The calcium-sensing receptor and its interacting proteins

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Abstract

Seven membrane-spanning, or G protein-coupled receptors were originally thought to act through heterotrimeric G proteins that in turn activate intracellular enzymes or ion channels, creating relatively simple, linear signalling pathways. Although this basic model remains true in that this family does act *via* a relatively small number of G proteins, these signalling systems are considerably more complex because the receptors interact with or are located near additional proteins that are often unique to a receptor or subset of receptors. These additional proteins give receptors their unique signalling 'personalities'. The extracellular Ca-sensing receptor (CaR) signals *via* $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$, but its effects *in vivo* demonstrate that the signalling pathways controlled by these α subunits are not sufficient to explain all its biologic effects. Additional structural or signalling proteins that interact with the CaR may explain its behaviour more fully. Although the CaR is less well studied in this respect than other receptors, several CaR-interacting proteins such as filamin, a potential scaffolding protein, receptor activity modifying proteins (RAMPs) and potassium channels may contribute to the unique characteristics of the CaR. The CaR also appears to interact with additional proteins common to other G protein-coupled receptors such as arrestins, G protein receptor kinases, protein kinase C, caveolin and proteins in the ubiquitination pathway. These proteins probably represent a few initial members of CaR-based signalling complex. These and other proteins may not all be associated with the CaR in all tissues, but they form the basis for understanding the complete nature of CaR signalling.

Keywords: calcium-sensing receptor • G protein-coupled receptor • interaction • scaffold • filamin• channel • RAMP • cell signalling

Introduction

The calcium-sensing receptor (CaR), a G protein-coupled receptor that signals through $G\alpha_i$, $G\alpha_q$ and sometimes $G\alpha_{12/13}$ pathways, is best known and understood for its role in regulating the secretion and synthesis of parathyroid hormone in response to extracellular Ca in the parathyroid glands [1]. Like

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many other G protein-coupled receptors, the CaR signals through a defined set G proteins, but also interacts with other proteins that probably give it its unique 'signalling personality'. This receptor was first cloned as an extracellular Ca sensor using a xenopus oocyte expression system. Since that time, it has been detected in most epithelial and mesenchymal cell types including renal and gastrointestinal epithelium, endothelial cells, keratinocytes, breast tissue, osteoblasts, cardiac myocytes and cells of the central and peripheral nervous systems where it can be expressed in non-polarized cells and polarized cells on either the apical or basolateral membrane [2]. The CaR appears to have distinct functions in these different cell types, although the functions are not precisely defined. For example, the CaR can either stimulate cell division (rat-1 cells) or inhibit division and promote differentiation (colonocytes or parathyroid cells) [3–6]. Additionally, its biologic effects in tissues such as the kidney or parathyroid glands cannot be explained completely on the basis of known second messenger signalling effects [1, 7].

The original model of signalling by G protein-dependent receptors was relatively simple. The receptors and G proteins through which the intracellular second messenger systems are activated are attached to the plasma membrane of the cell. These proteins could be relatively free in the membrane or loosely associated with each other. Upon activation of a receptor, the protein interactions change, G proteins are activated, which in turn activate effector molecules such as enzymes or ion channels, to generate intracellular signals (Fig. 1). As techniques to identify protein–protein interactions have been developed and as more precise analysis of cell signalling has become possible, the inadequacy of the original model has become clear through demonstration that G protein-coupled receptors interact with many intracellular proteins in addition to G proteins. These interacting proteins include RGS proteins (Regulator of G protein Signalling), scaffolding and structural proteins, ion channels, additional signalling proteins, chaperone and trafficking proteins and others that may not have defined functions yet [8–11]. Receptors that act through similar sets of G proteins may have different signalling or biologic effects in different regions of a cell and in different cells. This finding suggests that they may have common activities based on the G proteins with which they interact, but that they may also have distinct functions based on the unique sets of other proteins with which they interact as well as their unique locations within a cell. Although no one receptor in a native tissue has been characterized completely, enough work has been done with different receptors including the $\alpha2$-, β-adrenergic and metabotropic glutamate receptors in various experimental systems to indicate that such a scenario is not only plausible, but likely (Fig. 1) [9–11].
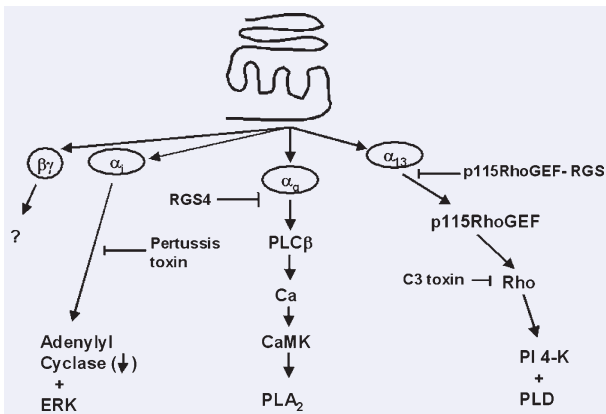
Signalling by the CaR

Most work on the signalling pathways controlled by the CaR has focused on traditional G protein-coupled pathways, $G\alpha_i$, $G\alpha_q$, and in some cases $G\alpha_{12}$ or $G\alpha_{13}$ [1, 12–16] (Fig. 2). Through $G\alpha_i$, the CaR inhibits adenylyl cyclase and activates extracellular signal-regulated kinase (ERK), through $G\alpha_q$ it activates phospholipase C, increases Ca_i and DAG (diacyl glycerol) levels, and activates phospholipase A_2, and through $G\alpha_{12/13}$, it activates Rho and phospholipase D [17]. Although the full physiologic significance is not understood, activation of the CaR initiates Ca oscillations *via* a $G\alpha_q$-dependent mechanism that when prolonged or forming a plateau, inhibit adenylyl cyclase activity co-ordinately with $G\alpha_i$ activation [14]. This system serves as an active turn-off system for cAMP signals and depends on the forms of adenylyl cyclase expressed in different tissues. In the intestine, the CaR inhibits cholera toxin and *E. coli* heat stable enterotoxin-stimulated fluid secretion *via* activation of cyclic nucleotide phosphodiesterases and inhibition of NKCC1 (sodium, potassium, 2Cl transporter-1) activity [18]. In keeping with studies of other G protein-coupled receptors, the CaR transactivates, the epidermal growth factor receptor presumably *via* a matrix metalloproteinase [19, 20]. In different regions of the nephron and gastro-intestinal tract, the CaR is expressed on apical or basolateral membranes of epithelial cells where it is likely to come into contact with distinct sets of proteins that should give the CaR different signalling characteristics and biologic effects. Similarly, the CaR is expressed in many different cell types with different functions, so its signalling and biologic functions could vary from cell type to cell type.

Distinct effects of angiotensin II and Ca receptors

A good example of the unique signalling and physiologic activities of the CaR is found in the distal
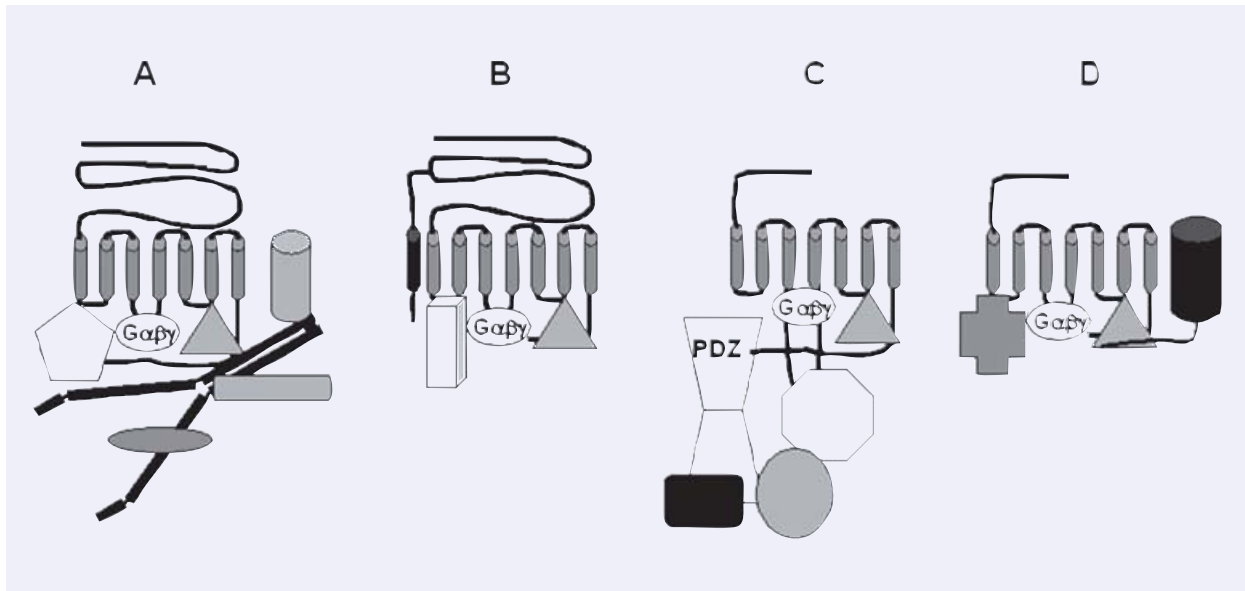
Fig. 1 Four scenarios in which all G protein-coupled receptors interact with G$\alpha\beta\gamma$ subunits that regulate standard second messenger generation and other common proteins (grey triangle, *e.g.* arrestin). They also interact with additional different proteins that give them unique signalling personalities. In **A**, the receptor interacts with filamin that itself binds additional proteins. The receptor shown in **B** interacts with an accessory protein (*e.g.* a RAMP) as well as another unique protein. The receptor shown in **C** has a long third intracellular loop that interacts with a unique protein (octagon), and a long C-terminus with a PDZ domain that binds a PDZ protein that itself brings additional proteins into the complex. In the scenario shown in **D**, the C-terminus of another membrane protein (*e.g.* a channel) interacts with the C-terminus of the receptor, and the receptor binds an additional protein.

Fig. 2 A schematic diagram of the principal second messenger signalling pathways that have been described for the CaR. Most of these studies pathways were identified in heterologous expression systems, and may not all exist in all cells where the CaR is expressed at all times.

nephron of the kidney where activation of the CaR results in significant Na, K, Ca, Mg, Cl and H_2O loss. Although the CaR acts *via* Gα_i, Gα_q and G$\alpha_{12/13}$, its biologic effects in the distal nephron cannot be explained solely on this basis. A number of G protein-coupled receptors that also act *via* Gα_i, Gα_q and G$\alpha_{12/13}$ are also expressed in the distal nephron, including those for angiotensin II (AT1), bradykinin (B2) and endothelin (ET$_B$). All of these receptors inhibit cAMP production, stimulate phospholipase C (with increases in Ca$_i$ and protein kinase C activity, and decreases in PIP$_2$ levels), and stimulate phospholipase A$_2$ (with increases in 20-HETE and other arachidonic acid metabolites). In all studies and experimental systems, activation of the CaR results in physiologically significant inhibition of Na transport in the distal nephron, while activation of the AT1 receptor (presumably AT1) can stimulate or inhibit transport depending on the concentration, experimental system and study and the effects are minor

Table 1 Proteins with which the CaR interacts. Table 1 lists the CaR interacting proteins that have been described so far alphabetically in the first column. The second column lists the function of the interacting protein. The third column lists the experimental basis for the interaction of a protein with the CaR, Y2H for yeast two hybrid, Co-IP for co-immunoprecipitation, GST for GST pull-down assays and functional if expression or mutagenesis studies indicate that the interaction is functionally significant. The fourth column (CaR domain) identifies the region of the CaR that interacts with the protein shown at left. The fifth column states whether co-localization studies of the CaR were performed with the interacting protein. Native means that studies were performed in native tissue, and Het means that studies were performed in heterologous expression systems. The sixth column provides essential references for the observations

Interacting Protein	Function	Interaction	CaR Domain	Co-localization	Reference
AMSH	Trafficking/de Ub enzyme	Y2H, GST	C-term		(80)
β-Arrestin	Trafficking/signalling	Functional	?		(77)
Caveolin-1	Structural/scaffolding	Co-IP	?	Native, Het	(81; 84)
E3 Ub ligase	Trafficking	Y2H, IP, Functional	C-term	Het	(79)
Filamin	Scaffolding/structural/-trafficking	Y2H, Co-IP, GST, functional	C-term	Native, Het	(15; 40; 41)
GRK-2	Signalling	Functional	?		(77)
GRK-4	Signalling	Functional	?		(77)
Kir4.1	K channel	Y2H, Co-IP, functional	C-term	Native, Het	(54)
Kir4.2	K channel	Y2H, Co-IP, functional	C-term	Het	(54)
PI-4-kinase	Signalling	Co-IP	?		(71)
PKC	Signalling	Functional	C-term		(77)
RAMP1	Structural/trafficking	Co-IP, Functional	?ECD and TM	Het	(37)
RAMP3	Structural/trafficking	Co-IP, Functional	?ECD and TM	Het	(37)
RGS proteins	Signalling	Functional	?		(16; 71)
Rho	Signalling	Co-IP	?		(71)

[21–31, 31]. These differences between the responses of the distal nephron to Ca and AII indicate that the two receptors have distinct effects on cells despite the fact that they stimulate production of the same second messengers.

The unique signalling characteristics and biologic effects of the CaR may be explained by its interactions with proteins in addition to G protein α subunits (Table 1). G protein-coupled receptors have four domains that are exposed to the intracellular space and that are available to interact with other proteins, three intracellular loops that connect transmembrane (TM) domains 1 and 2 (intracellular loop 1 or IC1), TM domains 3 and 4 (IC2), TM domains 5 and 6 (IC3) and the C-terminus. The intracellular loops, particularly IC2 and 3 interact with G proteins as well as other proteins including proteins involved in sig-

nalling, such as the arrestins or spinophilin [10]. In G protein-coupled receptors, the size of the loops and C-termini are highly variable. In the CaR, IC1 and 3 are 14 AA, IC2 is 24 AA, all relatively short and the C-terminus is 220 AA, relatively long. Because of its size, the C-terminus is easier to use for techniques to identify interacting protein such as yeast two hybrid cloning. For the CaR, as well as the better-characterized metabotropic glutamate receptors (both have a similar structures), interacting proteins have generally been identified using yeast two hybrid assays with tissue-specific cDNA libraries and the C-terminus as bait. The interactions are verified with co-immunoprecipitation and the functional interactions are characterized in relatively generic expression systems, such as HEK-293 cells. Although these CaR-interacting proteins have not been characterized completely

in native tissue, enough data exist to believe that the interactions are real, and that the biologic functions they contribute are also likely to be real. Interactions with other proteins have been identified presumptively based on the fact that they are receptor interacting proteins and affect the behaviour of the CaR. A number of these interacting proteins such as β-arrestin or receptor kinases are common to many other G protein-coupled receptors, or are involved in trafficking and degradation, and so are unlikely to be responsible for distinct signalling features of the CaR. Consequently, we will focus on three sets of proteins, receptor activity modifying proteins (RAMPs), filamin, a potential scaffolding protein, and two inwardly rectifying potassium channels, Kir4.1 and Kir4.2 that through their interactions with the CaR, could begin to explain some of its unique signalling characteristics.

RAMPS (Receptor Activity Modifying Proteins)

The affinity of the CaR for its agonists or calcimimetics (agents that sensitize the CaR to activation by agonists), appears to depend on the cell type where it is expressed. In the parathyroid gland and parathyroid cells, the EC_{50} for Ca is approximately 1.0 mM, while for the CaR expressed in cultured cells, the EC_{50} for Ca is on the order of 3.5 mM [32, 33]. In keratinocytes, the EC_{50} for induction of a differentiation marker, involucrin (presumably a CaR-mediated event), by extracellular Ca is approximately 0.1 mM [3]. Parathyroid cells are forty times more sensitive to cinacalcet for inhibition of parathyroid hormone secretion than are thyroid C cells to stimulation of calcitonin release [34]. This variability in CaR responsiveness could be explained by its interaction with different sets of intracellular proteins or accessory proteins such as RAMPs , a family of proteins that affect trafficking, glycosylation, ligand specificity and second messenger production by the receptors with which they interact [35].

The RAMP family currently has three members, RAMPs1–3. RAMP1 was discovered using xenopus oocyte expression cloning to identify an accessory protein for the calcitonin-like receptor (CLR) that could explain differences in ligand binding and signalling observed *in vivo* and in different expression systems [35]. RAMP1 is a 148 AA protein with a long extracellular domain, a single TM domain and a short cytoplasmic tail that interacts with both the calcitonin receptor (CTR) and CLR. In these settings, RAMP1 acts as a chaperone permitting cell surface expression and alters the affinity as well as the selectivity of these receptors for agonists. RAMP2 and RAMP3 were subsequently identified using database searches and have similar structures and effects on the CTR and CLR. RAMP3 has a C-terminal PDZ domain, so it may have different functions from RAMP2 and 3. The RAMPs interact with their target receptors *via* their extracellular N-termini and TM domains. The reasons why RAMPs interact with some GPCRs and not others are not known.

This family of proteins is relatively new and has not been studied extensively, so their tissue distributions, subcellular localizations and catalogue of receptors with which they interact are not fully defined, although RAMPs are expressed in all tissues studied to date [35, 36]. The level of RAMP expression changes in physiologic states and disease models, so receptor activity could be modulated by this mechanism [36, 37]. Most work has focused on the CTR, the CLR and their ligands calcitonin, adrenomedullin, calcitonin gene-related peptides (CGRP) 1 and 2 and amylin. Adrenomedullin and the CGRP affect vascular tone and blood pressure [38, 39]. RAMPs interact with other class 2 GPCRs, the vasopressin/pituitary adenylate cyclase-activating peptide (VPAC)-1, parathyroid hormone1, parathyroid hormone 2 and glucagons receptors, but the functional and physiologic consequences of these interactions are not known [35].

The CaR, a class 3 G protein-coupled receptor, interacts with RAMP1 and RAMP3 [37]. The initial observation was that the CaR could not reach the cell surface in COS7 cells. Subsequently, the investigators found that COS7 cells do not express any of the known RAMPs. Interaction of the CaR with one of these RAMPS is required for cell surface expression. In the absence of RAMP1 or 2, the CaR is trapped in the endoplasmic reticulum in an immature core glycosylated form. The RAMPs facilitate its exit from the endoplasmic reticulum, and its transit to the Golgi where it is glycosylated and then moves to the cell surface. The functional consequences of interaction with RAMP1 or RAMP3 were not tested, so it is possible that interaction with the two different RAMPs leads to different CaR activation kinetics. The fact that RAMP3 has a C-terminal PDZ domain means

that it could also contribute to localization of the CaR in the cell and with other signalling proteins. For example, RAMP-3 interacts with N-ethylmaleimide-sensitive factor (NSF) *via* its PDZ domain. Interaction with NSF is required for recycling of the agonist-occupied CLR and β2-adrenergic receptor [9, 35].

Filamin

At least two groups identified filamin as a CaR interacting protein that could serve as a scaffold for other signalling proteins [40, 41]. Filamin is a homodimer made up of 280 kD proteins that contain N-terminal actin-binding domains, 24 96 AA IgG-like repeats, a C-terminal dimerization domain (repeat 24) and two hinge regions [42, 43]. Filamin was originally described as an actin cross-linking protein that provides mechanical strength to the actin cytoskeleton. Subsequently, it was found to interact with a number of TM proteins (many of them signalling proteins) and anchor them and the plasma membrane to the cytoskeleton. A partial list of these proteins includes β integrins, Ca and K channels, a subset of G protein-coupled receptors (the D2 dopamine receptor, the CTR, some metabotropic glutamate receptors, and the CaR), and the insulin receptor [11, 42]. Filamin also interacts with intracellular signalling proteins including MAP kinases, Rho GTPases, Rho guanine nucleotide exchange factors (GEFs), Rho kinase, SMADS and phosphatases [42, 43]. Filamin appears to be involved in organization of these proteins in the cell and in their trafficking in that under the correct experimental conditions, loss of filamin or interference with its interaction with the protein of interest results in altered membrane expression and lost or reduced function [44, 45].

A problem in understanding the function of filamin is that its distribution in various differentiated cells has not been fully determined. Despite the fact that filamin is generally considered a cytoskeletal protein, most of it is found in the soluble fraction of hepatocytes, endothelial cells and presumably other cell types [46–48]. This distribution suggests that filamin may have a role in processes, such as protein trafficking in addition to anchoring proteins to the cytoskeleton and plasma membrane. One recent study indicates that in polarized epithelial cells, filamin may be preferentially expressed in the apical membrane, suggesting that in polarized cells, filamin's scaffolding function may be more important at the apical surface [49].

The mid-portion of the CaR C-terminus interacts with the region of filamin that contains repeats 15–17 and hinge 1 based in studies utilizing yeast two hybrid, co-immunoprecipitation and GST-fusion protein-binding assays. In the absence of filamin or when the interaction of filamin and the CaR is blocked, the CaR does not activate ERK or Jun N-terminal kinases (JNK) appropriately [40, 41, 50]. This interaction is also important for CaR-mediated Rho activation because expression of peptides that interfere with the CaR-filamin interaction block CaR-mediated Rho activation [15]. The simplest explanation for these observations is that filamin contributes to cell surface expression of the CaR. This is certainly the case, but even in the absence of filamin some CaR reaches the cell surface [51]. Evidence that filamin also provides an important scaffolding function comes from studies of Ca or Phe-induced CaR-mediated intracellular Ca oscillations [12]. Ca and Phe stimulate Ca oscillations with different patterns. The oscillations stimulated by Phe require $G\alpha_{12/13}$, Rho, TrpC1, an intact cytoskeleton and filamin, while the Ca-stimulated oscillations persist with disruption of the interactions of filamin and the CaR [12, 52]. Although the precise mechanism by which filamin is involved in CaR-mediated inhibition of parathyroid hormone secretion on the apical surface of parathyroid cells is not defined, an intact cytoskeleton is required [53]. If filamin is primarily found on the apical surface of epithelial cells, its interaction with the CaR may be important for those cell types (*e.g.* renal proximal tubule, intestinal and parathyroid cells) where the CaR is expressed on the apical surface. Other interacting proteins may contribute to its function in other cell locations.

Potassium channels

The C-terminus of the CaR interacts with and inactivates two inwardly rectifying K channels, Kir4.1 and Kir4.2 in the kidney that are expressed in the distal nephron (thick ascending limb of Henle and distal convoluted tubule) as well as other tissues [54]. Four Kir subfamilies, Kir2.x, Kir4.x, Kir5.x and Kir7.x, are expressed on the basolateral membrane of the distal

nephron where the CaR is also expressed [55–58]. A number of channels undoubtedly contribute to the distal nephron basloateral K conductance, but studies indicate that the biophysical properties of a component of it are compatible with homomeric Kir4.1, Kir4.2, or heteromeric Kir4-Kir5.1 channels [55, 59, 60]. These channels are probably involved in recycling K for Na,K-ATPases (and possibly H,K-ATPases) and regulating membrane potential.

G protein-dependent signalling systems regulate Kir channels by a number of mechanisms that involve protein–protein interactions and second messengers including release of $G\beta\gamma$ subunits that interact directly with Kir3 channels (GIRK) to activate them, and inhibition of Kir3 channels by RGS (regulator of G protein signalling) proteins [61–69]. The β_2 adrenergic and dopamine D_2 and D_4 receptors interact directly with heteromeric Kir3 channels (Kir3.1/3.4 and Kir3.1/3.2), but the physiologic consequences of these interactions are not defined [70]. Control of inositol lipid metabolism by PLC and PI kinases regulate Kir channel activity by determining the level of PIP_2, a lipid that is required for channel activity [69].

The CaR interacts directly and selectively with Kir4.1 and Kir4.2 as demonstrated with yeast two hybrid assays and co-immunoprecipitation. The channels can be co-immunoprecipitated from kidney cortex, and Kir4.2 and the CaR can be co-immuno-precipitated from heart and liver [54]. The reason for this association may be for regulation of channel activity by direct interaction with the receptor, to localize PIP_2 metabolism near the channel, (the CaR interacts with and regulates PI4-kinase and regulates PLC) or for trafficking to affect the stochiometry of the receptor channel complex [54, 71]. Kir4.1 and Kir4.2 have C-terminal PDZ domains, and may be organized in multi-protein complexes on that basis.

Other CaR-interacting proteins

Although frequently overlooked in the category of interacting proteins, G protein-coupled receptors interact with each other to form homodimers and with other receptors to form heterodimers [10]. These interactions are important for cell surface expression and determining sensitivity to agonists. The CaR forms homodimers in the ER *via* interactions of cystienes in the extracellular domain and this dimer-ization is important for cell surface expression [72–74]. Hetrodimerization with other receptors has not been demonstrated.

Interaction of the three intracellular loops of the CaR with other proteins has not been specifically demonstrated, but can be inferred from functional studies and knowledge of the behaviour of the potential interacting proteins. The CaR must make contact with the G protein α subunits through which it acts, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ [1]. Mutagenesis of the second and third intracellular loops and naturally occurring mutants demonstrate that these loops contribute to activation of, and presumably interact directly with $G\alpha_q$ and $G\alpha_i$ because mutants are defective in PLC activation, a $G\alpha_q$-dependent event [75, 76]. These loops are usually sites of phosphorylation by G protein receptor kinases (GRKs) in other receptors, and sites for interaction with proteins like arrestin and spinophilin [10]. GRKs recognize, bind to and phosphorylate the agonist-occupied receptor. Arrestins then bind to the phosphorylated receptor, block interaction with G proteins and initiate termination of the signal as well as internalization of the receptor [9, 10]. Arrestins are also capable of activating the ERKs *via* Src kinase in a G protein-independent manner. Based on the fact that over-expression of GRK 2 or 3 and β-arrestins 1 or 2 reduces CaR signalling, these proteins probably interact with the CaR, but the sites of phosphorylation and of interaction were not defined, and interaction was not specifically demonstrated directly [77]. Precisely how interactions with GRKs and arrestins affect CaR signalling and trafficking have not been studied.

In addition to filamin and the K channels, Kir4.1 and Kir4.2, a number of other proteins interact with the CaR C-terminus. The C-terminus is phosphorylated by, and so must interact with protein kinase C. This phosphorylation at Thr 888 results in reduced intracellular Ca store release [78]. PKC activity is required for PLC-mediated Ca oscillations [12]. PKC-mediated CaR phosphorylation may also be important for mediating the effects of β-arrestins because signalling by a mutant CaR that lacks PKC phosphorylation sites is not affected by expression of β-arrestins [77]. The degradation of the CaR is mediated by the ubiquitin system through interactions with the E3 ubiquitin ligase, dorfin and AMSH (Associated Molecule with the SH3 domain of STAM), a deubiquitinating enzyme [79, 80]. Both interactions were initially identified using yeast two hybrid cloning and

co-immunoprecipitation or GST pull-down assays. As expected and consistent with effects of ubiquitination of other G protein-coupled receptors, over-expression of dorfin resulted in reduced levels of CaR protein while a dominant negative dorfin construct increased CaR protein. Dorfin can mediate degradation of immature CaR protein from the ER [79]. Although expression of AMSH would be expected to increase CaR protein expression, it had the opposite effect [80].

The CaR interacts with several other proteins, but the region of the CaR responsible for the interaction is not defined. Caveolin, a protein that also interacts with filamin, co-immunoprecipitates with, and co-localizes with the CaR in parathyroid cells [81, 82]. Caveolae are membrane microdomains enriched in cholesterol and glycosphingolipids that in contrast to lipid rafts also contain caveolin. These structures concentrate the components of G protein signalling systems, and caveolin may be involved in stable membrane expression of these proteins as well as endocytosis and sorting [83]. Precisely how its presence in caveolae or its interactions with caveolin affect CaR function are not defined, but the correlation of reduced caveolin and CaR expression in parathyroid adenomas suggests that the interaction may be important functionally [84]. Filamin may also contribute to these interactions [82]. The CaR co-immunoprecipitates with PI-4-kinase and rho and stimulates PI-4-kinase activity [71]. This arrangement of proteins could result in a signalling module that regulates the metabolism of inositol lipids in a co-ordinated manner and in a limited region of the cell to precisely control processes such as channel activity, or it could contribute to protein trafficking that can also depend on inositol lipid metabolism [9, 54, 69]. Regulators of G protein signalling (RGS) proteins have been implicated in CaR signalling through expression studies, but interactions of the CaR or a CaR-based signalling complex with specific RGS proteins have not been demonstrated [16, 71].

Conclusions

G protein-coupled receptors act not only through heterotrimeric G proteins, but though additional proteins that may interact directly with the receptor or be brought into proximity by scaffolding or structural proteins. A long-standing question has been why so many receptors exist to signal through the same G proteins. Part of the answer is that signalling by receptors through subsets of G proteins represents a common characteristic of many receptors, but that the unique signalling and biologic characteristics of receptors are determined by the additional restricted, and possibly unique proteins with which an individual receptor interacts.

Studies of proteins that interact with the CaR are less advanced than studies of $\alpha 2$-, β-adrenergic or metabotropic glutamate receptors, but some common themes are becoming clear. G protein-coupled receptors interact with each other in the ER, an interaction that is important for trafficking and cell-surface expression. These receptors also interact with a restricted set of G proteins, although the location for the initial interaction is not established. Signalling by this class of receptors involves RGS proteins that participate in signal termination, can initiate signals (*e.g.* Rho GEF), and that can contribute to the specificity of G protein-receptor interactions. G protein-coupled receptors interact with GRKs and second messenger-dependent kinases (*e.g.* protein kinase C and protein kinase A), and arrestins as a part of the signal termination process and for receptor trafficking. The placement of these receptors in cells is specific and is determined by interactions with other proteins that may have structural, scaffolding, signalling, or mixed functions, and that may contain motifs such as PDZ domains. Many interacting proteins such as arrestins have multiple functions that include signalling and trafficking, so these aspects of receptor biology may not be separable.

Based on current information, several interactions may distinguish the CaR from other $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$-coupled receptors. Filamin appears to serve as a scaffolding protein that also affects CaR trafficking. The scaffolding function is probably important in all cells, but particularly so on the apical surface of polarized cells were filamin is enriched. Although the significance of the interaction with RAMPs1 and 3 is not fully established, these proteins have the potential to affect localization and signalling by the CaR. The ability of the CaR to interact directly with ion channels (Kir4.1 and Kir4.2) in tissues could permit tight control over channel activity through direct protein–protein contact or with minimal dispersion of second messengers in the cell. Undoubtedly, additional interacting proteins will be discovered that will

provide a more complete understanding of the function of the CaR and other similar receptors.

Acknowledgements

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