Ion-channel regulation by G proteins

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Ion channels are end-targets (effectors) in a large number of regulatory pathways that are initiated by G protein-coupled neurotransmitters and hormones. Modulation of ion channels by G proteins can be indirect (via second messengers and protein kinases) or direct, via physical interactions between G-protein subunits and the channel protein. These direct physical interactions are the focus of this review. A direct regulation has been firmly established for several voltage-dependent Ca^{2+} channels and the G protein-activated K^+ channels. In these ion-channel families, the G protein βγ subunits (Gβγ) are the active regulators, whereas the role of the α subunits (Gα) remains poorly understood. Accumulating evidence suggests that intricate relationships between the receptor, Gα, Gβγ and the ion channel play a major role in determining the specificity and magnitude of the overall regulation.

Ion channels, encoded by several hundred genes in humans, differ widely in molecular structure, selectivity to ions and mechanisms of operation. In spite of their diversity, these proteins share a general structural motif: a pore formed by and enclosed within the transmembrane segments of the channel protein, through which ions traverse the plasma membrane (Box 1). The conformation of a channel protein alternates dynamically between open (activated) and closed state(s) in a process called gating. It involves the movement of gates, which are specific parts of the channel molecule[1]. The pore of K^+ channels is the best-studied and probably contains the main gates of the channel[2]. Most mammalian ion channels also feature large extracellular and cytosolic parts, which are the targets and the transducers of the modulatory effects of intracellular signals, but in most cases, it is still unclear exactly how they affect gating.

Most mammalian ion channels are regulated by neurotransmitters and hormones via G protein-coupled receptors (GPCRs). GPCRs activate heterotrimeric G proteins (Gαβγ) by promoting the exchange of GDP for GTP at the Gα subunit, followed by dissociation of Gα–GTP and Gβγ (Ref. 3). Both Gα–GTP and Gβγ activate or inhibit a variety of target proteins (effectors), and can regulate ion channels indirectly (via second messengers and protein kinases) or directly, via physical interactions between G-protein subunits and the channel protein[4].

Although direct modulation by G proteins has been proposed for many ion channels, it has only been firmly established for two families (Box 1): (1) some voltage-activated Ca^{2+} channels (Ca_v), which are inhibited by Gβγ; and (2) G protein-activated inwardly rectifying K^+ channels (GIRKs, or Kir3), which are activated by Gβγ. Previous work has been extensively reviewed[4–12], and the focus here is on recent work and remaining problems.

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GIRKs – the best-studied ion channels directly gated by Gβγ

Gβγ is the mediator of action of neurotransmitters that activate GIRK

GIRKs mediate postsynaptic inhibitory effects of various $G\alpha_{i/o}$-activating transmitters in neurons (dopamine, somatostatin, opioids and others) and underlie a large part of the negative chronotropic parasympathetic regulation in the heart (reviewed in Refs 5,7). The initial studies on activation of GIRK by G-protein subunits were controversial, but now it is firmly established that Gβγ, rather than Gα, mediates the GPCR-induced activation of GIRK by a direct interaction with the channel. The accumulated experience helped to set experimental criteria that need be fulfilled before a similar mechanism for any ion channel is accepted (Box 2). For the GIRK, all criteria have been satisfied: coexpression of Gβγ in heterologous systems activates the GIRK; sequestration of Gβγ inhibits activation by transmitters; purified Gβγ activates GIRK in excised patches (see Fig. I in Box 2); Gβγ binds glutathione S-transferase fusion proteins that encompass parts of GIRK subunits (reviewed in Refs 4,5,7); Gβγ interacts with purified GIRK subunits[13,14]; and mutations within a Gβγ-binding site of GIRK remove activation by neurotransmitters[15]. In addition to Gβγ, GIRK gating depends crucially on the presence of membrane phosphatidylinositol 4,5 bisphosphate (PIP_2)[16].

Binding of Gβγ to GIRK subunits: stoichiometry and relationship to basal and agonist-evoked activity

Half-maximal effect of Gβγ in excised patches occurs at 5–15 nM (Refs 17–19). However, half-saturating concentration of Gβγ in direct binding to purified GIRK is 50–55 nM (Ref. 13). The difference could be the result of accumulation of Gβγ in the patch membrane (giving an overestimate of affinity); alternatively, the detergent present in biochemical assays might alter the affinity of interaction[13]. Mutational mapping of Gβ shows that residues of Gβ that interact with GIRK are found both within and outside its Gα-binding surface[19,20].

Direct binding and peptide-competition experiments demonstrated two or three separate Gβγ-binding segments in each GIRK subunit (Box 1), with up to 12 Gβγ-binding segments per channel, although they probably form fewer actual 3D binding sites. Cross-linking experiments suggest the binding of four Gβγ per purified GIRK1/4 or GIRK4 tetramer[14], supporting earlier electrophysiological data (reviewed in Ref. 7). Although it is tempting to assume binding of one Gβγ per GIRK subunit[14], it

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Box 1. The two classes of ion channels directly regulated by Gβγ

The mammalian G protein-activated inwardly rectifying K^+ channel (GIRK) family includes 4 subunits, GIRK1–4, and functional channels are homotetramers of GIRK2 or GIRK4 or heterotetramers of GIRK1/2, GIRK1/3, GIRK2/3 or GIRK1/4 (Refs a,b). Fig. Ia presents a side view showing two of the four subunits that form the channel. The structure of the pore region[c] is based on a high-resolution structure of a bacterial K^+ channel[d] that is probably shared by most other K^+ channels.

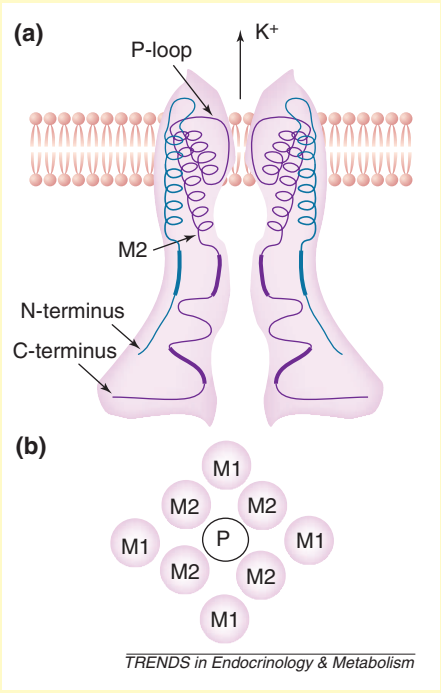

Fig. I. The structure of a GIRK channel. In each subunit, the core transmembrane domain contains 2 membrane-spanning α-helices (M1 and M2) and a re-entrant helix-P-loop structure that forms the selectivity filter, the narrowest part of the pore. The exact folding of the large cytosolic domain (>60% of total protein mass), consisting of N- and C-termini, is unknown and is speculative. The approximate location of Gβγ-binding segments is shown by thick lines. The N-terminal Gβγ-binding segment and the distal C-terminal segment are present in all GIRK subunits. The proximal C-terminal Gβγ-binding segment is found in GIRK4 (aa ~209–245) but not in GIRK1 (Ref. g). (b) A GIRK channel viewed from above, showing the four subunits surrounding the central pore (P). The arrangement of M1 and M2 segments around P is shown as in Ref. d and probably corresponds to a closed state of the channel[c,h].

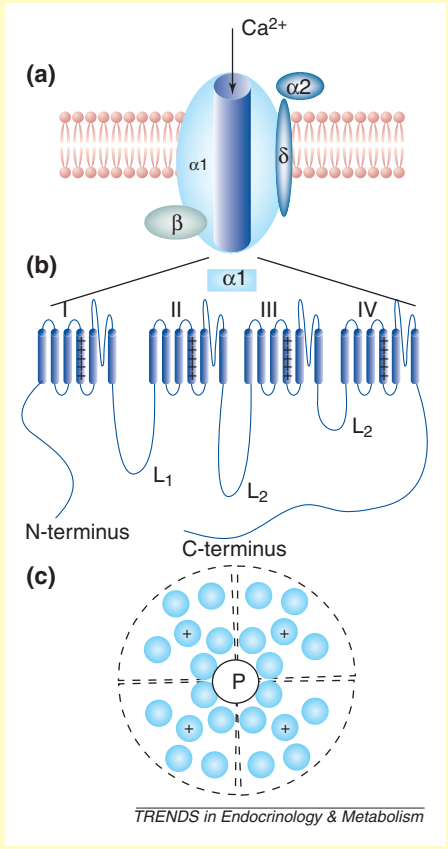

Fig. II. The structure of a voltage-dependent Ca^{2+} channel. (a) The quaternary structure of a 'high-voltage' mammalian Ca_v channel consists of a pore-forming subunit (α_1) and at least two auxiliary subunits ($\alpha_2\delta$ and β; Ref. e). The structure of the main subunit of voltage-dependent Na^+ channels is similar to that of α_1 (Ref. f). (b) The structure of the α_1 subunit, unfolded to demonstrate the underlying components: four homologous membrane domains (repeats) numbered I–IV, and five intracellular segments: N- and C-termini and three linkers between the domains, often called loops L_1, L_2 and L_3. (c) A scheme of the α_1 subunit viewed from above. The four repeats outlined by the dotted lines surround the pore (P).

Fig. II shows a minimal structure of a voltage-dependent Ca^{2+} channel (Ca_v). In the membrane, the four repeats are wrapped around a central pore. Each repeat is homologous to one subunit of a voltage-dependent K^+ channel and contains six transmembrane α-helixes and a re-entrant P-loop. The pore is probably lined by the two rightmost α helixes (S5 and S6) and the P-loop of all repeats[e]. One of the α-helixes (S4) carries a net positive charge and presents the voltage sensor whose movement, dictated by changes in membrane voltage, controls the state of the gate(s)[f]. The exact mechanism of coupling between the voltage sensor and the gates, and the exact folding of transmembrane and cytosolic parts of α_1, have not yet been resolved.

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remains possible that segments from two or more subunits form the actual binding sites.

A clear functional role has been assigned only to the distal C-terminal Gβγ-binding segment: it is a part of a low-affinity site underlying agonist-induced activation[15]. Mutations within it eliminate GPCR-induced activation but leave the Gβγ-dependent basal activity intact, implying that the latter is determined by a separate Gβγ-binding site[15]. It is possible that this site is formed by the proximal C-terminal Gβγ-binding segment, maybe together with the binding segment at the N-terminal (D. Logothetis, pers. commun.). The relationships between the high- and low-affinity Gβγ-binding sites, basal and agonist-evoked activity of GIRK, and Gβγ-binding stoichiometry still need to be better understood.

Specificity of signaling in the GPCR–Gα–Gβγ–GIRK cascade

All tested combinations of Gβ and Gγ similarly activate the GIRK (Refs 18,21). Therefore, it is unlikely that selectivity of coupling is determined by

Gβγ. However, in cardiac and neuronal cells, only pertussis toxin (PTX)-sensitive G proteins activate GIRK (Ref. 4), implying a role for Gα (PTX uncouples $G\alpha_{i/o}$ from GPCRs). Earlier studies suggested some selectivity even within the $G\alpha_{i/o}$ family[5], but heterologous expression experiments with PTX-insensitive $G\alpha_{i/o}$ mutants and GPCR–Gα fusion proteins showed no substantial preference in GIRK activation by all $G\alpha_{i/o}$ types[22,23]. It appears that the specificity lies mainly in receptor–Gα rather than in Gα–GIRK coupling[22].

The specificity of signaling from GPCRs to GIRK is far from absolute: upon overexpression of $G\alpha_s$ or $G\alpha_z$, these PTX-insensitive G proteins become major donors of Gβγ for GIRK activation[23–27]. Thus, factors such as colocalization might be involved, as is supported by kinetic considerations regarding the speed of channel activation[28]. $G\alpha_{i1}$ binds to the N-terminus of GIRK1, and it has been proposed that the Gαβγ heterotrimer, anchored at the N-terminus of a GIRK subunit, releases free Gβγ after interaction with an agonist-bound receptor, thus allowing fast and targeted interaction of Gβγ with its binding sites[29]. Although attractive, this model needs further experimental support.

How binding of Gβγ causes GIRK opening (gating)

Two recent studies[30,31] shed light on the gating mechanism by identifying amino-acid residues within the pore that, if mutated, render the channel constitutively active and Gβγ-insensitive. Thus, binding of Gβγ allosterically regulates the gating apparatus in the pore. It is proposed[30,31] that binding of Gβγ results in conformational rearrangement of the transmembrane M2 segment and a proximal part of the C-terminus that mediate open–close transitions by widening or narrowing the cytosolic outlet of the pore and allowing long-lasting periods of channel activity (bursts)[30,31], as is proposed for other K^+ channels (Ref. 32 and references therein). The allosteric effect also alters the permeation and/or gating at the selectivity filter[30,31], which might itself be a gate determining the fast open–closed transitions[33]. The details of these conformational changes and their relationship to the different Gβγ-binding segments remain to be elucidated. Interestingly, the constitutively active, Gβγ-insensitive mutant channels remain sensitive to PIP_2, suggesting that the mechanisms of gating by Gβγ and PIP_2 might be different[30].

Voltage-dependent Ca^{2+} channels: inhibitory modulation by Gβγ

Voltage-dependent inhibition of neuronal Ca^{2+} channels: the role of Gβγ

High-voltage-activated Ca_v channels consist of a pore-forming subunit (α_1) and at least two auxiliary subunits, ($\alpha_2\delta$ and β; see Fig. II in Box 1). The neuronal N- and P/Q-type channels, based on α_{1B} ($Ca_v2.2$) and α_{1A} ($Ca_v2.1$), respectively, are crucial for neurotransmitter release. These channels are inhibited by many GPCRs via voltage-independent (VI) and voltage-dependent (VD) mechanisms. VI inhibition proceeds via multiple pathways, mostly second messenger-mediated (reviewed in Refs 9,10,12). Depending on neurotransmitter, cell type and location within the neuron, the VI inhibition can be mediated by $G\alpha_q$, $G\alpha_i$ and/or by Gβγ (Refs 34–36). Accumulating evidence suggests that some types of VI inhibition are the result of a direct interaction of Gα or Gβγ with the N-type and P/Q channels[37–39], but many important criteria remain to be fulfilled before this is firmly established.

The VD inhibition is fast and is relieved by depolarization. It occurs in P/Q, N and R (α_{1E}, or $Ca_v2.3$) channels, but not in L-type ($\alpha_{1C} = Ca_v1.1$ and $\alpha_{1D} = Ca_v1.3$; reviewed in Refs 9–12). A wealth of evidence suggests that this modulation is mediated by a direct interaction with Gβγ: it is membrane delimited, as shown by the cell-attached recording test (see Fig. Ia in Box 2); coexpression of Gβγ induces the VD inhibition, whereas coexpression of Gβγ scavengers, such as the C-terminus of the β-adrenergic receptor kinase, suppresses it; Gβγ binds to fusion proteins encompassing parts of α_1 subunits; and mutations within a Gβγ-binding site remove the VD inhibition caused by neurotransmitters[9–11]. However, modulation by purified Gβγ in excised membrane patches has yet to be demonstrated.

Most neurotransmitters produce VD inhibition via PTX-sensitive G proteins, usually $G\alpha_o$. However, almost any Gαβγ heterotrimer can contribute Gβγ for the VD inhibition; $G\alpha_o$ could be the preferred donor of Gβγ simply because it is the most abundant Gα in the nervous system (reviewed in Ref. 10). Similarly, all Gβ subunits can cause VD inhibition[40] (but see Ref. 41). Therefore, the extent of selectivity of signaling from various G proteins to neuronal Ca^{2+} channels in the VD pathway remains unclear.

Binding of Gβγ to α_1 subunits: stoichiometry and relation to VD inhibition

Initial studies identified a Gβγ-binding segment in L_1 (Box 1), the same loop that binds the β subunit of the channel, in α_{1A}, α_{1B} and α_{1E}. Mutagenesis and peptide-competition experiments suggest that this interaction is crucial to VD modulation (reviewed in Ref. 11). An additional Gβγ-binding site has been found in the C-terminus of α_1 (Ref. 42); its contribution to VD inhibition seems to differ among N, P/Q and R channels[37,42,43]. Finally, a stretch in the N-terminus with a putative Gβγ-binding sequence has been shown (by mutagenesis) to be indispensable for VD inhibition[44,45]; however, physical binding of Gβγ to this segment has not been yet demonstrated. Although direct binding studies between full-length α_1 subunits and Gβγ have not been performed, indirect kinetic evidence suggests the presence of a single Gβγ-binding site per channel[46]. Therefore, it appears that, like in GIRKs, the actual binding site for Gβγ might be formed by Gβγ-binding segments from different parts of the α_1 subunit.

Box 2. How to determine whether a G protein directly regulates an ion channel

The proposed experimental criteria are based on experience accumulated from studies of regulation of G protein-activated inwardly rectifying K^+ (GIRK) and voltage-dependent Ca^{2+} (Ca_v) channels. Their full or partial fulfillment, often in conjuction with additional tests, is necessary before deciding which G-protein subunit mediates the effect of a neurotransmitter, and whether it is a direct regulation. Only some criteria would need be fulfilled for a (hypothetical) regulation which is direct but independent of activation of a G protein-activated receptor (GPCR), such as modulation of an ion-channel assembly by Gβγ (Ref. a).

Criterion 1. Coexpression or direct application of the G-protein subunit mimics the effect of transmitter; sequestration or knockdown of the G-protein subunit removes the effect of the transmitter.

These experiments establish which subunit is crucial for the regulation. Coexpression of the channel with Gβγ or with constitutively active mutants of Gα subunits in mammalian cells or *Xenopus* oocytes, to examine which one mimics the effects of neurotransmitters, is widely used to assess the roles of Gα or Gβγ (Refs b–d). However, it does not provide information about the nature of regulation (overexpressed G-protein subunits might cause indirect effects by affecting diverse signaling pathways), and it is often difficult to discard long-term effects such as changes in the metabolism of the cell.

Selective reduction in the concentration of one of the endogenous G-protein subunits by the addition of a scavenger, by antisense or transgenic knockdown, or by an antibody provides valuable information about which subunit regulates the effector. Sequestration of Gβγ by GDP-bound Gα subunits or Gβγ-binding peptides or polypeptides has been especially efficient (e.g. Ref. b). Caution should be exercised, however, because downregulation of Gα levels can be accompanied by changes in Gβγ and vice versa. Other effects can interfere. For example, sequestration of Gα by specific antibodies eliminated Ca_v modulation, and the initial assumption was that Gα inhibits the channel. However, later studies showed that the channel is inhibited by Gβγ, and the Gα antibodies often merely disrupted the signaling from GPCR to the G protein (reviewed in Ref. e). Therefore, to clarify which G-protein subunit participates in the modulation under study, concomitant application of the overexpression and knockdown methodologies is recommended.

Criterion 2. The protein mimics the effect of the neurotransmitter (hormone) in a membrane-delimited fashion.

This is best examined by monitoring channels trapped in an excised 'inside out' patch of plasma membrane at the tip of a micropipette (Fig. Ib). The purified G-protein subunit is added to the bath solution, to which the cytosolic side of the membrane is exposed to examine whether it mimics the effect of the neurotransmitter (e.g. Refs f,g). If it does, one can rule out the involvement of diffusible cytosolic ingredients (but not the involvement of membrane-soluble second messengers, such as diacylglycerol or cytoskeletal elements that remain attached to the patch). This kind of experiment also provides strong evidence for the involvement of a particular G-protein subunit in a modulation. A less unequivocal test involves the infusion of purified G-protein subunits into the cell via the recording patch pipette during a whole-cell recording, in which most but not all of the diffusible cytosolic contents are exchanged for the pipette solution (Fig. Ic). This method has been employed[h–j] in Ca_v channels whose activity in excised patches is notoriously unstable. The absence of an effect of a purified G-protein subunit in an excised patch or a whole cell does not necessarily refute function: the purified protein might need a specific lipid modification[k] or another ancillary protein[j] for correct membrane insertion or contact with the channel.

A complementary method that reveals indirect modulations is to monitor channels sealed by the micropipette within a cell-attached membrane patch. The seal

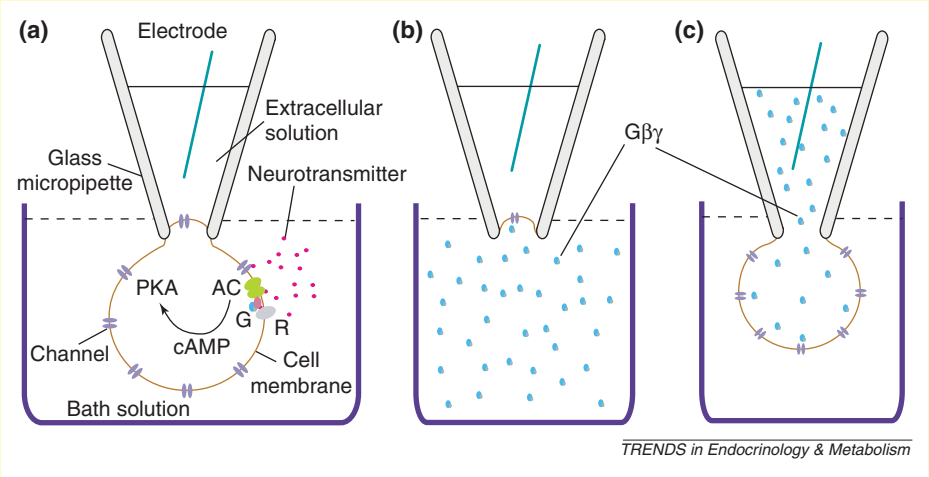

Fig. I. The use of patch-clamp recording methods to study ion-channel modulation by G proteins[n]. (a) Cell-attached recording. The metal electrode immersed into the pipette solution is connected to the voltage-clamp amplifier (not shown), which measures the potential and injects the currents. In this example of indirect channel modulation, a neurotransmitter activates adenylyl cyclase (AC) via a receptor (R)–G protein (G) interaction, cyclic AMP (cAMP) is produced, protein kinase A (PKA) is activated and phosphorylates the channel. (b) An excised (inside-out) membrane patch is formed by pulling the micropipette upwards to detach the patch shown in (a) from the rest of the cell. A channel located in the patch is gated in a membrane-delimited manner by Gβγ added to the bath solution. (c) Whole-cell recording becomes possible when the membrane of the cell-attached patch in (a) is ruptured by suction. Total current flowing through all the channels found in the cell membrane is recorded. Gβγ is shown diffusing from the pipette into the cytosol.

Additional subunits and proteins can affect binding of Gβγ or its effect on gating. First, the β subunit of the channel appears to be indispensable for Gβγ-induced VD modulation[47,48], but the mechanism of this interaction is still unclear. Second, syntaxin (a ubiquitous protein, crucial for transmitter release) modulates N and P/Q channels by binding to the L_2 loop of the α_1 subunit (reviewed in Ref. 12). Syntaxin

between the pipette and the membrane is impermeable for molecules from the external solution. A neurotransmitter added to the cell outside the pipette can modulate the channel only if the modulation is mediated by a small molecule (e.g. cAMP) that diffuses via the cytosol or within the membrane (Fig. Ia). Therefore, if a neurotransmitter modulates the channel when added to the pipette solution but does not modulate it when added outside the pipette, the modulation is considered membrane-delimited. However, this interpretation should be applied with caution, because highly localized production of a diffusible second messenger within a multiprotein signaling complex anchored to the channel could also give this phenomenology[l].

Criterion 3. The G-protein subunit physically interacts with the channel. *In vitro* binding of purified proteins or their parts is an excellent instrument to establish an interaction and to locate the binding sites, but it cannot show that the interaction takes place *in vivo*. Coprecipitation of a channel with a G protein from native cells or tissues is a good indication but not proof of functional interaction. A powerful method to demonstrate both close apposition and functional interaction of proteins *in vivo*, the fluorescence resonance energy transfer (FRET), has not yet been used to study G protein–ion channel interactions.

Criterion 4. Prevention of G-protein binding to its established binding site (by competition and/or by mutation of the binding site) abolishes the regulation. The strongest evidence for a direct regulation is a demonstration that the mutation within the G-protein binding site of a channel eliminates G-protein binding, in addition to modulation of the channel, both by purified/coexpressed G-protein subunit and by the neurotransmitter (e.g. Ref. m).

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concomitantly interacts both with Gβγ and the α_1 subunit and supports the Gβγ-induced VD inhibition, suggesting that it normally contributes to colocalization of α_1 and Gβγ (Ref. 49). Importantly, Gβγ applied via the pipette in a whole-cell recording produced VD inhibition only in the presence of coexpressed syntaxin[36]. Finally, $G\alpha_o$ and $G\alpha_i$ subunits physically interact with N and P/Q-type Ca_v channels[37,39,50]; potentially, they might serve as donors of Gβγ and could even modulate the interaction of the channel with Gβγ. The relationships between Gβγ and Gα, the β subunit of the channel, and syntaxin in their binding to α_1, and their effects on function of Ca_v channels are complex and remain to be resolved.

How Gβγ affects the gating of Ca_v channels?

A hallmark of the VD modulation is a slowing in Ca^{2+}-current activation caused by the inhibitory neurotransmitter and the voltage-dependent facilitation, whereby a depolarizing prepulse accelerates channel opening and relieves inhibition. This modulation is best described in terms of a model[51] in which the conformation of the channel alternates between willing (W) and reluctant (R) modes of gating. In the R mode, promoted by the inhibitory neurotransmitters, stronger depolarization is required for opening, whereas depolarization promotes the conversion into the W mode[51], possibly by reducing the affinity of the channel to Gβγ (Refs 46,52). P/Q-type channels and splice variants of the N-type channel show substantial differences in modal behavior[48].

The first transmembrane domain of α_1 (domain I) plays a crucial role both in voltage gating of Ca_v channels and in Gβγ-induced VD inhibition[53,54]. Importantly, the shift between R and W modes depends on a residue in the S3 α-helix of domain I and is affected both by Gβγ and the β subunit of the channel[48,55]. Thus, like in the GIRKs, the modulatory effects of Gβγ, in addition to the β subunit of the channel, are initiated by interactions with cytosolic parts of α_1 but are transmitted allosterically to the gating apparatus within the transmembrane parts of the channel[48,55].

Are L-type Ca^{2+} channels modulated by Gβγ?

L-type Ca^{2+} channels that contain the 'cardiac' α_{1C} and 'endocrine' α_{1D} subunits are ubiquitous and regulate heartbeat, smooth muscle tonus, hormone secretion and gene expression in the brain. They are inhibited by neurotransmitters acting via PTX-dependent G proteins in many cell types. However, these modulations are not voltage-dependent, and the L_1 loop of α_{1C} does not bind Gβγ (reviewed in Ref. 12). In smooth muscle cells, purified Gβγ subunits infused from the pipette in whole-cell recording (in the presence of Ca^{2+} chelators) enhance L-type via an indirect, phosphorylation-related pathway[56,57]. Therefore, α_{1C} has been assumed to lack interaction with Gβγ. However, recent studies suggest that a membrane-delimited modulation might exist. Inhibition of L-type channels by opioids and ATP in chromaffin cells, mediated by PTX-sensitive G proteins, is membrane-delimited as suggested by the

cell-attached patch-recording tests[58] (Box 2). Gβγ binds directly to *N*- and C-termini of α_{1C}; coexpression of Gβγ reduces the Ca^{2+}-channel current in a Ca^{2+}–calmodulin-dependent manner[59]. Unfortunately, attempts to reconstitute any (direct or indirect) modulation of α_{1C} or α_{1D} channels by PTX-sensitive G proteins in heterologous expression systems have failed[59,60], suggesting that additional cellular components might be needed and making it difficult to study the role of Gβγ. The possibility that L-type channels are regulated by a direct interaction with Gβγ in a physiological context remains poorly substantiated but open.

Other ion channels – candidates for direct modulation by G-protein subunits

Direct interaction of Gβγ with several ion channels has been reported. Thus, Gβγ binds directly to parts of a voltage-dependent K^+ channel (Kv1.1) and Gβγ coexpression or scavenging alter the inactivation of the channel in opposite ways. It has been proposed that Gβγ acts directly, mainly by altering the interaction between Kv1.1 and its β subunit (Kvβ1.1) that regulates the extent of inactivation[61]. IRK3 (Kir2.3), an inwardly rectifying K^+ channel that does not belong to the GIRKs, interacts directly with Gβγ and is inhibited by Gβγ coexpression[62]. In both cases, the physiological role of the Gβγ modulation is unclear.

An outstanding example of a well-documented but poorly understood regulation by G-protein subunits is that of the ATP-sensitive K^+ (K_{ATP}) channels. These inwardly rectifying K^+ channels, normally inhibited by resting levels of cytosolic ATP, play a protective role during metabolic stress and regulate insulin secretion and cardiac excitability (reviewed in Ref. 63). They are also activated by several GPCRs via PTX-sensitive G proteins[64,65]. K_{ATP} is composed of four Kir6.x subunits (similar to those of GIRK, Box 1) that form the core of the channel and underlie the regulation by ATP. The core is surrounded by four subunits of the SUR1 or SUR2 type (ATP-binding cassette-type proteins) that modulate the interaction of Kir6 with ATP (Ref. 8).

The action of G proteins is membrane delimited[17,64–66] and achieved by antagonizing the ATP-dependent inhibitory gating[17,66]. Previous work suggested that the effects of neurotransmitters are mediated by $G\alpha_i$ subunits, since $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ (but not $G\alpha_s$) activated K_{ATP} in native cells and a heterologous expression system[17,64–67], whereas Gβγ was reported to inhibit $G\alpha_i$-activated K_{ATP} (Ref. 17) or had no effect[67]. However, direct interaction between $G\alpha_i$ and K_{ATP} has never been demonstrated and binding sites have not been mapped, leaving the most important criteria for a direct regulation unfulfilled.

Recent work[68] challenges the role of Gα and suggests that Gβγ modulates K_{ATP} by a direct interaction. Gβγ directly binds to SUR and activates Kir6.2/SUR channels expressed in COS cells by reducing ATP inhibition. Furthermore, mutation of a single Arg residue in one of the binding segments fully eliminated the effect of Gβγ (Ref. 68). This fulfils some (but not all) of the most important criteria to establish Gβγ as a direct mediator of G-protein action on K_{ATP}. However, it does not provide strong evidence against the role of $G\alpha_i$. It is possible that both arms of the G-protein pathway, Gα and Gβγ, regulate K_{ATP} by distinct or interrelated mechanisms. Notably, the affinity of Gβγ revealed by experiments in excised patches is very high: 5–10 pM already caused substantial activation, suggesting sensitivity to Gβγ at least by two orders of magnitude higher than that of GIRK. Similarly, the apparent affinity for $G\alpha_i$ is reportedly 30–50 pM (Ref. 65), which is three orders of magnitude higher than for the best-studied effector of $G\alpha_i$, adenylyl cyclase[69]. The outstanding sensitivity of K_{ATP} to G-protein subunits leaves open the question of whether this channel might be already saturated by G-protein subunits at their 'basal' levels, when the other cellular targets of these G proteins are barely regulated. It is obvious that more work is needed for a better understanding of regulation of this channel by G proteins.

Conclusions

Modulation by direct interaction with Gβγ, unequivocally demonstrated in two ion-channel families (GIRK and Ca_v), occurs after binding of Gβγ to sites probably formed by several cytosolic segments of the channel protein. The binding allosterically affects the gating machinery within the transmembrane segments. Additional proteins play distinct roles in positioning Gβγ or regulating its effect on gating. The main role of Gα subunits is to provide the specificity of signaling and to serve as Gβγ donors, but could extend beyond these functions. Thus, signaling from GPCRs to these channels probably occurs within highly regulated, dynamic multimolecular complexes. Several other ion channels appear to be modulated by direct interactions with G proteins, but more studies are needed before this is firmly established.

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Brassinosteroid signaling in plants

Carsten Müssig and Thomas Altmann

In animals and humans, steroid hormones (SHs) regulate gene transcription via the binding of nuclear receptors. In addition, rapid nongenomic effects of steroids occur and appear to be mediated by plasma-membrane receptors. Plants also use steroids as signaling molecules. These brassinosteroids (BRs) show structural similarity to the SHs of vertebrates and insects. Plant mutants defective in brassinosteroid biosynthesis or perception exhibit dwarfism and reduced fertility, and reveal the need for BRs during growth and development. BR signaling in *Arabidopsis thaliana* and rice (*Oryza sativa*) – dicotyledonous and monocotyledonous models, respectively – is mediated by the receptor kinases BRI1 and OsBRI1. The extracellular domain of BRI1 perceives BRs and the signal is mediated via an intracellular kinase domain that autophosphorylates Ser and Thr residues and apparently has the potential to phosphorylate other substrates. BRI1 transduces steroid signals across the plasma membrane and mediates genomic effects.

Like animal and human steroid hormones (SHs), such as steroid sex hormones, vitamin D or the insect moulting hormone ecdysone, brassinosteroids (BRs) are cholestane derivatives (Fig. 1). Brassinolide[1] (BL) is considered to be the end-product of BR biosynthesis, because it shows the highest biological activity among BRs. In general, BRs such as BL with a 6,7-lacton function, possess higher biological activity than do 6-keto steroids. BRs lacking B-ring oxygen function show minor activity. The biological activities of intermediates correspond to their place in the biosynthetic pathway[2,3]. BRs elicit various physiological responses and are essential for male fertility and xylem differentiation[4,5]. Their growth-promoting effect results primarily from the stimulation of cell elongation and includes induction of the expression of genes encoding proteins such as xyloglucan endotransglycosylases[6,7] (XETs), which are probably involved in cell-wall metabolism and loosening. Moreover, effects such as cell-wall space acidification, appear to contribute to BR-induced growth stimulation. RNA-synthesis inhibitors (e.g. actinomycin D) and protein-synthesis inhibitors (e.g. cycloheximide) interfere with BR-induced growth[8]. BR effects on cell division are less clear; however, the induction of *CycD3* transcription by epi-brassinolide might represent a mechanism by which BRs can drive cell division[9].

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Steroid hormones trigger genomic and nongenomic effects in animals

A steroid effect that occurs within seconds to minutes that is not blocked by inhibitors of transcription or translation and that is mediated by steroids tethered to a membrane-impenetrable carrier protein is likely to be a nongenomic effect. Nongenomic actions of SHs in animals include ion-flux effects, neurotransmitter signaling, changes in second-messenger levels and changes in protein-kinase activities[10–12]. In addition to functional evidence, membrane-binding sites for several steroids have been described, exposing binding features compatible with an involvement in rapid steroid signaling. However, direct evidence of signal transmission and the mediation of specific effects is scarce for any of the proteins. SHs can apparently act through nongenomic and genomic pathways simultaneously. Both pathways are required (e.g. for calcitriol signaling[13]) and might interact (e.g. via second messenger-related modulation of some steroid-induced transcriptional processes).

Genomic steroid actions are characterized principally by delayed responses (i.e. responses taking several minutes or even hours), because synthetic events that alter transcript or protein levels are required. Inhibitors of transcription or translation, such as actinomycin D and cycloheximide, should block genomic effects. Because of their lipophilic nature, SHs pass through the cell membrane by simple diffusion and bind to SH receptors (SHRs) within target cells. SHRs not bound to their ligands are associated with chaperones, keeping them in ligand-friendly conformations. Upon activation by the steroid ligand, SHRs move into the nucleus, bind (mainly as homodimers) to palindromic DNA sequences termed hormone-response elements and activate transcription by interaction with general transcription factors, transcription intermediary factors and other coactivators[14,15]. Some members of the nuclear receptor superfamily (which includes the SHRs) not only stimulate gene expression, but also repress transcription[16–18]. SHRs are regulated by phosphorylation[19], and agents such as peptide growth

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