Adrenergic signaling molecular complexes

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Abstract

Adrenaline and noradrenaline bind to membrane receptors of the superfamily of G protein-coupled receptors (GPCR) in target cells, where they modulate physiological responses such as metabolism, vasoconstriction, vasodilation and proliferation. Alteration in their function is associated with conditions such as hypertension, benign prostatic hyperplasia and cardiac hypertrophy. In response to adrenaline, receptors form signaling complexes, which enables adrenergic action to be specific, rapid and efficient. These signaling complexes or signalosomes are composed of kinases, phosphatases, and adapter and scaffold proteins, which together modulate the receptor function. Manipulation of each protein-protein interaction of the adrenergic signaling complex emerges as a promising therapeutic strategy for the design of drugs that modulate adrenergic action and help to define its pathophysiological significance. An important biological model to perform these investigations is the heart, since it expresses all adrenergic receptors; to date, several heart signalosomes have been described. Mass spectrometry (proteomics), genetic manipulation and biochemical assays, such as two-hybrid and co-immunoprecipitation assays, are tools that are used in these studies.

KEY WORDS: Adrenaline. Noradrenaline. Adrenergic receptors. G protein-coupled receptors. Signaling complexes. Signalosomes.

Introduction

Adrenaline (ADRE) and noradrenaline (NA), hormone and neurotransmitter, respectively, are produced from L-tyrosine at two sources in the body: sympathetic nerve terminals, which release NA on effector organs after stimulation, and adrenal medulla chromaffin cells, which actually are postganglionic sympathetic neurons and main source of ADRE and NA synthesis, storage and release under stimulation of acetylcholine nicotinic receptors.¹

Both catecholamines (ADRE and NA), called by this name because they contain a catechol ring (1,2-dihydroxybenzene) in their chemical structure, mediate a large variety of functions that are essential to the body, including vasodilation, vasoconstriction, proliferation, blood pressure and cardiac systolic and diastolic pressure (Figure 1A).²⁻⁷ These catecholamines are released in situations of rage, stress or anxiety and by allergic or hypotension reactions, mediated by

hormones such as histamine, angiotensin II and bradykinin;¹ they exert their effects by binding to adrenergic receptors or adrenoceptors (Figures 1B and 1C). These are distributed in the central and autonomous system, as well as in peripheral tissues such as the iris radial muscle in the eye, the skin and blood vessel mucosa in the heart, the trigonum, bladder sphincter, obturator and skeletal muscles, adrenal medulla, pituitary gland, pineal gland and male sex organs, among others.²⁻⁷ The importance of these chemical mediators is such, that alterations in the synthesis and release of catecholamines –which normally circulate in blood in the order of nanomoles¹– or in the form they generate a cell response when they bind to their specific receptors⁸ can give rise to cardiac and prostatic hyperplasia, cardiac hypertrophy, hypertension or cancer.²

Adrenoceptors on the cell plasma membrane are activated by both catecholamines' binding and generate specific cell responses via several signaling

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Figure 1. Adrenergic signaling system. Catecholamins: adrenaline (hormone) and noradrenaline (neurotransmitter) bind to target receptors of 7 transmembrane domains known as G protein-coupled receptors (GPCR), which are composed, in addition, of 3 extracellular loops, 3 intracellular loops, extracellular amino terminus (N) and intracellular carboxyl terminus (C). Plasma membrane-located GPCR (β_2 -adrenergic), when activated by the agonist, couples to a heterodimeric G protein that activates an effector protein in the plasma membrane, which generates molecules named “second messengers” that are diffused in the cytoplasm until activating protein kinases that amplify the signal by phosphorylating their substrates (transcription factors), which regulate the expression of genes that participate in different cell responses.

pathways, where elements such as heterotrimeric G proteins, membrane enzymes such as phospholipase C and adenylyl cyclase (AC) are involved and produce molecules known as “second messengers”, which when diffused in the cell, activate cytosolic proteins such as kinases, which are proteins that transfer phosphate groups to amino acid residues that are specific to target proteins, such as transcription factors (Figure 1B). This way, and in a specific activation order, participating proteins amplify the extracellular signal by modulating the expression of genes related to different types of cell response (Figure 1B).⁸

Agonist (ADRE and NA) binding to its specific receptors induces a conformation change that promotes for them to interact with and recruit different elements of the signaling pathway by means of a platform or scaffold protein known as A kinase anchoring protein (AKAP), to which more components are added, giving rise to a multiprotein signaling complex (Figure 2A).⁹⁻²⁰ AKAP contains several protein-protein interaction

specific domains within its structure (Figure 2B), by means of which it recruits by affinity elements that participate in receptor regulation (Table 1), such as kinases and phosphatases, which maintain its phosphorylation status in balance, elements that participate in its ubiquitination and breakdown, as well as those participating in its internalization and in vesicular trafficking (Figures 2B and 2C).¹³⁻²⁰

The knowledge generated on adrenergic signaling molecular complexes, as well as the study of the interaction domains of the proteins that compose them, will enable having potential therapeutic targets for pathophysiological conditions where these receptors participate, such as cardiovascular-origin diseases, cardiac hypertrophy, myocardial ischemia, diabetic and malnourishment cardiomyopathy, dilated cardiomyopathy and congenital alterations.²¹⁻²⁷

Here, the classification of adrenergic receptors, their structural characteristics and signaling pathways are briefly described. The most representative

Figure 2. β_2 -adrenergic receptor (β_2 -AR) signaling complex. Binding of adrenaline to β_2 -AR increases its interaction with the scaffold protein (AKAP250/gravin/AKAP12). AKAP250 has protein-protein interaction domains, including 3 AKAP motifs preserved among AKAPs, one region of interaction with F-actin towards N-terminus and 2 PXXP regions (proline-X-X-proline; X = any amino acid) similar to SH3 domains by means of which it interacts with Src.

Table 1. Protein-protein interaction domains of some elements that compose the β_2 -adrenergic signalosome

Protein	Interaction domain	Domain localization	Interacting protein	Function
AKAP	Amphipathic helix	N-terminus	PKA	Signal amplification
AKAP79	Polybasic domains: 31-52, 77-101, 116-145 amino acids	N-terminus	PKC, CaM, F-actin, Cadherin, AC5	Recruits elements that participate in GPCR pathway
	315-360	C-terminus	PP2B	Signal arrest
	392-413		PKA	Signal amplification
AKAP250	18 amino acids forming an amphipathic helix with hydrophobic residues	N-terminus	PKA	Signal amplification
GRK2	α -D helix, α -F and α -G loops	N-terminus	GPCR	GPCR phosphorylation and signal inhibition
		N-terminus	Gby dimer	G α inactivation
		N-terminus	MEK, PKB, ezrin, PI3K γ , GIT	Migration
β -arrestin			Mdm2, MAPK, Src	GRK2 breakdown
G α protein	α -N helix	N-terminus	GPCR, by dimer and effectors (phospholipase C)	Signaling
GPCR		Intracellular loops and C-terminus	G protein, β -arrestin, GRK2	Signaling and desensitization

CAM, calmodulin; MEK, MAPK kinase; PKB, protein kinase B; PI3K, phosphoinositol 3-kinase; GIT, G protein-coupled receptor kinase interactor.^{14,16,45}

proteins of the β_2 -adrenergic receptor signaling complex, which is adrenergic receptors prototype, as well as its interaction domains are mentioned. Examples are given of signaling complexes identified in normal

and pathologic physiology of the heart. Finally, the methodological tools used in the study of protein interactions are referred.

Adrenergic receptor classification

Based on the pharmacology and affinity (effective dose 50, ED_{50}) presented by adrenergic receptors to different drugs and synthetic agents (agonists and antagonists), they are classified in 3 groups: β , α_1 and α_2 , a nomenclature based on the identity of their primary sequence.^{2-5,8} Each group has 3 subtypes: β_1 , β_2 , β_3 , α_{1A} , α_{1B} , α_{1D} , α_{2A} , α_{2B} , and α_{2C} , all of them products of different genes (International Union of Basic and Clinical Pharmacology, IUPHAR). The first classification was developed by pharmacologist Raymond Perry Ahlquist (1914-1983), who divided α -adrenergic and β -adrenergic receptors, based on a series of sympathomimetic amines on a variety of tissues. α -adrenergic receptors have vasoconstrictor effect and produce excitation on the uterus, heart and blood vessels and only have relaxation effect in the intestine. β -adrenergic receptors mainly have vasodilator effect and only vasoconstrictor effect in the uterus and excitatory in the myocardium.²⁸

In 1958, the work by Earl Wilbur Sutherland (1915-1974), who was awarded the Nobel Prize in Physiology and Medicine in 1971, contributed to define hormones mechanism of action. Sutherland and Ted Rall (1963) characterized receptor function using radiolabeled compounds such as adenosine triphosphate [$8\text{-}^{14}\text{C}$] ATP. They observed that cyclic adenosine monophosphate (cAMP), which originates in ATP cyclization, is an intermediary in the action of hormones such as ADRE, which acts on the phosphorylase that participates in the formation of glucose from glycogen.²⁹ In 1967, two types of β -adrenergic receptors were identified due to differences in their affinity for ADRE, which activates the β_2 -adrenergic subtype with 100-fold more strength than the β_1 -adrenergic subtype. The β_3 -adrenergic subtype was discovered later and was considered to be atypical, since it is insensitive to β_1 and β_2 typical antagonists.⁷

On the other hand, β_1 -adrenergic receptors classification started with the use of prazosin, a selective antagonist.²⁻⁴ With WB4101, another antagonist, two α_1 -adrenergic receptor subtypes were clearly identified: α_{1A} and α_{1B} . The first cloned gene was that of α_{1B} receptor; later, α_{1A} -adrenergic receptor was cloned, initially with some controversy, since it had been previously named α_{1C} -adrenergic ($\alpha_{1A/C}$). Subsequently,

the α_{1D} -adrenergic receptor ($\alpha_{1A/D}$) was identified and cloned,² with nomenclature finally remaining as α_{1A} , α_{1B} and α_{1D} . On the other hand, with yohimbine, an antagonist, the group of α_2 -adrenergic receptors was identified,³⁰ out of which two subtypes were identified: α_{2B} and α_{2C} , characterized with agonists and antagonists in functional and biochemical radioactive ligand to receptor binding assays known as "binding".³⁻⁶

Currently, the importance of each adrenergic receptor subtype in human physiology, the characterization of elements that participate in their signaling pathways and their biochemical and molecular regulation is being studied.

Adrenergic receptors characteristics

Adrenergic receptors belong to the G protein-coupled receptors or GPCR superfamily. They are composed of 7 hydrophobic regions or transmembrane (TM) helices, 3 extracellular loops that are important to ligand binding and 3 intracellular loops, as well as an amino terminus that is extracellular and a carboxyl terminus that is found within the cell (Figure 1C). They are called presynaptic adrenoceptors if they are expressed in sympathetic neurons and postsynaptic adrenoceptors if they are expressed in postsynaptic neurons.^{3,4} Their expression in target tissues or organs is tissue/organ-specific.²

Adrenergic receptors respond to environmental changes and, therefore, to determinate physiological conditions, such as an increase in ADRE and NA concentration in blood circulation.¹ Agonist binding to the receptor induces a conformation change in it that enables its coupling to the heterotrimeric G protein, which when activated exchanges guanosine diphosphate (GDP), which is bound to subunit α at its inactive state, for guanosine triphosphate (GTP). GDP release enables subunit α , which contains GTP, dissociation from $\beta\gamma$ dimer. GTP hydrolysis to GDP by subunit α (which has GTPase activity) enables subunits α and $\beta\gamma$ association; the G protein activation/deactivation cycle is initiated again when the receptor is activated.⁸

There are four families of heterotrimeric G proteins: G_s (stimulatory), G_i (inhibitory), $G_{q/11}$ and $G_{12/13}$.^{8,26} As a consequence of G protein activation ($G_{q/11}$ and G_s), plasma membrane effector enzymes such as phospholipase C, which produces inositol 1,4,5-triphosphate (IP_3) and 1,2 diacylglycerol (DAG), are activated, by means of which the release calcium ions from the endoplasmic reticulum and cAMP-producing AC

is observed.⁸ These “second messengers” (IP₃, DAG, calcium and cAMP) activate extracellular signal-amplifying proteins such as calcium-dependent protein kinase (PKC) and cAMP-dependent kinase (PKA)⁸ (Figure 1B). In turn, by de-phosphorylating their substrates from the phosphate groups’ monoester bonds by means of hydrolysis, phosphatase proteins regulate extracellular signal amplification.³¹

The investigation carried out by Robert J. Lefkowitz and Brian K. Kobilka at the Universities of Duke and Stanford in the United States on the GPCR structure, as well as on the regulation of the function of this superfamily of receptors, led them to obtain the Nobel Prize in Chemistry in 2012.³² The findings obtained over four decades by both these research groups have made way towards one of the most important goals of medicine: the design of specific drugs (agonists and antagonists) for the numerous GPCRs involved in different diseases. Currently, approximately 30 GPCRs have been characterized, with β_2 -adrenergic receptor, both at inactive³³ and active state,³⁴ the muscarinic receptor M3,³⁵ the δ -opioid receptor³⁶ and rhodopsin (light receptor)³⁷ standing out. GPCR crystals X-ray diffraction analysis and protein modelling with computing programs provide the receptor structures at atomic level, so that the function, regulation form, ligand binding sites and domains of interaction with proteins can be studied with more detail and accuracy.

Adrenergic signaling complexes

In signal transduction (transformation of an extracellular signal into intracellular signaling mediated by receptors) (Figures 1A and 3A), multiprotein complexes dynamic formation is key for cell response to be specific, efficient and quick (Figure 2A).^{38,39} There are preformed complexes in the absence of a stimulus, owing to cytoplasmic proteins simple diffusion and random encounter.⁴⁰ Recently, constitutive multiprotein complexes have been suggested to be assembled during protein biosynthesis.⁴¹ Integration requires regions that are affine to each other, i.e. with protein-protein interaction domains. Some described domains are the tyrosine residue kinase Src homology domains (Rous sarcoma virus): single-Src domain SH4, SH1 that is tyrosine kinases catalytic center, SH2 docked to phosphotyrosine residues and SH3, polyproline binding site.⁴²⁻⁴⁴ Others, such as PTB (p-Tyr-binding), bind to phosphotyrosines; PH (pleckstrin homology) is phosphoinositides binding site;

MH2 (N-terminal DNA binding) is a site that recognizes and binds to the pSer-X-pSer sequence;⁴⁰ and PDZ (PSD-95, Dlg and ZO-1, the 3 proteins where it was initially identified) is a site that recognizes 4 residues towards carboxyl terminus in target proteins,⁴⁵ are some examples. In particular, the domains of interaction of some elements of the adrenergic signaling complex are polybasic regions and hydrophobic regions (Table 1).

β_2 -adrenergic receptor signaling complex is the best characterized.¹⁴⁻¹⁶ In response to the agonist, β_2 receptor interacts with scaffold protein AKAP250, also known as gravin, or with AKAP12, by means of which it recruits proteins that participate in β_2 -adrenergic response (Figure 2A).⁴⁶ For example, G protein-coupled kinases 2 and 3 (GRK2/3), which are specific kinases that phosphorylate serine or threonine residues from intracellular loops or carboxyl-terminus of the receptor, phosphates that are recognized by proteins of the endocytic machinery (β -arrestin, AP2, clathrin), internalize it in early endosomes, by means of which the homologous desensitization process (loss of receptor response by agonist prolonged action) is triggered^{11,13,15,17} (Figure 3A). Similarly, other kinases (PKA and PKC) can be part of the complex (Figure 2A), participating in heterologous desensitization (loss of receptor response owing to endogenous agonist different stimuli).⁴⁷ Other proteins of the complex include phosphatases PP1, PP2A and PP2B (Figure 2A), which in addition participate in the “re-sensitization” process, since the internalized receptor returns to the plasma membrane without phosphates, where it can be activated again by the ligand (Figure 3A).⁴⁸ Src can be part of the complex as well (Figure 4), and participate both in receptor internalization and recycling or as a modulator of the adrenergic response, via growth factor receptors.⁴⁹

Some proteins that are part of the signaling complex do not bind to AKAP, but directly interact with the receptor or with adaptor proteins such as cAMP phosphodiesterase (PDE4), which decreases cAMP levels by breaking it down,⁵⁰ adaptor protein 2 (AP-2), clathrin and β -arrestin, which participate in receptor traffic,⁵¹ kinases of the mitogen-activated protein kinase (MAPK) family such as extracellular stimuli response kinase 1 and 2 (ERK1/2) and murine double minute 2 (Mdm2) ubiquitin ligase, which participates in receptor breakdown⁵³ (Figures 3A and 3C). It is important mentioning that β_2 -adrenergic receptor can form oligomers with other GPCRs, which are

Figure 3. GPCR- β -arrestin interaction. Agonist binding to β_2 -AR induces, among other effects, GRK activation, which phosphorylates the receptor. GPCR phosphorylation makes it more affine to interaction with β -arrestin, which is a versatile adaptor protein. On one hand, it interferes with β_2 -AR-G protein coupling, and on the other, it recruits proteins that participate in receptor internalization and traffic, as well as elements of the MAPK pathway. β -arrestin has a modular structure, the site of interaction with the receptor is at the N-terminus, it has two regulator domains, one is the site of clathrin (Clat) recognition, and the other is for adaptin 2 (AP2). β -arrestin has one or more PXXP recognition domains for Src and JNK3 at N-terminus, and for NSF at C-terminus. β -arrestin 2 has one JNK3 and Mdm2 binding site.

Figure 4. β_2 -AR interaction with adaptor and scaffold proteins and GPCR. β_2 -AR has protein-protein interaction domains for β -arrestin, scaffold proteins (AKAP), Src and Na⁺/H⁺ transporter (NERF1), among others, and this way it modulates different cell responses. β_2 -AR can form dimers and heterodimers, which are interactions that impact on its function.

Table 2. Adrenergic receptor oligomers. The role of interaction in internalization and its effect on different cell responses is indicated

Adrenergic receptor	Oligomerization	Internalization	Cellular effect
		Doesn't allow β_2 internalization	Decreases ERK activation
		Yes	More stable internalization
		Doesn't allow β_2 internalization	No coupling with Gi
	olfactory	Olfactory receptor in the membrane and co-internalization	Not known
	δ -opioid	Co-internalization	Not known
	κ -opioid	Doesn't allow β_2 internalization	Endocytosis in response to insulin
	Angiotensin I (AT1R)	Internalization inhibition by AT1R antagonists	Calcium currents
	Serotonin (5HT4)	Not known	Potassium current
	Cannabinoid I (CB1R)	CB1R constitutive internalization/co-internalization	Analgesia/bone formation inhibition by adrenergic action
		No	Not known
		Co-internalization	Not known
		No	Not known
		α_{1D} expression in the membrane	Decreases affinity for α_{1D} ligands
		Co-internalization	α_{1D} expression in the membrane
		Not known	Signaling increase
		Co-internalization	α_{2A} phosphorylation
		Not known	Both receptors signaling increase
	μ -opioid	Not known	Both receptors signaling increase
	δ -opioid	Not known	δ -opioid receptor-mediated neurites growth
		α_{2C} expression in the membrane	Increases α_{2C} -mediated ERK signaling

Table modified from references 2 and 14

interactions that have a functional impact. For example, β_2 -receptor internalized as homodimer is more stable to breakdown.^{54,55} On the other hand, β_2 -adrenergic action on responses such as bone formation is negatively regulated by type I cannabinoid receptor (CB1R),⁵⁶ a receptor whereby it forms heterodimers^{56,57} (Table 2). To date, at least 20 proteins that are part of β_2 -adrenergic signalosome have been described. Five proteins are known to interact with α_2 -receptor and 8 with α_1 -receptor (Tables 2 and 3).

Receptor structural characteristics and adrenergic signaling complex scaffold and adaptor proteins

The protein-protein interaction receptor domains, and the adaptor (β -arrestin) and scaffold proteins (AKAP) that constitute the adrenergic signaling complex have been widely studied.

Adrenergic receptors

β_2 -adrenergic receptor canonical interactions with Gs protein and β -arrestin, as well as interactions between two receptors, have been recently described with the support of X-ray crystallography and with crystal diffraction data acquisition, since it this allows observing their structure with high resolution.³⁴ β_2 -receptor-Gs protein co-crystal shows that the contact surface between both proteins (interface) involves TM V and VI amino-acids of the receptor, whereas the third intracellular loop and TM V participate in the receptor- β -arrestin interface; in addition, in order for this interaction to have more affinity, phosphorylation by GRK of both the receptor third intracellular loop and carboxyl terminus is required.³⁴ On the other hand, TM I, II, IV and V participate in β_2 -adrenergic receptor homodimer interface^{34,58} (Figure 1B).

Table 3. Proteins that directly interact with adrenergic receptors

Adrenergic receptor	Interaction protein	Site of interaction with receptor	Cellular effect
β_1	Endophilins	Third intracellular loop (rich in prolines)	Endocytosis
	PSD-95	C-terminus (ESKV)	Endocytosis/Association with NMDA receptor
	MAGI-2	C-terminus (ESKV)	Endocytosis/association with b-catenin
	GIPC	C-terminus (ESKV)	ERK inhibition
	CAL	C-terminus (ESKV)	Decreases membrane expression
	RasGEF	C-terminus (ESKV)	Ras activation
	GASP	C-terminus (ESKV)	Not known
	Golgin-160	Third intracellular loop	Increases membrane expression
	14-3-3	Phosphorylated sites	K ⁺ channels regulation
	PDE4	Not known	cAMP levels regulation
β_2	AKAP 250 (gravin)	C-terminus	Endocytosis/desensitization/re-sensitization
	AKAP79	Not known	GRK association
	AKAPLc	Not known	Calcium currents
	Grb ₂	Tyr350/354	Endocytosis in response to insulin
	Cav1.2	Not known	Calcium currents
	BKCa	Not known	Potassium current
	pVHL EGLN3	Pro382/395	Ubiquitination in response to oxygen
β_3	NERF 1	C-terminus (DSL1)	Na ⁺ /H ⁺ exchanger regulation
	Src	Third intracellular loop and proline-rich C-terminus	ERK activation
α_{1A}	Matrix metalloproteinase	Not known	Membrane expression
α_{1B}	RGS2	Third intracellular loop	Gq decrease
	Spinophilin	Third intracellular loop	Recycling
	Ap50	C-terminus	Endocytosis
α_{1D}	Ezrin	C-terminus	Recycling
	Syntrophins	C-terminus (ETDI)	Receptor stabilization in the membrane
α_{1A}, B, D	nNOS	Not known	Not known
α_{1B}, D	gC1qR	C-terminus (Arg)	Intracellular localization
α_{2A}	Uch-L1	Third intracellular loop	Decreases MAPK
α_{2A}, B, C	Spinophilin	Third intracellular loop	Not known
	14-3-3 z	Third intracellular loop	Not known

The site of interaction with the receptor and the function of such interaction is indicated.

MAGI-2, membrane-associated guanylate kinase inverted-2; GIPC, G α -interacting protein, C-terminus; CAL, cystic fibrosis transmembrane conductance regulator-associated ligand; Ras-GEF, GTP exchange factor; GASP, G protein coupled receptor-associated protein; NERF-1, Na⁺/H⁺ exchange regulatory factor; nNOS, neuronal nitric oxide synthetase; Uch-L1, ubiquitin carboxyl-terminal hydrolase-L1; BKCa, calcium activated potassium channel; pVHL, von Hippel-Landau protein; RGS, regulator of G protein signaling; gC1qR, globular C1q-domain receptor. In some cases, interaction site or function is yet not known. Table modified from reference 2

The “receptor-AKAP” interaction biochemical analysis shows that PDZ-type domains, out of which there are several classes with the X-[Ser/Thr]-X-[Val-Ile-Ile] consensus sequence (where X is any amino-acid), participate in this association.⁴⁵ Different PDZ domains have been characterized in adrenergic receptors, such as small glutamic-serine-lysine-valine sequences (ESKV) in β_1 -adrenergic receptor carboxyl-terminus; aspartic-serine-leucine-leucine (DSLL) in β_2 -adrenergic receptor and glutamic-aspartic-threonine-isoleucine (EDTI) in α_{1D} -adrenergic receptor. Numerous proteins that have PDZ domains, such as potassium channels and endophilin, bind to these domains^{2,14} (Table 3).

β -arrestin

β -arrestin was identified by Robert Lefkowitz group as a protein similar to visual arrestin, which participates in adaptation to light in the rhodopsin system. This protein was known for long time as the arrest of GPCR signals transduction because it physically interferes with the coupling of the receptor and Gs protein (Figure 3A).^{59,60} However, the same group observed that β -arrestin is also able to associate with other proteins during desensitization, acting as an adaptor protein that recruits the endocytic machinery.⁶¹ In addition, it can interact with other β -arrestins forming homo- and heterodimers (Figures 3B and 3C). Both β -arrestin isoforms have a polar nucleus in the amino-terminus with approximately 12 amino acids that recognizes phosphorylated GPCR.^{62,63} In this same region, β -arrestin 2 has the interaction site with kinases of the MAPK family, such as cJun N-terminal kinase 3 (JNK3), which are relevant to cell proliferation, response to stress and survival, as well the binding site of Mdm2 and κ chain-enhancer of activated B cell transcription factor inhibitor (I- κ B)^{62,63} (Figure 3B), and in both β -arrestins carboxyl-terminus, clathrin and AP2 binding domains (Figure 3B). β -arrestin has been shown to be able to interact with more proteins and whereby regulate several cell responses (Figure C).

A-kinase anchoring protein (AKAP)

There are nearly 700 scaffold proteins (AKAP), whose name mainly derives from their interaction with PKA, specifically with regulatory RII subunits. They express mainly in the plasma membrane,^{9,10,11,16,17} but their localization extends to other cell compartments.²⁰

AKAP5 1-107 residues (AKAP79/150) and AKAP250 1-912 residues are important to the interaction with GPCR; both are β_2 -adrenergic response scaffold proteins.⁶⁴ In AKAP5 amino-terminus, the sites of interaction with Src, a kinase involved in β_2 -adrenergic receptor recycling towards the plasma membrane, as well as the PKC, PP12 and PP2B sites were identified, and towards the carboxyl-terminus, the PKA and AKAP250 sites, by means of which it forms hetero-oligomers.⁶⁵

In AKAP250250 amino-terminus, an amphipathic region known as receptor binding domain (RBD), which works in the recognition and binding to GPCR, was identified, as well as 4 domains that allow it to anchor to the plasma membrane: one N-myristoylation site and three small, positively-charged sites (PCD) that electrostatically bind to the plasma membrane internal side¹⁶ (Table 1).

Adrenergic signaling complexes clinical importance

Some cardiovascular-origin physiopathologies, such as cardiac hypertrophy and myocardial ischemia, among other serious health problems in the world, are related to adrenergic action. The heart expresses all adrenergic receptor subtypes and several AKAP isoforms, and it is therefore considered to be a very important model to study the molecular mechanisms of signaling complexes formation in cardiac normal physiology and in physiopathological conditions hoping to discover therapeutic targets that help the treatment of diseases. Cardiomyocytes have been described to express β_1 and β_2 -adrenergic receptors in plasma membrane regions enriched with cholesterol and other lipids (lipid rafts), and to form invaginations in caveolin-containing caveolae; in addition, subtype β_1 is found in regions not containing cholesterol, even in the nuclear membrane, whereas subtype β_3 has not been observed in the plasma membrane, but it has in the nuclear membrane, together with subtype β_1 . These cells also express α_{1A} and α_{1B} -adrenergic receptors in plasma and nuclear membranes.^{10,14,66,67}

As previously mentioned, the heart expresses several AKAPs that act as adrenergic signaling integrators: mA-KAP, AKAP18, AKAP79, D-AKAP1/2, AKAP95, AKAP220, AKAP-LBC, AKAP250/gravin, ezrin, yotiao, synemin, SPHKAP and BIG2.^{12,15,21,22} In cardiac muscle contraction during each heartbeat, β_1 and β_2 -adrenergic receptors activation by sympathetic innervation-released catecholamines increases the

kinetics of the excitation-contraction cycle, where electric current turns into a mechanical force that results in a rhythmic cycle of calcium increase between cytosol and the sarcoplasmic reticulum (SR), events where a large number of AKAP18-recruited proteins participate.⁹ Positive chronotropic and inotropic effects are generated mainly via the classical route: G-protein-cAMP-pKA-phosphorylation of the contractile machinery and of accessory proteins such as calcium channels, ryanodine receptor, troponin-I and phospholamban.^{12,15,68} In this cycle, contraction depends on calcium and it starts with cation entering via L-type channels, which, owing to their localization in the sarcolemma, act on ryanodine receptors (RR); local calcium concentration increase in the RR surroundings is sufficient to activate calcium-induced calcium release and emptying the SR storage. Calcium robust wave in the cytosol enables cation binding to microfilaments, specifically to troponin C, and thereby starting myocyte contraction.^{12,15,68}

Chronic activation of this cascade has been observed to lead to progressive cardiac dysfunction, hypertrophy and heart failure. PKA prolonged activity deregulates some proteins, including β_1 -adrenergic receptor, and it was therefore regarded as the first therapeutic target in the treatment of these conditions, with the use of antagonists to inhibit its function. However, since these drugs produce side effects, the study and characterization of cardiac signaling complexes and interaction domains promises to identify more specific targets. For example, one of these targets might be the interaction between AKAP18 and calcium phospholamban-ATPase, since synthetic peptides that compete for binding to AKAP have been shown to decrease β -adrenergic action by 50 %. Another possible pharmacological action target is AKAP interaction with different phosphatases (PP1, PP2A and PP2B),²² since these are modulators of the phosphorylation/activation status of the complex's proteins during cardiac muscle relaxation, which requires the removal of the cytosolic calcium captured by the SR via calcium-dependent ATPase and $\text{Na}^+/\text{Ca}^{+2}$ exchanger and other channels, which restores the resting potential. ATPase inhibition results in negative inotropic and lusitropic effects; on the other hand, if AKAP-PKA interaction is affected, both in vivo and ex vivo, the heart normal function is also negatively affected; heartbeat duration (chronotropic effect) and strength (inotropic effect), as well as relaxation velocity (lusitropic effect), are altered.

Other signaling complexes generated in response to β_1 -adrenergic action and that are important to heart physiology are AKAP15-PKA- $\text{Ca}_v1.2$, AKAPLcvb-PKA-PKC-PKD-Rho-14-3-3 and Yotiao-PKA-KCNQ1-PD-E4D-PP1.^{21,22} Heart behavior was experimentally shown to be affected by the absence of any of the proteins that are part of signalosomes by point mutations or inhibition of their expression: the absence of the voltage-dependent calcium channel ($\text{Ca}_v1.2$) induces a severe cardiac hypertrophy phenotype and leads to perinatal death;³¹ the absence of AKAP-Lcb (AKAP13) induces myocardial thinning by cell growth inhibition, which is a phenotype that was been shown to be lethal in mouse embryos; and, finally, the lack of KCNQ1 potassium channel or Yotiao (AKAP9) generates heart arrhythmias.

A complex generated in response to α_{1D} -adrenergic receptor activation named dystrophin-associated protein complex (DAPC), which contains dystrophin and syntrophin, was characterized in mouse epicardium coronary arteries;⁶⁸ it is related to Duchenne's muscle atrophy, where a mutation in the dystrophin gene produces a defective protein that results in progressive muscle degeneration, affecting cognitive or cardiac functions.

Although multiprotein complexes and their importance have been described in some cardiac pathologies, such as the aforementioned, other heart conditions of elevated incidence, such as diabetic cardiomyopathy, malnutrition-induced cardiomyopathy, dilated cardiomyopathy and congenital alterations, still need to be addressed. The development of new experimental methods has substantially supported these studies.⁶⁹

Experimental methods for the study of protein-protein interactions

Some biochemical and molecular biology methods that are used for the study of protein interactions are the following:

- *Two-hybrid system*: it is based on the presence of two domains (A and B) of a transcription factor; two gene libraries are generated, one with genes fused to domain A, and the other to domain B. Plasmids with these genes are integrated in yeasts and only when proteins of both groups interact, both transcription factor domains bind and induce "reporter gene" (LacZ) expression, which is regulated by the factor and is detected by the activity of an enzyme (beta-galactosidase).

- *Fluorescence resonance energy transfer (FRET)*: is a technique based on excitation of a fluorochrome fused to a protein that is transferred to another nearby (10-100 Å) and that is detected as fluorescence.
- *Bioluminescence resonance energy transfer (BRET)*: it monitors protein interactions in real time. It is based on heterologous co-expression of two fusion proteins, with the first one being fused to a bioluminescent donor and the second to an acceptor fluorophore. Energy transfer can be detected as acceptor fluorescence.
- *Co-immunoprecipitation*: it is based on endogenous protein clearance and on detection of one or more associated proteins by means of Western blot assays using specific antigens.
- *Confocal microscopy*: it enables observing, with high resolution, the co-localization of two proteins, one fused to fluorophore X (green) and the other to fluorophore Y (red). If the proteins interact, when both images are overlapped, a different color than the original is observed, Z (yellow), due to the combination of both.
- *Mass spectrometry*: it identifies peptide masses in a protein that has been purified and fragmented with enzymes, as well as in associated proteins, and compares them with the theoretical masses of the series of peptides expected for the sequences stored in a data base.

Discussion

The formation of multiprotein complexes in GPCRs signal transduction systems, a family to which adrenergic receptors belong, is key to the organization of elements that participate in the transduction pathway in response to extracellular stimulus, providing spatial-temporal specificity and rapidness in the response.^{9-11,13,18,19}

Proteins that work as a scaffold (AKAP) are the place where kinases, phosphatases, phosphodiesterases, ion channels and adaptor proteins, among others, are recruited in order to regulate adrenergic receptors and many other GPCRs function. The study of AKAPs enabled observing that signaling complexes alteration, specifically in β_2 -adrenergic receptors, represents a key factor in the development of diseases of the heart and other organs where adrenergic receptors are expressed and participate. The design of more specific drugs that modulate interactions between the members of a complex is visualized as a promising therapeutic strategy.

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