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The type 2 inositol 1,4,5-trisphosphate receptor, emerging functions for an intriguing Ca^{2+}-release channel

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Abstract

The inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) type 2 (IP_3R2) is an intracellular Ca^{2+}-release channel located on the endoplasmic reticulum (ER). It displays in many cell types a predominantly perinuclear or even nuclear localization. IP_3R2 is characterized by a high sensitivity to both IP_3 and ATP and is biphasically regulated by Ca^{2+}. Interestingly, ATP stimulates IP_3R2 independently of the cytosolic [Ca^{2+}]. Furthermore, IP_3R2 is modulated by phosphorylation events mediated by e.g. protein kinase A, Ca^{2+}/calmodulin-dependent kinase II and protein kinase C. In addition to its regulation by protein kinase A, IP_3R2 forms a complex with adenylate cyclase 6 and is directly regulated by cAMP, thereby linking in a new way Ca^{2+}-dependent and cAMP-dependent signalling. Finally, in the ER, IP_3R2 is less mobile than the other IP_3R isoforms, while its functional properties appear dominant in heterotetramers. These properties make the IP_3R2 a Ca^{2+} channel with exquisite properties for setting up intracellular Ca^{2+} signals with unique characteristics. IP_3R2 plays a crucial role in the function of secretory cell types (e.g. pancreatic acinar cells, hepatocytes, salivary gland, eccrine sweat gland). In cardiac myocytes, the role of IP_3R2 appears more complex, because, together with IP_3R1, it is needed for normal cardiogenesis, while its aberrant activity is implicated in cardiac hypertrophy and arrhythmias. Moreover, IP_3R2 expression is driven by IP_3-induced Ca^{2+} release leading to a self-perpetuating system of cardiac hypertrophy. Most importantly, its high sensitivity to IP_3 makes IP_3R2 a target for anti-apoptotic proteins (e.g. Bcl-2) in B-cell cancers. Disrupting IP_3R/Bcl-2 interaction therefore leads in those cells to increased Ca^{2+} release and apoptosis. Intriguingly, IP_3R2 is not only implicated in apoptosis but also in the induction of senescence, another tumour-suppressive mechanism. These results were the first to unravel the physiological and pathophysiological role of IP_3R2 and we anticipate that further progress will soon be made in understanding the function of IP_3R2 in various tissues and organs.

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1. Introduction

The inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) are ubiquitously expressed intracellular Ca^{2+}-release channels. These channels are tetrameric in structure and predominantly localized in the endoplasmic reticulum (ER). IP_3, produced by phospholipase C after cell activation by hormones, growth factors or neurotransmitters, diffuses into the cytosol, binds to and activates the IP_3R, leading to Ca^{2+} release from the ER. This Ca^{2+} release is instrumental in the formation of the spatio-temporal Ca^{2+} signals, fundamental for the regulation of multiple cellular processes, including proliferation, differentiation, metabolism, secretion, cell fate and memory [1-3].

In all vertebrate organisms, three different genes encode IP_3Rs, leading to three main types of IP_3Rs, IP_3R1 (first fully cloned in 1989 [4]), IP_3R2 (first fully cloned in 1991 [5]) and IP_3R3 (first fully cloned in 1993 [6]). Each monomer is about 2700 amino acids in length and consequently has a predicted molecular mass of approximately 300 kDa. The IP_3R proteins are structurally and functionally divided into 5 distinct domains: the N-terminal coupling domain (a.k.a. suppressor domain), the IP_3-binding core, the internal coupling domain (a.k.a the modulatory and transducing domain), the channel domain and the C-terminal coupling domain (a.k.a. the gatekeeper domain) [7]. The three IP_3R isoforms share only 60-80 % overall similarity at the amino acid level, but the similarity is much higher in certain defined regions (e.g. the IP_3-binding site, the 5^{th} and 6^{th} transmembrane regions, putative ER-retention signals) while much lower in others, allowing for the existence of very distinct properties between the isoforms (see section 2). Finally, a further diversity can result from alternative splicing and the formation of heterotetramers [1, 8, 9].

The vast majority of cell types express more than one IP_3R isoform but their relative proportion can be highly variable [10-14]. Moreover, in various cases it was shown that the relative expression levels depended on the differentiation or developmental state of the cells or could be modulated by specific treatments (e.g. [14-21]).

The participation of the IP_3 Rs in establishing distinct patterns of Ca^{2+} signals resulting in different cellular outcomes depends therefore on the complement of the various IP_3R (splice) isoforms expressed, their intracellular location, the presence of regulatory factors, including associated proteins, and their phosphorylation status [1, 14, 22-25].

On the basis of their almost exclusive expression of a single IP_3R isoform, some cell types have been used as model system for the analysis of the role of the different IP_3R isoforms [8]. However, only a rather limited set of cell types expresses predominantly IP_3R2 (Table 1).

Interestingly, in many cells IP_3R2 is expressed at a different subcellular location than the other IP_3R isoforms. For example, in bovine aortic endothelial cells, bovine adrenal

glomerulosa cells and COS-7 cells [26], the HepG2 liver cell line [27] and the hippocampal cell line HT22 [28] IP_3R2 displayed a predominantly nuclear localization, while in hepatocytes IP_3R2 is confined to the apical pole of the cell, near the canalicular membrane [29].

The nuclear localization of IP_3R2 is particularly interesting with respect to the role of Ca^{2+} signalling in the nucleus, e.g. for gene transcription. Although the presence of IP_3Rs in the inner leaflet of the nuclear envelope remains the subject of debate [30-32], there is at least strong evidence that a subset of the IP_3R2 is facing the nucleoplasm in HepG2 cells [27], in SKHep1 cells [33] and in atrial myocytes [34] where they can control Ca^{2+} release directly into the nucleus.

Information concerning the regulation of IP_3R2 expression is likewise still quite limited. The 5′-flanking region of murine IP_3R2 has been sequenced and contained at least 7 transcription initiation sites with an upstream promoter containing no conventional TATA box but a GC box [35]. To the best of our knowledge, only two recent studies described pathways involved in the regulation of IP_3R2 expression. First, in the heart, direct binding of nuclear factor of activated T cells (NFAT) c1 to the IP_3R2 promoter drives IP_3R2 expression [36]. Second, in dendritic cells, IP_3R2 expression is controlled by the transcription factor ETS1, which itself depends on protein kinase B (AKT/PKB) 2 [37]. Finally, although not yet understood at the mechanistic level, in the HT22 cell line, oxidative stress leads to a specific upregulation of IP_3R2 [38] (see section 5).

Splicing of IP_3R2 is much less documented than that of IP_3R1, but two different splice variants have been described. One appears to be muscle-specific and is limited to the N-terminal 175 amino acids of IP_3R2 supplemented with 6 additional amino acids; although a regulatory role has been proposed, its function has not yet been elucidated. [39]. The second splice variant uses the same splice acceptor site and is lacking amino acids 176-208. The deletion is localized fully within the suppressor domain, which plays an important role in both the regulation of IP_3 binding and in the coupling of the IP_3R N-terminus to the channel region [40]. As a consequence, the resulting protein is defective in both IP_3 binding and Ca^{2+} release. However, its expression in cells prevents the agonist-dependent clustering of the endogenous IP_3Rs, probably via heterotetramerization, and can therefore impact intracellular Ca^{2+} signalling [41].

Finally, also at the protein level, IP_3R2 levels appear to be regulated in a different manner when compared to the other isoforms. While all IP_3R isoforms are downregulated under conditions of chronic stimulation [12, 42, 43], IP_3R2 appeared the least susceptible [12].

In spite of the unique molecular properties displayed by IP_3R2 (see sections 2.1-2.5), this protein is much less investigated than IP_3R1 or indeed, even IP_3R3. This apparent lack of progress was probably in retrospect multifaceted. The low general abundance of IP_3R2 and the lack of good model systems for its investigation partially explain the fewer studies directed specifically towards the IP_3R2. Additionally, but no less importantly, it appeared that it was historically difficult to make IP_3R2 expression constructs. Moreover, the quality of the antibodies raised against IP_3R2 was also generally poor. Finally, a recent analysis of

classically used IP_3R inhibitors demonstrated that IP_3R2 expressed in DT40 triple knockout (DT40 TKO) chicken B lymphocytes was the least sensitive of all the IP_3R isoforms to heparin, caffeine and 2-aminoethoxydiphenyl borate [44]. This latter observation may explain why pharmacological approaches to discern IP_3R2 function have been largely unsuccessful.

Notwithstanding these issues, recent work has begun to unravel the significance of IP_3R2 in a number of physiological settings. The aim of this review is therefore to highlight these important functions of IP_3R2 and to so stimulate further research in the field.

2. Specific molecular and cellular properties of IP_3R2

IP_3R2 has a high sequence and structural homology with the other IP_3R isoforms and consequently shares a large number of properties with IP_3R1 and IP_3R3. In this chapter, we will therefore focus on specific properties in which the IP_3R2 clearly differs from the other isoforms (Figure 1).

2.1 IP_3 affinity

Binding of IP_3 to the IP_3-binding core is the key step needed for the induction of IP_3-induced Ca^{2+} release. As stated above (see section 1), the three IP_3R isoforms all have a very similar structure [45]. For example, all IP_3R isoforms contain their IP_3-binding core towards their N-terminus, preceded by the so-called suppressor domain [46].

A striking property of the IP_3R2 is its much higher affinity for IP_3 when compared with the two other IP_3R isoforms. This was first observed in IP_3-binding experiments, which under various conditions demonstrated a rank-order of IP_3 affinities $IP_3R2 > IP_3R1 > IP_3R3$ [5, 10, 47]. Subsequent studies conclusively demonstrated that the IP_3-binding cores of each of the IP_3R isoforms display a similar affinity for IP_3 (about 2 nM), but as demonstrated by the analysis of normal and chimeric N-terminal domains of the different IP_3R isoforms the presence of the suppressor domain determines the specific IP_3 affinity of the isoform (50, 14 and 163 nM for IP_3R1, IP_3R2 and IP_3R3, respectively). These results underpin the importance of the suppressor domain for IP_3R function [46].

The difference in IP_3 affinity is also reflected in functional experiments. Analysis of IP_3-induced Ca^{2+} release in DT40 TKO cells expressing a single IP_3R isoform demonstrated that DT40 TKO cells heterologously expressing a single IP_3R isoform could sustain Ca^{2+} oscillations for an extended period after stimulation by an anti B-cell receptor antibody only if the expressed isoform was IP_3R2 [48]. Very similar results were obtained in vascular myocytes, whereby only the cells expressing IP_3R2 in addition to IP_3R1 displayed a Ca^{2+} oscillation pattern [49, 50]. Comparison of native IP_3R1 (from cerebellum) and IP_3R2 (from heart) [51] or the comparative analysis of each of the three IP_3R isoforms heterologously expressed in Sf9 insect cells [52] also confirmed the rank-order of the sensitivity of channel opening towards IP_3 as $IP_3R2 > IP_3R1 > IP_3R3$, when measured after incorporation in planar lipid bilayers.

It must however be pointed out that a number of studies found a rank-order of affinities that is different from that mentioned above [53, 54]. Although this discrepancy has never been fully clarified, it can be assumed that variability in the experimental conditions (e.g. pH, [Ca^{2+}]) as well as in the state of the IP_3Rs (e.g. redox state, phosphorylation state, associated proteins, existence of heterotetramers) could explain this variability [1].

Taken together, most of the available evidence points to IP_3R2 as being the most sensitive IP_3R isoform. This observation raises the intriguing potential that IP_3R2 can be active in the presence of basal, resting IP_3 levels.

2.2. Regulation by cytosolic Ca^{2+}

It has long been recognized that IP_3R activity can be biphasically regulated by cytosolic Ca^{2+}, meaning that a relatively low [Ca^{2+}] (usually less than 0.3 μM) potentiate IP_3-induced Ca^{2+} release, while higher [Ca^{2+}] lead to an inhibition of the IP_3R. Plotting IP_3R activity against [Ca^{2+}] therefore leads to a typical bell-shaped curve. The original observations were obtained in smooth muscle [55], neurons [56, 57] and oocytes [58], all tissues later shown to be particularly rich in IP_3R1. It was therefore a long standing question whether this property was uniquely related to this isoform or whether IP_3R2 and IP_3R3 shared this property.

Although the stimulatory effect of Ca^{2+} on IP_3R2 (and IP_3R3) activity has never been in doubt, the inhibitory action of a high [Ca^{2+}] was not always clearly observed. The group of Mignery published single-channel data demonstrating that both IP_3R2 endogenously expressed in heart [51] or recombinantly expressed IP_3R2 [59] displayed a much broader bell-shaped dependence towards Ca^{2+}, meaning that on the one hand the stimulatory phase starts at a much lower [Ca^{2+}] than for IP_3R1, and on the other hand that IP_3R2 remains active at a [Ca^{2+}] already fully inhibiting IP_3R1. In contrast to these reports, the group of Bezprozvanny found quite similar (and narrow) [Ca^{2+}] response curves for each of the three IP_3R isoforms heterologously expressed in Sf9 insect cells and investigated in planar lipid bilayers [60].

As will be further explained below (see section 2.3), the sensitivity of the IP_3R2 towards Ca^{2+} is not dependent on the presence of ATP, but the latter will increase the likelihood of IP_3R2 being in an open state at all [Ca^{2+}] (Figure 2).

Taken together, these results indicate that IP_3R2, similarly to the other IP_3R isoforms, is regulated in a biphasic way by the cytosolic [Ca^{2+}], though that depending on the exact state of the receptor, different sensitivities to Ca^{2+} can be observed.

2.3. Regulation by ATP

2.3.1. Regulation of IP_3R2 is distinct from other IP_3Rs—Adenine nucleotides were recognized by early studies [61-68] as important regulators of IP_3-induced Ca^{2+} release, raising the attractive possibility that channel activity could be fine-tuned to match the metabolic status of the cell. The diversity of cell types in which ATP modulates IP_3-induced Ca^{2+} release is consistent with a regulation affecting all IP_3R family members. However, two initial studies reported that in contrast to IP_3R1 and IP_3R3, IP_3R2 was not subject to modulation by adenine nucleotides [48, 52]. Specifically, studying individual mammalian

isoforms reconstituted in planar lipid bilayers, Bezprozvanny and colleagues reported that under conditions optimal for channel activity IP_3R2 had no requirement for ATP [52]. This observation was independently confirmed studying Ca^{2+} release from DT40 cells expressing a single IP_3R isoform following genetic ablation of the other family members [48]. These reports are, in hindsight, important because they provide the first indication that ATP regulation of IP_3R2 was distinct from the other family members. A subsequent detailed analysis of individual mammalian isoforms expressed in the DT40 TKO IP_3R *null* background confirmed these earlier reports [69]. IP_3R2 was indeed, in contrast to other IP_3R family members, insensitive to ATP at maximal [IP_3]. Nevertheless, it was demonstrated that IP_3R2 activity, measured as Ca^{2+} release, or at the single channel level in "on-nucleus" patch clamp recordings, was markedly enhanced at sub-saturating [IP_3] [69]. Moreover, the sensitivity of ATP regulation of IP_3-induced Ca^{2+} release also differed between individual isoforms under identical conditions with IP_3R2 being strikingly more sensitive than IP_3R1 or IP_3R3 (EC_{50} 40 μM, 100 μM and 500 μM for IP_3R2, IP_3R1 and IP_3R3 respectively) [69, 70]. An issue posed by these data is the concentration range of ATP that might be expected to dynamically regulate IP_3R activity. In turn, this raises the question whether modulation occurs at physiological levels of nucleotides or is only relevant under pathological conditions when ATP is depleted. Given the cellular levels of MgATP (~1 mM) and "free" ATP^{3-} and ATP^{4-} (10-100 μM) the answer is fundamentally dependent on the "species" of ATP that regulates IP_3R channel activity. Several studies have addressed this issue and have reached disparate conclusions [67, 71-73] and thus this important issue as well as the consequences of the high functional affinity of IP_3R2 remains to be resolved.

2.3.2. Putative peptide motifs in IP_3R2 responsible for ATP regulation—

Modulation of IP_3R activity is widely believed to occur by ATP binding to glycine-rich domains (Gly-Xaa-Gly-Xaa-Xaa-Gly), reminiscent of Walker type A repeats, present in a number of proteins that utilize ATP in a catalytic manner [74-77]. Consistent with this idea, a number of studies using either photo-affinity or fluorescent ATP probes have demonstrated binding to regions of IP_3R or glutathione S-transferase-recombinant fragments harbouring these putative recognition sites [69, 75-77]. The primary sequence of IP_3R2 contains one such motif, Gly-Leu-Gly-Leu-Leu-Gly, spanning amino acids 1969-1974, which has been termed the "ATPB" site (Figure 1). Mutagenesis of three Gly residues to Ala in the motif eliminated binding of ATP and nucleotide regulation of Ca^{2+} release, confirming the functional importance of the ATPB site in IP_3R2 [69]. Moreover, in cells expressing IP_3R2 with an ATP binding-deficient ATPB motif, the frequency and amplitude of B cell receptor-activated Ca^{2+} oscillations were markedly reduced compared with wild-type IP_3R2, suggesting strongly that nucleotide regulation of Ca^{2+} release is at least, constitutively required to shape cytosolic Ca^{2+} signals at physiologically relevant ATP levels [69]. Unexpectedly, mutations of all known Walker A motifs in IP_3R1 and IP_3R3 failed to abrogate nucleotide modulation [70]. The somewhat surprising conclusion is therefore, that ATP regulation of IP_3R1 and IP_3R3 is independent of known ATP-binding motifs, and thus the identity of molecular sites of nucleotide regulation in these IP_3R remains to be elucidated. Consequently, the ATPB site in IP_3R2 is unique as the only molecular locus for regulation of IP_3R family members by adenine nucleotides that is defined unequivocally.

2.3.3. Mechanism of ATP regulation of IP_3R2—Several studies have investigated the biophysical basis for ATP regulation of IP_3R channel activity. In accordance with the singular features of IP_3R2 when compared with other family members, it also appears that ATP regulates IP_3R2 in a similarly distinctive manner. Using "on-nucleus" patch clamp single channel recordings of both endogenous *Xenopus* IP_3R1 or rat IP_3R1 expressed in DT40 TKO cells, elevating ATP levels increased the channel open probability (P_o) by modulating the sensitivity of the channel to both activating and inhibitory [Ca^{2+}], essentially left-shifting the bell-shaped [Ca^{2+}] versus P_o relationship at a given [IP_3] [78, 79].

In contrast, while IP_3R2 displays an identical biphasic Ca^{2+} sensitivity when exposed to saturating [IP_3] (conditions in which IP_3R2 is insensitive to ATP), at low [IP_3], the Ca^{2+} sensitivity of mouse IP_3R2 was not altered by increasing ATP [79]. Elevating ATP simply dramatically enhanced P_o, resulting in a marked increase in activity. A detailed kinetic analysis of the channel gating also indicated that IP_3R2 displayed "bursting" activity with properties distinct from IP_3R1 (Figure 2). Specifically, with elevated ATP, the number of bursting episodes of relatively constant duration was increased, while IP_3R1 bursts simply lengthened in time. By analogy to a gear change in a car, we have termed this the transition from 'park' into a 'drive' mode. A minimal scheme to describe the channel kinetics at sub-saturating [IP_3] suggests that both channels transition between single open and closed states during drive mode with relatively constant kinetics and then are "parked" in a longer-lived closed state in the interburst intervals [79]. In the case of the IP_3R1, increasing Ca^{2+} and ATP facilitates bursting by facilitating both the transition out of the parked state and also by decreasing the likelihood it will return to this state. In contrast, [Ca^{2+}] does not influence the time the IP_3R2 spends in drive mode but simply destabilizes the parked state to initiate activity (Figure 3). Increasing ATP then appears to markedly increase overall channel P_o by prominently decreasing the amount of time in the parked state [79]. This unique property results in dissociation of the modulation of IP_3R2 activity by ATP from the [Ca^{2+}] in its immediate environment and likely allows added flexibility for tuning Ca^{2+} signals to the needs of the cell.

2.3.4. Dominance of IP_3R2 ATP regulatory characteristics—An important question exists as to how the distinct features of individual IP_3R subtypes are reflected in the overall characteristics of Ca^{2+} release from heterotetrameric channels. Specifically, are the properties simply a blended integration of the individual subtypes or can a particular subtype dominate the overall characteristics? Studies investigating ATP regulation of Ca^{2+} release in cells expressing multiple IP_3R isoforms indicate that the latter possibility occurs, specifically when IP_3R2 is expressed. For example, the characteristics of ATP regulation of IP_3R2 (albeit the lack of regulation at saturating [IP_3]) were observed in DT40 cells engineered to express only IP_3R2, or in cells expressing both IP_3R2 and IP_3R1 or IP_3R3 [48]. Similarly, in salivary and pancreatic acinar cells [80, 81], which natively express IP_3R2 and IP_3R3 to approximately equal extents, the features of ATP regulation precisely match those documented for IP_3R2 stably expressed in isolation in either DT40 TKO [69] or in AR42J pancreatoma [80] cells (*i.e.* absence of regulation at saturating [IP_3] and EC_{50} for ATP ~40 μM). Notably, similar experiments in pancreatic and parotid acinar cells prepared from IP_3R2 *null* animals revealed identical properties to IP_3R3 (*i.e.* regulation at all [IP_3]

and EC_{50} for ATP ~500 μM) [80, 81]. Conversely, "rescue" experiments ectopically expressing IP_3R2 in RINm5F insulinoma cells which predominately express IP_3R3, converted IP_3R3 characteristics to IP_3R2 [80]. While these data clearly indicate the dominant influence of IP_3R2 expression and are consistent with this occurring as a function of heterotetramer formation, these data could also formally be explained by an *inter*molecular interaction between clusters of homotetrameric IP_3R. This issue has recently been tackled by generating tetrameric IP_3R from concatenated IP_3R dimers connected by short flexible linkers [82]. Expression of dimers results in the assembly of tetramers where the subunit composition can be unequivocally defined. Expression of dimers of IP_3R1 or IP_3R2 exhibited the distinctive properties of ATP regulation typical of channels assembled from their respective monomeric parent subtype. Remarkably when heterodimers of IP_3R1 and IP_3R2 were expressed, resulting in assembly of channels consisting of equal numbers of IP_3R1 and IP_3R2 subunits, ATP regulation was indistinguishable from IP_3R2, thus recapitulating the dominant effects seen in cells expressing native receptors [82]. These data indicate that IP_3R2 in the context of a heterotetrameric channel exerts a dominant influence. Further work is needed to establish the number of monomers of IP_3R2 necessary to exert this influence and whether IP_3R2 similarly is the principle monomer that dictates the overall channel properties when subjected to other forms of regulation.

2.4. Regulation by phosphorylation

Like for many other ion channels, phosphorylation/dephosphorylation reactions provide a versatile, reversible form of acute regulation of IP_3R activity. IP_3Rs have been shown to be biochemical substrates for numerous families of serine/threonine and tyrosine directed kinases. In a more limited number of cases, a comprehensive documentation of the phosphorylation event, including location of the substrate motif and the subsequent functional consequences have been detailed. These studies have largely focused on IP_3R1 as a template. With some notable exceptions, for example the AKT/PKB site conserved in the C-termini of each IP_3R [83, 84], the amino acid motifs subject to phosphorylation events are not generally preserved between IP_3R subtypes (Figure 1). Therefore, this form of regulation has the capacity to provide modulation of activity in an IP_3R sub-type specific manner. Below we highlight reports that have specifically focused on regulation of IP_3R2 activity. The interested reader is directed to Vanderheyden et al. [23] and Betzenhauser and Yule [85] for detailed discussion of IP_3R phosphorylation and its functional consequences.

2.4.1. Regulation of IP_3R2 by protein kinase A (PKA)—Historically, perhaps the most exhaustive investigation of IP_3R modulation relates to PKA phosphorylation of IP_3R1. Indeed, IP_3R1 was identified as a major brain phosphorylated substrate even prior to the protein being appreciated as the receptor for IP_3 [86]. Subsequent studies demonstrated that IP_3R1 is phosphorylated at serine residues within two canonical consensus motifs (Bas-Bas-Xaa-Ser/Thr, where Bas = a basic residue) [87, 88] and phosphorylation is associated with markedly enhanced Ca^{2+} release [89, 90]. To complete the strong case for IP_3R1 being a functionally important PKA substrate, mutation of Ser^{1589} and Ser^{1755} to non-phosphorylatable alanine residues completely abrogates phosphate incorporation and the functional effects of PKA activation [90-92]. However, while PKA activation in cells that predominately express IP_3R2 such as hepatocytes, parotid acinar cells and AR42J similarly

results in enhanced Ca^{2+} release [93-95], the PKA substrate motifs present in IP_3R1 are not conserved in IP_3R2 [5]. In addition, while PKA activation results in IP_3R2 phosphorylation, phosphate incorporation is non-stoichiometric and indeed much reduced in comparison to IP_3R1 [96]. Nevertheless, IP_3R2 contains approximately 30 serine or threonine residues, which constitute minimal PKA consensus motifs consisting of basic residues preceding the phosphorylated amino acid at the −2 and −3 positions (Bas-Bas-Xaa-Ser/Thr). Using an approach based on expressing consecutive domains of IP_3R2 with N-terminal epitope tags, it was shown that PKA could only specifically phosphorylate *in vitro* a peptide fragment consisting of amino acids 920-1583 [97]. Mutation of Ser^{937} to alanine ($Ser^{937}Ala$) abrogated all phosphorylation of the fragment, pinpointing this residue as the PKA target site (Figure 1). Subsequently, it was shown that an antibody raised against phospho-Ser^{937} recognized IP_3R2 after forskolin treatment in cells expressing IP_3R2 but not cells expressing a mutant full-length receptor harbouring a $Ser^{937}Ala$ mutation [97]. Notably, Ser^{937} was independently identified as a phosphorylated residue in a proteomic screen of hepatocytes [98]. Importantly, PKA activation markedly potentiated IP_3-induced Ca^{2+} release in DT40 TKO cells expressing IP_3R2 but not Ser^{937} Ala IP_3R2 thus establishing this motif as likely solely responsible for the PKA-mediated phospho-regulation of IP_3R2 [97]. The Ca^{2+} signalling machinery is a rich source of substrates responsible for cross-talk between cAMP and Ca^{2+} signalling which ensure fine-tuning of the Ca^{2+} signal and appropriate activation of effectors [99]. PKA phosphorylation of IP_3R2 likely is an important site of this interaction in cells such as astrocytes, cardiac myocytes, hepatocytes and acinar cells that prominently express this family member.

2.4.2. Regulation of IP_3R2 by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)—CaMKII are a family of serine/threonine kinases assembled as either homo- or heteromultimers derived from the products of four closely related genes [100]. As a Ca^{2+}/calmodulin-regulated enzyme, this kinase is an important primary effector of IP_3R-induced Ca^{2+} release and accordingly plays prominent roles in regulating various signal transduction pathways including the translocation of transcription factors and activity of ion channels [101, 102]. Notably, IP_3Rs are substrates for the kinase, which provides a regulatory loop following Ca^{2+} release. Early work suggested that IP_3R1 was a substrate for CaMKII *in vitro* and that the sites were distinct from those phosphorylated by PKA [103], however the functional consequences were poorly defined. Subsequently, studies based largely on pharmacology, concluded that Ca^{2+} release from Xenopus oocytes and HeLa cells was attenuated following CaMKII activation [104, 105]. More recently, a thorough characterization of the molecular sites and functional consequences of the CaMKII-mediated phopho-regulation of IP_3R2 has been reported. Using a similar approach to that used to identify PKA sites, the ability of CaMKII to phosphorylate IP_3R2 fragments *in vitro* was assessed. It was initially demonstrated that a candidate residue was present within a fragment encompassing the initial 1078 amino acids [106] and further refinement narrowed the potential phosphor-acceptor residue to within residues 134-338 [107]. Subsequent mutagenesis of potential serines/threonine residues in CaMKII consensus motifs (Ser/Thr-Xaa-Asp) within this region [107] identified Ser^{150} as phosphorylated by CaMKII (Figure 1). This site is conserved in mammalian IP_3R family members and ryanodine receptor (RyR) 2, suggesting a common mode of regulation in these channels. When incorporated in

bilayers, CaMKII phosphorylation reduced the P_o of IP_3R2 and this effect was reversed by the CaMKII inhibitor KN62. Importantly, the reduced channel activity was absent in Ser^{150}Ala IP_3R2, indicating that the site was functionally relevant [107]. Notably, CaMKII colocalizes and interacts with IP_3R2 in the nuclear envelope of cardiac myocytes [106, 108]. This interaction has been proposed to be functional important for cardiac remodelling during hypertrophy [109] (see section 4).

2.4.3. Regulation of IP_3R2 by protein kinase C (PKC)—Cerebellar IP_3R was also initially identified as a substrate for PKC with phosphorylation sites independent of those sites modified by PKA [103]. Interestingly, however, it was demonstrated that phosphorylation by PKC was enhanced by prior PKA phosphorylation indicating a potential additional layer of cross-talk between these prominent cellular signalling systems [110]. Similarly to CaMKII, because at least conventional PKC family members are regulated by an elevation in Ca^{2+}, PKC phosphorylation provides a potential feedback loop to regulate IP_3R activity. To date however, the functional effects of PKC phosphorylation and the sites of phosphorylation are relatively poorly defined. Unfortunately despite the general appreciation of the amino acid motifs that constitutes a PKC consensus sequence and the presence of multiple such templates in IP_3R, none have been experimentally defined. Furthermore, while PKC activation results in enhanced Ca^{2+} release from liver nuclei, presumably reflecting IP_3R1 and IP_3R2 activity [111], Ca^{2+} release is inhibited in AR42J cells, which predominately express IP_3R2 [112]. These disparate findings may reflect subtype-specific regulation of IP_3R given that the PKC consensus motif numbers and location are different in each family member [9]. However an additional consideration is that numerous proteins in the signalling pathway from plasma membrane receptor occupation to the generation of Ca^{2+} signals are substrates for PKC and thus caution must be taken in interpreting data generated from indirect measurements of IP_3R activity. Our own experience is that IP_3R2 single channel activity recorded in either DT40 cell nuclei or in DT40 plasma membranes was unaffected by phorbol ester treatment or recombinant PKC exposure (Wagner, Chandrasekhar and Yule; unpublished observations). These data might indicate that IP_3R is not a direct substrate for PKC. Yet, we cannot formally exclude the possibility that a scaffolding or anchoring protein necessary for activity is absent from the DT40 system. Hence, further work is required to characterize the impact of PKC on IP_3R activity in general and on IP_3R2 in particular.

2.5. Other characteristics of IP_3R2

All the characteristics discussed above (see sections 2.1-2.4) have been the subject of extensive investigations. There are however, a few less studied properties, which nevertheless might be very interesting for understanding the cellular function of IP_3R2.

2.5.1. Regulation of IP_3R2 by cAMP—Similarly to the other IP_3R isoforms, many accessory proteins interact with, and modulate IP_3R2 function. These proteins include regulatory and structural proteins, many of which were also reported to interact with IP_3R1 and/or IP_3R3 [1, 2, 14]. An interesting exception is the interaction described between IP_3R2 and type 6 adenylate cyclase (AC6) [113]. In HEK 293 cells stably transfected with the type I parathyroid hormone receptor, a complex is formed between IP_3R2, AC6 and $G\alpha s$ [113,

114]. This close association facilitates an exquisite regulation of IP_3R2 by cAMP, and in addition, Ca^{2+} released through IP_3R2 may control AC6 in a negative feedback loop. Importantly, the regulation by cAMP does not require the canonical ATP-binding site or the activity of PKA. Moreover, although all IP_3R isoforms are potentially sensitive to cAMP, only IP_3R2 has been unequivocally linked to a cAMP-producing enzyme (AC6) [114]. This mechanism can be of great general importance, since it provides a novel example of cross-talk between the cAMP- and the Ca^{2+}-dependent pathways.

2.5.2. Clustering and mobility of IP_3R2—All IP_3R isoforms, including IP_3R2 [115] are known to cluster in an agonist-dependent way [116] but a punctate distribution of IP_3R2 has also been observed for native IP_3R2 [117] and heterologously expressed IP_3R2 [41] in resting cells. This property can be correlated to the higher affinity of IP_3R2 for IP_3 (see section 2.1), which may allow the clustering to occur at basal, resting [IP_3]. Interestingly, also other differences in behaviour were found between the different IP_3R isoforms. A recent study performed in COS-7 cells confirmed that heterologously expressed IP_3R2 showed a punctate distribution, in contrast to IP_3R1 and IP_3R3 that were uniformly distributed [118]. Moreover, IP_3R2 appeared much less mobile than either the other IP_3R isoforms or than other proteins involved in intracellular Ca^{2+} handling, such as RyR1 or sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) 1. In addition, its mobility depended on its intracellular localization with the IP_3R2 located in the perinuclear region having the lowest mobility. As the IP_3R2 has the highest sensitivity to IP_3 (see section 2.1), its lesser mobility may determine the initiation sites for intracellular Ca^{2+} signals.

3. The function of IP_3R2 in secretory cells

IP_3R2 exhibits prominent expression in classical secretory cells (Table 1), including exocrine cells of the pancreas [119-121], salivary glands [81, 120-123], lacrimal gland [124], olfactory glands [125], liver [29], eccrine sweat glands [126] and the secretory epithelia of the biliary tree [127] and the intestine [18] and the goblet cells of the small intestine [128]. A common feature of these epithelial cells is that they are morphologically and functionally polarized to secrete fluid and protein across their apical pole into a lumen forming a duct. Notably, the Ca^{2+} signal is centrally important to the primary secretory function of these cells by virtue of directly activating ion channels and the exocytotic machinery necessary for vectoral fluid and protein secretion [129, 130]. In exocrine glands, IP_3R2 and IP_3R3 are expressed in approximately equal numbers [12] and both family members are co-localized to a region immediately below the apical plasma membrane [119, 120]. This region has been termed “the trigger zone” because Ca^{2+} signals are invariably initiated in this region prior to the signal spreading as a Ca^{2+} wave towards the basal aspects of the cell [131-133]. There appears to be substantial functional redundancy between IP_3R2 and IP_3R3 in exocrine cells as mice *null* for either IP_3R in isolation have no obvious phenotype. However, compound knockouts of IP_3R2 and IP_3R3 have severe exocrine deficiency manifested as dry mouth [121], dry eye [124], pancreatic insufficiency [121] and attenuated mucus secretion [125]. As such, double knockouts are born normally but demise soon after weaning [121]. Indeed a detailed analysis of these mice have shown that Ca^{2+} signals in pancreatic, salivary, lacrimal and mucus glands are essentially unaltered in IP_3R3 *null* mice [121, 124, 125] and only reduced to a modest degree at low [IP_3] in IP_3R2 *null*

mice [80, 81, 121, 124, 125]. These data suggest that IP_3R2 is not generally essential for overall exocrine function.

A possible exception to this idea has been highlighted by a recent study, which investigated the cause of a severe congenital sweating defect in a Pakistani family [126]. Ca^{2+} signalling is known to be important for sweat secretion and both IP_3R2 and IP_3R3 are expressed in the secretory cells of the sweat gland. A screen based on identifying regions of autozygosity in the genome of afflicted individuals revealed a mutation ($Gly^{2498}Ser$) targeting an amino acid predicted to be critical to the function of the selectivity filter in the pore region of IP_3R2. This mutation rendered the channel completely inactive when expressed in DT40 TKO cells, thus potentially explaining the defect observed in the patients. Consistent with this idea, subsequent studies showed that mice lacking IP_3R2 exhibited a decreased ability to sweat although the effect was more modest than observed in humans. The differences in severity between the phenotype observed between mouse and human may reflect the relative levels of these subtypes in mouse versus human. Alternatively, the relatively mild phenotype in the mouse might be related *solely* to the knockout of IP_3R2, reflecting some degree of compensation by the residual IP_3R3. In this scenario the more severe effect in human could be due to the combined effect of ablation of IP_3R2 pore function and a possible dominant negative effect of the mutant IP_3R2 when incorporated into heterotetramers containing IP_3R3.

The primary secretory function of hepatocytes is the secretion of bile and changes in intracellular Ca^{2+} play important regulatory roles in this process. Hepatocytes express predominantly IP_3R2 (Table 1) with smaller amounts of IP_3R1 and virtually no IP_3R3. However, in contrast to exocrine acinar cells, each isoform exhibits a distinct sub-cellular localization and therefore the isoforms appear not to have redundant functions. IP_3R2 is enriched at the canalicular membrane, whereas IP_3R1 has a more uniform distribution throughout structures in the cytosol [29]. Consistent with the sensitivity of IP_3R2, agonist-induced Ca^{2+} signalling is initiated through IP_3R2 localized to the canalicular membrane [29, 134, 135] and Ca^{2+} release through this isoform is necessary for trafficking of the bile salt export pump to the canalicular membrane [134].

4. The function of IP_3R2 in the heart

As indicated (Table 1), cardiomyocytes are one of the cell types in which IP_3R2 are highly expressed. Both atria and ventricles express IP_3R2 [136] and consequently IP_3R2 channels have been implicated in both physiological and pathophysiological signalling in the heart.

At the physiological level, Mikoshiba and co-workers showed that IP_3R2 channels, together with IP_3R1 channels, are critical for normal cardiogenesis [137]. Consistent with this, IP_3R2 and IP_3R1 are co-expressed in different parts of the embryonic heart, including atria, ventricle and atrioventricular canal, and in different cell types, including endothelial cells and cardiomyocytes, although timing differences in the appearance of IP_3R2 versus IP_3R1 exist. IP_3R1/IP_3R2 double knockout mice die *in utero* at embryonic stage E11.5 with major heart defects at the level of the ventricles (thin myocardial wall and poor trabeculation) and the atrioventricular canal (reduced number of cells). These defects were associated with a

decrease in endocardial and myocardial cell proliferation. Furthermore, mesenchymal cells were lacking at the level of the developing atrioventricular canal. The authors hypothesized that this phenotype was due to the absence of IP_3R-mediated Ca^{2+} signalling, downstream of the activation of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin and of the translocation of NFATc to the nucleus. Indeed, in an *ex vivo* epithelial-mesenchymal transition (EMT) assay, the defect in EMT in atrioventricular explants derived from IP_3R1/IP_3R2 double knockout mice could be restored by transducing constitutively active calcineurin. Moreover, the phenotype of these mice resembled well the phenotypes of mice knockouts for calcineurin B [138] or for NFATc3/NFATc4 [139]. Interestingly, the defect in endocardial cells could also be observed in developing zebrafish exposed to calcineurin inhibitors, such as FK506 or cyclosporine A. Hence, from this study, it is clear that IP_3R1 or IP_3R2 channels are needed for activating calcineurin/NFATc signalling and endocardial cell proliferation in vertebrates. It is important to note that the presence of either IP_3R1 or IP_3R2 is sufficient to drive normal cardiac development, indicating redundant functions for these channels in this process. In addition to the cardiac crescent, or first heart field, giving rise to a linear beating tube, there is a second source of myocardial cells, which is termed the second heart field. However, with respect to the latter, it appears that there is a redundant role for IP_3R1 and IP_3R3 [140]. IP_3R1/IP_3R3 double knockout mice are characterized by hypoplasia of the outflow tract and the primitive right ventricle at E8.5-9.5, probably due to a defective Mef2c-Smyd1 transcriptional pathway.

At the functional level, IP_3Rs were first shown to impact contractility and arrhythmias. Atrial myocytes express much higher levels of IP_3R2 than ventricular myocytes [136]. They are activated in response to elevated extracellular agonist concentrations, e.g. after ischaemia or during disease. Endothelin-1-induced IP_3R activation promotes the inotropy and the occurrence of arrhythmic events in atrial myocytes [141, 142]. While in basal conditions, the cardiac function of IP_3R2 knockout mice was similar to one of wild-type mice, the positive inotropic effect and the arrhythmic events induced by endothelin-1 were absent in IP_3R2 knockout mice [143]. Hence, the presence of IP_3R2 appears to be not essential for the normal functioning of the rodent heart, which is in line with the normal phenotype of the IP_3R2 knockout mice generated by Chen and co-workers [143] and by Mikoshiba and co-workers [121].

In the ventricle, far fewer IP_3Rs are present but may still contribute to Ca^{2+} regulation under baseline conditions [144-147]. Moreover, a study by Roderick and co-workers revealed that increased IP_3R2-mediated Ca^{2+} signalling, in response to enhanced IP_3 signalling, is responsible for inducing hypertrophic pathways after prolonged endothelin-1 exposure of neonatal or adult rat ventricular cardiomyocytes [148]. Endothelin-1 triggered the expression of atrial natriuretic factor (ANF), a marker for hypertrophy. Endothelin-1-induced hypertrophy was independent of excitation-contraction coupling, but required IP_3 signalling and downstream IP_3R-mediated Ca^{2+} release. The latter occurred in the perinuclear region, but not in the cytosol, while Ca^{2+} transients linked to excitation-contraction coupling occurred throughout the cardiomyocyte. Specifically buffering nuclear Ca^{2+} by nuclear-targeted calbindin prevented endothelin-1-induced ANF expression. The nuclear Ca^{2+} signal

was mediated by IP_3R2 channels, which were enriched in the perinuclear region and led to the activation of calcineurin and downstream NFATc1, which accumulated in the nucleus.

The IP_3R/calcineurin/NFATc1 hyperactivity also seems to be operative in response to prolonged β-adrenergic signalling, which occurs during workload-induced cardiac hypertrophy via enhanced excitation-contraction coupling. Interestingly, this model led to increased endothelin-1 signalling. This involved the release of endothelin-1 and autocrine/paracrine-mediated hyperactivation of its receptor (ET_AR), thereby triggering downstream IP_3 signalling and Ca^{2+}-dependent calcineurin activation. In hypertrophic cardiomyocytes (e.g. derived from spontaneous hypertrophic rats or from aortic-banded mice), IP_3R2 not only played a role in the nucleus, where its hyperactivation via increased IP_3 signalling downstream of ET_AR led to calcineurin/NFATc1 activation and ANF expression, but also became upregulated in the junctional sarcoplasmic reticulum [146]. Here, localization of IP_3R2 coincides with RyR2 channels, thereby augmenting Ca^{2+} transients associated with excitation-contraction coupling or endothelin-1 exposure. As a consequence, the IP_3R2-mediated Ca^{2+} rise during diastole may activate or sensitize RyR2 channels, resulting in spontaneous extra-systolic Ca^{2+}-release events and the occurrence of arrhythmias [147]. This increased “extra-nuclear” expression of IP_3R2 was also found in human heart samples derived from patients with heart failure after ischemic dilated cardiomyopathy [146].

The upregulation of IP_3R2 channels during cardiac hypertrophy was mediated via a dynamic and Ca^{2+}-dependent regulation of miRNA-133a [149]. In normal physiological conditions, miRNA-133a expression is highly expressed in cardiomyocytes, thereby targeting the 3′ untranslated region of the IP_3R2 mRNA. As a consequence miRNA-133a reduces the basal expression of IP_3R2 and thereby avoids hypertrophy or arrhythmias resulting from excessive Ca^{2+} signalling. Interestingly, limiting the expression of miRNA-133a using an antagomir led to hypertrophic signalling (evident from the increased ANF expression), which was dependent on IP_3-induced Ca^{2+} release, since degrading IP_3 using IP_3 5-phosphatase limited the increase of ANF by miR-133a antagomir.

The role of miR-133a in controlling IP_3R2 expression and the initiation of hypertrophic markers was found both *ex vivo* and *in vivo*. In isolated, hypertrophic cardiomyocytes from spontaneous hypertensive rats, IP_3R2 levels were elevated, while miRNA-133a was downregulated [149]. Overexpression of miRNA-133a in these hypertrophic cardiomyocytes reduced ANF expression to levels similar as control cardiomyocytes. In addition, *in vivo* application of miRNA-133a antagomir caused IP_3R2 upregulation and hypertrophic signalling. Interestingly, increased IP_3-induced Ca^{2+} release was also involved in the decreased miRNA-133a expression in hypertrophic models. Indeed, lowering IP_3 signalling by IP_3 5-phosphatase transduction blunted the endothelin-1-induced decrease in miRNA-133a and the concomitant increase in IP_3R2 protein levels. Collectively, these findings indicate that during pathophysiological conditions associated with increased endothelin-1, increased IP_3 signalling can lead to downregulation of miRNA-133a. The latter will lead to upregulation of IP_3R2 protein levels, thereby further driving the downregulation of miRNA-133a by boosting IP_3-induced IP_3R2-mediated Ca^{2+} signalling. This perpetual feedback cycle will establish a new signalling network that favours the

expression of hypertrophic genes like ANF (via hyperactivation of calcineurin/NFATc1) and the occurrence of arrhythmic events.

The mechanism by which IP_3-induced Ca^{2+} release controls miRNA-133a expression seems to involve transcription factors like the serum response factor (SRF), which is negatively regulated by the homeodomain-only protein. SRF induces miRNA-133a expression and subsequent IP_3R2 downregulation. However, during hypertrophy, IP_3-induced Ca^{2+} release may increase homeodomain-only protein expression, thereby recruiting class I histone deacetylase (HDAC) and limiting transcriptional activity of SRF.

The importance of IP_3 signalling and IP_3R2 has also been elegantly addressed by Molkentin and co-workers by the generation of transgenic mice overexpressing an IP_3 sponge, which represents a mutated, high-affinity form of the IP_3-binding core (to blunt endogenous IP_3-induced Ca^{2+} release by trapping IP_3), or overexpressing IP_3R2 (to boost IP_3-induced Ca^{2+} release) in cardiomyocytes [150]. Mice overexpressing the IP_3 sponge displayed reduced cardiac hypertrophy in response to chronic β-adrenergic stimulation and angiotensin II stimulation. In contrast, IP_3R2-overexpressing mice displayed only a mild cardiac hypertrophic phenotype under basal conditions. However, when cardiac hypertrophy was induced (e.g. using transverse aortic constriction, chronic β-adrenergic stimulation or overexpression of Gαq, an upstream phospholipase C activator) mice expressing high IP_3R2 levels demonstrated increased hypertrophic responses. Under these conditions, mice expressing low levels of IP_3R2 (except for transverse aortic constriction) also displayed enhanced cardiac hypertrophy. Moreover, IP_3R2 channels, which already display high sensitivity to IP_3, may be further sensitized by increased PKA signalling downstream of β-adrenergic receptor stimulation leading to hyperphosphorylation of IP_3R2 at Ser^{937} [97] (see section 2.4.1). The increased sensitivity of IP_3R2-expressing mice to cardiac hypertrophy-inducing conditions could be linked to increased calcineurin and NFAT signalling. Consistent with this, the augmented cardiac hypertrophic response in IP_3R2-overexpressing mice were completely blunted when these mice were crossed with calcineurin B-knockout mice, indicating an essential role of calcineurin/NFAT signalling in response to hyperactive IP_3R2-mediated Ca^{2+} signalling. While from the above studies, calcineurin emerged as the downstream target of increased IP_3R-mediated Ca^{2+} signalling, it is important to note that also nuclear CaMKIIδ has been implicated in altered transcription in response to cardiac hypertrophic endothelin-1 signalling [109]. Increased IP_3 signalling in response to endothelin-1 triggers a unique nuclear Ca^{2+} signalling that does not occur during excitation-contraction coupling but activates CaMKII, which together with protein kinase D results in the phosphorylation and nuclear export of class II HDAC5, a transcriptional repressor. In healthy conditions, nuclear HDAC5 forms a complex with the transcription factor MEF2, thereby preventing the transcription of hypertrophic genes. In hypertrophic conditions, HDAC5 is exported from the nucleus, leading to de-repression of MEF2 and the induction of hypertrophic genes. Interestingly, blocking IP_3Rs using chemicals like 2-aminoethoxydiphenyl borate or using IP_3R2-knockout mice, prevents the nuclear export of HDAC5 and subsequent activation of the hypertrophic transcription program.

All these studies are consistent with a critical role for Ca^{2+} signalling via IP_3R2 in cardiac hypertrophy, being in the nucleus and required for driving transcription of hypertrophic

genes and in the junctional sarcoplasmic reticulum being responsible for driving extra-systolic Ca^{2+} rises and contractions. Moreover, these studies all support the concept of distinct Ca^{2+} signalling compartments in cardiomyocytes, either in the cytosol during physiological excitation-contraction coupling driven by RyR2 channels or in the nucleus during pathophysiological hypertrophic signalling driven by IP_3 and IP_3R2 channels [151].

5. The role of IP_3R2 in cell death and in senescence

Over the last 20 years, IP_3R channels have emerged as key regulators that control cell death and survival in a variety of cellular systems [14, 152-154]. T cells deficient in IP_3R1 are resistant to a variety of apoptotic triggers, including chemical stimuli, like corticoids, and biological stimuli, including excessive T-cell receptor stimulation and exposure to Fas ligand [155]. Interestingly, susceptibility to T-cell receptor stimulation could be restored by artificially rising the cytosolic [Ca^{2+}] using the SERCA inhibitor, thapsigargin. Also, a role for IP_3R3 has emerged in pro-apoptotic Ca^{2+} signalling [156], because some studies proposed that this channel may be preferentially located in the mitochondrial ER-associated membranes. As such, IP_3R3 channels are thought to be part of the "quasi-synaptic" Ca^{2+}-transport complex between the ER Ca^{2+} stores and the mitochondria that can involve IP_3Rs, GRP75 and VDAC1 [157, 158]. Nevertheless, it is becoming increasingly clear that all IP_3R isoforms participate in apoptotic Ca^{2+} signalling and/or influence the susceptibility of cells towards apoptotic stimuli. This can mean two things: i) not only IP_3R3, but also IP_3R1 and IP_3R2 channels can be part of the ER-mitochondrial junction complexes, and ii) not only direct Ca^{2+} transfer into the mitochondria, but also other downstream Ca^{2+}-dependent signalling pathways participate in triggering mitochondrial outer membrane permeabilization, the point-of-no-return in apoptosis. Furthermore, it is important to emphasize that a complex interaction exists between IP_3Rs and proteins from the B-cell lymphoma (Bcl)-2 family involved in the control of apoptosis, whereby several interaction sites for such proteins have already been identified on the IP_3R [159-162].

IP_3R-mediated Ca^{2+} release can lead to calcineurin activation, which dephosphorylates the pro-apoptotic "sensitizer" BH3-only protein, Bad [163, 164]. Phosphorylated Bad is neutralized due to its scaffolding with 14-3-3 proteins and therefore it cannot form a complex with anti-apoptotic Bcl-Xl [165]. Dephosphorylation of Bad by calcineurin, e.g. in response to increases in cytosolic [Ca^{2+}] mediated by IP_3Rs [164], results in Bad release from 14-3-3 proteins and its translocation from the cytosol to the mitochondrial membranes. Here, it can bind to and inhibit anti-apoptotic Bcl-Xl proteins [163], thereby displacing Bim/tBid, which then can activate Bax/Bak and induce apoptosis.

These data indicate that dampening the IP_3R-mediated Ca^{2+} rise, either by lowering IP_3R levels or altering the IP_3R-expression profile, by inhibiting the Ca^{2+}-flux properties of IP_3Rs, or by lowering the ER Ca^{2+} content, which decreases the driving force for Ca^{2+} release into the cytosol upon IP_3R activation, will be cytoprotective [162]. Not surprisingly, different pro-survival signalling mechanisms, which are often oncogenic, appear to have exploited this concept to promote cell survival, including the survival of malignant or altered cells. In many cases, different mechanisms can be simultaneously operative. For instance, oncogenic KRAS mutations appear to switch the expression from IP_3R3 into IP_3R1 and to

lower the ER Ca^{2+}-store content, together suppressing agonist-induced Ca^{2+} release and mitochondrial Ca^{2+} accumulation and thus protecting cells against menadione exposure [166]. AKT/PKB phosphorylates all three IP_3R isoforms, thereby suppressing their pro-apoptotic Ca^{2+}-release function [84, 156]. This mechanism is also exploited by tumour suppressors like the promyelocytic leukemia protein, which enhance IP_3R3 activity by counteracting PKB-mediated IP_3R3 phosphorylation [167]. Other survival/anti-apoptotic proteins, like Bcl-2, have been reported to lower ER Ca^{2+} store-content by sensitizing IP_3Rs to basal IP_3 levels and to directly suppress IP_3R-mediated Ca^{2+} release, thereby preventing toxic mitochondrial Ca^{2+} overload [168]. Evidently, these mechanisms will also result in reduced calcineurin activation, thereby limiting Bad dephosphorylation and its subsequent inhibitory effects on the anti-apoptotic Bcl-2 proteins.

While most studies have addressed the role of IP_3R1 and IP_3R3 channels in apoptosis, there is emerging evidence that IP_3R2 channels play a crucial role in mediating proapoptotic Ca^{2+} signalling. Definitely, IP_3R2 with its high sensitivity to IP_3 (see section 2.1) may actually be a very critical regulator of cell survival versus cell demise by rendering cells sensitive to basal IP_3 signalling. The role of IP_3R2 in cell death has been elucidated in different studies and using different approaches.

First of all, there is evidence that cell death triggered by cellular exposure to cytotoxic compounds or agents that induce oxidative stress has been associated with an increase in IP_3R2 levels and activity. lncreasing oxidative stress in a neuronal cell line exposed to sub-lethal concentrations of tert-butyl hydroperoxide-mediated oxidative stress led to prominent upregulation of IP_3R2 mRNA and protein levels, while IP_3R1 and IP_3R3-expression levels remained unaltered [38]. Consistent with elevated IP_3R expression levels, Ca^{2+} release from the nucleoplasm in response to a cell-permeable IP_3 ester was strongly potentiated in tert-butyl hydroperoxide-treated cells. Also, the nephrotoxic compound uranyl acetate has been shown to increase IP_3R2 mRNA and protein levels in human epithelial kidney cells, thereby increasing the basal cytosolic [Ca^{2+}] and apoptosis levels [169]. Similar findings have been reported in HeLa cells exposed to fast H_2S donors, although in this case IP_3R1 expression levels were also increased [170]. Interestingly, IP_3Rs may also be directly affected by reactive oxygen species (ROS) [171]. In intact DT40 cells, superoxide anions caused Ca^{2+} release from the ER, likely via a mechanism that sensitizes IP_3Rs to basal levels of IP_3 signalling. In these DT40 cells, the presence of IP_3R2 and IP_3R1 isoforms, but not of IP_3R3, was required for superoxide anion-induced [Ca^{2+}] rise in the cytosol.

The role of IP_3R2 channels in apoptotic Ca^{2+} signalling was also identified in B-cell cancer cells, in particular in a subset of “primed to death” diffuse large B-cell lymphoma cell lines [172]. Cells expressing high IP_3R2 levels seem “addicted” to the presence and recruitment of anti-apoptotic Bcl-2 proteins at the ER and especially in the IP_3R protein complex [173]. By interacting via its BH4 domain with the modulatory and transducing domain of the IP_3Rs, Bcl-2 inhibits IP_3-induced Ca^{2+} release [174-176]. The binding site for the BH4 domain of Bcl-2 (Figure 1) has been identified [175] and is conserved between the three IP_3R isoforms [177]. Importantly, a peptide tool designed to disrupt IP_3R/Bcl-2-complexes by targeting Bcl-2’s BH4 domain (see [175-178]) was very effective in inducing intracellular Ca^{2+} overload and provoking cell death in DL-BCL cells that express high

levels of IP_3R2, like SU-DHL-4 cells [172]. In contrast, cells that expressed very low levels of IP_3R2 were virtually resistant to this peptide tool. The apoptotic resistance of these cells to this peptide was not due to a general defect in the initiation or execution of apoptosis, since staurosporine or BH3-mimetic drugs were very effective in these cells. We hypothesize that anti-apoptotic Bcl-2 is required at the ER to associate with the IP_3R2 to prevent its hyperactivity in response to the ongoing IP_3 signalling downstream of the B-cell receptor [173]. It remains to be elucidated whether these findings translate into primary B-cell cancer cells. In any case, disrupting IP_3R/Bcl-2 complexes results in excessive Ca^{2+}-signalling patterns and apoptotic cell death in primary peripheral mononuclear blood cells (mainly B cells) isolated from chronic lymphocytic leukaemia patients [179]. Remarkably, a gene expression profile analysis using the GeneSapiens microarray database revealed an upregulation of IP_3R2 at the mRNA level in chronic lymphocytic leukaemia samples [173].

IP_3R2 channels are not only implicated in apoptosis but also play a role in cellular senescence. Stable cell cycle arrest is a key feature of cellular senescence, which is activated in response to cellular stress. Factors include oncogenic stress following loss of PTEN function, DNA damage or telomere attrition, oxidative stress and replicative stress [180]. The arrest in proliferation depends on the major tumour suppressor pathways involving p53/p21 and p16/Rb [180, 181]. At the physiological level, cellular senescence contributes to ageing at the level of the organism [182]. However, cellular senescence can also function as an important “health keeper” fighting pathophysiological conditions associated with oncogenic stress [183, 184]. As such, cellular senescence, in addition to apoptosis, is one of the pathways that counteract cancer cell initiation and tumour development [185, 186]. For instance, in pre-malignant hepatocytes, senescence led to the secretion of chemo- and cytokines, resulting in their clearance by $CD4^+$ T cells [187]. Loss of immune surveillance caused the progression of the pre-malignant hepatocytes into hepatocellular carcinomas. Recently, Wiel et al. [188] performed an elegant shRNA-based screen to identify which “loss-of-function” genes can cause escape from oncogene-induced senescence in immortalized human mammary epithelial cells (HEC). Interestingly, the gene coding for IP_3R2 was identified as a prominent modulator of this form of senescence. These findings correlated with an analysis performed by the authors using the Oncomine database, which indicated that many malignant tumours displayed a decrease in IP_3R2 mRNA levels. IP_3R2 shRNAs alleviated the growth arrest in HEC exposed to oncogenic stress. Prolonged incubation of these cells with cell-permeable IP_3 repressed cell growth and induced pre-mature senescence. Oncogenic stress-induced senescence led to an increase in the Ca^{2+} accumulation in the mitochondria, a process that did not occur in the IP_3R2 shRNA-treated cells, and also boosted IP_3-induced mitochondrial Ca^{2+} uptake. This mitochondrial Ca^{2+} uptake was proposed to be responsible for the decrease in mitochondrial potential observed during oncogene-induced senescence, because shRNA against the IP_3R2 or against the mitochondrial Ca^{2+} uniporter prevented this decline in mitochondrial potential. Interestingly, chemical induction of mitochondrial depolarization blocked cell growth and induced pre-mature senescence. The role of IP_3R2 and of the subsequent mitochondrial Ca^{2+} accumulation was linked to an increase in ROS production, since anti-oxidants promoted oncogene-induced senescence escape. Finally, these concepts may not be limited to oncogene-induced senescence, but may also be applicable in models of replicative

senescence. IP_3R2 knockdown counteracted the increase in mitochondrial Ca^{2+} and the decline in mitochondrial potential observed during replicative senescence, thereby delaying the occurrence of senescence in these models.

6. Conclusions

IP_3R2 is characterized by a number of important and specific properties, including, but not limited to, its high sensitivity to IP_3 and ATP. Other properties such as its regulation by protein kinases, its interaction with adenylate cyclase to couple to cAMP production, its ability to recruit associated proteins and its low mobility in the ER, remain underexplored. At the functional level, it is clear that IP_3R2 is not only important for regulating secretion, but also is implicated in health and disease, including prominent roles in cardiac function and tumour growth. The available evidence indicates that tumour cells either downregulate IP_3R2 expression or dampen its activity via Bcl-2, since IP_3R2 can promote senescence and/or apoptosis. It is now anticipated that further research will elucidate additional important functions of IP_3R2 in other tissues and organs and further that developing tools specifically targeting or impacting IP_3R2 will allow modulating its function in disease states.

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Abbreviations

AC6	Adenylate cyclase type 6
AKT/PKB	Protein kinase B
ANF	Atrial natriuretic factor
Bcl	B-cell lymphoma
BH	Bcl-2 homology domain
CaMKII	Ca^{2+}/calmodulin dependent protein kinase II
DT40 TKO	DT40 triple knockout
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ET_AR	Endothelin receptor type A
HDAC	Histone deacetylase
HEC	Immortalized human mammary epithelial cells

IP_3	Inositol 1,4,5-trisphosphate
$IP_3R(1, 2, 3)$	Inositol 1,4,5-trisphosphate receptor (type 1, type 2, type 3)
NFAT	Nuclear factor of activated T cells
PKA	Protein kinase A
PKC	Protein kinase C
P_o	Open probability
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SRF	Serum response factor

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Highlights

* The understanding of IP_3R2 has long lagged behind that of the other IP_3R isoforms

* IP_3R2 is an intracellular Ca^{2+}-release channel with important and unique properties

* IP_3R2 performs crucial physiological functions in various cell types

* IP_3R2 is implicated in health and disease, including cardiac hypertrophy and cancer

* IP_3R2 forms an important target for further research

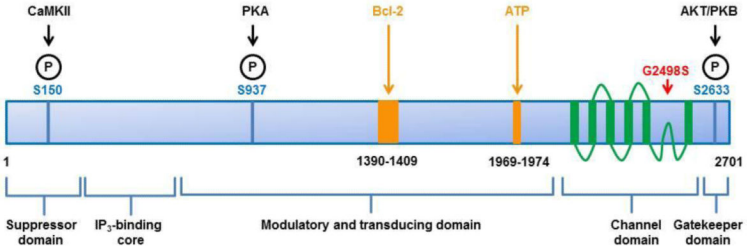

Figure 1. Linear representation of the IP_3R2 based on the human sequence demonstrating the interaction sites for its major regulators

The IP_3R2 is represented in blue; the 5 functional domains are indicated. In the channel domain, the 6 transmembrane helices as well as the connecting loops are depicted in green. The specific regulatory mechanisms discussed in the text are shown: identified phosphorylation sites are represented in dark blue, interaction sites for ATP and for Bcl-2 are in orange and the recently described $Gly^{2498}Ser$ mutation in the pore domain affecting IP_3R2 function [126] is depicted in red.

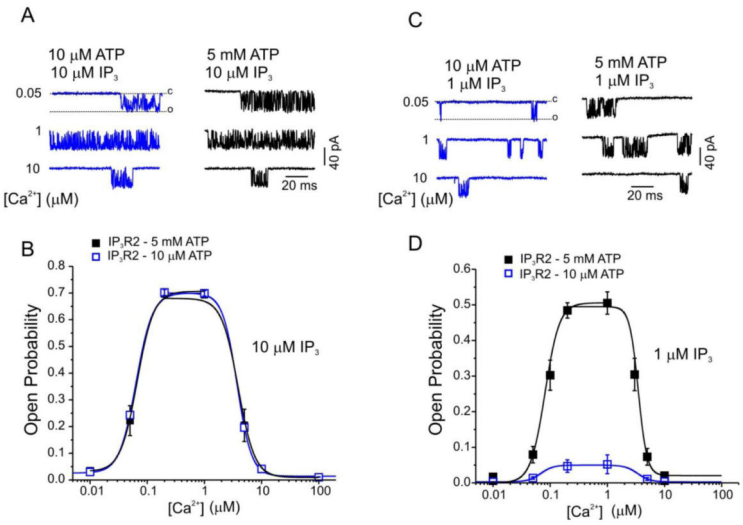

Figure 2. Modulation of IP_3R2 single-channel activity by IP_3, Ca^{2+} and ATP

Representative single channel recordings of IP_3R2 expressed in DT40 TKO cells using the "on-nucleus" configuration of the patch-clamp technique. In A, channel activity was stimulated with a maximal [IP_3] (10 μM) at the indicated [Ca^{2+}] and [ATP] (10 μM, in blue; 5 mM, in black). The pooled data in B reveal that channel activity stimulated by maximal [IP_3] is modulated by [Ca^{2+}] in a biphasic manner and that this relationship is unaffected by increasing the [ATP]. In C, channel activity was stimulated with a sub-maximal [IP_3] (1 μM) at the indicated [Ca^{2+}] and [ATP] (10 μM, in blue; 5 mM, in black). The pooled data in D demonstrate that while channel activity is also biphasically regulated by [Ca^{2+}] at sub-maximal [IP_3], the maximally achievable open probability, at each [Ca^{2+}], is, in contrast to what happens at a maximal [IP_3], markedly potentiated in the presence of a high [ATP]. Modified from [79], with permission.

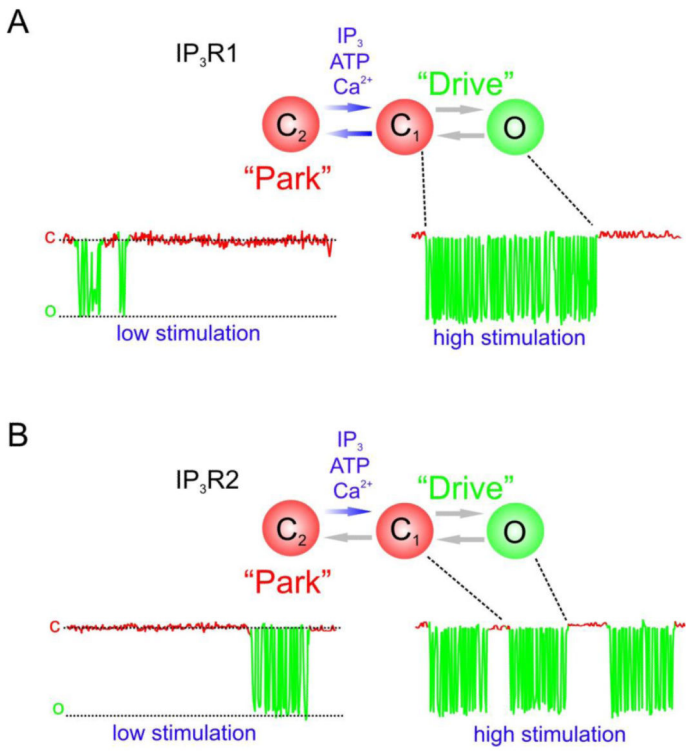

Figure 3. "Park and Drive" model for IP_3R1 and IP_3R2 gating

An increase in IP_3R1 (A) and IP_3R2 (B) channel activity in the presence of activating ligands is characterized by an increase in channel "bursting" without altering the intraburst kinetics. The bursts have subtype specific characteristics. A gating scheme for both channels can minimally be described by three states; one open state (in green) and two closed states (in red). Bursting activity is represented by rapid transitions between the open state (O) and a short-lived closed state (C_1) representing the "Drive Mode" of the channel. In the interburst intervals, the channel is effectively "Parked" in a long-lived closed state (C_2). For both IP_3R1 (A) and IP_3R2 (B) increasing the concentrations of activating ligands solely alters the transition from C_2 to C_1. However, ligands both increase the likelihood that IP_3R1 will leave the parked state to drive mode, as well as reciprocally decreasing the chances it will return to this state, thus extending the period of bursting (A). In the case of IP_3R2, ligands only destabilize the parked state resulting in an increase in bursting episodes of relatively constant duration (B).

Table 1

Cell types or tissues predominantly expressing IP_3R2.

Cell type/tissues ***(in alphabetical order)***	**Relevant references**	**Specific remarks**
AR42J	[11, 12]	Pancreatoma cell line
Cardiac myocytes	[12, 13, 136, 189]	
Glia	[117, 190]	
Hepatocytes	[11-13, 29]	Polarized expression IP_3R2
Intercalated cells of renal collecting duct	[191]	
Pancreatic acinar cells	[12]	$IP_3R3 \approx IP_3R2$
RBL-2H3	[11, 115, 192]	Mucosal mast cell line