1. General: The role of InsP3R in pancreatic acinar and other cells

The classical pathway linking stimulation by neurotransmitters and hormones to changes in phosphoinositide metabolism and the subsequent InsP3 induced Ca2+ release was in large part documented by work carried out in exocrine cells, in particular, acinar cells isolated from the exocrine pancreas (49). In seminal work using permeabilized rat pancreatic acini, Streb and colleagues demonstrated that the addition of InsP3 resulted in Ca2+ release from a non-mitochondrial Ca2+ store (43). This store was later identified to be the endoplasmic reticulum (ER) through experiments where sub-cellular fractions from pancreatic cells were isolated and exposed to InsP3 (4, 42). Ca2+ release is rapid, occurring as quickly as 1s following secretagogue stimulation of acinar cells. Ca2+ release is also isomer specific, with other structurally related inositol phosphates shown to be far less efficacious in releasing Ca2+ (43). The target protein for InsP3 binding was later identified as the inositol 1,4,5-trisphosphate receptor (InsP3R) (45). Strikingly, the secretion of digestive enzymes from pancreatic acinar cells has been shown to be entirely dependent on the activation of InsP3Rs and the resulting elevation in intracellular [Ca2+] (15). The receptor is encoded by 3 distinct genes in mammalian cells (ITPR1, ITPR2, ITPR3) that generate 3 monomeric isoforms (R1, R2, R3 respectively) that share 60-70% sequence homology (36).

Originally purified and cloned from rat cerebellum, the full length InsP3R forms a tetrameric cation selective channel in vivo (28). The three isoforms of InsP3R exhibit overlapping patterns of expression, with most cells and tissues expressing more than one isoform (50). Western blotting and quantitative PCR has revealed that there is relatively equal expression of the R2 and R3 isoforms in pancreas, with R1 only constituting ~3% of the total InsP3R (50). The importance of R2 and R3 isoforms to pancreatic Ca2+ signaling is most evident in studies employing InsP3R knockout (KO) mice. Although single R2 or R3 KO mice were found to have no observable ill effects or phenotypic alterations, double R2/R3 KO mice typically gained less body weight post-natally compared to single KO or WT mice. Furthermore, double KO mice tended to lose weight rapidly post weaning and typically died 4 weeks after birth. These observations were attributed to the key role
the R2 and R3 isoforms play in the secretion of saliva and exocytosis of digestive enzymes from exocrine glands and the resulting inability of double KO mice to swallow or properly digest dry adult food. Indeed, double KO mice were rescued and reached body weights comparable to WT when a fed pre-digested wet mash diet or an elemental diet. Pancreatic acinar cells from R2/R3 double KO mice were also found to accumulate zymogen granules that failed to secrete zymogen granules on stimulation with secretagogues. The study also highlights the inability of R1 to rescue double R2/R3 KO mice (15).

The fidelity and specificity of the Ca\textsuperscript{2+} signal required for exocytosis is thought to be largely determined by the differential expression, localization and modulation exhibited by the 3 InsP\textsubscript{3}R isoforms (14). For the most part, InsP\textsubscript{3}Rs are predominantly localized to the ER, although the golgi, nucleus, plasma membrane, peroxisomes and endolysosomal vesicles have also been reported to express small levels of InsP\textsubscript{3}Rs. Pancreatic acinar cells are highly polarized, both functionally and morphologically and InsP\textsubscript{3}Rs are predominantly expressed in ER.

**Figure 1. Localization of InsP\textsubscript{3}Rs in pancreatic acinar cells.** Immunofluorescence localizations in pancreatic lobules of InsP\textsubscript{3}Rs by confocal microscopy demonstrate the localization of InsP\textsubscript{3}R1, R2, R3 and amylase (A,B,C,D respectively) respectively. All 3 isoforms are predominantly localized to the apical pole of acinar cells directly abutting the plasma membrane (arrows in panels A-C). R1 and R3 also appeared to localize to perinuclear structures. (Scale bar = 10 \textmu m). From (57).
extensions in the apical regions juxtaposed to the acinar lumen (22, 24, 32, 57). Using fluorescence imaging techniques, studies have shown that stimulating InsP₃ generation in these cells results in the initiation of Ca²⁺ signals in the apical regions, followed by the propagation of a Ca²⁺ wave that is facilitated by peripheral InsP₃Rs. Stimulation of basal secretagogue receptors as well as uncaging InsP₃ in various regions of the cell also confirm that Ca²⁺ signals initiate in apical regions of the pancreatic acinar cell (3, 13, 21, 47, 53).

2. Structural features of the InsP₃R

Structurally, the InsP₃R monomer is conventionally divided into 3 functional domains: an N-terminal ligand binding domain (LBD), a coupling/modulatory domain and a C-terminal transmembrane domain (TMD) that contains the channel (14) (Figure 2). InsP₃ binding is mediated by the ‘core’ ligand binding domain, which constitutes amino acids (AA) 224-578 of the LBD. This region contains 10 conserved positively charged arginine and lysine residues (3 critical, R265, K508, R511) that are thought to allosterically coordinate the negatively charged PO₄³⁻ groups of InsP₃ in a binding pocket (55). The three isoforms have differing InsP₃ binding affinities that are regulated by the first 223 AA of the LBD, termed the suppressor domain (SD). Specifically, competitive InsP₃ binding assays using GST fusion constructs encoding AA 1-604 demonstrate that R2 has an 11-fold greater affinity (Kd) for InsP₃ compared to R3 (14nM vs. 163nM), with R1 having an intermediate affinity (49.5nM) (19). Similar studies in pancreatic acini revealed a Kd of 6nM (17), comparable to that of R2, while permeabilized Ca²⁺ release assays in acinar cells demonstrated an EC₅₀ for InsP₃ of 0.8µM (34) (Figure 3).

![Figure 2. Illustration of InsP₃R-1 depicting location of suppressor domain, ligand binding core, regulation / coupling domain and channel domain.](image-url)
Deletion of the suppressor domain (SD) results in the loss of distinct \(\text{InsP}_3\) affinities between the isoforms and a 10-100 fold increase in \(\text{InsP}_3\) affinity (19). Despite an increased \(\text{InsP}_3\) binding affinity, deletion of the SD also results in the loss of channel activity, indicating that the SD is required for inducing \(\text{InsP}_3\)R activation and \(\text{Ca}^{2+}\) release. Based on hydrophobic plots, the TMD is similar in structure to that of RyRs and voltage gated \(\text{K}^+\), \(\text{Na}^+\) and \(\text{Ca}^{2+}\) channels and constitutes 6 putative transmembrane regions (TM1-6) (12). The TMD is responsible for the ER targeting (33, 35) and for the oligomerization of the \(\text{InsP}_3\)R into tetramers, which occurs co-translationally (20). Lastly, TM5 and 6 forms the pore through which \(\text{Ca}^{2+}\) is conducted (37). The loop between TM5 and 6 contains a selectivity filter (GVGD; similar to the super family of cation selective channels) that provides some degree of cation selectivity to the \(\text{InsP}_3\)R (14). However, it is poorly \(\text{Ca}^{2+}\) selective and allows conduction of monovalent cations (\(\text{Ca}^{2+:K}^+ = 6:1\)). In fact, it is believed that “functional” \(\text{Ca}^{2+}\) selectivity of \(\text{InsP}_3\)R is primarily determined by virtue of SERCA being a highly selective \(\text{Ca}^{2+}\) pump and \(\text{Ca}^{2+}\) being by far the most abundant cation in the ER. To “gate” and open the channel, evidence suggest that the SD interacts with the cytosolic loop between TM4-5, and that \(\text{InsP}_3\) binding results in a conformational change that moves TM5 away from TM6 and opens the channel (38). The CT tail (last 160 AA) and the large (1700 AA) but less conserved modulatory domain contains putative binding sites for the numerous modulators of \(\text{InsP}_3\)R activity (56). These modulators, which include \(\text{Ca}^{2+}\), ATP and PKA, all contribute in distinct ways to the differential \(\text{Ca}^{2+}\) release profiles encoded by the 3 isoforms.

3. Modulation of the \(\text{InsP}_3\)R

Cytosolic \(\text{Ca}^{2+}\) is the most important regulator of \(\text{InsP}_3\)Rs, modulating activity in a biphasic manner (11). Numerous putative \(\text{Ca}^{2+}\) binding sites have been identified, and \(\text{Ca}^{2+}\) has been shown to induce dramatic conformational changes in R1 (1, 2, 18, 39, 40). For the most part, studies have shown that in the presence of \(\text{InsP}_3\), low to optimal \([\text{Ca}^{2+}]\) (300nM) stimulates channel activity while higher cytosolic \([\text{Ca}^{2+}]\) inhibits it (11). To explain this biphasic regulation, Foskett and collaborators have proposed a model in which \(\text{InsP}_3\)R activity is regulated by two distinct \(\text{Ca}^{2+}\) binding sites: a stimulatory and an inhibitory site. Under resting conditions, the inhibitory site has a higher affinity for \(\text{Ca}^{2+}\) than the stimulatory site. Accordingly, this site is occupied at resting conditions.
conditions and inhibits InsP₃R activity. The binding of InsP₃ decreases the inhibitory sites' affinity for Ca²⁺, thereby allowing Ca²⁺ to bind to the stimulatory site and positively regulating channel activity. The downstroke of the biphasic curve is the result of Ca²⁺ binding to the inhibitory sites due to cytosolic [Ca²⁺] being elevated beyond 300nM (46). In effect, they suggest that Ca²⁺ is only an essentially InsP₃R co-agonist, with InsP₃'s sole role being simply to modulate Ca²⁺ sensitivities (25).

Cytosolic ATP has also been shown to differentially modulate the activity of all 3 isoforms in an allosteric manner. This mode of regulation is thought to link the metabolic status of the cell to the Ca²⁺ release. Specifically, studies show that R2 is only modulated by ATP at sub-maximal [InsP₃] while R1 and R3 activity is affected irrespective of [InsP₃], implying that ATP is required for maximal activity of R1 and R3 (6, 7). Each isoform also differs in its affinity for ATP, with R2 having 3 fold and 10 fold higher affinities than R1 and R3, respectively (6). ATP binding was originally believed to occur at Walker A like motifs (G-X-G-X-X-G) that exist in each isoform. One such motif, called ATPB, is conserved across all 3 isoforms. Additionally, R1 contains 2 other sites: ATPA and ATPC, the latter only being found in the S2- variant which is expressed in peripheral tissues (48). Recently, mutagenesis studies have demonstrated that only the ATPB site in R2 is important in mediating the modulatory effects of ATP (34) and this regulation is important for defining the sensitivity of Ca²⁺ release in pancreatic acinar cells (17). In contrast, the ATPB sites in R1 and R3, in addition to the ATPA and ATPC sites in R1, play no role in modulating InsP₃R activity through ATP.

Lastly, protein kinase A (PKA) mediated phosphorylation has been shown to directly increase InsP₃R Ca²⁺ flux and single channel activity of R1 and R2 albeit by phosphorylation at different residues. Specifically, studies show that PKA mediated phosphorylation at S1598 and S1755 on R1 and S937 on R2 significantly enhance Ca²⁺ release and single channel activity (5, 9, 56). Conversely, no effects on Ca²⁺ release through R3 have been observed after PKA stimulation, despite evidence that R3 is phosphorylated by PKA at 3 sites in vivo (41, 56). Thus far, no single channel studies have been performed on R3 to rule out any PKA mediated effects on channel activity.

InsP₃Rs are also regulated and bound by numerous other kinases and accessory proteins, including cGMP protein kinase (PKG), Akt kinase, FK506 binding protein, calmodulin, CaBP, IRBIT, Bcl-2/XL, cytochrome C, RACK and Erp44 (14).

Lastly, due to the differences in modulation and Ca²⁺ signals generated by each isoform, it is entirely plausible that the formation of heterotetrameric InsP₃Rs would add another layer to the diversity of generated Ca²⁺ signals. To date, co-immunoprecipitation (IP) studies that utilize isoform specific antibodies have primarily provided evidence for the formation of heterotetramers (31, 52). Such experiments have been performed on the AR42J rat pancreatoma cell line (52) and pancreatic acinar cells (51) and have shown that such cells are capable of forming heterotetramers.

4. Tools available to study the InsP₃R

a. Antibodies

A multitude of commercially available isoform specific antibodies can be utilized to probe for each isoform of the InsP₃R. Additionally, there are antibodies that detect all 3 isoforms.

- InsP₃R1 Rabbit polyclonal (H-80) from Santa Cruz (sc-28614); epitope corresponding to AA 1894-1973 in the cytosolic modulatory domain.
- InsP₃R1, UC Davis/NIH NeuroMab Facility, clone L24/18; epitope against AA 2680-2749 in C-terminus.
- InsP₃R3 Goat polyclonal (C-20) from Santa Cruz (sc-7277); epitope corresponding to C-terminus.
- InsP₃R3 purified mouse monoclonal from BD Transduction Laboratories (Cat: 610313).
- InsP₃R1/2/3 Rabbit polyclonal (H-300) from Santa Cruz (sc-28613); epitope corresponding to AA 2402-2701 in C-terminus of InsP₃R-2 (human).

b. cDNA
Accession number:
InsP₃R1: rat, 55925609.
InsP₃R2: mouse, 60593031.
InsP₃R3: rat, 6981109.

c. Cell Lines
SHSY5Y Neuroblastoma: 99% InsP₃R1 (50).
AR42J Rat pancreatoma: 86% InsP₃R2; 12% InsP₃R1; 2% InsP₃R3 (50).
RINM5F Mouse insulinoma: 96% InsP₃R3; 4% InsP₃R1 (50).
DT40 triple knockout cell lines: Kurosaki and colleagues have generated a DT40 chicken B lymphocyte cell line with the endogenous InsP₃Rs stably knocked out (44). These triple knockout cells can subsequently be stably transfected with constructs encoding individual mammalian InsP₃R isoforms, allowing the study of activity and regulation of each isoform in isolation. These cell lines are available on request from our laboratory.

d. Mouse Lines
InsP₃R-1 knockout mice (27).
InsP₃R-2 (23) and R-3 single and double knockout mice (15).

e. Agonists/Antagonists
Commercially available InsP₃: D-myo-inositol 1,4,5-trisphosphate hexapotassium salt; Enzo Life Sciences (cat: ALX-307-00). Adenophostin is a high affinity analogue available from SIGMA (cat: A5094). Various chemically caged forms of InsP₃ are available which have no activity until a masking group is liberated by UV light exposure; e.g NPE-InsP₃ is available from Invitrogen (cat: I-23580). A cell permeable version of caged InsP₃ is available from Sichem (cat: cag-iso-145).

Cells are often stimulated with Gq coupled GPCR agonists to stimulate InsP₃ production. Agonists include acetylcholine, trypsin and cholecystokinin.

Antagonists
Only poorly selective/specific antagonists are currently available, these include Heparin (54); Caffeine (10); 2-Aminoethoxydiphenyl borate (2-APB) (26, 30); Xestospongin (16).

f. Techniques used to study InsP₃R function

5. References


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