertussis toxin-sensitive rise in nuclear calcium [53]. The expression of proinflammatory genes iNOS and cyclooxygenase-2 is associated with augmented ERKs phosphorylation and NFκB binding to the DNA consensus sequence (Table 1) [53].

Moreover, nuclear PAF receptor is responsible for the upregulation of unique set of growth factors, including vascular endothelial growth factor, *in vitro* and *ex vivo* [54]. Three potential internalization motifs in the C terminus of the PAF receptor, including a classical NLS, have been identified in primary human retinal microvascular endothelial cells. The results show that 311-330 amino-acids residues are essential for its nuclear localization (Table 2). Furthermore, nuclear localization of PAF receptor is independent of exogenous PAF stimulation, as well as intracellular PAF biosynthesis [54].

Lysophosphatidic Acid Receptor

Specific nuclear localization of lysophosphatidic acid receptor-1 (LPA1R) has been demonstrated in pCMVECs, LPA1R stably transfected HTC4 rat hepatoma cells, and rat liver tissue [55]. LPA1Rs is dually sequestrated in caveolin-1 and clathrin sub-compartments in pCMVECs, whereas in nuclear fractions LPA1R is expressed primarily in caveolae. In pCMVECs and in rat liver nuclei, lysophosphatidic acid (LPA) stimulation increases [Ca^{++}] as well as the expression of cyclooxygenase-2 and iNOS, which are prevented by pertussis toxin, phosphoinositide 3-kinase/Akt inhibitors, Ca^{++} chelator and channel blockers (Table 1) [55].

LPA1R traffics from cell membranes to the nucleus in response to LPA and cell-matrix interaction plays a key role in regulating nuclear localization of LPA1R [56]. In fact, the RGDS peptide, which blocks cell matrix-induced integrin clustering and cytoskeletal rearrangement, reduces the number of cells containing LPA1R in the nucleus. The same results are obtained by pre-treating cells with Rho kinase or myosin light chain kinase inhibitors [56]. The addition of LPA to isolated nuclei expressing LPA1R induces the phosphorylation of several proteins (Table 1) suggesting that nuclear LPA1R may be involved in regulating intranuclear protein phosphorylation and signaling.

Prostaglandin E Receptors

Prostaglandin E receptors (EP) have been detected in nuclear fractions isolated from porcine brain, myometrium, brain (cortex), neurons, pCMVECs, HEK 293 cells stably overexpressing EP1 and endothelial cells [57]. EP3 and the EP4 receptors are also localized on isolated nuclear membrane fractions of neonatal porcine brain and adult rat liver [58]. All the nuclear EP receptors are functional. In fact, their stimulation modulates intranuclear calcium concentration and the transcription of genes such as iNOS, through a pertussis toxin-sensitive mechanism (Table 1) [58]. A selective EP3 receptor agonist also induces increased phosphorylation of ERKs and Akt, which is prevented by a prostaglandin transporter blocker, actinomycin D, channel blockers, protein-kinase inhibitors and a NFκB inhibitor, in nuclei purified from endothelial cells [59,60].

Gonadotropin-Releasing Hormone Receptors

Nuclear localization of Gonadotropin-Releasing Hormone Receptors (GnRHRs) has been shown initially in *C. elegans* [61]. The mammalian type I GnRHR is a structurally unique GPCR that lacks cytoplasmic tail sequences and displays inefficient plasma membrane expression. Compared to its murine counterparts, this receptor is inefficiently folded and retained in the endoplasmic reticulum (ER) leading to a further reduction on the membrane. In two human cell lines (HEK 293 and HTR-8/SVneo) GnRHR-1 is expressed in the cytoplasm and is associated with the ER and nuclear membrane [62]. A molecular analysis of the receptor protein sequence shows a putative monopartite NLS (KKEKGKK) in the first intracellular loop of GnRH-RI (Table 2). However, the deletion or the mutation of this sequence does not alter its spatial distribution on nuclear membranes but is able to prevent Inositol Phosphate formation in HEK 293 cells. Gonadotropin-Releasing Hormone treatment of nuclei isolated from HEK 293 cells stably transfected with GnRH-RI triggers a significant increase in the acetylation and phosphorylation of histone H3, thereby revealing that the nuclear-localized receptor is functional (Table 1) [62].

Nuclear Opioid Receptors

Nuclear opioid binding sites and nuclear opioid receptors have been identified in NG108-15 neurohybrid cells and in highly purified nuclei isolated from hamster ventricular myocardial cells [63,64]. The exposure of isolated nuclei to dynorphin B, a natural agonist of kappa opioid receptors, markedly increased opioid peptide gene transcription. The transcriptional effect is mediated by nuclear PKC activation and occurs at a higher rate in nuclei from cardiomyopathic myocytes than in nuclei isolated from normal cells (Table 1) [64].

Leukotriene receptors

In colorectal adenocarcinomas an increased nuclear localization of the cysteinyl leukotriene 1 (CysLT1) receptor, which binds luekotriene D4 (LTD4), has been demonstrated [65]. It is mainly expressed in the outer nuclear membrane in colon cancer cells and in the non-transformed epithelial cell lines. CysLT1 protein contains a possible bipartite NLS (310-RKHSLSSVTYVPRKK-324) in its carboxyl tail region (Table 2). In contrast, no NLS sequence is present in the CysLT2 receptor. Mutagenesis analysis indicates that the predicted NLS domain is necessary for proper LTD4-induced nuclear translocation of the CysLT1 receptor [65]. LTD4 stimulation of nuclear CysLT1 receptor induces proliferative ERKs signaling and an increase of [Ca^{++}] in the nucleus (Table 1). The significance of these experimental findings is supported by the observed correlation between the proliferative marker Ki-67 and nuclear CysLT1 receptor localization in colorectal adenocarcinomas [65].

In human coronary artery smooth muscle cells, lipopolysaccharide significantly up-regulates the perinuclear expression of CysLT1 receptor and significantly enhances the changes in intracellular calcium induced by leukotriene C4 (LTC4) [66]. Microarray analysis reveals, among a number of significantly up-regulated genes after stimulation with LTC4, a significant increase in mRNA levels for plasminogen activator inhibitor (PAI)-2 (Table 1) [66]. Oxygen-glucose deprivation (OGD) induces the translocation of CysLT1 receptor from the cytoplasm to the nucleus in a time-dependent manner, in EA.hy926 cells. This suggests that in endothelial cells CysLT1 receptor can translocate to the nucleus in a ligand-independent manner [67].

PTH/PTHrP receptor

The type 1 PTH/PTH-related peptide receptor (PTH1R) is associated with the importin family of transport regulatory proteins [70]. Serum starvation favors nuclear localization of PTH1R, whereas serum addition or treatment with PTHrP induces its cytoplasmic localization. In MC3T3-E1 cells chromosomal region maintenance 1 (CRM1) and PTH1R form a complex *in vivo* and the treatment with leptomycin B, a specific inhibitor of CRM1-mediated nuclear export, induces PTH1R accumulation in the nucleus [68-70]. This suggests that PTH1R shuttles from the nucleus to the cytoplasm under normal physiological conditions and that this transport is dependent upon importins and CRM1 [70]. A putative bipartite NLS (471-RKSWSRWTLALDFKRKARIN-488) in the C-terminus of rat protein sequence of the PTH1R, has been identified (Table 2). This signal is functional and plays a role in targeting the PTH1R the nucleus [71].

Glutamate receptors

Metabotropic glutamate receptor, mGlu5, is localized to nuclear membranes in heterologous cells as well as midbrain and cortical neurons [72]. Nuclei isolated from mGlu5-expressing HEK cells and neuronal nuclei respond to the addition of glutamate with rapid, oscillatory [Ca^{2+}] elevations which are prevented by mGlut5 antagonists or calcium chelators (Table 1) [72].

Extracellular ligands such as glutamate and quisqualate reach nuclear receptors via both sodium-dependent transporters and cystine glutamate exchangers. Inhibition of either transport systems blocks agonist uptake, as well as agonist-induced nuclear Ca^{2+} changes [73]. In isolated striatal nuclei, nuclear receptor activation results in the de novo appearance of phosphorylated CREB protein. Thus, activation of nuclear mGlu5 receptors initiates a signaling cascade that alters gene transcription and regulates many paradigms of synaptic plasticity [73].

Formyl-peptide Receptors

The Formyl-peptide Receptor (FPR2), which is coupled to pertussis toxin-sensitive Gi proteins, is the last member of the GPCR family that has been localized on the nuclear membranes. In fact, FPR2, but not FPR1 and FPR3, is expressed in nuclei isolated from human lung carcinoma CaLu-6 and human gastric adenocarcinoma AGS cell lines, as demonstrated by western blot analysis, immunofluorescence experiments and radioligand binding assays [74]. Furthermore, in AGS cells stably transfected with a short hairpin RNA targeting FPR2 (AGS shFPR2), nuclear FPR2 expression is significantly reduced [74]. FPR2 sequence has been analyzed for the search of NLS by using cNLS mapper program (http://nls-mapper.iab.keio.ac.jp) [75] and the results show a stretch of basic aminoacids (227-KIHKK-231) in the third cytoplasmic loop of the receptor (Table 2). Apelin Receptor and Urotensin II receptor also show the presence of a NLS in the third cytoplasmic loop [20]. Multiple alignment of the FPR sequences shows that K231 of FPR2, within the sequence KIHKK, is replaced by the polar aminacids Q in FPR1 and N in FPR3. K230 of FPR2 is replaced by another basic aminoacid (R) in FPR3. Additionally, a HomoloGene/NCBI database search for the NLS within FPR2 reveals that the KIHKK sequence has been conserved among species. K230 and K231 are replaced by the basic residues RR in mouse and rat [74].

Nuclear FPR2 is a functional receptor and can respond to a specific agonist (WKYMVm), since it participates in intra-nuclear Signaling, as assessed by decreased Gi protein-FPR2 association upon stimulation of intact nuclei from CaLu-6 and AGS cells with WKYMVm [74]. Effectors associated with post G protein GPCR signaling, including mitogen-activated protein kinase (MAPK) pathway, are also present in the nucleus and p42MAPK and p44MAPK are nuclear and cytoplasmic protein kinases. In both compartments MAPK signal transduction system is regulated by serine/threonine phosphorylation. In isolated intact CaLu-6 and AGS nuclei stimulated with WKYMVm, a time-dependent phosphorylation of nuclear p42MAPK, but not of p44MAPK, is observed, which is prevented by preincubation with a FPR2 antagonist (Table 1) [74].

Activated MAPK phosphorylate and activate several transcription factors, such as c-Jun and c-Myc. In nuclei purified from CaLu-6 and AGS cells stimulated with WKYMVm, a time-dependent phosphorylation of the two transcriptional factors in both type of cells is observed (Table 1) [74]. The nuclear signaling triggered by FPR2 is not prevented by pertussis toxin, presumably because of its affinity for

nuclear receptors, lipophilic properties or specific structures assumed by nuclear receptors [74].

Single (K230A) and multiple (H229A/K230A/K231A) mutagenesis of the 227-KIHKK-231 show that nuclear localization or translocation of FPR2 depends on the integrity of the H229 and K231 residues within the NLS. Accordingly, nuclear expression of FPR1 and FPR3, where K231 is replaced by the polar aminoacids Q and N, respectively, has not been observed [74].

Conclusions

GPCRs may be inserted into nuclear membranes for prolonged periods, compose distinctive signaling units there, and respond to specific intracellular ligand by transducing nuclear transcriptional signals that differ from those sent by their plasma membrane complexes [13]. Several mechanisms according to which GPCRs can significantly influence nuclear events, have been proposed: i) activation of the membrane GPCR complex triggers second messengers which enter in the nucleus and induce processes of cellular modification; ii) nuclear translocation of protein fragments of GPCRs that are able to induce ligand-independent signals, or intact GPCRs that active transcription factors which directly induce or regulate transcriptional events; iii) constitutive nuclear GPCRs expression. Molecular mechanism(s) responsible of nuclear localization of GPCRs include nuclear import of cleaved receptor fragments and chaperone proteins which facilitate the removal of the receptor from a membrane-embedded state. Currently, the most plausible explanation for GPCRs in the nucleoplasm is through transport within micelles, which would retain a membrane-embedded GPCR suitable for further Signaling in cells nuclei [76]. The most studied are the molecular mechanism(s) through which intact GPCRs localize in nuclear membranes of activated cells, bind their respective cognate agonists in the nuclear domain, and transduce nuclear transcriptional signals different from those sent to the nucleus by the same GPCRs in the plasma membrane. For instance, T cell proliferation by S1P has been linked to signals from type 1 GPCR for S1P (S1P1) in the plasma membrane, whereas nuclear S1P1 transduces intracellular S1P suppression of proliferation [77]. The potential biological importance of the difference between nuclear GPCRs signal transcriptional events and the corresponding plasma membrane may be considered on different levels: i) GPCRs operate differently in pre- and post-cell activation stages, both in terms of their mechanisms of signal transduction and of functional consequences; ii) prior to cell activation by a GPCR ligand, plasma membrane GPCRs send one type of nuclear signal, whereas after cell activation and GPCR nuclearization the subsequent nuclear signals may have different or even opposite cellular effects [78]. Most of the downstream molecules normally associated with GPCR signalling at the plasma membrane have also been found in the nucleus. These include α subunit and βγ complexes of G-proteins, a number of effector molecules (cAMP, PLA2, PLCβ1, PLCδ1) and a variety of ion channels [14-18]. Moreover, the second messengers associated with downstream signalling events and many of the systems involved in regulation of GPCR signalling (RGS proteins, β-arrestin-1, GRKs) are also found in the nucleus. This indicates that nuclear GPCR signalling is tightly regulated, in a manner analogous to their cell surface counterparts, and hence reinforces the idea that these receptors are functionally relevant. Nuclear GPCRs can be constitutively activated or activated by specific ligands, which are synthesized within the target cell or are internalized from the extracellular space (intacrine ligands). The potential actions of intracellular ligands include: i) binding to intracellular membrane receptors and generation of second messengers; ii) binding to nuclei and nucleolus; iii) binding to chromatin; iv) binding to receptors on the endoplasmic reticulum; v) binding to intravescicular receptors with consequent generation of second messengers [78]. The diverse functions exerted by many of these ligands suggest that intracrine signalling may play important roles distinct from those of the same receptors activated at the cell surface. The discovery of nuclear receptors open new avenues in the biology of GPCRs, notably in the molecular mechanisms involved in the control of gene transcription. Several GPCR agonists do not trigger nucleoplasmic second messengers and gene expression when acting on nuclear GPCRs, which play major roles in the effects of prolonged exposure to a specific ligand. On the other hand, plasma membrane GPCRs play a more definite role in acute response to stimuli [78]. However, the profile of genes expressed in response to activation of plasma membrane GPCRs may differ from that induced by activation of nuclear counterpart. Further investigations are needed for a clear knowledge of the biological role of nuclear GPCRs in both physiological and pathological conditions since final results from these studies may have a major impact on new therapeutic drug development.

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