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Supramolecular Assemblies and Localized Regulation of Voltage-Gated Ion Channels

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Abstract

This review addresses the localized regulation of voltage-gated ion channels by phosphorylation. Comprehensive data on channel regulation by associated protein kinases, phosphatases, and related regulatory proteins are mainly available for voltage-gated Ca^{2+} channels, which form the main focus of this review. Other voltage-gated ion channels and especially $K_v7.1$-3 (KCNQ1-3), the large- and small-conductance Ca^{2+}-activated K^+ channels BK and SK2, and the inward-rectifying K^+ channels $K_{ir}3$ have also been studied to quite some extent and will be included. Regulation of the L-type Ca^{2+} channel $Ca_v1.2$ by PKA has been studied most thoroughly as it underlies the cardiac fight-or-flight response. A prototypical $Ca_v1.2$ signaling complex containing the β_2 adrenergic receptor, the heterotrimeric G protein G_s, adenylyl cyclase, and PKA has been identified that supports highly localized via cAMP. The type 2 ryanodine receptor as well as AMPA- and NMDA-type glutamate receptors are in close proximity to $Ca_v1.2$ in cardiomyocytes and neurons, respectively, yet independently anchor PKA, CaMKII, and the serine/threonine phosphatases PP1, PP2A, and PP2B, as is discussed in detail. Descriptions of the structural and functional aspects of the interactions of PKA, PKC, CaMKII, Src, and various phosphatases with $Ca_v1.2$ will include comparisons with analogous interactions with other channels such as the ryanodine receptor or ionotropic glutamate receptors. Regulation of Na^+ and K^+ channel phosphorylation complexes will be discussed in separate papers. This review is thus intended for readers interested in ion channel regulation or in localization of kinases, phosphatases, and their upstream regulators.

I. Introduction

The past decade has revealed an unanticipated number of protein-protein interactions that fundamentally changed our view of the localization and functional interactions of proteins inside cells. Signaling pathways are no exception. Proximity of the relevant control elements including protein kinases and phosphatases is critical for fast, efficient, and specific signaling by many different pathways (310). These targeting mechanisms are especially prevalent at the plasma membrane, where incoming signals may be relayed and integrated with high specificity. Spatial restriction is not only limited to kinases. Second messengers and especially cAMP can

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Note Added in Proof: After acceptance of this manuscript, Lemke et al. (234a) reported on the functional relevance of $Ca_V1.2$ serine-1928 phosphorylation in response to β-adrenergic signaling within cardiac myocytes. Mutating serine-1928 to alanine in knock-in mice did not affect basal L-type calcium currents, β-adrenergic-dependent upregulation with isoproterenol, or PKA-dependent stimulation with forskolin in isolated ventricular myocytes. Furthermore, neither basal nor isoproterenol-stimulated cardiac contractility was affected in the knock-in animals. These results indicate that serine-1928 is not the main PKA phosphorylation site that mediates the strong upregulation in $Ca_V1.2$ current. However, more modest contributions of serine-1928 phosphorylation to $Ca_V1.2$ regulation cannot be ruled out at this point. For instance, a recent abstract by Rankovic et al. (323a) suggests that PKA regulates calcium-dependent inactivation of L-type channels in thalamocortical relay neurons in response to β-adrenergic stimulation.

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also act in a highly localized manner (78,155,329,455). This article reviews mechanisms that promote localized and thereby selective regulation of voltage-gated ion channels by kinases and phosphatases. The localization of the protein kinases cAMP-dependent protein kinase (PKA); protein kinase C (PKC); Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII); Src; the phosphatases PP1, PP2A, and PP2B; their adaptor proteins [e.g., A kinase anchor proteins (AKAPs)]; and their regulators [e.g., G protein-coupled receptors (GPCRs) and G proteins] near or at ion channels are discussed in depth. Relevant aspects of the structures and anchoring mechanisms of the different kinases and phosphatases are included. This review largely focuses on those mechanisms for which interactions between ion channels and their regulators have been identified and ideally verified on a molecular level. Even with this limitation, it is not possible to discuss every contribution to this field. We apologize to those colleagues whose work could not be mentioned.

The most studied example of ion channel regulation by phosphorylation is the stimulation of the L-type Ca^{2+} channel $Ca_v1.2$ in the heart by signaling via cAMP and PKA. β-Adrenergic stimulation increases heart rate and contractility as part of the fight-or-flight response. Although many questions remain, various aspects of the control mechanisms of this channel have emerged over the last few years. $Ca_v1.2$ assembles the β_2 adrenergic receptor (AR), the heterotrimeric G protein G_s, adenylyl cyclase (AC), PKA, and the counteracting phosphatase PP2A into a prototypical signaling complex (11,78,81). Similar complexes are formed by inward-rectifying $K_{ir}3$ channels (231,325) and the AMPAR GluR1 subunit (M. Joiner, D. Hall, Z. Malik, M. Lise, Y. Chen, A. Burette, R. Weinberg, A. El-Husseini, and J. Hell, unpublished data). The pioneering work on these complexes indicates that signaling from the β_2 AR via cAMP to $Ca_v1.2$ is locally restricted (78). The discussion of the $Ca_v1.2$ complex is combined with an overview of findings that support the notion of spatially restricted cAMP pools and of stimulus-independent preassembly of G proteins with their cognate GPCRs or their downstream effectors for selective and effective signaling.

The review of Ca^{2+} channel regulation is interwoven with examples of analogous localized control of other ion channels. The type 2 cardiac ryanodine receptors (RyR2) in the sarcoplasmic reticulum will be discussed because it anchors PKA, PP1, and PP2A independently of $Ca_v1.2$, although it is in close proximity and functionally linked to $Ca_v1.2$. AMPARs and NMDARs constitute another group of ion channels that are regulated by anchored kinases and phosphatases. They are colocalized with $Ca_v1.2$ at postsynaptic sites and will be discussed in that context. Molecular and functional aspects of interactions of Na^+ and K^+ channels and other Ca^{2+} channels with kinases and phosphatases will be compared with those of $Ca_v1.2$. These channels include $K_v7.1$-3 (KCNQ1-3), the large-conductance Ca^{2+}-activated K^+ channel BK, the small-conductance Ca^{2+}-activated K^+ channel SK2, and the inward-rectifying K^+ channels of the $K_{ir}3$ family.

II. Structure and Function of Ion Channels

A. Ca^{2+} Channels

Ca^{2+} is a potent second messenger that controls a variety of cellular functions (44,66,137). As a major source of Ca^{2+} influx, voltage-gated Ca^{2+} channels fulfill critical roles in Ca^{2+} signaling. L-type Ca^{2+} channels regulate muscle contraction, hormone secretion, neuronal excitability, and gene expression. P/Q-, N-, and to some degree R- and L-type Ca^{2+} channels trigger neurotransmitter release at nerve terminals and other locations. T-type channels support neuronal burst firing and relaxation in coronary smooth muscle (56,98,134,143,248,264,383, 447) (see Refs. 52,313 for most recent reviews). Ca^{2+} channels consist of a central α_1 subunit, which forms the ion-conducting pore and defines the channel type (see below). The α_1 subunit has four homologous domains, I-IV, each consisting of six transmembrane segments and a P-loop between segments 5 and 6 (Fig. 1). The auxiliary subunits α_2-δ, β, and γ directly interact

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with α_1 They modulate surface expression and biophysical properties such as channel activation and inactivation (6,52,83). The α_2-δ subunit is created from a single transcript by proteolytic cleavage of the original polypeptide into two fragments. Four distinct genes encode α_2-δ-1 through α_2-δ-4, which are further diversified by differential splicing (83). The δ subunit consists of a short cytosolic COOH terminus, a single transmembrane segment, and a short extracellular domain, which is linked via a disulfide bridge to the heavily glycosylated and much larger (~200 kDa) α_2 polypeptide. The intracellular COOH terminus of δ is 1–15 residues long (83) and is unlikely to be phosphorylated by protein kinases. Coexpression of α_2-δ generally increases surface expression of Ca^{2+} channels and influences to some degree their biophysical properties (83).

In contrast to α_2-δ, β is localized exclusively at the cytosolic face of the channel. The existence of four different β genes (β_1-β_4) and extensive differential splicing, especially of β_1 and β_2 transcripts, give rise to multiple isoforms (120). Recent structural studies demonstrate that β subunits consist of two protein-protein interaction domains, an SH3 domain, and a GK domain (62,308,402). Five sequential β strands constitute the core of the β SH3 domain analogous to canonical SH3 domains. However, the loops between strands 1 and 2 and strands 4 and 5 are much longer than in classic SH3 domains, in which the first loop contains several residues that form contacts with proline-rich domains. This arrangement is similar to the SH3-HOOK-GK motif in PSD-95 and its homologs (272,389). The HOOK domain in PSD-95 corresponds to the large loop between strands 4 and 5 of the SH3 domain of β and has been suggested to obstruct access of proline-rich sequences to the unconventional SH3 domain (272).

The main interaction site on α_1 for β subunits is a sequence of 18 residues in the loop between domain I and II (loop I/II) called the α interaction domain or AID, which binds to a hydrophobic grove in the GK domain of β (62,308,402). Additional interaction sites for β subunits have been identified in the NH_2- and COOH-terminal regions of different α_1 subunits (87,407). The GK domain is important for Ca^{2+} channel trafficking to the cell surface likely by masking an ER retention signal in loop I/II (26,238). Recent evidence, however, indicates that the SH3 domain mediates other functional effects of β subunits on channel activity including channel gating. The SH3 domain can act independently of the GK domain by binding to loop I/II (residues 520-532 in $\alpha_1$1.2) downstream of AID (residues 458–475 in $\alpha_1$1.2) (69,238,259, 273). Splice variants that mainly consist of the SH3 domain and lack the GK domain have been described for all four β isoforms (120,174,191,286). The respective SH3 splice variant of the β_1 subunit (β_{1d}) does not support surface trafficking of $\alpha_1$1.2 but increases mean open probability of the limited number of channels that is present at the surface in the absence of a GK-containing β subunit (69). PKA, PKC, and CaMKII can regulate Ca^{2+} channel activity at least in part via mechanisms that involve β subunits (see sects. III*B2*D, IV*B*, and V*A*).

Eight genes encode γ_1-γ_8, which share four putative transmembrane segments (NH_2 and COOH termini are intracellular) and a signature motif (GLWXXC) as well as a pair of conserved cysteine residues in the first extracellular loop (reviewed in Ref. 213). These features are also characteristic for the otherwise more distantly related claudin family members, which are critical for formation of tight junctions (213,397). The γ_2, γ_3, γ_4, and γ_8 subunits are more closely related to themselves than to the other family members, including the original γ subunit, γ_1. In contrast to the other γ isoforms, γ_2-γ_4 and γ_8 (as well as claudins) possess a PDZ domain binding consensus sequence at their very COOH termini that mediates interaction with PSD-95 and its homologs. Although interactions between the γ_2 subfamily members and Ca^{2+} channels have been observed (213), their most prominent role is to support surface expression of AMPARs (see sect. III*B4*B and Fig. 10).

As γ_2-γ_4 and γ_8 are critical for postsynaptic targeting of AMPARs, they may also steer Ca^{2+} channels to this location, thereby fostering the colocalization and perhaps functional interaction

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of glutamate receptors and Ca^{2+} channels (215). Like glutamate receptors, L-type Ca^{2+} channels are clustered at dendritic spines, which constitute the postsynaptic sites of excitatory synapses (78,163,303). In fact, inhibition of L-type channels reduces maintenance, though not necessarily initial induction, of LTP (Lim and Hell, unpublished results). LTP refers to a stable increase in synaptic transmission that is at least in part mediated by a lasting elevation of glutamate receptor activity (31,256–258). Back-propagating and locally generated dendritic action potentials contribute to LTP induction when occurring shortly after (10–50 ms) an excitatory postsynaptic potential (EPSP) at a given synapse. They do so by promoting Ca^{2+} influx through voltage-gated Ca^{2+} channels, including L-type channels, into dendritic spines (139,262,449). However, no γ subunits have been detected yet in the neuronal L-type channel complex (6,52), although γ_2 has been observed to coimmunoprecipitate with neuronal $Ca_v2.1$ and $Ca_v2.2$ and to affect channel activity in heterologous expression systems (213,214).

Ca^{2+} channels are divided into high- and low-voltage-activated channels (HVA and LVA, respectively). LVA channels require less depolarization for activation and subsequent inactivation than HVA channels. L-type channels are HVA channels and are pharmacologically defined by their sensitivity to dihydropyridines and other so-called organic Ca^{2+} channel blockers. The four L-type channels $Ca_v1.1$-1.4 incorporate $\alpha_1$1.1-1.4 (previously α_{1S}, α_{1C}, α_{1D}, and α_{1F}; for nomenclature, see Refs. 54,55,110). The other HVA family contains the P/Q-, N-, and R-type channels ($Ca_v2.1$-2.3 consisting of $\alpha_1$2.1–2.3 also known as α_{1A}, α_{1B}, and α_{1E}). P- and Q-type channels are created from $\alpha_1$2.1 transcripts by differential splicing (35). They are selectively, but with different potency, inhibited by the funnel web spider toxin ω-AgaIVA and the cone snail toxin ω-CTx-MVIIC (35). N-type currents are quasi-irreversibly blocked by the cone snail toxin ω-CTx-GVIA and some, though not all, R-type currents are inhibited by the tarantula toxin SNX482. $Ca_v2.3$, which is selectively affected by SNX482, underlies a portion of the R-type current (295,317,430). LVA currents are mediated by the three T-type channels $Ca_v3.1$-3.3, which are formed by the related $\alpha_1$3.1–3.3 subunits, (α_{1G}, α_{1H}, and α_{1I}).

B. Na^+ Channels

The structure of the pore-forming α subunit of voltage-gated Na^+ channels mirrors that of Ca^{2+} channels with four homologous domains each consisting of six transmembrane segments and a reentry P loop (Fig. 2) (51). The auxiliary β_1 and β_2 subunits consist of an extracellular immunoglobulin-like domain, a single transmembrane segment, and a short intracellular domain. The β_1 subunit binds to the extracellular segment of the α subunit that precedes transmembrane segment IVS6 (Fig. 2). Their coexpression with the α subunit accelerates activation and inactivation of the resulting Na^+ currents (51). As suggested by their structural relationship to the large family of cell adhesion molecules, β subunits also regulate the subcellular distribution of Na^+ channels (e.g., Ref. 271). Nine different α subunit genes encode $Na_v1.1$–1.9 (53). Na^+ channels contain one α subunit and either no β subunit, β_1, β_2, or both β subunits.

C. K^+ Channels

1. K_v7/KCNQ K^+ channels—K_v7 channels are part of the large voltage-gated K^+ channel family. Each of the K_v channels consists of four subunits that are homologous to each other and also to the individual four domains of Na^+ and Ca^{2+} channels (Fig. 3A) (223,453). In addition, $K_v7.1$/KCNQ1 assembles with the single transmembrane segment protein KCNE1/MinK to form the slow K^+ current I_{Ks} in the heart (12,150,346). This current is critical in repolarization of the cardiac action potential. Loss of function mutations prolong the Q-T interval, which leads to arrhythmias. Upregulation of I_{Ks} in the heart is important during sympathetic stimulation of the heart rate to ensure faster repolarization.

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$K_v7.2$ and $K_v7.3$ (KCNQ2/3) are mainly found in the nervous system, where they combine to form heteromeric channels that mediate the M-current (150,408). This current received its name because it inactivates upon stimulation of muscarinic receptors. Muscarinic activation of phospholipase C (PLC) leads to depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2), which otherwise binds directly to $K_v7.2/3$ to activate the channel (184,377).

2. Large-conductance Ca^{2+}-activated K^+ channel BK—Large-conductance Ca^{2+}-activated K^+ channels (BK, or "big-K"; also called $K_{Ca}1.1$, maxi-K, or slo) are activated by depolarization; intracellular Ca^{2+} reduces the degree of depolarization required for channel opening (37). The pore is formed by 4 α subunits encoded by a single gene (*slo*) first cloned from the *Drosophila Slowpoke* locus (9). The α subunit sequence is basically homologous to other K^+ channel α subunits with six transmembrane segments but contains an additional transmembrane segment towards the NH_2 terminus, which places the NH_2 terminus on the extracellular side (Fig. 3B). Four homologous genes encode the auxiliary β1–4 subunits, which modulate Ca^{2+} sensitivity, voltage dependency, and gating kinetics to various degrees (37). BK critically contributes to the rapid phase of the afterhyperpolarization that follows action potentials.

BK channel activation can be induced by Ca^{2+} influx through L-type channels (24,319,379), P/Q-type channels (24,319), N-type channels (264,379), and NMDAR (199) but not R-type $Ca_v2.3$ Ca^{2+} channels (24). Biochemical analyses indicate that BK can form physical complexes with L-type $Ca_v1.2$ and $Ca_v1.3$, P/Q-type $Ca_v2.1$, and N-type $Ca_v2.2$ channels (24,148,249). The interaction between $Ca_v1.2$ and BK is stabilized in HEK293 cells by the β_2 AR, which binds to both ion channels (249) as further discussed below [but see the interaction of BK with $Ca_v1.2$ in CHO cells transfected only with the $\alpha_1 1.2$ and BK α subunit, which appeared to be β_2 AR-independent (24)]. These interactions place BK channels within a few nanometers of the Ca^{2+} channel pores ("nanodomains") as required for their highly localized stimulation by Ca^{2+} influx due to their relatively low affinity for Ca^{2+} (10 μm and higher is required for effective BK activation under physiological conditions; Refs. 37,293). Because of this proximity, it would be conceivable that the $Ca_v1.2$-associated signaling molecules including β_2 AR, G_s, AC, and PKA can regulate BK not only indirectly by affecting $Ca_v1.2$ activity but also by directly phosphorylating BK. Interestingly, BK independently assembles itself a similar signaling complex as discussed in the next section.

3. Small-conductance Ca^{2+}-activated K^+ channel SK—Small-conductance Ca^{2+}-activated K^+ channels (SK) are activated by intracellular Ca^{2+} but not voltage (34). The pore-forming subunits are encoded by four different genes. SK1-3 are found in various brain regions, but the related IK1 (SK4) isoform is mainly expressed in peripheral tissue. Similar to other K^+ channels, SK channels consist of four homologous domains, each containing six transmembrane segments and a P loop between segments 5 and 6 (Fig. 3C). The four SK subunits form pairs, each of which firmly binds two CaM molecules (355). During periods of depolarization, voltage-gated Ca^{2+} channels and NMDARs mediate influx of Ca^{2+}, which binds to CaM in the SK complex, thereby causing conformational changes that open the SK channel pore. SK channels thereby mediate slow afterhyperpolarization and spike-frequency adaptation during trains of action potentials in neurons (34).

SK channels form functional units with various Ca^{2+} channels including L-type channels (264) and NMDARs (114,296). SK channels can also be activated by Ca^{2+} influx through P-, N-, and T-type Ca^{2+} channels (106,431). Recent biochemical and physiological experiments provide circumstantial evidence for α-actinin physically linking SK channels in general and specifically SK2 to $Ca_v1.2$ and $Ca_v1.3$ (250; see also Ref. 343). We also observed earlier that SK2 channels coimmunoprecipitate with α-actinin using two different SK2 antibodies (Fig. 4). However, more rigorous studies are required to firmly establish these α-actinin interactions.

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If SK channels are linked to L-type Ca^{2+} channels, signaling molecules associated with $Ca_v1.2$ including the β_2 AR, G_s, AC, and PKA might be shared. Although there is no evidence that this signaling pathway regulates directly SK channels, regulation of SK-coupled $Ca_v1.2$ obviously will translate in altered SK channel activity.

4. Inward rectifying $K_{ir}3$ K^+ channels—The pore-forming α subunits of G protein-gated inward rectifying K^+ channels of the $K_{ir}3$ family are encoded by four different genes, $K_{ir}3.1$-3.4 (formerly GIRK1–4) (223,453). As for other K^+ channels, the gene products can assemble into tetramers in various combinations. However, $K_{ir}3$ and in general $K_{ir}\alpha$ subunits only contain two transmembrane segments, which flank the P-loop, similar to the S5/S6 region of the other K^+ channel α subunits (Fig. 3D). These channels thus lack the voltage-sensing S4 transmembrane segment. $K_{ir}3$ channels are largely activated by direct interactions of $G\beta\gamma$ with the channel (185,328,427). Several studies indicate that G proteins are preassociated with $K_{ir}3$ channels and undergo conformational changes upon activation of G proteins (325,331, 332). Although $G\alpha_i$ does not mediate regulation of $K_{ir}3$ by G proteins, it directly binds to their NH_2 and COOH termini. These findings further support the model that trimeric G proteins are preassociated with $K_{ir}3$. Especially prominent is the function of the K_{ACh} channel in the heart, which is formed by $K_{ir}3.1$ and 3.4. Parasympathetic release of acetylcholine leads to activation of the muscarinic m_2 receptor G_i, and ultimately, via $G\beta\gamma$ released from G_i, K_{ACh}. Activation of this channel reduces cardiac excitability and thereby the heart rate.

III. Regulation of Ion Channels by Protein Kinase A

A. Regulation and Targeting of PKA

PKA is a tetramer consisting of two regulatory (R) and two catalytic (C) subunits. Distinct genes encode four R (RIα,*β*, RIIα,*β*) and three C subunits (*Cα*, *β*, and *γ*). C subunit catalytic activity is suppressed when associated with a homodimeric R core until release by cAMP binding to R (36). RII and to some degree RI dimers are recruited to certain substrates by AKAPs (46,340,433). AKAPs are a structurally diverse family of proteins, which share an amphipathic α helix that binds the R dimer. The Ht31 peptide derived from the RII binding site on the AKAP Ht31 disrupts all tested AKAP-RII interactions and is a powerful tool in delineating the functional importance of PKA anchoring by these AKAPs. Because some AKAPs are lipid-modified or can directly bind to certain phospholipids, earlier thinking assumed that AKAPs are recruited to defined subcellular compartments by their interactions with lipid membranes (52,90,126). However, it is a now well-established notion that AKAPs are more precisely targeted by binding to specific proteins (for early work, see Refs. 79,426).

B. Regulation of Ca^{2+} Channels by PKA

1. $Ca_v1.1$ regulation by PKA—$Ca_v1.1$ is specifically expressed in skeletal muscle. Upon depolarization, $Ca_v1.1$ induces Ca^{2+} release from the sarcoplasmic reticulum (Ca^{2+}-induced Ca^{2+} release) by the type 1 RyR (RyR1) and thereby contraction (excitation-contraction coupling). $Ca_v1.1$ likely activates the RyR1 (green structure in background in Fig. 5) via direct physical interaction by a Ca^{2+}-independent mechanism (276,322,330). However, the increase in contraction force by epinephrine via ARs and cAMP signaling depends on extracellular Ca^{2+} and is, therefore, at least in part due to elevated Ca^{2+} entry through $Ca_v1.1$. Supporting this notion, PKA phosphorylates $\alpha_1 1.1$ and its β subunit and upregulates the activity of $Ca_v1.1$ (52,74,119). Phosphorylation of full-length $\alpha_1 1.1$ (1873 residues) by PKA occurs mostly at serine-1757 and serine 1854 in vitro and in myotubes (281,339). The Ca^{2+}-activated protease calpain removes these sites by cleaving $\alpha_1 1.1$ between residues 1685 and 1699 (84,86,193). The main phosphorylation site of the truncated $\alpha_1 1.1$ is serine-687 in the loop between domains II and III (337) though it is unclear whether this site is actually effectively regulated in intact cells (339). The prevailing β subunit in skeletal muscle is β_1, which is phosphorylated by PKA

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in vitro on serine-182 and threonine-205 (84,342). The phosphorylation sites that regulate channel activity of $Ca_v1.1$ have not been defined on a functional level.

PKA-mediated potentiation of $Ca_v1.1$ activity upon depolarization occurs quickly (in the range of tens of milliseconds) (e.g., Ref. 208). It likely reflects very fast phosphorylation events, which would best be accomplished if PKA would be anchored near $Ca_v1.1$. In fact, the Ht31 peptide, which universally disrupts AKAP-PKA RII interactions (see sect. IIIA), inhibits this rapid potentiation of $Ca_v1.1$ channel activity by PKA in skeletal myotubes (208). AKAP15 [also named AKAP18 (126) or AKAP7 (433)] was identified and cloned as $Ca_v1.1$-associated protein (144,145). AKAP15 interacts with $Ca_v1.1$ by binding to a leucine zipper (LZ) -like motif close to the very COOH terminus of $\alpha_1$1.1 (Table 1). A peptide derived from the LZ-like motif on AKAP15 inhibits depolarization-induced potentiation of $Ca_v1.1$ in skeletal myotubes (192). Collectively these observations indicate that AKAP15 mediates PKA binding to $Ca_v1.1$ and that this interaction is important for fast and effective regulation of the channel activity.

In vitro binding of cAMP to the R-subunit dimer releases and thereby activates the C subunits, which are inhibited when tightly complexed with the R subunits under basal conditions. Unless there are additional anchoring mechanisms for C subunits, this mechanism should lead to a loss of C subunits from the AKAP-RII complexes and phosphorylation of the ultimate target proteins. In fact, there is some evidence that continued stimulation of PKA-dependent phosphorylation ultimately results in a reduction of the potentiation of $Ca_v1.1$ by PKA consistent with the possibility that the PKA C subunit becomes ultimately displaced from the channel-AKAP15-R subunit complex (208). However, C subunits can phosphorylate substrates without complete dissociation from RII subunits upon cAMP addition (404,446). Such an incomplete dissociation mechanism might contribute to anchoring C near its substrates more permanently. RII seems to be anchored by AKAPs to a larger degree than RI isoforms, which releases its C subunit during substrate phosphorylation in vitro (404). Additional work is required to better understand C-subunit behavior upon stimulation by cAMP.

2. $Ca_v1.2$ regulation by PKA

A) Physiological Role of $Ca_v1.2$ and its Regulation by PKA: The regulation of $Ca_v1.2$ has been extensively studied because of its central role in cardiac function. Ca^{2+} influx through $Ca_v1.2$ sparks Ca^{2+} release from the sarcoplasmic reticulum by the type 2 RyR (RyR2) and subsequent contraction in the heart. The increase in heart rate and contractility during the fight-or-flight response is mediated to a substantial degree by β adrenergic stimulation of L-type Ca^{2+} channels (20,309,327) and the RyR2 (263,267). Upon catecholamine binding, β ARs activate the stimulatory heterotrimeric G protein G_s by inducing the exchange of GDP for GTP on $G\alpha_s$. $G\alpha_s$ dissociates from its $G\beta\gamma$ partners to stimulate AC and cAMP production, which in turn activates PKA. Dysregulation of $Ca_v1.2$, RyR2, and thereby Ca^{2+}-induced Ca^{2+} release contributes to the contractile dysfunction in heart failure (140,160,353,395).

Biochemical and functional studies indicate that $Ca_v1.2$ accounts for at least 75–80% of L-type channels in the brain (165,167,221,364,376). In neurons, $Ca_v1.2$ is concentrated at postsynaptic sites of asymmetric dendritic, presumably glutamatergic synapses and at somatic synapses, which are likely GABAergic (78,163,303). The postsynaptic localization of L-type channels is also supported by functional Ca^{2+} imaging studies of dendritic spines (32,178,449).

B) Function and Structure of the Cytosolic Cooh Terminus of $\alpha_1$1.2: Like $\alpha_1$1.1, $\alpha_1$1.2 exists in a long and a short form of ~250 and 220 kDa (164,167). The short form is created in intact neurons by proteolytic cleavage of the COOH terminal region by the Ca^{2+}-activated protease calpain upon Ca^{2+} influx through NMDARs (163) (Fig. 6, black X in COOH terminus). Similar processing of $\alpha_1$1.2 occurs in heart (85,128,129). In vitro phosphorylation of $\alpha_1$1.2 isolated from rat brain is solely detectable in the long but not short form (164,167).

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This observation indicates that the main phosphorylation site of $\alpha_1$1.2 is in its COOH-terminal region, analogous to $\alpha_1$1.1. In fact, at this point, the only observable phosphorylation site of $\alpha_1$1.2 is serine-1928 (85,281,312), which is downstream of the calpain cleavage site (163). This COOH-terminal fragment can remain associated and functionally interacts with the rest of the channel after cleavage (128,135,196). Currents through $\alpha_1$1.2 expressed in HEK293 cells are severalfold higher when the COOH-terminal region is truncated by insertion of stop codons around the predicted calpain cleavage site (128,135,196,278,417). This effect is at least in part due to an increase in coupling efficiency between the voltage sensor and the opening of the channel pore.

Coexpression of COOH terminally truncated $\alpha_1$1.2 with the COOH-terminal 350 residues as an independent polypeptide reverses the disinhibition caused by the truncation of $\alpha_1$1.2; it actually overcompensates, leading to sixfold stronger inhibition compared with full-length $\alpha_1$1.2 (196). Three negatively charged residues near the very COOH terminus (E2103, E2106, D2110) are critical for the COOH-terminal effect (196). These three residues interact with two positively charged residues in the membrane-proximal portion (R1696 and R1697), which are also required for the inhibitory effect of the distal COOH terminus (196). Similarly, injection of a polypeptide covering the very COOH-terminal 144 residues of $\alpha_1$1.2, which contain E2103, E2106, D2110, also reversed the disinhibition otherwise observed when $\alpha_1$1.2 was truncated 147 residues upstream of its COOH terminus (128). Additional evidence for an interaction between the NH_2- and COOH-terminal portion of the full-length COOH terminus of L-type channels comes from the finding that the unique extension of the COOH terminus of $\alpha_1$1.4 by 60 residues abrogates the Ca^{2+}- and CaM-dependent inactivation of Ca_v1.4 and also Ca_v1.3 if added to the $\alpha_1$1.3 COOH terminus (363,406). This is an important mechanism for ensuring long-lasting dark currents by Ca_v1.4, which is only known to be present in photoreceptors and mediates the tonic neurotransmitter release of photoreceptors. Why the mechanism for Ca^{2+}- and CaM-dependent inactivation of $\alpha_1$1.4 has not been directly abrogated remains a puzzle. Perhaps the machinery mediating Ca^{2+}- and CaM-dependent inactivation has additional yet to be discovered functions.

An attractive though currently speculative model is that the interaction of the very COOH terminus with the rest of the channel reduces ion conduction activity and that phosphorylation of serine-1928 (see sect. ıı*B2*ᴄ), which is 249 residues upstream of the COOH terminus of full-length $\alpha_1$1.2, releases this inhibitory interaction. In support of this model, both COOH-terminal truncation of $\alpha_1$1.2 and phosphorylation by PKA, lead to a left shift in the current-voltage curve. Functionally, such a left shift means that the channel opens more effectively at lower depolarization levels when the driving force for Ca^{2+} influx is higher, resulting in currents through the individual channels (128,135,195,196,278,417). Like COOH-terminal truncation, PKA also increases the coupling efficiency between gating and pore opening as indicated by single-channel recording. These recordings show that Ca_v1.2 exists in three main modes. Ca_v1.2 is not available for activation in mode 0, exhibits short frequent openings in mode 1, and long-lasting openings with brief closings in between in mode 2 (173). β AR stimulation leads to transition from mode 0 to mode 1 or mode 2 in frog ventricular cells (20). Thus modifications by PKA and COOH-terminal truncation have comparable effects. It should be noted that earlier reports indicate that the majority of $\alpha_1$1.2 in heart may exist in the cleaved state (85,129). However, when high concentrations of calpain inhibitor I and II and of EGTA are present and all solutions and instruments are precooled to 0°C during rapid extraction procedures, immunoblotting typically shows that ~50% and sometimes more of the detectable $\alpha_1$1.2 is in its long form in extracts from brain (79,81,163,166) and heart (Fig. 7).

C) Role of $\alpha_1$1.2 Serine-1928 in Ca_v1.2 Regulation by PKA: How PKA regulates Ca_v1.2 is not unequivocally established as it proved difficult if not impossible for a number of experienced investigators to reliably and reproducibly reconstitute regulation of Ca_v1.2 in

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heterologous cell lines without injection of exogenous PKA (52,171,278,464). These difficulties proved also true even if AKAP79/150, which recruits PKA to $Ca_v1.2$ (see below) was coexpressed (76) [AKAP79 is the human homolog of AKAP150 but is missing 36 imperfect octapeptide repeats of unknown function that are present in the rodent AKAP150 (340)]. Perhaps additional but largely untested factors such as ahnak, a 700-kDa protein that can associate with $Ca_v1.2$, are important as well (151). In our hands, in 7 of 14 recordings from HEK293 cells transfected to transiently express $Ca_v1.2$ application of the cell-permeable phosphodiesterase resistant cAMP analog *S*p-5,6-dichloro-1-*β*-D-ribofurano-sylbenzimidazole-3′,5′-cyclic monophosphorothioate (DClcBIMPS) lead to a >20% increase in Ca^{2+} currents (Fig. 8). Others made similar observations (M. J. Davis, Univ. of Missouri, Columbia, MO; personal communication). In some cases sequential recording of two cells within the same culture found one cell to be responsive to DClcBIMPS while another one was not. We do not know which factors determine whether DClcBMPS upregulates $Ca_v1.2$ currents. We can only speculate that the cell cycle status or other signaling mechanisms set the level of $Ca_v1.2$ phosphorylation before DClcBIMPS application in our system so that some but not other cells can respond possibly due to near-maximal basal phosphorylation of the relevant PKA site(s).

Nevertheless, it has been reported that currents through $\alpha_1 1.2$ coexpressed with β_{1b} and $\alpha_2\delta$ in the HEK293-derived tsA-201 cells were increased by the membrane-permeable cAMP analog 8-Br-cAMP for wild type but not serine-1928 to alanine mutant $\alpha_1 1.2$ (149) (serine-1928 corresponds to serine-1901 in rat neuronal $\alpha_1 1.2$ investigated in Ref. 149). Others using BHK6 cells stably transfected with β_{1a} and $\alpha_2\delta$ for transient expression of wild-type or serine-1928 to alanine mutant $\alpha_1 1.2$ (288) did not observe an increase in currents through wild-type $Ca_v1.2$ upon stimulation of AC with forskolin but observed a left-shift in the current-voltage curve, which allows $Ca_v1.2$ to open in response to smaller depolarization. This shift is another hallmark of the effect of PKA and was absent in the serine-1928 to alanine mutant in support of a role of serine-1928 in regulation of $Ca_v1.2$ by PKA.

Others expressed $\alpha_1 1.2$ and β_{2a} together with AKAP150 (AKAP5, Ref. 433) in tsA-201 cells and described a potentiation of resulting currents by PKA; this potentiation was absent when serine-1928 in $\alpha_1 1.2$ was mutated to alanine, AKAP150 omitted, or binding of PKA to AKAPs inhibited by the Ht31 peptide (130). In more recent work, HEK293 cells expressing $\alpha_1 1.2$, β_{2b}, and $\alpha_2\delta 1$ were pretreated with forskolin for maximal phosphorylation of $Ca_v1.2$ by PKA to provide equalized starting points before Ca^{2+} currents through these channels were monitored (304). Over time, the Ca^{2+} currents decreased mainly because the incoming Ca^{2+} stimulated the Ca^{2+}-activated serine-threonine phosphatase PP2B, which, like PKA, is also anchored by AKAP79/150 (see sect. VII*C*). No rundown was observed if serine-1928 had been mutated to alanine or the membrane-permeable stearylated Ht31 peptide was present (which disrupts anchoring of PKA but not PP2B by AKAPs) likely because either manipulation prevented any initial upregulation of $Ca_v1.2$ channel activity by forskolin and PKA. These results suggested that serine-1928 as well as PKA anchoring by AKAP79/150 are critical for potentiation of the channel activity by PKA. However, neither Gui et al. (149) nor Naguro et al. (288) (see previous paragraph) ectopically expressed AKAP150. Perhaps the level of endogenous AKAP79 in tsA-201 and other cells varies and was sufficient in the latter two studies. A recent publication reports substantial amounts of endogenous AKAP79 in HEK293 cells used by these authors (133). Whether AKAP150 is present in BHK6 cells is unknown.

It is, therefore, still not firmly established that serine-1928 phosphorylation is largely responsible for the PKA-dependent increase in channel activity. In fact, Ganesan et al. (127a) provided evidence that serine-1928 is not absolutely critical for PKA-mediated upregulation of $Ca_v1.2$ activity. The authors used cardiomyocytes as an endogenous system to express $\alpha_1 1.2$ with two point mutations (T1066Y/E1089M) to make it insensitive to

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dihydropyridines. This elegant strategy allowed them to inhibit endogenous wild-type $Ca_v1.2$ with dihydropyridines and selectively measure currents through the ectopically expressed channel. Stimulation of β ARs with isoproterenol increased the activity of the ectopically expressed T1066Y/E1089M $\alpha_1$1.2 by 50%. When serine-1928 was mutated to alanine in this $\alpha_1$1.2 construct, the increase was 35%. Thus, in this system, serine-1928 may only play a secondary role in β-adrenergic stimulation of $Ca_v1.2$. However, isoproterenol increased the activity of the endogenous L-type channel (measured in the absence of dihydropyridines and without ectopic expression of $\alpha_1$1.2) by >300% rather than 50%. Accordingly, only a small fraction of $Ca_v1.2$ regulation is reconstituted in this system. Serine-1928 phosphorylation might be responsible for a sizable portion of the missing 250% of $Ca_v1.2$ regulation.

Be that as it may, there is no question that serine-1928 is phosphorylated in vivo as a phosphorylation-state specific antibody against the phosphorylated serine-1928 site reacted with the $\alpha_1$1.2 long but not short form isolated from heart (85) and brain (79–81) and showed increased staining of dissociated cardiomyocytes upon stimulation of either β_1 or β_2 AR (195). Quantitative analysis indicates that 16.7 ± 1.8% of $\alpha_1$1.2 is phosphorylated on serine-1928 under basal conditions in rat brain hippocampi (80). This phosphorylation increases to ~40– 60% upon activation of PKA by forskolin application in acutely prepared hippocampal slices (166) or in vivo by administration of isoproterenol (153). This upregulation of serine-1928 phosphorylation is blocked by coadministration of the β AR antagonist propranolol and absent in AKAP150 KO mice in vivo (153) (see NOTE ADDED IN PROOF and Ref. 234a).

D) Role of β_2 Serines-478 and -479 in $Ca_v1.2$ Regulation by PKA: When $\alpha_1$1.2 is expressed alone in COS cells, Ba^{2+} currents through this channel are increased severalfold upon application of the active catalytic subunit of PKA in whole cell or excised inside-out patch recordings. These results show that $\alpha_1$1.2 can be regulated by PKA independent of $\alpha_2\delta$, β, or γ (147,356). However, other evidence suggests that PKA also phosphorylates at least one of the cardiac Ca^{2+} channel β subunits in vitro and in vivo upon β-adrenergic stimulation (152). PKA can phosphorylate serines-459, -478, and -479 in the cardiac β_{2a} subunit (136). These sites are conserved in most other β_2 subunit splice forms but not in β_{2c} or in β_1, β_3, or β_4. PKA increases Ba^{2+} currents through a mutant $\alpha_1$1.2 form that is truncated 265 residues upstream of its natural COOH terminus to eliminate serine-1928 and is expressed together with β_{2a} in tsA-201 cells. Mutating serines-478 and -479, but not -459, to alanines in β_{2a} eliminates this increase. These results suggest that phosphorylation of serine-478 or serine-479 but not serine-459 contributes to PKA-mediated regulation of $Ca_v1.2$ (39) (Fig. 6). As several different β subunits can interact with $\alpha_1$1.2 (24,49,50,99,292,316,418) and other β subunits do not have analogous phosphorylation sites, it appears likely that other phosphorylation sites in $Ca_v1.2$ can mediate PKA-induced upregulation of current activity. Furthermore, given the difficulties with regard to reproducibility of the regulation of $Ca_v1.2$ after ectopic expression in cell lines (see above), additional evidence for the role of serine-478 and -479 phosphorylation in $Ca_v1.2$ regulation is necessary especially in the context of full-length $\alpha_1$1.2 (perhaps with serine-1928 mutated to alanine to accentuate effects that are independent of serine-1928 phosphorylation).

3. The β_2-adrenergic receptor-$Ca_v1.2$ signaling complex—PKA and cAMP had been assumed to diffuse with minimal hindrance throughout the cell. PKA would thereby gain access to most of its substrates. However, the past 10 years revealed that various kinases and phosphatases are anchored at or, upon activation, recruited to many of their substrates for fast, effective, and selective signaling (310). Furthermore, the coexistence of multiple GPCRs that are positively coupled to cAMP production prompted earlier considerations that signaling by these GPCRs may not be purely redundant but rather linked to different signaling pathways (for review, see Ref. 324). Such selectivity would require spatially restricted cAMP signals. Stimulation of either β_1 or β_2 ARs leads to increased Ca^{2+} influx through $Ca_v1.2$ into ventricular

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cardiomyocytes (5,20,441). However, only β_1 but not β_2 AR activation effectively stimulates PKA throughout the myocyte resulting in a global phosphorylation of phospholamban to foster Ca^{2+} sequestration in the sarcoplasmic reticulum, glycogen phosphorylase kinase to regulate glycogen hydrolysis, and troponins I and C to control contraction and relaxation (441). These observations suggest that PKA acts locally at the plasma membrane and specifically near $Ca_v1.2$ upon β_2-AR stimulation but globally upon β_1-AR stimulation. Activation of the glucagon receptor or the prostaglandin E_1 receptor also results in locally restricted cAMP production (334,371). In contrast to β_2 AR activation, which leads to more prominent cAMP/PKA responses at the sarcolemma including a positive inotropic response, prostaglandin E_1 preferably stimulates cytosolic PKA (159,348,416).

Assembly of critical signaling molecules into macromolecular signaling complexes may foster pathway selectivity and localized signaling by cAMP and PKA. $Ca_v1.2$ forms the core of such a complex or signalosome (11,78,79,81,153). Coimmunoprecipitation of functionally active PKA and an AKAP with $Ca_v1.2$ provided initial clues for the existence of a signaling complex assembled around $Ca_v1.2$ (79). A systematic search for additional signaling components upstream of PKA in this complex was further inspired by earlier indications for β_2 AR-regulated localized cAMP signaling in heart (see above; Refs. 371,441). β-AR stimulation with isoproterenol increased serine-1928 phosphorylation of $Ca_v1.2$ in vivo in the rat and mouse brain by more than twofold; this effect is prevented by co-administration of the β-AR antagonist propranolol (153). In dissociated cardiomyocytes, stimulation of either the β_1 AR or β_2 AR increased serine-1928 phosphorylation (195). Coimmunoprecipitation studies from rat brain and heart revealed that the β_2 AR, G_s, and AC are constitutively associated with $Ca_v1.2$ (11, 78). The existence of a β_2 AR-$Ca_v1.2$ signaling complex was further supported by immunofluorescence colocalization of the β_2 AR with $Ca_v1.2$ at postsynaptic sites in the brain (78). In vitro pull-down experiments with bacterially expressed fusion proteins indicate that the COOH terminus of β_2 AR directly binds to $\alpha_1$1.2 (78) (Fig. 6). How G_s and AC are linked to $Ca_v1.2$ is unclear. However, AKAP150 selectively associates with AC V and VI (17). This interaction could recruit one or both AC isoforms to $Ca_v1.2$.

A) Localized Signaling from the β_2 AR to $Ca_v1.2$: The assembly of the β_2 AR-$Ca_v1.2$ signaling complex might foster localized cAMP signaling. During cell-attached patch-clamp recording from somata of hippocampal pyramidal neurons in culture with Ba^{2+} as charge carrier, application of the β_2 AR-selective agonist albuterol resulted in a more than twofold increase in L-type-mediated current when applied inside, but not outside, the recording pipette electrode (78). Analogous findings were obtained for localized $Ca_v1.2$ regulation by β_2-AR stimulation in cardiomyocytes (63). This stimulation is mediated by PKA as PKA inhibitors block β_2-adrenergic upregulation of L-type currents in cardiomyocytes (5,440,441,462) and neurons (178). The requirement for localized β_2-AR stimulation suggests that cAMP signaling can be restricted to submicrometer dimensions. Synthesis of cAMP occurs presumably in the immediate surrounding outside the pipette when albuterol is added, as the β_2 AR-$Ca_v1.2$ complex is distributed throughout the plasma membrane in these neurons (79). The wall of the pipette used for the cell-attached patch recordings is typically several hundred nanometers thick. The plasma membrane usually forms an Ω-shaped structure inside the tip of the patch pipette with the narrow neck of the Ω-structure closely attached to the glass wall inside the pipette for a few hundred nanometers. The lack of channel potentiation by albuterol applied outside the pipette suggests that cAMP does not reach concentrations that are high enough to stimulate PKA-mediated phosphorylation of $Ca_v1.2$ channels inside the patch, which are only several hundred nanometers away from the β_2 AR outside the electrode. Accordingly, cAMP signaling is restricted to an area of <1 μm around the β_2 AR.

Electrophysiological studies also provide evidence that signaling by the β_1 AR in cardiomyocytes and neurons is much less locally restricted than β_2 AR signaling (63,78). These

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findings support the earlier biochemical studies mentioned above (371,441) that demonstrated a more local signaling by β_2 AR versus a more global one by β_1-AR stimulation. Fluorescence resonance energy transfer imaging provided further support for the idea that cAMP is not freely distributing throughout cardiomyocytes but rather concentrating at the Z-line level upon β-adrenergic stimulation (455). The observed gradient, however, was exacerbated in these studies by the overexpression of the fluorescent RII-derived cAMP sensor, as PKA itself can buffer cAMP concentrations (348), and this cAMP sensor itself was preferably localized to membrane-associated AKAPs (416).

B) Spatial Restriction of Camp Signaling: ***I) Spatial restriction of cAMP by G_i:*** The β_2 AR can couple not only to G_s but also to G_i, especially upon prolonged stimulation (8,371,440, 441). At least in fibroblast cell lines, this switch can be induced by PKA-mediated phosphorylation of the β_2 AR (75,456). The β_2 AR can thus limit cAMP production by inhibiting AC after switching from coupling to G_s to coupling to G_i. This mechanism appears as an effective means to temporally restrict cAMP production.

II) Spatial restriction of cAMP by AC: A second such mechanism is based on the inhibition of AC V and VI by PKA (60,204). AC V and VI themselves can associate with AKAP150 (17). When AC V and the tonically active $G\alpha_s$ mutant Q227L are coexpressed in HEK293 cells, coexpression of AKAP79 induces AC V phosphorylation by PKA and reduces cAMP production (17). S676 in AC V is homologous to S674 in AC VI, which is critical for inhibition by PKA (60). Mutating S676 to alanine prevents the reduction in cAMP production induced by AKAP79 overexpression in this system (17). This regulation creates a negative feedback for cAMP production. Although it is unknown which ACs are present in the β_2 AR-$Ca_v1.2$ complex, AC V and VI could be associated with the $Ca_v1.2$ complex via AKAP150 (17) (see sect. IIIB4A), and their phosphorylation by PKA could provide such inhibitor feedback.

III) Spatial restriction of cAMP by phosphodiesterase: A third mechanism that can restrict the effective radius of cAMP is its hydrolysis by phosphodiesterases (PDEs) that compartmentalize cAMP in cardiomyocytes (159,210,234,334,335,348,371,440,441). More specifically, PDE inhibitors delocalize spatially restricted β_2-adrenergic regulation of $Ca_v1.2$ (176,210). Although evenly distributed PDEs would be sufficient to reduce the lifetime of cAMP and thereby its effective radius, some PDEs associate with AKAPs for localized reduction of cAMP (22) (see next paragraph). Recruitment of different PDE4D isoforms to activated β_2 ARs via β-arrestin is yet another mechanism that can contribute to reduced cAMP signaling (10,314). In general, β-arrestin binds to the β_2 AR upon receptor stimulation. It is not known whether (β-arrestin also associates with the β_2 AR in the $Ca_v1.2$ complex, but if so, this mechanism would further contribute to the spatiotemporal restriction of cAMP signaling in the vicinity of such a complex.

Both cGMP-inhibited cAMP PDE3 and cAMP-specific PDE4 isoforms can contribute to spatially restricted cAMP signaling (334). Certain GPCRs including the α_1 AR regulate cAMP degradation by stimulating specific PDEs (371) for localized cAMP signaling. Furthermore, PDE4D selectively counteracts upregulation of the cardiac contraction rate by the β_2 but not β_1 AR via PKA (437). The abundant PDE4D3 isoform is recruited to various subcellular compartments by directly or indirectly binding to mAKAP (95,96), AKAP350 (AKAP9) (386), and gravin [also designated AKAP250 and AKAP12 (433)] (429). These AKAPs act in part to assemble PKA and PDE4D into a complex in which localized phosphorylation of PDE4D by PKA results in its stimulation (71,96). Gravin can directly bind to the COOH terminus of the β_2 AR, which is important for agonist-induced internalization of the β_2 AR as well as recovery of the β_2 AR from this desensitization (116,244,360). This binding requires phosphorylation of the PKA binding domain of gravin itself by the anchored PKA (385). How the gravin-PKA complex promotes recycling of internalized β_2 ARs to the plasma membrane

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is unclear. This mechanism might be analogous to the β_1 AR, which requires association of the AKAP150-PKA complex with its COOH terminus for recycling. In this latter case, AKAP150-anchored PKA phosphorylates S312, which is required for receptor recycling (132,133). The analogy between gravin and AKAP150 is especially noticeable as these two AKAPs share a number of features including anchoring of PKC and PP2B in addition to PKA and binding to negatively charged phospholipids via three positively charged segments in their NH_2-terminal regions, which is antagonized by CaM in the presence of Ca^{2+} (90,141,384, 409). Whether PDE4D or another PDE binds to the β_2 AR-$Ca_v1.2$ complex via gravin, AKAP150, or, upon β_2-AR activation, β-arrestin is untested.

mAKAP also binds PDE4D (95) (see sect. IIIB4D). More specifically, it interacts with PDE4D3 but not PDE4D5, two of nine splice variants encoded by the PDE4D gene. In PDE4D knockout mice, isoproterenol-induced cAMP accumulation is increased in the heart with no change in total cAMP under resting conditions. Furthermore, PKA phosphorylates serine-54 of PDE4D3, which increases its hydrolytic activity, thereby reducing cAMP in the vicinity of this complex (358). PKA also phosphorylates serine-13 in the binding site of PDE4D3 for mAKAP. This phosphorylation increases the PDE4D3-mAKAP interaction, thereby increasing the presence of PDE4D3 in mAKAP complexes (45). The extracellular signal-regulated kinase ERK5 and potentially ERK2 are associated with the mAKAP complex via binding to a KIM and a FQF docking site upstream and downstream of serine-579 on PDE4D3, respectively (95,253). Both ERKs phosphorylate PDE4D3 on serine-579, which reduces its PDE activity (95,253). ERK5 in turn is downregulated by cAMP via the cAMP effector Epac1 but not PKA (95).

It is unclear how cAMP is targeted to the $Ca_v1.2$-associated PKA rather than diffusing away from the complex. Analogous to substrate channeling in certain metabolic enzyme complexes, we therefore proposed that analogous channeling could occur for cAMP from the $Ca_v1.2$-associated AC to PKA (155). This is the more conceivable as AC V or VI might be rather closely localized to PKA in the $Ca_v1.2$ complex due to a direct link by AKAP150. It is also worth considering in this context that one of the three AKAP150 attachment sites on $\alpha_1$1.2 is ~150 residues downstream of the main PKA phosphorylation site, serine-1928 (153,304). Accordingly, the COOH-terminal portion of $\alpha_1$1.2 together with AKAP150 could bring AC V or VI and PKA into close proximity to each other and to the PKA substrate site.

C) Association of Trimeric G Proteins with Signaling Complexes: Rodbell and co-workers provided early evidence for G proteins being part of large signaling complexes based on radiation inactivation and other methods (e.g., Ref. 350). Kinetic studies also argue that G proteins may remain associated with their immediate effectors (e.g., AC, Ref. 239) or their receptors (e.g., G_q interactions with the muscarinic M_1 receptor, perhaps in conjunction with PLCβ_1,Ref. 28) beyond the brief encounters that were postulated by the original collision-coupling model. Live interaction studies using bioluminescence resonance energy transfer (BRET) studies provide further evidence for the notion that G protein-GPCR interactions exist for an extended time period (127,300), including G protein interactions with the β_2 AR. Also, an increasing body of evidence indicates that GPCRs associate with trimeric G proteins in the early secretory pathway (103,104).

Many cells have a variety of G$\beta\gamma$ heterodimers, which act as pathway-selective signal transducers in conjunction with specific Gα subunits (see Refs. 294, 324 and citations therein). For example, muscarinic M_4 and somatostatin receptors inhibit voltage-gated Ca^{2+} channels in GH3 cells through $G\alpha_{o1}\beta_3\gamma_4$ and $G\alpha_{o2}\beta_1\gamma_3$, respectively (see Ref. 220 and citations therein). It is difficult to envision how this selectivity could be maintained if Gα and G$\beta\gamma$ completely dissociate from each other and from their cognate receptors upon stimulus-induced GDP/GTP exchange of Gα find all their binding partners including their cognate receptors and targets, and, after hydrolysis of GTP on Gα to GDP find each other again by random collision. Because

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different Gα subunits have similar affinities for most G$\beta\gamma$ dimers in vitro, there must be mechanisms in vivo that ensure reassociation of the original combinations. Analogously, different G$\beta\gamma$ complexes can activate the inward-rectifying muscarinic K^+ channel ($K_{ir}3$/GIRK; activated by m_2 muscarinic receptors) (185, 225, 328, 427). Biochemical and biophysical evidence indicate that not only G$\beta\gamma$ but also Gα can stably interact with $K_{ir}3$, further supporting the notion that heterotrimeric G_i can form a quite steady complex with $K_{ir}3$ (185, 203, 298, 325). It should be noted, however, that binding of Gα_i to $K_{ir}3$ reflects at least in part an additional direct regulatory mechanism of $K_{ir}3$ channel activity by Gα_i (311).

Kinetic and biochemical evidence indicates that both Gα_s and G$\beta\gamma$ are rather stably associated with its effector AC independent of their activation status (239,261; see also Ref. 419). More recent FRET and BRET studies suggest that activation of G_i by coexpressed α_{2A} AR upon agonist application does not lead to dissociation of Gα_i from G$\beta\gamma$ but rather to a rearrangement of the trimeric complex (38,124,127). Similarly, total internal reflection fluorescence combined with FRET indicate that heterotrimeric G proteins are preassociated with $K_{ir}3.1/3.4$ channels at the cell surface and undergo a conformational change upon activation by an upstream $G_{o/i}$-linked GPCR (331,332).

The members of the K^+ channel $K_{ir}3$ family are activated by direct binding of G$\beta\gamma$ to the NH_2 and COOH termini of their subunits (27,67,202,225). Selectivity for G$\beta\gamma$ appears to be minimal, but Gα_{i2} and Gα_{i3} seem to be the preferred donors of G$\beta\gamma$. This selectivity for the Gα subunit may be in part due to direct binding of Gα_{i2} and Gα_{i3} to $K_{ir}3$ subunits (203,311), which may lead to preassociation of trimeric G proteins with these two subunits and thereby to effective and selective signal transduction by those G proteins. It is tempting to speculate that a similar direct interaction recruits G_s to $Ca_v1.2$, although the stable association of G_s could equally well be due to constitutive binding of G_s to other proteins in the $Ca_v1.2$ complex including the β_2 AR or AC.

D) Caveolae and Membrane Rafts as Platforms for Signaling Complexes: An alternative for the assembly of signaling complexes at the plasma membrane by direct protein-protein interactions is the colocalization within distinct membrane domains such as Triton X-100-insoluble rafts, caveolae, or postsynaptic density fractions. Caveolae and postsynaptic densities may not be a structural requirement for the β_2 AR-$Ca_v1.2$ complex as respective marker proteins are undetectable in this complex after Triton X-100 solubilization and immunopurification from rodent brain (78). However, cardiac β_2 AR and $Ca_v1.2$ colocalize by immunofluorescence microscopy and co-fractionate during sucrose density gradient centrifugation with caveolin-3 (11). In fact, caveolin-3 coimmunoprecipitates with the β_2 AR-G_s-AC-PKA-$Ca_v1.2$ complex from heart. Furthermore, disruption of caveolae by depletion of cholesterol with methyl β-cyclodextrin and siRNA knockdown of caveolin-3 abrogated β_2-adrenergic upregulation of L-type current in mouse ventricular myocytes without affecting β_1-adrenergic upregulation (11). These observations indicate that caveolin-3 and more generally caveolae fulfill in some cells a critical supportive role in either the formation of the β_2 AR-$Ca_v1.2$ complex or its colocalization with other signaling or structural components.

4. Role of AKAPs in regulating $Ca_v1.2$ and colocalized ion channels

A) Role of Akap in the Regulation of $Ca_v1.2$ by Pka: The first AKAP that was found to be associated with $Ca_v1.2$ was MAP2B (79), a microtubule-associated protein known to bind PKA (400). It directly binds to three different site of the $\alpha_1 1.2$ subunit (153). However, the question of whether MAP2B mediates PKA binding to $Ca_v1.2$ proved difficult to answer because heterologous overexpression of MAP2B is detrimental to cells due to its strong microtubule bundling effect, because $Ca_v1.2$ and MAP2B are large proteins with at least three interaction sites, and because of other technical issues (Davare, Dong, Rubin, and Hell, unpublished

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observations). Like $Ca_v1.2$, MAP2B had been detected in dendritic spines (40), but it is mainly localized in dendritic shafts and is not a prevalent AKAP in the spines.

In contrast to MAP2B, AKAP150 is enriched at postsynaptic sites, perhaps in part due to its interactions with the structural postsynaptic protein PSD-95 (which interacts with AMPA receptors via stargazing/γ_2; see sect. II*A*) and its homolog SAP97 (which directly binds to the AMPAR GluR1 subunit) (41,70,141,236,388). Furthermore, according to an earlier report (130) (see sect. III*B2*c), PKA-mediated potentiation of $Ca_v1.2$ expressed in heterologous tsA-201 cells also required coexpression of AKAP150 (see also Ref. 304). Although AKAP79/150 was initially not detectable in coimmunoprecipitation experiments with $Ca_v1.2$ despite repeated efforts (79; Davare and Hell, data not shown), AKAP150 reproducibly coprecipitates with $Ca_v1.2$ from brain when 150 mM NaCl is added to the solubilization buffer (153; see also Ref. 304). Furthermore, coimmunoprecipitation of PKA with $Ca_v1.2$ from brain extracts is drastically reduced but not absent in AKAP150 knockout mice. Isoproterenol-induced phosphorylation of serine-1928 is eliminated in AKAP150 knockout mice in vivo (153). These findings indicate that AKAP150 is the major but not only AKAP that recruits PKA to neuronal $Ca_v1.2$ complexes.

MAP2B shows little if any expression outside the nervous system. In contrast, AKAP15 (see set. III*B1*) is abundant in heart, where it coimmunoprecipitates and colocalizes with $Ca_v1.2$ (194). Furthermore, Ca^{2+} currents through $Ca_v1.2$ are increased upon PKA activation when $Ca_v1.2$ is coexpressed with AKAP15 in tsA-201 cells, and this increase is not observed when AKAP15 is omitted (126). Finally, a peptide derived from the leucine-like zipper motif in AKAP15 that mediates its binding to $Ca_v1.1$ and inhibits upregulation of $Ca_v1.1$ by PKA (see sect. III*B1*) also blocks β-adrenergic stimulation of L-type Ca^{2+} currents in cardiomyocytes (194). Although this peptide may prevent not only AKAP15 but also other AKAPs from binding to $Ca_v1.2$ and the AKAP15-dependent increase in $Ca_v1.2$ activity in tsA-201 cells is rather modest (18%) (126), collectively these data suggested that AKAP15 recruits PKA to at least a sizeable $Ca_v1.2$ population for fast and efficient signaling in the heart. Because AKAP15 is present in brain and specifically in dendrites, it may help recruit PKA to a certain neuronal $Ca_v1.2$ population as well (194).

AKAP79/150 was thought to be absent or of low abundance in the heart. However, there is a detectable AKAP150 pool in cardiac tissue, and AKAP150 coimmunoprecipitates with $Ca_v1.2$ from cardiac extracts (Fig. 7). It thus could contribute to PKA anchoring in the heart. Furthermore, one of the three AKAP150 binding sites on $\alpha_1 1.2$ lies within the last 125 residues (153), which contain the LZ-like motif that anchors AKAP15 (194). Disrupting this motif with point mutations reduces the interaction between $\alpha_1 1.2$ and AKAP150 and inhibits regulation of $Ca_v1.2$ by PKA (304). The peptide derived from the LZ-like motif in AKAP15 as described in Reference 194 might thus also affect the AKAP150-$\alpha_1 1.2$ interaction.

AKAP150 binds not only to $\alpha_1 1.2$ but also the cytosolic COOH terminus and to a lesser degree the third intracellular loop of the β_2 AR (Fig. 9) (125). AKAP150 could, therefore, fulfill an auxiliary role in the association of the β_2 AR with $Ca_v1.2$. However, earlier evidence clearly shows also a direct interaction between the COOH termini of the β_2 AR and $Ca_v1.2$ (78).

Finally, AKAP79 promotes cell surface expression of $Ca_v1.2$ in HEK293 cells (3). Although loop II/III, the intracellular connection between domains II and III, of $\alpha_1 1.2$ was implicated in this effect, there is actually no evidence that AKAP150 binds directly to this loop. Rather, AKAP150 interacts with the relatively short NH_2 terminus, the loop I/II, and the distal COOH terminus of $\alpha_1 1.2$ (153) (Fig. 6). Intriguingly, MAP2B shows the same interaction pattern (153). It is unclear whether AKAP150 regulates $Ca_v1.2$ surface expression reported in Reference 3 by signaling via its binding partners PKA, PKC, or PP2B or by other means.

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AKAP150 binds with its NH_2-terminal region not only PKC but also PIP_2, F-actin, and cadherin, and might through these interactions foster $Ca_V1.2$ surface expression (90,141,142). It is also conceivable that it stabilizes a certain conformation of $\alpha_1$1.2 by binding to its three different attachment sites. Although it is unclear whether AKAP150 simultaneously binds to two or three sites, such interactions could regulate the overall structure of $\alpha_1$1.2. If AKAP150 concurrently interacts with the distal COOH terminus and one of the other two sites, it could stabilize the association of the distal COOH terminus with the rest of the channel after cleavage of the COOH terminus as discussed in section III*B2*B.

B) Role of Akap79/150 in Regulating the Ampar Glur1 Subunit: $Ca_V1.2$, AMPAR, and NMDAR are coclustered if not intermingled at postsynaptic sites yet contain their own AKAPs (Fig. 10). This molecular organization suggests once more that PKA is specifically targeted to individual channel complexes for phosphorylating defined substrates with high specificity and spatial restriction well below the dimensions of postsynanptic sites, which are formed by dendritic spines that are ~1 μm diameter, reaching dimensions of the size and distances of individual channel complexes (a few nanometers). For this reason, postsynaptic PKA anchoring to AMPAR and NMDAR will be described in more detail.

AMPARs consist of four homologous subunits encoded by four different genes (GluR1-4), with GluR1/R2 and GluR2/R3 being the prevalent combinations in adult rodent cortex and hippocampus. Each subunit contains a large extracellular NH_2-terminal domain, a transmembrane segment M1 that is followed by a reentry loop M2 and two other transmembrane segments M3 and M4, and the intracellular COOH terminus (Fig. 10). The NH_2 terminus forms the clamshell-like glutamate binding site together with the extracellular loop between M2 and M3. In addition, AMPARs associate with stargazing/γ_2 and its homologs γ_3, γ_4, and γ_8 (see sect. II*A*), which are necessary for surface expression and synaptic clustering (57), the latter depending on PSD-95 (351). They also promote and prolong ligand-induced opening of the receptor channel (280,297,321,396) and have been named TARPs (397). Recently, γ_7 was also shown to act as TARP (217). γ_2 was originally identified as stargazin, which is defective in the stargazer mouse. γ_2 is the only TARP expressed in the cerebellum, and the resulting cerebellar ataxia is due to the absence of surface expression of AMPARs in the cerebellum. Other brain regions express other members of the γ_2 subfamily facilitating proper synaptic AMPAR expression (397).

AKAP150 binds to the SH3 domains of PSD-95 and SAP97 (70,388). PSD-95 and its homologs SAP97, SAP102, and PSD-93/CHAPSYN110 consist of three PDZ domains, which typically bind to the very COOH termini of certain proteins, followed by an atypical SH3 domain and a GK domain, the latter two resembling Ca^{2+} channels β subunits (see sect. II*A*). PSD-95 interacts with the COOH termini of TARPs including stargazing/γ_2. As mentioned above, SAP97 directly binds to the COOH terminus of the AMPAR GluR1 subunit (41,70,141,236, 388).

Reconstitution of GluR1 regulation by PKA in HEK293 cells requires coexpression of AKAP79 (388). Accordingly, AKAP79/150 targets individually PKA to AMPAR and $Ca_V1.2$ even if the different channel complexes are in close proximity to each other. AKAP79/150 also interacts with PKC and PP2B. AKAP79/150-anchored PP2B counterbalances PKA-mediated stimulation of GluR1 channel activity (68,388) (see also sect. VII*A*). Anchoring of PKC via AKAP79/150 (219) is required for effective phosphorylation of GluR1 at serine-831 by PKC (387), which can otherwise also be phosphorylated by CaMKII (260).

C) Role of Yotiao in Regulating NMDAR: NMDARs consist of four subunits that are homologous to AMPAR subunits (see sect. III*B4*B) with an extracellular NH_2 terminus, four membrane segments including the M2 reentry loop, and an intracellular COOH terminus. All

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NMDAR contain two NR1 subunits, which bind the coagonist glycine rather than glutamate, and two glutamate-binding NR2 subunits encoded by four different genes (NR2A-D, with NR2A and -2B being most common in the cortex and hippocampus). Yotiao, a ~230-kDa splice form of AKAP350/450 (AKAP9), binds to the C1 cassette in the NR1 COOH terminus and links PKA and also the counteracting phosphatase PP1 physically and functionally to the NMDAR at postsynaptic sites (426). $Ca_V1.2$, which is localized in close proximity to postsynaptic NMDARs, does not coimmunoprecipitate Yotiao (153). The COOH termini of NR2 subunits bind to the first two PDZ domains of PSD-95, which can interact with AKAP150 (see sect. ιιι*B4*ʙ). However, it is unclear whether PSD-95 recruits AKAP150 to the NMDAR.

D) Role of Makap in Regulating the Ryanodine Receptor RyR2: The cardiac RyR (RyR2) is in close proximity and functionally coupled to $Ca_V1.2$ in cardiomyocytes but associates with PKA via its own AKAP. Localized regulation of the RyR2 versus $Ca_V1.2$ will therefore be discussed in more detail. Three genes encode RyR1–3. RyR1 is mainly expressed in skeletal muscle, where it is linked to $Ca_V1.1$ (see sect. ιιι*B1* and Fig. 5). RyR2 is the RyR in the heart, which releases Ca^{2+} upon a modest amount of Ca^{2+} influx through juxtaposed $Ca_V1.2$ (Ca^{2+}-induced Ca^{2+} release). All three RyR are ~5,000 residues long and consist of a very large cytosolic foot domain, four transmembrane segments, and a short cytosolic COOH terminus (Fig. 5).

RyR2 associates with mAKAP via a LZ-like motif in the foot domain (residues 3003–3039; Table 1) (266). mAKAP was originally named AKAP100 (269) but later renamed mAKAP after full-length cDNA was obtained due to its expression in muscle (216). More recently, it was also designated as AKAP6 (433). Like RyR2, mAKAP is enriched at the Z-line level at the t-tubule/junctional sarcoplasmic reticulum (443). Because RyRs and $Ca_V1.2$ are juxtaposed, PKA anchored via mAKAP to RyR2 would be in close proximity to $Ca_V1.2$; however, mAKAP does not coimmunoprecipitate with $Ca_V1.2$, at least not from brain, where RyR2 and mAKAP are also expressed at substantial levels (153) (Davare and Hell, data not shown). That PKA is linked to the RyR2 and $Ca_V1.2$ by different AKAPs suggests once more that within a radius that is likely substantially <100 nm, AKAP anchored PKA cannot effectively reach neighboring protein complexes. In fact, the identified AKAP binding sites on $Ca_V1.1$, $Ca_V1.2$, RyR1, RyR2, and type 1 IP_3 receptors (IP_3R1) are quite close to identified PKA phosphorylation sites on these different ion channels (Table 1), suggesting the requirement for a rather limited interaction range for PKA, its AKAPs, and its targets.

mAKAP also binds the PDE4D splice isoform PDE4D3 (see sect. ιιι*B3*ʙ*III*) (95). As mAKAP binds to the cardiac RyR2, it might recruit PDE4D3 to the RyR2 (234). In PDE4D knockout mice phosphorylation of serine-2808, the PKA site of the RyR2, is elevated. PDE4D association with the RyR2 is reduced and serine-2808 phosphorylation increased in human heart failure. Serine-2808 phosphorylation increases the open probability of RyR2 possibly because it destabilizes binding of FKBP12.6 (calstabin2) to the RyR2, which renders the RyR leaky, thereby contributing to heart failure (266,267; but see Ref. 438). Binding of PDE4D3 to RyR2 brings this PDE in close proximity of $Ca_V1.2$ in cardiomyocytes. RyR2-anchored PDE4D3 thus could contribute to locally restricting cAMP in a manner that affects not only the RyR2 but also $Ca_V1.2$.

C. Regulation of Na^+ Channels by PKA

PKA and PKC synergistically regulate neuronal Na^+ channel activity by reducing peak current and enhancing slow inactivation (48,61,77,241). PKA phosphorylates four serine residues in loop I/II of the $Na_V1.2$ α1.2 subunit (Fig. 11), the predominant Na^+ channel in forebrain (287). PKC phosphorylates serine-1506 of α1.2 near the inactivation gate in loop III/IV (241, 423). Na^+ channels interact with PKA via AKAP15 (394). Regulation by PKA requires

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anchoring via AKAP15 (42). AKAP15 in turn binds to the NH_2-terminal segment of loop I/II in α1.2 (residues 446– 453) via a modified LZ motif (Fig. 11) (43,118). This motif is shorter than established for other LZ motifs and contains a phenylalanine in the first position, which is canonically occupied by leucine. This motif is well conserved among all nine Na^+ channel α subunits. A peptide derived from the LZ of AKAP15 prevents downregulation of Na^+ channel peak currents upon stimulation of the dopamine $D_{1/5}$ receptor in hippocampal neurons (118).

D. The Yotiao-K_v7.1 Complex and β_2-AR Regulation

PKA is recruited to K_v7.1 via the AKAP Yotiao (265). Yotiao binds to the LZ motif in the COOH terminus of K_v7.1 that is formed by residues 588–616 (Fig. 12). PKA anchoring by Yotiao is critical for phosphorylation of serine-27 in K_v7.1, which leads to increased channel activity (265). The PP1/PP2A blocker okadaic acid enhances this increase presumably because in its absence Yotiao-anchored PP1 rapidly reverses serine-27 phosphorylation.

In addition to its role in PKA anchoring, binding of Yotiao itself increases K_v7.1 currents, but only if serine-27 is phosphorylated or replaced with the phosphomimetic aspartate (58,227). Although Yotiao binding per se mediates such an increase independent of PKA or PKC activity (227), phosphorylation of serine-43 of Yotiao magnified the increase without altering binding of Yotiao to the channel or its phosphorylation on serine-27 by Yotiao-anchored PKA (58).

Two different binding sites in Yotiao for K_v7.1 have been identified, one near its NH_2 terminus (residues 29–46) and the other one near its COOH terminus (residues 1574–1643) (59). A missense mutation in the COOH-terminal binding site (S1570L) in a subpopulation of humans with long Q-T syndrome reduces binding of Yotiao to K_v7.1 and cAMP-induced phosphorylation and regulation of K_v7.1 (59). AKAP150 also interacts with K_v7 channels but appear mainly to function in anchoring PKC rather than PKA to these channels (see sect. IV*D*).

I_{Ks} slow K^+ conductance currents are upregulated upon β-adrenergic stimulation. FRET between secondary antibodies labeling K_v7.1 and the β_2 AR indicates close proximity of those two proteins in cardiomyocytes (94). Interestingly, overexpression of the β_2 AR increases phosphorylation and channel activity of K_v7.1 independent of agonist stimulation of the β_2 AR (94). Overexpression of the β_2 AR did not increase L-type channel Ca_v1.2 activity under basal conditions. It appears that the β_2 AR is especially intimately associated with K_v7.1 so that signaling from the receptor to the channel is spatially restricted and does not translate into stimulation of Ca_v1.2. A lack of effect on Ca_v1.2 is puzzling, however, as the β_2 AR is structurally and functionally closely associated with Ca_v1.2 (see sect. III*B3*). Perhaps the β_2 AR behaves differently when associated with the K_v7.1 versus Ca_v1.2 complex.

E. The PKA-β_2 AR-BK Complex

1. PKA binding to BK—PKA activity can either increase or decrease BK channel activity depending on the splice isoform of BK. PKA increases the activity of BK channels (224) that lack the exon STREX-1 at splice site 2 by direct phosphorylation of serine-869 (serine-899 in some isoforms) in the COOH terminus of the α subunit (Fig. 13) (290,393).

PKA-mediated phosphorylation of the serine in position 4 of STREX-1 leads to BK channel inhibition independent of the presence of serine-869 (393). Whereas an increase in channel activity requires phosphorylation of all four serine-869 residues in the tetrameric channel, phopshorylation of a single serine residue in position 4 of the STREX-1 insert is sufficient to reduce channel activity (392).

Functional studies of native BK channels reconstituted into lipid bilayers or in membrane patches excised from BK-transfected *Xenopus* oocytes and HEK293 cells provided initial

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evidence for a tight association of PKA with BK (65,112,393). Subsequent biochemical studies indicate that the catalytic C subunit of PKA by itself but not when associated with regulatory R subunits or with the inhibitory PKI peptide can directly bind to the COOH terminus of the *Drosophila* BK α subunit dSlo (410,461). Residues 922–956 in the BK COOH terminus are required for this binding and contain the PKA phosphorylation site serine-942, which is homologous to serine-869 in mammalian BK. Several experiments indicate that the association of the PKA C subunit with the BK COOH terminus is not simply mediated by a catalytic site-substrate interaction (461).

Direct binding of free PKA C subunit to dSlo is different from PKA targeting via AKAPs. In mammalian BK channels, one of two LZ-like motifs in the COOH terminus of BK (LZ1 but not LZ2 further COOH terminal) is required for coimmunoprecipitation of PKA with BK (391). LZ1 is also necessary for PKA-mediated upregulation of channel activity of STREX-1-lacking BK as well as for downregulation of STREX-1-containing BK (391). Although LZ-like motifs can anchor a number of different AKAPs (see above), regulation of BK by PKA is not inhibited by the Ht31 peptide, which blocks signaling by AKAP-anchored PKA by displacing PKA from the different AKAPs in numerous other systems (391). In contrast to dSlo (see previous paragraph), no interaction of the region surrounding serine-869/899 of mammalian BK with PKA C subunit was detectable by pull-down experiments (391). It appears that an unknown adaptor protein that interacts with LZ1 is required for functionally recruiting PKA holoenzyme to the channel complex.

2. The interaction of the β_2 AR with BK—The β_2 AR and BK coimmunoprecipitate from rat brain and colocalize in neurons (249). The β_2 AR directly binds with its third intracellular loop (i3) to the rat BK α subunit (Fig. 13) (249). AKAP150, which can directly interact with the β_2 AR (125), is recruited to the β_2 AR-BK complex through its interaction with the β_2 AR (249). At first glance, the β_2 AR/AKAP150 complex could be the missing link that connects PKA to BK, but in this case, BK regulation by PKA should be Ht31 sensitive. Although this interaction could contribute to BK regulation, an alternative PKA anchoring mechanism remains sufficient.

The main binding region for AKAP150 in the β_2 AR is its cytosolic COOH terminus, which binds to the NH_2-terminal half of AKAP150 (Fig. 9). This arrangement can easily accommodate simultaneous binding of the β_2 AR loop i3 to BK (Fig. 13). In cell-attached recordings, the β_2-selective agonist salbutamol increased BK channel activity largely only if both the β_2 AR and the AKAP-150 homolog AKAP79 were coexpressed in *Xenopus* oocytes (249). Importantly, bath application of salbutamol increased BK activity to a significantly lesser degree. These findings indicate that signaling from the β_2 AR to BK via cAMP/PKA is restricted to domains in the range of several hundred nanometers and possibly less, similar to signaling from the β_2 AR to $Ca_v1.2$ (see above and Refs. 63,78).

IV. Regulation of Ion Channels by Protein Kinase C

A. Regulation and Targeting of PKC

Stimulation of G_q-coupled receptors leads to activation of PLCγ. This enzyme cleavages PIP_2, generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 activates IP_3Rs, thereby inducing Ca^{2+} release from internal stores. Diacylglycerol can either alone or in conjunction with Ca^{2+} stimulate most PKC isoforms. Ten different PKC genes have been identified and their protein products divided into three different classes (282,299). Conventional PKC isoforms (cPKC: PKCα, PKCβI and its splice isoform PKCβII, PKCγ) are activated when diacylglycerol or Ca^{2+} bind to the conserved C1 and C2 domains, respectively. Diacylglycerol and Ca^{2+} are required for full cPKC activity. Novel PKC isoforms (nPKC: PKCδ, PKCε, PKCη, PKCθ) contain only a C1 domain and require solely diacylglycerol for

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stimulation. Atypical PKC isoforms (aPKC: PKC*ι*, PKC*λ*, PKCζ; human PKC*ι* and mouse PKC*λ* may be orthologs) lack C1 and C2 domains. Their regulation is not well understood but may be in part mediated by arachidonic acid, ceramide, and other lipids.

Most PKC isoforms redistribute upon activation from one specific subcellular location including the cytosol and nucleus to another, depending on the PKC isoforms and cell type (101,282). Ca^{2+} binding to the C2 domain induces its interaction with acidic phospholipids such as phosphatidylserine, thereby fostering recruitment of cPKC isoforms to membrane surfaces, especially the plasma membrane, although activation of cPKC can also lead to their localization at intracellular structures such as the nucleus or perinuclear region (101,282). Subsequent binding of their C1 domains to diacylglycerol causes further activation and may stabilize membrane anchoring via the V1 region. The V1 region is upstream of the C1 domain and varies among PKC isoforms. It interacts with the catalytic domain, thereby acting as pseudosubstrate inhibitor. Diacylglycerol binding to C1 releases V1 from the catalytic domain. After dissociation from the catalytic domain, the V1 region itself may mediate membrane interactions, possibly fostering membrane association and targeting of PKC (302).

Differential recruitment of the various PKC isoforms to certain subcellular compartments is further fine-tuned by their interactions with specific PKC adaptor proteins named RACKs upon their activation (366). This mechanism is best characterized for PKC*β* and PKC*ε*. Subtype-specific sequences in the C2 domain and the V5 domain at the very COOH terminus (PKC*β*) or the V1 domain (PKC*ε*) become accessible for RACK interaction upon enzyme activation (366,369). PKC*β* selectively binds to RACK1 (338). Two different peptides derived from the C2 region of PKC*β* inhibit this interaction. Both peptides antagonized L-type channel inhibition by phorbol esters in ventricular myocytes (459). Of the cPKC isoforms in the heart, only activated PKC*β*II is prominent at the surface of cardiomyocytes together with its PKC*β*-specific RACK, RACK1. PKC*β*I and PKC*α*, which could also be affected by C2 domain-derived peptides, accumulate at other locations. PKC*γ* is not detectable in heart. It appears that PKC*β*II downregulates $Ca_V1.2$ upon its activation and subsequent recruitment by RACK1 to $Ca_V1.2$ complexes that are present in the plasma membrane (101). Stimulation of PKC*ε* induces its translocation to Z lines in ventricular myocytes, placing it near L-type channels in t tubules (186,333). Although this spatial proximity does not necessarily have to translate into a functional interaction between L-type channels and PKC*ε*, it is tempting to speculate that different PKC isoforms may regulate $Ca_V1.2$ in opposite ways (see sect. IV*B*) that may in part depend on the exact localization of the $Ca_V1.2$-PKC complex, in this case the plasma membrane versus t tubules.

Various adaptor proteins that can constitutively recruit PKC to defined protein complexes have been described. For example, InaD consists of five PDZ domains and assembles TRP ion channels, PLC*β*, and PKC into a signaling complex that is a critical part of the light-sensing system of *Drosophila* (398). Interaction of the *Drosophila* eye PKC with its PDZ domain binding site at its very COOH terminus to the fourth PDZ domain of InaD appears activation independent. Mammalian PKC*α* binds in a similar way to the multi-PDZ domain proteins PSD-95 and SAP102 (242) and to the PDZ domain in the scaffolding protein PICK1, which can form homo-oligomers (367). PICK1 in turn interacts with AMPAR GluR2 and GluR3 subunits (436) and the acid-sensing ion channels ASIC1 and -2 (181). Furthermore, PSD-95 and SAP102 bind to NMDAR NR2 subunits (283) and to stargazin and its homologs, which might thus recruit PSD-95-PKC complexes to AMPAR (see sects. II*A* and III*B4*B). It is therefore conceivable that PICK1, PSD-95, or SAP102 can target PKC*α* to AMPAR or NMDAR. $Ca_V1.2$ also possesses a PDZ domain binding consensus sequence at its very COOH terminus that interacts with PDZ domain-containing proteins including NIL-16 (228). However, it is unknown whether PKC associates with the $Ca_V1.2$ via adaptor proteins similar to NIL-16 or, after activation, RACK1.

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B. Regulation of $Ca_v1.2$ by PKC and Its Anchoring by AKAP150

In vitro experiments indicate that $\alpha_1$1.2 and at least one β subunit can be phosphorylated by PKC (164,167,205). Activation of PKC via the G_q-linked α_1AR, endothelin receptor, or angiotensin II receptor or by direct activators such as diacylglycerols or phorbol esters leads to upregulation, downregulation, or biphasic effects on currents through cardiac $Ca_v1.2$ channels (reviewed in Refs. 52,212). The complexity of these results may in part be due to effects not mediated by PKC. For example, extracellular application of dioctanoylglycerol inhibited the Ca^{2+} currents by a PKC-independent mechanism, whereas intracellular photorelease of caged dioctanoylglycerol yielded a robust PKC-dependent stimulation (161).

Two alternatively spliced exons encode the initial segment of the NH_2 terminus of $\alpha_1$1.2, which is either 46 (exon 1a) or 16 (exon 1b) residues long and had originally been cloned from rodent heart and brain, respectively, (279,365); both splice forms have been described in both organs (33,361). PKC decreased Ba^{2+} currents through $Ca_v1.2$ expressed in HEK 293-derived tsA-201 cells by phosphorylating threonines-27 and -31 of the $\alpha_1$1.2 isoform with the longer NH_2 terminus (Fig. 14) (274). These residues are missing in isoforms with the shorter NH_2 terminus, which does not show inhibition by PKC. Alanine but not aspartate substitution of either threonine prevented the PKC effect. These results indicate that phosphorylation of both threonines is necessary for the PKC effect, with aspartate effectively mimicking the negative phosphate charge. The first 20 residues of the long NH_2 terminus of $\alpha_1$1.2 are also important for the increase in channel activity by PKC as observed upon expression in *Xenopus* oocytes (361). However, this effect does not seem to involve direct phosphorylation of this segment by PKC but perhaps entails phosphorylation of another site in the $Ca_v1.2$ complex that functionally interacts with the very NH_2 terminus (361). Similar to the decrease in channel activity of the $\alpha_1$1.2 isoform with the longer NH_2 terminus described above, phosphorylation of serine-81 in the NH_2 terminus of $\alpha_1$1.3 by PKC decreases the activity of $Ca_v1.3$ in tsA-201 cells ectopically expressing this channel (14). It is thus possible that analogous inhibitory effects by PKC on $Ca_v1.2$ and $Ca_v1.3$ exist.

It is unknown how PKC increases $Ca_v1.2$. PKC is capable of phosphorylating serine-1928 in vitro and in intact HEK293 cells (445), which could upregulate channel activity (see sect. III*B2*c). However, phosphorylation by PKC appears to be much less prominent than by PKA (79).

As mentioned in section IV*A*, inactive PKC can be cytosolic or preassociate with defined subcellular structures (101,282). In fact, cPKC*α*, cPKC*β*, and cPKC*γ* but not nPKC*δ* or nPKC*ε* coimmunoprecipitate from brain with $Ca_v1.2$ (445). PKC*β* also coimmunoprecipitates with $Ca_v1.2$ from heart. Two different GST fusion proteins covering the membrane proximal and distal part of $\alpha_1$1.2 COOH terminus (residues 1509–1905 and 1906–2170, respectively) pull down recombinant PKC*α* (445). Accordingly, cPKC can directly bind to $\alpha_1$1.2 (Fig. 14). PKC also binds to the NH_2-terminal region of AKAP150 (219). Because AKAP150 is associated with $Ca_v1.2$ (153,304), it could help to recruit PKC to the $Ca_v1.2$ complex. In arterial smooth muscle cells, PKC*α* increases Ca^{2+} influx through $Ca_v1.2$ and thereby Ca^{2+} sparklets, leading to increased blood pressure. This regulation requires recruitment of PKC*α* but not of PKA by AKAP150 to $Ca_v1.2$ (291).

C. Antagonistic Regulation of $Ca_v2.1$ and $Ca_v2.2$ by G*βγ* and PKC

N- and P/Q-type channels fulfill various functions, but perhaps most prominent is the presynaptic Ca^{2+} influx that triggers neurotransmitter release (383). Regulation of their activity has profound effects on synaptic transmission (382). $\alpha_1$2.1 (P/Q-type), $\alpha_1$2.2 (N-type), and $\alpha_1$2.3 (R-type) are phosphorylated by PKA, PKC, CaMKII, and PKG in vitro and in intact neurons (162,164,166,344,452). However, little is known about the physiological functions of

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these different in vitro phosphorylations except that PKC can increase activity of N-type channels (368,444), which potentiates fast synaptic transmission (382), and of R-type channels (368).

PKC acts by antagonizing the downregulation of channel activity by G proteins (381,382). This downregulation by G proteins is membrane-delimited, i.e., it does not involve a freely diffusible second messenger but rather direct interactions with the G$\beta\gamma$ dimer upon its release from G proteins (88,121,169,175,197,457). The respective G proteins must be in close proximity to the channels because the membrane-delimited signaling prevents access to the channels for G proteins outside a cell-attached recording electrode. The upregulation of N- and P/Q-type channel activity by PKC is tightly interwoven with its downregulation by G$\beta\gamma$ (recently reviewed in Refs. 100,113). Because channels are to some degree tonically inhibited by G$\beta\gamma$, PKC activation can lead to an increase in channel activity even under basal conditions, i.e., without previous pharmacological activation of trimeric G proteins.

Loop I/II of the Ca^{2+} channel α_1 subunits is the major binding site for not only the auxiliary β subunits (see sect. IIA) but also for the $\beta\gamma$ dimer upon its release from $G\alpha$ (Fig. 15). β Subunits bind to a stretch of 18 residues that is 16 residues downstream of IS6, the last transmembrane segment of domain I. G$\beta\gamma$ dimers bind to two different segments in loop I/II. Each segment is ~20 residues long. One partially overlaps with the NH_2-terminal portion of the β-subunit binding site and one is ~40 residues downstream of the β-subunit binding site (88,457). Regions in the NH_2 and COOH termini of these channels have also been implicated in G$\beta\gamma$-mediated regulation (52,100,323), but details are less established than for loop I/II, which clearly plays a central role in these regulatory mechanisms. G$\beta\gamma$ binding results in a slowing of ion current activation kinetics, which likely mirrors the voltage-dependent release from inhibition. Strong depolarization to positive potentials release G$\beta\gamma$ from the channel, thereby increasing channel activity.

PKC phosphorylates threonine-422 in the second G$\beta\gamma$ binding site in loop I/II of rat $\alpha_1$2.2, thereby antagonizing binding of G$\beta\gamma$ to this site and its inhibitory effect on channel activity (157,457). It appears that all five Gβ isoforms Gβ_{1-5} can functionally interact with Ca_v2.1 and Ca_v2.2 but may differentially affect channel activity (7,131,341). PKC-mediated phosphorylation antagonizes Ca_v2.2 channel inhibition specifically through $G\beta_1$ but not other Gβ isoforms (72,97). This functional difference between Gβ_1 and other Gβ subunits is due to a pair of aspartate residues that is only present in $G\beta_1$.

The homologous position of threonine-422 in rabbit $\alpha_1$2.2 is alanine and cannot be phosphorylated, yet PKC still antagonizes channel inhibition by G$\beta\gamma$ (25). Thus additional PKC phosphorylation sites can control channel inhibition by G$\beta\gamma$ (100). Furthermore, PKC as well as CaMKII can phosphoryalte loop II/III of $\alpha_1$2.2 in the so-called synprint region. This region mediates binding of different presynaptic proteins involved in synaptic vesicle exocytosis including syntaxin 1A. This interaction reduces Ca_v2.2 channel activity. Phosphorylation of synprint prevents syntaxin 1A binding, thereby counteracting the inhibitory effect of syntaxin 1A on channel activity (450,451).

Enigma homolog (ENH) is an adaptor protein that consists of a PDZ domain followed by three LIM domains, which are cysteine-rich double zinc finger motifs. It interacts through one or more of its LIM domains with the COOH terminus of $\alpha_1$2.2 but not $\alpha_1$2.1 (254). More precisely, ENH binds to a region within 150 residues upstream of the alternative splice site that creates the two main splice isoforms of $\alpha_1$2.2 (254). Different LIM domains can interact with PKC, and all three LIM domains of ENH bind to PKCβI (226). PKCα, -βI, and -ζ but not PKCγ, -δ, and -ε also showed binding to ENH in vitro (226). However, only PKCε but not PKCα, PKCβII, and PKCγ coimmunoprecipitated with ENH from brain extracts (254). The differences

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in isoform binding to ENH versus coimmunoprecipitation with ENH might be due to variable assay sensitivity or brain-specific mechanisms that lead to different selectivity for ENH-PKC isoform interactions. PKCε also coimmunoprecipitated with $Ca_v2.2$ (254). Coexpression of ENH with $Ca_v2.2$ in *Xenopus* oocytes accelerated the increase in channel current as observed upon stimulation of endogenous PKC with the phorbol ester phorbol 12,13-dibutyrate (PDBu) (254). This effect depended on the COOH terminus of $\alpha_1 2.2$ consistent with the model that an ENH-PKCε complex binds to this region. A peptide derived from the NH_2 terminus of PKCε that inhibits translocation of PKCε from the cytosol to the periphery blocked coimmunoprecipitation of ENH with PKCε. This peptide also inhibited phorbol ester-induced potentiation of N-type currents in neurons (254). Indirect evidence suggests that PKCδ can also be recruited to $\alpha_1 2.2$ upon activation by G_q-coupled receptors (362), although this interaction might be independent of ENH as ENH does not bind PKCδ in vitro (but see above for PKCε) (226).

An interesting twist with respect to membrane-delimited inhibition of N-type channels by G$\beta\gamma$ is the formation of a physical complex between the nociceptin receptor ORL1 and $Ca_v2.2$ (21). ORL1 suppressed constitutive N-type activity in a G$\alpha_{i/q}$/G$\beta\gamma$-dependent manner (21). This mechanism appears at least in part to be kinase independent. However, prolonged stimulation of ORL1 with nociceptin induced internalization of $Ca_v2.2$ in a PKC-dependent manner (4).

D. Regulation of $K_v7.2/3$ by AKAP150-Anchored PKC

The NH_2-terminal region of AKAP150 (residues 1–143), which interacts with PKC, also binds to the membrane proximal region of the COOH terminus of $K_v7.2$ (residues 321–499), i.e., immediately downstream of the sixth (last) transmembrane segment (180). Coexpression of the $K_v7.2$ fragment spanning residues 321–499 or of mutant AKAP150 constructs lacking its PKC binding site with full-length $K_v7.2$ in CHO cells strongly reduces inhibition of currents induced by stimulation of the stably expressed muscarinic m_1 receptor, which acts via G_q and PKC. Inhibitors that act on the diacylglycerol binding site of PKC (calphostin C and safingol) reduced muscarinic inhibition as well as phosphorylation of $K_v7.2$, but catalytic site inhibitors of PKC (bisindolylmaleimide and chelerythrine) did not (180). Perhaps AKAP150-anchored PKC is only accessible for the former but not latter inhibitors. It should be emphasized that depletion of PIP_2, which can lead to PKC stimulation via IP_3-mediated intracellular Ca^{2+} release, is primarily responsible for inactivation of the M current in native cells independent of PKC, with PKC only playing a modulatory role for this current (184,377). In native superior cervical ganglion cells, however, knockdown of AKAP150 reduces muscarinic but not bradykinin-induced inhibition of endogenous M currents (179). The m_1 receptor but not the bradykinin B_2 receptor coimmunoprecipitated with AKAP150 from HEK293 cells when cotransfected (179). AKAP150 might thus be important for recruiting m_1 to the endogenous $K_v7.2$ complex.

E. PKC Anchoring at the BK Signaling Complex

PKC inhibits BK channel activity at least in part by counteracting PKA-induced channel activation (13,230). Recordings of BK channel activity in excised membrane patches or after reconstitution into planar lipid membranes imply that PKC is constitutively associated with the channel (29,326). The PKC adaptor protein RACK1 binds to the COOH terminus of BK; this interaction might be direct or potentially mediated by other proteins present in the cell extract used by Isacson et al. (200). However, these authors were unable to detect coimmunoprecipitation of PKC with BK. Nevertheless, overexpression of RACK1 with BK in *Xenopus* oocytes shifted its activation curve towards more positive potentials in the absence but not presence of ectopically expressed channel β subunits. The activation time constant was decreased by RACK1 overexpression in the presence but not absence of β1. Although it is

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unclear whether and how PKC is associated with BK, these data suggest RACK1 as a likely link.

F. GPCR-$K_{ir}3$ Complexes

Dopaminergic $D_{2/3/4}$ receptors act via $G_{i/o}$ and activate $K_{ir}3$ channels (198,422). Although regulation of $K_{ir}3$ by $G\beta\gamma$ is typically initiated by $G_{i/o}$-coupled receptors, β_2 AR can also stimulate K_{ir} (243,420), possibly in part because it can switch from G_s to $G_{i/o}$ (8,371,420, 440,441). D_2 and D_4 receptors and the β_2 AR coimmunoprecipitate with different $K_{ir}3$ subunits but not $K_{ir}2.1$ upon coexpression in HEK293 and COS7 cells (231). Elegant BRET experiments confirmed the close proximity of these proteins with each other and with $G\alpha_s$, $G\beta_1$, and $G\gamma_2$ (231,325). $K_{ir}3.2$ coimmunoprecipitates with D_2 receptor and β_2 AR from brain detergent extracts after partial clearance of nonsoluble material by intermediate speed centrifugation (231). These complexes also incorporate AC II when coexpressed in cell lines; AC V or VI was detected in immunoprecipitates from brain extracts. These interactions were not sensitive to agonist treatment as determined by BRET, indicating that they are constitutive rather than transient (231,325).

$G\beta$ coimmunoprecipitates with $K_{ir}3.1$ from solubilized atrial membranes after rigorous ultracentrifugation (298). Contrasting to some degree the findings described in the previous paragraphs, exposure of atrial membranes to GDP, which will promote reassociation of $G\beta\gamma$ with $G\alpha$, prevented $G\beta$ coimmunoprecipitation with $K_{ir}3.1$. This $K_{ir}3.1$ complex also contained PKA, PP1, PP2A/C, and RACK1. RACK1 directly binds $G\beta\gamma$ (89) and PKC (338). Addition of PKC to inside-out patches of rat atrial myocytes inhibited K_{ACh} currents (298). However, the role of RACK1 was not tested in this system. PKC phosphorylates $K_{ir}3.1$ in vitro (275). PKA increases K_{ACh} currents (284,285) and PP2A decreases them (275,285).

Activation of $K_{ir}3$ channels by $G_{i/o}$-coupled $GABA_B$ receptors is well established (222,252). FRET in combination with total internal reflection fluorescence indicates that GABA B_1 and B_2 subunits both are in close proximity with and likely directly linked to $K_{ir}3.2/3.4$ channels at or near the cell surface (122). This interaction is independent of the activation status of the $GABA_B$ receptor and thus constitutive. Similarly, BRET, supplemented by coimmunoprecipitation experiments, suggests close proximity of GABA B_1 and $K_{ir}3.1$ (82). This interaction was observed independent of GABA B_2 (82).

V. Regulation of Ion Channels by Calcium/Calmodulin-Dependent Protein Kinase II

A. Regulation of Ca^{2+} Channels by CaM and CaMKII

Ca^{2+} influx through $Ca_v1.2$ regulates channel activity via CaM in several different ways. During long depolarizing test pulses, Ca^{2+} currents through $Ca_v1.2$ decrease due to distinct voltage- and Ca^{2+}-dependent inactivation processes starting within 50 ms and extending over a period of up to several seconds. Ca^{2+}-dependent inactivation is usually measured as the difference in inactivation observed with Ca^{2+} versus Ba^{2+} as charge carriers. Inactivation observed with Ba^{2+} is largely voltage-dependent inactivation. Ca^{2+} binding to CaM likely induces a conformational change in the complex preformed between Ca^{2+}-free apo-CaM and the membrane proximal region of the COOH terminus of $\alpha_1 1.2$ (Fig. 16) (109,218,315; for review, see Ref. 156). CaM binding to the NH_2 terminus provides a further modest and typically masked contribution to Ca^{2+}-dependent inactivation (92,201).

Multiple short depolarizing pulses can lead to facilitation of certain Ca^{2+} channels, i.e., the peak current amplitude increases with pulse numbers (91,232). This facilitation is especially prominent for $Ca_v2.1$ and in this case also mediated by CaM. Ca^{2+}-dependent inactivation of

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$Ca_v2.1$ requires the two EF hands in the N domain of CaM, whereas Ca^{2+}-dependent facilitation may depend either on the two EF hands in the C domain or on all four EF hands (91,232). Ca^{2+}-dependent facilitation is rather small, if at all detectable, for $Ca_v1.2$ because it is overshadowed by Ca^{2+}-dependent inactivation; it can be unmasked by mutating isoleucine-1654 in $\alpha_1$1.2, one of the residues important for Ca^{2+}-dependent inactivation, to alanine (465). The small facilitation of $Ca_v1.2$ observed in cardiomyocytes and smooth muscle can be inhibited by CaMKII-specific blockers (105,268,439; see also Ref. 233). Addition of constitutively active CaMKII to excised inside-out patches prolonged single-channel open times and thereby overall current conduction by $Ca_v1.2$ (105). Ca^{2+}-dependent facilitation of $Ca_v1.2$ is thus mediated mainly by CaMKII.

1. Regulation of $Ca_v1.2$ by CaMKII—CaMKII phosphorylates $\alpha_1$1.2 and at least one of the β-subunit isoforms in vitro (164,167,205). To observe Ca^{2+}-dependent facilitation by whole cell patch recording from HEK293 cells transfected with $\alpha_1$1.2 and β_{2a}, coexpression of CaMKII is necessary (233). Facilitation was prevented by KN93, which inhibits CaMKII as well as several other targets. In this system, mutating serine-1512 or serine-1570 to alanine, either individually or together, abolished the CaMKII-dependent facilitation, suggesting that these two residues are important for CaMKII-mediated facilitation. Mutating both sites reduced overall phosphorylation of full-length $\alpha_1$1.2 and nearly abolished phosphorylation of a fusion protein spanning this region by CaMKII (233; see also Ref. 111). Similarly, inhibition of calcineurin leads to an increase in L-type currents by increasing mode 2. This increase is likely due to CaMKII as it is prevented by KN62, a KN93 analog, and after mutating serine-1512 to alanine (111). Collectively, these findings indicate that serine-1512 and -1570 are phosphorylated by CaMKII and contribute to increased channel activity by facilitation and transition to mode 2 gating. It is noteworthy that serine-1512 and -1570 flank the EF-hand motif located between the last transmembrane segment and the CaM binding region of $\alpha_1$1.2 (Fig. 16). This motif does not appear to bind Ca^{2+}, which is in agreement with general findings that single EF-hand motifs typically do not bind Ca^{2+}. It rather is thought to play a structural role in mediating Ca^{2+}-dependent inactivation that is triggered by Ca^{2+} binding to CaM associated with $\alpha_1$1.2 downstream of the EF hand (e.g., Ref. 466).

To observe upregulation of the open probability of $Ca_v1.2$ by CaMKII in excised patch recordings from tsA293 cells, coexpression of β_{2a} (or perhaps another β subunit) with $\alpha_1$1.2 is necessary (147). In these experiments, constitutive active CaMKII was applied to the cytosolic face of the patches. Mutating threonine-498 in β_{2a} (Fig. 16) to alanine blocked the CaMKII effect (147). It also reduced (but did not abolish) phosphorylation of β_{2a} by CaMKII. Furthermore, ectopic expression of this mutant β_{2a} subunit in rat cardiomyocytes abolished facilitation of L-type currents (147). These results indicate an important role of threonine-498 in β_{2a} in the excised patch recordings and in intact cardiomyocytes. It is unclear at this point whether phosphorylation of serine-1512 and -1570 in $\alpha_1$1.2 and threonine-498 in β_{2a} (or on the homologous sites in β_1 and β_3) all have to occur for effective regulation of $Ca_v1.2$ by CaMKII as suggested by these two studies. Alternatively, the different expression and recording systems might reveal two different regulatory mechanisms of $Ca_v1.2$ by CaMKII.

2. CaMKII targeting to $Ca_v1.2$ and the NMDAR—Four closely related genes encode CaMKIIα, -β, -γ, and -δ (189). CaMKIIα and -β are the main isoforms in neurons and CaMKIIδ in heart. Immunofluorescence staining indicates that CaMKII (presumably δ) is enriched along Z-lines in cardiomyocytes (301,435). This observation suggests the existence of anchoring mechanisms for CaMKII. The first identified anchoring protein that recruits CaMKII to a defined subcellular site is αKAP in skeletal muscle (19). αKAP is identical to the COOH-terminal portion of CaMKIIα called the association domain, which mediates multimerization of full-length CaMKII subunits to dodecamers. It is generated from an alternative promoter in the CaMKII coding region. Alternative splicing leads to addition of a

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hydrophobic segment at the NH_2 terminus of αKAP. This segment targets αKAP-CaMKII complexes to the endoplasmic reticulum in skeletal muscle, where CaMKII may contribute to the regulation of RyRs (19).

Whether αKAP is critical for CaMKII anchoring in the vicinity of $Ca_v1.2$ is unclear. However, CaMKII and $Ca_v1.2$ coimmunoprecipitate from heart extracts (190,233) and from HEK cells transfected with $\alpha_1$1.2 plus β_{2a} (233). In vitro interaction studies indicate that CaMKIIα can directly bind to multiple sites on $\alpha_1$1.2 (188) and to residues 410–505 in β_{2a} downstream of its GK domain (Fig. 16) (147). One of the $\alpha_1$1.2 sites is immediately upstream of and partially overlapping with the third CaM binding site in the membrane-proximal region of the $\alpha_1$1.2 COOH-terminal region, which contains the IQ motif (residues 1644–1649 in cardiac $\alpha_1$1.2 from rabbit; Fig. 16) (188). The functional relevance of this site was tested in $\alpha_1$1.2 with isoleucine at position 1654 in the IQ motif mutated to alanine. This $\alpha_1$1.2 mutant shows clear Ca^{2+}-dependent facilitation, which is abolished by additional mutation of the CaMKII binding site formed by residues 1644–1649 (188). However, whether this mutation abrogated Ca^{2+}-dependent facilitation by preventing CaMKII binding to this site or by disrupting the precise interactions between CaM and $\alpha_1$1.2 is unclear because this CaMKII binding site overlaps with residues critical for CaM binding including phenylalanine 1648 (115,401) (Fig. 16). Furthermore, this region of $\alpha_1$1.2 also binds to the NH_2-terminal half of the β-subunit SH3 domain and the mutation might affect this interaction (458). Finally, overall binding of CaMKII to $Ca_v1.2$ and phosphorylation of $\alpha_1$1.2 by CaMKII in vitro were not affected by mutating residues 1644–1649 (188).

Whether binding to residues 410–505 of β_{2a} is necessary for CaMKII anchoring at $Ca_v1.2$ is untested. However, threonine-498 is clearly critical for CaMKII-mediated effects on $Ca_v1.2$ (147). The region surrounding threonine-498 exhibits striking similarity to the autoinhibitory domain of CaMKII and also to the CaMKII binding sites in the COOH terminus of the NMDAR NR2B subunit (18,235,237,373,374) and the *Drosophila* K^+ channel Eag (dEag) (380) (Fig. 16, top). Serine-1303 in NR2B and threonine-286 in the autoinhibitory CaMKII segment are phosphorylated by CaMKII to inhibit their binding to the so-called T site of CaMKII, which is formed in part by tryptophan-237 and valine-298 (Fig. 16, top). Disruption of the interaction of the autoinhibitory CaMKII segment with the T site either by threonine-286 phosphorylation or by binding of the NR2B or the homologous Eag site allows CaMKII to maintain its activity after Ca^{2+}/CaM has been removed (18,247,380). Finally, CaMKII binding to NR2B depends on induction of an active CaMKII conformation by either the presence of Ca^{2+}/CaM or by T286 autophosphorylation (18,235,237,373,374). Therefore, β_{2a} may similarly regulate CaMKII activity at $Ca_v1.2$.

3. CaMKII interactions with $Ca_v2.1$—When expressed in the HEK293-derived tsA-201 cells, $Ca_v2.1$ shows faster voltage-dependent inactivation in the presence of two different CaMKII inhibitors (the organic compund KN93 and ectopic expression of the endogenously occurring inhibitory polypeptide CaM-KIIN) than under control conditions (207). This effect is independent of interactions between CaM and either the IQ-like CaM binding motif (IQ domain) or the downstream CaM binding domain (CBD). The IQ-like motif is homologous to the third CaM binding site in the COOH terminus of $\alpha_1$1.2, but a CBD that would functionally be equivalent to the CBD in $\alpha_1$2.1 has not been identified in $\alpha_1$1.2. However, a peptide derived from the $\alpha_1$2.1 region that is homologous to the CaMKII binding region of $\alpha_1$1.2 located upstream and partially overlapping with the IQ-like motif (residues 1897–1912 in $\alpha_1$2.1) blocked pull-down of CaMKII by the COOH terminus of $\alpha_1$2.1. The peptide also accelerated voltage-dependent inactivation of $\alpha_1$2.1, similar to the effects of KN93 and CaM-KIIN (207). Because CaMKII coexpression increases inactivation when Ba^{2+} was used as charge carrier instead of Ca^{2+}, which could otherwise activate CaMKII, the authors hypothesize that CaMKII might mainly act via binding to $\alpha_1$2.1 and not by phosphorylating it. In fact, the AIP peptide,

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which is derived from the autoinhibitory domain of CaMKII and typically also a very effective and specific CaMKII blocker, had no effect on $Ca_V2.1$ currents obtained in the presence of ectopically expressed CaMKII. However, neither KN93 nor CaM-KIIN inhibited binding of CaMKII to the $\alpha_1$2.1 COOH terminus, although they blocked the CaMKII effect. Although it is possible that CaMKII has to be in a certain conformation that is similar to the active one to exert its effect on $Ca_V2.1$ and that this conformation is prevented by either inhibitor, it is equally possible that AIP did not effectively gain access to the $\alpha_1$2.1-CaMKII complex, whereas KN93 and CaM-KIIN did and thus might have acted by actually blocking basal CaMKII activity. This latter possibility is quite conceivable because CaMKII binding to NMDAR and dEAG has been found to induce autonomous activity in CaMKII (see sect. v, *A2* and *B*) (18,247, 380).

4. Regulation of $Ca_V3.2$ by CaMKII—T-type channels are activated by relatively small depolarizations with optimal activity when membrane potentials are around −50 mV. At such low potentials, the driving force for Ca^{2+} influx into cells is high. T-type channels can, therefore, mediate a substantial amount of Ca^{2+} influx. Three different genes encoding α_1 subunits that form T-type channels exist: $\alpha_1$3.1, $\alpha_1$3.2, and $\alpha_1$3.3. CaMKII selectively increases the activity of $Ca_V3.2$ but not $Ca_V3.1$ by shifting the half-activation potential to hyperpolarized potentials (421,432). In other words, CaMKII lowers the magnitude of depolarization required to open $Ca_V3.2$, thus increasing the number of open channels at more negatively polarized potentials, which in turn provide a higher driving force for Ca^{2+} influx. This effect is mediated by phosphorylation of serine-1198 in the intracellular loop that connects domains II and III of the pore-forming channel portion (421,448). Endogenous CaMKII also regulates $Ca_V3.2$ during excised membrane patch recordings, suggesting that CaMKII is anchored near or on the channel complex (15). Biochemical evidence indicates that CaMKIIγ can directly bind to loop II/III of $\alpha_1$3.2 but not $\alpha_1$3.1 (448). This interaction is not increased by activation of CaMKII in contrast to CaMKII binding to the NMDAR (18,235,237,372,373) and the eag K^+ channel in *Drosophila* (see Fig. 4) (380). It is unclear whether CaMKII has to be associated with $Ca_V3.2$ for its regulation. In fact, activation of CaMKII by Ca^{2+}/CaM and ATP leads to phosphorylation of serine-1198, which in turn results in the release of CaMKII from loop II/III. Accordingly, CaMKII can associate with $Ca_V3.2$ under basal conditions when Ca^{2+} concentrations are low but dissociates from the channel after stimulation and phosphorylation of serine-1198.

B. CaMKII Targeting to *Drosophila* K^+ Channel Eag

dEag was first cloned from the *Drosophila* genetic ether-a-go-go locus (414). It is homologous to mammalian K_V10 channels, which belong to a superfamily of K^+ channels that includes Erg (K_V11) and Elk (K_V12) channels (415). Its overall structure is similar to Shaker-type K^+ channels (see also Fig. 3A). CaMKII phosphorylates dEag on threonine-787 in its COOH terminus (413). Inhibition of CaMKII and mutation of threonine-787 to alanine reduced dEag current amplitude and accelerated inactivation (413). CaMKII binds to a segment in the COOH terminus of dEag that is homologous to its binding site on NR2B (residues 773–794 in Eag; Fig. 16, top). As for CaMKII binding to NR2B (see sect. v*A2*), dEag binding requires active CaMKII as induced either by the presence of Ca^{2+}/ CaM or by T286 autophosphorylation (380). Furthermore, binding of CaMKII to dEag keeps CaMKII in a constitutively active conformation (380) analogous to its binding to NR2B (see sect. v*A2*) (18).

Given that dEag also binds Slob (for Slowpoke binding protein) (352), Eag forms an intriguing signaling complex that could be regulated by phosphorylation-induced recruitment of 14-3-3ζ to Slob, although no further evidence supporting this hypothesis is currently available. Slob was originally identified as a protein associated with the *Drosophila* BK channel dSlo. Slob directly binds and recruits 14-3-3ζ to dSlo (460). As with most other 14-3-3 interactions, this interaction is mediated by phosphorylated serine residues. There are two different sites in

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Slob that can independently bind 14-3-3ζ: serine-54 and serine-79. Both sites are phosphorylated by CaMKII. Overexpression of constitutive active CaMKII leads to increased coimmunoprecipitation of Slob with 14-3-3ζ, and ectopic expression of a CaMKII inhibitory peptide causes decreased coimmunoprecipitation (460). 14-3-3ζ decreases dSlo channel activity by shifting the current-voltage curve to the right. Accordingly, channel activity will be lower in the presence of 14-3-3ζ at a given depolarization level (460).

VI. Regulation of Ion Channels by Src

A. Exemplary Regulation of the NMDAR by Src

Src-mediated stimulation of NMDAR activity is quite well characterized. This stimulation depends on the association of Src and its upstream activator Pyk2 with the NMDAR complex (187,251,345,357,454). Src associates with the NMDAR via two different proteins. The NADH dehydrogenase subunit ND2 is a mitochondrial protein that exists outside mitochondria at postsynaptic sites where it links Src to the NMDAR (138). Src binds with its unique domain located between the NH_2-terminal SH4 and the SH3 domain to residues 239–321 of ND2. Src also interacts with its SH2 domain with the NH_2 terminus of PSD-95 in an unusual phosphotyrosine-independent manner (211). This interaction inhibits Src activity but localizes it next to the tyrosine kinase Pyk2, which binds to the SH3 domain of PSD-95 (357). Autophosphorylation of Pyk2, which is also targeted to the NMDAR via PSD-95 (357), on tyrosine-402 creates a Src binding site, which recruits Src and activates it upon binding in other systems (93). Upon its activation, Pyk2 could reposition Src from its preassociated site on the NH_2 terminus of PSD-95 under resting conditions to phosphorylated tyrosine 402 on Pyk2.

B. Regulation of $Ca_V1.2$ by Src

Regulation of voltage-gated ion channels by tyrosine phosphorylation has received more modest attention. Src increases current activity of L-type channels in smooth muscle and other cells including neurons (108,183,375,434). Src-mediated upregulation of L-type channel currents can be triggered by integrin signaling (149,434), perhaps via the Pyk2-related focal adhesion kinase FAK, although no direct experimental evidence that supports the latter notion is available. Tyrosine-2122 in the COOH-terminal region of rat $\alpha_1$1.2 has been identified as a critical residue for phosphorylation by Src and the ensuing upregulation in $Ca_V1.2$ activity (23,149). Similar to the main PKA site serine-1928 (see sect. IIB2A), tyrosine-2122 is cleaved off in the short form of $\alpha_1$1.2 (23), although the COOH-terminal fragment may still functionally interact with $Ca_V1.2$ (see sect. IIIB2B). Of interest with respect to kinase targeting is the finding that Src specifically bound in vitro to a fusion protein of the COOH-terminal region of $\alpha_1$1.2 downstream of the cleavage site (23). This COOH-terminal region contains two sites that match the consensus binding sequence for the SH3 domain of Src (RPLPXXP). $Ca_V1.2$ might coimmunoprecipitate with Src from colonic smooth muscle cells, but it is unclear whether the coimmunoprecipitation was specific because no negative control was exhibited (183). Whether the reported Src-$Ca_V1.2$ interactions reflect constitutive recruitment of Src to the channel complex perhaps via its SH3 domain or a catalytic site-substrate interaction and whether this interaction is necessary for effective phosphorylation and regulation of $Ca_V1.2$ by Src remains to be demonstrated. It should be noted that tyrosine-2122 is not conserved in rabbit $\alpha_1$1.2 and might thus not be the only Src phosphorylation site in $\alpha_1$1.2.

C. Binding of Src to BK and $K_V1.5$

Src coimmunoprecipitates with the *Drosophila* BK channel dSlo when expressed in HEK293 cells (410). Whether Src binds directly or through adaptor proteins is unknown. It is unclear how Src regulates dSlo, but initial preliminary data suggest that it regulates the subcellular distribution of BK (410). Overexpression of Src with mammalian BK in HEK293 cells increases channel activity by shifting the current-voltage curve to the left but only in the

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presence of 1–120 μM Ca^{2+} (246). Furthermore, Src phosphorylates BK, likely on tyrosine-766, because mutation of this tyrosine to phenylalanine prevented the Src-induced phosphorylation and increase in channel activity (246). Pyk2 might be upstream of Src activation in this system (245).

Src also directly binds a proline-rich domain in the NH_2 terminus of human $K_v1.5$ (177). This domain is not conserved in rat $K_v1.5$, which does not bind Src. Src decreases channel activity of human $K_v1.5$ (177).

VII. Regulation of Ion Channels by Anchored Serine/Threonine Phosphatases

A. Serine/Threonine Phosphatase Targeting to Ion Channels Exemplified by AMPAR and RyR

Known mammalian phosphatases that dephosphorylate serine and threonine residues are designated as PP1, PP2A, PP2B, PP2C, PP4, PP5, PP6, and PP7. The catalytic subunits of these phosphatases are structurally related and form the PPP family (for “protein phosphatase of phosphorylase”). The exception is PP2C and related nuclear phosphatases, which form the PPM family (for “protein phosphatase requiring magensium”). Similar to kinases, phosphatases are associated with some if not many of their substrates (206,405), often via scaffolding proteins that also anchor kinases (16). For example, AKAP79/ 150 does not only anchor PKA but also PP2B (calcineurin) (68,305). The association of AKAP75/150 with the AMPAR via SAP97 and PSD-95/stargazin (see sect. III*B4*B) regulates dephosphorylation of serine-845, the PKA phosphorylation site on the GluR1 subunit of AMPAR (70,388). The AKAP Yotiao constitutes another example that recruits both PKA and, in this case, the protein phosphatase PP1 to the NMDAR (426).

Monomeric PP1 consists of a catalytic subunit that exists in four isoforms encoded by three different genes (PP1 $\alpha, \beta, \gamma 1$, and $\gamma 2$; the latter two arise by differential splicing from the same gene; PP1β had also been named PP1δ). PP1 binds mainly with a hydrophobic groove opposite its catalytic site to (R/K)(V/I)XF motifs present in anchoring proteins that recruit PP1 to other substrates, although additional interactions provide further stabilization and selectivity for one versus the other PP1 isoforms (47,107,390). For example, neurabin and the related spinophilin/ neurabin 2 (2,289,347) recruit PP1 to various targets including ion channels. Spinophilin is critical for regulation of dynamic phosphorylation and dephosphorylation of AMPAR and therefore postsynaptic AMPAR function (182,442). Spinophilin has an LZ motif that mediates binding to other targets including RyR2 (266). The (R/K)(V/I)XF motif also mediates PP1's inhibitory interaction with its endogenous blockers, inhibitor 1, DARPP-32, and perhaps also inhibitor 2 (229). Yotiao, which docks PKA and PP1 at the NMDAR, contains a KVXF sequence, but this sequence is apparently not necessary for its interaction with PP1 (426). However, PP1 binding to AKAP149 (also known as D-AKAP1 or AKAP1, Ref. 433) and AKAP220 (AKAP11) is mediated by this or a similar motif (349,370). Although evidence indicates that PP1 regulates Ca^{2+} channels including $Ca_v1.2$, none of the PP1 adaptor proteins discussed in this paragraph has been reported to interact with a Ca^{2+} channel, and it is unclear whether and how PP1 is targeted to those channels.

PP2A is intimately associated with several of its substrates including RyR2 (266), β ARs (318), and CaMKIV (424). PP2A forms complexes with other kinases such as casein kinase II (168) and the p70 S6 kinase (425). PP2A is a heterotrimer composed of catalytic (C), structural (A), and regulatory (B) subunits (171,206,320,359). To date, over 15 PP2A B subunits have been identified that are subdivided into three classes (B, B′, and B″) (73,206, 270,320). The substrate specificity of PP2A is at least in part determined by the B subunit present in the holoenzyme (320). The B” subunit PR130 (463) mediates PP2A binding to RyR2 by forming a leucine/isoleucine zipper interaction with the second leucine/isoleucine zipper segment of RyR2 (266). The first leucine/isoleucine segment of this receptor binds to PP1 (see

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previous paragraph) and the third to mAKAP (see sect. III*B4*D) (266). The first and third but not second leucine/isoleucine segments are present in the sekletal muscle RyR1, which also interacts with PP1 and PKA but not PP2A (266).

B. Regulation of $Ca_V1.2$ by PP1 and PP2A

Rundown of L-type channel currents in inside-out patches after excision from rabbit ventricular myocytes is reversed by endogenous and also purified PKA and inhibited by the phosphatase inhibitor okadaic acid (306). Okadaic acid also strongly increases currents through porcine ventricular L-type channels when reconstituted into lipid bilayers, although it is unclear how phosphatase inhibition can actually lead to such an increase in the absence of ATP (411). These results suggest that, like PKA, a phosphatase must be docked near or at the channel that antagonizes PKA-mediated regulation of $Ca_V1.2$ (81). Several pieces of evidence collectively indicate that PP2A and in some systems PP1 are involved in reversing PKA-mediated phosphorylation (171). For instance, application of purified PP1 and PP2A inhibits whole cell L-type currents in guinea pig and rat heart cells that have been upregulated by β-adrenergic stimulation; neither phosphatase affected basal currents in these cases (172). These findings suggest that in guinea pig heart PP1 and PP2A antagonize PKA-mediated upregulation of L-type currents because the rise in channel activity upon β-adrenergic stimulation is PKA dependent (see sect. III*B3*). In frog heart, PP1/PP2A inhibitors also reverse or occlude PKA-mediated increases in L-type currents (123,158). In mouse ventricular myocytes, it is mainly PP1 that counteracts the stimulatory action of PKC on L-type currents (102).

A more detailed analysis indicates that PP1 and PP2A differentially regulate channel availability and length of openings. As discussed in section III*B2*B, $Ca_V1.2$ exists in three major modes. It shows no openings in mode 0, shows brief frequent openings in mode 1, and long openings and short closings in mode 2 (173). β-Adrenergic stimulation induces a switch from mode 0 to mode 1 or mode 2 in frog ventricular cells (20). Okadaic acid inhibits switching from mode 2 to mode 1 in cell-attached patch recordings from cardiac and smooth muscle cells while PP2A application to inside-out patches from human umbilical vein smooth muscle cells promotes such a switch (146,307,428). In guinea pig ventricular myocytes, the transition from mode 1 to 0 is less sensitive to okadaic acid. Calyculin A, on the other hand, inhibits the switch from mode 1 to 0 more effectively than the switch from mode 2 to 1 (428). Okadaic acid inhibits PP2A with higher potency than PP1, but calyculin A is about equipotent towards both phosphatases. Those results, therefore, indicate that PP2A plays a predominant role in the reversal of mode 2 to mode 1 and PP1 in that of mode 1 to mode 0. In rat heart, the situation may be reversed, with PP2A being more critical for switching $Ca_V1.2$ from mode 1 to 0 and PP1 for the mode 2 to 1 transition (307).

Initial experiments to determine the phosphorylation status of $\alpha_1 1.2$ in intact neurons before and after PKA stimulation revealed that $\alpha_1 1.2$ phosphorylation was unstable even during advanced purification steps unless a cocktail of serine/threonine phosphatase inhibitors including 2–4 μM microcystin LR was continuously present (Hell, unpublished observations; Ref. 166). Furthermore, okadaic acid prevents rundown of L-type channel currents in inside-out patches (306) (see above). These observations suggest that a phosphatase is associated and copurifies with $Ca_V1.2$. In fact, a more careful analysis shows that PP2A but not the γ isoforms of PP1 coimmunoprecipitates with $Ca_V1.2$ (81). This endogenous phosphatase reverses PKA-mediated phosphorylation of serine-1928 on $\alpha_1 1.2$ (81). Bacterially expressed fusion proteins of the catalytic subunit of PP2A (PP2A/C) binds directly to fusion proteins of the COOH terminus of $\alpha_1 1.2$ (81). This association is not due to a rather long-lasting catalytic site interaction as microcystin, which prevents access to the PP2A/C catalytic site, does not inhibit the PP2A/C-$\alpha_1 1.2$ COOH-terminal interaction. More recent studies show that PP2A/C binds ~30–50 residues downstream of serine-1928 (154), nesting PP2A between this PKA

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phosphorylation site and the COOH-terminal AKAP150 binding site (see sect. IIIB2C;Fig. 6). These studies also indicate that binding to that site is critical for efficient dephosphorylation of serine-1928 in intact HEK293 cells. In general, B-type subunits are thought to dock PP2A at its targets. However, the work on the PP2A/C-$\alpha_1$1.2 interaction unraveled an unexpected way of PP2A/C anchoring that is completely independent of the scaffolding A subunit or a B-type subunit (81,154).

C. Regulation of $Ca_V1.2$ by PP2B

Although earlier work did not detect any effects of PP2B inhibition on L-type currents in the heart (123,170) or neurons (403), more recent work indicates otherwise (304,412). Furthermore, there is also evidence that $Ca_V1.2$ in smooth muscle cells is downregulated by PP2B (354). Especially well worked out are details for PP2B targeting to and regulation of $Ca_V1.2$ in neurons (304). PP2B interacts with AKAP79/150 (68,305). AKAP79/150 is associated with Cav1.2 (153) (see sect. IIIB4A) and recruits PP2B to the $Ca_V1.2$ complex (304). Manipulation of the binding site for PP2B on AKAP79/150 and for AKAP79/150 on $\alpha_1$1.2 indicates that tethering of PP2B to $\alpha_1$1.2 by AKAP79/150 is important for highly dynamic and localized reversal of PKA-mediated upregulation of $Ca_V1.2$. However, it is somewhat surprising that Ca^{2+} influx through $Ca_V1.2$ activates PP2B without causing a more pronounced Ca^{2+}-dependent inactivation via $Ca_V1.2$-associated CaM as seen by other investigators (see sect. VA).

D. Reversal of PKC-Mediated $Ca_V2.2$ Phosphorylation by $Ca_V2.2$-Anchored PP2C

The phosphatase PP2C shares little sequence similarity with other main serine/threonine phosphatases. Because no effective inhibitor is established for this phosphatase, little is known about its function. Recent work showed that PP2C directly binds to the COOH termini of $\alpha_1$1.2, $\alpha_1$2.1, and $\alpha_1$2.2 (240). PP2C binds with its own COOH-terminal region, which is downstream of its catalytic domain, to a site that is likely within residues 1708–1864 in $\alpha_1$2.2, right after the IVS6 segment (240). Among the four main phosphatases (i.e., PP1, 2A, 2B, and 2C), PP2C is most effective in dephosphorylation of PKC-phosphorylated $\alpha_1$2.2 loop II/III (240). However, PKC-phosphorylated loop I/II was only partially dephosphorylated by PP2C. Overexpression of PP2C diminishes the phorbol ester-induced potentiation of $Ca_V2.2$ ectopically expressed in tsA201 cells. Ectopic expression of the COOH-terminal PP2C fragment acts as a dominant negative construct by increasing potentiation of $Ca_V2.2$ in neurons upon treatment with phorbol ester, which likely acts through PKC in this case (240). These data indicate that PP2C counteracts the stimulatory effect of PKC by dephosphorylation of loop II/III residues and perhaps one or more specific sites in loop I/II while other loop I/II phosphorylation sites are dephosphorylated by other phosphatases. As discussed above, PKC can prevent the inhibitory binding of syntaxin 1A to loop II/III and of G$\beta\gamma$ to loop I to the channel. Either of these mechanisms could be antagonized by $Ca_V2.2$-anchored PP2C.

E. Association of Casein Kinase 2 and PP2A With SK2

1. Association of casein kinase 2 with SK2—SK2 directly recruits casein kinase 2 (CK2), along with PP2A, which in turn regulate CaM phosphorylation and thereby its Ca^{2+} sensitivity in the SK2 complex (1,30,255). CK2 directly binds to three different SK2 segments, one in the NH_2 terminus and two in the COOH terminus (1). The interaction between a positively charged segment in the NH_2-terminal domain (specifically lysine-121) and CK2 promotes activation of CK2, which is otherwise accomplished by small basic molecules such as spermine. CK2 phosphorylates CaM on threonine-80. This phosphorylation occurs only when SK2 is in the closed state. It reduces the affinity of CaM in the SK2 complex for Ca^{2+}, thereby reducing activation of SK2. It also leads to faster deactivation of SK2. SK2 also mediates the reduction of SK2 activity upon noradrenergic stimulation (255).

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SK2 is in close proximity and tightly functionally coupled to the NMDAR at postsynaptic sites (114,296). Although there is no evidence that CK2 is associated with the NMDAR complex, CK2 can phosphorylate serine-1480 of the NR2B subunit (64). This residue is critical for binding of NR2B to PDZ domains of PSD-95 and its homologs and its phosphorylation disrupts these interactions, thereby reducing NR2B surface expression (64). In this signaling complex, CK2 is directly or indirectly activated by CaMKII. CaMKII is directly associated with the NMDAR NR1 and NR2B subunits (237,277,373). It is conceivable that SK2 shares CK2 with the NMDAR complex.

2. Association of PP2A with SK2—PP2A binding to SK2 is mediated by a direct interaction of the structural PP2A A subunit with the COOH terminus of SK2 (30). As discussed in section VII*A*, PP2A targeting is typically mediated by one of more than a dozen B subunits. The A subunit functions to support the interaction of the catalytic C subunit with a B-type subunit. A direct interaction of the A subunit with a PP2A target is yet another example of targeting of PP2A to an ion channel independent of the B subunit (see the direct binding of the C subunit to $Ca_V1.2$; see sect. VII*B*). PP2A binding to SK2 requires the sequence EQRK (residues 469–472) in the SK2 COOH terminus (1,255). Mutating this sequence to AEAA not only inhibited binding of PP2A to SK2 but also functionally prevented PP2A from counteracting CK2-mediated acceleration of channel deactivation. Although the catalytic CK2*α* subunit can also directly bind PP2A (168), these findings clearly show that PP2A has to be directly bound to SK2 for dephosphorylation of CaM in the SK2 complex. Consistent with this notion is that SK2 binds both the CK2*α* and the regulatory CK2*β* subunit which form heterotetrameric CK2 complexes that do not by themselves bind PP2A (168).

VIII. Conclusion

Only those structural and functional interactions of kinases and phosphatases with voltage-gated ion channels that are well established by coimmunopreciptiation and in vitro interaction assays could be discussed in this review. These interactions likely constitute only the tip of the iceberg, and many more such interactions will turn out to be critical for a variety of physiological functions. Similar complexes exist for other ion channels and, if at all, could only be mentioned in passing. Examples include the Trp-InaD complex in the *Drosophila* eye (see sect. ivA) and the signaling complex assembled by the NMDAR (see sects. III*B4*c, V*A2*, and VI*A*).

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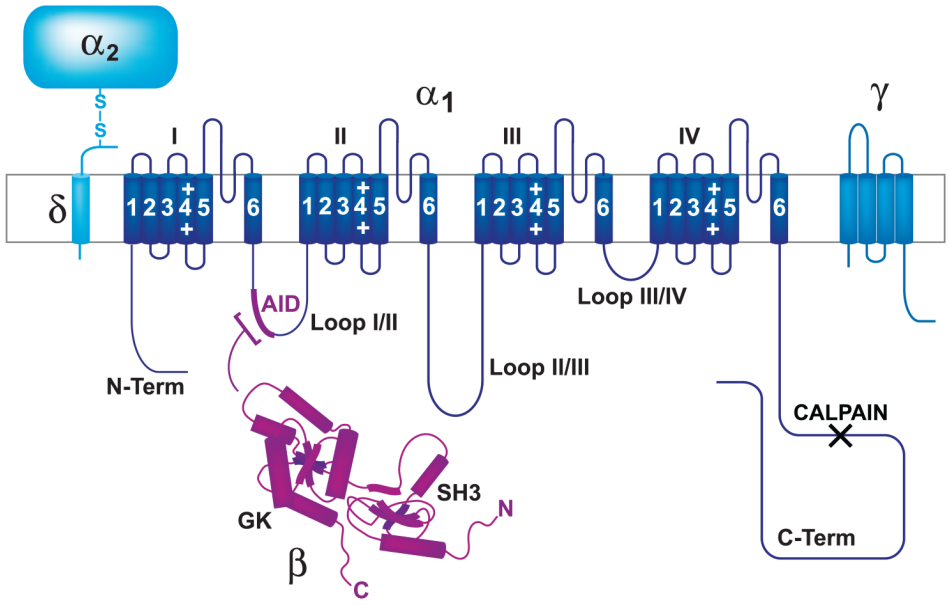

FIG. 1.

Membrane topology of Ca^{2+} channels. The central pore-forming subunit α_1 (dark blue) consists of the four homologous domains I–IV that are linked to each other by the intracellular loops I/II, II/III, and III/IV, each containing six transmembrane segments and a P-loop between segments 5 and 6. The auxiliary subunits α_2-δ (light blue) and β (magenta; Refs. 62,308,402) directly interact with α_1 [the precise interaction sites of α_1 with α_2-δ and γ (medium blue) have not been defined]. Magenta: β subunits generally bind with their GK domains to loop I/II connecting domains I and II (AID); black X: calpain cleavage region.

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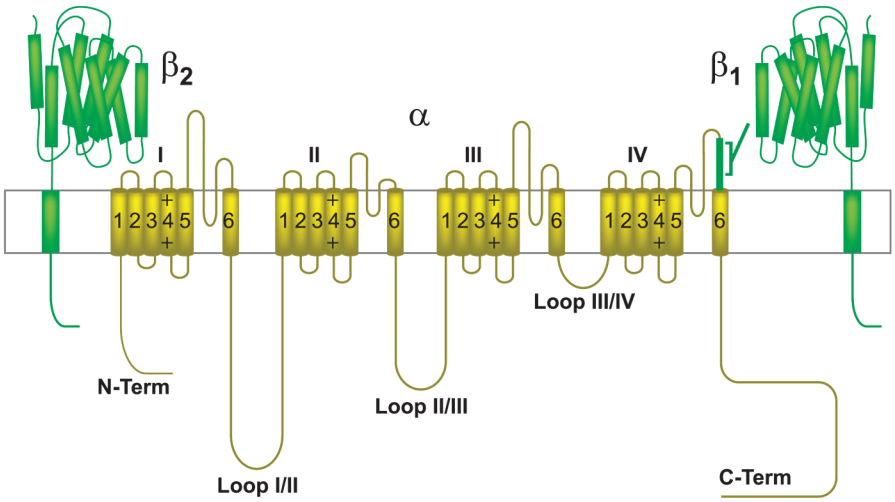

FIG. 2.

Membrane topology of Na^+ channels. The central pore-forming subunit α (yellow) consists of the four homologous domains I–IV that are linked to each other by the intracellular loops I/II, II/III, and III/IV, each containing six transmembrane segments and a P-loop between segments 5 and 6. Some complexes contain the auxiliary subunits β_1 and β_2, which span the plasma membrane once (green). The extracellular interaction of β_1 with the exctracellular segment preceding IVS6 is indicated (bracket).

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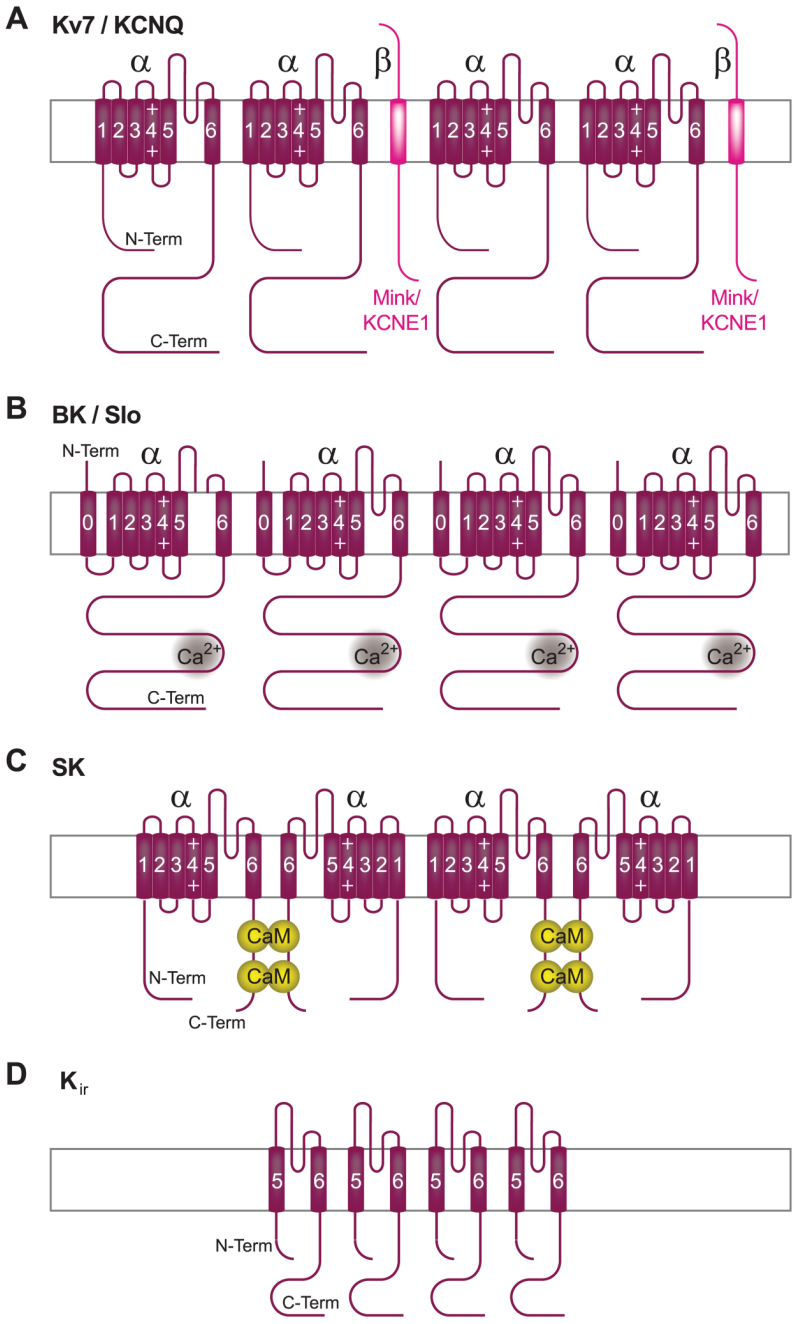

FIG. 3.

Membrane topology of K^+ channels. The pore is formed by four homologous *α* subunits (purple), which interact with each other via their NH_2 termini. *α* Subunits of K_v (*A*), BK (*B*), and SK (*C*) are formed by six transmembrane segments and a P-loop between segments 5 and 6, whereas K_{ir} (*D*) lacks S1–S4. BK channel subunits contain an additional transmembrane segment (S0) NH_2 terminal to the conserved S1 segment. K_v7/KCNQ channels (*A*) are typically associated with the auxiliary single transmembrane MinK/KCNE subunit (magenta), BK channels bind Ca^{2+} with their COOH termini (*B*), and SK channel *α* subunits dimerize through binding two CaM molecules (*C*).

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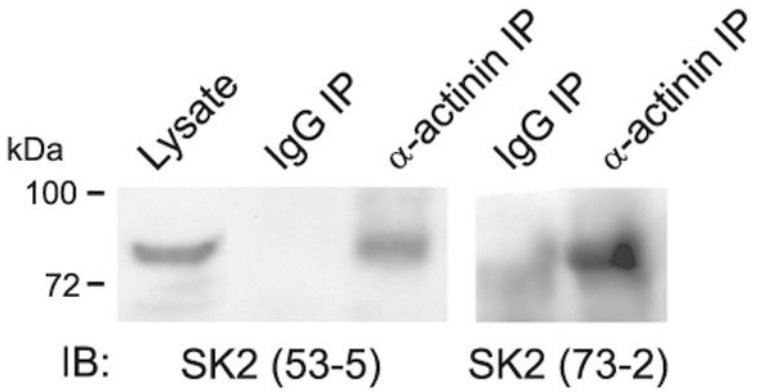

FIG. 4.

α-Actinin coimmunoprecipitates with SK2 from rat brain. Rat forebrains were homogenized in 1% Triton X-100, and nonsolubilized proteins were removed by ultracentrifugation before immunoprecipitation with anti-*α*-actinin or control IgG and immunoblotting with two different antibodies against SK2 (53–5 and 73–2) (see Refs. 153, 154 for more technical details). Both SK2 antibodies detected a single band of the expected molecular mass in anti-*α*-actinin but not control precipitates and in total lysate.

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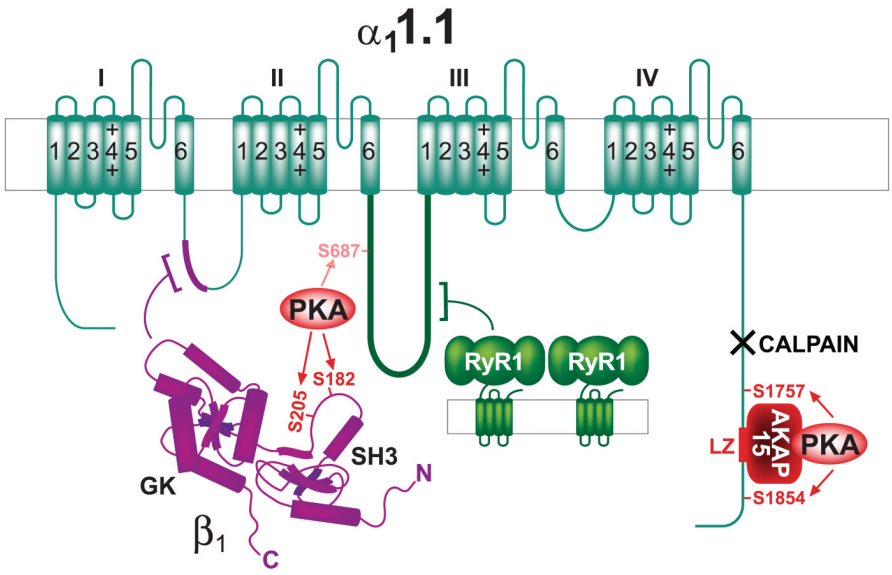

FIG. 5.

The $Ca_v1.1$-AKAP15-PKA complex. Shown are the subunits $\alpha_1 1.1$ (dark green) and β_1 (magenta). Dark red, AKAP15 and AKAP15 leucine zipper binding site on $\alpha_1 1.1$ (LZ); light red, PKA and identified phosphorylation sites for PKA on α_1 and β_1 (arrows). The main PKA sites in full-length $\alpha_1 1.1$ (serines 1757 and 1854) are removed by calpain (black X, calpain cleavage region). Magenta, β_2 and its interaction with $\alpha_1 1.1$. The main in vitro PKA site of the truncated $\alpha_1 1.1$ is serine 687. Green, RyR1 (for simplicity only 2 of the 4 subunits that form one pore complex in the sarcoplasmic reticulum are shown). The large cytosolic foot structure of RyR1 directly interacts with $\alpha_1 1.1$.

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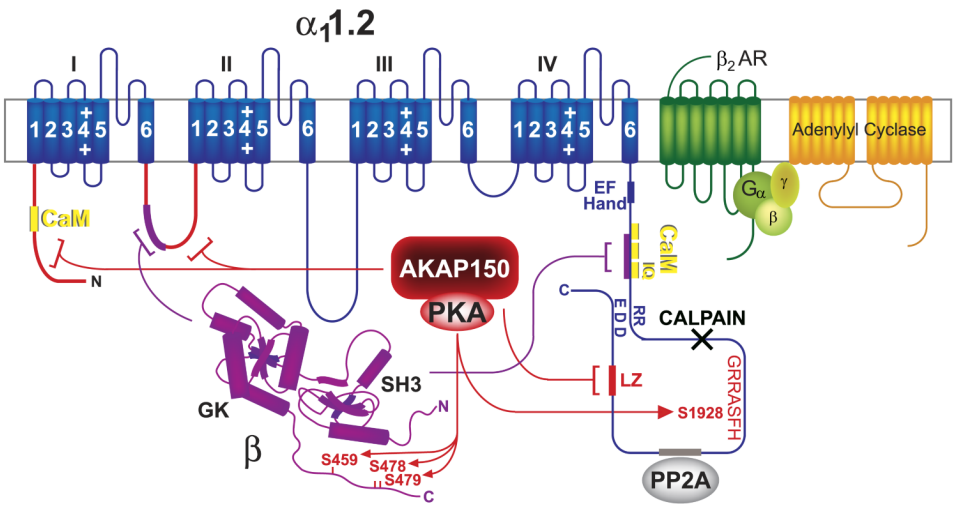

FIG. 6.

The $Ca_v1.2$-AKAP150-PKA complex. Blue, $\alpha_1$1.2; magenta, β_2 and its interactions with $\alpha_1$1.2; yellow, CaM binding sites on $\alpha_1$1.2 [there is one binding site in the NH_2 terminus and three binding sites in tandem in the COOH terminus; the latter region also interacts with β subunits (magenta bracket and segment)]; red, AKAP79/150 binding sites (LZ, brackets, and segments), PKA, and PKA phosphorylation sites on α_1 and β_2 (arrows); gray, PP2A binding site; black X, calpain cleavage region; green, β_2 AR; yellow-green, heterotrimeric G protein complex; yellow-orange, adenylyl cyclase.

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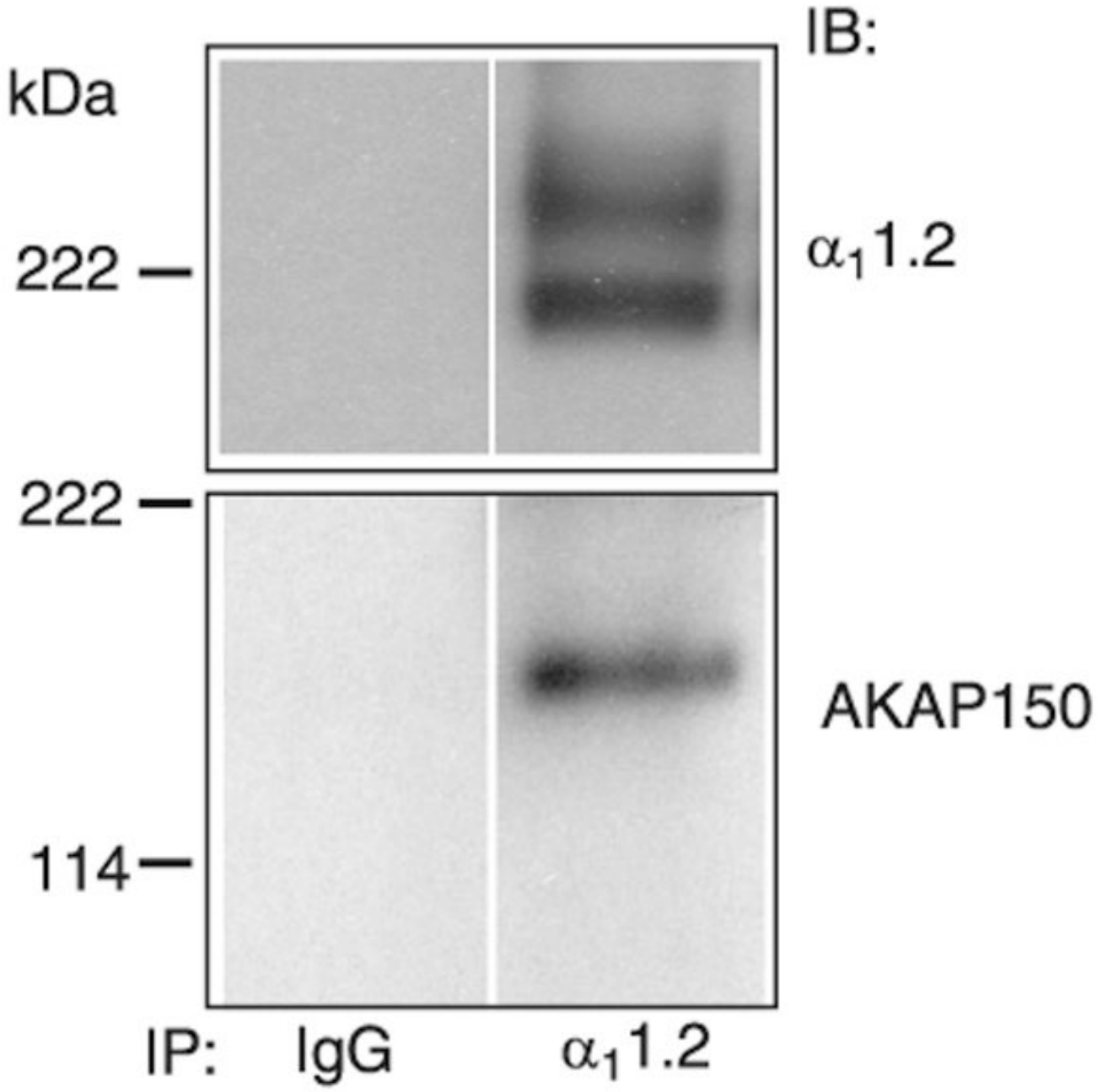

FIG. 7.

Cardiac $\alpha_1$1.2 and its association with AKAP150. Ca_v1.2 was solubilized from rat heart extracts with 1% Triton X-100 before ultracentrifugation to remove nonsolublized material, immunoprecipitation (IP) with anti-$\alpha_1$1.2 or control antibody (IgG), and immunoblotting (IB) with antibodies against $\alpha_1$1.2 and AKAP150. *Top:* $\alpha_1$1.2 long and short forms are present in a ratio of ~1:1. Note the rather diffuse appearance of the two $\alpha_1$1.2 bands, which suggests heterogeneity likely due to minor variations by differential splicing and other factors. *Bottom:* AKAP150 is prominent in total rat heart extract (data not shown) and coprecipitates with Ca_v1.2.

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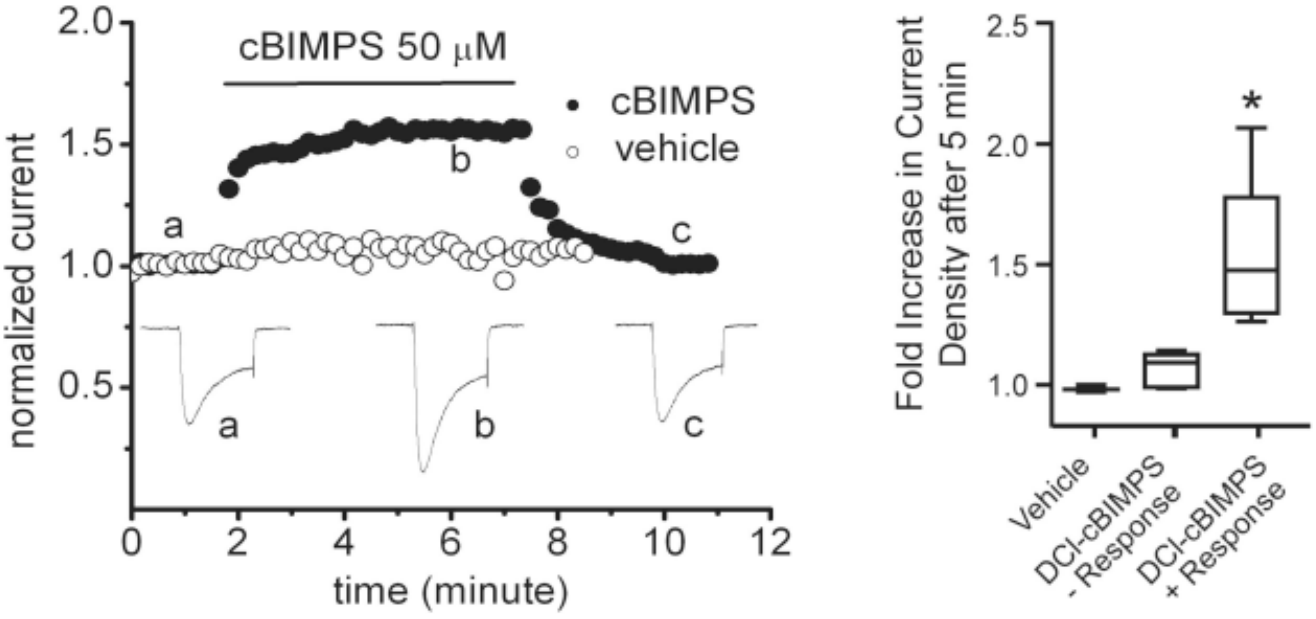

FIG. 8.

Exemplary $Ca_v1.2$ currents and their regulation by cAMP/PKA in HEK293 cells. HEK293 cells were grown on poly-D-lysine-coated coverslips in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum at 37°C under 5% CO_2 and transfected with full-length cardiac $\alpha_1 1.2$, β_{2a} with FuGENE 6. Whole cell patch Ca^{2+} currents were elicited by depolarization from a holding potential of −70 to 0 mV for 200 ms at 24°C (leak-subtracted using p/4; extracellular: 125 mM NaCl, 10 mM tetraethylammonium chloride, 5 mM $CaCl_2$, 5.4 mM CsCl, 1 mM 4-aminopyridine, 1 mM $MgCl_2$, 10 mM HEPES-NaOH, 10 mM glucose, pH 7.4; intracellular: 120 mM CsCl, 10 mM tetraethylammonium chloride, 10 mM EGTA, 1 mM $MgCl_2$, 3 mM MgATP, 0.5 mM Na_3GTP, 10 mM HEPES-CsOH, pH 7.3). DClcBIMPS (closed circles) but not vehicle (open) induced in 7 of 14 experiments (see box blots) an increase in current of >20% (compare *a* and *b*), which readily reversed upon wash-out (*c*).

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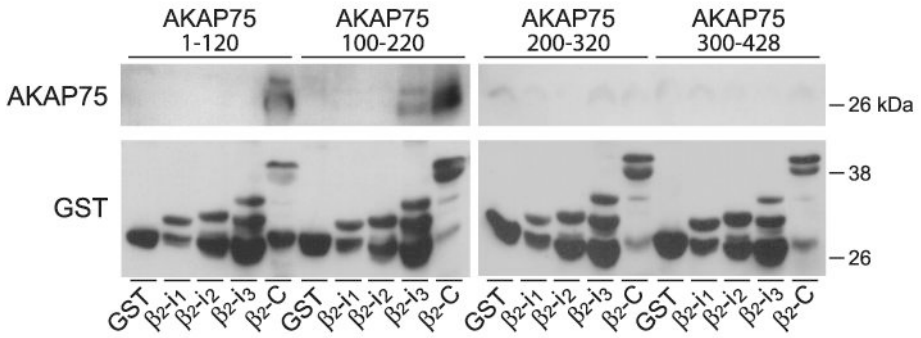

FIG. 9.

Direct interactions between the NH_2-terminal half of AKAP150 and the β_2 AR. Fusion proteins were expressed in *E. coli* and extracted using sarcosyl as described (81,153,236). GST fusion proteins of the first, second, and third intracellular loops of the β_2 AR (β_2-i_1, -i_2, -i_3, respectively) or its cytosolic COOH terminus (β_2-C), or GST alone (GST) were immobilized on glutathione Sepharose. Resins were incubated with *E. coli* lysates of 6xHis- and V5-tagged AKAP79 fragments encoding residues 1–120, 100–220, 200–320, and 300–428 (these constructs cover the full length of AKAP75). *Top:* immunoblotting with antibodies against V5 showed specific binding of the first two AKAP75 fragments to the COOH terminus of the β_2 AR. Weak binding of the second fragment to the third intracellular loop of the β_2 AR (β_2-i_3) was also detectable to variable degrees. *Bottom:* reprobing with anti-GST demonstrated that comparable amounts of the full-length GST fusion proteins were present, although loop fragments were also substantially degraded.

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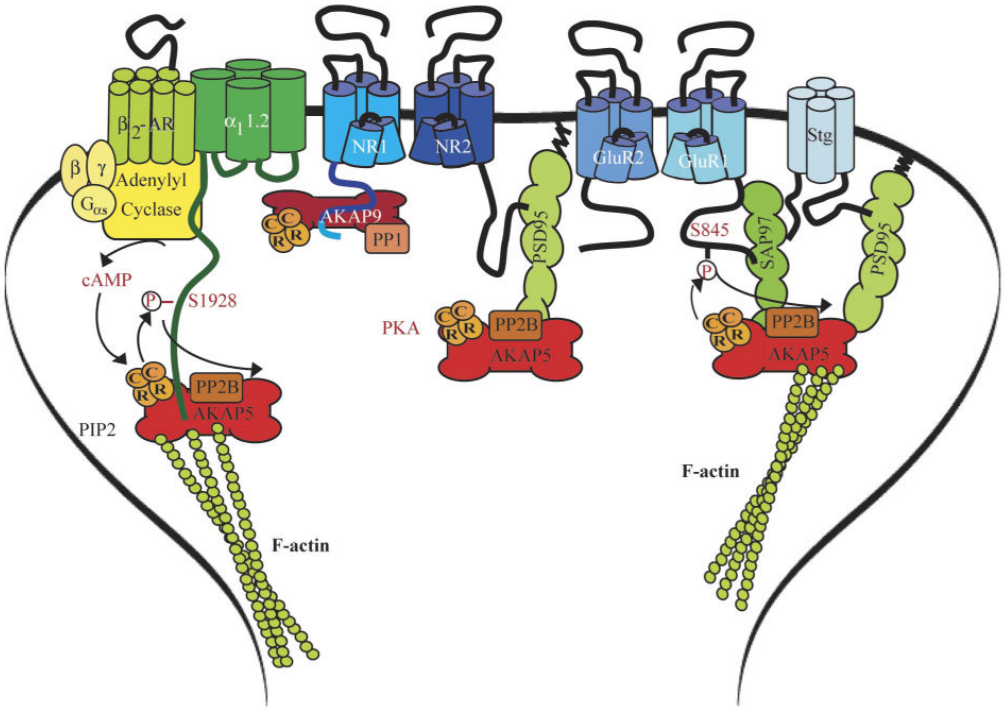

FIG. 10.

Postsynaptic A kinase anchor protein (AKAP)/PKA complexes. *Left:* the $Ca_V1.2$ signaling complex containing the β_2-AR, adenylyl cyclase (AC), G_s, and the PKA holoenzyme (2C plus 2R), which is linked to the complex via AKAP150 (AKAP5). The PKA phosphorylation site serine-1928 is indicated. *Middle:* the NMDAR complex with Yotiao (AKAP9; binds to the C1 segment in the NR1 COOH terminus) and AKAP150. Yotiao functionally links not only PKA but also the counteractive phosphatase PP1 to the NMDAR. AKAP150 interacts with PSD-95 (or its homologs), which in turn bind to the very COOH-terminal ESDL-COO^- motif of NR2A and 2B. A potential function of AKAP150 in NMDAR regulation is not known. *Right:* anchoring of PKA and PP2B by AKAP150 via SAP97 and via PSD-95/stargazin (stg) to the AMPAR GluR1 subunit. SAP97 and PSD-95 bind AKAP150 with their COOH-terminal portion containing SH3 and GK (the exact interaction sites have proven difficult to dissect). PKA and PP2B phosphorylation and dephosphorylation are indicated at serine-845 on GluR1.

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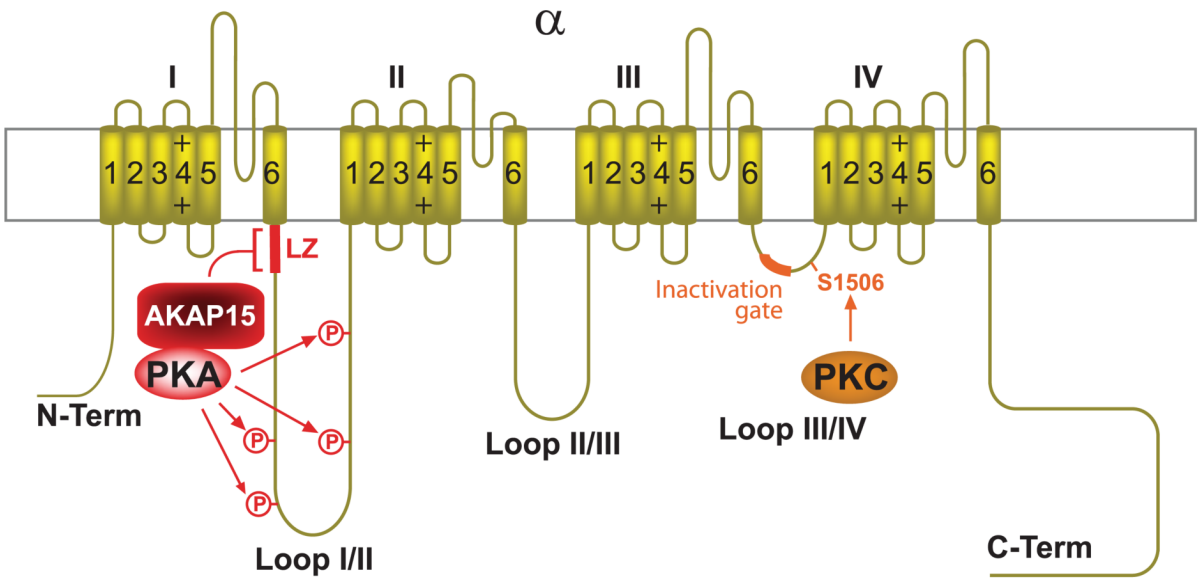

FIG. 11.

The $Na_V1.2$-AKAP15-PKA complex. Yellow, sodium channel $\alpha 1.2$ subunit; dark red, AKAP15 binding site (LZ, bracket and segment); light red, PKA, four phosphorylation sites for PKA within loop I/II (arrows); orange, PKC, PKC phosphorylation site serine-1506, and the inactivation gate sequence within loop III/IV.

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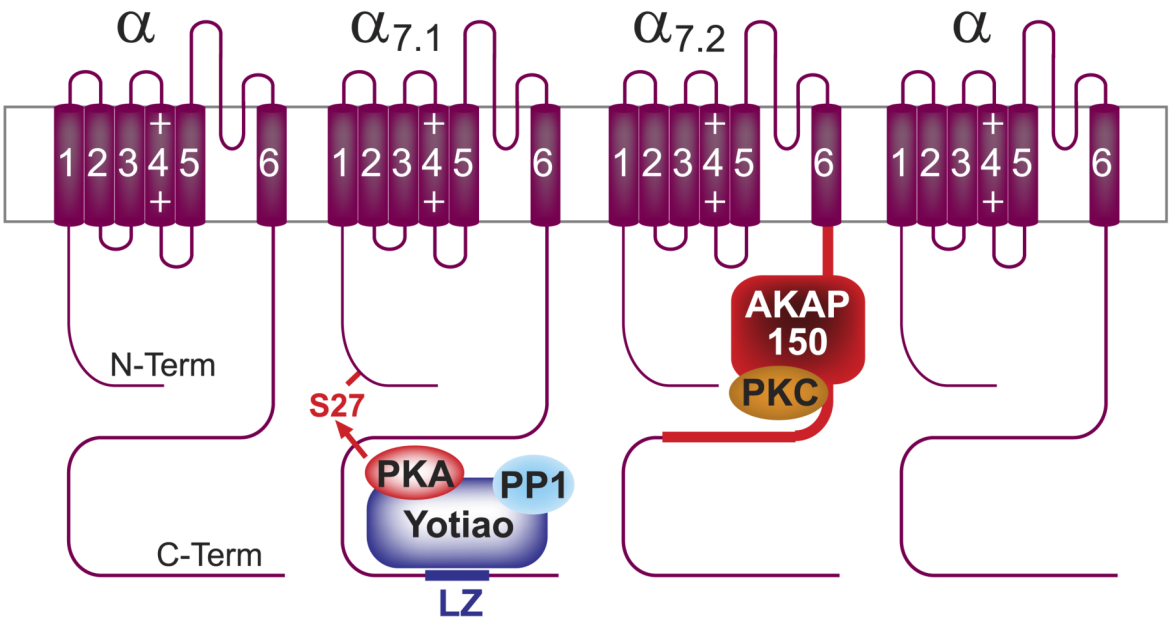

FIG. 12.

The $K_V7.1$-Yotiao-PKA/PP2A and $K_V7.2$-AKAP150-PKC complexes. Purple, α7.1/7.2; blue, Yotiao binding site on α7.1 (LZ); magenta, AKAP150 binding site on α7.2; bright red, PKA, identified phosphorylation site for PKA in NH_2 terminus of α7.1; dark red, AKAP150 and AKAP150 binding sites on α7.1; orange, PKC binding to AKAP150.

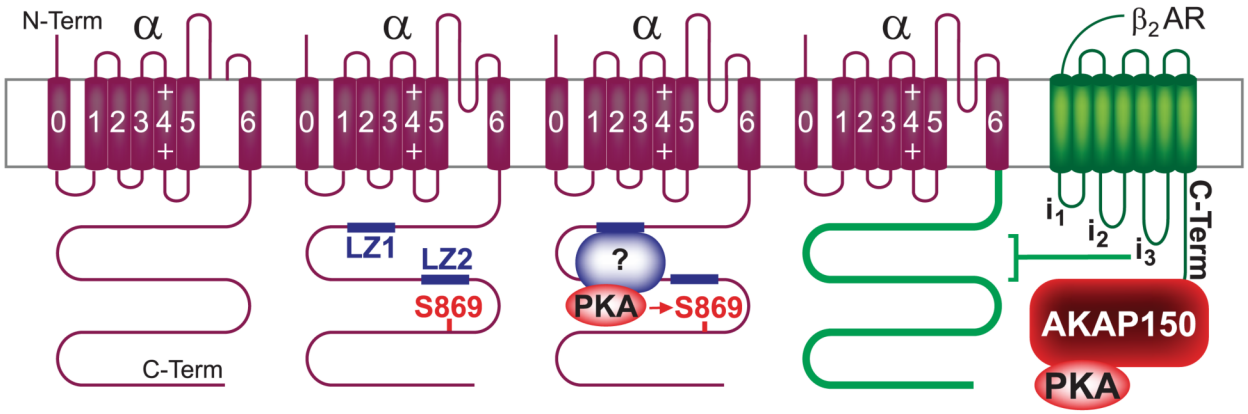

FIG. 13.

The BK-PKA complex. Purple, α subunit of BK; red, PKA, identified PKA phosphorylation sites; blue, leucine zipper motifs (LZ) within BK, unidentified AKAP that recruits PKA for serine-869 phosphorylation; green, β_2 AR and its interactions with the BK α subunit; dark red, AKAP150.

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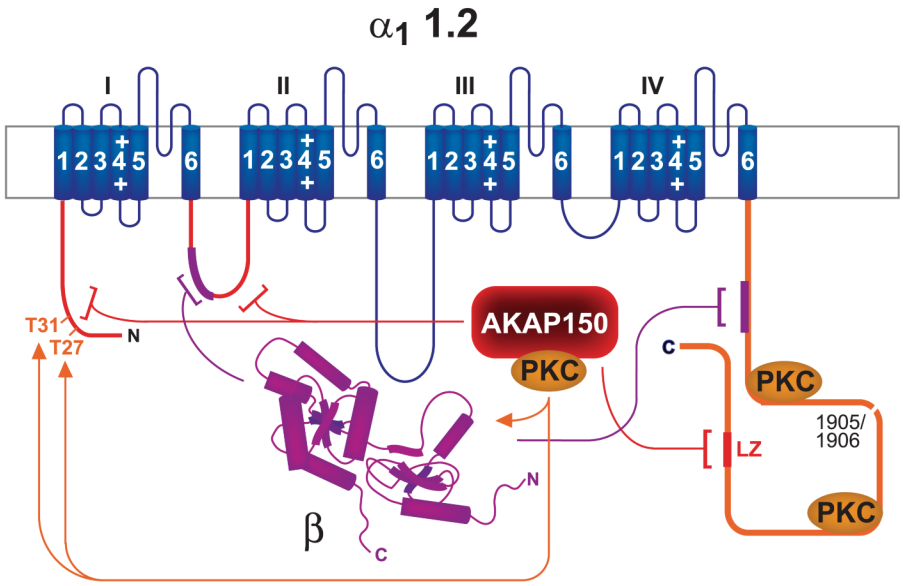

FIG. 14.

The $Ca_v1.2$(-AKAP150-)PKC complex. Blue, $\alpha_1$1.2; red, AKAP79/150 binding sites (brackets and segments); orange, identified direct binding sites independent of AKAP150 (segments) and phosphorylation sites (arrows, residues followed by number) for PKC on α_1. Two separate fragments of the α_1 COOH terminus (1509–1905, 1906–2170; Ref. 445) bind PKC. Magenta, β_2 and its interactions with $\alpha_1$1.2.

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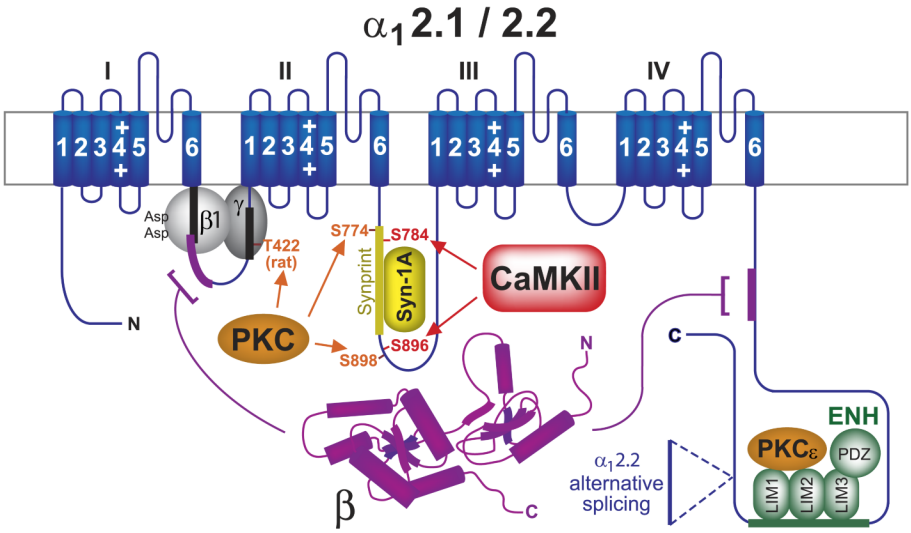

FIG. 15.

$Ca_v2.2$ PKC complexes. PKC increases channel activity by antagonizing the donwregulation by binding of $G_{\beta\gamma}$ to loop I/II and of syntaxin to the synprint region in loop II/III. Blue, $\alpha_1 2.2$; orange, PKC phosphorylation site threonine-422 in loop I/II; this site is present in rat but not rabbit $\alpha_1 2.2$ and specifically antagonizes inhibition by $G_{\beta 1}$, which carries two unique aspartates, but not other trimeric G_β subunits in rat. Other unidentified phosphorylation sites are responsible for the antagonistic action of PKC with respect to other $G_{\beta\gamma}$ interactions. Grey, β_1 and γ subunits of G proteins; black, segments within loop I/II that bind G protein β_1 and γ subunits. Also shown in orange are PKC phosphorylation sites in and near the synprint region in loop II/III. Yellow, synprint region within loop II/III and inhibitory interacting protein Syntaxin 1A (Syn-1A); red, CaMKII, identified CaMKII phosphorylation sites in and near the synprint region in loop II/III; green, ENH binds with its LIM domains to both the COOH terminus of $Ca_v2.2$ (but not $Ca_v2.1$) and to PKC-ε (orange), thereby recruiting PKC-ε to the channel complex.

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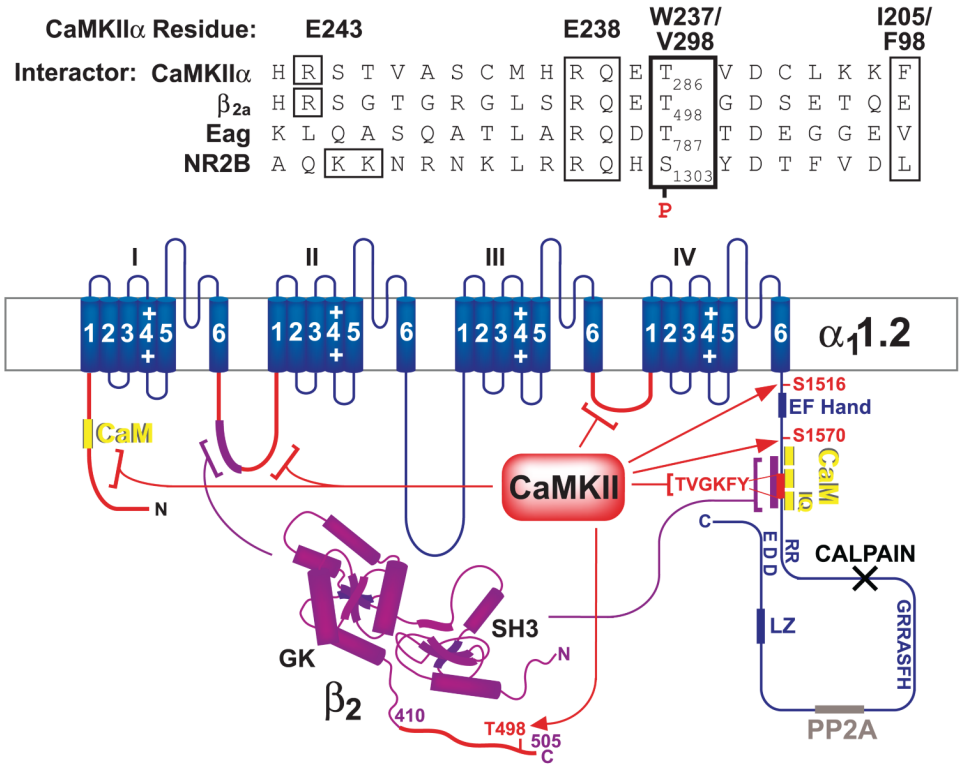

FIG. 16.

Interaction of CaMKII with $Ca_v1.2$ and related targets. Blue, $\alpha_1 1.2$; magenta, β_2 and its interactions with $\alpha_1 1.2$; red, CaMKII binding sites (brackets and segments) and defined phosphorylation sites (arrows and residues followed by number). Evidence for functional importance is available for CaMKII binding inside the CaM binding region (TVGKFY) and the COOH terminus of β_2. Yellow, one CaM binding site has been identified in the NH_2 terminus and a cluster of three sites in the COOH terminus; the cluster also interacts with β subunits (magenta arrow). Gray, PP2A binding site; black X, calpain cleavage region. *Top*: sequence alignment of the autoinhibitory domain of CaMKII with CaMKII binding sites on ion channel subunits. Residues at the top refer to interactions between residues in the segment of the CaMKII autoinhibitory domain that interacts with the P site under unstimulated conditions and residues in the large lobe of CaMKII. I205 is also important for binding of CaMKII to the NMDA receptor NR2B subunit (18). Boxes indicate residues that interact (or are homologous to other proteins) with CaMKII residues above.

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Table 1
Identified PKA phosphorylation sites and AKAP binding sites

Ion Channel	Phosphorylation Site(s)	AKAP Binding Region	AKAP	Reference Nos.
$Ca_v1.1$	S1757/S1854	L1786–L1814	AKAP15	192,339
$Ca_v1.2$	S1928	I2073–L2094	AKAP15	85,194
RyR type 1	S2843	I3039–L3075	mAKAP	266,378
RyR type 2	S2808	V3003–L3039	mAKAP	266,336
IP_3R	S1589/S1755	N1251–I1287	Yotiao	117,399

PKA, cAMP-dependent protein kinase; AKAP, A kinase anchor protein; RyR, ryanodine receptor; IP3R, inositol 1,4,5-trisphosphate receptor.