REVIEW ARTICLE

Protein–protein interactions in intracellular Ca^{2+}-release channel function

John J. MACKRILL[1]

Department of Biochemistry, University College Cork, National University of Ireland, Cork, Ireland

Release of Ca^{2+} ions from intracellular stores can occur via two classes of Ca^{2+}-release channel (CRC) protein, the inositol 1,4,5-trisphosphate receptors (InsP_3Rs) and the ryanodine receptors (RyRs). Multiple isoforms and subtypes of each CRC class display distinct but overlapping distributions within mammalian tissues. InsP_3Rs and RyRs interact with a plethora of accessory proteins which modulate the activity of their intrinsic channels. Although many aspects of CRC structure and function have been reviewed in recent years, the properties of proteins with which they interact has not been comprehensively surveyed, despite extensive current research on the roles of these modulators. The aim of this article is to review the regulation of CRC activity by accessory proteins and, wherever possible, to outline the structural details of such interactions. The CRCs are large transmembrane proteins, with the bulk of their structure located cytoplasmically. Intra- and inter-complex protein–protein interactions between these cytoplasmic domains also regulate CRC function. Some accessory proteins modulate channel activity of all CRC subtypes characterized, whereas other have class- or even isoform-specific effects. Certain accessory proteins exert both direct and indirect forms of regulation on CRCs, occasionally with opposing effects. Others are themselves modulated by changes in Ca^{2+} concentration, thereby participating in feedback mechanisms acting on InsP_3R and RyR activity. CRCs are therefore capable of integrating numerous signalling events within a cell by virtue of such protein–protein interactions. Consequently, the functional properties of InsP_3Rs and RyRs within particular cells and subcellular domains are 'customized' by the accessory proteins present.

Key words: accessory proteins, inositol 1,4,5,-trisphosphate receptor, ryanodine receptor.

INTRODUCTION

Release of Ca^{2+} ions from intracellular membrane systems is a key step in many signal-transduction processes and plays a role in the aetiology of certain pathological states [1]. Two multigene families of intracellular Ca^{2+}-release channel (CRC) proteins have been extensively characterized over the past decade. The mechanisms by which extracellular stimuli activate such CRCs are summarized in Figure 1. Ryanodine receptors (RyRs) are gated by allosteric coupling to plasmalemmal voltage-operated Ca^{2+} channels (VOCCs), or by Ca^{2+} itself (Figure 1a), and bind the neutral plant alkaloid ryanodine, a toxin used extensively in the biochemical and functional characterization of these channel proteins [2]. Ryanodine binding is used as an indirect measure of the RyR channel activity, since antagonists/agonists influence both the interaction of this alkaloid and the opening of these CRCs in a similar manner. Inositol 1,4,5-trisphosphate receptors (InsP_3Rs) are so named because they are activated by InsP_3 (Figure 1b), a second-messenger molecule [3]. The primary structure of SCaMPER (sphingolipid Ca^{2+}-release-mediating protein from endoplasmic reticulum), a third candidate class of CRC, has recently been deduced [4], although the properties of this channel have not been thoroughly investigated.

The RyRs and InsP_3Rs display approx. 70% amino acid identity between isoforms within each family, as well as limited identity between the two families. InsP_3R monomers consist of about 2700 amino acid residues, whereas RyR polypeptides are composed of approx. 5000 residues. Both associate as tetrameric complexes of these high-molecular-mass protein monomers, forming high-unitary-conductance, low-selectivity cation channels. Both channel families are activated by Ca^{2+} itself in a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (Figure 1), although different classes and isoforms display distinct Ca^{2+}-dependencies for this response [5]. The three mammalian InsP_3R isoforms characterized show a broad tissue distribution, whereas the three RyR subtypes were originally thought to be expressed only in skeletal muscle (type 1 RyR), heart (type 2 RyR) and brain/epithelial cells (type 3 RyR) [6]. However, it has become apparent that RyRs are also expressed in a diverse range of tissues [7]. Both InsP_3Rs and RyRs are present in numerous cell types [8,9], indicating that there are important differences between these two channel families in terms of cell function, or that there is redundancy in cellular Ca^{2+}-release mechanisms. Further structural diversity of CRCs arises from alternative splicing of the mRNAs encoding them, as well as assembly of heterotetrameric complexes in the case of the InsP_3Rs [10].

In some tissues, CRCs are located in highly specialized domains of the endomembrane system. For example, in striated muscles, RyRs are highly enriched in endings of the sarcoplasmic reticulum (SR) called terminal cisternae (TC), which are closely apposed to infoldings of the plasmalemma termed t-tubules. Such membrane junctions ('dyads' and 'triads', consisting of single/paired TC

Abbreviations used: AKAP, protein kinase A anchor protein; cADPr, cyclic ADP-ribose; CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; CICR, Ca^{2+}-induced Ca^{2+} release; CRC, Ca^{2+}-release channel; CT1, cardiac triadin 1; DHPR, dihydropyridine receptor; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid; ER, endoplasmic reticulum; FKBP, FK506-binding protein; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HCP, histidine-rich Ca^{2+}-binding protein; InsP_3, inositol 1,4,5-trisphosphate/receptor; InsP_3R, InsP_3 receptor; InsP_4, inositol 1,3,4,5-tetrakisphosphate; InsP_4R, InsP_4 receptor; KAP, kinase anchor protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TC, terminal cisternae; 7-TMR, seven-transmembrane-segment receptor; VOCC, voltage-operated Ca^{2+} channel.

[1] e-mail STBI8042@ucc.ie

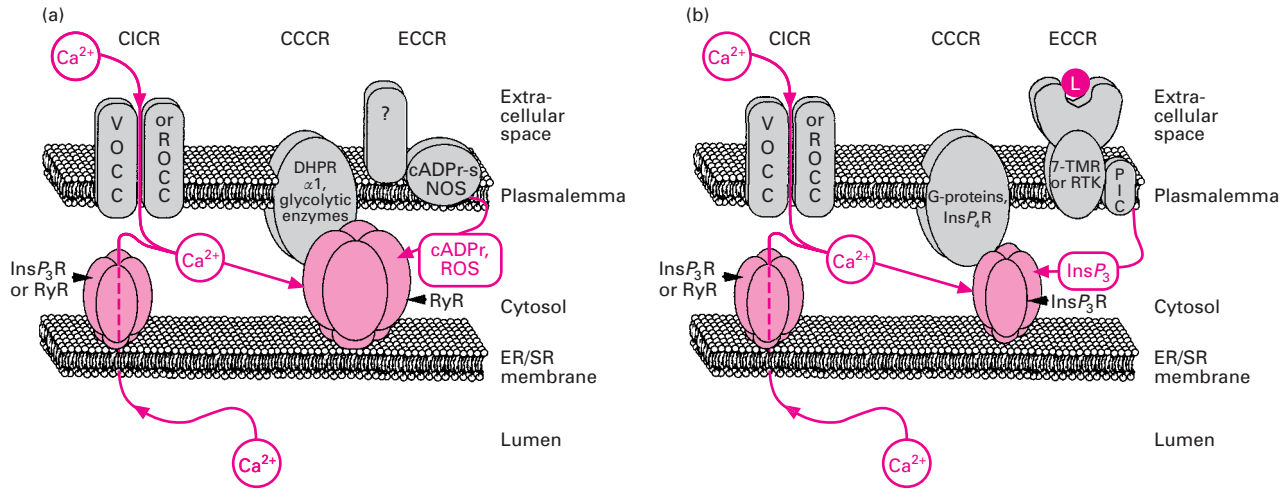

Figure 1 Potential mechanisms by which (a) RyRs and (b) InsP_3Rs are coupled to extracellular signals

CRCs are coupled to extracellular signals by three distinct mechanisms. (i) In CICR, CRCs are activated by 'triggering Ca^{2+}' released from the SR/ER lumen via RyRs or InsP_3Rs, or entering from the extracellular space via receptor-operated Ca^{2+} channels (ROCCs), voltage-operated Ca^{2+} channels [VOCCs, as in the dihydropyridine receptor (DHPR) in heart] or other plasmalemmal Ca^{2+} channels. (ii) Conformationally coupled Ca^{2+} release (CCCR) involves the stimulation of CRCs via direct or indirect allosteric interactions with plasmalemmal proteins, such as the skeletal-muscle DHPR α1 subunit, G-proteins or InsP_3Rs. (iii) In enzymically coupled Ca^{2+} release (ECCR), enzymes linked to signalling events at the plasmalemma increase the intracellular concentrations of signalling species, such as InsP_3, NO, reactive oxygen species (ROS) and cyclic ADP-ribose (cADPr). This type of transduction is typified by InsP_3, whose production is catalysed by phosphoinositidase C (PIC) isoenzymes coupled to the binding of extracellular ligands (L) either to plasmalemmal 7-transmembrane receptors (7-TMRs) via heterotrimeric G-proteins or to receptor tyrosine kinases (RTKs) via protein phosphorylation events, and which diffuses through the cytoplasm to activate InsP_3Rs. Abbreviation: NOS, NO synthase. CADPr-s is cyclic ADP-ribose synthase.

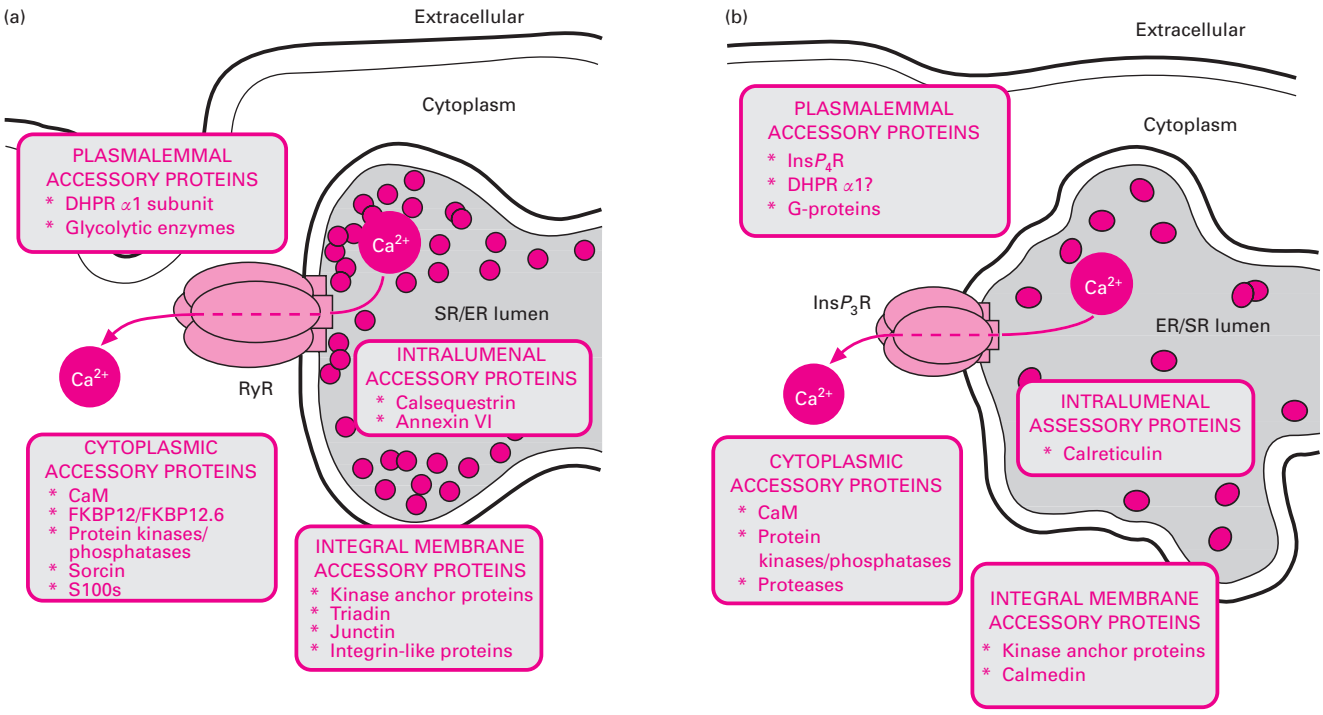

Figure 2 Categorization of CRC accessory proteins according to subcellular localization

CRC accessory proteins are categorized according to their reported subcellular locations. Many of these protein modulators are shared by both RyRs and InsP_3Rs, whereas others only regulate one particular class, or even one particular isoform, of CRC. In other cases, the effects of accessory proteins have only been examined for one type of CRC.

apposed to t-tubules) permit functional (in heart) or allosteric (in skeletal muscle) coupling between plasmalemmal dihydropyridine receptor (DHPR) VOCCs and RyRs in the SR. The InsP_3Rs and RyRs possess similar transmembrane topology, as predicted from their primary amino acid structures. Both families are suggested to have a small cytoplasmic C-terminus, between four and twelve segments spanning the endoplasmic reticulum (ER) or SR, and the N-terminal bulk of the protein ($\sim 80\%$ of the structure) forming a large cytoplasmic domain. This N-terminal domain of the CRCs is a major site of interaction with

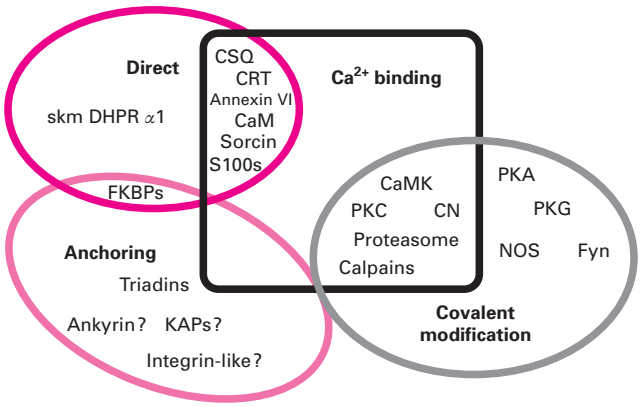

Figure 3 Categorization of CRC accessory proteins according to function

Proteins which modulate CRCs covalently modify them, mediate effects by direct protein–protein interactions, anchor other accessory proteins within their vicinity or respond to changes in Ca^{2+} concentration. This categorization is limited, because some accessory proteins influence CRC activity by several mechanisms. Abbreviations: CN, calcineurin; CRT, calreticulin; CSQ, calsequestrin; NOS, NO synthase; skm, skeletal muscle.

modulators of channel function, such as solutes and accessory proteins. Many of these modulators are shared by both channel families, whereas others display class- or even isoform-specific interactions.

Although the pharmacological, structural, cell-biological and electrophysiological aspects of CRCs have been reviewed extensively in recent years, the properties of the accessory proteins that modulate their activity have not been comprehensively surveyed. This is despite extensive current research into the roles of such CRC-associated proteins. The aim of this review is to examine the roles of protein–protein interactions in CRC function. Such interactions include not only those with accessory proteins, but also those between and within individual CRC complexes. The diversity of interactions between CRCs and their associated proteins suggests that they represent key sites for the integration of intracellular signals. In this review, CRC accessory proteins are categorized according to their subcellular localization (Figure 2). CRC modulatory proteins might also be grouped according to their functional features, but this is problematic, since several have multiple effects on these channel proteins (Figure 3).

ER/SR INTRALUMENAL MODULATORY PROTEINS

Calsequestrins and calreticulin

Calreticulin and calsequestrins are structurally related high-capacity, moderate-affinity, non-EF-hand Ca^{2+} storage proteins, predominantly located within the lumen of endomembrane systems [11]. These proteins bind up to 40 mol of Ca^{2+} per mol of protein via stretches of acidic amino acids, and undergo large conformational changes upon doing so. Calreticulin and calsequestrins also possess single high-affinity, low-capacity Ca^{2+}-binding sites, postulated to mediate regulatory functions. Within striated muscles, calsequestrins are highly enriched within TC, leading to the hypothesis that they sequester Ca^{2+} to sites of Ca^{2+} release.

The rate of Ca^{2+} release from skeletal-muscle SR is dependent on the Ca^{2+} loading of these membranes, which in turn is largely dependent on Ca^{2+} binding to calsequestrin. When skeletal-muscle SR is treated with RyR agonists, there is a transient increase in the intralumenal free Ca^{2+} concentration, which precedes the expected decrease as Ca^{2+} is released, suggesting reciprocal coupling between the RyRs and intralumenal Ca^{2+}-storage proteins [12]. These changes in intralumenal Ca^{2+} concentration are abolished by removal of calsequestrin from the SR and are partially restored by its re-addition. Furthermore, the intensity of fluorescence from skeletal muscle TC proteins labelled with a conformation-dependent probe is partially dependent on the presence of calsequestrin, which is not in itself labelled. One of these fluorescently tagged proteins corresponds to the type 1 RyR [13], indicating communication between calsequestrin and this CRC. More directly, micromolar concentrations of calsequestrin increase the open-probability of purified type 1 RyRs in the presence of millimolar Ca^{2+}, when added to the *trans* side of planar lipid bilayers, corresponding to the intralumenal environment [14]. Overexpression of cardiac calsequestrin in transgenic mice results in a phenotype with severe cardiac hypertrophy [15]. Cardiomyocytes from these mice display decreased spontaneous or Ca^{2+}-influx-induced RyR activity, but Ca^{2+} release induced by the RyR agonist caffeine is of 10-fold greater amplitude than in controls. These results indicate that cardiac calsequestrin plays pivotal roles both in Ca^{2+} storage and in regulation of Ca^{2+} release via the type 2 RyR. Calsequestrin might bind directly to RyR channels, and/or via intermediate proteins, such as the triadins and junctin. Calsequestrin co-sediments with the type 1 RyR upon solubilization and sucrose-density-gradient centrifugation, indicating that these proteins might be physically associated [16]. Indeed, calsequestrin can be chemically cross-linked on to the type 1 RyR in skeletal-muscle triad junctions [17].

The roles of calreticulin in Ca^{2+} storage and CRC regulation are controversial. In addition to its putative role in Ca^{2+} storage, calreticulin modulates integrin function at the plasmalemma and transcription factors within the nucleus, and also acts as a molecular chaperone within the ER [18]. This indicates that calreticulin is not strictly confined to the ER subcellular compartment. In overlay assays, ^{125}I-calreticulin binds specifically to membrane proteins of $\sim$ 20–38 kDa and $\sim$ 50 kDa in liver ER, as well as to a 30 kDa protein in skeletal-muscle SR [19]. Although the identity of these proteins has not been investigated, they might represent triadin/junctin homologues, anchoring calreticulin to sites of Ca^{2+} release. In some experimental systems, the $Ins P_3R$ channels, like the RyRs, show dependence on intralumenal Ca^{2+} loading. Calreticulin inhibits repetitive $Ins P_3$-dependent oscillations in the cytosolic Ca^{2+} concentration when overexpressed in *Xenopus* oocytes, indicating that it might be coupled to $Ins P_3Rs$ [20]. This inhibition appears to be mediated via calreticulin's high-affinity regulatory Ca^{2+}-binding site, rather than by its low-affinity, high-capacity Ca^{2+} storage sites. Overexpression of calreticulin in mammalian cell lines leads to enhanced Ca^{2+} accumulation within stores sensitive to both $Ins P_3$ and the SR/ER Ca^{2+}-ATPase (SERCA) pump inhibitor thapsigargin, as well as decreased 'capacitive Ca^{2+} influx' upon depletion of these pools [21,22]. In contrast, genetic ablation of calreticulin in transgenic mice results in no detectable change in intracellular Ca^{2+} stores, but disrupts integrin-mediated Ca^{2+} signalling at the cell surface [23].

Annexin VI

The annexins are a family of Ca^{2+}-dependent phospholipid-binding proteins [24]. The physiological functions of these proteins have not been defined conclusively, although they might play roles in secretion or act as plasmalemmal ion channels. Annexin VI is a 67 kDa member of this family which activates

skeletal-muscle RyR channels at nanomolar concentrations by increasing their mean open time and open-probability in a Ca^{2+}-dependent manner [25]. Targeted overexpression of annexin VI results in altered cardiomyocyte Ca^{2+} signalling in transgenic mice, although these effects could be attributed to the actions of this protein on plasmalemmal Ca^{2+} pumps and exchangers, rather than on the type 2 RyR [26]. Although the annexins are generally associated with the plasmalemma, an immunofluorescence study indicated that annexin VI is located within Ca^{2+}-sequestering organelles [27]. In support of this observation, annexin VI only exerts its stimulatory effects on the intralumenal face of RyRs incorporated into planar lipid bilayers for single-channel recordings [28].

Chromogranin A

Chromogranin A is the major lumenal Ca^{2+}-binding protein in the secretory vesicles of adrenal chromaffin cells, although it is also present in most neuronal and endocrine cells [29]. Chromogranin A binds to secretory-vesicle integral membrane proteins, including an InsP_3R subtype, in a pH-dependent manner [30]. Furthermore, tetramerization of chromogranin A and its subsequent binding to a peptide corresponding to a postulated intralumenal loop of the type 2 InsP_3R (residues 2502–2521) are thermodynamically favoured in low-pH (pH 5.5)/high-$CaCl_2$-concentration (35 mM) buffers, which resemble conditions within secretory vesicles [31]. This peptide sequence is highly conserved among InsP_3R isoforms, but is absent from the RyRs. These results raise the possibility of reciprocal communication between InsP_3Rs and chromogranin A in secretory vesicles, reminiscent of that between RyRs and calsequestrin in the SR. Although ryanodine-sensitive CRCs are also present in secretory vesicles, their association with chromogranin A is as yet uncharacterized.

SR/ER INTEGRAL MEMBRANE PROTEINS

Triadins and junctins

Triadin is a major integral membrane glycoprotein of the junctional SR of skeletal-muscle triads. A rabbit triadin with an apparent molecular mass of ~ 95 kDa was demonstrated to bind both the skeletal-muscle RyR and the DHPR α1 subunit using ligand-overlay and protein cross-linking analyses [32]. An ~ 94 kDa glycoprotein, sharing many characteristic biochemical features with the ~ 95 kDa triadin, was localized to the junctional TC in rabbit skeletal muscle using immunofluoresence and immunoelectron-microscopy techniques [33]. Molecular cloning of this ~ 94 kDa rabbit triadin revealed that it consists of 706 amino acids, has a predominance of basic residues (calculated pI 10.18) and possesses a single membrane-spanning domain [34]. This transmembrane topology predicts that only 47 amino acid residues are present in the N-terminal cytoplasmic domain, casting doubts on the ability of triadin to interact directly with the t-tubular DHPR α1 subunit. Use of a fluorescent probe to label cysteine residues indicates that these are important for the interaction between triadin and the type 1 RyR. Labelling with this probe, as well as the formation of a high-molecular-mass complex between the RyR and triadin, is inhibited by RyR agonists and is activated by antagonists of these CRCs [34–36]. Using fusion protein affinity chromatography, the C-terminus of triadin was found to interact both with the predicted intralumenal face of the RyR and with the Ca^{2+}-storage protein calsequestrin [37]. The interaction of the triadin C-terminus with calsequestrin is inhibited by increasing Ca^{2+} concentrations, whereas its binding to the RyR is Ca^{2+}-independent. No triadin-derived fusion protein was capable of binding the DHPR. From these findings, it was postulated that, rather than coupling the RyR to the DHPR, triadin anchors calsequestrin to the skeletal-muscle CRC, such that its stored Ca^{2+} is in the vicinity of these Ca^{2+}-release sites.

The topology of triadin in skeletal muscle remains an area of intense debate. One anti-triadin monoclonal antibody inhibits depolarization-induced Ca^{2+} release from skeletal muscle triads [38], whereas another binds a site on the cytoplasmic side of these vesicles [39]. The epitopes that these antibodies recognize map to residues 673–706 and 110–163 respectively on the basis of their interaction with fusion proteins derived from rabbit triadin. These antibodies bind triadin in intact vesicles in the absence of detergent, indicating that their epitopes are cytoplasmic. Furthermore, a fusion protein corresponding to the DHPR α1 subunit II–III cytoplasmic loop interacts with several triadin-derived fusion proteins [40], in contrast with a previous study. Thus triadin might contain more than one transmembrane domain, such that regions communicating with the DHPR α1 subunit would be cytoplasmic. An unusual biochemical feature of skeletal muscle triadin is that it forms homopolymers under non-reducing conditions. These homopolymers are formed by disulphide bridges between the cysteine residues at position 270 or 671, although not both, between monomers, lending support to a four-transmembrane-domain model of triadin [40]. However, use of site-specific anti-peptide polyclonal antisera against the N- and C-terminal ends of triadin, in combination with the dependence of proteolysis of this protein within vesicles on the presence of detergent, supports the model of a single transmembrane topology [41]. Despite this controversy, the single-transmembrane-topology model of triadin is widely accepted, and is supported by structural predictions of non-skeletal-muscle triadin subtypes as well as of the related junctins.

Various anti-triadin antibodies recognize an ~ 95 kDa protein in the junctional SR of rat ventricular muscle [42,43]. Several antibodies against skeletal-muscle triadin specifically detect proteins of ~ 35 kDa (cardiac triadin 1; CT1), 40 kDa (CT2) and 92 kDa (CT3) in rabbit cardiac microsomes. Molecular cloning of the cDNAs encoding these three proteins indicates that they share an identical N-terminal 264-amino-acid stretch with each other and with the skeletal-muscle triadin subtype, but diverge at their C-termini [37]. Since their 5′ nucleotide sequences are identical, it was suggested that these four triadin subtypes are the products of alternative splicing of a common mRNA. All triadin subtypes have a similar predicted single transmembrane topology, and a fusion protein derived from their homologous intralumenal domain is capable of binding cardiac-muscle calsequestrin. Proteolysis studies of cardiac-muscle triadins supports their predicted transmembrane topologies, in that their C-termini are only susceptible to digestion upon detergent treatment of heart microsomes. The triadin gene has been proposed as a candidate gene for a familial dilated cardiomyopathy, since both the gene and the disease locus map to chromosome 6q22-q23 [44].

Calsequestrin-binding proteins in heart junctional SR include CT1 and CT2, as well as a doublet of ~ 26 kDa and a fourth protein of apparent molecular mass ~ 30 kDa [45]. The ~ 26 kDa protein doublet was purified from cardiac muscle, demonstrated to be highly enriched in junctional SR using membrane subfractionation and immunofluoresence techniques, and was detected in both cardiac and skeletal muscle, but not in liver. Molecular cloning of this ~ 26 kDa canine 'junctin' revealed that it consists of 210 amino acid residues, has a net basic charge (calculated pI 9.37) and displays a high degree of sequence identity with the triadins, as well as with ER aspartyl β-hydroxylase [46]. Like the triadins, the ~ 26 kDa junctin is

predicted to possess a short (22 amino acid) cytoplasmic N-terminus and a single transmembrane segment, with the majority of the protein being intralumenal. The susceptibility of the ~26 kDa junctin to proteolysis in intact and detergent-permeabilized SR vesicles is consistent with this predicted topology. Junctin from cardiac-muscle SR is capable of interacting with calsequestrin, as well as with triadin, the RyR and itself, as assessed by ligand-overlay, affinity-chromatography and co-immunoprecipitation techniques [47]. The association of junctin with calsequestrin is inhibited by increasing the Ca^{2+} concentration to millimolar levels, whereas the interaction of this protein with the RyR or triadin is Ca^{2+}-independent. Binding between triadin and junctin might be mediated by 'polar zippers' formed between 'KEKE' motifs present in their primary structures. On the basis of these observations, it was proposed that the RyR, junctin, calsequestrin and triadin form a quaternary complex, which is involved in the accumulation and release of Ca^{2+} from the SR.

The ~26 kDa junctin consists of an immunologically and biochemically related doublet of proteins, suggesting that it might be post-translationally modified. It is unlikely that this involves glycosylation, since junctin lacks consensus sequences for such modification. The calsequestrin-binding protein of ~30 kDa present in cardiac and skeletal muscle might be a junctin isoform [48]. The channel activity of skeletal-muscle RyRs is increased by 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (DIDS), an agent which inhibits anion channels by covalently modifying their amino acid residues. [^{3}H]DIDS labels a calsequestrin-binding protein of ~30 kDa in rabbit skeletal-muscle SR, rather than the RyR itself, suggesting that this modifying agent increases the activity of these CRCs via their interaction with the junctin-like protein. Association of triadins or junctins with CRCs in non-striated muscle tissues has not been reported to date.

CYTOSOLIC MODULATORY PROTEINS

cAMP-dependent protein kinase (protein kinase A; PKA)

PKA is a heterodimeric serine/threonine protein kinase whose activity is stimulated by cAMP. It consists of a catalytic subunit in combination with a regulatory subunit which is localized either to the cytoplasm (RI type) or to membrane systems via protein kinase A anchor proteins (AKAPs) (RII type) [49]. Intracellular cAMP levels are increased by adenylate cyclase isoenzymes at the plasmalemma, which are coupled to seven-transmembrane-segment receptors (7-TMRs) via stimulatory $G_s\alpha$ or inhibitory $G_i\alpha$ subunits of heterotrimeric G-proteins. This second messenger is degraded by phosphodiesterases. Many of these cyclase and phosphodiesterase isoenzymes are regulated by intracellular Ca^{2+}, thereby participating in feedback mechanisms between PKA activity and CRC function. The effects of PKA on CRC function are complex, since this kinase not only phosphorylates the channel proteins directly, but also modifies accessory proteins, such as the DHPR α1 subunit and sorcin. Examples of both direct and indirect regulation of CRCs by PKA are represented diagrammatically in Figure 4.

All three mammalian RyR isoforms contain multiple consensus sites for phosphorylation by PKA. RyRs in canine heart SR (mainly the type 2 isoform) are phosphorylated by exogenous PKA at between 1 and 4 mol of phosphate per mol of tetrameric CRC [50], which results in a 30% increase in the density of ryanodine-binding sites in these membranes [51]. Skeletal muscle RyRs (mainly type 1) are not significantly phosphorylated under similar conditions, although in a separate study exogenous PKA phosphorylated this protein at ~1 mol of phosphate per mol of CRC subunit. Phosphopeptide mapping indicates that PKA phosphorylates a single serine residue at position 2483 in the rabbit type 1 RyR [52]. PKA-mediated phosphorylation of the type 2 RyR coupled to β-adrenergic receptor stimulation has been demonstrated in intact newborn-rat cardiomyocytes, indicating that this modification might be physiologically relevant [53]. Similarly, treatment of fetal rat neurons with a membrane-permeant cAMP analogue enhances phosphorylation of their type 2 RyR population and increases the number of cells displaying intracellular Ca^{2+} elevations in response to caffeine, consistent with PKA activating these CRCs [54].

Type 1 and type 3 InsP_3Rs contain two consensus sites for PKA-mediated phosphorylation, whereas the type 2 isoform bears only one. The reported effects of PKA on InsP_3R-mediated Ca^{2+} signalling are conflicting, depending on the experimental system employed. Phosphorylation of InsP_3Rs by exogenous PKA in cerebellar microsomes results in a decrease in the potency of InsP_3 in mobilizing $^{45}Ca^{2+}$ from these membranes, without altering their binding properties for this ligand [55]. In contrast, phosphorylation by PKA increases both the rate and the extent of Ca^{2+} influx into proteoliposomes bearing purified cerebellar InsP_3Rs [56]. These discrepant observations suggest that some of the effects of PKA on InsP_3R Ca^{2+} signalling are mediated indirectly. In permeabilized hepatocytes, PKA increases the activity of InsP_3Rs both directly, by sensitizing them to InsP_3, and indirectly, by increasing the Ca^{2+} loading of intracellular stores by stimulating SR/ER Ca^{2+}-ATPase pumps [57]. Glucagon stimulates the phosphorylation of InsP_3Rs in intact hepatocytes by PKA and activates these CRCs by increasing their sensitivity to Ca^{2+} [58]. In a variety of cell lines, agonists that stimulate cAMP formation lead to increased phosphorylation of the InsP_3R population. Although immunoprecipitated type 1 InsP_3R is a good substrate for exogenous PKA, with other subtypes being weakly phosphorylated, all isoforms are modified in this manner in intact, agonist-stimulated cells. Finally, PKA is reported to enhance InsP_3-induced Ca^{2+} release in all cell lines examined, regardless of their InsP_3R isoform expression profile [59].

Protein kinase C (Ca^{2+}-dependent protein kinase; PKC)

PKC is the generic name for a family of structurally related protein kinases that are typically activated by elevations of intracellular Ca^{2+} in combination with increased plasmalemmal levels of diacyglycerol, a product of phospholipid hydrolysis. These co-activators often stimulate a concomitant translocation of PKC from the cytosol to cell membranes. However, not all PKC isoenzymes are regulated in this manner [60]. Phorbol esters are frequently used to activate PKC isoenzymes, probably by acting as diacylglycerol mimics. Exogenous PKC phosphorylates purified cardiac-muscle RyR, albeit at a lower rate than PKA or Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII), increasing its ryanodine binding capacity by about 15% [61]. The Ca^{2+}-uptake rate in skeletal-muscle TC is decreased about 2-fold by exogenous PKC, concomitant with phosphorylation of the type 1 RyR to a stoichiometry of about 1 mol of phosphate per mol of receptor [50]. Futhermore, PKC is associated with junctional t-tubular membranes in skeletal muscle [62], placing it in a suitable microdomain for modification of substrates involved in RyR-mediated Ca^{2+} signalling. PKC might play a role in regulating RyR function in physiological processes. For example, phorbol esters reduce the frequency of 'Ca^{2+} sparks' occurring by RyR activation in cerebral arterial myocytes, thereby reducing activation of plasmalemmal Ca^{2+}-dependent K^+ channels and blocking membrane hyperpolarization [63].

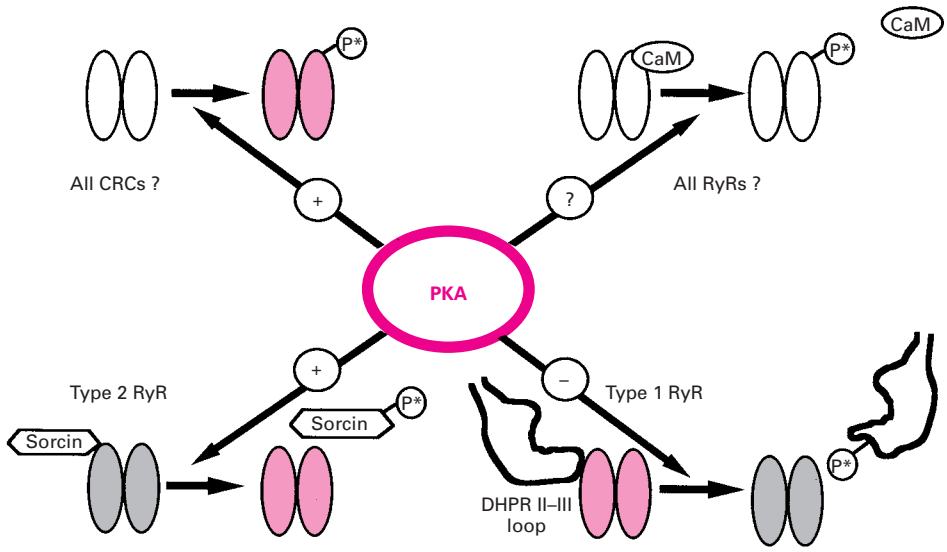

Figure 4 Multiple effects of PKA on CRC function

PKA can exert both direct and indirect effects on the activity of CRCs. This kinase stimulates (red shading) possibly all known CRCs by direct phosphorylation of the channel proteins. The interaction between RyRs and CaM might be abolished by PKA-mediated phosphorylation of the PM1 binding domain (residues 2937–3225 of the type 1 RyR), although the functional consequences of this have not been determined. Phosphorylation of sorcin by PKA blocks its inhibitory effect (grey shading) on the type 2 RyR. PKA-mediated phosphorylation of the II–III loop of the skeletal muscle DHPR α1 subunit prevents its association with the type 1 RyR, thereby abolishing its CRC-stimulating action.

The type 1 InsP_3R contains one or more consensus sites for PKC phosphorylation, depending on mRNA splicing [64], and serves as a substrate for this kinase *in vitro* [65]. Phosphorylation of this channel protein by PKC, PKA and CaMKII is additive, occuring at three distinct serine residues. Phorbol ester treatment of permeabilized pancreatic acinar cells leads to a reduction in both the potency and the maximal response of exogenous InsP_3 in releasing Ca^{2+} from intracellular stores, without modifying the Ca^{2+}-uptake properties of these pools [66]. Both the rate and extent of $^{45}Ca^{2+}$ release from isolated liver nuclei in response to exogenous InsP_3 are enhanced by PKC activation using phorbol esters [67]. This stimulation of Ca^{2+} release by PKC appears to be a consequence of the phosphorylation of two proteins that are immunologically related to the cerebellar InsP_3R. The reasons for the opposing effects of PKC on InsP_3R activity in pancreatic acini and liver nuclei are unclear, but might involve the expression of distinct CRC subtypes, or might be due to experimental differences. The expression of PKC is reported to be both spatially and temporally [68] different from that of InsP_3Rs, although this might permit distinct patterns of InsP_3R modulation, rather than eliminating the possibility of physiological regulation of these CRCs by the kinase.

cGMP-dependent protein kinase (protein kinase G; PKG)

cGMP is formed by transmembrane guanylate cyclases which are activated by the binding of extracellular ligands, or by cytosolic forms of these enzymes which are stimulated by nitric oxide (NO). Many effects of cGMP are mediated by two forms of protein kinase, type I being soluble and the type II form being membrane-associated as a consequence of N-terminal myristoylation [69]. Both skeletal (type 1) and cardiac (type 2) muscle RyRs are phosphorylated by exogenously added PKG [61]. Again, PKG phosphorylates the serine residue at position 2483 of rabbit type 1 RyRs [52], or the analogous amino acid at position 2809 in the type 2 isoform [70]. Phosphorylation of the type 2 RyR by PKG leads to an approx. 10% increase in ryanodine binding-site density, although the physiological relevance of this has not been ascertained.

Type 1 InsP_3R purified from rat cerebellum is an excellent substrate for PKG. Phosphopeptide mapping indicates that this modification occurs at Ser-1755 in this CRC, which is also the major substrate for PKA [71]. PKG phosphorylates InsP_3Rs in rat aortic smooth-muscle cells in response to atrial natriuretic peptide, a hormone which stimulates cGMP accumulation. Phosphorylation of InsP_3Rs in intact rat aorta in response to activators of cAMP or cGMP accumulation is selectively blocked by inhibitors of, and selectively enhanced by activators of, PKG [72]. This indicates that PKG catalyses InsP_3R phosphorylation in response to both cGMP and cAMP, possibly because the activation of this kinase is not very cyclic-nucleotide-specific. Unlike PKA, PKG is reported to be localized to the SR in vascular smooth muscle [73], placing it in a suitable vicinity for phosphorylating InsP_3Rs. In intact platelets, activators of cGMP accumulation block Ca^{2+} elevations in response to thrombin, a phosphoinositidase C-linked agonist [74]. Exogenous cAMP or cGMP also blocks Ca^{2+} release triggered by exogenous InsP_3 in permeabilized platelets and gastric muscle cells [75]. This inhibition of Ca^{2+} responses appears to be a consequence of InsP_3R phosphorylation, rather than of activation of Ca^{2+} uptake and extrusion mechanisms. In contrast with these observations, activation of PKG by cell-permeant analogues of cGMP elicits Ca^{2+} oscillations in hepatocytes [76]. These Ca^{2+} transients appear to be a consequence of PKG-mediated phosphorylation of InsP_3Rs at sites shared with PKA, leading to increased sensitivity of these CRCs to InsP_3, resulting in their activation at resting levels of this ligand.

CaMKII

The CaMKIIs are a family of protein kinase isoenzymes that are dependent on CaM and Ca^{2+} for activity. Functional CaMKII is multimeric and, like other serine/threonine protein kinases, undergoes autophosphorylation. Unlike PKC, PKA or PKG,

autophosphorylation drastically decreases the activity of CaMKII and also renders it independent of Ca^{2+}/CaM. CaMKII isoenzymes are ubiquitous among mammalian cells, but are highly enriched in brain [77]. CaMKII might play a key role in responding to oscillating intracellular Ca^{2+} levels, since its activity can be regulated by both the frequency and the amplitude of such signals [78]. A 60 kDa Ca^{2+}/CaM-dependent protein kinase is associated with skeletal-muscle TC and phosphorylates several resident substrates, including itself and the type 1 RyR [79]. This modification was reported to have no effect on the CICR properties of these membranes [79,80]. Incubation of patch-clamped 'blebs' of frog skeletal-muscle SR under conditions permissive for protein phosphorylation inhibits RyR CRC activity. This block is reversed by the addition of acid protein phosphatase or a specific CaMKII inhibitor, indicating that phosphorylation of the RyR or modulatory proteins by an endogenous CaMKII-like kinase inhibits channel activity in native membranes [81]. However, interpretation of these observations is complicated by the co-expression of two distinct RyR isoforms (α and β) in amphibian skeletal muscle [2]. CaMKII phosphorylates mammalian type 1 RyR to a similar extent to PKA, up to 1 mol of phosphate per mol of CRC, at the same serine residue (Ser-2483) [61]. This reduces the rate of $^{45}Ca^{2+}$ uptake by skeletal muscle TC approx. 2-fold [50]. In planar lipid bilayers, CaMKII- or PKA-mediated phosphorylation prevents the blockade of type 1 RyR channels by Mg^{2+}, an endogenous inhibitor [82].

Treatment of cardiac muscle SR with CaM and ATP reduces ryanodine binding by about 50 %. It also results in phosphorylation of the type 2 RyR at a serine residue distinct from that modified by PKA, PKC or PKG [61]. This phosphorylation appears to be isoform-specific and maps to Ser-2809 of the type 2 RyR in heart and brain [83]. CaMKII phosphorylates the type 2 RyR to a greater degree than either PKG or PKA, to a stoichiometry of 2 mol of phosphate per mol of tetramer. Furthermore, a Ca^{2+}/CaM-dependent kinase activity, which is inhibited by a CaMKII-specific peptide, remains associated with the type 2 RyR through several purification procedures [70]. This suggests that CaMKII not only plays a central role in modulating type 2 RyR function, but is also tightly bound to this CRC complex.

Use of a microsyringe technique to apply protein phosphatases or kinases to cardiac muscle RyRs incorporated into planar lipid bilayers revealed that Mg^{2+} (an endogenous RyR inhibitor) block of these CRCs is prevented by CaMKII or PKA [82]. This relief of Mg^{2+} inhibition could be reversed by acid protein phosphatase. In contrast, acid protein phosphatase treatment of cardiac muscle SR enhances ryanodine binding by increasing the affinity, without altering the binding-site density [84]. Furthermore, acid phosphatase increases the open-probability of type 2 RyRs by increasing the opening rate and by inducing a longer-lasting open state. These alterations in ryanodine binding and channel properties are reversed by treatment with exogenous CaMKII. The discrepancies in the effects of CaMKII on RyR in different studies might be a consequence of the technique used to assess CRC function, or perhaps of whether the CaMKII was added exogenously or was present endogenously. Exogenous CaMKII might phosphorylate a site distinct from that modified by endogenous kinase, thereby exerting distinct effects.

CaMKII phosphorylates cerebellar $Ins P_3$Rs to a greater extent than either PKA or PKC, to a stoichiometry of 1 mol of phosphate per mol of receptor monomer. This phosphorylation occurs at a residue distinct from that phosphorylated by either PKA or PKC [65]. CaMKII co-localizes with the type 3 $Ins P_3$R in gastrointestinal mucosal cells, as assessed using immunofluoresence and immunoelectron-microscopy techniques [85]. Hormone-stimulated Ca^{2+} oscillations in 3T6 fibroblasts and HeLa cells [86] appear to be a consequence of phosphorylation/dephosphorylation cycles mediated by a CaM/Ca^{2+}-dependent kinase and a CaM/Ca^{2+}-dependent phosphatase. Pharmacological analyses indicate that CaMKII is likely to be the kinase activating $Ins P_3$Rs in these systems, whereas a calcineurin-like phosphatase is responsible for the dephosphorylation phase of the cycle.

Non-receptor tyrosine kinases

Genetic ablation of *syk* (a member of the Src family of non-receptor tyrosine kinases) in B-cells leads to a loss of $Ins P_3$ accumulation and Ca^{2+} mobilization in response to antigen receptor stimulation. In contrast, elimination of the *fyn* Src kinase results in reduced Ca^{2+} release without detectable modification of inositol polyphosphate accumulation [87]. This indicates that *fyn* is involved in mobilization of intracellular Ca^{2+} distal to the production of $Ins P_3$. Since the human T-cell type 1 $Ins P_3$R bears two consensus sites for tyrosine phosphorylation, at positions 478–482 (EDLV**Y**) and 2612–2617 (DSTE**Y**), it was speculated that this modification alters the activity of these CRCs [88]. In support of this, *fyn* opens type 1 $Ins P_3$R channels incorporated into planar lipid bilayers at sub-activating concentrations of $Ins P_3$, in the presence of ATP. Furthermore, phosphorylation of $Ins P_3$Rs in mouse T-cells coupled to antigen receptor stimulation was reduced in cells in which *fyn* was genetically ablated [89]. The tyrosine residue at position 482 is unique to the type 1 $Ins P_3$R, whereas that at position 2617 is conserved in the type 2, though not the type 3, isoform.

Protein phosphatases

The dynamic regulation of proteins by phosphorylation demands the action of protein phosphatases in addition to protein kinases. The importance of phosphatases in the regulation of CRCs is apparent from the previous sections on protein kinases. The only endogenous protein phosphatase involved in CRC regulation that has been unambiguously identified is calcineurin, also known as protein phosphatase 2B. Calcineurin is a ubiquitous serine/threonine protein phosphatase [90], which is highly enriched in brain and is dependent on Ca^{2+}/CaM for activity.

FK506-binding proteins (FKBPs)

The FKBPs are a family of binding proteins for the immunosuppressant drugs FK506 and rapamycin. Although several members of this family have been characterized, a 12 kDa isoform known as FKBP12 predominates in mediating the immunosuppressive actions of these drugs. FKBP12 displays a broad tissue distribution, is mainly located in the cytosol and catalyses peptidylpropyl-*cis–trans*-isomerization, believed to be involved in protein folding. Although FK506 or rapamycin inhibit this isomerase activity, they act by altering the association of FKBP12 with other proteins, rather than by preventing protein folding [91]. The interaction between FKBP12 and CRCs was first identified by comparison of amino acid sequences deduced from the cDNA encoding the type 1 RyR with those obtained from analysis of proteolytic digestion products of the native RyR complex purified from skeletal muscle. One amino acid sequence obtained from the native CRC complex which was absent from the deduced primary structure of the type 1 RyR corresponds to the N-terminus of FKBP12, suggesting a robust association between these two proteins [92]. The interaction between FKBP12

and skeletal-muscle RyR was verified biochemically, with the isomerase co-purifying at a ratio of four molecules per tetrameric CRC [93]. This association can be disrupted by treatment with FK506 or rapamycin, permitting investigation of the effect of FKBP12 on RyR function. Removal of FKBP12 from type 1 RyRs in TC vesicles results in an enhanced sensitivity of $^{45}Ca^{2+}$ release to the CRC agonists Ca^{2+} and caffeine [94]. In planar lipid bilayers, skeletal-muscle RyRs stripped of FKBP12 display long-lasting subconductance states. Similar subconductance states are observed in type 1 RyRs heterologously expressed in insect cells [95]. Addition of FKBP12 to FKBP12-deficient RyRs restores channel gating to predominantly fully open or fully closed openings, rather than subconductance states. FKBP12 might also play a role in rectification of the type 1 RyR channel, preventing retrograde movement of cations from the myoplasm into the SR [96].

Cerebellar InsP_3Rs (predominantly the type 1 isoform) are associated with the Ca^{2+}-dependent protein phosphatase calcineurin, as determined by co-purification and co-immunoprecipitation. This association is inhibited by FK506 or rapamycin, indicating that calcineurin is anchored to the InsP_3R via an FKBP [97]. Inhibition of calcineurin results in enhanced sensitivity of $^{45}Ca^{2+}$ release from cerebellar microsomes in response to InsP_3, as a consequence of increased phosphorylation of InsP_3Rs by protein kinases, particularly PKC. Cerebellar RyRs (predominantly the type 2 isoform) are also associated with calcineurin in a manner which is disrupted by FK506 treatment. An FKBP12 binding site has been identified as a leucine–proline dipeptide in the central modulatory domain of the type 1 InsP_3R (residues 1400–1401), using yeast two-hybrid technology. Similar motifs are present in the primary structures of all InsP_3R isoforms, as well as in the RyRs, and are postulated to resemble the molecular moiety within FK506 that disrupts the FKBP12–CRC interaction. Use of yeast three-hybrid methodology supports the proposed formation of ternary complexes between CRCs, FKBP12 and calcineurin [98]. However, the mechanism of FKBP12 action on the type 1 RyR might not be dependent on calcineurin, since re-addition of purified FKBP12 (without calcineurin) is sufficient to restore normal gating properties to these CRCs after depletion with FK506. FKBP12 binding sites have been mapped to the periphery of the type 1 RyR, approx. 12 nm distant from the putative channel pore, using three-dimensional models reconstructed from cryo-electron micrographs [99]. This implies that the isomerase exerts its effects on CRC activity by causing long-range conformational changes.

Type 2 RyRs in heart muscle appear to interact with a distinct FKBP isoform, known as FKBP12.6. *In vitro*, skeletal muscle RyR is capable of interacting with either FKBP12 or FKBP12.6, whereas cardiac muscle RyR selectively binds FKBP12.6 [100]. A study using single-channel recording, $^{45}Ca^{2+}$ uptake and ryanodine binding to assay RyR activity demonstrated that removal and re-addition of FKBP12.6 from cardiac muscle SR had no discernable effects on type 2 RyR channel activity [101]. In contrast, FK506 treatment increased the duration of spontaneous or depolarization-evoked Ca^{2+} transients in intact cardiomyocytes and induced long-lasting subconductance states in heart RyRs in planar lipid bilayers [102]. Genetic ablation of FKBP12 in transgenic mice results in a phenotype with apparently normal skeletal muscle, but with severe dilated cardiomyopathy. RyRs from both skeletal and cardiac muscle in these FKBP12-deficient mice display long-lasting subconductance states, similar to those observed in FK506-treated RyRs from normal animals [103]. The lack of gross abnormalities in the skeletal muscle of FKBP12-deficient mice indicates that this isomerase is not essential for regulation of Ca^{2+} release in this tissue, suggesting that there might be additional mechanisms controlling this process, such as interaction with the DHPRs. The severe cardiomyopathy observed in FKBP12-deficient mice indicates that this protein is essential for regulation of the type 2 RyR in heart, contrary to earlier studies *in vitro*.

CaM

CaM is a ubiquitous ~ 17 kDa Ca^{2+}-binding protein, consisting of two pairs of EF-hands linked by a flexible hinge region, whose primary structure is highly conserved amongst protozoans. In the presence of micromolar free Ca^{2+}, micromolar concentrations of CaM were found to reduce the rate of RyR-mediated $^{45}Ca^{2+}$ mobilization from skeletal [104] and cardiac [105] muscle SR. CaM modulates RyR channels from both skeletal and cardiac muscles by reducing their mean open duration, without modifying single-channel conductance. This modulation involves a direct CaM–RyR interaction, since it is not dependent on ATP (essential for protein kinase activity) and occurs between purified proteins [106]. Mapping of CaM-binding sites using ligand overlay on type 1 RyR-derived bacterial fusion proteins identified six distinct sequences, which lie in close proximity to candidate Ca^{2+}-binding sites in this CRC [107]. In contrast, Menegazzi et al. [108] detected only three CaM-binding sites in the type 1 RyR, named PM1, PM2 and PM3, corresponding to residues 2937–3225, 3610–3629 and 4534–4552 respectively. These sites are highly conserved between the three mammalian RyR isoforms and are in the vicinity of predicted transmembrane segments of these CRCs. Ultrastructurally, binding sites for CaM have been localized to the periphery of the skeletal-muscle RyR complex, approx. 10 nm from the putative entrance of the putative channel pore, by transmission electron microscopy and image reconstruction techniques [99].

Several studies have indicated that the effect of CaM on type 1 RyRs has a biphasic dependency on the cytoplasmic free Ca^{2+} concentration, with stimulation of channel activity by CaM occurring at nanomolar levels of this ion [109–111]. This biphasic Ca^{2+}-dependency might be a consequence of distinct Ca^{2+}-dependencies of CaM association with different sites within the type 1 RyR. Indeed, interaction of CaM with a fusion protein encompassing the PM3 sequence was dependent on the presence of micromolar Ca^{2+} (500 μM), unlike that with proteins containing the PM1 or PM2 sites [112]. Peptides derived from the PM1, PM2 and PM3 sites of distinct RyR isoforms display different affinities for CaM, with PM1 showing the highest affinity in all subtypes. The PM3 peptide of the type 2 RyR also binds CaM with high affinity, unlike that of the type 1 or type 3 isoforms. Phosphorylation of the PM1 peptide by PKA abolishes its interaction with CaM, potentially increasing the level of complexity in the modulation of RyR-channel activity by CaM (Figure 4).

CaM also modifies channel activity via indirect pathways (Figure 5). As described above, protein phosphorylation by CaMKII regulates both RyRs and InsP_3Rs. In addition, CaM is an essential cofactor for the activity of calcineurin, a protein phosphatase. Although sea-urchin egg homogenates release Ca^{2+} in response to cyclic ADP-ribose, microsomal preparations from the same source do not. A factor present in the homogenates conferring Ca^{2+} responses to cADPr was identified as CaM [113]. This potentiation is blocked by specific inhibitors of CaMKII and is mimicked by the addition of a constitutively active form of CaMKII, without the requirement for exogenous CaM. This suggests that phosphorylation of RyRs or associated proteins by CaMKII is necessary for sensitivity to cADPr.

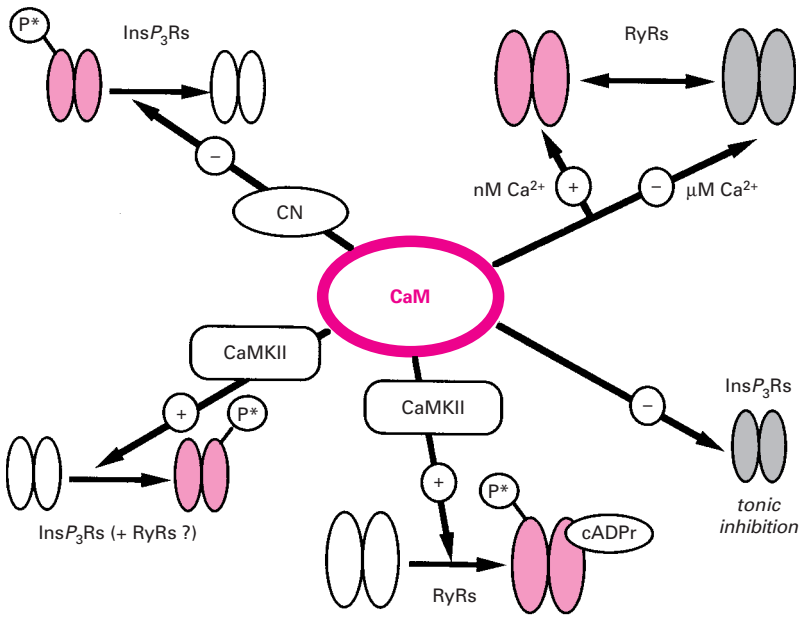

Figure 5 Multiple effects of CaM on CRCs

CaM directly activates RyRs at nanomolar Ca^{2+} concentrations (red shading), but reduces channel activity at micromolar levels of cytoplasmic Ca^{2+} (grey shading). In contrast, CaM might tonically inhibit the InsP_3Rs under some circumstances. CaM sensitizes RyRs to the putative second messenger cADPr, possibly via a direct or indirect CaMKII phosphorylation event. CaMKII directly activates InsP_3Rs and possibly RyRs, by direct phosphorylation. Finally, the CaM/Ca^{2+}-dependent phosphatase calcineurin (CN) reverses the stimulation of InsP_3R by protein kinases, in particular by dephosphorylating sites modified by PKC.

The regulation of InsP_3R function by CaM is less well characterized. CaM antagonists are reported to inhibit InsP_3-mediated Ca^{2+} release in rat liver epithelial cells [114], whereas they have no effect on this process in sea-urchin eggs [113]. Use of the scintillation proximity assay in combination with conventional radioligand binding assays showed that CaM partially inhibited InsP_3 binding to, as well as $^{45}Ca^{2+}$ flux via, cerebellar InsP_3Rs in a Ca^{2+}-independent manner [115]. These data also indicate that CaM interacts with the type 1 InsP_3R at multiple sites with distinct affinities. A CaM-binding site within the central regulatory domain of the type 1 InsP_3R (residues 1564–1585) has been detected by ligand overlay on fusion proteins derived from this channel [116]. A similar CaM-binding site is also present in the type 2 InsP_3R, although it is absent from the type 3 isoform, indicating CRC-subtype-specific regulation by this modulator [117]. It is suggested that high concentrations of CaM tonically inhibit the high densities of type 1 InsP_3Rs in cerebellar Purkinje cell dendritic spines, which could otherwise respond to extremely small changes in the number of InsP_3 molecules present within this spatially restricted microdomain.

Sorcin

Sorcin is a 22 kDa Ca^{2+}-binding protein that is up-regulated in multidrug-resistant cell lines [118]. Sorcin displays a wide tissue distribution and is associated with the type 2 RyR in cardiomyocytes, as determined by co-immunoprecipitation and co-localization by immunofluorescence [119]. At nanomolar concentrations, sorcin inhibits type 2 RyR channel activity in a Ca^{2+}-independent manner, by binding at a site distinct from those that bind CaM. This inhibition is reduced by the PKA-mediated phosphorylation of sorcin [120], indicating that this kinase mediates indirect as well as direct modulation of the type 2 RyR (Figure 4). Sorcin also associates with the C-termini of DHPR $\alpha 1$ subunits from both skeletal muscle and heart, possibly mediating inter-channel communication between these VOCCs and CRCs [121]. The effects of sorcin on other RyR isoforms or on the InsP_3Rs are unknown, despite its presence in skeletal muscle and brain. Sorcin-like immunoreactivity, detected using immuno-electron microscopy, co-localizes with RyRs in rat caudate putamen [122], suggesting that this protein might play a role in the modulation of CRC activity in neurons.

S100s

The S100s are a family of small ($\sim$ 10–12 kDa), predominantly cytoplasmic, EF-hand Ca^{2+}-binding proteins, which can form homo- or hetero-dimers [123]. At submicromolar cytoplasmic Ca^{2+} concentrations, nanomolar levels of the S100A1 isoform activate rabbit skeletal muscle RyRs by increasing their mean open-probability. This effect is not observed when the cytoplasmic Ca^{2+} concentration is raised to micromolar levels. Using optical biosensor techniques, S100A1 was shown to interact directly with the type 1 RyR in a Ca^{2+}-independent way, at three potential sites [124]. One of these S100A1-binding sites in the type 1 RyR primary structure overlaps with a putative CaM-binding site (PM3; residues 4534–4552). S100A1 also associates with two unidentified proteins of apparent molecular mass $\sim$ 100 kDa and $\sim$ 60 kDa in skeletal muscle TC. S100 isoforms have been reported to interact with a variety of proteins present in the triad junction of skeletal muscle, including aldolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the annexins [123], some of which are putative CRC accessory proteins. Although the interaction of S100 proteins with other RyR isoforms has not been characterized, levels of the S100A1 isoform are altered in some human cardiomyopathies, suggesting a role for this protein in Ca^{2+} handling in the heart [125].

Association of S100 isoforms with InsP_3Rs has not been reported to date.

PROTEASES

Calpains

The calpains constitute a family of cysteine proteases which have an absolute requirement for Ca^{2+} and neutral pH optima [126]. Treatment of cardiac- or skeletal-muscle SR with purified calpain II does not dramatically alter their ryanodine-binding characteristics, despite cleaving their RyRs into fragments of $\sim$ 315 kDa and $\sim$ 150 kDa [127]. However, calpain treatment blocks the inactivation of these RyRs in planar lipid bilayers, presumably by altering the influence of an inhibitory segment of the protein on the channel domain. Calpain II-mediated proteolysis of the RyR of skeletal muscle is inhibited by CaM, leading to the proposal that this protease interacts near CaM-binding sites in these CRCs [128], at so-called PEST sequences [129], i.e. sequences enriched in proline, glutamate, aspartate, serine and threonine residues. Skeletal-muscle TC bear an endogenous calpain-like enzyme, which cleaves their RyRs, does not alter the ryanodine-binding properties of these membranes [130], but increases the rate of $^{45}Ca^{2+}$ efflux from passively loaded vesicles [131]. This endogenous protease has a neutral pH optimum and cleaves the RyR of skeletal muscle, but not those of cardiac muscle or brain, into fragments of $\sim$ 375 kDa and $\sim$ 150 kDa. Digestion of skeletal muscle RyRs by this endogenous calpain does not modify their ryanodine-binding parameters, but decreases their channel activity in planar lipid bilayers by increasing the frequency of a subconducting state [132]. This discrepancy with earlier studies might be a consequence of differences in the calpain isoform employed, or in the extent of proteolysis attained. Cerebellar InsP_3Rs are also excellent substrates for exogenously added calpain [133], presumably since they contain numerous PEST motifs. Experimental evidence suggests that calpain-like proteases might underlie the degradation of InsP_3Rs *in vivo* in response to extracellular cues [134], at least in some cell types. In addition, calpains potentially modulate CRC function by interacting with certain accessory proteins, such as PKC [135] and the DHPR α1 subunit [136].

Proteasomal and lysosomal pathways

In SH-SY5Y human neuroblastoma cells, prolonged stimulation of muscarinic acetylcholine receptors at the cell surface leads to accelerated degradation of their type 1 InsP_3R proteins, with a consequent decrease in the sensitivity of $^{45}Ca^{2+}$ release from their intracellular Ca^{2+} pools in response to exogenous InsP_3 [137]. Under these conditions, levels of the type 2 RyR protein expressed in SH-SY5Y neuroblastomas are not altered [9], indicating that there is specificity in the mechanism of 7-TMR-linked degradation of InsP_3Rs. A similar phenomenon is observed in AR4-A2J pancreatoma cells chronically stimulated with the hormone cholecystokinin, in which the three InsP_3R isoforms expressed are down-regulated at different rates [138]. In methacholine (a muscarinic acetylcholine receptor agonist)-stimulated SH-SY5Y cells, type 1 InsP_3R down-regulation appears to be mediated by a Ca^{2+}-dependent, calpain-like cysteine protease [134]. However, InsP_3R degradation in WB rat liver epithelial cells under resting conditions is predominantly via the lysosomal pathway. Furthermore, persistent angiotensin II stimulation of these cells leads to enhanced polyubiquitination of their type 1 and type 3 InsP_3Rs, targeting them for degradation via the multicatalytic proteasomal pathway [139]. The polyubiquitin/proteasomal degradation pathway is believed to play a central role in antigen presentation, cell-cycle regulation and selective proteolysis of intracellular proteins [140]. The specificity of this pathway is determined mainly by the enzymes involved in the polyubiquitination of target proteins. CRCs in skeletal muscle might also be degraded via the proteasomal pathway, since the sequence of a proteolytic peptide ('KC5') from the isolated, native skeletal muscle RyR complex, which is not present in the primary structure of this ion channel [92], corresponds closely to part of S5a [141], a regulatory subunit of the 26 S proteasome which recognizes polyubiquitinated substrates. The relative contributions of the lysosomal, calpain and proteasomal pathways in the degradation of distinct CRC subtypes in different cell types and in response to various extracellular stimuli awaits further characterization.

PLASMALEMMAL ACCESSORY PROTEINS

DHPRs

The DHPRs are a family of plasmalemmal binding proteins for dihydropyridine and phenylalkylamine 'Ca^{2+} antagonists', which function as 'L-type' VOCCs. DHPR isoforms and mRNA splicing subtypes exist in a wide range of tissues, but are present at greatest abundance in skeletal muscle, heart and brain. In skeletal muscle, DHPRs are localized to the junctional t-tubular membrane, and consist of five distinct protein subunits: α1, α2, β, γ and δ, whereas DHPRs from other tissues lack a δ subunit. The α1 subunit acts as both a voltage sensor and a VOCC, as well as bearing binding sites for 'Ca^{2+} antagonists' [142]. The cDNA sequence of the skeletal-muscle DHPR α1 subunit encodes a protein of 1873 amino acid residues, with a predicted topology consisting of four repeats of six hydrophobic segments linked by cytoplasmic loops of variable length [143], as well as cytoplasmic N- and C-termini (Figure 6). The cardiac muscle DHPR α1 protein displays 66% amino acid identity with the skeletal-muscle form [144], but has a longer C-terminal domain.

Subcellular fractionation, co-immunoprecipitation [145] and chemical cross-linking [146] experiments indicate that the skeletal-muscle DHPR α1 subunit and type 1 RyR interact physically with one another. Furthermore, the activities of these channel proteins are reciprocally coupled, such that the presence of the type 1 RyR stimulates the skeletal-muscle DHPR α1 subunit and vice versa [147]. Distinct DHPR α1 subunits are believed to underlie the different mechanisms of excitation–contraction coupling in skeletal and cardiac muscles, as described in Figure 1(a). Dissection of the structural basis of these distinct coupling mechanisms has been greatly assisted using the mutant mouse lines *dysgenic* [148], which lacks the skeletal-muscle DHPR α1 subunit, and *dyspedic* [149], an engineered mutant with the type 1 RyR deleted. Excitation–contraction coupling is ablated in skeletal-muscle myocytes from these mice, but is rescued by transfection with the skeletal-muscle DHPR α1 subunit or the type 1 RyR respectively. Transfection of *dysgenic* skeletal myotubes with the cardiac muscle DHPR α1 subunit results in heart-muscle-like coupling, which is dependent on influx of Ca^{2+} from the extracellular environment [150]. Transfection of *dysgenic* myotubes with chimaeras between skeletal and cardiac muscle DHPR α1 subunits indicates that the II–III cytoplasmic loop (between the II and III transmembrane repeats) is a critical determinant of skeletal-muscle-type excitation–contraction coupling [151].

Nanomolar concentrations of synthetic peptides corresponding to the II–III loops of both cardiac and skeletal-muscle DHPR α1 subunits activate type 1 RyRs, but not the type 2 isoform, as assessed by ryanodine binding and single-channel recording [152]. PKA-mediated phosphorylation of the skeletal-muscle

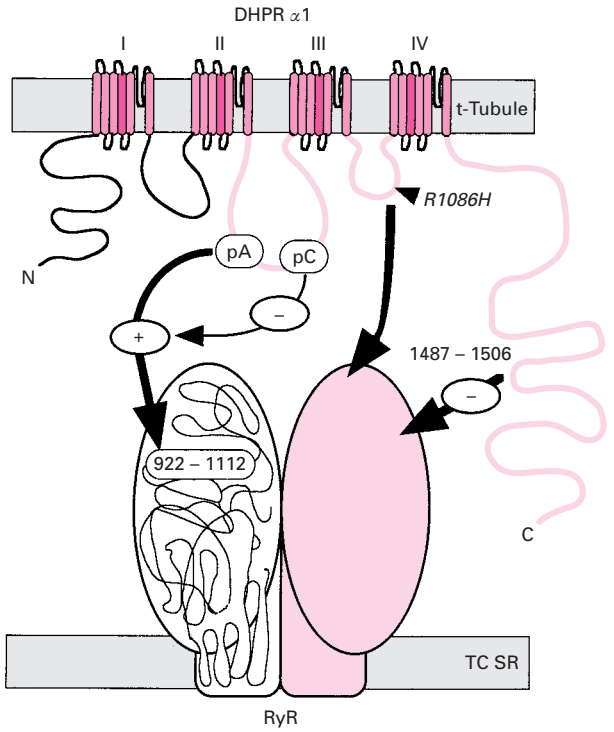

Figure 6 Interactions between cytoplasmic loops of the skeletal-muscle DHPR α1 subunit and the type 1 RyR

The t-tubular DHPR α1 subunit of skeletal muscle has a characteristic topology, consisting of four transmembrane-spanning repeats (I, II, III and IV), with the fourth segment of each (darker pink) forming the voltage sensor of this VOCC. Conformational changes in response to plasmalemmal depolarization are transduced to the type 1 RyR in the TC SR via interactions with cytoplasmic regions of the α1 subunit. The II–III loop contains a region, peptide A (pA; residues 681–690), which activates the CRC by interaction with a region corresponding to residues 922–1112 of the RyR protein. This interaction is abolished by a peptide corresponding to a second region of the II–III loop, peptide C (pC; residues 724–760). Genetic analysis indicates that a mutation in the DHPR α1 subunit III–IV region, R1086H, interferes with the function of the type 1 RyR. Finally, a peptide corresponding to residues 1487–1506 of the DHPR α1 subunit C-terminal tail inhibits the channel activity of both type 1 and type 2 RyRs.

II–III loop peptide at an amino acid corresponding to Ser-687 reduces its ability to activate the type 1 RyR [153], indicating that this kinase might modulate the interaction between these channel proteins (Figure 4). A truncated version of this peptide, corresponding to Thr-671 to Leu-690 ('peptide A'), was sufficient to stimulate skeletal-muscle RyRs [154], whereas a second sequence from the II–III loop ('peptide C'; Glu-724 to Pro-760) blocks this triggering effect (Figure 6). Truncation of peptide A revealed the sequence RKRRKMSRGL (residues 681–690) as the minimal essential unit sufficient to activate the skeletal-muscle RyR [155].

Mapping of the site(s) of interaction of the type 1 RyR with the skeletal muscle DHPR α1 subunit has yielded different results, depending on the techniques employed. Transfection of *dyspedic* skeletal-muscle myocytes with deleted type 1 RyR constructs, or with type 1/type 2 isoform chimaeras, indicated that the so called 'D2' region of the type 1 RyR, corresponding to residues 1303–1406, is essential for skeletal-muscle-type excitation–contraction coupling [156]. However, other regions of the type 1 RyR must also be critical in determining skeletal muscle excitation–contraction coupling, since this mechanism was restored in chimaeras containing the type 2 isoform D2 region in a type 1 RyR background. Of numerous *in vitro* translation products derived from the N-terminal four-fifths of the type 1 RyR, only a fragment corresponding to Leu-922 to Asp-1112 could specifically associate with the DHPR α1 II–III loop. A homologous fragment from the type 2 RyR did not bind to this loop. Analysis of chimaeras between type 1 and type 2 RyRs localized this interaction to residues Arg-1076 to Asp-1112 of the CRC [157]. It was suggested that ionic interactions are important in the association between the type 1 RyR and the DHPR α1 subunit, since a double mutation of Lys residues at positions 677 and 682 of the II–III loop dramatically reduced its ability to bind the CRC fragment. Chimaeras between type 1 and type 2 RyRs expressed in *dyspedic* myotubes appear to be reciprocally coupled to the DHPR α1 subunit by interactions at two sites, encompassed by residues 1635–2636 and 2659–3720 of the type 1 RyR [158]. However, this study did not address the possibility that the type 2 RyR might also interact physically with the DHPR α1 subunit under some circumstances.

Other cytoplasmic regions of the DHPR α1 subunit might be involved in its modulation of RyRs (Figure 6). Malignant hyperthermia is a genetic disorder in which patients undergo potentially fatal hyperthermia and skeletal-muscle rigidity in reaction to volatile anaesthetics and depolarizing relaxants. This condition is genetically heterogeneous, but in many cases is linked to point mutations in the type 1 RyR. However, malignant hyperthermia has been linked in one family to an Arg-1086 → His mutation in the III–IV loop of the skeletal muscle DHPR α1 subunit, implying that this cytoplasmic domain might also interact with the type 1 RyR [159]. Micromolar concentrations of a peptide corresponding to residues Asn-1487–Leu-1506 of the DHPR α1 subunit C-terminal tail inhibit ryanodine binding to both skeletal and cardiac muscle SR membranes, by slowing both association and dissociation time constants [160]. This peptide also decreases the open-probability of skeletal muscle RyRs in planar lipid bilayers. Since a sequence similar to this peptide is also present in the heart DHPR α1 subunit, it is not unexpected that this C-terminal region might also influence the activity of the cardiac muscle RyR. Thus multiple protein–protein interactions, including those with the accessory proteins sorcin, PKA and AKAPs, determine the efficiency of coupling between DHPRs and RyRs.

DHPRs and other VOCCs might directly regulate CRC activity in systems other than skeletal muscle. As mentioned above, the DHPR and type 2 RyR of cardiac muscle display tight functional coupling, although the mechanistic basis of this interaction is unknown. One possibility is that cardiac muscle DHPRs and RyRs display skeletal-muscle-type coupling in some situations. Functional coupling between DHPRs and RyRs in cerebellar granule cells is enhanced by glutamate receptor stimulation, via an undetermined mechanism [161]. DHPRs might also regulate the activity of InsP_3Rs. Sub-plasmalemma populations of these CRCs have been identified from a number of sources, including brain, liver [162] and SH-SY5Y neuroblastomas [134], using membrane fractionation and immunocytochemical techniques. Such InsP_3Rs are associated with the plasmalemma via cytoskeletal interactions, and often co-purify with dihydropyridine-binding sites. Using a number of pharmacological approaches, the initial Ca^{2+} responses of adrenal glomerulosa cells to the peptide hormone angiotensin II were proposed to be mediated by activation of InsP_3Rs by DHPRs, independently of InsP_3 production or Ca^{2+} influx [163]. The structural basis of this allosteric coupling has not been determined.

Inositol 1,3,4,5-tetrakisphosphate receptors (InsP_4Rs)

One mechanism of InsP_3 metabolism within cells is its phosphorylation to higher inositol polyphosphates, such as InsP_4

[164]. It is hypothesized that plasmalemmal InsP_4Rs might act as Ca^{2+} channels, allosterically coupled to InsP_3Rs in the subplasmalemmal ER. Such reciprocal coupling occurs between plasmalemmal InsP_4Rs and intracellular InsP_3Rs in lobster olfactory neurons [165]. An InsP_4-binding protein present in platelet plasma membranes is reported to stimulate Ca^{2+} influx in this system [166]. Furthermore, the synergistic effect of InsP_4 on Ca^{2+} release in L1210 cells induced by a metabolically resistant analogue of InsP_3 is mediated indirectly by an InsP_4R protein distinct from the InsP_3Rs [167]. InsP_4 does not stimulate Ca^{2+} release or influx in mouse lacrimal acinar cells, but instead inhibits Ca^{2+} mobilization induced by InsP_3 [168]. The mechanisms by which InsP_4 regulates CRCs await more extensive characterization, although this is complicated by the range of proteins that bind this inositol polyphosphate, as well as its susceptibility to metabolism within cells.

G-proteins

G-proteins can directly regulate the gating of ion channels. In saponin-permeabilized pulmonary artery smooth muscle, stimulation of G-proteins by the non-hydrolysable analogue guanosine 5′-[γ-thio]triphosphate activates Ca^{2+} release via InsP_3Rs by two distinct mechanisms, only one of which is dependent on InsP_3 production [169]. In vascular smooth muscle, thrombin induces Ca^{2+} mobilization via InsP_3R activation. Thrombin receptors are coupled to InsP_3R activation by a G-protein which is slowly ribosylated by pertussis toxin. Ribosylation blocks thrombin-induced Ca^{2+} release, although this does not appear to be a consequence of alterations in InsP_3 production. In permeabilized pancreatic acinar cells, low concentrations of phosphoinositidase C-coupled receptor agonists, or of guanosine 5′-[γ-thio]triphosphate, stimulate InsP_3Rs, with small or undetectable increases in cellular InsP_3 levels [170]. In vascular smooth muscle, $G_i\alpha$ co-localizes with the InsP_3R, and pertussis-toxin treatment inhibits InsP_3-induced Ca^{2+} release, indicating that heterotrimeric G-proteins can regulate these CRCs directly [171]. Effects of G-proteins on RyR activity have not been determined to date.

OTHER ACCESSORY PROTEINS

Calmedin

A detergent-soluble factor from brain microsomal membranes which confers Ca^{2+} sensitivity of InsP_3 binding to purified cerebellar InsP_3Rs is a protein of $\sim$ 300 kDa native molecular mass, termed ‘calmedin’ [172]. The primary structure of this protein has not been determined, nor has it been biochemically isolated.

Kinase anchor proteins (KAPs)

As their name suggests, KAPs anchor protein kinases to cellular microdomains where their substrates are located, restricting them to the two-dimensional plane of membranes rather than the three-dimensional space of the cytoplasm, thereby increasing their probability of encountering a target [173]. AKAPs usually associate with PKA via its RII regulatory subunit. Potentiation of skeletal-muscle DHPR α1 subunit channel activity by PKA is inhibited by a synthetic peptide which blocks AKAP anchoring. PKA is tightly associated with the native skeletal muscle DHPR complex, via its interaction with a 15 kDa AKAP which binds the biotinylated RII subunit of PKA in overlay assays [174]. A 100 kDa AKAP (AKAP100) is expressed at greatest abundance in skeletal and cardiac muscles, but is also present in brain [175]. AKAP100 targets PKA to the SR of both skeletal and cardiac muscle-derived cell lines, placing it in a suitable location for interaction with RyRs. Similary, a novel protein termed alphaKAP anchors CaMKII to the SR in skeletal muscle, thereby bringing the kinase into proximity with these CRCs [176].

Glycolytic enzymes

GADPH and aldolase are glycolytic enzymes which are peripheral membrane proteins that are enriched in the t-tubular system of skeletal muscle. Ligand overlay and cross-linking experiments indicate that these enzymes are specifically associated with both the DHPR α1 subunit and type 1 RyRs in this tissue, possibly mediating indirect interchannel communication [177]. The functional effects of GADPH and aldolase on type 1 RyRs have not been demonstrated, nor have these enzymes been shown to interact with other RyR isoforms or with the InsP_3Rs.

Ankyrins

The ankyrins are a family of proteins which link integral membrane proteins to the cytoskeletal network. In rat brain, a large proportion of InsP_3-binding sites are resistant to detergent solubilization. Insoluble InsP_3Rs have an 8-fold greater affinity for their ligand than those in microsomal membranes, although the two types are indistinguishable in terms of immunoreactivity and apparent size [178]. Insoluble InsP_3Rs are co-immunoprecipitated by an antibody against erythrocyte ankyrin, suggesting that they are linked to the cytoskeleton via an ankyrin-like protein. A non-neuronal splice form of the type 1 InsP_3R is located in a unique type of low-density intracellular vesicle from mouse T-lymphoma cells. Ankyrin inhibits InsP_3 binding to, and InsP_3-induced Ca^{2+} release from, these vesicles, binding to their InsP_3Rs with sub-nanomolar affinity [179]. These inhibitory effects are blocked by a peptide with the sequence GGVG-DVLRKPS, which corresponds to residues 2546–2556 of the mouse type 1 InsP_3R and is homologous to other ankyrin-binding motifs [180]. This candidate ankyrin-binding domain is highly conserved in all known InsP_3Rs, including that from *Drosophila melanogaster*, although it is not apparent in the RyRs. Mouse lymphoma light vesicles also contain a protein that is immunologically related to RyRs from other tissues and which sediments as a homotetrameric complex. Ankyrin binds to this lymphoma RyR with sub-nanomolar affinity, inhibiting its activation by cADPr [181]. The effects of ankyrin on CRCs from other sources have not been investigated. However, certain ankyrin subtypes appear to be expressed as integral membrane proteins of the skeletal-muscle SR, placing them in a suitable vicinity to interact with type 1 RyRs in this tissue [182].

BiP

BiP, also known as the 78 kDa glucose-regulated protein, is a molecular chaperone which binds Ca^{2+} at 1–2 mol per mol of protein and contributes up to 25 % of the Ca^{2+}-storage capacity of the ER. Overexpression of BiP in HeLa cells leads to increased amplitude of their Ca^{2+} responses to phosphoinositidase C-linked receptor stimulation [183]. Whether this enhanced InsP_3-induced Ca^{2+} release is merely a consequence of increased Ca^{2+} storage in the ER, or reflects coupling between BiP and the InsP_3Rs, remains to be investigated.

Integrin-like protein

A protein of $\sim$ 167 kDa apparent molecular mass was detected in skeletal and cardiac muscles, but not in other tissues, using an

antiserum against $\alpha_5\beta_1$-integrin [184]. This protein is biochemically and immunologically distinct from the integrins, has a unique N-terminal sequence, is highly enriched in the TC and is located intralumenally. The potential role of this novel protein in CRC modulation awaits investigation.

90 kDa protein

An approx. 90 kDa protein recognized by one of a panel of monoclonal antibodies generated against skeletal-muscle triad junctions is an integral membrane protein of the junctional SR, is a substrate for endogenous protein kinase activity and is detectable in skeletal-muscle, but not in heart or brain [185]. It is distinct from skeletal-muscle triadin in that it does not form homopolymers under non-denaturing conditions and is not recognized by anti-triadin antibodies. Although the $\sim$ 90 kDa protein shares a number of properties with the Ca^{2+}-binding molecular chaperone calnexin, it is distinct in terms of several biochemical properties, skeletal-muscle-specific expression and TC SR restricted subcellular localization [185].

Histidine-rich Ca^{2+}-binding protein (HCP)

Phosphorylation of an $\sim$ 160 kDa protein by an endogenous kinase in skeletal-muscle TC SR results in a decreased ryanodine binding-site density in these membranes, with no change in affinity for the alkaloid [186]. This $\sim$ 160 kDa protein is biochemically similar to HCP, which is predicted to be a peripheral membrane protein. However, the $\sim$ 160 kDa protein appears to be intralumenal, and is phosphorylated by endogenous casein kinase II within the SR [187]. Both HCP and triadin are reported to be phosphorylated by an endogenous membrane-bound, cytoplasmic-facing CaM-dependent protein kinase in TC SR [188]. Therefore the subcellular location of HCP, its relationship with other proteins of $\sim$ 160 kDa in skeletal muscle and the potential roles of these proteins in modulating RyR function remain to be fully clarified.

Intracellular K^+ channels

It is hypothesized that K^+ influx into the lumen of the ER/SR via intracellular channels is required to counteract the change in membrane potential resulting from the release of Ca^{2+} via CRCs [189]. The K^+-channel blocker apamin inhibits InsP_3-induced Ca^{2+} release from a platelet endomembrane preparation, this effect being prevented by the K^+/H^+ ionophore nigericin. A monoclonal antibody which recognizes an $\sim$ 63 kDa protein in various tissues and cells also blocks InsP_3-induced Ca^{2+} mobilization in platelets, cerebellum, aortic smooth muscle, HEL cells and sea-urchin eggs [190]. Since the effects of this antibody were circumvented by nigericin, it is postulated that the $\sim$ 63 kDa protein is an intracellular K^+ channel which is functionally coupled to the InsP_3Rs.

INTER- AND INTRA-COMPLEX INTERACTIONS

Intracomplex interactions

InsP_3Rs can form heterotetrameric complexes between distinct isoforms [10], whereas there is no experimental evidence to support such associations between RyR subtypes. This increases the structural diversity of the InsP_3Rs enormously, although the functional consequences of heterotetramer formation have not been elucidated. InsP_3Rs are also distinct from RyRs in that they undergo autophosphorylation [191], although the physiological relevance of this is not clear. In both classes of CRC, the C-terminal transmembrane region is critical for tetramer formation [191–193]. In the case of InsP_3Rs, the InsP_3-binding site is close to the N-terminus, and monomeric fusion proteins derived from this region can bind inositol polyphosphates [194]. These N-terminal fusion proteins undergo large conformational changes upon binding InsP_3, which might be transduced to the channel domain within the native CRC complex.

Monomeric RyRs do not bind ryanodine. The ryanodine-binding site of the skeletal-muscle RyR has been crudely mapped to the C-terminal 650 amino acid residues, either using a photoaffinity-labelled analogue of the alkaloid [195], or by tryptic digestion and isolation of complexes prelabelled with this ligand [16]. An mRNA encoding the C-terminal 656 residues of the type 1 RyR is specifically expressed in rabbit brain [196]. When overexpressed in CHO cells, the protein encoded by this mRNA is targeted to the ER. However, the presence and physiological role of this truncated RyR in mammalian tissues have not been demonstrated. An N-terminally truncated type 1 RyR protein of $\sim$ 130 kDa is enriched in the ER when overexpressed in CHO cells [197] and is capable of acting as a CRC, but is inactivated at higher Ca^{2+} concentrations than the full-length protein and, unlike the wild-type channel, displays inward rectification. Unlike the full-length type 1 RyR, the N-terminally truncated form is not stimulated by caffeine [198]. Functional analyses of mutated type 3 RyRs indicate that the glumate residue at position 3885, within the transmembrane region, is important for the Ca^{2+}-activation of their channels [199]. Co-expression of 3885 mutant and wild-type RyRs suggests that their Ca^{2+} sensor is dependent on quarternary structure, being composed of one glutamate residue from each CRC monomer within the tetrameric complex.

The cytoplasmic C-terminal tail of the RyRs is highly conserved between isoforms. This region is relatively resistant to degradation by proteolytic enzmes, suggesting that it is inaccessible as a result of intracomplex protein–protein interactions [200]. It is essential for type 1 RyR function, since deletion of the last three amino acids reduces ryanodine binding and channel activities, whereas a 15-residue deletion inactivates these CRCs, although it does not prevent tetramerization [201]. The extreme C-termini of InsP_3Rs are structurally divergent, but might play critical roles in channel function. A monoclonal antibody recognizing the C-terminal 12 amino acids of the type 1 InsP_3R inhibits Ca^{2+} release from cerebellar microsomes in response to exogenous InsP_3 [202].

The N-terminal four-fifths of CRCs appear to be the major site of interaction with ligands and modulators. Although numerous binding sites for modulatory proteins have been mapped to the cytoplasmic domains of CRCs, characterization of inter- and intra-subunit interactions occurring within these regions has been somewhat limited. A monoclonal antibody against skeletal muscle RyR recognizes an epitope a few residues N-terminal of Gly-341 and activates these CRCs by increasing their sensitivity to cytoplasmic Ca^{2+}, as assessed by the effects of this ion on ryanodine binding [203]. Using optical biosensor technology, a fusion protein (residues 163–522) recognized by this antibody was demonstrated to interact directly with purified type 1 RyR complex. In ligand-overlay experiments, this domain interacts with a type 1 RyR fusion protein corresponding to residues 2937–3225, which contains the CaM-binding site PM1 [204]. These observations suggest that intramolecular interactions between residues 163–522 and the PM1 CaM-binding site play a role in the regulation of type 1 RyR-channel activity. Similar interactions might also occur within InsP_3R complexes. Biochemical analyses indicate that the N-terminal $\sim$ 620 amino acids of the rat type 1 InsP_3R remain non-covalently associated with a region of $\sim$ 850 residues at the C-terminus following proteolysis with trypsin [205]. Interactions between these N- and

C-terminal regions are likely to be essential in CRC function, since they show a high degree of sequence conservation between InsP_3R and RyR families.

The skeletal-muscle RyR is activated by both endogenous (GSH, H_2O_2, reative oxygen species) and pharmacological oxidizing agents. NO has a bifunctional effect on the type 1 RyR, preventing oxidation of hyper-reactive thiol groups at low concentrations, but oxidizing them at higher concentrations [206]. The alkylating agent *N*-ethylmaleimide first inhibits type 1 RyR activity (phase 1), then restores it (phase 2), and finally lowers it again (phase 3), over time. In contrast, the oxidizing agent diamide activates these CRCs and protects against the phase 1 inhibition by *N*-ethylmaleimide. Diamide treatment results in intersubunit cross-links within the type 1 RyR which are not located within the N-terminal 170 kDa of the protein, since calpain-cleaved complexes are also modified. Diamide causes both intra- and inter-subunit cross-linking, at regions encompassed by residues 2100–2843 or 2844–4685, as determined using proteolytic mapping, site-directed antibodies and N-terminal sequencing of peptide fragments. Residues most susceptible to *N*-ethylmaleimide alkylation map to the N-terminal amino acids 426–1396. Alkylation of residues within this domain abolishes the formation of both inter- and intra-subunit cross-links [207]. Thiol oxidation of residues within the type 2 RyR also influences its function, first activating and then irreversibly inhibiting its channel activity. The type 2 RyR is progressively activated by NO as a consequence of *S*-nitrosylation of up to three thiol groups per subunit [208], these effects being reversed by denitrosylation. Oxidation of up to six thiol groups per type 2 RyR subunit has no effect on channel function, although oxidation of additional thiols results in irreversible activation. These results suggest that redox cycling of mutiple thiol residues involved in both intra- and inter-subunit cross-linking might be involved in gating of RyR channels.

The activity of InsP_3Rs is also influenced by redox reactions. The thiol-oxidizing reagent thimerosal enhances InsP_3 binding to cerebellar and liver microsomal membranes by increasing their affinity, without altering the maximal binding-site density [209]. Thimerosal stimulates InsP_3-induced Ca^{2+} fluxes through and InsP_3 binding to cerebellar InsP_3Rs directly, without the necessity of indirect action via modification of accessory proteins, as assessed using the isolated channel complex reconstituted into proteoliposomes [210]. Oxidized glutathione also increases the potency of InsP_3 in activating InsP_3R channels in the ER of permeabilized hepatocytes, although, unlike thimerosal, it increases the number of InsP_3-binding sites without altering their affinity [211]. Furthermore, oxidized glutathione does not alter the InsP_3-binding properties of solubilized, partially purified InsP_3Rs, suggesting that it might influence the function of these CRCs by an indirect mechanism. It was postulated that two conserved cysteine residues within a putative intralumenal loop in all InsP_3R isoforms are modified by an oxidized glutathione-dependent thiol–disulphide oxidoreductase within the lumen of the ER [211], thereby maintaining these CRCs in an appropriate conformation for activation by InsP_3. The type 3 InsP_3R is the main isoform expressed in bronchial-mucosa-derived 16HBE14o- cells. Unlike aorta-derived A7r5 cells, which predominantly express the type 1 InsP_3R, these mucosal cells do not display enhanced sensitivity of $^{45}Ca^{2+}$ release by InsP_3R in response to thimerosal, which was suggested to be a protective mechanism against CRC dysregulation within the oxidizing environment of the respiratory tract [212]. Mapping of the positions of key reactive thiol groups within distinct CRC subtypes will be necessary for understanding their roles in regulating the activity of these channels.

Intercomplex interactions

RyR complexes from both skeletal and cardiac muscles form ordered arrays or lattices in the junctional SR membrane [213]. Purified skeletal-muscle RyR complexes are capable of self-assembling *in vitro* into arrays resembling those in native SR membranes (F. A. Lai, personal communication). The physiological relevance of this is uncertain, although it might be responsible for the formation of Ca^{2+}-release channel units, which are groups of CRCs capable of co-ordinated opening, leading to the phenomenom of discrete 'Ca^{2+} spark' events [214]. Indeed, analyses of multiple skeletal-muscle RyRs incorporated into planar lipid bilayers reveal that these channels can display synchronized gating and that FKBP12 is necessary for such coupling between complexes [215].

InsP_3Rs also form arrays of CRC complexes. Within cerebellar Purkinje neurons, InsP_3Rs form bridging structures between adjacent cisternal stacks of smooth ER under certain conditions, via head-to-head contacts between opposing CRCs. Furthermore, cryoelectron micrographs of deep-etched, freeze-fractured cisternal stacks reveal that these InsP_3Rs are arranged in ordered arrays [216]. In a number of cell lines, elevations of intracellular Ca^{2+} triggered by 7-TMR activation, receptor tyrosine kinase stimulation or ionomycin lead to clustering of the InsP_3R population into large aggregates, as assessed by immunofluoresence and sucrose-density-gradient centrifugation techniques. This phenomenom is dependent on the presence of extracellular Ca^{2+}, occurs with all InsP_3R isoforms and is independent of any gross changes in endomembrane ultrastructure [217]. Such clustering of CRCs might lead to altered receptor/channel properties, such as the Ca^{2+}-induced conversion of hepatocyte InsP_3Rs from a high-channel-conductance, low-InsP_3-binding-affinity state, to a high-affinity, low-conductance conformation [218].

PERSPECTIVE: CRCs AS INTEGRATORS OF NUMEROUS CELLULAR SIGNALS

By virtue of direct interactions, as well as via their associations with accessory proteins, CRCs respond to a spectrum of intracellular signalling species, including Ca^{2+}, cAMP, cGMP, NO, reactive oxygen species, InsP_3, InsP_4 and cADPr. RyRs and possibly InsP_3Rs can respond to plasmalemmal depolarization via interactions with DHPRs. Consequently, CRCs are key integrators of such cellular signals, with the output being net release (by channel activation) or net uptake (by channel inhibition) of Ca^{2+} across the endomembrane system. Therefore CRCs represent key therapeutic targets for the treatment of diseases involving the dysregulation of intracellular Ca^{2+} homoeostasis, such as malignant hyperthermia and cardiomyopathies.

The activity of CRCs within a cell will be related to the distinctive subset, localization and activity of the accessory proteins that it expresses. Particular CRCs and accessory proteins might be co-expressed to suit specific roles within a particular cell type, this being exemplified by the predominance of the oxidant-insensitive type 3 InsP_3R in the oxidizing environment of bronchial mucosa cells, or the high concentrations of both InsP_3Rs and CaM within cerebellar Purkinje-cell dendritic spines. This tailoring of RyRs and InsP_3Rs to particular cellular environments by their protein–protein interactions explains why CRCs require accessory proteins and are not autonomous units, despite their large cytoplasmic domains. Reciprocal interactions between CRCs and modulatory proteins might explain discrepancies between biochemical and functional detection of these channels. For example, functional RyR-channel expression only becomes readily apparent in pancreatic β-cells [219] and in fetal-rat

neurons after activation of PKA. Furthermore, protein–protein interactions involved in CRC regulation are dynamic, such as the rapid clustering of InsP_3R complexes in response to extracellular signals, or the developmental increase in oligomerization of membrane proteins involved in Ca^{2+} handling in skeletal muscle [220]. Thus protein–protein interactions permit flexibility of CRC function in response to various intrinsic and extrinsic cues.

Many of the well-established CRC accessory proteins are highly abundant within cells, such as CaM, FKBP12, calsequestrin and triadin. However, it is also likely that low-abundance proteins are involved in CRC regulation, although the roles of few of these have been characterized. Such is the pace of research in this field, however, that known protein–protein interactions will be further clarified and novel CRC modulatory proteins will be identified during the course of publication of this article. Just as CRCs and their accessory proteins integrate numerous intracellular signals, a complete understanding of these interactions requires an integration of approaches. Although *in vitro* biochemical and molecular techniques resolve such associations in fine structural detail, they might not represent the situation *in vivo*, as exemplified by discrepancies between the behaviour of RyRs in FKBP12 knockout mice compared with those biochemically stripped of this isomerase. Conversely, observations of CRC activity in intact cells or whole organisms are frequently hampered by redundancy in signalling pathways, as well as by the lack of specific tools for pharmacological and molecular manipulations *in vivo*. Molecular genetic techniques used to study heritable defects in Ca^{2+} signalling often reveal structural detail of coupling between proteins involved in such transduction systems, but rarely yield direct information on the function of the mutated protein concerned. Consequently, the combination of such techniques is necessary in order to advance our understanding of the roles of CRCs and their accessory proteins in normal and diseased cell function.

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