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β_2 Adrenergic Receptor Complexes with the L-Type Ca^{2+} Channel $Ca_V1.2$ and AMPA-Type Glutamate Receptors: Paradigms for Pharmacological Targeting of Protein Interactions

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Abstract

Formation of signaling complexes is crucial for the orchestration of fast, efficient, and specific signal transduction. Pharmacological disruption of defined signaling complexes has the potential for specific intervention in selected regulatory pathways without affecting organism-wide disruption of parallel pathways. Signaling by epinephrine and norepinephrine through α and β adrenergic receptors acts on many signaling pathways in many cell types. Here, we initially provide an overview of the signaling complexes formed between the paradigmatic β_2 adrenergic receptor and two of its most important targets, the L-type Ca^{2+} channel $Ca_V1.2$ and the AMPA-type glutamate receptor. Importantly, both complexes contain the trimeric G_s protein, adenylyl cyclase, and the cAMP-dependent protein kinase, PKA. We then discuss the functional implications of the formation of these complexes, how those complexes can be specifically disrupted, and how such disruption could be utilized in the pharmacological treatment of disease.

Keywords

G_s; adenylyl cyclase; PSD-95; AKAP; cAMP; norepinephrine

1. INTRODUCTION

Innumerous signaling mechanisms govern most if not all of the molecular mechanisms of cellular functions. Given that many signaling pathways are simultaneously engaged, it is crucial for the specificity and efficacy of a particular pathway that all of its individual components are in close proximity (1, 2). The last 25 years revealed a plethora of protein-protein interactions that are critical for the transfer of information between the various modules and nodes within the myriad signaling pathways at work in individual cells. With approximately 800 members, G protein–coupled receptors (GPRCs) mediate the lion's share of cellular signaling (3). G_sPCRs activate the stimulatory trimeric G_s protein and thereby cAMP production by adenylyl cyclase (AC). Although cAMP is a freely diffusible second messenger, spatial restriction of cAMP production can result in differential signaling by

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G_sPCRs, even within the same cell (1,4, 5). For instance, stimulation of the β_1 adrenergic receptor (β_1 AR) in cardiomyocytes leads to phosphorylation of proteins by the cAMP-dependent protein kinase (PKA) throughout these cells, whereas stimulation of the β_2 adrenergic receptor (β_2 AR) is mostly restricted to the vicinity of the L-type Ca^{2+} channel (LTCC) $Ca_V1.2$ (4, 6).

The identification of signaling complexes formed between the β_2 AR, the LTCC $Ca_V1.2$ (5, 7, 8), and the AMPA-type glutamate receptors (AMPARs) (9, 10), which also contain G_s, AC, and PKA, was a milestone that significantly promoted our understanding of cAMP signaling. Selective interception of signaling pathways by ligands that specifically disrupt the respective protein-protein interactions has the potential to restrict their effects to one rather than several signaling cascades downstream of a particular GPCR. This approach expands other recent noteworthy efforts to affect signaling by GPCRs, and especially the β_2 AR, by ligands that bind to the intracellular portions of GPCRs rather than their orthosteric ligand sites (3). Pharmacological agents that disrupt binding of G_s to a GPCR impair many signaling cascades downstream of a given GPCR, whereas the specific displacement of a GPCR from its ultimate target will affect only one signaling cascade.

2. ION CHANNELS AS DRUG TARGETS

Voltage- and ligand-gated ion channels regulate neuronal, cardiovascular, endocrine, and numerous other functions. Thus, they constitute prime targets for the pharmacological treatment of a multitude of diseases, including anxiety, depression, epilepsy, hypertension, and diabetes. However, achieving selectivity is difficult given the homology and high degree of sequence conservation between members of various ion channel families. Due to their limited contact sites with their targets, this problem of selectivity is a major hurdle for the development of small organic compounds as therapeutics. Thus, biologics such as peptides and antibodies are increasingly pursued to pharmacologically target ion channels (11).

2.1. Signaling by Epinephrine and Norepinephrine via the β_2 Adrenergic Receptor in the Brain

Epinephrine and norepinephrine (NE) regulate numerous physiological functions throughout our body. The fight-or-flight response is perhaps the most obvious effect of these stress hormones that exert peripheral effects such as increased heart rate mediated by downstream β_1 AR signaling. In the brain, NE generally augments arousal, acuity of behavioral tasks, and learning of emotionally charged content (12–14). As detailed below, the β_2 AR is required for certain forms of synaptic plasticity and forms unique signaling complexes with $Ca_V1.2$ (7, 8) and the AMPAR (9, 10). We propose that these complexes are among the most important conduits of NE signaling in the brain and thus could be important drug targets for treating conditions such as post-traumatic stress disorder (PTSD) and attention-deficit hyperactivity disorder (ADHD) (15–18).

2.2. $Ca_V1.2$ Function in Health and Disease in the Brain

Ca^{2+} influx through $Ca_V1.2$ governs gene expression via CREB and NFAT (19, 20) and controls neuronal excitability via Ca^{2+}-activated K^+ channels (21, 22). $Ca_V1.2$ constitutes

about 80% of L-type channels in the brain (23, 24). $Ca_V1.2$ mediates a portion of long-term potentiation (LTP) induced by 200-Hz tetanic stimuli (25), particularly during aging. Function of $Ca_V1.2$ is essential for mGluR-dependent long-term depression (26) and LTP induced by a prolonged theta tetanus (PTT) (PTT-LTP) at 5–10 Hz for 90–180 s (27, 28), a rhythm naturally occurring in the brain. Both PTT-LTP and $Ca_V1.2$ are relevant for spatial learning (29, 30).

Animal studies implicated increased $Ca_V1.2$ channel activity in anxiety disorders, depression, and self-injurious behavior (24) and in the etiology of senile symptoms and Alzheimer's disease (31–33). Haploinsufficiency in the *CACNA1C* gene encoding the central pore-forming α_1 1.2 subunit of $Ca_V1.2$ leads to deficits in prosocial ultrasonic communication in mice, suggesting that $Ca_V1.2$ plays a significant role in regulating socio-affective communication in rodents (34). Multiple genome-wide association studies uncovered variants in the *CACNA1C* gene as major risk factors for schizophrenia (SCZ), bipolar disorder (BPD), and autism spectrum disorder (ASD) (35–40). Single-nucleotide polymorphisms (SNPs), including the high-risk allele *s1006737*, are within a 100-kb region of *CACNA1C*'s third intron. As this is a noncoding region, one hypothesis is that these SNPs somehow interfere with proper splicing or enhancer activity. Sequence variants in a human-specific 30-bp tandem repeat in this region that exhibit decreased enhancer activity are associated with a similar disease risk as the flanking SNPs previously linked to BPD and SCZ (41).

3. β_2 ADRENERGIC RECEPTOR COMPLEXES THAT ENABLE LOCALIZED SIGNALING

3.1. The β_2 Adrenergic Receptor–$Ca_V1.2$ Signaling Complex in the Brain

Earlier considerations that signaling by cAMP could be spatially restricted for the specific activation of subsets of cAMP signaling cascades by the proximity of G_sPCRs with their ultimate targets inspired multiple searches for putative G_sPCR signaling complexes. $Ca_V1.2$ was the first target shown to form a complex with a GsPCR that also contained all other proteins in the classic cAMP cascade. Indeed, $Ca_V1.2$ associates with the β_2 AR, G_s, AC, and PKA for highly localized regulation via cAMP in the brain (7, 42). Here, the A-kinase anchor protein 5 (AKAP5) (rodent AKAP150, human AKAP79) binds to three different sites in α_1 1.2, i.e., the N terminus, the loop between domains I and II, and the distal C terminus (42–44) (Figure 1). In turn, the AKAP5 N terminus interacts with different ACs (45, 46), while a short motif near its C terminus interacts with the regulatory RII subunits of PKA (Figure 1). The cytosolic C terminus of the β_2 AR binds to the distal C terminus region of α_1 1.2 encompassing S1928 (27). S1928 is the main PKA phosphorylation site of α 1.2 (42,47) and is essential for the upregulation of $Ca_V1.2$ activity in neurons (28) and vascular smooth muscle cells (VSMCs) (48) but, remarkably, not in the heart (49). It is unclear whether the β_2 AR directly contacts S1928, potentially occluding access for PKA. Given that upregulation of $Ca_V1.2$ activity by β_2 AR signaling requires not only S1928 phosphorylation but also phosphorylation of the β_2 AR itself on its PKA site S261/S262 (50), it is tempting to speculate that phosphorylation of S261/S262 results in a conformational change that renders S1928 accessible. The β_2 AR is displaced from $Ca_V1.2$

upon S1928 phosphorylation for about 5 min. During this period, $Ca_V1.2$ is refractory to the enhancement of its activity by a second β_2 AR stimulation (27). How G_s is attached to the β_2 AR–$Ca_V1.2$ complex is unclear, but it is potentially via preassociation with the β_2 AR (51).

The $Ca_V1.2$ complex also contains the serine/threonine phosphatases PP2A and PP2B. PP2A binds with its catalytic C subunit directly to residues 1965–1971, less than 40 residues downstream of S1928, to counteract S1928 phosphorylation and the upregulation of LTCC activity (52–54). PP2B binds to α_1 1.2 immediately upstream of the PP2A binding site to augment $Ca_V1.2$ activity through an unknown mechanism (55). Another PP2B molecule anchored to AKAP5 regulates α_1 1.2 to curb $Ca_V1.2$ activity, in part, by governing Ca^{2+}-dependent inactivation (44, 56, 57).

Application of the β_2 AR–selective agonist albuterol in the cell-attached recording configuration increases channel open probability greater than twofold when applied inside the patch electrode but not at all when administered to the outside of the electrode. Despite the fact that over 99% of the cell surface and thus the vast majority of β_2 ARs are accessible in the latter arrangement, their stimulation does not translate into upregulation of the activity of those channels that are physically occluded by the electrode (7; see also 58). Accordingly, cAMP signaling from the β_2 AR to $Ca_V1.2$ is limited to less than 200 nm, the estimated dimension of the distance between β_2 AR and $Ca_V1.2$ inside and outside the patch. A peptide consisting of α_1 1.2 residues 1923–1942 (Pep1923–1942) displaces the β_2 AR from $Ca_V1.2$ and prevents β_2 AR stimulation of $Ca_V1.2$, demonstrating that β_2 AR binding to $Ca_V1.2$ is required for the upregulation of channel activity and providing further evidence for the notion that this cAMP-mediated signaling is highly localized, potentially to the dimension of individual β_2 AR-$Ca_V1.2$ complexes (27).

That the β_2 AR and $Ca_V1.2$ colocalize within a complex at postsynaptic sites makes this signaling node a prime conduit for NE signaling (7). This notion is supported by the findings that PTT-LTP requires β_2 AR and $Ca_V1.2$ activity, their association, and the phosphorylation of $Ca_V1.2$ on S1928 by PKA downstream of the β_2 AR (27, 28, 59). Thus the β_2 AR–$Ca_V1.2$ complex constitutes a potentially important target for the pharmacological treatment of conditions related to impaired signaling by NE or $Ca_V1.2$.

3.2. The β_2 Adrenergic Receptor–$Ca_V1.2$ Signaling Complex in the Cardiovascular System

$Ca_V1.2$ controls our heart beat as well as the excitability of VSMCs, where it controls arterial diameter and thus blood pressure. Therefore, $Ca_V1.2$ has been established as a major drug target (60) in the control of cardiovascular diseases. Dihydropyridines preferentially bind to LTCCs under the depolarizing conditions that occur in VSMCs, particularly during vasospasm, and are particularly effective for the initial treatment of hypertension (61). Phenylalkylamines and benzothiazepines are use-dependent LTCC blockers. Accordingly, they inhibit cardiac LTCCs, especially during increased action potential frequencies, and are thus useful for treating arrhythmias (61). Hence, these general $Ca_V1.2$ inhibitors will likely have central and peripheral effects. To treat mental disorders, it would be important to selectively target brain-specific isoforms of $Ca_V1.2$ and largely spare $Ca_V1.2$ in the cardiovascular system. Importantly, the regulation of $Ca_V1.2$ by β ARs fundamentally differs between the brain and heart. As discussed above, the functional regulation of $Ca_V1.2$

by β ARs in neurons occurs through β_2 AR and requires α_1 1.2 S1928, whereas β_1AR signaling regulates $Ca_V1.2$ function in the heart where α_1 1.2 S1928 phosphorylation is dispensable (27, 28, 59, 62, 63). However, $Ca_V1.2$ associates with the β_2 AR, G_s, AC, and PKA for localized cAMP signaling in not only the brain (7, 42) but also the heart (8, 58). During normal cardiac physiology the heart beat is controlled by the β1 AR, whereas under pathological conditions the β_2 AR is upregulated and becomes more prominent in the regulation of $Ca_V1.2$ (62, 63). Thus, these mechanisms potentially constitute an additional pharmacological target site for therapeutics aimed at dampening β_2 AR–$Ca_V1.2$ signaling.

Much like in neurons, macromolecular protein complexes play key roles in modulating the function of VSMCs and therefore have a major influence on vessel diameter. Control of VSMC excitability can be regulated by a myriad of signaling inputs, including protein kinases and phosphatases. The effects of these ubiquitous signaling molecules are often dependent on scaffold proteins that provide a platform for targeting and compartmentalization signaling events to specific substrates (1,2). In the next section, we describe the composition and physiological relevance of specific macromolecular complexes controlling VSMC excitability.

In VSMCs, $Ca_V1.2$, transient receptor potential (TRP) channels, and Ca^{2+}- and voltage-gated potassium channels (BK and various K_V channels) regulate cell excitability by controlling membrane potential (E_m) and the magnitude of intracellular Ca^{2+} (64). These channels exist in complexes with signaling molecules, including PKA, protein kinase C (PKC), and cGMP-dependent protein kinase as well as protein phosphatases such as PP2B, which are organized by multivalent scaffold proteins such as AKAPs (64). AKAP5, which binds PKA, PKC, and PP2B, interacts with $Ca_V1.2$ (48, 65, 66) (Figure 2) and TRPV4 channels (67) in VSMCs. In these cells, AKAP5 may provide structural support to control the activity and location of $Ca_V1.2$ (68). Here, some $Ca_V1.2$ channels in VSMCs showed stochastic activity with low Ca^{2+} flux and event duration, whereas others showed persistent activity characterized by increased Ca^{2+} flux and events with a prolonged open time produced by the opening of two or more channels (68). The occurrence of persistently active $Ca_V1.2$ is restricted to discrete regions of the surface membrane and is highly dependent on the activity of PKC as well as the expression of AKAP5 (65, 68). Moreover, the activity of phosphatases such as AKAP5-associated PP2B counteracts anchored kinase activity and curbs persistent LTCC events (65). These results point to an important role for AKAP5-anchored PKC and PP2B activity in modulating basal persistent $Ca_V1.2$ events. The physiological significance of these findings stems from the fact that persistent $Ca_V1.2$ events account for 50% of the total dihydropyridine-sensitive (e.g., $Ca_V1.2$) Ca^{2+} influx at physiological E_m (68), a process critical for VSMC contractility (Figure 2).

During the pathological condition of angiotensin II–induced hypertension, PKC activity and persistent $Ca_V1.2$ events were increased in an AKAP5-dependent manner (65). Indeed, AKAP5 knockout mice were hypotensive and did not develop angiotensin II–induced hypertension. An increase in persistent LTCC events was also observed in VSMCs during diabetes (69), a pathological condition that unexpectedly is linked to the activation of AKAP5-anchored PKA (48). The finding that PKA induces the LTCC activity that promotes VSMC contraction in response to diabetic hyperglycemia suggests the specific engagement

of pools of PKA that could be in close proximity to $Ca_V1.2$ along with a GPCR mediating its activation. Indeed, the purinergic receptor $P2Y_{11}$, the only known P2Y receptor coupled to G_s, is closely associated with subpopulations of $Ca_V1.2$ and PKA and can be activated by elevations in extracellular glucose to promote $Ca_V1.2$ activity and vasoconstriction (70). Whether AKAP5 interacts with a GPCR that could mediate the angiotensin II and/or glucose effects in VSMCs is unknown.

By linking PP2B within the same signaling complex as $Ca_V1.2$, AKAP5 orchestrates a signaling module for optimal activation of the phosphatase in VSMCs during hypertension and diabetes, signaling that ultimately results in the activation of the transcription factor NFATc3 (68) (Figure 2). Among the many genes that are altered by NFATc3 activation during hypertension and diabetes, downregulation of the BK β1 and $K_V2.1$ subunits is a prominent feature, resulting in changes in channel activity that ultimately affect E_m (66, 71, 72). Importantly, the AKAP150-mediated anchoring of PP2B seems essential for activation of NFATc3 and subsequent gene expression remodeling because the aforementioned changes in BK β1 and $K_V2.1$ subunit expression are not observed in mice expressing an AKAP150 that cannot bind PP2B (65, 66, 72). Thus, the AKAP150-mediated complex may be a distinctly critical site of action for signal transduction in VSMCs (Figure 2) that could be exploited as a potential therapeutic target for treating vascular complications associated with several pathologies, including hypertension and diabetes.

TRPV4 channels have also been found complexed with AKAP150 in VSMCs (67, 73). The association between these two proteins is critical for regulation of the ion channel by G_q-PKC signaling (67). TRPV4, AKAP150, and PKC could form a macromolecular signaling complex to regulate Ca^{2+} signaling. Intriguingly, optimal AKAP150-anchored PKC modulation of TRPV4 activity is highly dependent on the distance between the targeted kinase and the ion channel, with a suggested distance between them of ~200 nm (73). Whether AKAP150-anchored PKA and PP2B also regulate TRPV4 channels in VSMCs and the relevance of the AKAP150/PKC/TRPV4 complex in VSMCs during pathological conditions remain to be determined.

Although β adrenergic stimulation of PKA promotes K^+ channel activity and VSMC relaxation (64) (Figure 2), the involvement of an intermediary that could link all components of the signaling complex has been unclear. Recently, the scaffold protein postsynaptic density 95 (PSD-95), which was thought to be a neuron-specific protein, was observed in VSMCs (74). Functionally, PSD-95 was necessary for the basal and isoproterenol-induced, PKA-mediated activation of $K_V1.x$ channels that promotes arterial smooth muscle relaxation (74, 75). This was due to the formation of a distinctive PSD-95-mediated signaling complex involving the β_2 AR, PKA, and specifically $K_V1.2$ channels. Although PSD-95 is associated with AKAP150 in neurons (76), whether a PSD-95-AKAP150 complex is involved in β adrenergic regulation of VSMC excitability is unknown and therefore an area for further investigation.

3.3. The β_2 Adrenergic Receptor–AMPAR Signaling Complex

More than 80% of the synapses in the cortex use glutamate for fast neurotransmission. AMPARs mediate most of the basal postsynaptic response upon presynaptic glutamate

release. They are potential pharmacological targets for the treatment of diseases that involve dysregulation of glutamatergic synapses, including the overexcitation that occurs during epilepsy (77) and the neuronal damage caused by ischemic conditions, nerve damage, and other insults (78–81). Like $Ca_V1.2$, AMPARs form signaling complexes with β_2 ARs that also contain G_s, AC, and PKA. Accordingly, those AMPAR complexes could be additional pharmacological targets to treat disorders associated with the dysregulation of NE–β_2 AR signaling such as ADHD and PTSD. Furthermore, the β_2 AR has been identified as a target for the β amyloid peptide 1–42 ($\beta AP_{1–42}$) (10), which is thought to be the main pathogen in Alzheimer's disease (82, 83). It is tempting to speculate that $\beta AP_{1–42}$ acts in part by dysregulating AMPAR function by the associated β_2 AR, which could lead to a loss of synaptic strength and plasticity (84). Moreover, as evidence indicates that amyloid β (Aβ) oligomers bind near GluA2-containing complexes and AMPAR antagonists can inhibit Aβ oligomer binding and synaptic loss, it is quite plausible that Aβ affects AMPAR trafficking by binding directly to the GluA2 protein complex (85). Finally, the antidepressant and cognitive enhancer tianeptine augments synaptic AMPAR function and antagonizes impairment of synaptic function following stress (86), suggesting that AMPARs could be pharmacological targets for the treatment of depression and anxiety-related disorders, including PTSD.

PSD-95 is the central organizer of glutamatergic postsynaptic sites, where it anchors AMPARs by binding with its first two PDZ domains to the cytosolic C termini of auxiliary AMPAR subunits called TARPs (transmembrane AMPAR regulatory proteins) (87) (Figure 3). PSD-95 links the β_2 AR to AMPARs by binding with its third PDZ domain to the C terminus of the β_2 AR (9, 10); it also binds AKAP5, but it is unclear whether PSD-95 helps to recruit AC and PKA to AMPARs (76). Rather, the PSD-95 homolog synapse-associated protein 97 (SAP97) binds to the C terminus of the AMPAR GluA1 subunit (88) and is required to recruit PKA and PP2B to AMPARs, as demonstrated in HEK293 cells (76, 89). AKAP5 also links ACs to AMPARs (45, 46, 90) and binds to the SH3-GK module of PSD-95 and SAP97 (76), but the specific interaction sites have not been defined.

PTT-LTP depends not only on the activation of the β_2 AR and $Ca_V1.2$ but also on AKAP5-mediated PKA anchoring and phosphorylation of the AMPAR subunit on S845 (59, 90). S845 phosphorylation augments the channel activity of AMPARs (91), amplifying depolarization at postsynaptic sites. β_2 ARs, AMPARs, and $Ca_V1.2$ are colocalized at postsynaptic sites of glutamatergic neurons (7, 9). There, they likely form a functional unit such that, upon presynaptic glutamate release, the local AMPAR-driven depolarization can activate $Ca_V1.2$ (28) (Figure 4).

4. PHARMACOLOGICAL TARGETING OF β_2 ADRENERGIC RECEPTOR PROTEIN-PROTEIN INTERACTIONS

4.1. Cell-Penetrating Peptides as Biologics

The use of biologically active peptides targeting protein-protein interactions has greatly expanded into a broad range of therapeutic areas (11, 92–94). Importantly, the potential for small organic compounds to disrupt protein-protein interactions is generally thought to be

low because the interaction surfaces between proteins are typically much larger than their interaction surfaces with small drugs (11, 92, 93). Peptides have larger interaction surfaces and can acutely disrupt protein interactions in a highly effective and specific manner. However, the hydrophilic nature of many peptides results in low membrane permeability and prevents their access to intracellular targets, which would, without additional modifications, limit their therapeutic value. In the specific case of using peptides for the treatment of brain disorders, delivery of the hydrophilic peptides across the blood–brain barrier (BBB) must be achieved.

The HIV-1 protein transactivator of transcription (TAT) and the *Drosophila melanogaster* Antennapedia protein (Ant) were found to efficiently cross the plasma membrane (94–97). The translocation properties of Ant were narrowed down to a short, 16-amino-acid peptide corresponding to the third helix of the Antennapedia homeodomain, called pAntp or Penetratin (98). In the case of TAT, a minimal 9-residue-long basic sequence (residues 49–57) was found to mediate its cellular uptake (99). Many short cell-penetrating peptides (CPPs) varying from 5 to 30 residues in length have been identified (92, 93).

CPPs can pass through cell membranes via energy-dependent and energy-independent mechanisms but do not seem to require specific receptors (92). Passive, energy-independent entry of peptides into cells occurs by transient membrane disruption or spontaneous translocation. Energy-dependent internalization of a CPP occurs via endocytosis. CPPs can essentially be categorized into three main classes: mostly cationic (e.g., TAT- and pAntp-derived), amphipathic [with alternating regions of hydrophilic (here, cationic lysine and arginine residues) and hydrophobic residues (valine, leucine, isoleucine, and alanine)], and mostly hydrophobic (92).

TAT peptide conjugates have been found to disrupt protein binding in many systems (100). Disruption of postsynaptic interactions is accomplished in cultured hippocampal neurons (101), acute brain slices (102), and in vivo (103). Even full-length recombinant proteins are typically membrane-permeant when carrying the TAT sequence (94, 100, 104). Many TAT fusion proteins have been expressed in *Escherichia coli,* purified and successfully used to disrupt protein-protein interactions in mammalian cells (94, 104, 105). TAT proteins can also penetrate various tissues, including an intact BBB (94,105, 106). An alternative to the actual TAT sequence is a stretch of 11 arginine residues, which is as effective in rendering peptides membrane-permeant as TAT itself (9, 107, 108). In addition, the attachment of lipid moieties, e.g., myristoylation (27, 109) or stearylation (92), can also facilitate the penetration of macromolecules into cells. In fact, myristoylation of an already TAT-tagged peptide makes the peptide more membrane-permeant (110).

Numerous biochemical entities have been developed for targeting protein-protein interactions. Below, we describe in detail the paradigmatic pepducins before discussing the nature and potential use of nanobodies (Nbs)/intrabodies (Ibs) and nucleic acid aptamers. Finally, we discuss the use of peptides conjugated to the TAT and related poly-arginine segments that can disrupt β_2 AR complexes with $Ca_V1.2$ and AMPARs.

4.2. GPCR Signaling and Biased Agonism: Paradigmatic Targets for Biologics Affecting Protein-Protein Interactions

The intracellular regions of GPCRs have been identified as new target sites for drugs that modify interactions between downstream effectors and modulators such as G_s, GPCR kinases (GRKs), and arrestins. Recent examples include peptides (pepducins), cellularly expressed antibody derivatives (Ibs, Nbs), and RNA- or DNA-based aptamers. Ligand binding to GPCRs can activate signaling that depends on G proteins and arrestins (111). Agonist binding to β_2 AR causes a conformational change that promotes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on $G_{\alpha s}$ within the heterotrimeric stimulatory G_s protein, causing $G_{\alpha s}$ to dissociate from the obligate heterodimeric $G_{\beta\gamma}$ subunit. $G_{\alpha s}$ activates ACs, and $G_{\beta\gamma}$ modulates effectors such as the K^+ channel $K_{ir}3$, phospholipase C, AC, and voltage-gated Ca^{2+} channels via direct interaction with these proteins (112).

G_s-independent signaling involves arrestins and GRKs (113, 114). Upon GPCR activation, the PH domain of GRKs binds to isoprenylated, membrane-anchored $G_{\beta\gamma}$, leading to GRK translocation to the plasma membrane (115). GRKs phosphorylate serine/threonine residues on the GPCR, creating binding sites for arrestins (116). Arrestin binding causes homologous desensitization of the activated GPCR by sterically hindering $G_{\alpha s}$ binding (117), whereas heterologous desensitization of other GPCRs occurs through inhibition of $G_{\alpha s}$ coupling by PKA and PKC (113). β-Arrestin-2 recruits phosphodiesterase 4D to phosphorylated β_2 AR to dampen the increase in cAMP levels induced by the restimulation of β_2 AR (118). In fact, heterologous phosphorylation allows for the occurrence of β-arrestin-dependent effects in the absence of homologous receptor activation (119). β-Arrestins also function as adapters for clathrin-mediated endocytosis, with clathrin, AP-2, ARF-6, and NSF facilitating GPCR internalization and recycling and trafficking of the internalized GPCR (113, 114). Finally, arrestins recruit MAP kinases (120, 121) and Src (122). Binding of Src to arrestin upon β_2 AR stimulation activates Src, which in turn stimulates activation of ERK1/2 (122). In addition, β-arrestins can transactivate EGFR in a GRK5- and GRK6-dependent manner, leading to ERK activation (123). Arrestins also serve as adapters for a complex consisting of JNK1/2 and upstream kinases MKK4 and MKK7 to facilitate the phosphorylation of JNKs (124).

Ligands can bias GPCR signaling to either $G_{\alpha s}$ or arrestin (113). GPCRs exist in their active and inactive forms in multiple dynamically interconverting conformations (125). The conformational differences induced by certain ligands are propagated to transducers and regulatory proteins to impart differing signaling consequences (126–130). It has been suggested that GRKs recognize the different conformations of ligand-receptor complexes and endow specific phosphorylation patterns onto GPCRs, leading to distinct conformations of transducers and regulatory proteins through these apparent phosphorylation barcodes (131, 132). The phenomenon of biased signaling has therapeutic value because defined GPCR ligands can be designed to tweak signaling to a desired pathway by inducing a specific GPCR conformation that yields the desired pharmacological effect and minimizes unwanted side effects.

4.2.1. Pepducins.—Pepducins are short, lipidated (e.g., palmitoyl- or myristoyl-conjugated) CPPs with sequences derived from intracellular loops (ICLs) of GPCRs. They can act as agonists, antagonists, or biased agonists of the cognate GPCR. A systematic analysis identified four classes of pepducins targeting β_2 AR signaling with differential signaling properties: (*a*) partial agonists promoting both $G_{\alpha s}$ signaling and β-arrestin recruitment; (*b*) β-arrestin-biased pepducins, mainly derived from ICL1; (*c*) receptor-dependent $G_{\alpha s}$-biased pepducins (e.g., pepducin ICL3–9); and (*d*) receptor-independent, $G_{\alpha s}$-biased pepducins (e.g., pepducin ICL3–8) (133). ICL3–8 and ICL3–9 are derived, respectively, from the proximal and central portions of the third ICL of the β_2 AR and do not promote β-arrestin recruitment and β_2 AR internalization. ICL3–8 is receptor-independent, i.e., it increases cAMP production by binding to and directly activating G«s. ICL3–9 stimulates cAMP production by stimulating $G_{\alpha s}$ recruitment to β_2 AR. ICL3–9 induces a β_2 AR conformation different from that induced by the full β_2 AR agonist isoproterenol but allows interaction with $G_{\alpha s}$, albeit with a binding mode distinct from that stimulated by isoproterenol.

The β-arrestin-biased pepducin ICL1–9 increases cardiomyocyte contractility (134). The efficacy of β-arrestin recruitment to the β_2 AR by ICL1–9 is about 50%, relative to isoproterenol, but it does not induce cAMP production or bind to the orthosteric site. Although ICL1–9 triggers less GRK-dependent β_2 AR phosphorylation and receptor internalization than isoproterenol, it induces more prolonged ERK activation with higher efficacy than isoproterenol and with faster kinetics than the β-arrestin-biased orthosteric agonist carvedilol (134). By stabilizing a β_2 AR conformation that is favorable for β-arrestin binding, ICL1–9 increases contractility in murine cardiomyocytes in an unconventional cAMP-dependent manner that does not require Ca^{2+} mobilization (134). The specificity or promiscuity of signaling modulation by pepducins depends on their mechanism of action. For example, ICL3–8 functions by directly activating $G_{\alpha s}$ and increasing cAMP production in conjunction with several G_sPCRs (133). On the other hand, ICL3–9 functions by inducing a distinct β_2 AR conformation, which stimulates $G_{\alpha s}$ in conjunction with β_2 AR and the closely related β_1AR but not the prostaglandin E2 receptor PGE_2R (133).

4.2.2. Nanobodies and intrabodies.—Camelids produce antibodies lacking light chains. The variable region of these heavy-chain antibodies (VHH domain) can be recombinantly expressed. The ~15-kDa products are Nbs (135), which can be expressed exogenously in mammalian or insect cells as Ibs. Different Nbs were developed that specifically bind to the intracellular domains of active (agonist-bound) or inactive (antagonist-bound) β_2 AR, stabilizing the corresponding conformations (136). When coexpressed with β_2 AR in HEK293 cells as Ibs, Nbs specific to inactive β_2 AR inhibit cAMP production as well as β-arrestin recruitment and reduce β_2 AR expression. Several active conformation-stabilizing Ibs also inhibit cAMP production and β-arrestin recruitment by stabilizing a conformation that is not conducive to the binding of $G_{\alpha s}$ and GRKs, the steric hinderance of β-arrestin access to the β_2 AR, and enhanced β_2 AR–G_i coupling by active conformation-stabilizing Ibs.

Antibody antigen-binding fragments (Fabs) can be converted to single-chain antibodies (135) and expressed as Ibs. Fabs that bind to the active conformation of β-arrestin coupled to

the vasopressin receptor modulate interactions of β-arrestin with ERK and clathrin with variable and biased effects (137). One of them, Fab9, augments the binding of β-arrestin to ERK but not to clathrin (137), demonstrating that Fabs can exert an overall stimulatory effect. Fab5/Ib5 inhibits clathrin-mediated receptor internalization without affecting ERK (137). Steric hinderance of clathrin binding to β-arrestin and allosteric modulation of β-arrestin could mediate this effect. However, Ib5 nonselectively inhibits β-arrestin-mediated endocytosis of a wide range of GPCRs, including the β_2 AR; muscarinic M2 receptor; dopaminergic D1, 2, 3, and 4; and μ-opioid receptor (137).

4.2.3. Aptamers.—Aptamers are DNA or RNA strands exhibiting secondary structures that facilitate their binding to target molecules with high specificity and affinity. Aptamers are typically identified through an iterative screening process called SELEX (systematic evolution of ligands by exponential enrichment) (138, 139). Nucleic acids can be readily subjected to chemical modification (140), including myristoylation, to render them membrane-permeant (141). As aptamers that specifically bind to the active β_2 AR conformation inhibit isoproterenol-stimulated cAMP production in β_2 AR–expressing HEK293 cells and inactive conformation-specific aptamers have no effect, it is thought that active conformation-specific aptamers act by blocking the access of $G_{\alpha s}$ (142). The effect of the stabilized active conformation on other arms of the signaling cascade and the functional consequences of this remain to be established.

Pepducins, intrabodies, and aptamers intercepting β_2 AR signaling may find their therapeutic use in the treatment of a broad range of diseases, including congestive heart failure (CHF), hypertension, asthma, chronic obstructive pulmonary disorder, and ASD. They are predicted to exhibit fewer side effects compared to the β-blockers currently in clinical use. For example, although β_1AR is an important therapeutic target in CHF, β_2 AR may also contribute to cardiac pathology due to the upregulation of heterotrimeric G_i, to which the β_2 AR is also coupled (143, 144). Conventional β_2 AR blockade inhibits G_i to restore inotropy, but one undesirable consequence is that this also inhibits the prosurvival effects mediated by $G\beta\gamma$ (145). Specific targeting of β-arrestin signaling, as demonstrated for ICL1–9, is likely to stimulate both contractility and cell survival and may prove to be a promising therapeutic approach in the future. Nevertheless, as β-arrestin signaling activates diverse pathways in different cell types, the signaling consequences of its targeting must be examined in each case.

4.3. Pharmacological Targeting of the Interactions of the β_2 Adrenergic Receptor and Phosphatases with $Ca_V1.2$ and AMPA-Type Glutamate Receptors

The β_2 AR–G_s-AC-PKA-$Ca_V1.2$ and β_2 AR-G_s-AC-PKA-GluA1 complexes are so far the only known complexes that assemble a G_sPCR (i.e., β_2 AR) with all intermediaries (G_s, AC, PKA) and the final target ($Ca_V1.2$, GluA1). Nearly all interactions have been mapped out except for G_s association (1, 7–9, 27, 28, 42–44, 90, 146) (Figures 1 and 3). However, most interactions are not unique to the $Ca_V1.2$ or AMPAR complex. For instance, AKAP5 links PKA to a number of different proteins (1, 2). Accordingly, using peptides that displace either AC or PKA from AKAP5 will affect various signaling cascades. Displacing AKAP5 from PSD-95 or SAP97 could potentially provide a more selective effect; however, their

interactions are currently not defined in sufficient detail for designing peptides that would accomplish such displacements. Rather, targeting the direct binding of the β_2 AR to residues 1923–1942 in the α_1 1.2 C terminus has the potential to be quite selective, as no analogous interaction is currently known (27). In fact, a membrane-permeant myristoylated peptide derived from residues 1923–1942 in α_1 1.2 displaces the β_2 AR from α_1 1.2 and not from the AMPAR complex when applied to acute forebrain slices (27). Consistently, it only affects β_2 AR–triggered phosphorylation of α_1 1.2 but not the AMPAR GluA1 subunit at the respective PKA site in the slices. The converse is true for a peptide that displaces the β_2 AR from the AMPAR complex and is alternatively either 11-Arg-conjugated (9, 27) or myristoylated (B. Lee & J.W. Hell, unpublished data). However, this peptide might affect some other function related to PSD-95 because it interferes with the binding of β_2 AR with the third PDZ domain of PSD-95, its link to the AMPAR, and this PDZ domain mediates binding of other proteins as well (9). Still, an 11-Arg-conjugated version of this peptide effectively prevents upregulation of GluA1 phosphorylation and its otherwise consequent surface accumulation in hippocampal cultures (9) and spike-time-dependent plasticity in acute cortical slices (14).

$Ca_V1.2$ also has different phosphatases linked to it. Remarkably, PP2B directly interacts with residues 1943–1971 immediately downstream of the PKA phosphorylation site S1928 and of the β_2 AR binding site in the C terminus of α_1 1.2 (55) (Figure 1). This PP2B does not dephosphorylate S1928 but rather augments $Ca_V1.2$ activity, possibly by dephosphorylating a hypothetical inhibitory phosphorylation site. A second PP2B attachment site is provided by AKAP5, which is important for Ca^{2+}-dependent inactivation of $Ca_V1.2$ (44, 57). Finally, the PP2A catalytic C subunit, rather than one of its targeting B-type subunits, directly binds immediately downstream of PP2B to residues 1965–1971 (52–54). PP2A dephosphorylates S1928, and displacing PP2A with a corresponding peptide augments channel activity, consistent with a role of PP2A in counteracting cAMP-mediated upregulation of $Ca_V1.2$ (54). Although phosphatase targeting to $Ca_V1.2$ is complex, some interactions such as the direct binding of the PP2A C subunit to α_1 1.2 have the potential to be unique and thus constitute prospective drug targets for controlling $Ca_V1.2$ activity.

5. CONCLUSION AND PERSPECTIVE

Biologics are rapidly emerging as promising therapeutics under development and in the clinic. Membrane-permeant peptides have vast pharmacological potential given their ability to specifically and selectively target physiologically relevant protein-protein interactions among the multitude of protein signaling complexes in the cellular milieu. We envision the development of a number of peptides that can precisely target and disrupt protein-protein interactions in defined complexes that modulate the function of $Ca_V1.2$ and glutamate receptors in specific subcellular compartments. As the target ion channels serve widespread and multiple functions, such peptides possibly could exert their effects at a quasi-microsurgery molecular level and thus limit the off-target side effects elicited by many of the existing small-molecule drugs in the clinic.

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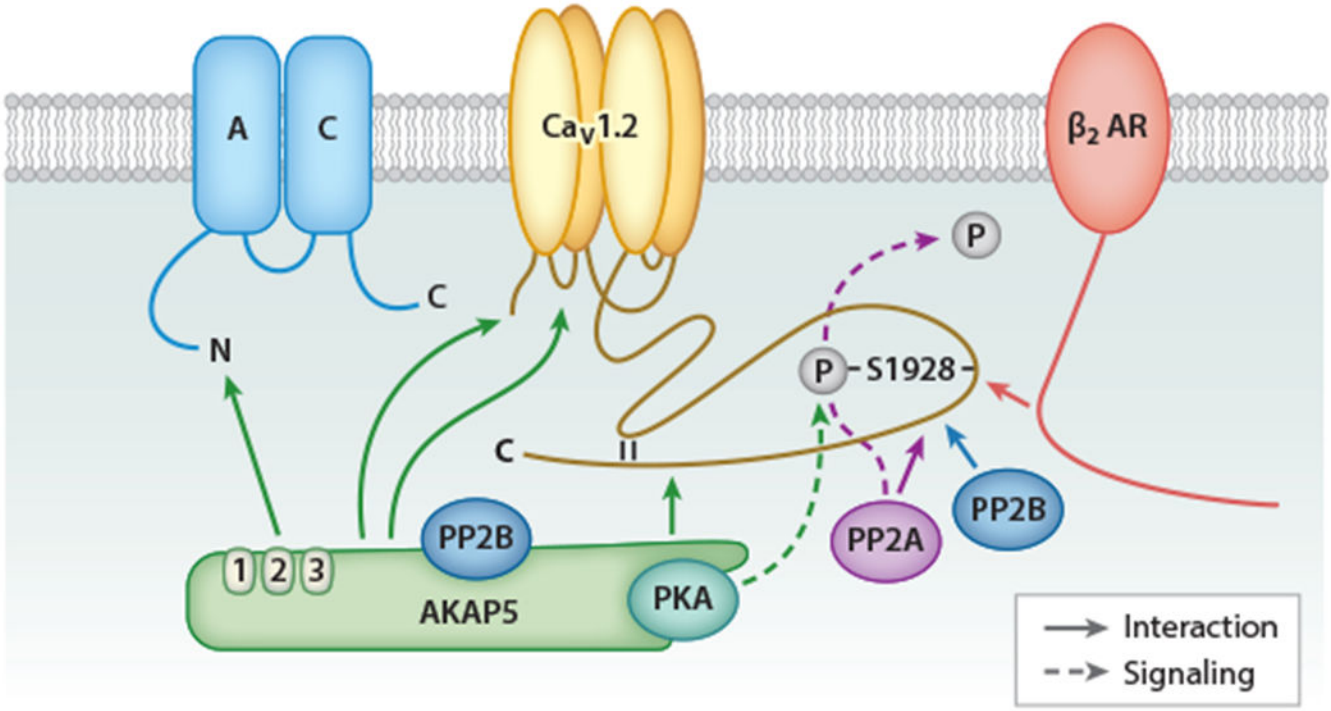

Figure 1.

The β_2 AR–AC-PKA-$Ca_V1.2$ complex. Green arrows indicate binding of the N terminus of AKAP5 to the N terminus of AC, the C terminus of AKAP5 to the distal C terminus of α_1 1.2, and the so far undefined regions of AKAP5 to the N terminus and the loop between domains I and II of α_1 1.2. AKAP5 links in this way AC, PKA, and PP2B to $Ca_V1.2$. The β_2 AR binds with its C terminus to the region around S1928 in the distal C terminus of α_1 1.2 (*red arrow*). PP2A and PP2B also bind directly to α_1 1.2 about 40 and 50 residues downstream of S1928 (*purple and blue arrows*). Activation of β_2 AR–G_s-AC-cAMP-PKA signaling leads to S1928 phosphorylation by PKA (*dashed green line*) and upregulation of $Ca_V1.2$ activity, both of which are reversed by the $\beta_1$1.2-associated PP2A (*dashed purple line*). Abbreviations: AC, adenylyl cyclase; AKAP5, A-kinase anchor protein 5; AR, adrenergic receptor; PKA, cAMP-dependent protein kinase; PP, protein phosphatase.

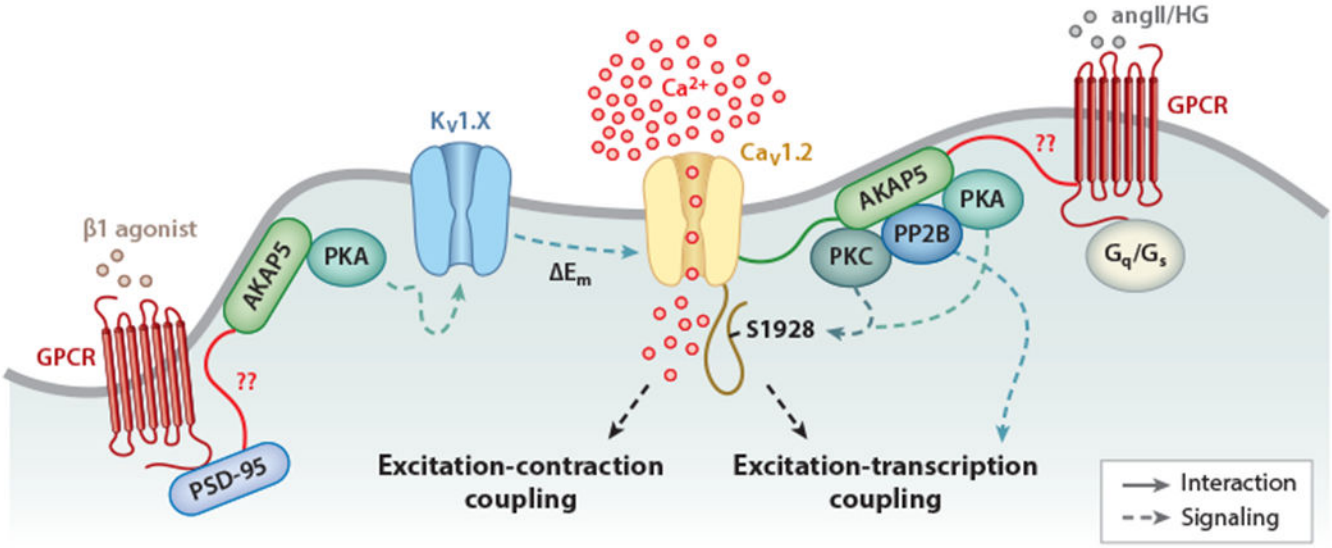

Figure 2.

Proposed model for the regulation of vascular smooth muscle cell (VSMC) excitability by macromolecular complexes. The magnitude of Ca^{2+} influx via $Ca_V1.2$ is critical for the control of excitation-contraction and excitation-transcription coupling in these cells. Under physiological conditions, K^+ channels oppose pressure-induced depolarization to limit $Ca_V1.2$ activity and VSMC contractility. The activity of K^+ and $Ca_V1.2$ channels can be regulated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and the protein phosphatase PP2B, which are targeted to the specific channels and G protein-coupled receptors (GPCRs) by AKAP5 and/or PSD-95, and their function may be altered during pathological conditions. Both PKA and PKC can phosphorylate $Ca_V1.2$ on S1928 (*dashed blue lines*) but are regulated by different GPCRs. PKA can also regulate K^+ channels of the K_V1 family. In turn, K_V1 channels negatively control $Ca_V1.2$ activity. Whether the hypothesized interactions (*solid red lines*), including those involving the GPCRs that mediate angiotensin II (angII) and high glucose (HG) signaling, PSD-95, and AKAP5, occur in native VSMCs is unclear.

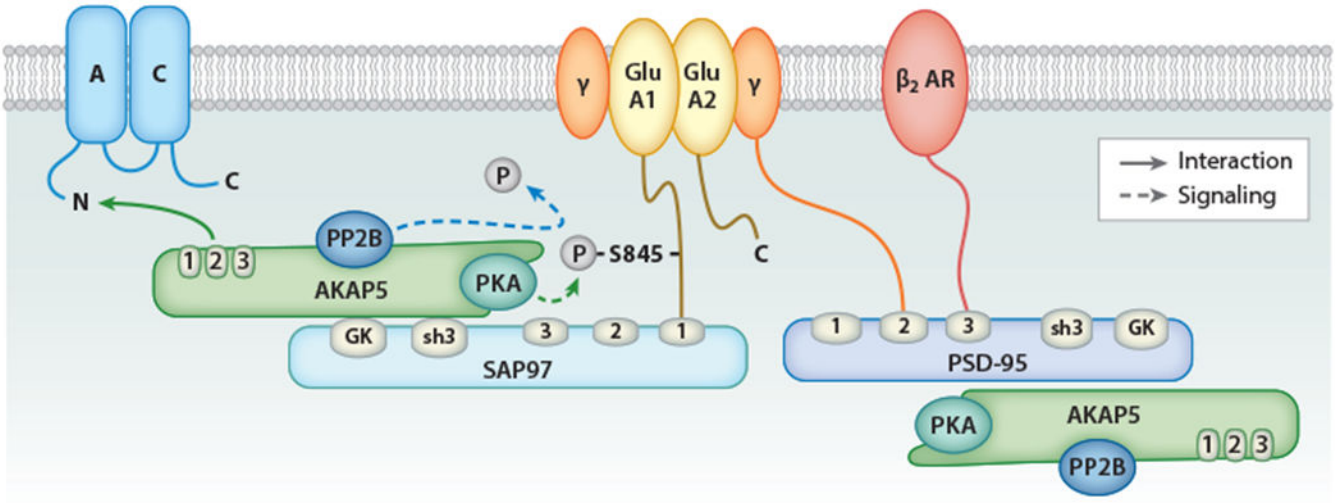

Figure 3.

The β_2 AR–G_s-AC-PKA-GluA1 complex. PSD-95 is a highly prevalent and central structural protein of excitatory glutamatergic synapses. AMPARs are linked to PSD-95 via the binding of their auxiliary TARP (γ) subunits to the first two PDZ domains of PSD-95, whereas the β_2 AR binds to the third PDZ domain of PSD-95. Both PSD-95 and its homolog, SAP97, bind to AKAP5 via their SH3-GK modules. SAP97 binds to the C terminus of the GluA1 subunit of AMPAR, recruiting PKA and PP2B to the vicinity of AMPAR. Through the SAP97-AKAP5 interaction, AC is also localized close to GluA1. Stimulation of β_2 AR induces cAMP increase and PKA activation, increasing S845 phosphorylation on GluA1 and, consequently, AMPAR activity. Abbreviations: AC, adenylyl cyclase; AKAP5, A-kinase anchor protein 5; AMPAR, AMPA-type glutamate receptor; AR, adrenergic receptor; PKA, cAMP-dependent protein kinase; PP, protein phosphatase; PSD-95, postsynaptic density 95; SAP97, synapse-associated protein 97; TARP, transmembrane AMPAR regulatory protein.

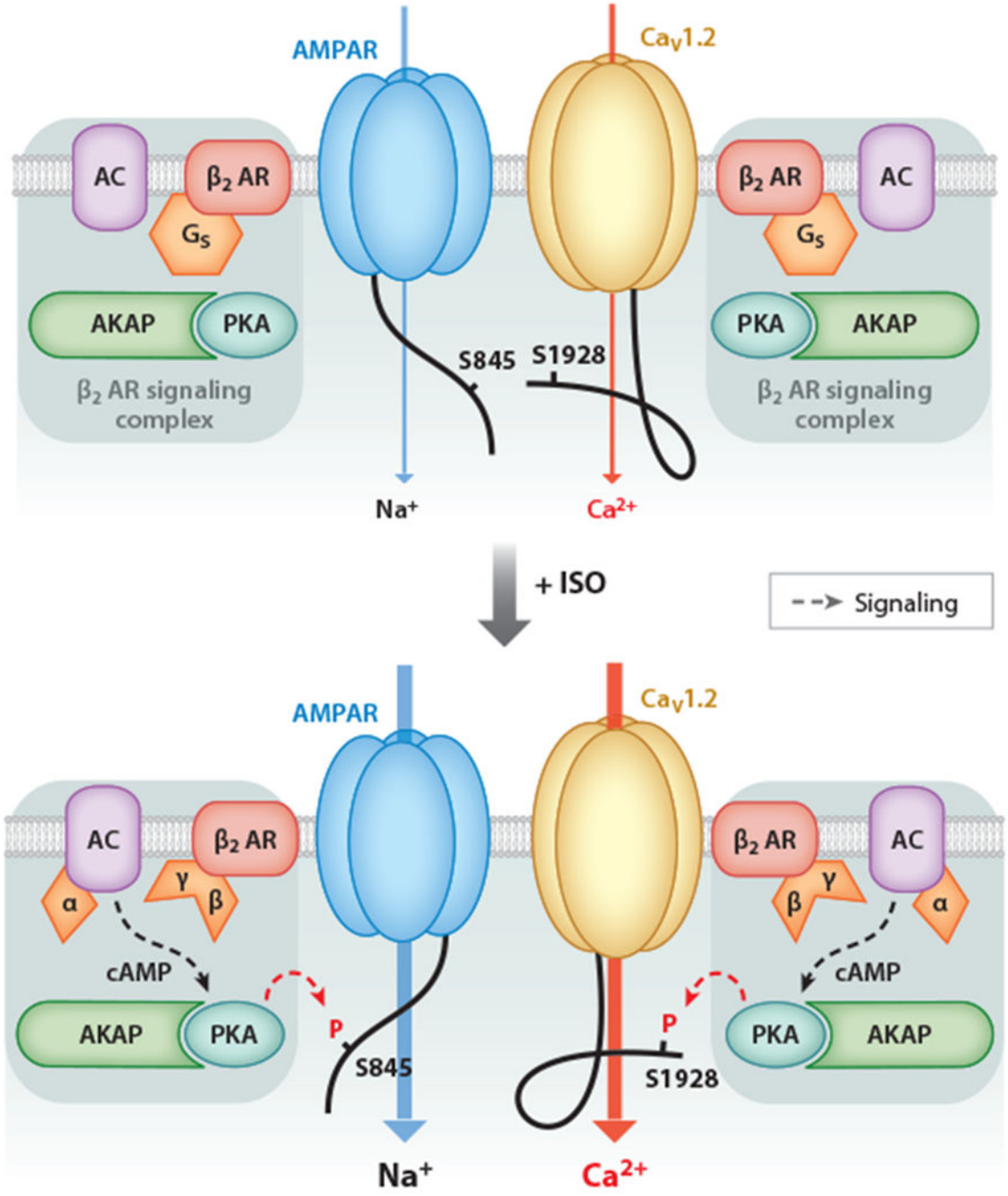

Figure 4.

The β_2 adrenergic receptor (β_2 AR)–$Ca_V1.2$ and β_2 AR–GluA1 signaling complexes participate in prolonged theta tetanus long-term potentiation (PTT-LTP). β_2 AR activation is required in the induction of PTT-LTP. Stimulation of β_2 AR augments $Ca_V1.2$ and AMPA-type glutamate receptor (AMPAR) channel activity via phosphorylation of $Ca_V1.2$ on S1928 and GluA1 on S845, respectively, by A-kinase anchor protein (AKAP)-anchored cAMP-dependent protein kinase (PKA). The upregulation in AMPAR activity increases depolarization upon synaptic transmission and thereby increases $Ca_V1.2$ activation, in

addition to the increased open probability of $Ca_V1.2$ due to S1928 phosphorylation. Phosphorylation of both S845 and S1928 is required for the induction of PTT-LTP. Adapted from Reference 28 with permission from AAAS.