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Activation of ryanodine receptors in the nuclear envelope alters the conformation of the nuclear pore complex

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Abstract

Nuclear pore complexes (NPCs) are supramolecular protein pores that traverse the nuclear envelope and form the only known direct route of transport between the cytoplasmic and nuclear spaces. Detailed studies have identified both active and passive mechanisms of transport through the NPC and structural studies have revealed its three-dimensional architecture. Under certain conditions, structural studies have found evidence for a mass in the central pore of the NPC whose identity remains unclear. Some studies suggest this mass represents cargo caught in transit, while others suggest it is an integral component of the NPC, the position of which is sensitive to sample conditions. Regardless of its identity, previous studies have shown that the central mass location within the NPC pore is influenced by the presence of calcium in the cisternal spaces of the nuclear membrane. Specific depletion of these calcium stores through inositol 1,4,5-trisphosphate (IP_3) receptor activation leads to the apparent displacement of the central mass towards both the cytoplasmic and nucleoplasmic sides of the NPC. Whether the central mass is cargo or a NPC component, these observations may offer interesting insights linking transport and calcium signaling pathways. Here, we show that ryanodine (Ry) receptors are also present in the nuclear envelope of *Xenopus laevis* oocytes, and their specific activation can affect the conformational state of the NPC. Although previously undetected, Western blot analysis of isolated oocyte nuclei reveals the presence of Ry receptors in the nuclear envelope, albeit in low abundance. Extensive atomic force microscopy (AFM) studies at the single pore level of isolated, fixed nuclei reveal changes in the NPC conformational state following treatments that stimulate Ry receptor activity. At resting calcium levels (~200 nM Ca^{2+}), the central mass within the lumen of the NPC is recessed 5.3 nm below the cytoplasmic rim of the NPC. Following treatment with 10 nM ryanodine, the central mass displaces towards the cytoplasmic face occupying a new position only 2.9 nm below the cytoplasmic rim. Interestingly, at high ryanodine concentrations (20 μM), which are reported to deactivate Ry receptors, the central mass is observed to return to the recessed position, 5.4 nm below the cytoplasmic rim. Treatments with caffeine also lead to large changes in the NPC conformation, confirming the link to specific activation of Ry receptors. These observations are consistent with a new mechanism of NPC regulation in which specific activation of Ry receptors located in the nuclear envelope can modulate cisternal calcium levels, leading to changes in the NPC conformation. Together with previous studies, it now appears that both IP_3 and Ry receptors are present in the nuclear envelope of *Xenopus* oocytes and are capable, through activation, of indirectly influencing the conformational state of the NPC.

Keywords: Nuclear pore complex; Calcium; Ryanodine receptors; Atomic force microscopy; Central mass

1. Introduction

Calcium acts as one of the most important second messengers within the cell and regulates a myriad of processes from fertilization to apoptosis. All eukaryotes use calcium in some form to relay intracellular signals. The transient release of intracellular calcium is modulated through both agonist-gated channels and voltage-gated channels in excitable cells. The former includes inositol 1,4,5-trisphosphate (IP_3) and ryanodine (Ry) receptors, which can be found individually or together depending on the particular cell type.

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While the endoplasmic reticulum (ER) forms the largest calcium store in the cell, other intracellular compartments are also known to store and release calcium transiently through agonist-gated channels. One such store resides in the inner bilayer region of the nuclear envelope. The nuclear envelope is composed of a double bilayer structure, with the outer bilayer contiguous with the ER and the inner bilayer defining the nuclear compartment. The cisternal space between these two bilayers has been shown to store and release calcium through the activation of specific receptors located in the nuclear envelope [1].

Recently, it was shown that specific activation of IP_3 receptors in the nuclear envelope releases calcium stores and can lead to a conformational change in nuclear pore complexes (NPCs) [2–6]. NPCs are large ~125-MDa protein complexes, which form the only known channel traversing both bilayers of the nuclear envelope. They exhibit an eight-fold rotational symmetry in the plane of the nuclear envelope and are composed of cytoplasmic and nucleoplasmic rings connected by a spoke-like complex [7–14]. Filaments extend off both ring structures into the respective cellular compartments, with the nucleoplasmic filaments terminating in a basket-like structure that extends approximately 120 nm into the nucleoplasm [15,16].

High-resolution electron microscopy (EM) [7,8,16,17] and atomic force microscopy (AFM) measurements of nuclear envelopes from *Xenopus laevis* oocytes have found evidence of a mass in the center of the NPC, whose function and identity remains controversial. This mass is often referred to as the "central plug" [4,17], "central granule" [4,17,18] or "transporter" [7,8]. Some evidence suggests that this central mass is cargo partially caught in transit between the nucleus and cytoplasm, while others indicate that it may be an intrinsic part of the NPC structure.

While the identity and role of the central mass within the pore remains poorly understood, its location within the lumen of the pore has been shown to be sensitive to changes in cisternal calcium concentrations within the nuclear envelope. Treatments that specifically release or chelate nuclear envelope calcium stores lead to an outward displacement of the "central granule" toward the cytoplasmic face of the NPC [2,4,6]. While it has been suggested that these changes can modify NPC permeability, it has yet to be directly related to function. Alternatively, the conformational change could alter access to phenylalanine-glycine rich repeat (FG repeats) sites within the lumen of the pore, which have been shown to interact with transported cargo.

Given the demonstrated link between IP_3 receptor activation and NPC conformation, it is interesting to ask if any other calcium signaling pathways might also be involved in regulating NPC conformation. One obvious possibility would be through activation of Ry receptors [19–22]. Ry receptors are homotetrameric calcium channels found mainly in the sarcoplasmic reticulum (SR) and ER, but have also been observed in the nuclear envelopes of some species [23–27]. Ry receptors are activated by a number of agonists and exhibit a bell-shaped activation dependence with calcium levels, similar to that of IP_3 receptors [20]. Surprisingly, Ry receptors have not previously been detected in *X. laevis* oocytes [28,29] although their presence in oocytes from both lower and higher order species has been reported [30–34].

In this report, Western blot analyses reveal that indeed Ry receptors are located in the nuclear envelope of *X. laevis* oocytes, albeit in low numbers. Extensive AFM measurements on single NPCs of isolated nuclear envelopes reveal a displacement of the central mass towards the cytoplasmic face of the NPC following activation of Ry receptors with either ryanodine or caffeine. Together, these measurements indicate a ~3-nm displacement of the central mass towards the cytoplasmic side of the NPC, following activation of Ry receptors in the nuclear envelope. These results are discussed in terms of a new calcium-mediated pathway for regulating permeability or translocation through the NPC.

2. Materials and methods

2.1. Nuclear envelope preparation for AFM

Stage VI oocytes were surgically removed from adult female *X. laevis* (Xenopus Express, Plant City, FL) using a modified procedure outlined by Marcus-Sekura and Hitchcock (1987) with ethyl 3-aminobenzoate methanesulfonate salt (MS-222) (Sigma-Aldrich, St. Louis, MO) used as the anesthetic [35]. The oocytes were stored in Barth's solution containing: 10 mM HEPES (Sigma-Aldrich), 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ (Fisher Scientific, Pittsburgh, PA) and 200 units penicillin/streptomycin (Cellgro, Herndon, VA). The buffer was adjusted to a pH of 7.4 using concentrated NaOH. For all measurements reported here, oocytes were only used on the same day that they were surgically removed.

Nuclei were manually removed from the oocytes and placed in a modified mock intracellular buffer solution containing: 90 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1.1 mM EGTA (Sigma-Aldrich), 0.75 mM $CaCl_2$, 10 mM HEPES. This solution was adjusted to a pH of 7.3 with concentrated NaOH, which leads to a free calcium concentration of approximately 200 nM [6]. The nuclei were incubated in the modified mock intracellular buffer solution for 10 min before being transferred to a treatment solution of modified mock internal solution containing either ryanodine (Calbiochem, San Diego, CA) or caffeine (Sigma-Aldrich) in the appropriate concentrations. The nuclei were incubated in these solutions for 10 min prior to fixation.

The treated nuclei were transferred to 12-mm glass coverslips (Fisher Scientific) and immersed in 5% paraformaldehyde (Sigma-Aldrich) solution made from the modified mock intracellular buffer solution. The intact nuclei were allowed to incubate for approximately 15 min

before they were gently pulled apart with fire-polished glass micropipettes. The nucleoplasm was carefully removed and the membranes were flattened onto the coverslip and air-dried. The membranes were washed two times with distilled water and allowed to air dry.

2.2. Atomic force microscopy

AFM measurements were performed using a Digital Instruments Nanoscope IIIa (Santa Barbara, CA) controller and a multimode head operating in contact mode. For each membrane treatment, a series of 1.5×1.5 μm (512×512 pixel) areas were collected at a scan rate of 3.05 Hz. All imaging was performed using ultra-sharp silicon nitride AFM tips (MikroMasch USA, Portland, OR).

To quantify the central mass location within each NPC for the various treatments, individual pore depth measurements were conducted on each NPC in the AFM measurement. As described previously [2], each NPC was subjected to 24 line cuts separated at 15° intervals, which were then averaged together to provide a representative cross section. This method provides the best measure of central mass location, free from complications arising from the symmetry of the pore or the complicated morphology of the membrane. The central mass location was calculated by taking the average difference in height between the two outer rims of the NPC and the center of the central mass.

2.3. SDS-PAGE and Western blotting

Approximately 120–150 nuclei were manually removed from the oocytes and placed in a modified mock intracellular buffer containing: 100 μM leupeptin, 100 μM pepstatin A and 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The nuclei were then centrifuged briefly to form a small pellet and the supernant was removed. A lysis buffer consisting of 50 mM Tris–HCl, 1.0 mM EDTA, 10 μM leupeptin, 10 μM pepstatin, 100 μM PMSF, 1 mM β-mercaptoethanol, 3.0% Triton X-100 and 17 mM sodium dodecyl sulfate (SDS), was added to the nuclei pellet. Chicken skeletal microsomes (2.5 mg/ml) which were utilized for the positive control and the Ry receptors were detected using the monoclonal anti-avian skeletal muscle ryanodine receptor antibody 34C [36]. This antibody has been shown to recognize all three isoforms of the Ry receptors in many species [36–39]. The chicken skeletal microsomes and 34C antibody were both generously provided by the laboratory of John L. Sutko (University of Nevada, Reno). The nuclei and chicken skeletal microsomes were placed in a sample buffer containing: 0.06 M Tris–HCl (pH 6.8), 3.4 M glycerol, 5% β-mercaptoethanol, 0.07 M SDS, 0.19 mM bromophenol blue (Sigma-Aldrich) and 100 mM DL-dithiothreitol (DTT) (Sigma-Aldrich), at a 1:2 dilution for the nuclei sample and a 1:10 dilution of the chicken skeletal microsome sample. The nuclei sample was then placed in a boiling hot water bath for ~5 min and allowed to cool prior to running the SDS-PAGE.

Samples were run on a 4–20% Tris–HCl SDS-PAGE gradient gel (Bio-Rad, Hercules, CA) using a Mini-Trans Blot Cell (Bio-Rad). The proteins were transferred to a polyvinylidene fluoride (PVDF) (Bio-Rad) membrane at ~4 °C, in a recommended transfer buffer from the manufacturer which was modified slightly to only use 10% methanol. The proteins were transferred to the PVDF membrane using a voltage routine consisting of 100V for ~1 h followed by an overnight transfer at 30 V.

After transfer, the PVDF membrane was washed for 10 min in Tris-buffered saline (TBS) (Bio-Rad) and then blocked with 5% nonfat milk (Bio-Rad) for 1 h. The PVDF membrane was washed two times with Tween-20 Tris-buffered saline (TTBS) (Bio-Rad) and incubated in a solution of monoclonal antibody 34C (1:15 dilution) in TTBS for 2 h. The PVDF membrane was washed again with TTBS and incubated with goat anti-mouse IgG alkaline phosphate antibody (1:1000 dilution) (Bio-Rad) for 2 h. After washing, the PVDF membrane was developed using a colorimetric alkaline phosphatase solution (Bio-Rad) to detect the presence of ryanodine receptors.

3. Results

Western blot analysis of isolated nuclei revealed evidence for the presence of Ry receptors in the nuclear envelope. The presence of Ry receptors was detected using the monoclonal antibody 34C [36], which recognizes all three isoforms of the Ry receptor in many species. In Fig. 1A, recognition of the Ry receptors by the 34C antibody is

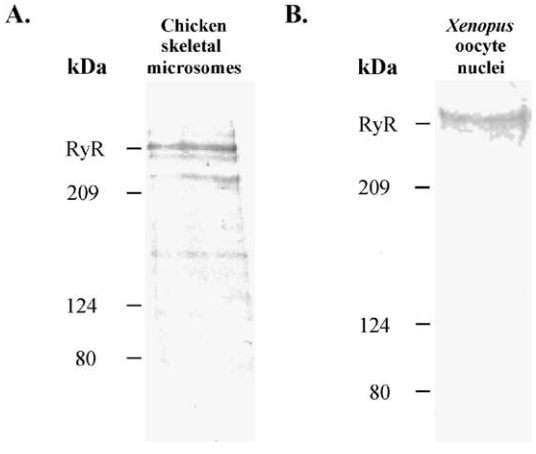

Fig. 1. SDS-PAGE and Western blotting of chicken skeletal microsomes (A) and nuclei removed from *Xenopus* oocytes (B). The presence of the Ry receptors was detected using the monoclonal anti-avain skeletal muscle ryanodine antibody 34C. This antibody detects all three isoforms of the Ry receptor in a number of species. The positive control lane is shown in A. The faint band observed in the nuclei lane in B indicates the presence of Ry receptors in the nuclei from *Xenopus* oocytes. Due to the long exposure times needed to detect the presence of the Ry receptors in the nuclear sample, separate runs are shown.

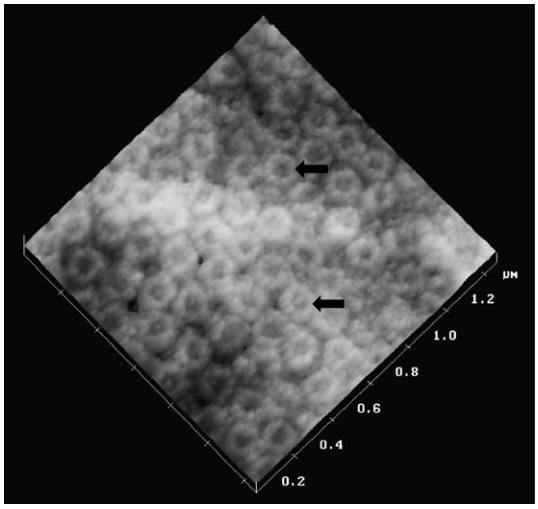

Fig. 2. Field of NPCs in the nuclear membrane measured using AFM. Visible in the topography image is the eight-fold symmetry of the pores and the channel in the center through which species pass. Arrows denote NPCs with the central mass recessed (upper arrow) and occluded (lower arrow) in which the central mass is displaced towards the outer rim.

observed in the chicken skeletal microsome sample, which is rich in Ry receptors. Fig. 1B displays the results from nuclei extracted from *Xenopus* oocytes, in which a faint band is observed, indicating the presence of Ry receptors. Separate runs are shown in Fig. 1 because the long exposure times needed to detect the presence of Ry receptors in the *Xenopus* nuclei sample, resulted in excess labeling of bands in the chicken skeletal microsome sample. In fact, the large number of nuclei and increased exposure times required to detect the faint Ry receptor band suggests that the receptors are present in low abundance. Non-specific binding was not observed in control samples of nuclei treated with secondary antibody only.

Having established the presence of Ry receptors within the nuclear envelope, extensive AFM measurements were carried out to determine if activation of Ry receptors influenced NPC conformation. Fig. 2 shows a typical AFM image of a nuclear envelope, which has been fixed and dried onto a glass coverslip. In agreement with previous SEM and AFM studies, the NPCs appear as toroidal structures approximately 120 nm in diameter. Clearly observable in the AFM image is the distribution of eight-fold rotational symmetry of the complexes and, in some pores, the presence of a central mass.

AFM images similar to that shown in Fig. 2 were analyzed to quantify the position of the central mass within the NPC following treatments that activate the Ry receptors. Because of the eight-fold NPC symmetry and complex topography of the membrane, single line cuts through the pores were insufficient to characterize the central mass location. Instead, a series of 24 line cuts through each individual pore were utilized as briefly discussed in the experimental section and elsewhere [2]. All depths reported here were taken from the cytoplasmic rim of the NPC to the center of the central mass, with negative numbers indicating that the central mass was recessed below the cytoplasmic rim of the NPC.

Fig. 3 plots the observed central mass location following various treatments of ryanodine to activate the Ry receptors in the nuclear membrane. At resting calcium concentrations (~200 nM Ca^{2+}) and no added ryanodine, the distribution of pores appear to have the central mass recessed ~5 nm below the cytoplasmic rim [2]. Upon treatment with 10 nM ryanodine, however, a large displacement of the central mass was observed. The distribution of pores now appears more occluded with an ~3 nm displacement of the central mass towards the cytoplasmic face of the NPC. Treatments with increasing concentrations of ryanodine revealed a complicated behavior that generally reduced the extent of the central mass displacement. Eventually, at the highest concentration studied, 20 μM ryanodine, the central mass was recessed and its location returned to a configuration that was similar to that observed under resting calcium level conditions. The measured distribution of pore depths, along with the standard error at each treatment, were: -5.3 ± 0.1 nm (n=429, 3 nuclei) at ~200 nM Ca^{2+}; -2.9 ± 0.1 nm (n=795, 3 nuclei) at 10 nM ryanodine; -4.5 ± 0.1 nm (n=599, 3 nuclei) at 100 nM ryanodine; -2.9 ± 0.1 nm (n=598, 3 nuclei) at 1.0 μM ryanodine; -4.4 ± 0.1 nm (n=760, 3 nuclei) at 10 μM ryanodine; and -5.4 ± 0.1 nm (n=793, 3 nuclei) at 20 μM ryanodine.

Activation of Ry receptors with caffeine was also studied. Caffeine is a potent agonist of Ry receptors and

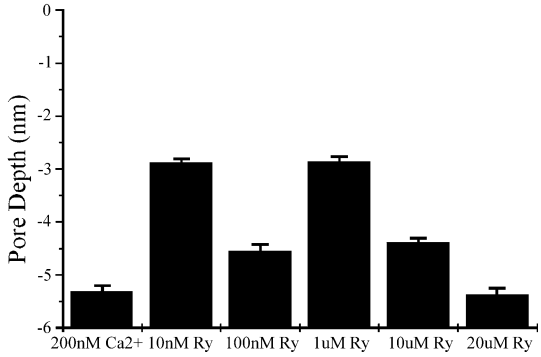

Fig. 3. Bar graphs of NPC pore depth measured with AFM following activation of Ry receptors with ryanodine. Zero is defined as the central mass position that is even with the cytoplasmic rim of the NPC. The negative numbers at each treatment, therefore, reflect a central mass position recessed from the cytoplasmic rim. The measured NPC pore depths, along with the standard error, at each treatment are: -5.3 ± 0.1 nm (n=429, 3 nuclei) at ~200 nM Ca^{2+}; -2.9 ± 0.1 nm (n=795, 3 nuclei) at 10 nM ryanodine; -4.5 ± 0.1 nm (n=599, 3 nuclei) at 100 nM ryanodine; -2.9 ± 0.1 nm (n=598, 3 nuclei) at 1.0 μM ryanodine; -4.4 ± 0.1 nm (n=760, 3 nuclei) at 10 μM ryanodine; and -5.4 ± 0.1 nm (n=793, 3 nuclei) at 20 μM ryanodine. At resting calcium conditions, the pores appeared to have the central mass maximally recessed in the lumen of the pore. At low concentrations of ryanodine, stimulation of the Ry receptors leads to a displacement of the central mass toward the cytoplasmic rim. This is reversed at high ryanodine concentrations where the Ry receptors are locked in a closed state and the NPC pores once again appeared to have the central mass recessed below the cytoplasmic rim.

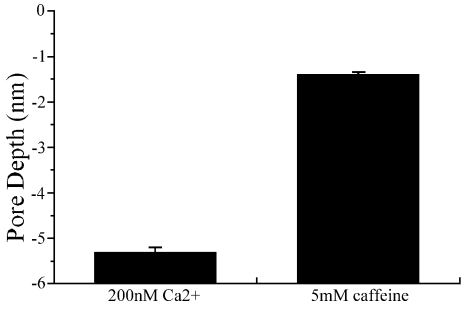

Fig. 4. Bar graphs of NPC pore depth measured with AFM following activation of the Ry receptors with caffeine. The measured NPC pore depths at each treatment are: -5.3 ± 0.1 nm (n=429, 3 nuclei) at ~200 nM Ca^{2+} and -1.4 ± 0.1 nm (n=581, 2 nuclei) following treatment with 5 mM caffeine. The large displacement of the central mass to a occluded configuration reflects the effective release of nuclear envelope calcium stores following caffeine stimulation of the Ry receptors.

Fig. 4 plots the measured NPC pore depths following treatments with 5 mM caffeine. At the 5 mM caffeine treatment, the central mass was maximally displaced towards the cytoplasmic rim with a measured pore depth of -1.4 ± 0.1 nm (n=581, 2 nuclei). As before, this displacement was compared to the ~200 nM Ca^{2+} treatment in which the pores appear to have the central mass recessed to a position of -5.3 ± 0.1 nm (n=429, 3 nuclei) below the cytoplasmic rim of the NPC.

4. Discussion

The detection of Ry receptors in *Xenopus* oocytes has, to date, proven elusive. Previous immunological and biochemical manipulations from *Xenopus* oocytes failed to detect their presence, while IP_3 receptors were found to be abundant [28,29]. The Western blot data shown in Fig. 1 clearly establishes the presence of Ry receptors within the nuclear envelope of *Xenopus* oocytes, although in very low abundance. Their low number is evidenced by the large number of nuclei needed and the prolonged developing times necessary for their observation in the Western blot analysis.

While IP_3 receptors are generally found in the nuclear envelopes of cells, expression of Ry receptors is more tissue dependent. Where they have been found, they are usually present in low abundance. For instance, they have been identified in the nuclear envelope from cardiac cells, but at a much lower abundance than that found in the sarcoplasmic reticulum [24]. For *Xenopus* oocytes, the presence of IP_3 receptors in the nuclear envelope is well established. With the data shown in Fig. 1, it now appears that both IP_3 and Ry receptors are present in the nuclear envelope of *Xenopus* oocytes. Having previously shown that activation of nuclear IP_3 receptors can release calcium from the nuclear envelope and that this specific activation leads to a conformation change in the NPC, it is interesting to understand the role, if any, that Ry receptor activation may play in these processes.

To characterize the state of the NPC following Ry receptor activation, AFM measurements were taken on the cytoplasmic side of nuclei to characterize the state of the NPC. Previously, we have carried out similar studies on nuclear envelopes following IP_3 receptor activation [2]. Fig. 2 shows a typical field of NPCs with the distribution of central masses in both the recessed and exposed conformations, as depicted by the arrows. To understand the role of Ry receptor activation, nuclei were exposed to various treatments of ryanodine, fixed and imaged with AFM to characterize the state of the central mass of the NPC. The images have been collected in contact mode, which provides for higher resolution than compared to tapping mode [6]. The samples have been fixed to be able to obtain the highest resolution possible. This allows for the monitoring of small nanometer sized changes to be observed, such as seen with the displacement of the central mass.

As shown in Fig. 3, for nuclei prepared at resting calcium concentrations (~200 nM Ca^{2+}), the central mass is recessed below the cytoplasmic rim approximately 5.3 nm. However, upon the addition of 10 nM ryanodine to the intracellular buffer, a large displacement of the central mass towards the cytoplasmic rim of the NPC was observed. Analysis of the AFM data indicate that on average the central mass was located 2.9 nm below the cytoplasmic rim in these preparations. This nearly 2.5-nm shift in central mass location within the NPC lumen clearly indicates that Ry receptor activation influences the NPC conformation through the release of cisternal calcium stores in the nuclear envelope.

Activation of Ry receptors is known to have complicated dose–response characteristics, which is reflected in the bar graph shown in Fig. 3. In general, ryanodine binds with high affinity and induces slow gating (frequency of opening) with a reduced ion conductance [20,40–42]. However, at low doses (~10 nM ryanodine) normal conductance levels are recovered along with faster gating times. Intermediate ryanodine concentrations (~1 μM ryanodine) lead to the slow gating with lower conductance levels mentioned above while high doses (~100 μM ryanodine) appear to lock the channel in the closed state. In Fig. 3, the central mass location within the NPC at various ryanodine doses, similarly followed a complicated response. At low doses (10 nM ryanodine), the NPCs appeared occluded with the central mass maximally displaced towards the cytoplasmic rim. At 100 nM ryanodine, the central mass was displaced back towards a more recessed configuration, while at 1 μM ryanodine, the central mass again appeared to be displaced toward the cytoplasmic rim. As the ryanodine doses were further increased, the central mass gradually displaced back towards a more recessed state.

At low ryanodine concentrations where the frequency of opening and conductance levels are high, Ry receptors are expected to efficiently release calcium from the cisternal spaces of the nuclear envelope. As previously seen, release of these calcium stores in the nuclear envelope results in a

conformational change in the NPC in which the central mass displaced towards the cytoplasmic rim of the pore, which is reflected in Fig. 3 [2–6]. At high ryanodine concentrations, where previous studies have shown that the Ry channels become locked in a closed state, efficient calcium release is not expected and the NPC central mass should be recessed. This too is reflected in the data tabulated in Fig. 3. The behavior observed in the mid-range of ryanodine concentrations is more complicated. In this concentration range, we find that the central mass displaces from an intermediate position at 100 nM ryanodine to an occluded position at 1 μM ryanodine before being displaced back to a recessed configuration gradually as the dose was increased.

While generally consistent with results observed in other studies on Ry receptor activation, the biphasic behavior in the mid-dose range was somewhat unexpected. However, it should be emphasized that details of Ry receptor activation are still being explored and the results reported here may be complicated by the fact that we are measuring an event secondary to their activation, namely the effect that their activation has on NPC conformation. Moreover, Ry receptors, like IP_3 receptors, are known to be influenced by the presence of free calcium in a calcium-induced calcium-release mechanism (CICR). CICR results in a bell-shaped activation curve as calcium concentrations increase, which may be influencing the results of the NPC conformation presented in Fig. 3.

Finally, to further confirm that specific activation of Ry receptors is responsible for the NPC conformational changes, the effects from caffeine stimulation were studied. Caffeine can stimulate Ry receptors in the presence or absence of ryanodine and enhances potentiation through the CICR mechanism [21]. Fig. 4 compares the NPC central mass location under conditions of resting calcium levels and that observed upon the addition of 5 mM caffeine. As before, under resting calcium conditions the central mass within the NPC was recessed 5.3 nm below the cytoplasmic rim. Upon caffeine stimulation, the pores appeared more occluded with a central mass location 1.4 nm below the pore rim. These results confirmed that specific activation of Ry receptors with caffeine leads to the central mass displacement in the NPC.

In previous studies, we found that stimulation of IP_3 receptors leads to a displacement of the central mass towards both the cytoplasmic and nucleoplasmic faces of the NPC [2]. The maximum amplitude of the displacement towards the cytoplasmic NPC rim was found to be ~3.4 nm compared to its position under resting calcium conditions [2]. Despite the lower abundance of Ry receptors, as evidenced through the Western blot analysis, we find similar displacements of the central mass following Ry receptor activation. Treatments with either ryanodine or caffeine lead to shifts in the central mass towards the cytoplasmic face of the NPC, with a maximum amplitude of ~3.9 nm compared to resting calcium conditions. These results suggest that both IP_3 and Ry receptors can release nuclear envelope calcium stores and affect changes in the NPC conformation. While a direct link with NPC permeability remains to be established, it now seems clear that Ry receptors are present in the nuclear envelope and their activation can influence the conformational state of the NPC.

5. Conclusions

Western blot analysis of large numbers of nuclei surgically removed from *Xenopus* oocytes revealed the presence of Ry receptors in the nuclear envelope, which have previously gone undetected. However, the large number of nuclei required and lengthy exposure times required for their observation suggests that they are present in low abundance. Stimulation of Ry receptors with either ryanodine or caffeine leads to a large displacement of the central mass in the NPC. At low ryanodine doses, the central mass displaced toward the cytoplasmic rim. This was consistent with previous studies, which have found evidence for an outward displacement of the central mass following depletion of the nuclear envelope calcium stores following IP_3 receptor activation. At high doses, where ryanodine locks the Ry receptors into a closed state, the central mass remains recessed, consistent with calcium remaining in the cisternal stores. The role of Ry receptors in these processes was further confirmed through their specific activation with caffeine, another potent agonist of Ry receptors. Treatments with 5 mM caffeine resulted in NPCs with the central mass displaced towards the cytoplasmic rim. The results presented here, when combined with earlier studies, suggest that activation of either Ry or IP_3 receptors in the nuclear envelope can release nuclear envelope calcium stores, leading to the outward displacement of the NPC central mass. These observations may offer interesting avenues through which NPC permeability is modified through secondary messenger activity. Further experiments to directly link the central mass location with permeability are currently underway.

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