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Bilayer Measurement of Endoplasmic Reticulum Ca^{2+} Channels

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Abstract

Reconstitution of ion channels into planar lipid bilayers (also called black lipid membranes or BLM) is the most widely used method to conduct physiological studies of intracellular ion channels, including endoplasmic reticulum (ER) calcium (Ca^{2+}) channels. The two main types of Ca^{2+} release channels in the ER membrane are ryanodine receptors (RyanRs) and inositol(1,4,5)-trisphosphate receptors ($InsP_3Rs$). Use of the BLM reconstitution technique enabled the initial description of the functional properties of $InsP_3R$ and RyanR at the single-channel level more than 20 years ago. Since then, BLM reconstitution methods have been used to study physiological modulation and to perform structure–function analysis of these channels, and to study pathological changes in the function of $InsP_3R$ and RyanR in various disease states. The BLM technique has also been useful for studies of other intracellular Ca^{2+} channels, such as ER Ca^{2+} leak presenilin channels and NAADP-gated lysosomal Ca^{2+} channels encoded by TPC2. In this article, basic protocols used for BLM studies of ER Ca^{2+} channels are introduced.

Introduction

Studies of plasma membrane ion channels have been greatly facilitated by the development of the patch-clamp technique (Sakmann and Neher 1983). However, membranes of the endoplasmic reticulum (ER) and other intracellular compartments are not accessible for traditional patch clamp experiments. Application of the patch-clamp technique to nuclear patches provided an opportunity to conduct some studies of intracellular ion channels (Mak and Foskett 1997), but this technique (see Patch-Clamp Electrophysiology of Intracellular Ca^{2+} Channels [Mak et al. 2013]) is only applicable to certain types of cells and preparations and has a number of additional technical limitations. For these reasons, reconstitution of ion channels into planar lipid bilayers (also called black lipid membranes or BLM) is the most widely used method to conduct physiological studies of intracellular ion channels, including ER Ca^{2+} channels. General methods for making bilayers and for ion channel reconstitution into BLM have been extensively described in an excellent manual (Miller 1986). In this article, the focus will primarily be on the technical issues specific for BLM studies of ER Ca^{2+} channels.

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There are two types of Ca^{2+} release channels in the ER membrane—ryanodine receptors (RyanRs) and inositol(1,4,5)-trisphosphate receptors ($InsP_3Rs$). There are single isoforms of $InsP_3R$ and RyanR in *Drosophila melanogaster* and *Caenorhabditis elegans* and three mammalian isoforms for both the $InsP_3R$ and RyanR families (Bezprozvanny 2005; Foskett et al. 2007; Mikoshiba 2007; Lanner et al. 2010; Capes et al. 2011). These tetrameric channels are very large, with subunits of $InsP_3R$ having a mass of about 260 kDa and subunits of RyanR having a mass of 560 kDa (Bezprozvanny 2005; Foskett et al. 2007; Mikoshiba 2007; Lanner et al. 2010; Capes et al. 2011). The large size of these channels enabled direct structural studies using particle electron microscopy and image analysis (Hamilton and Serysheva 2009; Serysheva and Ludtke 2010).

$InsP_3Rs$ are gated by the second messenger inositol (1,4,5)-trisphosphate ($InsP_3$), which is generated following phospholipase C-mediated cleavage of the lipid precursor phosphatidylinositol 4,5-bisphosphate (PIP_2). All $InsP_3R$ isoforms have a conserved amino-terminal domain that forms a high affinity $InsP_3$-binding site (Bezprozvanny 2005; Foskett et al. 2007; Mikoshiba 2007). The crystal structure of the $InsP_3$-binding domain from $InsP_3R1$ was solved in both $InsP_3$-bound and apo ($InsP_3$-free) forms (Bosanac et al. 2002; Bosanac et al. 2005; Lin et al. 2011). Skeletal muscle RyanR1s are gated mechanically by direct movement of voltage-sensors in plasma membrane $Ca_V1.1$ channels (DHPR) (Lanner et al. 2010; Capes et al. 2011). The mechanical coupling between DHPR and RyanR1 is facilitated by a specialized triad structure in skeletal muscle, which brings the sarcoplasmic reticulum and plasma membrane in close proximity to each other. RyanR2 is a predominant isoform in the heart and brain. RyanR2 is gated by an increase in Ca^{2+} levels and supports Ca^{2+}-induced Ca^{2+} release (CICR). RyanR3 is expressed in brain, smooth muscle, and several other tissues and also functions as a Ca^{2+}-gated Ca^{2+} channel. Activation of RyanRs by a novel messenger, cyclic-ADP ribose (cADPR), has been proposed, but cADPR does not bind directly to RyanR, and the issue of RyanR activation by cADPR remains controversial (Venturi et al. 2012).

BLM EXPERIMENTS TO STUDY $InsP_3R$ AND RyanR

Both $InsP_3Rs$ and RyanRs play a key role in control of cytosolic Ca^{2+} concentrations in cells. Due to the central role played by these channels in Ca^{2+} signaling, both proteins are subject to multiple levels of regulation. BLM recordings of native and recombinant $InsP_3R$ and RyanR played a key role in understanding the physiological modulation of these channels. Initial bilayer recordings of native skeletal muscle RyanR1 was achieved in 1985 (Smith et al. 1985, 1986), native smooth muscle $InsP_3R1$ in 1988 (Ehrlich and Watras 1988), and native cerebellar $InsP_3R1$ and RyanR in 1991 (Bezprozvanny et al. 1991). The main procedures used in these initial publications have been used with only minor changes for more than 20 years now to describe physiological properties and modulation of $InsP_3R$ and RyanR in bilayers. Using bilayer techniques, it was shown that both $InsP_3R$ and RyanR are modulated by cytosolic Ca^{2+} levels (Smith et al. 1986; Bezprozvanny et al. 1991). However, in the physiological Ca^{2+} range, skeletal muscle RyanR1 and cardiac RyanR2 function as Ca^{2+}-gated Ca^{2+} channels (Smith et al. 1986), whereas cerebellar $InsP_3R1$ displays very narrow bell-shaped Ca^{2+} dependence (Bezprozvanny et al. 1991). The activity of both skeletal muscle RyanR1 and cerebellar $InsP_3R1$ are potentiated by cytosolic levels of

ATP (Smith et al. 1986; Bezprozvanny and Ehrlich 1993). Additionally, RyanR and $InsP_3R$ form high conductance nonselective cation-permeable channels (Tinker and Williams 1992; Bezprozvanny and Ehrlich 1994). Direct modulation of RyanR and $InsP_3R$ by phosphorylation was investigated in bilayers (Hain et al. 1994; Tang et al. 2003b). Modulation of $InsP_3R1$ gating by intraluminal Ca^{2+} levels (Bezprozvanny and Ehrlich 1994) and modulation of RyanR1 by cytosolic and luminal pH (Laver et al. 2000) was studied in BLM. The phenomenon of "adaptation" of RyanR to rapid changes in cytosolic Ca^{2+} levels was discovered in BLM experiments (Gyorke and Fill 1993; Valdivia et al. 1995). The laboratories involved in these studies used a number of variations on the procedures used to obtain BLM recordings of native $InsP_3Rs$ and RyanRs, but the general outline of these procedures has remained the same since pioneering work by Smith et al. (1988). In the associated protocols, I provide an outline of these basic protocols as used in our studies of cerebellar $InsP_3R$ function together with Dr. Barbara Ehrlich at the University of Connecticut Medical Center (Bezprozvanny et al. 1991; Bezprozvanny and Ehrlich 1993, 1994) and later in my own laboratory in UT Southwestern Medical Center (Lupu et al. 1998; Tang et al. 2003b). See Preparation of Microsomes to Study Ca^{2+} Channels (Bezprozvanny 2013a) and Reconstitution of Endoplasmic Reticulum $InsP_3$ Receptors into Black Lipid Membranes (Bezprozvanny 2013b).

Cloning of the $InsP_3R$ and RyanR genes created an opportunity for structure–function analysis of these channels. Once again, the BLM reconstitution technique was very useful for these studies. Wild-type and mutant RyanRs were expressed in mammalian cell lines, purified, and reconstituted in BLM (Chen et al. 1993, 1997). A similar approach was also initially taken with $InsP_3R$ structure–function studies (Kaznacheyeva et al. 1998; Ramos-Franco et al. 1998), but expression of wild-type and mutant $InsP_3R$ in Sf9 cells by baculoviral infection provided a more abundant source of recombinant $InsP_3R$ for BLM studies. Using this approach, my laboratory compared the functional properties of three mammalian $InsP_3R$ isoforms (Tu et al. 2005b), described channel properties of *Drosophila* $InsP_3R$ (Srikanth et al. 2004), and mapped structural determinants responsible for $InsP_3R$ modulation by Ca^{2+} (Tu et al. 2003; Tu et al. 2005a). The procedures used by our laboratory at UT Southwestern Medical Center in these studies are described in the accompanying protocols. See Preparation of Microsomes to Study Ca^{2+} Channels (Bezprozvanny 2013a) and Reconstitution of Endoplasmic Reticulum $InsP_3$ Receptors into Black Lipid Membranes (Bezprozvanny 2013b).

In addition to studies of the basic functional properties of RyanR and $InsP_3R$, the BLM reconstitution technique was also useful for studies of the pathophysiology of these channels. This application of the BLM technique has become particularly useful in recent years, as more disease-relevant molecular data have become available for both $InsP_3R$ and RyanR. Functional effects of a number of malignant hyperthermia (MH) mutations in RyanR1 and effects of the volatile anesthetic halothane on the mutant RyanR1 have been characterized in BLM (Jiang et al. 2008). BLM recordings were used to characterize the phenotype of point mutations in RyanR1 linked with muscle weakness and central core disease (CCD) (Ghassemi et al. 2009; Loy et al. 2011) and point mutations in RyanR2 linked to ventricular arrhythmia and sudden death (Jiang et al. 2007; Jones et al. 2008).

These results led to the hypothesis that dysfunction of the store-overload-induced Ca^{2+} release (SOICR) mechanism plays a key role in cardiac arrhythmia (Priori and Chen 2011). BLM recordings have been used to investigate changes in RyanR2 functional states in the model of exercise-induced sudden cardiac death and during heart failure (Marx et al. 2000; Wehrens et al. 2003). In addition, the BLM technique was used extensively to study pathogenic interactions of neuronal $InsP_3R1$ with mutant Huntingtin, ataxin-2, and ataxin-3 proteins (Tang et al. 2003a; Chen et al. 2008; Liu et al. 2009), and the results obtained form the basis for the hypothesis that abnormal Ca^{2+} signaling plays a role in polyglutamine expansion neurodegenerative disorders (Bezprozvanny 2009, 2011). Thus, BLM studies of RyanR and $InsP_3R$ have provided key mechanistic insights about mechanisms of disorders affecting skeletal muscle, the heart, and the brain.

OTHER USES FOR BLM METHODS

The BLM techniques developed for studies of RyanR and $InsP_3R$ can be easily adapted to studies of other Ca^{2+}-permeable channels. For example, BLM methods have been used to show that Aβ42 oligomers forms Ca^{2+}-permeable channels in membranes (Arispe et al. 1993). These findings form the basis for the hypothesis that the ion channel forming activity of Aβ42 oligomers may be responsible for amyloid toxicity in Alzheimer's disease (AD) (Pollard et al. 1995). BLM recordings with recombinant presenilins were used to show their ability to support ER Ca^{2+} leak and to show that most familial AD mutations in presenilins disrupt their leak function (Tu et al. 2006; Nelson et al. 2007). Obtained results provided strong support to the hypothesis that aberrant Ca^{2+} signaling plays a role in AD (Bezprozvanny and Mattson 2008; Bezprozvanny 2009; Supnet and Bezprozvanny 2011). BLM recordings were used to confirm the recent discovery that the TPC2 ion channel functions as a NAADP-gated lysosomal Ca^{2+} channel and to study regulation of this channel by lysosomal Ca^{2+} and pH (Pitt et al. 2010).

In summary, BLM reconstitution of Ca^{2+} channels continues to provide an opportunity to gather unique mechanistic information highly relevant for the basic biology of these channels and for better understanding of the pathogenesis of diseases implicating these channels.

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