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Studying isoform-specific inositol 1,4,5-trisphosphate receptor function and regulation

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Abstract

Inositol 1,4,5-trisphosphate receptors (InsP₃R) are a family of ubiquitously expressed intracellular Ca²⁺ channels. Isoform-specific properties of the three family members may play a prominent role in defining the rich diversity of the spatial and temporal characteristics of intracellular Ca²⁺ signals. Studying the properties of the particular family members is complicated because individual receptor isoforms are typically never expressed in isolation. In this article, we discuss strategies for studying Ca²⁺ release through individual InsP₃R family members with particular reference to methods applicable following expression of recombinant InsP₃R and mutant constructs in the DT40-3KO cell line, an unambiguously *null* InsP₃R expression system.

Keywords

Ca²⁺ release; inositol 1,4,5-trisphosphate receptors; DT40-3KO cells; permeabilized cells; flash photolysis

Introduction

Inositol 1,4,5-trisphosphate receptors (InsP₃R) are a family of intracellular Ca²⁺ channels (for review see [1–3]. Three genes code for distinct Ca²⁺ release channels of molecular mass ~300 kDa and are named the InsP₃R-1, InsP₃R-2 and InsP₃R-3 [4–6]. Additional diversity is generated by alternative splicing of the InsP₃R-1 and InsP₃R-2 genes [7,8]. InsP₃-triggered elevations in cytosolic Ca²⁺ control a vast and diverse array of physiological processes. InsP₃-induced Ca²⁺ release is also subject to “fine tuning”, often in an isoform specific manner by events including Ca²⁺ binding, post translational modification and by interaction with protein partners. These regulatory inputs define the spatial and temporal characteristic of the Ca²⁺ signal. Historically, InsP₃R function has been monitored as the composite signal from the complement of receptors expressed in a particular cell type. The Ca²⁺ release properties have then been studied by stimulating phospholipase C coupled receptors or following direct exposure to InsP₃. Typically, Ca²⁺ release is monitored using fluorescence techniques or alternatively electrophysiologically by means of a Ca²⁺ activated surrogate reporter. However, the ubiquitous expression of the channel, together with the expression of multiple receptor isoforms in individual cells, represents a significant hurdle to designing

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experimental paradigms useful in studying individual isoform, or splice variant specific InsP₃R function and regulation. Given these issues, some insight into individual InsP₃R isoform activity has been inferred by approaches designed to isolate the activity of a particular InsP₃R isoform. Notwithstanding these efforts, the unambiguous interpretation of data from individual InsP₃R, in particular recombinant channels, requires expression on a truly *null* background. With the generation by Kurosaki and colleagues of a DT40 cell line, in which the genes for all three InsP₃R have been stably ablated (DT40-3KO) [9], a unique genetically tractable system for the study of InsP₃R structure and function is now available. This article will first review the general experimental systems/paradigms which have been used to investigate particular InsP₃R isoform function. Secondly, we will describe several techniques which are applicable to studying InsP₃R isoform specific function by optical techniques with particular reference to the utility of DT40-3KO InsP₃R *null* cell line.

Description of Method

Choice of cell/tissue to study endogenous InsP₃R isoform activity

Several studies have documented the relative expression of individual InsP₃R types in both native tissue and in immortalized cell lines [10–13]. These data have been exploited to study Ca²⁺ release from reasonably well defined populations of InsP₃R, and in particular from cells expressing at least a predominance of an individual isoform. While no native cell type expresses a single InsP₃R in absolute isolation [14,15], cerebellar purkinje neurons (PN) come close to this situation as they express almost exclusively InsP₃R-1 and at very high levels relative to other cells [10,16]. Optical studies of isolated PN have provided a platform for investigating how the properties of InsP₃R-1 impact cerebellar function [14,15,17]. The widespread use of PN for optically studying InsP₃R-1 specific activity has however, been rather limited, presumably based on the relative difficulty of isolating and culturing these neurons. In a similar fashion, acutely isolated, cultured hepatocytes express ~80 % InsP₃R-2 (remainder InsP₃R-1), and a number of studies have ascribed the Ca²⁺ release characteristics in this cell type to the InsP₃R-2 isoform [18,19].

Immortalized cell lines with fairly well defined populations of InsP₃R have also been widely used to compare and contrast the fundamental regulation of InsP₃R isoforms. Cell lines do exist which express an individual InsP₃R isoform in relative isolation. For example, SHSY5Y neuroblastoma or A7R5, smooth muscle derived cells, express a majority of InsP₃R-1 and have been used to study the regulation of isoform by factors including Ca²⁺, ATP and phosphorylation [20,21]. In a similar fashion, the rat pancreatoma cell line AR4-2J and insulinoma line RinM5F have been the cells of choice for monitoring InsP₃R-2 and InsP₃R-3 activity respectively [21–24]. An additional complimentary approach is to attempt to isolate a particular InsP₃R following specific reduction in expression of a particular isoform, using antisense or siRNAi technology [25–27]. It should be noted that, the absolute ablation of a particular isoform with these techniques is probably unrealistic and therefore the general caveat applies that a particular receptor is unlikely to be expressed in unambiguous isolation using any of the cell-line based paradigms described above.

While important contributions have been made using the above techniques, the generation by Kurosaki and colleagues of a series of DT40 based cell lines with defined populations of InsP₃R provides important tools to explicitly study the activity of a particular InsP₃R homotetramer [9,28]. Based on the high rate of homologous recombination of this cell line, genes can be readily targeted for ablation. Lines have been established which express all permutations of the endogenous chicken InsP₃R and importantly an unequivocally InsP₃R *null* cell line, in which all three chicken isoforms have been ablated. The so-called DT40-3KO cell line has provided a unique tool in which InsP₃R and mutant constructs can

be expressed [9]. Next, several optical paradigms are discussed which are designed to study InsP₃R activity in DT40 cells.

Intact cell $[Ca^{2+}]_i$ measurements in DT40 cells following agonist exposure

The generation of DT40 cell lines in which the genes for pairs of InsP₃R have been ablated allows the study of individual chicken InsP₃R isoforms in isolation [9]. Perhaps of more importance, a particularly useful approach is to express individual mammalian InsP₃R and mutants by transient or stable transfection into DT40-3KO cells. DT40 cells are relatively resistant to transfection, but acceptable efficiency can be achieved by electroporation and in particular by nucleofection (AMAXA, INC, Gaithersburg, MD). For detailed methods regarding transient and stable expression of constructs in DT40 cells see [29]. In intact cells, Ca^{2+} release can be monitored by routine digital imaging of Ca^{2+} sensitive fluorescent dyes such as Fluo-4 and Fura-2 [30–32]. In our case, we use a Till Photonics imaging system (Gräfelfing, Germany) consisting of a Polychrome IV monochromator capable of rapid wavelength switching, coupled through a light guide to an Inverted microscope with high NA objective. The emitted light is captured using a Cooke Sensicam QE camera (Romulus, MI). Wavelength switching and image capture is controlled by the Vision software suite. A variety of methods to increase InsP₃ levels have been employed in DT40 cells based on either stimulation of endogenous receptors or heterologous expression of phospholipase C coupled receptors. For example, the characteristics of the global $[Ca^{2+}]_i$ signals following activation of the endogenous B cell receptor (BcR) have been described in cells expressing individual InsP₃R isoforms or following transfection with mammalian isoforms [29–31,33]. An example is shown in Figure 1B and 1C where the characteristic of Ca^{2+} signals following BcR activation are compared in cells expressing wild type S2- InsP₃R-1 or charge mutations in protein kinase A (PKA) phosphorylation sites which mimic phosphorylation (“EE”) [30]. In studies utilizing transient transfection, the InsP₃R construct of interest is typically co-transfected with a fluorescent protein such as HcRed to facilitate identification of expressing cells. The degree of expression of the fluorescent co-transfected protein can also be used as a somewhat crude indicator of the degree of expression. An example is shown in figure 1A. BcR activation results in tyrosine phosphorylation and stimulation of phospholipase C γ activity, however, DT40 cells also endogenously express Gq/PLC β coupled protease activated receptor 2, which can be activated by trypsin [34]. Stimulation of either of these pathways takes advantage of activating endogenous DT40 signaling, but has the major shortcoming that activation of these receptors is practically irreversible.

Expression of exogenous cell surface receptors

To our knowledge there have been no further reports of the presence of any other Gq/11 coupled receptor in DT40 cells which could provide a convenient means of activation. A further approach is to express by transfection a receptor coupled to G α_q . We and others have used a plasmid encoding a muscarinic m3 receptor which is available commercially from Missouri S&T cDNA Resource Center (CDNA.org). Stimulation of Ca^{2+} release can then be monitored following exposure to a muscarinic agonist. In DT40-3KO cells the magnitude of the initial peak following agonist exposure appears to be a useful reflection of Ca^{2+} release because this parameter is largely unaffected by removal of extracellular Ca^{2+} or blocking Ca^{2+} influx with lanthanides or by blocking ryanodine receptors [30]. In addition, the muscarinic m3 appears not to desensitize appreciably allowing multiple exposures to agonist in a single experiment [30,31]. This allows an indirect measurement of the sensitivity of Ca^{2+} release and even given the variability of expression, can be used to compare the activity of particular InsP₃R constructs. An example is shown in figure 2A, illustrating the increase in apparent sensitivity to muscarinic stimulation of cells expressing a S2- InsP₃R-1 phosphomimetic construct compared to wild type. A normalized concentration vs. response relationship for these constructs is shown in Figure 2C. DT40-3KO cells can also be used to

create stable cell lines expressing either Gαq coupled plasma membrane receptors or InsP₃R constructs. Stable expression allows the investigator to establish DT40 cells with constant receptor levels and thus negate this source of variability associated with transient over expression. It should be noted that in the process of targeting both copies of the three InsP₃R genes, the DT40-3KO cells have acquired resistance to most commonly used selection agents [9]. Practically, the only marker available for selection is geneticin, and thus only one additional further gene, either plasma membrane receptor or InsP₃R, can be stably expressed.

Activation of InsP₃R by flash photolysis of caged-InsP₃ and derivatives

Perhaps a more direct method for stimulating Ca²⁺ release is through activation of InsP₃R following flash photolysis of chemically caged-InsP₃. InsP₃ “caged” using a nitrophenylethyl ester moiety is cell impermeant and thus typically introduced into cells by microinjection or via dialysis from a pipette in the whole cell mode of the patch clamp technique. Both these methods are somewhat invasive and at least in the latter case, Ca²⁺ buffering is imposed by the internal pipette solution which may not reflect native cellular buffering. Recently, Ci-InsP₃, (caged-isopropylidene-InsP₃) a caged analogue of InsP₃ has become available (SiChem, Bremen, Germany) which is rendered cell permeable as a result of effectively masking the three phosphates of InsP₃ with ester labile propionyloxymethyl groups (PM) [35]. The molecule can then be loaded into cells by simple passive diffusion avoiding the need to disrupt cellular integrity. An added advantage of these cell permeable probes, is that, in contrast to microinjection, incubation obviously results in all cells being loaded with cage. Photo-destruction of the cage to liberate active InsP₃ can be accomplished by irradiating with intense light at UV wavelengths either supplied by a xenon flash lamp over the whole field or by a laser. Typically, Ca²⁺ release is monitored by digital imaging of cells loaded with a visible wavelength fluorescent probe. A detailed description of such experiments using DT40-3KO cells expressing mammalian InsP₃R is described below.

DT40-3KO cells either stably expressing a particular InsP₃R or following transient expression can be simultaneously loaded by incubation with the visible wavelength indicator, such as fluo-4 (4 μM) and the cell permeable form of caged-inositol trisphosphate (ciIP₃/PM; 2 μM) for 30 min in HEPES-buffered physiological saline solution (HEPES-PSS) at room temperature. A further period of approximately 30 min is allowed for de-esterification of both dye and cage. The cells are then allowed to adhere to low volume perfusion chamber (Warner Instruments) prior to imaging. Cells are illuminated at 488 ± 5 nm and fluorescence collected through a 525 ± 25 nm band pass filter and captured using the Till Photonics Vision imaging suite. Photolytic release while continuously measuring fluorescence of the Ca²⁺ indicator dye can be achieved following brief discharge of UV light (360 ± 7.5 nm) from a pulsed Xenon arc lamp (Till Photonics) ported into the microscope through a dual-port epifluorescence condenser and reflected to the image plane via a 400nm dichroic mirror. The intensity of the flash, and thus the degree of photolysis can be controlled by varying the degree of charging of the multiple capacitance banks in the flash lamp controller (between 5–183 joules) and/or the flash duration (0.5–5 msec duration). Typically the fluorescence traces are displayed as % ΔF/F₀, where F is the recorded fluorescence and F₀ is the mean of the initial 10 sequential frames. A typical example is shown in Figure 3 in which Ci-InsP₃ loaded DT40-3KO cells transiently expressing either non-phosphorylatable (Figure 3A) or phosphomimetic S2- InsP₃R-1 (Figure 3B) constructs and illustrates the apparent enhanced functional sensitivity of phosphorylated InsP₃R-1 to InsP₃.

Other experimental considerations-flash photolysis

As an alternative to the protocol described above using a Xenon flash lamp, photolysis can be achieved by any light source which can provide sufficiently intense UV light. Examples in the literature include shuttered Xenon/Mercury burners with appropriate filters, UV lasers and multiphoton excitation [32,36]. The former method of illumination usually requires longer light exposure to achieve sufficient energy for photolysis and is therefore not suited to experiments where fine temporal resolution is required. UV lasers and multi-photon excitation photolysis allow focal, sub-cellular photolysis and the added spatial resolution this brings [32]. A consideration for experiments with cell permeable Ci-IP_3 is that cells are loaded with a finite amount of cage which could in experimental paradigms requiring multiple uncaging events become limiting. Further, Ci-IP_3 has been reported to be poorly metabolized and this has been reported to influence the kinetics of Ca^{2+} release and the number of consecutive release events which can be evoked [35].

Studying InsP_3R function in permeabilized cells

The experiments described above allow estimations of InsP_3R function in a cellular context, but access to the cell interior is required for more direct measurements of InsP_3R function especially when manipulation of the intracellular milieu is desired. Once access is gained by permeabilization, Ca^{2+} in InsP_3 -releaseable stores can be readily measured with Ca^{2+} 3 indicator probe trapped in the ER lumen with identical imaging equipment used for intact cell Ca^{2+} measurements. A major benefit of this approach is that InsP_3R function can be measured in native membrane environments under near physiological ionic conditions. In addition, ER Ca^{2+} measurements can be made from virtually any cell type including acutely isolated cells, primary cultures and immortalized cell lines. Typically, permeabilization of the plasma membrane is accomplished with agents such as digitonin, saponin, β -escin or streptolysin-O [37–40]. Fura-2 is routinely used for measurement of ER Ca^{2+} because the affinity of the dye for Ca^{2+} (reported range 15–36 μM) is ideally suited to report free Ca^{2+} in this compartment. Other low affinity Ca^{2+} indicators dyes, such as Fura-FF are also available and have been successfully used to monitor ER Ca^{2+} [37].

The first report of ER Ca^{2+} measurements using Fura-2 was from Hofer and Machen [41] who measured Ca^{2+} in InsP_3 -releaseable stores of intestinal epithelial cells. This approach, which has been described in great detail elsewhere [42,43], measures ER Ca^{2+} using buffers where SERCA activity is retained. Under these conditions, the balance between InsP_3R and SERCA activities determines ER Ca^{2+} levels. As such, the steady state levels of ER Ca^{2+} after InsP_3 application are used to estimate InsP_3R activity. Multiple groups have used this approach in a variety of cell types and recently, a high throughput variation of this method was described by Taylor and colleagues [44].

Hirose and Iino further refined Fura-2 measurements of ER Ca^{2+} by modifying buffers to allow a unidirectional measurement of InsP_3R -mediated Ca^{2+} flux [38]. Specifically, they removed MgCl_2 prior to activating InsP_3R , which effectively disables SERCA while Ca^{2+} is being released. Instead of using the Ca^{2+} level in the stores after InsP_3 application, the rate of Ca^{2+} release is used for comparisons. This minor modification allows effective measures of InsP_3R activity under various experimental conditions. InsP_3 sensitivity, biphasic Ca^{2+} regulation and ATP modulation of InsP_3R have all been observed using this assay [9,17,45]

Iino and colleagues observed isoform specific modulation of InsP_3R -1, InsP_3R -2 and InsP_3R -3 by InsP_3 , Ca^{2+} and ATP by utilizing these unidirectional ER Ca^{2+} measurements with DT40 cells expressing individual isoforms [9]. Mutated receptors and GFP-tagged InsP_3R have also been introduced into DT40-3KO [29,34]. Measurements of InsP_3R function in cells expressing mutated isoforms has been used to determine the role of putative

Ca^{2+} and ATP binding domains in $\text{InsP}_3\text{R-1}$ [29]. We have successfully used this approach with cells transiently expressing wild type and mutated InsP_3R . Nuclear-targeted hcRed was cotransfected into the cells with InsP_3R to help identify transfected cells [29]. A detailed description of permeabilized cell experiments using DT40-3KO cells stably expressing the S2-splice variant of rat $\text{InsP}_3\text{R-1}$ is described below.

Ca^{2+} indicator loading and permeabilization

DT40 cells from low to mid density cultures were washed in HEPES-PSS and resuspended in HEPES-PSS supplemented with 1% BSA containing 10 μM of Fura2-AM. Cells were then loaded at 37°C, 5% CO_2 for 30 minutes to facilitate sequestration of the dye in the ER lumen. After loading is complete, cells were allowed to adhere to a coverslip at the bottom of a small-volume perfusion chamber for 15 minutes. Permeabilization involves perfusing with a buffer solution approximating intracellular conditions (ICM: 125 mM KCl, 10 mM NaCl, 1 mM EGTA, 10 mM HEPES, pH 7.3) and containing 40 μM β -escin. Monitoring of the dye leaving the cytosol is crucial to allow proper timing of the permeabilization process. Care must be taken not to allow β -escin to permeabilize intracellular membranes.

An example of DT40 cell permeabilization is shown in figure 4. Cells loaded with Fura2 prior to β -escin treatment are shown in figure 4A. Cells were excited with 360 nm light (the isosbestic point for Fura2) in 5 second intervals. Permeabilization was initiated by application of ICM containing 40 μM β -escin and is washed off when the fluorescence falls below 20% of the original fluorescence. This process typically takes one to two minutes as shown in Figure 4C. Cells after permeabilization are shown in Figure 4B. After permeabilization, cells were washed with ICM for 15–20 minutes to allow passive depletion of Ca^{2+} from ER stores.

Monitoring intraluminal Ca^{2+} levels in InsP_3 -sensitive stores

An example of a typical intraluminal Ca^{2+} recording is shown in Figure 4D. Loading of the stores is accomplished by application of ICM supplemented with 1.4 mM MgCl_2 , 3 mM Na_2ATP and 650 μM CaCl_2 . Under these conditions, Mg_2ATP and free CaCl_2 are calculated to be 1.3 mM and 200 nM respectively. These conditions are optimal for activation of SERCA and the increase in fluorescence ratio indicates Ca^{2+} entering the lumen of the ER. The rise in intraluminal Ca^{2+} is typically complete within two to three minutes as evidenced by the stabilization of the fluorescence ratio. Removing MgCl_2 from the perfusion buffer for one minute prior to InsP_3 application disables SERCA activity and allows unidirectional measurement of InsP_3 -induced Ca^{2+} release. Subsequent application of buffer containing InsP_3 causes a rapid reduction in the fluorescence ratio.

A major benefit to this protocol is that multiple Ca^{2+} release events can be monitored from the same cells. Also, recordings from dozens of cells can be made simultaneously and averaged together. Bleaching of the dye however is a major concern if multiple release events are to be monitored from the same cells. In order to overcome this problem we typically use higher binning and lower exposure times (4×4 and 20–40 ms respectively) during image acquisition. In addition, acquisition rates are reduced to every 10 seconds during the “loading” phase when kinetic information is not required. This allows for more rapid acquisitions (every 0.5 to 1 second) during the “release” phase of the experiment. An example of multiple recordings from the same group of cells is shown in figure 4C. Even with these considerations, bleaching typically prevents recording of more than three or four release events per experiment.

Information about relative InsP_3R activity under various experimental conditions can be gained from these recordings by fitting the decrease in fluorescence ratios to exponential

functions. Limiting the fitting to the initial 20 to 30 seconds allows fits to be made with single exponentials. An example of the type of information gained using these conditions is shown in figure 4E. Ca^{2+} release rates from cells stably expressing $\text{InsP}_3\text{R1 S1-}$ using various $[\text{InsP}_3]$ were compared yielding an EC_{50} for InsP_3 of 300 nM.

Other experimental considerations-permeabilized cell Ca^{2+} release assays

Some additional experimental considerations are required for accurate determination of InsP_3R function using optical measurements of ER luminal Ca^{2+} . Besides the image acquisition issues outlined above, careful buffer preparation is essential. Buffer pH should be carefully determined since the affinity of EGTA for Ca^{2+} is highly dependent on pH. Furthermore, free $[\text{Ca}^{2+}]$ in buffers should be verified empirically using fluorimetry measurements with Fura-2 or with a Ca^{2+} electrode. In addition, rapid exchange of buffer solutions is necessary if kinetic information about Ca^{2+} release is desired. Under our conditions, flow rates are typically greater than 4 ml/minute and buffer volumes in the Warner chamber are less than 500 μl .

Concluding Remarks

In summary, using standard imaging techniques coupled with flash photolysis and the use of permeabilized cells, DT40-3KO cells provide a valuable platform for the investigation by optical techniques of Ca^{2+} release through defined populations of recombinant InsP_3R . These paradigms have great potential to reveal the isoform-specific characteristics of individual InsP_3R important for defining the patterns of $[\text{Ca}^{2+}]_i$ signals which ultimately encode the appropriate activation of physiological end-points.

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Abbreviations

$[\text{Ca}^{2+}]_i$	intracellular calcium concentration
InsP_3R	inositol 1,4,5 trisphosphate receptor
$\text{Ci-IP}_3/\text{PM}$	caged isopropylidene inositol1,4,5 trisphosphate pentoxymethylester
Ci-IP_3	caged-isopropylidene inositol1,4,5 trisphosphate, isopropylidene inositol1,4,5 trisphosphate, i- IP_3
PSS	physiological salt solution
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
ER	endoplasmic reticulum
PKA	protein kinase A

References

1. Foskett JK, White C, Cheung KH, Mak DO. *Physiol Rev.* 2007; 87:593–658. [PubMed: 17429043]
2. Patel S, Joseph SK, Thomas AP. *Cell Calcium.* 1999; 25:247–264. [PubMed: 10378086]
3. Patterson RL, Boehning D, Snyder SH. *Annu Rev Biochem.* 2004; 73:437–465. [PubMed: 15189149]
4. Furuichi T, Yoshikawa S, Miyawaki A, Wada K, Maeda N, Mikoshiba K. *Nature.* 1989; 342:32–38. [PubMed: 2554142]
5. Maranto AR. *J Biol Chem.* 1994; 269:1222–1230. [PubMed: 8288584]

6. Sudhof TC, Newton CL, Archer BT 3rd, Ushkaryov YA, Mignery GA. *Embo J*. 1991; 10:3199–3206. [PubMed: 1655411]
7. Danoff SK, Ferris CD, Donath C, Fischer GA, Munemitsu S, Ullrich A, Snyder SH, Ross CA. *Proc Natl Acad Sci U S A*. 1991; 88:2951–2955. [PubMed: 1849282]
8. Iwai M, Tateishi Y, Hattori M, Mizutani A, Nakamura T, Futatsugi A, Inoue T, Furuichi T, Michikawa T, Mikoshiba K. *J Biol Chem*. 2005; 280:10305–10317. [PubMed: 15632133]
9. Miyakawa T, Maeda A, Yamazawa T, Hirose K, Kurosaki T, Iino M. *Embo J*. 1999; 18:1303–1308. [PubMed: 10064596]
10. Wojcikiewicz RJ. *J Biol Chem*. 1995; 270:11678–11683. [PubMed: 7744807]
11. De Smedt H, Missiaen L, Parys JB, Bootman MD, Mertens L, Van Den Bosch L, Casteels R. *J Biol Chem*. 1994; 269:21691–21698. [PubMed: 8063813]
12. De Smedt H, Missiaen L, Parys JB, Henning RH, Sienaert I, Vanlingen S, Gijssens A, Himpens B, Casteels R. *Biochem J*. 1997; 322(Pt 2):575–583. [PubMed: 9065779]
13. Ross CA, Danoff SK, Schell MJ, Snyder SH, Ullrich A. *Proc Natl Acad Sci U S A*. 1992; 89:4265–4269. [PubMed: 1374893]
14. Khodakhah K, Ogden D. *Proc Natl Acad Sci U S A*. 1993; 90:4976–4980. [PubMed: 8506344]
15. Khodakhah K, Ogden D. *J Physiol*. 1995; 487(Pt 2):343–358. [PubMed: 8558468]
16. Supattapone S, Worley PF, Baraban JM, Snyder SH. *J Biol Chem*. 1988; 263:1530–1534. [PubMed: 2826483]
17. Fujiwara A, Hirose K, Yamazawa T, Iino M. *Neuroreport*. 2001; 12:2647–2651. [PubMed: 11522941]
18. Adkins CE, Taylor CW. *Curr Biol*. 1999; 9:1115–1118. [PubMed: 10531009]
19. Hirata K, Pust T, O'Neill AF, Dranoff JA, Nathanson MH. *Gastroenterology*. 2002; 122:1088–1100. [PubMed: 11910359]
20. Maes K, Missiaen L, De Smet P, Vanlingen S, Callewaert G, Parys JB, De Smedt H. *Cell Calcium*. 2000; 27:257–267. [PubMed: 10859592]
21. Wojcikiewicz RJ, Luo SG. *J Biol Chem*. 1998; 273:5670–5677. [PubMed: 9488697]
22. Arguin G, Regimbald-Dumas Y, Fregeau MO, Caron AZ, Guillemette G. *J Endocrinol*. 2007; 192:659–668. [PubMed: 17332533]
23. Chaloux B, Caron AZ, Guillemette G. *Biol Cell*. 2007; 99:379–388. [PubMed: 17373911]
24. Hagar RE, Burgstahler AD, Nathanson MH, Ehrlich BE. *Nature*. 1998; 396:81–84. [PubMed: 9817204]
25. Hattori M, Suzuki AZ, Higo T, Miyauchi H, Michikawa T, Nakamura T, Inoue T, Mikoshiba K. *J Biol Chem*. 2004; 279:11967–11975. [PubMed: 14707143]
26. Jayaraman T, Marks AR. *Mol Cell Biol*. 1997; 17:3005–3012. [PubMed: 9154798]
27. Mendes CC, Gomes DA, Thompson M, Souto NC, Goes TS, Goes AM, Rodrigues MA, Gomez MV, Nathanson MH, Leite MF. *J Biol Chem*. 2005; 280:40892–40900. [PubMed: 16192275]
28. Sugawara H, Kurosaki M, Takata M, Kurosaki T. *Embo J*. 1997; 16:3078–3088. [PubMed: 9214625]
29. Wagner LE 2nd, Betzenhauser MJ, Yule DI. *J Biol Chem*. 2006; 281:17410–17419. [PubMed: 16621795]
30. Wagner LE 2nd, Li WH, Joseph SK, Yule DI. *J Biol Chem*. 2004; 279:46242–46252. [PubMed: 15308649]
31. Wagner LE 2nd, Li WH, Yule DI. *J Biol Chem*. 2003; 278:45811–45817. [PubMed: 12939273]
32. Won J-H, Cottrell WJ, Foster TH, Yule DI. *Am J Physiol Gastrointest Liver Physiol*. 2007; 293:G1166–1177. [PubMed: 17901163]
33. van Rossum DB, Patterson RL, Kiselyov K, Boehning D, Barrow RK, Gill DL, Snyder SH. *Proc Natl Acad Sci U S A*. 2004; 101:2323–2327. [PubMed: 14983008]
34. Morita T, Tanimura A, Nezu A, Kurosaki T, Tojyo Y. *Biochem J*. 2004; 382:793–801. [PubMed: 15175012]
35. Dakin K, Li WH. *Cell Calcium*. 2007; 42:291–301. [PubMed: 17307252]

36. Echevarria W, Leite MF, Guerra MT, Zipfel WR, Nathanson MH. *Nat Cell Biol.* 2003; 5:440–446. [PubMed: 12717445]
37. Hajnoczky G, Thomas AP. *Embo J.* 1997; 16:3533–3543. [PubMed: 9218795]
38. Hirose K, Iino M. *Nature.* 1994; 372:791–794. [PubMed: 7997268]
39. Tojyo Y, Tanimura A, Matsumoto Y. *Biochem Biophys Res Commun.* 1997; 240:189–195. [PubMed: 9367908]
40. van de Put FH, Elliott AC. *J Biol Chem.* 1996; 271:4999–5006. [PubMed: 8617776]
41. Hofer AM, Machen TE. *Proc Natl Acad Sci U S A.* 1993; 90:2598–2602. [PubMed: 8464866]
42. Hofer AM, Schulz I. *Cell Calcium.* 1996; 20:235–242. [PubMed: 8894270]
43. Hofer AM. *Methods Mol Biol.* 2006; 312:229–247. [PubMed: 16422202]
44. Tovey SC, Sun Y, Taylor CW. *Nat Protoc.* 2006; 1:259–263. [PubMed: 17406242]
45. Miyakawa T, Mizushima A, Hirose K, Yamazawa T, Bezprozvanny I, Kurosaki T, Iino M. *Embo J.* 2001; 20:1674–1680. [PubMed: 11285231]

Figure 1. Activation of Ca^{2+} release in transiently transfected DT40-3KO cells via the endogenous BcR

In A, three DT40-3KO cells are shown in bright field (left panel), following excitation at 560 nm (middle panel) revealing expression of HcRed and thus presumably the InsP_3R construct of interest and following excitation at 360 nm (right panel) to excite Fura-2. In B. Cartoon above each trace indicates the amino acids within the two canonical PKA phosphorylation motifs in S2- InsP_3R . Individual DT40 cells expressing HcRed and S2- WT InsP_3R -1 are stimulated with a threshold concentration of IgM to stimulate the BcR. This results a transient increase in $[\text{Ca}^{2+}]_i$ in each cell. In C, cells expressing a phosphomimetic S2- InsP_3R -1 construct ("EE" InsP_3R -1) are stimulated with an identical [IgM] which results in repetitive Ca^{2+} oscillations typical of enhanced sensitivity of the phosphomimetic construct. Adapted from Ref [31], with permission.

Figure 2. Activation of Ca^{2+} release via transiently expressed M3 muscarinic Receptor
 In A, concentration vs Response relationship for an individual DT40-3KO cell expressing S2-InsP₃R-1 and the M3 muscarinic receptor. In B, a similar experiment is performed in a cell expressing a construct harboring phosphomimetic charge mutations (“EE” InsP₃R-1). C shows pooled data where the magnitude of the initial peak of a particular response is normalized to the maximal peak in an individual cell. Adapted from Ref [31], with permission.

Figure 3. Activation of Ca²⁺ Release via photolysis of cell permeable caged-InsP₃

In A, Ca²⁺ release events are shown from a DT40-3KO expressing a non-phosphorylatable S2- InsP₃R-1 and loaded with Fluo-4 and Ci-InsP₃, a cell permeable caged InsP₃ analogue. At short flash durations (constant intensity ~60 J) release events are refractory. Ca²⁺ release can be evoked with longer flash times. In B, cells expressing a phosphomimetic “EE” construct respond at shorter flash durations than “AA” expressing cells, consistent with this construct exhibiting enhanced sensitivity to InsP₃. Adapted from Ref [31], with permission.

Figure 4.

Panel A shows DT40 cells stably expressing rat S2- InsP₃R-1 after loading with fura-2. B shows the same cells after treatment with β-escin. Images were acquired following excitation at 360 nm. C, shows the time course of permeabilization. Application of β-escin causes a reduction in fluorescence at 360 nm, indicating dye leaving the cytoplasm during permeabilization. D–F, show the results of ER luminal Ca²⁺ measurements. In D, application of buffer containing MgCl₂, CaCl₂ and ATP allows activation of SERCA which results in Ca²⁺ entering the ER as indicated by a rise in the 340/380 ratio. Washout of MgCl₂ causes no change in the fluorescence ratio but application of InsP₃ in the continued absence of MgCl₂ causes a rapid decline in the ratio indicating Ca²⁺ exiting the ER lumen. The recording is an average of 30 cells from a single experiment. E, shows the results of representative InsP₃-induced Ca²⁺ release experiments with the indicated [InsP₃]. Each trace is the average of 30 cells. F, shows the pooled results from multiple experiments. Ca²⁺ release rates were calculated by fitting the initial 20 seconds of each trace to a single exponential function.