Does the nuclear envelope contain two types of ligand-gated Ca^{2+} release channels?

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Abstract The nuclear envelope is composed of two membranes deliminating a perinuclear space which displays functional properties similar to those of a Ca^{2+}-storing compartment. ATP-driven Ca^{2+} uptake and $InsP_3$-induced Ca^{2+} release processes have been described in isolated nuclei. Recently, it was reported that cADP-ribose and $InsP_3$ can trigger a nucleoplasmic Ca^{2+} increase. It was hypothesized that the inner nuclear membrane possesses Ca^{2+} channels that are regulated by ryanodine or $InsP_3$. Radio-ligand binding assays and Western blot experiments were performed in order to investigate their presence in sheep cardiac and rat liver nuclear envelopes. Ryanodine receptors (RyR) were not detected in liver nuclear envelopes by either binding assay or Western blot analysis. However, cardiac nuclear envelopes were found to retain a very low level of specific ryanodine binding, which was not detected on immuno-blots obtained with three types of isoform-specific RyR antibodies. In contrast, nuclear $InsP_3$-binding sites were consistently detected in both cardiac and liver nuclear envelopes. Altogether, these results provide evidence for the major contributor $InsP_3$-gated Ca^{2+} channels in control of Ca^{2+} release from the perinuclear space in liver and cardiac cells.

Key words: Cell nucleus; Nuclear Ca^{2+} channel; Ca^{2+} signaling; Inositol 1,4,5-trisphosphate; Ryanodine

1. Introduction

The cell nucleus is delimited by the nuclear envelope (NE) that is made of two membranes (i.e. the outer nuclear membrane, ONM; and the inner nuclear membrane, INM). Both membranes are thought to fuse at the nuclear pore complex (NPC), which controls the nucleo-cytoplasmic exchanges of macromolecules [1]. This double membrane system is separated by a perinuclear space that is structurally and functionally related to the endoplasmic reticulum [2]. Some of the NE properties are similar to those of a Ca^{2+}-storing compartment. A Ca^{2+}-Mg^{2+}-ATPase is present in the ONM [2], and is responsible for Ca^{2+} uptake into the perinuclear space [3–5]. Ca^{2+}-binding proteins similar to calreticulin and calnexin have been located in the lumen of the perinuclear space [6,7]. Finally, ionic channels activated by inositol 1,4,5-trisphosphate ($InsP_3$) are present in the ONM [8,9]. It has been reported that agonists promoting an increase of intracellular Ca^{2+} also triggered an increase of nucleoplasmic Ca^{2+} [10,11]. It was initially proposed that intranuclear Ca^{2+} variations result from the diffusion of cytoplasmic Ca^{2+} through the NPC [10,11]. However, resting nucleo-cytoplasmic gradients of Ca^{2+} have already been described [12–15] and NPC is known to limit the propagation of Ca^{2+} waves through the nuclear envelope [16]. Malviya and co-workers have described the existence of $InsP_3$ receptor ($InsP_3R$) in the INM [17] and $InsP_3$ was shown to trigger an increase of nucleoplasmic Ca^{2+} [5,18]. Lately cyclic ADP-ribose (cADPR), a putative regulator of the ryanodine receptor (RyR), was also reported to increase the nucleoplasmic Ca^{2+} in isolated liver nuclei [5]. However, biochemical evidence concerning the presence and the functional role of this specific family of intracellular Ca^{2+} channels are scant. In the present work, we have systematically investigated the presence of both nuclear RyR and $InsP_3R$ in cardiac and liver cells, using [^{3}H]ryanodine and [^{3}H]$InsP_3$-binding assays, as well as extensive Western blot analysis. Several microsomal membrane fractions were used as positive and negative controls for binding studies, and to confirm the specificity of antibodies raised against the various RyR isoforms. Our results indicate that $InsP_3$ receptors are consistently present in cardiac and liver nuclear envelopes; although their density was lower than the density of $InsP_3$-binding sites measured in brain microsomal fractions. In contrast, our data show that RyR is clearly absent in the liver NE, while it is present in very low amount in the NE of cardiac cells, when compared to its density in cardiac SR membranes.

2. Materials and methods

2.1. Purification of nuclei and nuclear envelope

Purification of nuclei and nuclear envelopes were routinely performed on the same day. All procedural steps were done at 4°C. Sheep cardiac and rat liver nuclei were purified according to the procedure described by Howell and Lefebvre [19] and modified by Rousseau et al. [20]. In brief, fresh rat liver and sheep heart were homogenized in (25% w/v) ice cold buffer containing: 0.32 M sucrose, 3 mM $MgCl_2$, 2 mM DTT, 20 mM K-HEPES (pH 7.2) and 50 μM Pefabloc, 1 μM pepstatin, 1 μM leupeptin, 1 μM Na-ascorbate and 2.4 UI aprotinin with an Ultra-Turrax T25 at low speed with two 30-s bursts. The homogenate was filtered twice through two and four layers of cheesecloth, respectively, and then centrifuged at $3800\times g$ (20 min). The pellet was resuspended in 2.4 M sucrose, 1 mM $MgCl_2$, 2 mM DTT, 5 mM K-HEPES (pH 7.2) and centrifuged at $50\,000\times g$ (90 min). The resulting pellet was gently resuspended in 150 mM KCl, 20 mM $MgCl_2$, 5 mM K-HEPES (pH 7.2), and centrifuged at $500\times g$ (20 min). Purified nuclei were finally resuspended in the same medium or in 0.3 M sucrose 1 mM $MgCl_2$, 5 mM K-HEPES (pH 7.2) for storage at −85°C.

The NE were prepared from nuclei suspensions sonicated by two 30 seconds bursts (W-375 sonicator, Heat System Ultrasonics Inc.) separated by one minute at 4°C. Chromatin was digested with DNase (10 μg/ml) and RNase (5 μg/ml) during 30 min at 30°C and nuclear membranes were washed with 1.8 M NaCl in order to prevent histones self aggregation [7]. Nuclear membranes were sedimented by a

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final centrifugation (100 000×g, 45 min), resuspended in either 50 mM KCl, 5 mM K-HEPES (pH 7.2) or 0.3 M sucrose 1 mM $MgCl_2$, 5 mM K-HEPES (pH 7.2) and kept at −85°C. The protein concentration was determined according to Lowry et al. [21], using bovine serum albumin as standard.

2.2. Preparation of cardiac and skeletal SR, as well as brain and liver microsomal fractions

Membrane fractions enriched in sarcoplasmic reticulum (SR) derived from rabbit skeletal, canine diaphragm, and canine cardiac muscles were prepared according to Rousseau et al. [22], Picher et al. [23] and Decrouy et al. [24], respectively. Canine brain microsomes (BM) were prepared according to reference [25]. Freshly isolated brain from which cerebellum has been removed was homogenized with an Ultra-Turrax T25 at low speed with two 30 seconds bursts, in the same ice cold buffer as described for the nuclei. The homogenate was filtered twice through two and four layers of cheesecloth, respectively, and centrifuged at 3800×g (20 min). The resulting supernatant was centrifuged at 5000×g (10 min) and then at 100 000×g (45 min). The final pellet containing the brain microsomal fraction was resuspended in either 150 mM KCl, 5 mM K-HEPES pH 7.2 or 0.3 M sucrose, 1 mM $MgCl_2$, 5 mM K-HEPES (pH 7.2) and kept at −85°C until used. The same procedure was used to prepare rat liver microsomal fractions.

2.3. [^{3}H]Ryanodine and [^{3}H]InsP3-binding assays

Ryanodine-binding assays were performed on NE vesicles as described previously [22] with sheep cardiac SR as positive controls. Briefly, vesicles (500 μg of proteins) were incubated with [^{3}H]ryanodine (2619.6 GBq/mmol) for 90 min at 37°C in the presence of 40 μM free Ca^{2+} with or without an excess of non-radioactive ryanodine. The mixture was then centrifuged twice to remove non-specific binding and the pellet radioactivity was determined by liquid scintillation. Specific [^{3}H]ryanodine (expressed in fmol mg^{-1} protein) was calculated by subtracting non-specific binding (5 nM [^{3}H]ryanodine+5 μM ryanodine) from total binding (5 nM [^{3}H]ryanodine alone). [^{3}H]$InsP_3$ binding was performed as described previously [26]. Membrane vesicles (500 μg of proteins) were incubated 30 min at 4°C in Tris-HCl 25 mM (pH 8.5), KCl 100 mM, NaCl 20 mM, KH_2PO_4 5 mM, EDTA 1 mM and BSA 0.1% (w/v) in presence of 10 nM [^{3}H]$InsP_3$ (770 GBq/mmol) or 10 nM [^{3}H]$InsP_3$ plus 10 μM $InsP_3$. The membrane suspensions were centrifuged and the pellet radioactivity was determined by liquid scintillation. Specific [^{3}H]$InsP_3$ binding (expressed in fmol mg^{-1} protein) was calculated by subtracting non-specific from total counts.

2.4. SDS-polyacrylamide gel electrophoresis and Western blot analysis

Membrane samples were solubilized in 2% SDS and proteins were separated on 10% SDS-PAGE under reducing conditions (β-mercaptoethanol 5 mM). All gels were run in parallel with broad molecular weight standards provided by BioRad. Proteins were stained by gel incubation in ethanol 50% (v/v), acetic acid 10% (v/v) and Coomassie blue 0.1% (w/v). Western blot analysis of the different membrane fractions was performed as follows: the proteins were electrotransferred onto a PVDF-membrane (BioRad) overnight at 35 V (4°C). The membranes were then incubated in the presence of a monoclonal mouse antibody raised against Lamin B1 [27]. Nuclei and microsomal preparations were also probed for the presence of protein specific for the NPC, using a monoclonal antibody (M Ab414) which recognizes a protein of 62 kDa [28]. In order to investigate the presence of RyRs, various membrane fractions were electrophoresed on 6% SDS-PAGE and transferred to nitrocellulose membranes at 30 V overnight at 4°C. The nitrocellulose membranes were washed 15 min in 200 mM Tris, 1.4 M NaCl+0.1% Tween 20, pH 7.6 (TBS-T), blocked with 5% non-fat dry milk in TBS-T and incubated with antibodies against various RyR isoforms. Antibodies against RyR I and RyR III isoforms were generous gifts from Dr. G. Meissner and Dr. W. Chen, respectively. Revelations were performed by enhanced chemiluminescence (Boehringer-Mannheim) or horseradish peroxidase (BioRad) using protein A or specific IgG as a secondary antibody.

2.5. Preparation of anti-cardiac ryanodine receptor polyclonal antibodies

The cardiac ryanodine receptor complex (RyR_2) was solubilized and purified to homogeneity by sucrose gradients and heparin-Sepharose affinity chromatography [22]. The proteins were concentrated on AMICON prior to deposition on immobilon fragments. Male rabbits were immunized by intrasplenic insertion of nanogram quantities of the purified canine cardiac RyR_2 absorbed to small immobilon fragments according to [29] with some modifications. The rabbits were anesthetized with 1% halothane and the spleen was carefully exposed by a 20 mm cutaneous incision, followed by incision of the abdominal wall and the peritoneum. The splenic capsule was punctured with a needle and the micro-fragment of immobilon (2 mm^2) inserted under the capsule. Bleeding was stopped by application of a 3 mm^2 SURGICEL patch. After intrasplenic deposition of the antigen, the abdominal wall and the skin were sutured separately. Intrasplenic immunization was repeated twice within a month without the use of adjuvant. Cardiac anti-RyR_2 antibodies were detected in rabbit serum two weeks after the second intrasplenic deposition by a standard ELISA procedure. Then the immunological response was improved by four successive immunizations performed once-a-month. The first immunization was a subcutaneous implant, while the others were simultaneous subcutaneous (150 μl) and intramuscular (50 μl) injections. The presence of cardiac anti-RyR_2 antibodies in the serum was confirmed by immuno-blot assays on purified RyR_2 and cardiac heavy SR membranes.

3. Results

The first part of this work was aimed at the characterization of the nuclear envelope (NE) fractions. Thus, in Fig. 1A, the protein profiles of liver and cardiac NE (lanes 2 and 4) were compared to the profile of liver microsomal and cardiac SR fractions (lanes 1 and 3), respectively. Although these Coomassie blue stainings of membrane fractions are likely to reveal protein bands of similar relative molecular weight, the nuclear and microsomal protein profiles displayed major differences. It should be further noted that the protein profile derived from liver and cardiac NE also displayed major dissimilarities. Indeed, cardiac NE harboured bands in the 200, 97 and 45-kDa regions that were not detected in the liver NE. To further characterize the NE, a typical intranuclear membrane (INM) marker was used. Lamins A, B, C proteins are known to form a highly organized nucleoplasmic network

Table 1
[^{3}H]Ryanodine and [^{3}H]$InsP_3$ specific binding in nuclear envelopes and microsomal fractions

Membrane fractions	[^{3}H]Ryanodine (fmol/mg of protein)		[^{3}H]$InsP_3$ (fmol/mg of protein)	
Liver nuclear envelope	0.5 ± 1.9	(n = 12)	187 ± 6.7	(n = 6)
Cardiac nuclear envelope	21.9 ± 1.7	(n = 6)	33.5 ± 4.4	(n = 6)
Cardiac sarcoplasmic reticulum	1972 ± 36.8	(n = 6)	0.57 ± 0.38	(n = 3)
Brain microsomes	34[a]		214.5 ± 16.4	(n = 6)

The purification of the different membrane fractions and subsequent binding assays were performed as indicated in Section 2. The values given for [^{3}H]ryanodine and [^{3}H]$InsP_3$ binding are expressed in fmol mg^{-1} protein and represent the mean ± S.E.M. of (n) independent experiments.
[a]From Padua et al., 1994 [33].

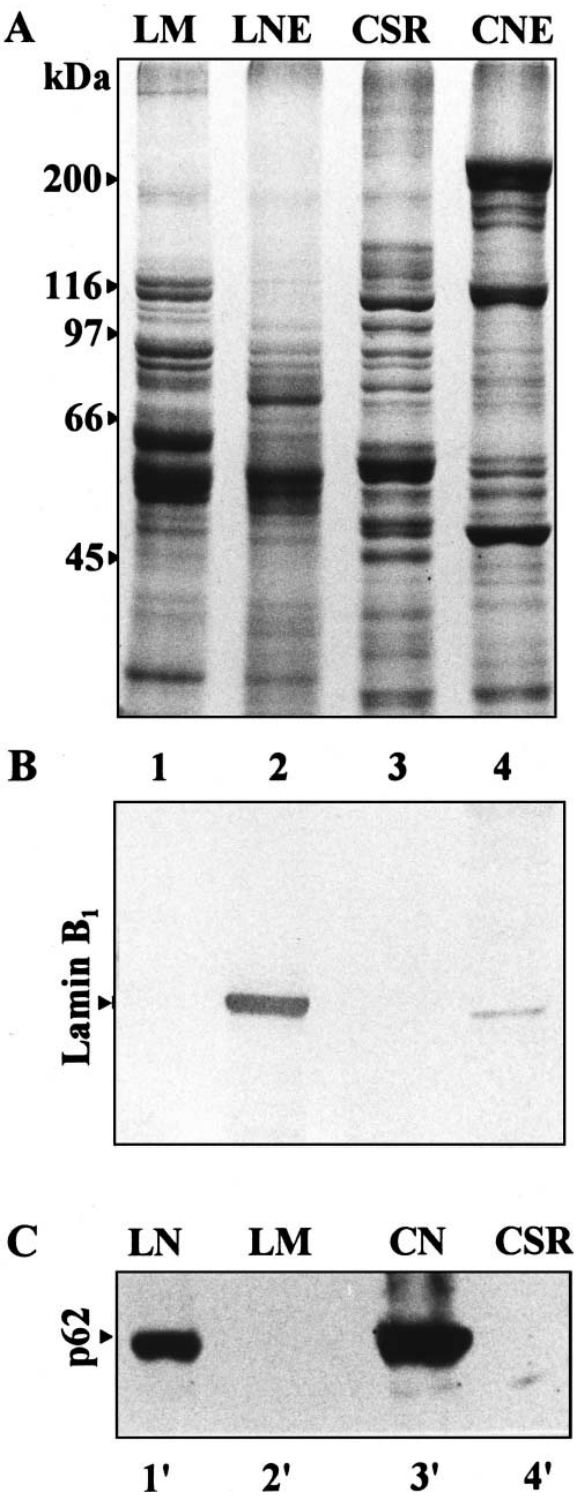

Fig. 1. Protein profile and immuno-detection of Lamin B1 in nuclear and microsomal membrane fractions derived from liver and cardiac cells. (A) Coomassie blue staining of the protein profile (20 μg of protein) from crude liver microsomes (lane 1), liver nuclear envelope (lane 2) cardiac heavy SR (lane 3) and cardiac nuclear envelope (lane 4) loaded on 10% SDS-PAGE. (B) Western blot analysis of the same membrane fractions, using a monoclonal anti-lamin B1 antibody as indicated in Section 2. (C) Immuno-detection of the 62 kDa protein (p62) of NPC using the monoclonal antibody MAb414: liver nuclei (lane 1′), crude liver microsomes (lane 2′) cardiac nuclei (lane 3′) and cardiac heavy SR (lane 4′). The p62 was absent from liver microsomal and cardiac SR fractions (lane 2′ and 4′, respectively). In contrast, liver and cardiac nuclei preparations were enriched in p62 (lanes 1′ and 3′), thus they were used to prepare the corresponding NE to perform subsequent binding and immunological assays.
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involved in nuclear structure and dynamics [30]. Lamins A and C are distributed across the whole nucleoplasm [31] while lamin B tightly interacts with a complex receptor that is located in the inner nuclear membrane [32]. Lamin B is considered to be a good marker of INM [20,27]. Western blot experiments using a monoclonal anti-lamin B1 antibody were performed in order to investigate the presence of lamin B in NE, microsomal and SR membrane fractions. The results are given in Fig. 1B. The antibody mainly recognized a 67-kDa protein in both liver and cardiac NE (lanes 2 and 4, respectively). This protein was neither detected in liver microsomal (lane 1), nor in cardiac SR (lane 3) fractions. We have consistently observed that the relative signal density generated by the anti-lamin B1 antibody was higher in liver than in cardiac NE, even though equivalent amounts of liver and cardiac proteins were loaded on the gel. This is likely to result from a higher lamin B1 expression in liver NE. Fig. 1C shows that the liver and cardiac nuclei fractions were both enriched in a 62-kDa protein known to be associated to the NPC [28] (lanes 1′ and 3′, respectively). In contrast, the liver microsomal and cardiac SR fractions were totally deprived of such protein band (lanes 2′ and 4′). Altogether, these results indicate that it is possible to prepare enriched NE fractions from rat liver or sheep cardiac nuclei. Moreover, these nuclear membrane fractions display the presence of a typical NE and INM markers p62 of the NPC and lamin B1, which were never detected in crude liver microsomal or in cardiac SR fractions.

3.1. Detection of nuclear ryanodine receptors

We have used experimental conditions that have been previously used for an efficient detection of specific [^{3}H]ryanodine-binding sites in skeletal, cardiac and atrial SR [22,24]. In the SR membrane preparations ryanodine binds to its receptor with an estimated K_d of 2.5 nM. [^{3}H]ryanodine-binding assays were performed on membrane vesicles derived from liver and cardiac NEs and were compared to the binding level in cardiac SR fractions as a positive control (Table 1). [^{3}H]ryanodine binding was not performed on brain microsomal fraction in this study. However, our brain microsomal fractions and binding conditions (i.e. high ionic strength) were similar to the one described by Padua et al. [33]. Thus, we assumed that the [^{3}H]ryanodine binding level of our brain microsomal fraction was equivalent (i.e. 34 fmol mg^{-1} protein) to the one determined by these authors. NEs purified from cardiac cells retained 21.9 ± 1.7 fmol [^{3}H]ryanodine mg^{-1} protein (n = 6). This value was low when compared to cardiac SR fraction (1972 ± 36 fmol mg^{-1} protein, n = 6) but was very close to the one we initially determined (20 fmol mg^{-1} protein) on cardiac nuclei fraction [20]. As discussed earlier, we cannot exclude that cardiac NE ryanodine binding originated from a residual contamination (less than 1.5%) of nuclei by heavy SR vesicles [20]. Surprisingly, the [^{3}H]ryanodine binding to liver NE was negligible (0.5 ± 1.9 fmol mg^{-1} protein, n = 6). Moreover, no specific ryanodine binding was detected on intact liver nuclei (data not shown)

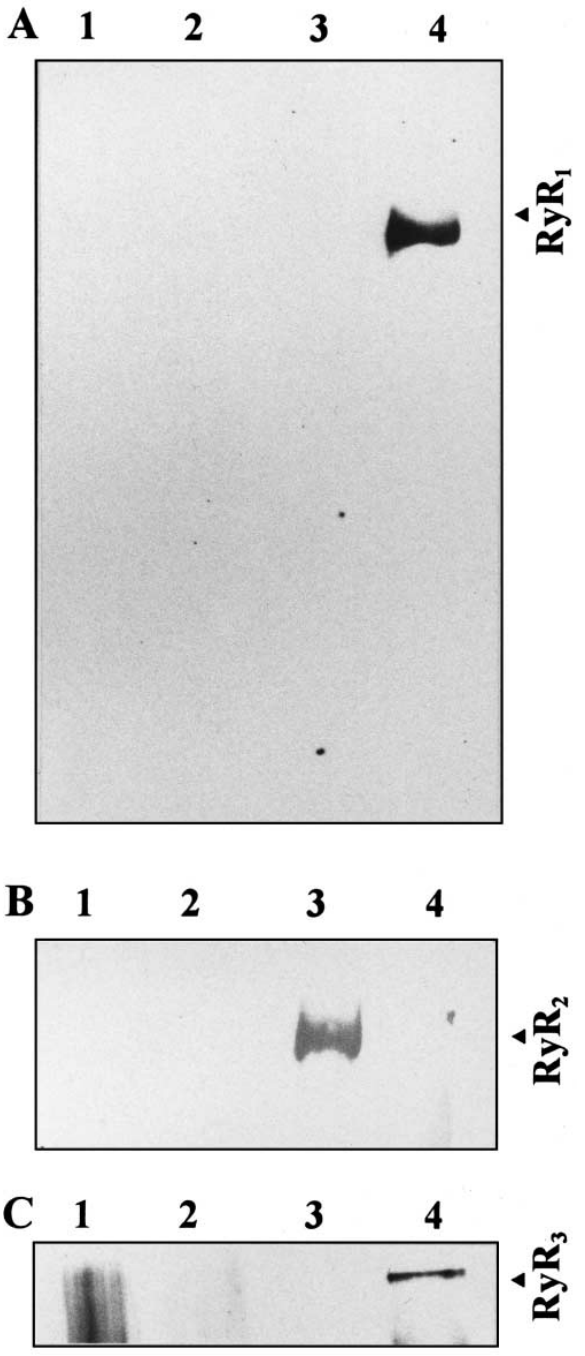

Fig. 2. Western blot analysis of the various RyR isoforms in nuclear envelopes and SR membrane fractions. (A) Membrane proteins of rat liver NE (lane 1), sheep cardiac NE (lane 2), canine cardiac SR (lane 3) and rabbit skeletal SR fractions (lane 4) were loaded and separated on 6% SDS-PAGE, transferred on nitrocellulose membranes and incubated in the presence of anti RyR_1 antibodies. The RyR_1 isoform was only detected in skeletal SR, as a single protein band of high molecular weight. (B) Same membrane fractions as in A probed with Anti RyR_2 antibodies. Note the absence of staining in the liver and cardiac NE (lanes 1 and 2), as well as in the skeletal SR (lane 4) fraction from fast-twitch muscles. A single band was detected in cardiac SR (lane 3). (C) Same membrane fraction as above in lanes 1, 2 and 3, while lane 4 contained SR membrane fractions derived from canine diaphragm muscles, used as positive control for the RyR_3 isoform. No specific staining was detected in the liver and cardiac NE (lanes 1 and 2), as well as in cardiac SR (lane 3), although the presence of a smear probably due to the presence of DNA was observed in lane 1. Note the high molecular weight band stained in the SR membrane from canine diaphragm (lane 4).
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which strongly suggest the absence of specific ryanodine-binding sites in the NE of liver cells.

Western blot analysis, using antibodies directed against various ryanodine receptor isoform – RyR_1, RyR_2 and RyR_3 – were performed in order to reveal the putative presence of RyR in the NEs of liver and cardiac cells. Fig. 2A indicates that the RyR_1 isoform was neither detected in liver (lane 1) nor in cardiac (lane 2) NEs. Furthermore, the RyR_1 antibody did not recognize the RyR_2 isoform from cardiac SR (lane 3), but clearly detected the RyR_1 isoform in skeletal SR membrane (lane 4), indicating that this antibody efficiently discriminated between RyR_1 and RyR_2 isoforms. Fig. 2B shows that the anti-RyR_2 antibody did not detect any protein band (RyR_2) in both liver (lane 1) and cardiac (lane 2) nuclear membranes, even when 80 μg of proteins were loaded instead of 20 μg, or in skeletal SR (lane 4). Under identical conditions, the anti-RyR_2 antibody recognized a single protein band in the cardiac SR (lane 3). A third set of western blot analysis was performed using an other antibody raised against a specific sequence of type III RyR. This isoform was previously detected in the terminal cisternae (junctional SR) from various mammalian skeletal muscles, and consistently in the diaphragm from different species [34]. Fig. 2C shows that the anti-RyR_3 antibody did not recognize any protein band in liver (lane 1) and cardiac (lane 2) NEs, as well as in the cardiac SR fraction (lane 3). However, this antibody detected a high molecular weight protein band in canine diaphragm preparations known to be enriched in RyR_3 isoform (lane 4); as well as in the SR fractions derived from rabbit hind limb rabbit skeletal muscles (data not illustrated). These results indicate that the RyR isoforms I, II and III are unlikely to reside in the nuclear envelope of liver and cardiac cells. If so, it must be below detectable level since similar results were obtained on freshly isolated nuclei (data not illustrated).

3.2. Detection of nuclear $InsP_3$ receptors

In the following experiments, we investigated the putative presence of $InsP_3R$ in cardiac and liver NE. We have determined the $[^3H]InsP_3$ binding ability of these two types of nuclear membrane fractions, while brain microsomal (BM) and cardiac SR vesicles were used as positive and negative controls, respectively. Table 1 indicates that BM bound 214 ± 16.8 fmol mg^{-1} protein, while cardiac SR did not exhibit any significant $[^3H]InsP_3$ binding (0.57 ± 0.38 fmol mg^{-1} protein). Liver and cardiac nuclear envelopes retained 187 ± 6 fmol mg^{-1} protein and 33 ± 4 fmol mg^{-1} protein, respectively. These results demonstrate the existence of $InsP_3R$ in both cardiac and liver nuclei. $InsP_3$-binding sites had already been described in the NE of liver cells [4]. They were located to the inner nuclear membrane and were correlated with the immuno-detection of a nuclear $InsP_3R$ [17].

4. Discussion

The present study shows direct biochemical and immunological evidences of the absence of ryanodine receptors in NE of liver nuclei, and minimizes the putative role of these recep-

tors in cardiac NE. Moreover, we provide new evidence that $InsP_3Rs$ could be involved in the control of nuclear Ca^{2+} in cardiac and liver cells, as shown previously in oocytes nuclei [8,9].

During the last decade, numerous studies have demonstrated that the nuclear envelope is involved in the regulation of nucleoplasmic ion concentrations [35]. Several ionic permeabilities have been characterized in both the inner and the outer nuclear membranes of different cell types (reviewed in [36]). The existence of nuclear Ca^{2+} channels was postulated from the pioneer works of [3] and [4] who, respectively, showed an $InsP_3$-induced Ca^{2+} release from isolated liver nuclei, and the presence of $InsP_3$-binding sites in the nucleus. $InsP_3$-gated channels have recently been functionally characterized in the outer nuclear membrane of *Xenopus* oocytes [8,9]. In liver nuclei, $InsP_3$-binding sites have been located in the inner nuclear membrane [17] and a $InsP_3$-induced Ca^{2+} release from the perinuclear space to the nucleoplasm was measured [5]. In the present study, we have confirmed the presence of $InsP_3$-binding sites in the nuclear envelope of liver cells. Significant $InsP_3$ specific binding was also found in the NE of cardiac cells, although its relative density was six times lower than in the liver NE. These findings confirm the previous immuno-localization of $InsP_3R$ isoform I in the perinuclear region of rat liver [37]. However, the amount of $InsP_3$ binding to the cardiac NE reported herein (33 fmol/mg of protein, Table 1) is relatively higher than previously determined (12 fmol/mg of protein) on crude cardiac nuclei [20].

Recently cADPR was reported to trigger Ca^{2+} release from the perinuclear space of liver nuclei via RyRs [5]. Our present results do not support this conclusion. Although several findings indicate that cADPR regulates intracellular Ca^{2+} release [38] it is still a matter of debate whether cADPR directly triggers the opening of RyR [39]. In the present study, we clearly demonstrated that liver nuclei possess no ryanodine receptor. This is also supported by the fact that messenger RNAs encoding RyaR isoforms I, II or III are not detected in liver extracts [40] and that ryanodine does not induce Ca^{2+} release from isolated nuclei [41]. It should be further mentioned that NAD^+, the metabolic precursor of cADPR, stimulates Ca^{2+}-uptake by the nuclear envelope [42]. These data and the ones presented herein indicate that the cADPR-triggered effects in liver nuclei is likely to be mediated through a mechanism that does not depend on a high molecular weight nuclear RyR. Furthermore, no trace of specific immuno-staining was detected in the cardiac NE using three different isoform specific RyR antibodies despite the low level of [^{3}H]ryanodine binding that might be due to a residual contamination of NE by heavy SR membrane vesicles as discussed previously [20]. One may point out that cADPR has been reported to interact with 100 and 140-kDa proteins, of which neither the functions nor the cellular distribution of which have been defined [43]. In these conditions, it is tempting to postulate that cADPR may trigger Ca^{2+} release in the cell nucleus through the activation of an unknown and somewhat more complex pathway. Nevertheless, in cardiac cells, no Ca^{2+} sparks which originate from clusters of SR-RyRs, have been reported to occur in close vicinity of the NE [44].

Intracellular Ca^{2+} regulation represents a signalling pathway through which the cell coordinates major biological functions [45]. Ca^{2+} has been involved in the regulation of typical nuclear functions such as gene expression, DNA breakdown, repair and replication through its binding to calmodulin [46]. Furthermore, intracellular Ca^{2+} transients are often associated with cell-cycle events such as DNA condensation and nuclear envelope breakdown [47] and it has been suggested that $InsP_3$ may be involved in the early events triggering Ca^{2+}-regulated nuclear envelope breakdown [48]. The contribution of the $InsP_3$ pathway has also been investigated in the case of post-mitotic nuclear envelope reformation. On the basis of the works of [49,50], it appears that the activation of $InsP_3R$ located in nuclear envelope vesicules and the subsequent release of Ca^{2+} from these vesicules represents a fundamental step preceding the fusion of nuclear vesicules and nuclear envelope reformation. Recently, the expression of a *c-fos* – a well known Ca^{2+}-regulated gene – was shown to be differentially regulated upon increasing cytosolic or nucleoplasmic Ca^{2+} [51]. This indicates that Ca^{2+} involved in nucleoplasmic Ca^{2+} increase may originate from different sites. It is accepted that the increase of nucleoplasmic Ca^{2+} can result from the diffusion of cytosolic Ca^{2+} through the NPCs, since NPCs restrict but do not prevent Ca^{2+} diffusion [16]. However, alternative pathways should be considered. Phosphatidyl-inositol 4,5 bisphosphate (PIP_2) is located in the nuclear envelope [52]. Phosphoinositidases C (β1, δ4) are also present in the nucleus [53,54]. Intranuclear production of $InsP_3$ from PIP_2 hydrolysis could activate the nuclear $InsP_3R$, which could also be stimulated by nuclear protein kinase C [42,55]. The current challenge is to demonstrate that ligand-gated nuclear ionic channels may play an important role in NE functions.

Lately, Jayaraman and Marks [56] have shown the T cells deficient in $InsP_3R$, are resistant to apoptosis induced by dexamethasone. This property was reversed by pharmacologically raising cytoplasmic Ca^{2+} levels. Moreover, receptor mediated apoptosis can be induced in Ca^{2+}-free media. These findings suggest that intracellular Ca^{2+} release, via $InsP_3R$, is a critical step controlling programmed cell death. Several reports show that the Ca^{2+} content of perinuclear space could also control the nucleo-cytoplasmic traffic of macromolecules. Indeed, closure of the nuclear pore complex and subsequent blockade of nucleo-cytoplasmic traffic was observed after emptying the perinuclear space of its Ca^{2+} content [57–59]. This shows that the elucidation of the mechanism(s) of regulation of nuclear Ca^{2+} fluxes is of primary importance in molecular cell physiology.

In conclusion, according to the data obtained in the present study, the odds would be in favor of a major role for $InsP_3R$, over the putative implication of RyRs, in controlling Ca^{2+} efflux from the perinuclear space. It is worth noticing that in a recent study using Ca^{2+} confocal imaging to analyse rapid and slow Ca^{2+} waves as well as pharmacological assays, Jainovish and Liberona [60] came up with the same conclusions regarding the absence of RyR and the presence of $InsP_3R$ in rat myotubes nuclei.

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