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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 ($InsP_3R$) are a family of ubiquitously expressed intracellular Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} release through individual $InsP_3R$ family members with particular reference to methods applicable following expression of recombinant $InsP_3R$ and mutant constructs in the DT40-3KO cell line, an unambiguously *null* $InsP_3R$ expression system.

1. Introduction

Inositol 1,4,5-trisphosphate receptors ($InsP_3R$)[1] are a family of intracellular Ca^{2+} channels (for review see [1–3]. Three genes code for distinct Ca^{2+} release channels of molecular mass ~300 kDa and are named the $InsP_3R$-1, $InsP_3R$-2 and $InsP_3R$-3 [4–6]. Additional diversity is generated by alternative splicing of the $InsP_3R$-1 and $InsP_3R$-2 genes [7,8]. $InsP_3$-triggered elevations in cytosolic Ca^{2+} control a vast and diverse array of physiological processes. $InsP_3$-induced Ca^{2+} release is also subject to "fine tuning", often in an isoform-specific manner by events including Ca^{2+} binding, post-translational modification and by interaction with protein partners. These regulatory inputs define the spatial and temporal characteristic of the Ca^{2+} signal. Historically, $InsP_3R$ function has been monitored as the composite signal from the complement of receptors expressed in a particular cell type. The Ca^{2+} release properties have then been studied by stimulating phospholipase C coupled receptors or following direct exposure to $InsP_3$. Typically, Ca^{2+} release is monitored using fluorescence techniques or alternatively electrophysiologically by means of a Ca^{2+} 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 experimental paradigms useful in studying individual isoform, or splice variant specific $InsP_3R$ function and regulation. Given these issues, some insight into individual $InsP_3R$ isoform activity has been inferred by approaches designed to isolate the activity of a particular $InsP_3R$ isoform. Notwithstanding these efforts, the unambiguous interpretation of data from individual $InsP_3R$, 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 $InsP_3R$ have been stably ablated (DT40-3KO) [9], a unique genetically tractable system for the study of $InsP_3R$ structure and function is now available. This article will first review the general experimental systems/paradigms which have been used to investigate particular $InsP_3R$ isoform function. Secondly, we will describe several techniques which are applicable to studying $InsP_3R$ isoform-specific function by optical techniques with particular reference to the utility of DT40-3KO $InsP_3R$ *null* cell line.

2. Description of method

2.1. Choice of cell/tissue to study endogenous $InsP_3R$ isoform activity

Several studies have documented the relative expression of individual $InsP_3R$ types in both native tissue and in immortalized cell lines [10–13]. These data have been exploited to study Ca^{2+} release from reasonably well defined populations of $InsP_3R$, and in particular from cells expressing at least a predominance of an individual isoform. While no native cell type expresses a single $InsP_3R$ in absolute isolation [14,15], cerebellar purkinje neurons (PN) come close to this situation as they express almost exclusively, $InsP_3R$-1 and at very high levels relative to other cells [10,16]. Optical studies of isolated PN have provided a platform

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[1] *Abbreviations used:* $[Ca^{2+}]_i$, intracellular calcium concentration; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; Ci-IP_3/PM, caged isopropylidene inositol 1,4,5-trisphosphate pentoxymethylester; Ci-IP_3, caged-isopropylidene inositol 1,4,5 trisphosphate; i-IP_3, isopropylidene inositol1,4,5-trisphosphate; PSS, physiological salt solution; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; ER, endoplasmic reticulum; PKA, protein kinase A.

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for investigating how the properties of $InsP_3R$-1 impact cerebellar function [14,15,17]. The widespread use of PN for optically studying $InsP_3R$-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% $InsP_3R$-2 (remainder $InsP_3R$-1), and a number of studies have ascribed the Ca^{2+} release characteristics in this cell type to the $InsP_3R$-2 isoform [18,19].

Immortalized cell lines with fairly well defined populations of $InsP_3R$ have also been widely used to compare and contrast the fundamental regulation of $InsP_3R$ isoforms. Cell lines do exist which express an individual $InsP_3R$ isoform in relative isolation. For example, SH-SY5Y neuroblastoma or A7r5, smooth muscle derived cells, express a majority of $InsP_3R$-1 and have been used to study the regulation of isoform by factors including Ca^{2+}, 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 $InsP_3R$-2 and $InsP_3R$-3 activity, respectively [21–24]. An additional complimentary approach is to attempt to isolate a particular $InsP_3R$ following specific reduction in expression of a particular isoform, using antisense or sRNAi 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 $InsP_3R$ provides important tools to explicitly study the activity of a particular $InsP_3R$ 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 $InsP_3R$ and importantly an unequivocally $InsP_3R$ *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 $InsP_3R$ and mutant constructs can be expressed [9]. Next, several optical paradigms are discussed which are designed to study $InsP_3R$ activity in DT40 cells.

2.2. 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 $InsP_3R$ have been ablated allows the study of individual chicken $InsP_3R$ isoforms in isolation [9]. Perhaps of more importance, a particularly useful approach is to express individual mammalian $InsP_3R$ 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 $InsP_3$ 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 $InsP_3R$ isoforms or following transfection with mammalian isoforms [29–31,33]. An example is shown in Fig. 1B and C where the characteristic of Ca^{2+} signals following BCR activation are compared in cells expressing wild type S2-$InsP_3R$-1 or charge mutations in protein kinase A (PKA) phosphorylation sites which mimic phosphorylation ("EE") [30]. In studies utilizing transient transfection, the $InsP_3R$ construct of interest is typically co-transfected with a cDNA encoding 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 Fig. 1A. BCR activation results in tyrosine phosphorylation and stimulation of phospholipase Cγ activity, however, DT40 cells also endogenously express Gq/PLCβ 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.

2.3. Expression of exogenous cell surface receptors

To our knowledge there have been no additional 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 Gαq. 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

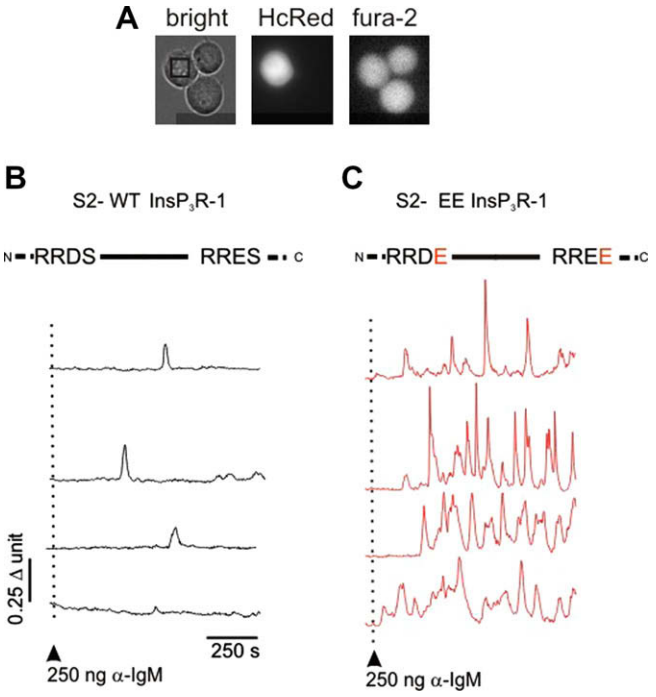

Fig. 1. Activation of Ca^{2+} release in transiently transfected DT40-3KO cells via the endogenous BCR. In (A) three DT40-3KO cells are shown in bright field (left panel), following excitation at 560 nM (middle panel) revealing expression of HcRed and thus presumably the $InsP_3R$ construct of interest 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 $[Ca^{2+}]_i$ 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.