Topic Introduction

Patch-Clamp Electrophysiology of Intracellular Ca^{2+} Channels

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The modulation of cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is a universal intracellular signaling pathway that regulates numerous cellular physiological processes. Ubiquitous intracellular Ca^{2+} -release channels localized to the endoplasmic/sarcoplasmic reticulum—inositol 1,4,5-trisphosphate receptor (InsP_3R) and ryanodine receptor (RyR) channels—play a central role in $[\text{Ca}^{2+}]_i$ signaling in all animal cells. Despite their intracellular localization, electrophysiological studies of the single-channel permeation and gating properties of these Ca^{2+} -release channels using the powerful patch-clamp approach have been possible by application of this technique to isolated nuclei because the channels are present in membranes of the nuclear envelope. Here we provide a concise description of how nuclear patch-clamp experiments have been used to study single-channel properties of different InsP_3R channels in the outer nuclear membrane. We compare this with other methods for studying intracellular Ca^{2+} release. We also briefly describe application of the technique to InsP_3R channels in the inner nuclear membrane and to channels in the outer nuclear membrane of HEK293 cells expressing recombinant RyR.

INTRODUCTION

Modulation of cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in response to external cues regulates numerous cellular physiological processes, including apoptosis, gene expression, secretion, immune responses, reproductive fertilization, muscle contraction, synaptic transmission, and learning and memory. The inositol 1,4,5-trisphosphate (InsP_3) receptor (InsP_3R), which is a Ca^{2+} -release channel (Foskett et al. 2007) localized mainly at the endoplasmic reticulum (ER) in all animal cell types (Taylor et al. 1999), plays a central role in this ubiquitous intracellular signaling pathway. Cytoplasmic InsP_3 generated in response to various extracellular stimuli activates the InsP_3R channel to release Ca^{2+} sequestered in the ER lumen into the cytoplasm, thus generating and modulating the intracellular $[\text{Ca}^{2+}]_i$ signals (Berridge 1993). InsP_3R -mediated Ca^{2+} release is intricately regulated by binding of ligands (Ca^{2+} and InsP_3) and ATP, interaction with proteins (e.g., CaBP1 , CIB1 , and presenilins), phosphorylation and redox modifications, clustering, and differential localization (Joseph 1996; MacKrell 1999; Patel et al. 1999; Jøhennig and Ehrlich 2002; Foskett et al. 2007; Betzenhauser et al. 2008; Kang et al. 2008; Wagner et al. 2008; Li et al. 2009; Rahman et al. 2009). The complex control of InsP_3R channel activity contributes significantly to the cell's ability to integrate stimuli from various sources by generating diverse Ca^{2+} signals (Bootman et al. 2002) with distinct forms (Thorn et al. 1993), frequencies (Woods et al. 1986), magnitudes (Tregear et al. 1991), and spatial localization (Thorn 1996).

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DIFFERENT METHODS FOR STUDYING SINGLE InsP_3R CHANNEL ACTIVITY

Because of its intracellular localization, studies of InsP_3R channel activity have been restricted for many years to measurements of Ca^{2+} fluxes from permeabilized cells or membrane vesicles, monitoring $[\text{Ca}^{2+}]_i$ or ER luminal free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{ER}}$) in intact or permeabilized cells by confocal microfluorimetry using Ca^{2+} -sensitive dyes, or recording electrical currents passing through InsP_3R channels reconstituted into lipid bilayers. As an alternate experimental approach to investigate the activity of single InsP_3R channels, we (Mak and Foskett 1994) and another group (Stehno-Bittel et al. 1995a) independently applied patch-clamp electrophysiology of isolated nuclei to study single InsP_3R channels, based on the observation that the ER is continuous with the outer membrane of the nuclear envelope (Dingwall and Laskey 1992).

Ca^{2+} flux measurements and Ca^{2+} microfluorimetry allow observation of the activities of a population of InsP_3Rs , whereas electrophysiological approaches (planar lipid bilayer and patch-clamp experiments) allow monitoring of the activity of single (or a few) InsP_3R channel(s), providing high-resolution kinetic information about channel gating behaviors. Recent technical advances in fluorescence microscopy have made it possible to image Ca^{2+} release by individual InsP_3R channels (Smith and Parker 2009). Such “optical patch-clamping” (Demuro and Parker 2005) allows monitoring of Ca^{2+} release by many individual InsP_3R channels simultaneously in intact cells (Parker and Smith 2010) but, to date, electrophysiological patch-clamping of InsP_3R channel gating still provides substantially higher temporal resolution (Hamill et al. 1981) and signal-to-noise ratio (Foskett et al. 2007).

The environment of the InsP_3R cannot be rigorously controlled during Ca^{2+} flux and microfluorimetry experiments because access to the cytoplasm is restricted. This makes it difficult to isolate the intrinsic behaviors of the InsP_3R channel from the effects of other variables such as intracellular Ca^{2+} buffering capacities. These approaches can only infer channel activity from changes in $[\text{Ca}^{2+}]_i$ or $[\text{Ca}^{2+}]_{\text{ER}}$, therefore they cannot rigorously control both $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_i$ simultaneously during experiments. In contrast, the nuclear patch-clamp approach provides relatively easy access to both the cytoplasmic and luminal sides of the InsP_3R so that ionic and ligand conditions can be rigorously controlled and precisely known throughout an experiment. This allows the InsP_3R to be studied under constant physiological ligand conditions to determine its steady-state gating kinetics (Mak and Foskett 1997; Mak et al. 2000; Boehning et al. 2001; Ionescu et al. 2006, 2007; Cheung et al. 2010; Vais et al. 2011; Wagner and Yule 2012) and ligand dependencies (Mak et al. 1998, 1999, 2001a,b,c, 2003a,b; Ionescu et al. 2006; Wagner and Yule 2012). This also allows the conductance properties of the InsP_3R channel and its relative permeabilities for various ions to be readily determined using symmetric and asymmetric ionic compositions on the two sides of the channel (Mak and Foskett 1998; Mak et al. 2000; Vais et al. 2010a). Combining different configurations of nuclear patch-clamp electrophysiology (described below) with rapid perfusion solution exchange techniques (Franke et al. 1987; Hinkle et al. 2003), the solution on one side (cytoplasmic or luminal) of the observed InsP_3R channels can be rapidly (with msec resolution), reliably, and repeatedly changed. This allows the *same* individual InsP_3R channel(s) to be monitored in the same patch-clamp experiment under different ionic and ligand conditions to provide better comparison of the gating and conductance properties of the InsP_3R channel in different environments (Vais et al. 2010a,b). This also allows the kinetics of the InsP_3R channel responding to rapid changes in cytoplasmic ligand conditions to be investigated (Mak et al. 2007).

Other electrophysiological approaches have also been used to study single-channel properties and regulation of InsP_3R . Of these, reconstituting purified or enriched InsP_3R into an artificial planar lipid bilayer formed over an aperture in the partitioning wall of an experimental chamber (Montal and Mueller 1972) has been most commonly employed. This method allows the solutions on both sides of the channel to be changed in the same experiments. A variation of this method is the reconstitution of the InsP_3R into a planar lipid bilayer formed over the tip of a micropipette (Thrower et al. 1996; 1998) by the “tip-dip” method (Coronado and Latorre 1983). Single InsP_3R channel gating has also been successfully observed by applying the traditional patch-clamp approach to giant liposomes containing

purified InsP_3R molecules (Dargan et al. 2002). However, these approaches disrupt the in situ membrane environment of the channel. Some direct (Hagar et al. 1998; Mak et al. 2001c; Swatton and Taylor 2002) and indirect (Picard et al. 2000; Cannon et al. 2003; Nagata et al. 2007) evidence suggests that this may affect normal gating of the channel (Taylor and Laude 2002; Cannon et al. 2003; Nagata et al. 2007). Furthermore, nuclear patch-clamping eliminates cumbersome procedures required to extract InsP_3R and enables rapid, reversible, and reliable exchange of solutions on one side of the InsP_3R channel.

Recently, the discovery that, in some cell types, InsP_3R channels are localized to the plasma membrane at very low densities (<5 per cell) has made it possible to perform single-channel studies on these channels by whole-cell patch-clamping (Dellis et al. 2006, 2008; Betzenhauser et al. 2008, 2009a; Schug et al. 2008; Wagner et al. 2008; Gin et al. 2009). However, the channels studied were in the plasma membrane, with protein and lipid environments different from those in the ER. Even in whole-cell and nuclear patch-clamp experiments performed by the same laboratory studying the same recombinant InsP_3R channels expressed in the same mutant DT40 cell line with no endogenous InsP_3R channel expression, there were significant differences in channel conductance properties and Ca^{2+} dependence observed. InsP_3R channels in the plasma membrane have substantially lower conductance (~ 200 pS) (Betzenhauser et al. 2008; Wagner et al. 2008) compared with those in the nuclear membrane (~ 380 pS) (Wagner and Yule 2012) under the same ionic conditions. The channels in the plasma membrane are also significantly more sensitive to Ca^{2+} inhibition (Gin et al. 2009) compared to those in the nuclear membrane (Wagner and Yule 2012) in comparable concentrations of InsP_3 and ATP. Furthermore, the whole-cell patch-clamp configuration precludes rapid and reliable exchange of solutions on the cytoplasmic side of the channels observed.

Besides being the only experimental approach to observe with high temporal resolution the single-channel gating behaviors of InsP_3R channels in their native membrane milieu, nuclear patch-clamping can be applied to study both endogenous and recombinant InsP_3R channels using nuclei isolated from a wide variety of cell types (Fig. 1). Culture cells used for nuclear patch-clamp experiments include: insect *Spodoptera frugiperda* Sf9 cells (Fig. 1A) (Ionescu et al. 2006), *Drosophila melanogaster* S2 cells, chicken lymphocyte DT40 cells (wild-type and mutant cells that express only recombinant InsP_3R ; Fig. 1B) (Li et al. 2007; Betzenhauser et al. 2009b; Rossi et al. 2009), rat neuronal PC12 cells (Fig. 1C), mouse fibroblast MEF cells (Cheung et al. 2010), neuronal N2a cells (Kopil et al. 2011), monkey fibroblast COS-7 cells (wild-type cells transfected to express recombinant InsP_3R) (Boehning et al. 2001), human neuronal SH-SY5Y cells, kidney HEK293 cells, and B lymphoblasts (Cheung et al. 2010). Nuclear patch-clamp experiments have also been performed using primary tissues including

FIGURE 1. Typical single-channel current traces of various InsP_3R channels recorded in on-nucleus patch-clamp experiments: (A) endogenous insect InsP_3R from cultured Sf9 cells with $[\text{InsP}_3] = 10 \mu\text{M}$, $[\text{Ca}^{2+}]_i = 1 \mu\text{M}$, $V_{\text{app}} = +20$ mV; (B) recombinant rat type 3 InsP_3R expressed in cultured DT40-KO cells with $[\text{InsP}_3] = 3 \mu\text{M}$, $[\text{Ca}^{2+}]_i = 2 \mu\text{M}$, $V_{\text{app}} = -40$ mV; (C) endogenous type 1 InsP_3R from cultured rat neuronal PC12 cells with $[\text{InsP}_3] = 10 \mu\text{M}$, $[\text{Ca}^{2+}]_i = 2 \mu\text{M}$, $V_{\text{app}} = -30$ mV; and (D) endogenous type 1 InsP_3R from primary mouse embryonic cortical neurons with $[\text{InsP}_3] = 10 \mu\text{M}$, $[\text{Ca}^{2+}]_i = 1 \mu\text{M}$, $V_{\text{app}} = +40$ mV. All pipette solutions contained 0.5 mM Na_2ATP . All bath solutions had free Ca^{2+} concentration $[\text{Ca}^{2+}]_f = 70 \text{ nM}$. All traces were recorded at room temperature, filtered at 1 kHz , and digitized at 5 kHz . Arrows indicate the closed channel current levels. Each trace is 3 sec long.

Xenopus laevis oocytes (Mak and Foskett 1994; Mak et al. 2000) and mouse embryonic cortical neurons (Cheung et al. 2010) (Fig. 1D). In fact, we have been able to perform nuclear patch-clamp experiments on nuclei from all the cell types we have tried so far.

Performing patch-clamp experiments on isolated nuclei is not significantly different from or substantially more difficult than performing regular patch-clamp experiments on the plasma membrane. The experiments can be performed with a regular patch-clamp setup using an inverted microscope with good optics, without any extra equipment or special modifications. Since isolated nuclei are generally smaller than cells, it is preferable to have higher magnification objectives. A 20× objective is sufficient for many nuclei, but a 60× one is necessary for patching nuclei from smaller cells like DT40 cells. In our experience, it has been less challenging to obtain giga-ohm seals (using a pipette with resistance ~10–25 MΩ in 140 mM KCl solution) when patching isolated nuclei than patching plasma membranes of most cells. It is less critical to avoid contaminating the tip of the patch-clamp pipette before it comes into contact with the nuclear membrane. The seals form more readily, with stable seal resistances (~1–10 GΩ) similar to those for plasma membrane patching.

Relative to planar lipid bilayer experiments, samples used for nuclear patch-clamping experiments (isolated nuclei) can be readily made using a simple protocol with little specialized equipment beyond a homogenizer (see **Isolating Nuclei from Cultured Cells for Patch-Clamp Electrophysiology of Intracellular Ca²⁺ Channels** [Mak et al. 2013a]). Samples can be made in <30 min on the same day of the experiment and therefore there is no need to store samples over long periods.

We have found relatively few types of ion channels localized to the nuclear membrane when the bath and recording pipettes contain primarily KCl solutions, and all of them have single-channel conductance values <100 pS in physiological 140 mM KCl solution, substantially lower than those observed for InsP₃R channels (320–550 pS) (Foskett et al. 2007; Vais et al. 2010a). Thus, even in the small fraction of nuclear patch-clamp current records in which non-InsP₃R channels are observed, the activities of those channels can be easily isolated from those of InsP₃R channels. Thus, no reagents are required to inhibit activities of non-InsP₃R channels, and solutions with physiological ionic compositions can be used on both sides of the isolated nuclear membrane in nuclear patch-clamp experiments to study the InsP₃R.

Different membrane patch configurations can be achieved in nuclear patch-clamping for various experimental purposes, as in regular patch-clamping of the plasma membrane. When the patch micropipette first comes into contact with the isolated nucleus and a giga-ohm seal is formed between the pipette and the outer nuclear membrane, the on-nucleus configuration (analogous to the on-cell configuration in plasma membrane patching [Hamill et al. 1981]) is achieved (Fig. 2A). With experience, a >90% success rate of obtaining the on-nucleus configuration can be achieved for nuclei of most cell types we have tried.

Nuclei can be isolated with the outer nuclear membrane intact (Nicotera et al. 1989, 1990; Gerasimenko et al. 1995; Stehno-Bittel et al. 1995b) so that the protein environment in the perinuclear space on the luminal side of the InsP₃R channel (Fig. 2A) is preserved in this configuration. We have not detected any membrane potential difference across the outer nuclear membrane after achieving the on-nucleus configuration. This is not surprising since our regular bath solutions contain no MgATP (see **Nuclear Patch-Clamp Electrophysiology of Ca²⁺ Channels** [Mak et al. 2013b]) so any ion pumps in the outer nuclear membrane are inactive. Various ionic leak(s) across the ER membrane (Camello et al. 2002; Le Gall et al. 2004; Lizak et al. 2008) should be able to equilibrate the ionic concentrations across the outer nuclear membrane after the nuclei are placed in the experimental bath solutions. This allows the ionic compositions on both sides of the isolated nuclear membrane patch to be precisely known.

PATCH-CLAMP STUDY OF SINGLE InsP₃R CHANNELS IN THE OUTER NUCLEAR MEMBRANE

FIGURE 2. Schematic diagram illustrating the orientation of InsP_3R channels in isolated nuclear membrane patches relative to the micropipette in various configurations of nuclear patch-clamping. (A) On-nucleus configuration with outer nuclear membrane intact, (B) excised luminal-side-out configuration, (C) excised cytoplasmic-side-out configuration, (D) on-nucleus configuration with outer nuclear membrane removed, (E) excised nucleoplasmic-side-out configuration, and (F) diagram showing how the two aspects of the InsP_3R channel are represented in this figure.

In this configuration, the cytoplasmic side of the isolated membrane patch and of any InsP_3R channel in that patch faces the pipette solution (Fig. 2A). With stimulatory $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]_i$ in the pipette solution, currents through active InsP_3R channels can be recorded (Fig. 3A) (Mak and Foskett 1994; Mak et al. 1998, 2000; Boehning et al. 2001; Ionescu et al. 2006). The probability of observing InsP_3R channel activity in an isolated nuclear membrane patch (P_d) and the number of channels observed in a patch (N_A) vary greatly with different cell types, the number of times the cultured cells have been passed, the time that has passed since the last cell passage and since nucleus isolation, and the experimental ligand concentrations ($[\text{Ca}^{2+}]_i$ and $[\text{InsP}_3]$) (Ionescu et al. 2006; Vais et al. 2010b).

To gain better access to the luminal side of the outer nuclear membrane patch, the patch can be excised (see **Nuclear Patch-Clamp Electrophysiology of Ca^{2+} Channels** [Mak et al. 2013b]) to achieve the luminal-side-out (lum-out) configuration (Fig. 2B). This is analogous to the inside-out configuration in regular patch-clamping (Hamill et al. 1981). This configuration allows the rapid and repeated changing of solution on the luminal side of the isolated membrane so that conductance, permeation, and gating characteristics of the *same* InsP_3R channels under different luminal ionic conditions can be compared (Fig. 3B) (Vais et al. 2010a,b). The success rate of excising an isolated nuclear membrane patch into the lum-out configuration is 40%–80%, varying mainly with the cell type used.

With a protocol similar to that used in regular patch-clamping (Hamill et al. 1981), the cytoplasmic-side-out (cyto-out) configuration (Fig. 2C) can be achieved by breaking through to the

FIGURE 3. Typical single-channel traces of endogenous insect InsP_3R channels from cultured Sf9 cells recorded in nuclear patch-clamp experiments with different configurations. (A) On-nucleus configuration. Pipette solution: $[\text{Ca}^{2+}]_i = 2 \mu\text{M}$ and $[\text{InsP}_3] = 110 \text{ nM}$. Bath solution: $[\text{Ca}^{2+}]_{\text{ER}} = 70 \text{ nM}$. $V_{\text{app}} = +20 \text{ mV}$. (B) Excised lum-out configuration. Pipette solution: $[\text{Ca}^{2+}]_i = 50 \text{ nM}$ and $[\text{InsP}_3] = 10 \mu\text{M}$. Bath perfusion solution: $[\text{Ca}^{2+}]_{\text{ER}}$ switched between 100 nM and 250 μM , and $[\text{KCl}]$ between 70 and 100 mM, as indicated by color bars at top. Channel current magnitude decreased at the solution switch because the concentration of free Ca^{2+} , which is a permeant channel blocker (Mak and Foskett 1998; Vais et al. 2010a), in the perfusion solution was increased and the $[\text{K}^+]$ gradient across the channel was reduced. Baseline leak current level was reduced at the solution switch because the increase in $[\text{Ca}^{2+}]_i$ in the perfusion solution increased the giga-ohm seal resistance (Priel et al. 2007). $V_{\text{app}} = +20 \text{ mV}$. (C) Excised cyto-out configuration. Pipette solution: $[\text{Ca}^{2+}]_{\text{ER}} = 2 \mu\text{M}$. Bath perfusion solution: $[\text{Ca}^{2+}]_i = 2 \mu\text{M}$, $[\text{InsP}_3]$ switched between 0 and 10 μM and $[\text{KCl}]$ between 70 and 100 mM, as indicated by color bars at top. Channel current magnitude and baseline leak current level changed at the solution switches because of the change in $[\text{KCl}]$. $V_{\text{app}} = +20 \text{ mV}$. (D) Excised nucleo-out configuration. Pipette solution: $[\text{Ca}^{2+}]_{\text{ER}} = 1.2 \mu\text{M}$ and $[\text{InsP}_3] = 10 \mu\text{M}$. $V_{\text{app}} = -40 \text{ mV}$. Upper trace: perfusion solution with $[\text{Ca}^{2+}]_i = 1 \mu\text{M}$ and no InsP_3 . Lower trace: same experiment, but after perfusion solution was replaced with one containing 1 μM InsP_3 . All solutions had $[\text{KCl}] = 140 \text{ mM}$ unless stated otherwise. All solutions on the cytoplasmic side of the InsP_3R channel contained 0.5 mM Na_2ATP . All current traces are 4 sec long.

perinuclear region with a high voltage zap followed by slow excision (see **Nuclear Patch-Clamp Electrophysiology of Ca^{2+} Channels** [Mak et al. 2013b]). This is analogous to the outside-out configuration in regular patch-clamping of the plasma membrane (Hamill et al. 1981). In this configuration, the solution on the cytoplasmic side of the isolated nuclear membrane patch can be rapidly and repeatedly changed to study the response of the InsP_3R channel to changes in the ionic and ligand

compositions on its cytoplasmic side (Fig. 3C) (Mak et al. 2007). The success rate of obtaining this configuration (10%–50%) is substantially lower than that of getting the lum-out configuration, mainly because of difficulty in getting the membrane to reseal during excision after the breakthrough.

The cyto-out nuclear membrane patch isolated at the tip of the micropipette after the breakthrough-excision maneuver is not the same patch of membrane that was isolated in the on-nucleus configuration before the maneuver. Thus, any InsP_3R channels observed in the cyto-out configuration are not the ones that might have been observed when the on-nucleus configuration was first achieved (Fig. 2A,C). In fact, N_A observed in a cyto-out membrane patch is generally significantly higher than that observed in the on-nuclear membrane patch before the breakthrough-excision maneuver, possibly because of the difference in the area of membrane isolated (Vais et al. 2011).

Giga-ohm seals obtained in nuclear patch-clamp experiments in various configurations are generally very stable, lasting over tens of minutes. The main factors that consistently affect the stability of the nuclear patch-clamp seals are the polarity and magnitude of the applied voltage (V_{app}). Relative to giga-ohm seals obtained in regular patch-clamp experiments, nuclear patch-clamp seals are much less tolerant to high magnitude V_{app} of either polarity. Sustained V_{app} of >60 mV can weaken the seals substantially. On the other hand, brief (<1 sec) applications of high V_{app} (up to 100 mV) in a voltage ramp can be made repeatedly (Vais et al. 2010a). Depending on the cell type used, nuclear patch-clamp seals can be more stable with higher V_{app} on the cytoplasmic side (*Xenopus* oocytes and Sf9 cells) (Mak and Foskett 1997; Ionescu et al. 2006) or on the luminal side (DT40 cells) (Vais et al. 2010b). For the same cell type, the favorable polarity for better seal stability is consistent for all nuclear patch-clamp configurations.

The seals generally have high resistance (2–10 G Ω) so the baseline current noise is low. This, together with the large conductance of the InsP_3R channel, gives high signal-to-noise ratio in most current records. This allows the current to be recorded with high anti-aliasing filter frequency (5–10 kHz), providing good temporal resolution, even with low V_{app} (<60 mV). Most of the current records we have obtained were filtered at 1 kHz and digitized at 5 kHz because of the relatively slow gating kinetics of the InsP_3R observed under most ligand conditions (Mak and Foskett 1997; Mak et al. 1998; Ionescu et al. 2006).

In constant ligand conditions, abrupt, stochastic inactivation of InsP_3R channels has been widely observed in on-nucleus and excised lum-out configurations in many different cell types (Mak and Foskett 1997; Boehning et al. 2001; Ionescu et al. 2006). When this behavior is observed, a minor fraction ($<10\%$) of the inactivated channels can be reactivated by a sharp change in the magnitude or polarity of V_{app} . Nevertheless, all active InsP_3R channels inevitably inactivate irreversibly if the giga-ohm seal remains stable long enough (Mak and Foskett 1997). This behavior limits the activity duration (T_{act}) during which InsP_3R channel activity can be monitored. The mechanism(s) behind apparent inactivation remain poorly understood, and no effective means has been found to prevent or even slow down the inactivation. The mean T_{act} can vary significantly among cell types (~ 40 sec for oocyte InsP_3R [Mak and Foskett 1997], ~ 100 sec for Sf9 InsP_3R [Ionescu et al. 2006]) and show a dependence on $[\text{Ca}^{2+}]_i$ (Boehning et al. 2001; Ionescu et al. 2006). For recombinant InsP_3R expressed in DT40-KO cells with no endogenous InsP_3R expression, inactivation was observed in some laboratories (mean $T_{\text{act}} \sim 140$ sec) (Vais et al. 2011) but not in others (Rahman et al. 2009), even though very similar experimental protocols and conditions were used (Vais et al. 2011). Inactivation rates for endogenous InsP_3R in Sf9 cells and recombinant rat InsP_3R in DT40-KO cells were reduced significantly in the cyto-out configuration, with the mean T_{act} an order of magnitude or more longer (Mak et al. 2007; Vais et al. 2010b).

PATCH-CLAMP STUDY OF SINGLE InsP_3R CHANNELS IN THE INNER NUCLEAR MEMBRANE

Although the inner nuclear membrane is distinct from the ER membrane, integral proteins in the outer and inner nuclear membranes can intermix when the nuclear envelope disassembles and reassembles

FIGURE 4. Representative on-nucleus single channel recordings from nuclei isolated from HEK293 cells stably expressing mouse recombinant RyR2. Pipette solution contained 250 mM KCl, 25 mM HEPES (pH 7.3), and 0.5 mM free ATP with (A) no caffeine, (B) 2 mM caffeine, and (C) 2 mM caffeine and 10 μ M ruthenium red. Current traces recorded in symmetrical 250 mM K^+ at +40 mV. Arrows indicate zero current level. All traces are 4 sec long.

during cell division (Marshall and Wilson 1997). Evidence indicates that $InsP_3R$ channels are present in the inner nuclear membrane (Humbert et al. 1996; Adebajo et al. 2000; Huh and Yoo 2003; Yoo et al. 2005). Together with the machinery for $InsP_3$ metabolism (Divecha et al. 1991; Koppler et al. 1993), these $InsP_3R$ channels may play a key role in the generation and modulation of Ca^{2+} signals in the nucleoplasm (Sullivan et al. 1993; Echevarria et al. 2003; Gerasimenko and Gerasimenko 2004).

The outer nuclear membrane of an isolated nucleus can be removed by chemical treatments (Gurr et al. 1963; Gilchrist and Pierce 1993; Marchenko et al. 2005), rendering the inner membrane accessible for patch-clamping. This approach allows the nucleoplasmic-side-out (nucleo-out) configuration to be obtained, which, because it is topologically equivalent to the cyto-out configuration, exposes the cytoplasmic aspect of the $InsP_3R$ to the bath by simple excision of the isolated membrane, without the breakthrough step required to achieve the cyto-out configuration using the outer membrane (Fig. 2D, E). Application of the patch-clamp technique to such treated nuclei showed definitively that functional $InsP_3R$ channels are localized to the inner nuclear membrane (Marchenko et al. 2005). A requirement for $InsP_3$ in the bath solution to activate the channels in the nucleo-out configuration confirms that the inner membrane was indeed the one patched (Fig. 3D) (Marchenko et al. 2005).

PATCH-CLAMP STUDY OF SINGLE RyR CHANNELS IN THE OUTER NUCLEAR MEMBRANE

Another intracellular Ca^{2+} -release channel that plays a central role in the generation and regulation of intracellular Ca^{2+} signals is the ryanodine receptor (RyR) channel (Smith et al. 1988; Fill and Copello 2002). Until recently, electrophysiological studies of the conductance and permeability properties (Smith et al. 1988; Lindsay et al. 1991; Tinker et al. 1992; Tinker and Williams 1992, 1993, 1995; Tu et al. 1994; Fill and Copello 2002; Kettlun et al. 2003) and ligand regulation (Ashley and Williams 1990; Meissner 1994; Schiefer et al. 1995; Fill and Copello 2002; Fill and Ramos 2004; Laver 2010; Liu et al. 2010) of single wild-type and mutant (Zhao et al. 1999; Gao et al. 2000; Du et al. 2001; Chen et al. 2002; Wang et al. 2005; Xu et al. 2006) RyR channels have been performed exclusively by reconstituting the channels into artificial planar lipid bilayers. However, like the $InsP_3R$, the RyR is localized to the nuclear envelope as well as the ER or sarcoplasmic reticulum (Gerasimenko and Gerasimenko 2004; Marius et al. 2006). Thus, it should also be possible to apply nuclear patch-clamping to examine single-channel properties of RyR in its native membrane milieu. A preliminary study has recently been attempted (Cheung, KH, unpublished data) using HEK293 cells expressing recombinant RyR (Jiang et al. 2007). Channels activated by caffeine and inhibited by ruthenium red were detected (Fig. 4).

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