

1 **IP₃ receptors – lessons from analyses *ex cellula***

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10 **Running title**

11 IP₃ receptors

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14 **Key words**

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16 Nuclear patch-clamp; Permeabilized cell; Radioligand binding; Ryanodine receptor.

17 **ABSTRACT**

18 Inositol 1,4,5-trisphosphate receptors (IP₃Rs) are widely expressed intracellular channels that
19 release Ca²⁺ from the endoplasmic reticulum (ER). We review how studies of IP₃Rs removed
20 from their intracellular environment (*‘ex cellula’*), alongside similar analyses of ryanodine
21 receptors, have contributed to understanding IP₃R behaviour. Analyses of permeabilized cells
22 demonstrated that the ER is the major intracellular Ca²⁺ store, and that IP₃ stimulates Ca²⁺
23 release from it. Radioligand binding confirmed that the 4,5-phosphates of IP₃ are essential for
24 activating IP₃Rs, and facilitated IP₃R purification and cloning, which paved the way to
25 structural analyses. Reconstitution of IP₃Rs into lipid bilayers and patch-clamp recording
26 from the nuclear envelope established that IP₃Rs have a large conductance and select weakly
27 between Ca²⁺ and other cations. Structural analyses are now revealing how IP₃ binding to the
28 N-terminus of the tetrameric IP₃R opens the pore ~7nm away from the IP₃-binding core
29 (IBC). Communication between the IBC and pore passes through a nexus of interleaved
30 domains contributed by structures associated with the pore and cytosolic domains, which
31 together contribute to a Ca²⁺-binding site. These structural analyses provide a plausible
32 explanation for the suggestion that IP₃ gates IP₃Rs by first stimulating Ca²⁺ binding, which
33 leads to pore opening and Ca²⁺ release.

34 **Introduction**

35 Inositol 1,4,5-trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyR) are the two
36 major families of intracellular Ca²⁺-release channels in animal cells (**Fig. 1A**). IP₃Rs are
37 expressed in most cells, whereas RyRs have a more restricted distribution. RyRs are most
38 abundant in excitable cells, notably in striated muscle, where they contribute to excitation-
39 contraction coupling (**Fig. 1A**) (Van Petegem, 2014). In this review, we focus on IP₃Rs, and
40 how methods applied to IP₃Rs removed from intact cells have contributed to our
41 understanding of IP₃R behaviour. Progress with understanding IP₃Rs and RyRs has
42 developed in parallel, and with this progress it became clear that the two families share
43 structural and functional features (Baker et al., 2017; Seo et al., 2012). Hence, despite our
44 focus on IP₃Rs, we draw also on evidence from analyses of RyRs.

45 Classic work by Sydney Ringer demonstrated that cardiac muscle contraction requires
46 extracellular Ca²⁺ (Ringer, 1883). This was, with benefit of hindsight, the first of many
47 studies to show that the contributions to physiological responses of extracellular Ca²⁺ and
48 Ca²⁺ held within intracellular stores are entangled. For cardiac muscle, depolarization of the
49 plasma membrane (PM) causes voltage-gated Ca²⁺ channels (Ca_v1.2) to open, and the local
50 increase in cytosolic free Ca²⁺ concentration [Ca²⁺]_c is then amplified by Ca²⁺-induced Ca²⁺
51 release (CICR) through type 2 ryanodine receptors (RyR2) in the sarcoplasmic reticulum
52 (Bers, 2002) (**Fig. 1A**). CICR and the local Ca²⁺ signalling that is required to avoid CICR
53 from becoming explosive have become recurrent themes in Ca²⁺ signalling (Rios, 2018).

54 Fluorescent Ca²⁺ indicators and optical microscopy now allow Ca²⁺ sparks, local Ca²⁺ signals
55 evoked by a small cluster of RyRs, to be measured with exquisite subcellular resolution in
56 cardiac muscles (Cheng and Lederer, 2008). However, it was studies of permeabilized cells
57 ('skinned' fibres) that provided the first evidence for CICR in muscle (Endo et al., 1970;
58 Fabiato and Fabiato, 1979). Analyses of RyRs that were reconstituted into planar lipid
59 bilayers first showed that RyRs form large-conductance cation channels that are biphasically
60 regulated by cytosolic Ca²⁺ (Lai et al., 1988; Meissner, 2017). Finally, analyses of RyR
61 fragments by X-ray crystallography (Van Petegem, 2014) and of complete RyRs by cryo-
62 electron microscopy (des Georges et al., 2016; Efremov et al., 2015; Peng et al., 2016; Yan et
63 al., 2015; Zalk et al., 2015) are revealing the structural basis of RyR behaviour.

64 Progress towards understanding the second major family of intracellular Ca²⁺-release
65 channels, the IP₃Rs, began with an influential review in which a causal link between receptor-
66 stimulated turnover of phosphatidylinositol and Ca²⁺ signalling was proposed (Michell,
67 1975). Subsequent work established that many receptors stimulate phospholipases C, which

68 cleave phosphatidylinositol 4,5-bisphosphate to produce IP₃ and diacylglycerol (Berridge,
69 1993) (**Fig. 1A**). IP₃ provides the link to Ca²⁺ signalling; not, as first envisaged by directly
70 stimulating Ca²⁺ entry across the PM (Michell, 1975), but by stimulating Ca²⁺ release from
71 the endoplasmic reticulum (ER) through IP₃Rs (Berridge and Irvine, 1984; Streb et al., 1983).
72 Another influential review suggested the link between IP₃-evoked Ca²⁺ release and Ca²⁺ entry
73 across the PM. This review proposed that loss of Ca²⁺ from the ER stimulated Ca²⁺ entry
74 (Putney, 1986). The workings of this store-operated Ca²⁺ entry (SOCE) pathway are now
75 clear: dissociation of Ca²⁺ from the luminal EF-hand motif of a protein embedded in the ER
76 membrane, stromal interaction molecule 1 (STIM1), causes STIM1 to oligomerize and
77 expose a cytosolic domain, through which it stimulates opening of a Ca²⁺-selective channel in
78 the PM (Feske et al., 2006; Prakriya and Lewis, 2015). The Ca²⁺ channel that mediates SOCE
79 is a hexameric assembly of Orai subunits (Hou et al., 2012; Yen and Lewis, 2018),
80 grandiloquently named from Greek mythology after the keepers of Heaven (Feske et al.,
81 2006).

82 IP₃Rs and RyRs are biphasically regulated by cytosolic Ca²⁺ (Bezprozvanny et al., 1991). For
83 IP₃Rs exposed to IP₃, a modest increase in [Ca²⁺]_c stimulates opening, whereas higher [Ca²⁺]_c
84 are inhibitory (Foskett et al., 2007; Iino, 1990). Hence IP₃Rs, at least once they have bound
85 IP₃ (Alzayady et al., 2016), can, like RyRs, mediate CICR (**Fig. 1B,C**). As with RyRs, IP₃Rs
86 assemble into clusters, within which opening of one IP₃R ignites the activity of its neighbours
87 to generate local ‘Ca²⁺ puffs’ (**Fig. 1C**) (Smith and Parker, 2009; Thillaiappan et al., 2017),
88 analogous to Ca²⁺ sparks in muscle. These behaviours illustrate some of the many similarities
89 between IP₃Rs and RyRs, which include their close structural relationship (Baker et al., 2017;
90 Seo et al., 2012; Van Petegem, 2014). Although IP₃Rs and RyRs are the major intracellular
91 Ca²⁺-release channels, they are not the only intracellular Ca²⁺ channels (**Box 1**).

92 The productive interplay between studies of minimally perturbed tissue, facilitated by a
93 plethora of Ca²⁺ indicators (Lock et al., 2015), fluorescent proteins (Rodriguez et al., 2017)
94 and fluorescence microscopy techniques (Thorn, 2016), alongside analyses of cellular
95 components has shaped our understanding of Ca²⁺ signalling. Here, we consider how
96 analyses of IP₃Rs conducted outside their normal intracellular environment (*ex cellula*) have
97 advanced our understanding of IP₃-evoked Ca²⁺ signals. We begin by considering how
98 analyses of permeabilized cells established that the ER is the major intracellular Ca²⁺ store
99 and that IP₃ releases Ca²⁺ from it. Radioligand binding analyses then both identified the sites
100 to which IP₃ binds to activate IP₃Rs and paved the way to structural studies, which we show
101 are now coming close to revealing how IP₃ binding causes the pore of the IP₃R to open. We

102 conclude by considering the contributions of electrophysiological recordings to our
103 understanding of IP₃R gating.

104

105 **Lessons from permeabilized cells**

106 Permeabilized cells allow the Ca²⁺ content of intracellular organelles to be measured under
107 conditions where the intracellular environment can be precisely controlled. To achieve this
108 control, the PM must be disrupted without unduly perturbing organelles (Schulz, 1990). The
109 permeabilized cells are then bathed in medium that mimics cytosol, notably in its low [Ca²⁺]_c
110 (~100 nM). Electroporation (Knight, 1981; Xie et al., 2013) and a variety of chemical means
111 have been used to selectively permeabilize the PM. The chemicals achieve their PM-
112 selectivity by interacting with cholesterol (e.g. saponin, digitonin, β-escin), which is enriched
113 in the PM (Wassler et al., 1987), or as pore-forming toxins (e.g. α-toxin, streptolysin-O) that
114 are too large to pass through their own pores (Schulz, 1990).

115 After a protracted controversy (Babcock et al., 1979; Dehaye et al., 1980), analyses of
116 permeabilized cells established that the ER, rather than mitochondria, is the major
117 intracellular Ca²⁺ store in animal cells (Burgess et al., 1983). In an elegant study, saponin-
118 permeabilized hepatocytes were bathed in cytosol-like medium with Ca²⁺ buffered to mimic
119 the [Ca²⁺]_c of an unstimulated cell. Each permeabilized cell was then shown to have the same
120 Ca²⁺ content as an intact cell, and critically all of that Ca²⁺ was in the ER (Burgess et al.,
121 1983). Hence, it is the ER from which most extracellular stimuli evoke Ca²⁺ release.

122 Analyses of insect salivary glands demonstrated that phosphoinositide turnover was required
123 for extracellular stimuli to evoke Ca²⁺ signals (Berridge and Fain, 1979), and showed that IP₃
124 was the first cytosolic product of receptor-stimulated phosphoinositide hydrolysis (Berridge,
125 1983). Hence, IP₃ emerged as the likely messenger that links receptors in the PM to Ca²⁺
126 release from the ER (**Fig. 1A**). Permeabilized cells again provided the decisive experiment:
127 addition of IP₃ to permeabilized pancreatic acinar cells stimulated release of Ca²⁺ from a non-
128 mitochondrial Ca²⁺ store (Streb et al., 1983). It is now universally accepted that most IP₃Rs
129 reside in ER membranes, but IP₃Rs can also mediate Ca²⁺ release from the Golgi apparatus
130 (Aulestia et al., 2015; Pinton et al., 1998), the nuclear envelope (Foskett et al., 2007; Rahman
131 et al., 2009; Stehno-Bittel et al., 1995) and perhaps from a nucleoplasmic reticulum
132 (Echevarria et al., 2003). In some cells, a few IP₃Rs (typically only 2-3 IP₃Rs per cell) are
133 also expressed in the PM, where they mediate Ca²⁺ entry (Dellis et al., 2006; Dellis et al.,
134 2008). In many studies, though not in all (Watras et al., 1991), the ER Ca²⁺ release evoked by
135 IP₃ was shown to be positively cooperative (eg, Champeil et al., 1989; Marchant and Taylor,

136 1997; Meyer et al., 1988), suggesting a need for IP₃ to bind to several IP₃R subunits before
137 the channel can open. A recent study using concatenated IP₃R subunits showed that a
138 defective IP₃-binding site in only one of the four subunits prevents IP₃R activation (Alzayady
139 et al., 2016), leading to the conclusion that all four subunits of an IP₃R must bind IP₃ before
140 the channel can open.

141 But IP₃ binding is not alone sufficient to stimulate Ca²⁺ release through IP₃Rs. Instead, IP₃
142 binding primes IP₃Rs to bind Ca²⁺, and Ca²⁺ binding then causes the channel to open (Adkins
143 and Taylor, 1999; Marchant and Taylor, 1997) (**Fig. 1B**). Hence, IP₃Rs require binding of
144 two ligands, IP₃ and Ca²⁺, to open. This dual regulation endows IP₃Rs with their capacity to
145 mediate regenerative Ca²⁺ signals through CICR. Again, it was analyses of permeabilized
146 cells that provided the first evidence that Ca²⁺ release through IP₃Rs is regulated by [Ca²⁺]_c
147 (Iino, 1987). High-resolution optical analyses of Ca²⁺ signals later revealed that within intact
148 cells, IP₃-evoked Ca²⁺ signals originate from elementary units that comprise a small cluster of
149 IP₃Rs (Smith and Parker, 2009; Thillaiappan et al., 2017). Opening of the first IP₃R within a
150 cluster is proposed to rapidly ignite the activity of some of its neighbours by CICR to
151 generate a Ca²⁺ puff (**Fig. 1C**). As the stimulus intensity increases, Ca²⁺ spreading from one
152 Ca²⁺ puff to another IP₃R cluster can initiate further Ca²⁺ puffs, allowing the signal to spread
153 across the cell as a regenerative Ca²⁺ wave (Marchant et al., 1999). The frequency of these
154 global signals then increases with stimulus intensity (Thurley et al., 2014).

155 Structure-activity relationships (SAR), established by comparing the activities of a range of
156 structurally-related chemical stimuli, are often used to probe the recognition properties of
157 receptors. SAR analyses of the effects of IP₃ analogues on Ca²⁺ release from permeabilized
158 cells provided the first evidence that dephosphorylation of IP₃ to (1,4)IP₂ terminates IP₃
159 activity (Burgess et al., 1984). The (1,3,4,5)IP₄ that is produced when IP₃ is phosphorylated
160 by IP₃ 3-kinase was proposed to regulate IP₃Rs (Loomis-Husselbee et al., 1996), but it is now
161 clear that this phosphorylation also inactivates IP₃ signalling through IP₃Rs (Bird and Putney,
162 1996; Saleem et al., 2012). Hence, both endogenous pathways for IP₃ metabolism effectively
163 inactivate IP₃ signalling to IP₃Rs (**Fig. 1A**). SAR analyses of many analogues of IP₃ and
164 adenophostin A, a fungal metabolite that binds with high-affinity to IP₃Rs (Takahashi et al.,
165 1994), established that a key feature of IP₃R agonists is the presence of a vicinal 4,5-
166 bisphosphate moiety (**Fig. 1D**) (Rossi et al., 2010; Rossi et al., 2009; Saleem et al., 2012). All
167 active inositol phosphate analogues have this 4,5-vicinal bisphosphate moiety (**Fig. 1D**).

168 There are no wholly selective antagonists of IP₃Rs. Some ligands (heparin, 2-
169 aminoethoxydiphenylborane (2-APB), Xestospongine C and caffeine) have utility, but they all

170 lack selectivity. Furthermore, heparin is not membrane-permeant, and results with
171 Xestospongin C are inconsistent (see Saleem et al., 2014). Addition of large substituents to
172 the 2-*O*-position of IP₃ produces partial agonists. Partial agonists are ligands that, once they
173 have bound to IP₃R, are less effective in causing the channel to open than full agonists like
174 IP₃ (Rossi et al., 2009). These SAR analyses of 2-modified analogues of IP₃, again relying
175 heavily on permeabilized cells, have both confirmed the importance of the extreme N-
176 terminal region of the IP₃R (the suppressor domain, SD, **Fig. 1E**) in IP₃R activation and they
177 suggest systematic strategies towards developing high-affinity antagonists of IP₃Rs.
178 There is, therefore, a long history of experiments using permeabilized cells illuminating our
179 understanding of IP₃-evoked Ca²⁺ release. These studies first identified ER as the major
180 intracellular Ca²⁺ store, they showed that IP₃ evokes Ca²⁺ release from the ER, and that IP₃Rs
181 are regulated by Ca²⁺. Furthermore, they defined the biochemical steps that inactivate IP₃
182 and, through SAR analyses, they have provided ligands that have contributed to
183 understanding the mechanisms of IP₃R activation.

184

185 **Analyses of IP₃ binding to IP₃Rs**

186 Binding of IP₃ to the four binding sites of the IP₃R initiates the conformational changes that
187 culminate in opening of the Ca²⁺-permeable pore (Alzayady et al., 2016; Chandrasekhar et
188 al., 2016). These IP₃ binding events are usually analysed by means of radioligand binding,
189 which allows determination of binding affinities (as equilibrium dissociation constants, K_D)
190 for ³H-IP₃ or any competing ligand, but there are a variety of other methods (**Fig. 2, Box 2**).
191 K_D values are important for comparison with functional analyses in revealing how ligands
192 activate IP₃Rs. Such analyses were, for example, critical in showing that the vicinal 4,5-
193 bisphosphate of IP₃ is essential for activity, whereas the 1-phosphate improves binding
194 affinity (**Fig. 1D**) (Nahorski and Potter, 1989). Comparisons of SAR with binding analyses
195 can also establish which bound ligands most effectively open the channel. Our comparisons
196 of functional and ³H-IP₃ equilibrium-competition binding analyses, for example, established
197 that whereas IP₃ is a full agonist that effectively gates the IP₃R, other modified analogues of
198 IP₃ bind with high-affinity to IP₃R, but they are much less effective in causing the channel to
199 open (Rossi et al., 2009). These partial agonists provide insight into the mechanisms of IP₃R
200 activation by demonstrating how large moieties at the 2-position of IP₃ attenuate IP₃R
201 activation, and they suggest strategies for development of analogues that bind without
202 activating IP₃Rs (i.e. antagonists).

203 Binding analyses also allow IP₃R properties to be addressed under conditions where IP₃-
204 evoked Ca²⁺ release is not retained. This opportunity is particularly important during
205 purification of IP₃R fragments for structural studies using either IP₃R fragments for X-ray
206 crystallography (Bosanac et al., 2002; Bosanac et al., 2005; Hamada et al., 2017; Lin et al.,
207 2011; Seo et al., 2012) or, after detergent-solubilization of complete IP₃R, for single-particle
208 analysis by cryo-EM (Fan et al., 2015; Paknejad and Hite, 2018). In subsequent sections, we
209 review progress towards understanding how IP₃ binding leads to opening of the IP₃R pore.

210

211 **IP₃ initiates IP₃R activation by binding to the IP₃-binding core**

212 The route to IP₃R structures began with the identification of specific, high-affinity,
213 intracellular ³²P-IP₃-binding sites with recognition properties that matched those expected of
214 the receptor through which IP₃ evoked Ca²⁺ release (Baukal et al., 1985; Spät et al., 1986).
215 Subsequent studies established that heparin competed with ³H-IP₃ for these binding sites
216 (heparin is a competitive antagonist of IP₃), and that the sites were abundant in Purkinje cells
217 of cerebellum (Worley et al., 1987). Together, these observations allowed IP₃R to be
218 purified from cerebellum using heparin-chromatography (Maeda et al., 1988; Supattapone et
219 al., 1988). Functional reconstitution of the purified protein then established that it was alone
220 sufficient to form an IP₃-gated Ca²⁺ channel (Ferris et al., 1989; Maeda et al., 1991). Many
221 additional proteins were later shown to associate with IP₃R and modulate their responses to
222 IP₃ (Prole and Taylor, 2016). Screening of cDNA libraries from cerebellum then provided the
223 complete primary sequence of IP₃R1 (Furuichi et al., 1989; Mignery et al., 1989), and soon
224 afterwards the other two IP₃R subtypes, IP₃R2 (Südhof et al., 1991) and IP₃R3 (Blondel et al.,
225 1993) were identified. Subsequent studies established that the three IP₃R subunits (IP₃R1-3)
226 assemble to form homo-tetrameric and hetero-tetrameric channels (Monkawa et al., 1995),
227 and they confirmed that the core properties of all IP₃R subtypes are similar: each forms a
228 large-conductance Ca²⁺-permeable channel that is gated by binding of IP₃ and Ca²⁺ (Foskett,
229 2010), and each generates Ca²⁺ puffs (Mataragka and Taylor, 2018). The subtypes are,
230 however, differentially expressed, and they differ in their affinities for IP₃ (Iwai et al., 2007),
231 sensitivity to Ca²⁺ regulation (Foskett, 2010) and in whether they are modulated by additional
232 regulators (Prole and Taylor, 2016). Furthermore, the functional consequences of mutant or
233 defective IP₃R differ among subtypes (see Terry et al., 2018). IP₃R1 has so far been the
234 major focus of the structural studies.

235 Deletion analyses (Mignery and Südhof, 1990) and expression of IP₃R fragments in bacteria
236 (Yoshikawa et al., 1996) established that each IP₃R subunit has a single IP₃-binding site

237 formed by residues, the IBC (residues 224-604), towards the N-terminal of the primary
238 sequence (~2750 residues) (**Fig. 1E**). The identification of four IP₃-binding sites in each
239 IP₃R, and the demonstration that all four are required for IP₃ to evoke Ca²⁺ release (Alzayady
240 et al., 2016), provided an explanation for the widely observed cooperative responses to IP₃
241 (Champeil et al., 1989; Meyer et al., 1988; Parker and Miledi, 1989). Subsequent studies
242 identified residues within the IBC that are required for IP₃ binding, notably the residues that
243 bind to the critical 4- and 5-phosphate groups of IP₃ (Furutama et al., 1996). These residues
244 are conserved in IP₃Rs, but not in RyRs (Bosanac et al., 2002; Seo et al., 2012). It was also
245 shown that the SD inhibits IP₃ binding (Uchida et al., 2003), which aligns with the
246 importance of the SD in coupling IP₃ binding to channel gating (Rossi et al., 2009): IP₃Rs
247 without an SD bind IP₃ with high affinity, but they do not release Ca²⁺ (Uchida et al., 2003).
248 High-resolution crystal structures of N-terminal fragments of the IP₃R directly revealed both
249 the determinants of IP₃ binding and the initial steps in IP₃R activation. The two domains (α
250 and β) of the IBC form a clam-like structure, within which conserved residues bind to IP₃
251 (Bosanac et al., 2002). The 1- and 5-phosphates of IP₃ interact predominantly with residues in
252 IBC- α , whereas the 4-phosphate interacts with IBC- β (**Fig. 1D,E**). Interaction of the critical
253 4- and 5-phosphates with opposing sides of the clam allows IP₃ to partially close the clam and
254 initiate IP₃R activation (Hamada et al., 2017; Lin et al., 2011; Paknejad and Hite, 2018; Seo
255 et al., 2012). That interpretation, which elegantly reveals the structural basis of the SAR, is
256 supported by results with an adenophostin A analogue in which an alternative contact with
257 the α -domain substitutes for loss of the usual phosphate (Sureshan et al., 2012).
258 In the isolated N-terminal domain, the SD is firmly anchored to IBC- α by an extensive
259 interface and more loosely associated with IBC- β (**Fig. 1E**). Hence, when IP₃ causes the IBC
260 clam to close, the SD moves with IBC- α and that was predicted to disrupt interaction of an
261 exposed SD loop, the 'hot spot' loop (Yamazaki et al., 2010) with IBC- β of a neighbouring
262 subunit (Seo et al., 2012). In RyR too, these inter-subunit interactions between N-terminal
263 domains are weakened during receptor activation (des Georges et al., 2016). The resulting
264 weakening of interactions between subunits may contribute to channel gating. This is
265 supported by evidence that Ca²⁺-binding protein 1 (CaBP1), which inhibits IP₃R gating,
266 rigidifies these interactions between IP₃R subunits (Li et al., 2013). However, within the
267 constraints of a full-length IP₃R, strong inter-subunit interactions between the SD and IBC- β
268 might constrain the SD, such that IBC- α moves when IP₃ closes the clam (Paknejad and Hite,
269 2018). Identification of the sites to which IP₃ binds, which relied heavily on radioligand

270 binding analyses, set the scene for the structural analyses that seek to understand how IP₃
271 binding opens the pore of the IP₃R. We consider recent progress with such structural analyses
272 in the next section.

273

274 **Structures of complete IP₃ and ryanodine receptors**

275 Structures determined by single-particle analysis of cryo-EM images of the complete IP₃R1
276 in a closed state (Fan et al., 2015), of IP₃R3 with and without IP₃ and Ca²⁺ bound (Paknejad
277 and Hite, 2018), and of RyRs in different states (des Georges et al., 2016; Efremov et al.,
278 2015; Peng et al., 2016; Yan et al., 2015; Zalk et al., 2015) have begun to reveal the workings
279 of the pore regions of these related channels. The results also tentatively suggest how IP₃
280 binding might lead to opening of the IP₃R pore.

281 The IP₃R has a structure reminiscent of a square mushroom. Much of the stalk is embedded
282 in the ER membrane and the cap, with a diameter of ~25 nm, extends at least 13 nm into the
283 cytosol (Fan et al., 2015). The large size is significant because it might exclude IP₃R from
284 the narrow junctions between ER and the PM (Thillaiappan et al., 2017), whereas at other
285 junctions, between ER and mitochondria for example (Csordas et al., 2018), it places the head
286 of the IP₃R, from which Ca²⁺ exits, very close to the neighbouring organelle.

287 The cytosolic entrance to the central cavity of the IP₃R is surrounded by the N-terminal
288 domains (SD and IBC-β, **Fig. 3**). IBC-α forms part of a larger domain (ARM1) that curves to
289 the edge of the cap and interacts with two large curved domains (ARM2 and ARM3) that
290 comprise most of the remaining cytosolic structure and form the underside of the mushroom
291 (**Fig. 3**). Within the ER membrane, there are 24 trans-membrane domains (TMDs), six from
292 each subunit (Fan et al., 2015). However, recent structural analyses of both IP₃R (Paknejad
293 and Hite, 2018) and RyR1 (des Georges et al., 2016) identified a pair of additional helices
294 (between TMD1 and 2 of IP₃R3) that challenge the accepted view that there are six TMDs
295 per subunit. The TMD region, similar in structure to voltage-gated ion channels, is very
296 similar (though not identical) (Baker et al., 2017) in RyRs and IP₃Rs. The ion-conducting
297 path is lined by the four tilted TMD6 helices and a short (~1 nm) ‘selectivity filter’ at the
298 luminal end through which hydrated cations must pass in single-file. The selectivity filter, its
299 supporting pore-loop helix and a flexible luminal loop are all formed by residues linking
300 TMD5 to TMD6. Near the cytosolic end of TMD6, a narrow hydrophobic constriction blocks
301 the movement of ions in the closed channel (Fan et al., 2015) (**Fig. 3**). The hydrophobic side
302 chains of these residues must move for the pore to open. Opening of the RyR is associated
303 with splaying and bowing of TMD6, such that the hydrophobic side-chain of a residue that

304 occludes the cytosolic end of the closed pore is displaced, opening a hydrophilic path that
305 allows passage of a hydrated Ca^{2+} ion. Similar mechanisms may be associated with opening
306 of the IP_3R pore.

307 TMD6 is supported by TMD5, which is buttressed by the TMD1-4 bundle of the adjacent
308 subunit. The short cytosolic TMD4-5 helical linker aligns along the ER membrane behind the
309 TMD6 helices holding them in place. In the closed RyR1 channel, this linker tightly encircles
310 the cytosolic end of the TMD6 bundle restricting its movement, but this grip is relaxed as the
311 channel opens freeing TMD6 to move, and allowing the pore to dilate (des Georges et al.,
312 2016). In both IP_3R and RyR, TMD6 extends well beyond the ER membrane (~ 1.5 nm in
313 IP_3R) and then terminates in a pair of short α -helices (the linker domain, LNK, in IP_3R) that
314 includes a Zn^{2+} -finger motif that aligns parallel with the ER membrane (des Georges et al.,
315 2016; Fan et al., 2015; Paknejad and Hite, 2018). In IP_3R , but notably not in RyR, the
316 entwined TMD6 helices then continue beyond the LNK domain to the cap of the mushroom,
317 where each contacts the IBC- β domain of a neighbouring subunit (Fan et al., 2015). Hence,
318 structures formed by the TMD5-6 loop guard the luminal entrance to the pore, whereas the
319 cytosolic vestibule is formed by the extended TMD6. Each of these regions is enriched in
320 acidic residues that probably contribute to the cation selectivity of IP_3R and RyR (des
321 Georges et al., 2016; Fan et al., 2015; Paknejad and Hite, 2018).

322 A conserved Ca^{2+} -binding site is present in both RyR (des Georges et al., 2016) and IP_3R
323 (Paknejad and Hite, 2018). The site is formed, in the case of IP_3R , by residues near the C-
324 terminal end of ARM3 and by another residue contributed by the LNK domain (**Fig. 3**). In
325 RyR, the equivalent residues are proposed to coordinate the Ca^{2+} required for stimulation (des
326 Georges et al., 2016). The same may hold true for IP_3Rs , but this has yet to be tested. A
327 conserved glutamate residue on the bottom surface of the ARM3 domain (Glu²¹⁰¹ in $\text{IP}_3\text{R1}$)
328 previously suggested to mediate Ca^{2+} regulation of IP_3R (Miyakawa et al., 2001) and RyR
329 (Fessenden et al., 2001), does not contribute to Ca^{2+} binding to this site, but it does stabilize
330 the interaction between the cooperating domains in RyR1 (des Georges et al., 2016). A
331 second Ca^{2+} -binding site was identified in the structure of $\text{IP}_3\text{R3}$, and again it is formed by
332 residues that are contributed by different domains (ARM3 and the α -helical domain linking
333 ARM1 to ARM2) (Paknejad and Hite, 2018) (**Fig. 3**). Formation of both Ca^{2+} -binding sites
334 requires movement of the contributing domains from their positions in the apo-state, so as to
335 bring the Ca^{2+} -coordinating residues into register (Paknejad and Hite, 2018). This important
336 observation is consistent with evidence that IP_3 controls IP_3R gating by regulating Ca^{2+}
337 binding (**Fig. 1B**).

338 Taken together, structures of full-length IP₃R_s have defined where IP₃ binds, identified Ca²⁺-
339 binding sites that may mediate Ca²⁺ regulation, and established that hydrophobic residues
340 projecting into the pore must move to allow Ca²⁺ to pass.

341

342 **Towards understanding how IP₃ and Ca²⁺ binding open the IP₃R pore**

343 The only contacts between the large cytosolic structures of the IP₃R and its channel region
344 are the C-terminal end of ARM3 and the LNK domain (**Fig. 3**) (Fan et al., 2015). There are
345 similar contacts in RyR (des Georges et al., 2016). In both IP₃R and RyR, this critical nexus
346 comprises a platform of interleaved structures: the C-terminus of the ARM3 domain (the
347 intervening lateral domain, ILD) forms a ‘thumb-and-fingers’ arrangement of an upper thumb
348 abutting the bulk of ARM3, and an α -helical pair of fingers lying below and forming a cavity
349 into which the LNK domain inserts (**Fig. 3**) (Fan et al., 2015). Mutations within the thumb
350 disrupt IP₃R function (Hamada et al., 2017). The LNK domain also wraps around the thumb
351 and contributes a residue to the Ca²⁺-binding site at the base of the ARM3 domain.

352 How, then, does IP₃ binding to the IBC cause hydrophobic pore residues some 7 nm distant to
353 move and allow Ca²⁺ to pass from the ER lumen to the cytosol (Fan et al., 2015)? Recalling
354 that IP₃ primes IP₃R_s to bind Ca²⁺, which then triggers channel opening (Adkins and Taylor,
355 1999) (**Fig. 1B**), it seems reasonable to speculate that IP₃ binding to the IBC is communicated
356 to the Ca²⁺-binding site at the ILD-LNK nexus and thence to the pore (Paknejad and Hite,
357 2018). IP₃ binding closes the clam-like IBC, and, with IBC- β held firmly in place by inter-
358 subunit interactions at the top of the mushroom, IBC- α moves and initiates conformational
359 changes throughout the associated ARM domains. These changes include disruption of inter-
360 subunit interaction between ARM1 and ARM2 domains, and rotation of the LNK domains
361 (Paknejad and Hite, 2018). Here, the need for the SD is attributed to its role in stabilizing
362 inter-subunit interactions to provide a fixed structure against which movement of IBC- α can
363 leverage conformational changes through the ARM domains (Paknejad and Hite, 2018).

364 Given the essential role of the SD in IP₃R activation, an alternative possibility was that the
365 direct contact between the SD and ARM3 might mediate communication between N-terminal
366 regions and the ILD. However, the SD-ARM3 interaction occurs through the handle of the
367 hammer-like SD, which can be deleted without impairing IP₃R function (Yamazaki et al.,
368 2010). Another possibility was that interaction between IBC- β and the CTD (which is unique
369 to IP₃R) might communicate IP₃ binding to the LNK domain. However, this scheme is
370 difficult to reconcile with functional IP₃R/RyR chimeras (Seo et al., 2012) since the RyR
371 structure does not have an extended CTD, and with evidence that deletion of residues within

372 the CTD that interact with IBC- β do not prevent IP₃-evoked Ca²⁺ release (Hamada et al.,
373 2017; Schug and Joseph, 2006). Whatever the exact path from IBC to the ILD-LNK nexus is,
374 IP₃-evoked conformational changes appear to reconfigure the Ca²⁺-binding site formed at the
375 LNK-ARM3 interface to allow Ca²⁺ binding (Paknejad and Hite, 2018), thereby providing a
376 plausible mechanism for IP₃ priming IP₃Rs to respond to Ca²⁺ (**Fig. 1B**).
377 We conclude that analyses of IP₃ binding contributed to defining the SAR for IP₃Rs and to
378 quantitative comparisons of the relationship between binding and channel activation, but
379 most significantly they allowed IP₃Rs to be identified during their purification, which paved
380 the way to cloning and molecular manipulation of IP₃Rs, and to structural studies. The latter
381 have established that IP₃Rs are huge tetrameric structures, wherein IP₃ binding closes a clam-
382 like IBC. That conformational change is communicated to a critical nexus between
383 interleaved structures from the cytosolic and channel domains. IP₃ binding probably stabilizes
384 Ca²⁺ binding to this nexus, leading to re-arrangement of the pore, such that occluding
385 hydrophobic residues are displaced to allow the passage of Ca²⁺ from the ER lumen to the
386 cytosol.

387

388 **Lessons from planar lipid bilayers and patch-clamp recording**

389 Electrical recordings from ion channels, most often by means of patch-clamp recording (**Box**
390 **3**) (Lape et al., 2008; Neher, 1992), allow the openings and closing of single channels to be
391 recorded with sub-millisecond resolution, and they allow their ion permeation properties to
392 be defined. Since the intracellular location of RyR and IP₃R in the ER presents a formidable
393 barrier to such recordings (Jonas et al., 1997), two alternative approaches have been used.

394

395 *Planar lipid bilayers*

396 The first approach, which involves reconstitution of ER vesicles or solubilized IP₃Rs into
397 planar lipid bilayers, provided the first measurements of currents through IP₃R
398 (Bezprozvanny et al., 1994; Bezprozvanny and Ehrlich, 1994; Bezprozvanny et al., 1991;
399 Ehrlich and Watras, 1988; Maeda et al., 1991). These analyses established that IP₃R, like
400 RyR, are large-conductance cation channels with relatively low-selectivity for Ca²⁺. Both
401 features are important in allowing IP₃R to generate large local cytosolic Ca²⁺ signals: the
402 large conductance allows an open IP₃R to pass ~500,00 Ca²⁺ per second (Foskett et al., 2007),
403 whereas the weak selectivity might allow a counter-flux of K⁺ to dissipate the electrical
404 gradient that is formed as Ca²⁺ leaves the ER and which would otherwise rapidly terminate
405 Ca²⁺ release (Zsolnay et al., 2018). The short, wide selectivity filter and large vestibules with

406 abundant acidic residues probably provide the structural basis of these ion permeation
407 properties (Fan et al., 2015). Bilayer analyses also confirmed the biphasic regulation of IP₃R1
408 by cytosolic Ca²⁺ (Bezprozvanny et al., 1991). A potential problem with recordings from
409 planar lipid bilayers is that solubilization and/or reconstitution could lead to loss of accessory
410 proteins or perturbation of structure. Maximal open probabilities recorded from IP₃R3 in
411 bilayers, for example, are much lower than in patch-clamp recordings, and bilayer recordings
412 of IP₃R2 and IP₃R3 failed to capture the inhibitory effect of cytosolic Ca²⁺ (Hagar et al.,
413 1998; Ramos-Franco et al., 2000).

414

415 *Patch-clamp recording*

416 The second approach to obtaining electrical recordings from IP₃R exploits the fact that the
417 ER is continuous with the outer nuclear membrane (ONM) (**Box 3**) (Dingwall and Laskey,
418 1992). Hence, patch-clamp recording from the ONM allows analysis of IP₃R in a native
419 membrane, albeit not the ER (Mak et al., 2013; Rahman and Taylor, 2010) (**Box 3**). These
420 recordings, which have been applied to both native and heterologously expressed IP₃R3
421 (Betzenhauser et al., 2008; Cheung et al., 2010; Foskett et al., 2007; Marchenko et al., 2005;
422 Rahman et al., 2009), confirmed their ion permeation properties and the biphasic regulation
423 of all IP₃R subtypes by Ca²⁺. They have also suggested complex gating schemes wherein IP₃
424 drives bursts of IP₃R activity by extending the duration of sequences of openings and
425 shortening the gaps between the bursts (Gin et al., 2009; Ionescu et al., 2007).

426 Another application of nuclear patch-clamp recording is provided by our work, where we
427 showed that IP₃R3s within patches that fortuitously contained several IP₃R3s behave differently
428 to patches with only a single IP₃R3 (Rahman et al., 2009). This led to our proposal that low
429 concentrations of IP₃, perhaps arising from occupancy of only some of the four IP₃-binding
430 sites, trigger IP₃R3 clustering (Rahman et al., 2009). The clustered IP₃R3s, we suggest, are
431 better placed than lone IP₃R3s to benefit from CICR when a near neighbour opens to release
432 Ca²⁺ and so provide the second stimulus that is needed for IP₃R3 opening (**Fig. 1B**). Effects of
433 clustering on the IP₃ and Ca²⁺ sensitivity of IP₃R3s reinforce the propensity of clustered IP₃R3s
434 to amplify Ca²⁺ signals by CICR. These proposals have been challenged (Rahman et al.,
435 2011; Smith et al., 2009; Vais et al., 2011) and our own recent work suggests that even in
436 unstimulated cells there are pre-existing clusters of IP₃R3s, each typically comprising about
437 eight IP₃R3s (Thillaiappan et al., 2017). Our revised proposal therefore envisages that IP₃R3s
438 are, as we have shown, loosely clustered in unstimulated cells (Thillaiappan et al., 2017) and

439 that IP₃ might then cause IP₃Rs within the cluster to huddle more closely and so be more
440 likely to respond to Ca²⁺ released by a neighbour.

441

442 **Concluding remarks**

443 Throughout the long history of analyses of intracellular Ca²⁺ signalling, there has been a
444 productive interplay between studies of intact tissues and of biological systems extracted
445 from intact cells (*ex cellula*). These approaches succeeded in showing that the ER is the
446 major intracellular Ca²⁺ store and they identified the enormous channels (RyR and IP₃R) that
447 mediate Ca²⁺ release from the ER. We are now fast approaching an understanding of how IP₃
448 binding leads, through its interactions with Ca²⁺ binding, to opening of the IP₃R. In parallel
449 with these approaches, developments in optical microscopy have provided opportunities to
450 examine IP₃-evoked Ca²⁺ release with exquisite temporal and spatial resolution in intact cells.
451 We can surely look forward to these analyses converging with structural analyses *in situ* to
452 provide a comprehensive understanding of IP₃Rs in living cells.

453

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457

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833 **Fig. 1: Ca²⁺ release by IP₃ and ryanodine receptors.** (A) Many receptors in the plasma
834 membrane (PM), including G-protein-coupled receptors (GPCRs) and receptor tyrosine
835 kinases (RTKs), stimulate phospholipases C (PLC), causing hydrolysis of the PM lipid,
836 phosphatidylinositol 4,5-bisphosphate, into diacylglycerol and IP₃. IP₃ binds to each of the
837 four IP₃-binding sites of the tetrameric IP₃R to initiate conformational changes that lead to
838 channel opening and release of Ca²⁺ from the ER. IP₃ is deactivated by phosphorylation to IP₄
839 or dephosphorylation to IP₂. RyRs are close relatives of IP₃Rs, but they are predominantly
840 expressed in the sarcoplasmic reticulum of skeletal (RyR1) and cardiac (RyR2) muscle. Each
841 RyR is activated when depolarization of the PM activates voltage-gated Ca²⁺ channels (Ca_v1).
842 RyR1 are directly activated by conformational coupling to Ca_v1.1, whereas Ca²⁺ entering
843 cardiac myocytes through Ca_v1.2 activates RyR2 through Ca²⁺-induced Ca²⁺ release (CICR).
844 Structures from Electron Microscopy Data Bank (IP₃R, EMD-5278 (Ludtke et al., 2011);
845 RyR1, EMD-1275 (Ludtke et al., 2005)). (B) IP₃ binding is not alone sufficient to activate
846 IP₃Rs. IP₃ binding primes IP₃Rs to bind Ca²⁺ and that leads to channel opening. All four IP₃-
847 binding sites must be occupied for the pore to open, but it is not yet known how many Ca²⁺-
848 binding sites must be occupied (we show four for simplicity). (C) Dual regulation of IP₃Rs by
849 IP₃ and Ca²⁺ allows them to propagate regenerative Ca²⁺ signals by CICR. Local CICR
850 activity within a small cluster of IP₃Rs generates a Ca²⁺ puff. (D) The vicinal 4,5-
851 bisphosphate moiety of IP₃ is essential for activity, whereas the 1-phosphate enhances
852 affinity. (E) IP₃ is recognised by the IP₃-binding core (IBC) of IP₃R. The essential 4- and 5-
853 phosphates of IP₃ interact with opposing sides of the clam-like IBC to cause clam closure.
854 The loop of the suppressor domain (SD) interacts with IBC-β of a neighbouring subunit (Seo
855 et al., 2012). A-C modified from Taylor et al. (2014), and D reproduced from Seo et al.
856 (2012).

857 **Fig. 2: Measuring IP₃ binding to IP₃Rs.** (A) Binding assays allow determination of the
858 equilibrium dissociation constant (K_D) (**Box 2**). Non-equilibrium measurements allow rate
859 constants (k_{+1} and k_{-1}) to be determined. (B) Commonly, radioactive IP₃ (typically ³H-IP₃) is
860 equilibrated with IP₃R before rapidly separating (usually by centrifugation) bound and free
861 ligands to determine the amount of ³H-IP₃ bound to its receptor. (C) By immobilizing IP₃R on
862 the surface of a bead that detects only immediately adjacent (i.e. bound) ³H-IP₃, scintillation
863 proximity assays (SPA) report bound ³H-IP₃ without separating bound from free ligand (Patel
864 et al., 1996). (D) A variety of methods, including surface-plasmon resonance (SPR),
865 fluorescence correlation spectroscopy (FCS) and fluorescence polarization (FP) rely on
866 detecting the large increase in apparent size of IP₃ as it binds to the IP₃R (or a fragment of it).
867 With FP, for example (illustrated), a fluorescent analogue of IP₃ rotates rapidly when free,
868 but less so when it has bound to a soluble IP₃R fragment. The difference can be measured,
869 without separating bound and free ligands, by recording the extent to which plane-polarized
870 light remains polarized (Ding et al., 2010). (E) Isothermal titration calorimetry (ITC)
871 measures the very small amounts of heat released or absorbed (ΔH) as IP₃ binds to purified
872 IP₃R by comparison with a reference cell (de Azevedo and Dias, 2008).

873 **Fig. 3: Towards understanding how IP₃ and Ca²⁺ open IP₃Rs.** (A) Single IP₃R1 subunit
874 showing key domains: the N-terminal suppressor domain (SD); the β and α domains of the
875 IP₃-binding core (IBC); the intervening lateral domain (ILD), which lies between ARM3 and
876 the first trans-membrane domain (TMD1); TMD6, which lines the pore and is occluded by
877 hydrophobic residues towards its cytosolic end in the closed state; the helical linker domain
878 (LNK); and the C-terminal α-helical domain (CTD), which is unique to IP₃Rs. The structure
879 was published in Fan et al. (2015) (Protein Data Base, PDB 3JAV). (B) Simplified scheme,
880 derived from structures of IP₃R1 (Fan et al., 2015) and IP₃R3 (Paknejad and Hite, 2018)
881 shows that the only contact between the cytosolic and pore region occurs at the nexus
882 between ARM3 with its C-terminal ILD domain and the C-terminal extension of TMD6
883 (LNK). These contacts form an interleaved structure, with residues from LNK and the base of
884 ARM3 cooperating to form a Ca²⁺-binding site. Binding sites for IP₃ (IBC-α and IBC-β) and
885 Ca²⁺ are formed by residues contributed from different domains, allowing rigid-body
886 movements of domains to reconfigure the sites. The first Ca²⁺-binding site assembles from
887 residues provided by ARM1 and the α-helical linker between ARM1 and ARM2. The second
888 Ca²⁺-binding site is structurally conserved in RyR, and assembled by residues from ARM3
889 and LNK domains. This second site may mediate the IP₃-regulated binding of Ca²⁺ that
890 precedes channel opening (see text for details) (Paknejad and Hite, 2018). Opening of the
891 pore requires movement of occluding hydrophobic residues that lie close to the cytosolic end
892 of TMD6; Ca²⁺ can then pass rapidly from the ER lumen to the cytosol.

893 **Box 1: IP₃Rs and RyRs are not the only intracellular Ca²⁺ channels**

894 IP₃Rs and RyRs are the major intracellular Ca²⁺-release channels in most cells and the major
895 links between extracellular stimuli and Ca²⁺ release from the ER or sarcoplasmic reticulum
896 (SR) (**Fig. 1A**), but they are not the only intracellular Ca²⁺ channels (Taylor et al., 2009).
897 Polycystin-2 (also known as TRPP2), a member of the transient receptor potential (TRP)
898 superfamily, is also expressed in the ER and is activated by Ca²⁺ (Koulen et al., 2002). A
899 variety of Ca²⁺-permeable channels are expressed in lysosomes, including those regulated by
900 luminal pH and ATP (P2X purinoceptor 4, P2X4) (Huang et al., 2014), cytosolic nicotinic
901 acid adenine dinucleotide phosphate (NAADP; two pore channel 2, TPC2) (Morgan and
902 Galione, 2013), and the lysosomal membrane lipid, phosphatidylinositol 3,5-bisphosphate
903 (transient receptor potential mucolipin 1 channel, TRPML1) (Cao et al., 2017). The
904 mitochondrial uniporters (MCU) comprise another important family of intracellular Ca²⁺
905 channels (Oxenoid et al., 2016; Patron et al., 2013). Opening of MCU is triggered by large
906 local increases in [Ca²⁺]_c, causing Ca²⁺ to flow rapidly from the cytosol across the inner
907 mitochondrial membrane and into the mitochondrial matrix, where Ca²⁺ regulates many
908 activities (Rizzuto et al., 2012). A recurrent theme in Ca²⁺ signalling is the importance of
909 interactions between Ca²⁺ channels in different membranes: store-operated Ca²⁺ entry is
910 activated after loss of Ca²⁺ from the ER through IP₃Rs; mitochondrial Ca²⁺ uptake is driven
911 by local Ca²⁺ release through IP₃Rs and RyRs (Csordas et al., 2018); NAADP-evoked Ca²⁺
912 release from lysosomes is amplified by CICR through closely apposed IP₃Rs or RyRs
913 (Morgan and Galione, 2013); and Ca²⁺ puffs and sparks are ignited by CICR triggering Ca²⁺
914 release within clusters of IP₃Rs or RyRs (**Fig. 1C**) (Cheng and Lederer, 2008; Rios, 2018;
915 Thillaiappan et al., 2017).
916

917 **Box 2: Analysis of IP₃ binding**

918 Analyses of IP₃ binding allow affinities of IP₃ or competing ligands to be determined (as
919 equilibrium dissociation constants, K_D, the concentration of IP₃ at which 50% of binding sites
920 are occupied) (**Fig. 2**). These analyses determine the relationship between the concentration
921 of a ligand and the amount bound to IP₃Rs. Radioligand binding, using ³H-IP₃, is the most
922 commonly used approach. Most methods used to determine specific binding of ³H-IP₃ to
923 IP₃Rs require rapid separation of bound and free ³H-IP₃, such that the equilibrium between
924 free ³H-IP₃, competing ligands and the IP₃R is not perturbed by the separation procedure
925 (filtration or centrifugation) (**Fig. 2A,B**). Measuring specific binding with different
926 concentrations of ³H-IP₃ allows the K_D for ³H-IP₃ to be determined, whereas measuring
927 specific binding of ³H-IP₃ in the presence of different concentrations of a competing ligand
928 allow the K_D of that ligand to be determined (Cheng and Prusoff, 1973). Advantages of these
929 ³H-IP₃ binding assays are their simplicity and applicability to IP₃Rs within membranes, after
930 detergent-solubilization or as IP₃-binding fragments (Rossi et al., 2009). Scintillation
931 proximity assays (SPA) avoid the need for separation steps because the SPA beads are
932 impregnated with a scintillant, such that when IP₃Rs are immobilized on the surface of the
933 bead, only ³H-IP₃ bound to an IP₃R is detected (**Fig. 2C**) (Patel et al., 1996). More
934 specialized methods allow analysis of ligand binding to IP₃Rs without using radioligands.
935 These methods include fluorescence polarization (FP), which uses a fluorescent analogue of
936 IP₃ to report the size of the molecule to which the fluorophore is attached. When free, the
937 fluorescent IP₃ is small and tumbles rapidly in solution, but when bound to a large IP₃R
938 fragment it tumbles more slowly. These changes can be detected using plane-polarized light
939 (**Fig. 2D**) (Ding et al., 2010; Rossi and Taylor, 2013). Isothermal titration calorimetry (ITC),
940 which measures heat exchange during IP₃ binding, is another means of measuring ligand
941 binding to IP₃Rs without using ³H-IP₃ (**Fig. 2E**) (de Azevedo and Dias, 2008). Limitations of
942 both FP and ITC include the need for both specialised equipment and large amounts of
943 purified protein.

944

945 **Box 3: Nuclear patch-clamp recordings can be applied to IP₃Rs**

946 Patch-clamp recording allows the opening and closing of single ion channels to be recorded
947 with exquisite sensitivity (Neher, 1992). Usually these recordings are made at the plasma
948 membrane (PM), but that is not applicable to single-channel recordings from IP₃Rs, most of
949 which are expressed in ER. However, the outer nuclear membrane (ONM) is continuous with
950 the ER membrane, and IP₃Rs are expressed in the ONM. A glass microelectrode applied to
951 the ONM of an isolated nucleus allows single-channel recording from IP₃Rs trapped within.
952 By excising the patch from the intact nucleus to provide an excised patch, it is possible to
953 make recordings with the IP₃-binding site of the IP₃R exposed to either the interior of the
954 patch-pipette patch or (with greater difficulty) to the bath solution (Mak et al., 2007). The
955 latter allows rapid application of IP₃ or Ca²⁺ to the cytosolic surface. K⁺ or Cs⁺ are commonly
956 used as charge-carriers for patch-clamp recording because they provide large currents and
957 they, unlike Ca²⁺, do not regulate IP₃R gating. These patch-clamp methods allow the ion
958 selectivity and conductance of IP₃Rs to be determined. By examining the sequence of channel
959 openings and closing, gating schemes can be developed that seek to explain how regulators of
960 IP₃Rs (like IP₃ and Ca²⁺) move the channel through different closed states to its open state
961 (Mak and Foskett, 2014; Rahman et al., 2009). Image reproduced, with permission, from
962 Rossi et al. (2012).

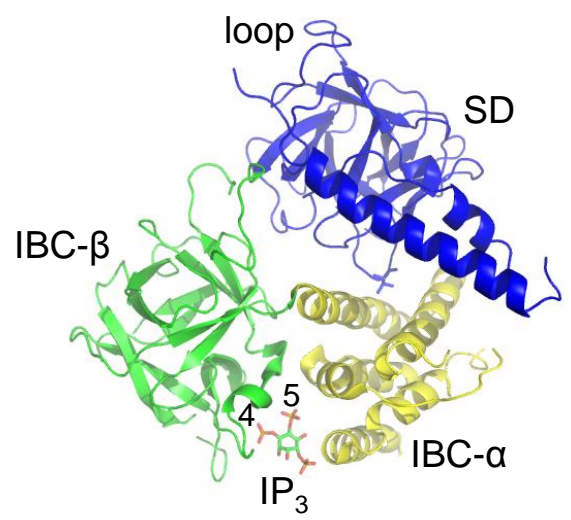
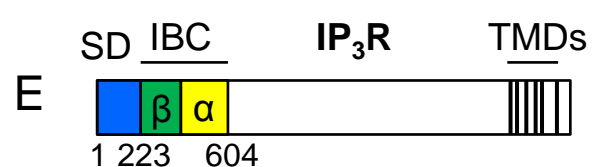
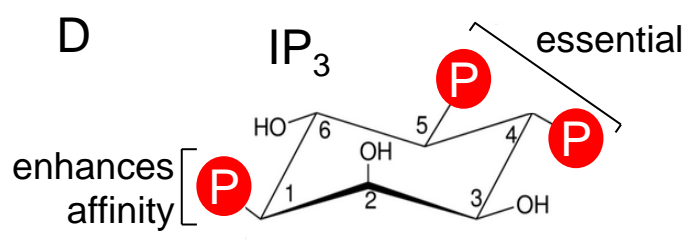
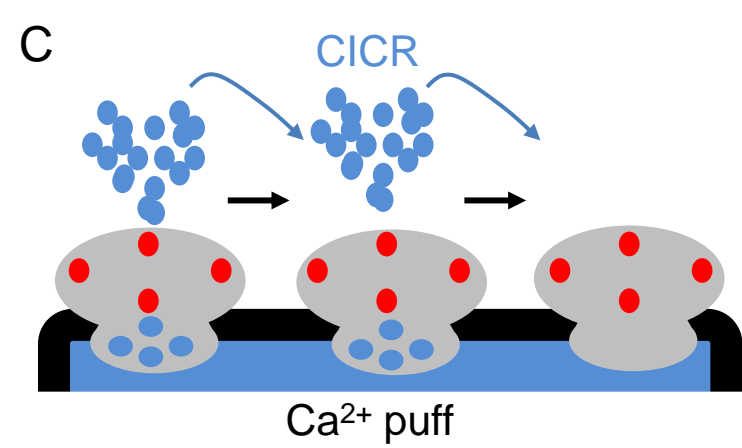
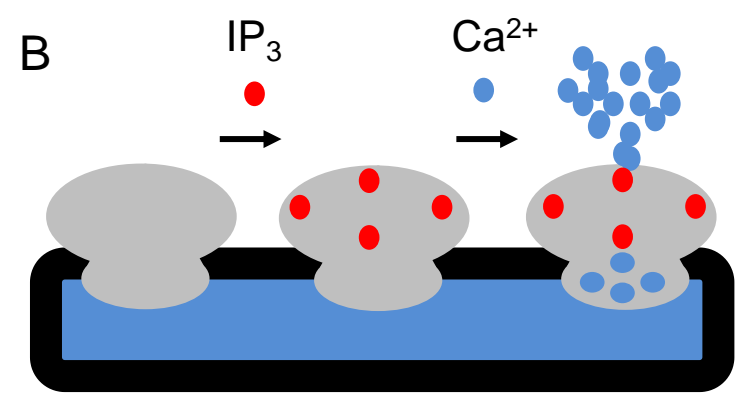
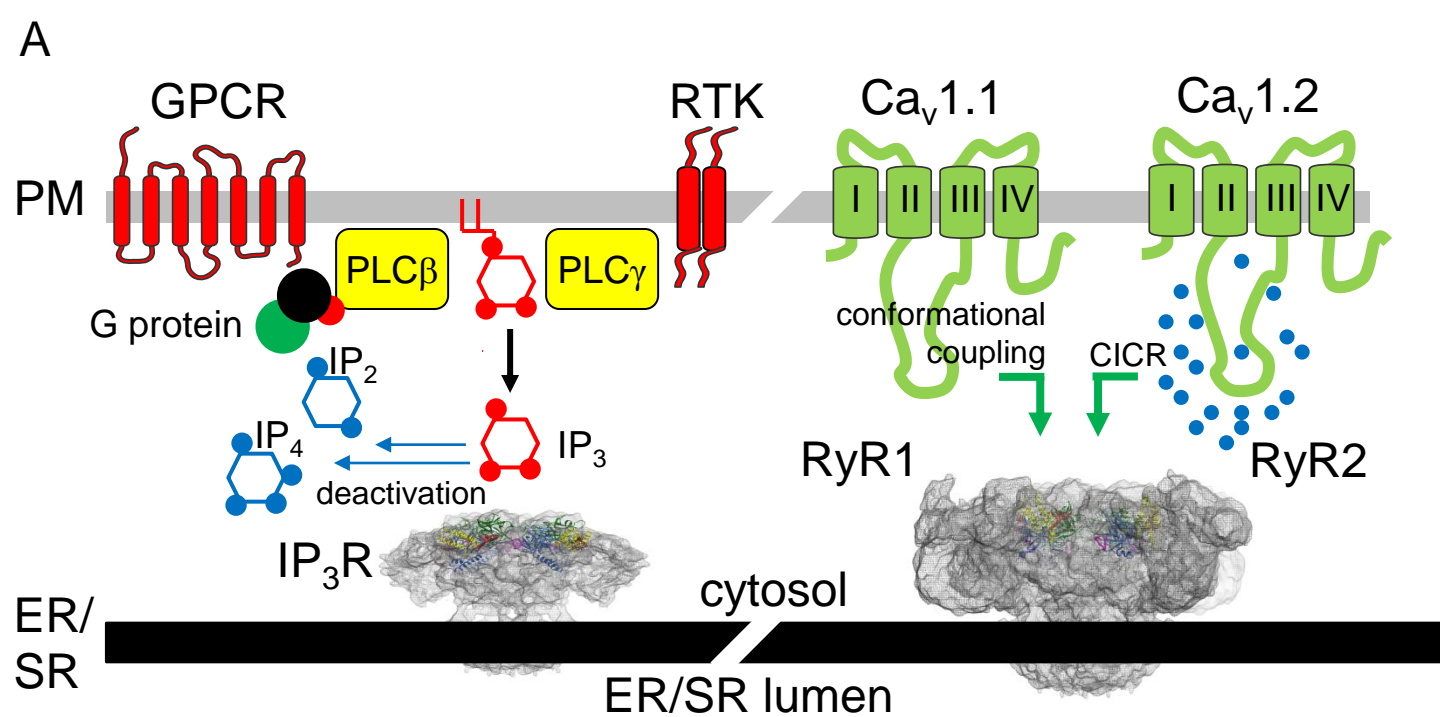
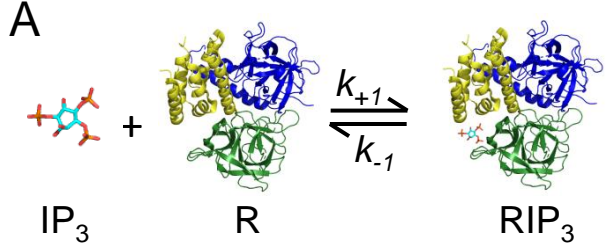


Figure 1



$$K_D = \frac{k_{-1}}{k_{+1}} = \frac{[IP_3][R]}{[RIP_3]}$$

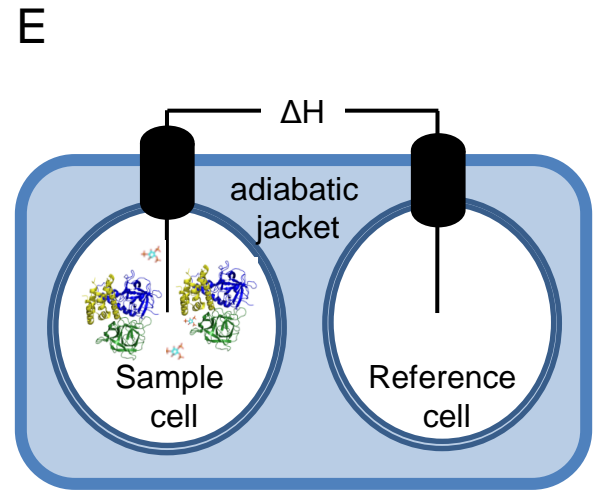
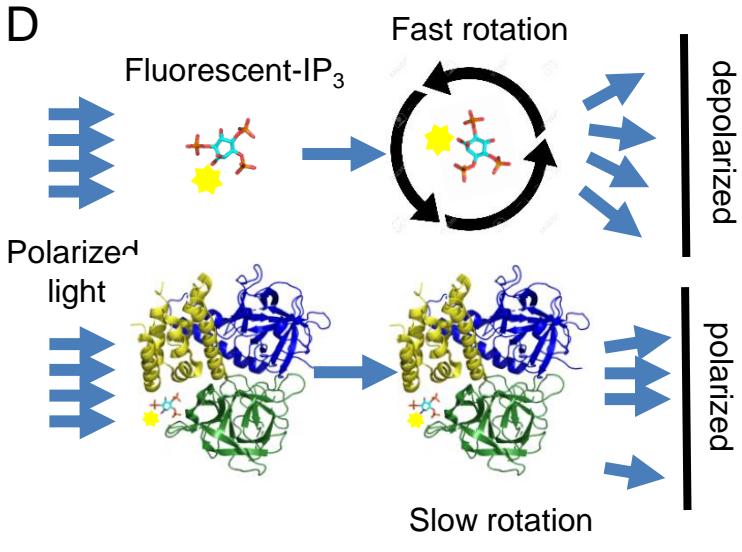
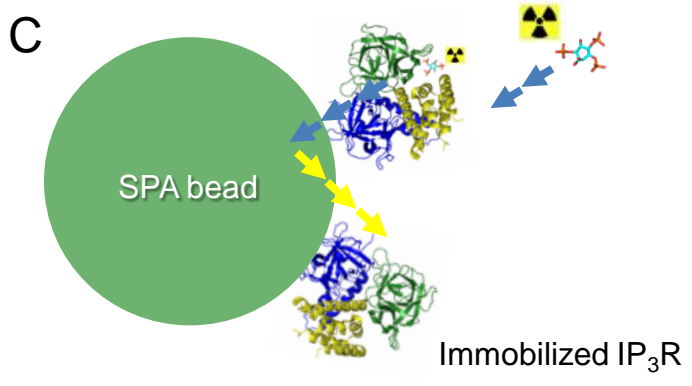
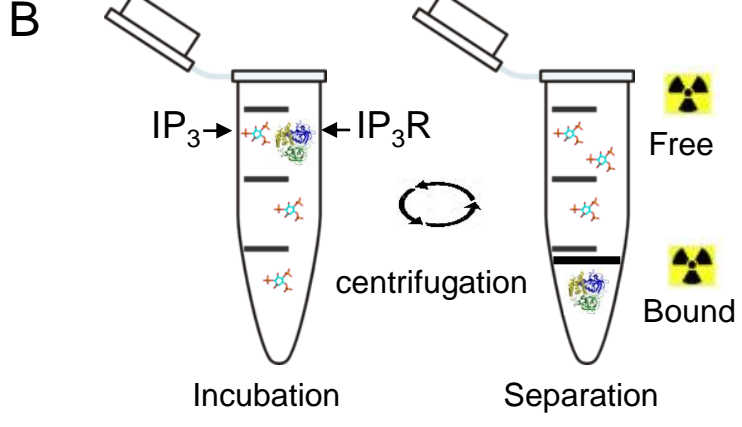
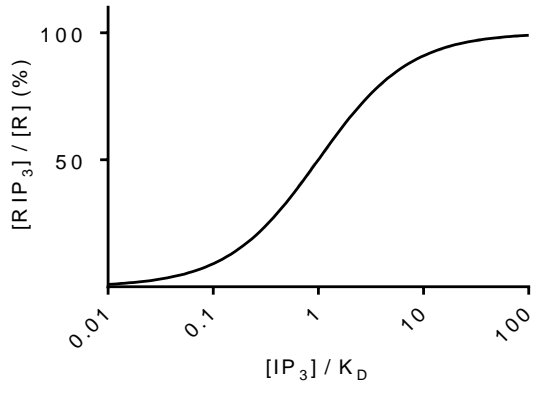


Figure 2

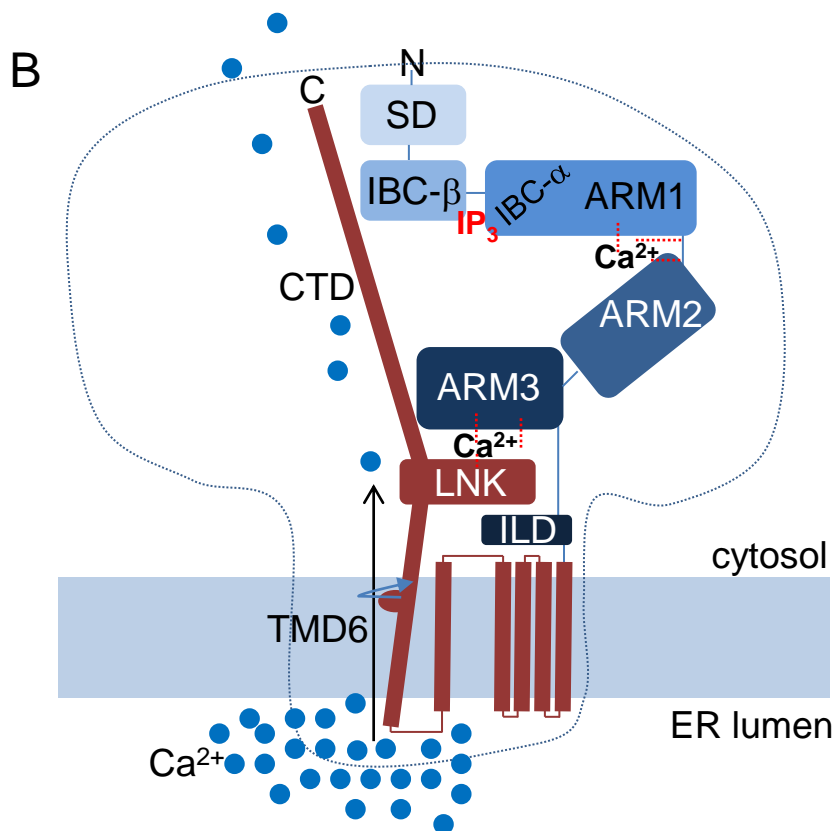
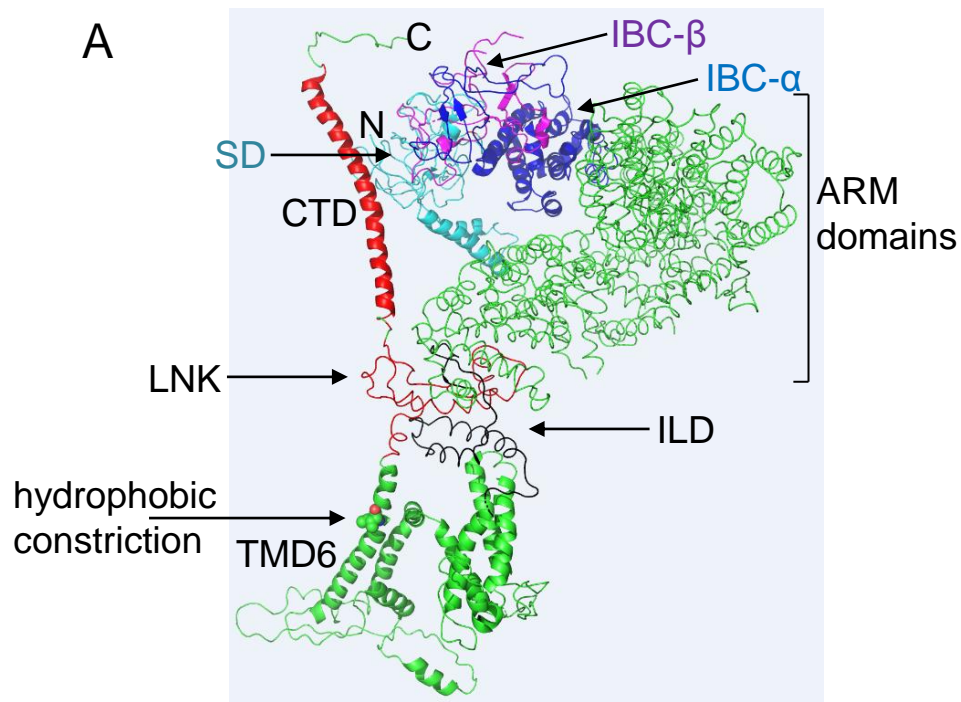


Figure 3

