

Reconstitution of Ionic Channels from Inner and Outer Membranes of Mammalian Cardiac Nuclei

Eric Rousseau, Caroline Michaud, Danie Lefebvre, Sonia Proteau, and Anne Decrouy

Le Bilarium, Department of Physiology and Biophysics, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

ABSTRACT Recent reports suggest that the nuclear envelope possesses specific ion transport mechanisms that regulate the electrolyte concentrations within the nucleoplasm and perinuclear space. In this work, intact nuclei were isolated from sheep cardiac cells. After chromatin digestion, the nuclear envelopes were sonicated and four nuclear vesicle populations were separated by sucrose step gradients (SF₁–SF₄). These fractions were compared by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and their protein content was analyzed by Western blot, using lamin and SEC 61 antibodies. The lamins, which are associated with the inner nuclear membrane, were present in three fractions, SF₂, SF₃, and SF₄, with a lower amount in SF₁. The SEC 61 protein, a marker of the rough endoplasmic reticulum, was detected in small amounts in SF₁ and SF₂. Upon fusion of vesicles into bilayers, the activities of nuclear ionic channels were recorded in 50 mM *trans*/250 mM *cis* KCl or CsCl, pH 7.2. Two types of Cl[−] selective channels were recorded: a large conducting 150–180-pS channel displaying substates, and a low conducting channel of 30 pS. They were both spontaneously active into bilayers, and their open probability was poorly voltage dependent at negative voltages. Retinoic acid (10^{−8} M) increases the p_o of the large Cl[−] conducting channel, whereas ATP modifies the kinetics of the low conductance anion selective channel. Our data also suggest that this anionic channel is mainly present in the SF₃ and SF₄ population. The presence of a 181 ± 10 pS cation-selective channel was consistently observed in the SF₂ population. The behavior of this channel was voltage dependent in the voltage range −80 to +60 mV. Furthermore, we report for the first time the activity of a channel exclusively present in the SF₃ and SF₄ fractions, shown to contain mainly inner membrane vesicles. This cation selective channel displays a 75-pS conductance in 50 mM *trans*/250 mM *cis* K-gluconate. It is concluded that the bilayer reconstitution technique is an attractive approach to studying the electrophysiological properties of the inner and outer membranes of the nuclear envelope.

INTRODUCTION

The nuclear envelope (NE) is characterized by a typical geometry with two unit membranes limiting the perinuclear space and merging at the nucleopores (NPs). The NP, which is formed by a large octo-trimeric protein complex, controls the transit of macromolecules between the nucleoplasm and the cytoplasm (Unwin and Milligan, 1982; Davis and Blobel, 1986; Davis, 1992; Dingwall and Laskey, 1992). The nuclear envelope contains a large number of NPs whose density depends on the type of cell nuclei and their stage of development. Average NP density oscillates between 11 pores/μm² in yeast (Aris and Blobel, 1989) and 80 pores/μm² in nuclei from *Xenopus* oocytes. These wide-open pores could provide pathways for ion fluxes, thereby preventing the formation of ionic concentration gradients and potentials across the nuclear envelope. Actually, no major difference in ionic composition was quantified between the cytoplasm and the nucleoplasm by concentration electron microscopy x-ray analysis (Kowarski et al., 1985), despite the fact that Ca²⁺ concentration rises into the nucleus during the contractions (Dr. A. Somlyo, personal communication). Nevertheless, the high input resistance of the NE,

which was measured by classical microelectrode techniques, suggests that ion fluxes are restricted (Loewenstein and Kanno, 1963; Ito and Loewenstein, 1965). It was shown that the input resistance of nuclei varies during the cell cycle (Ito and Loewenstein, 1965). Furthermore, recent reports have clearly established the presence of nuclear ionic channels (NICs) in the outer membrane of the nuclear envelope (Mazzanti et al., 1990, 1991; Tabares et al., 1991; Matzke et al., 1992; Bustamante, 1992, 1993, 1994a; Dale et al., 1994). The patch-clamp technique allowed the characterization of various types of conductance from different cell nuclei, namely large and low conducting Cl[−] channels as well as cation selective channels (reviewed by Bustamante, 1994b).

Although the physiological role and the factors involved in the regulation of the NICs remain to be identified, it is reasonable to suggest that they could directly control ion movements across the nuclear membranes, thus controlling the ionic concentrations in the perinuclear cisternae as well as their transmembrane potentials. The ionic composition of the perinuclear space remains to be ascertained. But it was reported by Kowarski et al. (1985) that in saponin-treated smooth muscle cells exposed to 1 μM Ca²⁺ and 5 mM oxalate, Ca²⁺ deposits were frequently observed around the nucleus. The existence of Ca²⁺ pumps in the nuclear membrane was reported (Lanini et al., 1992), and this supports the presence of a restricted exchange of ions between the cytoplasm and the perinuclear space. Such evidence indicates that the active transport of divalent cations is con-

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Address reprint requests to Dr. Eric Rousseau, Le Bilarium, Department of Physiology and Biophysics, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada. Tel: 819-564-5306; Fax: 819-564-5399; E-mail: e.rousseau@courrier.usherb.ca.

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trolled across the outer membrane of the NE. The presence of calmodulin and PKC in the nucleus also suggests a potential role for Ca^{2+} in the control of intranuclear processes (Bachs et al., 1992). In addition, specific binding of [^3H]inositol trisphosphate (IP_3) has been identified on the membrane of liver nuclei (Malviya et al., 1990), which suggests the presence of ligand-gated channels into the nuclear membranes. This assumption has recently been confirmed (Mak and Foskett, 1994; Stehno-Bittel et al., 1995). The results of Duke et al. (1994) indicate that divalent cations (Ca^{2+} , Mg^{2+}) might also indirectly control intranuclear processes. Furthermore, Gilchrist and Pierce (1993) have identified the presence of calnexin (p93), a calcium-binding protein, in the perinuclear space. Altogether, these data strongly suggest that the nuclear envelope is able to manipulate ionized Ca^{2+} . Likewise, in the case of the sarcoplasmic reticulum system, the nuclear membranes must contain parallel ionic pathways as counter-charge transport systems to maintain their potential away from the Ca^{2+} equilibrium potential, if Ca^{2+} movement ought to occur, and to maintain the electroneutrality of the system. Actually, if the outer membrane of the NE is considered as part of the rough endoplasmic reticulum, the electroneutrality principle has to be respected when newly synthesized proteins are translocated into the perinuclear space.

We hypothesize that both monovalent anion- and cation-selective channel proteins of the nuclear envelope control the charge movements across the nuclear membranes limiting the perinuclear space. Here we have prepared and characterized four microsomal fractions derived from the NE of cardiac nuclei and, for the first time, investigated the biophysical properties of various monovalent ionic channels of the inner and outer membranes of the NE upon fusion of microvesicles into planar lipid bilayers. We also present data confirming that Mg-ATP modulates the gating of a low-conductance anion-selective channel. Moreover, we provide evidence that retinoic acid modulates the gating of the large conducting Cl^- channel. Up to now, this compound was essentially known as a regulator of gene expression, via its successive association with retinol-binding proteins and selective binding of the retinol-protein complexes on specific DNA motif (Hudson et al., 1990). Finally, we report a differential localization of cation channels between the inner and outer membranes of the NE. Some of these findings appeared in a preliminary report (Rousseau et al., 1994).

MATERIALS AND METHODS

Preparation of the microsomal fractions derived from the nuclear membranes

Nuclei were isolated from sheep cardiac ventricular cells according to the procedure initially described by Widnell and Tata (1964), which is known to yield enzymatically active nuclei. Sheep hearts (100 g) were recovered from a local slaughterhouse and homogenized in (30% w/v) ice-cold buffer (mM): 320 sucrose, 3 MgCl_2 , 1 dithiothreitol (DTT), 20 K-HEPES (pH 7.2), plus 50 μM pefabloc, 1 μM pepstatin, 1 μM leupeptin, and 2.4 IU

aprotinin, with a Polytron at setting 3500 with three 30-s bursts. The homogenate was filtered twice through four layers of cheesecloth. The filtrates were then diluted 10-fold in the same buffer and centrifuged at $4000 \times g$ for 20 min in a type 16 rotor. The pellet was resuspended in 2.4 M sucrose containing 5 mM K-HEPES, 1 mM MgCl_2 , and 1 mM DTT (adjusted to pH 7.2 with KOH) and centrifuged at $50,000 \times g$ for 60 min in a Ti 35 rotor (Beckman). The nuclear pellet was resuspended in freshly prepared 0.25 M sucrose containing 1 mM MgCl_2 , 5 mM K-HEPES (pH 7.2), and 1 mM DTT and centrifuged at $1000 \times g$ for 10 min in a Ti 42.1 rotor. Microsomal fractions enriched in outer or inner membranes of the nuclear envelope were prepared according to the procedure described by Howell and Lefebvre (1989), with slight modifications. The nuclei were resuspended and sonicated in ice-cold digestion buffer (20 mM Tris-HEPES, 0.30 M sucrose, 1 mM MgCl_2 , 1 mM DTT, pH 7.2), to which a freshly made solution of DNase 1 (from Sigma) was added to give a final concentration of 20 $\mu\text{g}/\text{ml}$. The DNase 1 digestion lasted 20 min at 30°C . When chromatin digestion was completed, the suspension was centrifuged at $12,000 \times g$ for 10 min, and the pellets of NE were resuspended in 5 mM Tris-HEPES buffer containing 1 mM DTT (pH 7.2). This suspension was sonicated with a microprobe (Sonicator W375 from Ultrasonic, Inc.) and then layered onto discontinuous sucrose gradients consisting of 0.25, 1.5, 1.8, and 2.0 M sucrose. The gradients were centrifuged at $100,000 \times g$ for 90 min in a SW28 rotor. Four nuclear subfractions (SF_1 – SF_4) were recovered with a Pasteur pipette and then washed in 5 mM K-HEPES (pH 7.2) containing 1 mM DTT. The subfractions were then centrifuged at $120,000 \times g$ for 45 min in a Ti 35 rotor; resuspended in 300 mM sucrose, 5 mM K-HEPES, and 1 mM DTT (pH 7.2); and immediately frozen in liquid nitrogen to be stored at -85°C . Alternatively, step gradients of CsCl of identical density were used. The main advantage of this second procedure is to create a high ionic strength that depolymerizes the actomyosin filaments, which thereby facilitates the extraction of these cytosolic proteins, leading to the physical cleaning of the microsomal fractions directly derived from the NE. An identical procedure was used to prepare nuclear microsomal fractions from rabbit hearts. Crude and heavy SR microsomal fractions from sheep hearts were prepared as previously described in our laboratory (Rousseau et al., 1992).

Biochemical characterization of the nuclear membrane and microsomal fractions

The protein content of the subcellular fractions was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. DNA and RNA were measured using the Tri-Reagent kit from Molecular Research Center Inc. (Cincinnati, OH), using calf thymus DNA as the standard.

Binding assays

Ryanodine binding assays were performed using a procedure described previously (Rousseau et al., 1992). Briefly, SR vesicles (100 μg of protein) from each fraction were incubated with [^3H]ryanodine (with or without nonradioactive ryanodine) for 1 h 30 min at 37°C , in the presence of 50 μM free Ca^{2+} . This mixture was then centrifuged twice, and pellet radioactivity was determined by liquid scintillation. Specific [^3H]ryanodine binding was calculated by subtracting nonspecific binding (5 nM [^3H]ryanodine + 5 μM ryanodine) from total binding (5 nM [^3H]ryanodine alone). The [^3H]inositol-trisphosphate binding was determined as previously described by Guillemette et al. (1988).

Polyacrylamide gel electrophoresis and Western Blot analysis

Membrane samples (20 μg of protein) were solubilized in 2% sodium dodecyl sulfate (SDS), and proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), casted with a 3% stack-

ing gel. Gels were casted into a Mini-Protein II dual cell (Bio-Rad), and protein bands were stained with 0.1% Coomassie blue or silver. All gels were run in parallel with high-molecular-weight standards provided by Bio-Rad. Western blot analysis of the various nuclear fractions derived from the NE was performed as previously described by Lebel and Raymond (1984). In brief, the proteins were electrotransferred from the gel to immobilon membranes for 2 h at 70 V. The membranes were incubated overnight at 4°C with the primary antibody, either monoclonal mouse anti-lamin A and C or anti-lamin B antibodies. The secondary antibody was an anti-mouse IgG₁ coupled to horseradish peroxidase (HRP). A similar protocol was used to reveal the presence of the SEC 61 protein using antibodies provided by Dr. T. A. Rapoport (Harvard Medical School). This rabbit antibody was raised against the SEC 61 α -subunit C-terminus. In that case the secondary antibody was an anti-rabbit IgG, coupled to HRP.

Electron microscopy

Sheep purified nuclei and nuclear membrane microsomal fractions were diluted 1/10 in 250 mM sucrose and 5 mM K-HEPES buffer (pH 7.2). The pellets were fixed 2 h in ice-cold 2.8% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) and postfixed with 2% osmium tetroxide (OsO₄) in 0.1 M sodium cacodylate. They were then dehydrated in graded series of ethanol solutions and embedded in EPON. Ultrathin sections were stained with uranyl acetate followed by lead citrate treatment (Hayat, 1986). The preparations were examined at different magnifications with a Philips EM300 electron microscope, operated at 80 keV.

Planar lipid bilayer formation and vesicle fusion

The planar lipid bilayers (PLBs) were formed at room temperature with a lipid mixture containing phosphatidylethanolamine and phosphatidylcholine in a ratio of 1:1, if not specified otherwise. The final lipid concentration was 25 mg/ml decane. A 250- μ m diameter hole, drilled in a DELRIN cup, was pretreated with the same lipid mixture dissolved in chloroform (Rousseau et al., 1992). With a Teflon stick, a drop of the decane lipid mixture was gently spread across the hole to obtain an artificial membrane. Membrane thinning was monitored by applying voltage test pulses in the form of sawtooth waves (0.5 mV p-p). Typical capacitance values were 250–400 pF. Aliquots of nuclear microsomal fractions (typically 10–60 μ g protein) were added to the *cis* chamber in the proximity of the bilayer. This chamber contained 250 mM CsCl (or KCl), plus 109 μ M CaCl₂, 100 μ M EGTA (10 μ M of free Ca²⁺), and 10 mM Tris-HEPES (pH 7.4). The *trans* chamber contained 50 mM CsCl (or KCl) plus 10 μ M free Ca²⁺ and 10 mM Tris-HEPES. The fusions were either spontaneous or induced by applying large voltage pulses (\pm 80 mV) across the bilayer. They were monitored as discrete Cl[−] conductance increments due to the presence of Cl[−]-selective channels in the nuclear membranes. Freshly prepared nuclear membrane fractions were more fusogenic than frozen preparations.

Recording instrumentation and signal analysis

The currents were measured using a low-noise operational amplifier (Dagan 8900 from Dagan Corp., Minneapolis). They were then filtered (cutoff frequency 10 kHz) and recorded either on a videotape recorder (unitrade) through a pulse code modulation device or a digital audiotape (DAT.75ES song from Dagan Corp). The currents were simultaneously displayed on-line on a chart recorder (DASH II MT; Astro Med.) and an oscilloscope (Kikusui, 5040). Current recordings were played back, filtered at 500 Hz, and sampled at 2 kHz for storage on hard disk and for further analysis with an HP-VECTRA computer, with programs kindly provided by Dr. M. Nelson (University of Vermont). The open probability values (p_o) and time histograms were determined from data stored during a 40–300-s period, using the half-threshold discriminator method. Applied voltages were defined with respect to the *trans* chamber, which was held at virtual ground.

Chemical reagents

TRIZMA base (Tris), HEPES, PIPES, ATP, AMP-PCP (ATP analog), cAMP, cGMP, protease inhibitors, and retinoic acid were obtained from Sigma (St. Louis, MO). The radioligands used for binding assays were purchased from New England Nuclear (NEN-Dupont Canada, Inc.). Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). All other materials were of reagent grade. Water deionized on a Milli-Ro-Milli-Q-UF system from Millipore (18 ± 0.2 MW/cm²) was used to prepare all buffer solutions.

RESULTS

Identification of the cardiac nuclei

The sheep cardiac nuclei were mainly identified by electron microscopy on the basis of their size and ellipsoidal shape. Most nuclei were isolated with an intact nuclear envelope (NE) and without mitochondria (Fig. 1 A). The inner membrane was underlined by a lamin network as well as festoons of heterochromatin (Fig. 1 A). The outer membrane of the cardiac nuclei appears to be deprived of ribosomes. Furthermore, in contrast with the previous reports on liver nuclei (Tabares et al., 1991), our preparations contain basically no rough endoplasmic reticulum (RER) membrane system attached to the nuclei (Fig. 1 A). The nuclei represent about 95% of the biological material purified in the nuclear pellet, as determined by electron microscopy analysis.

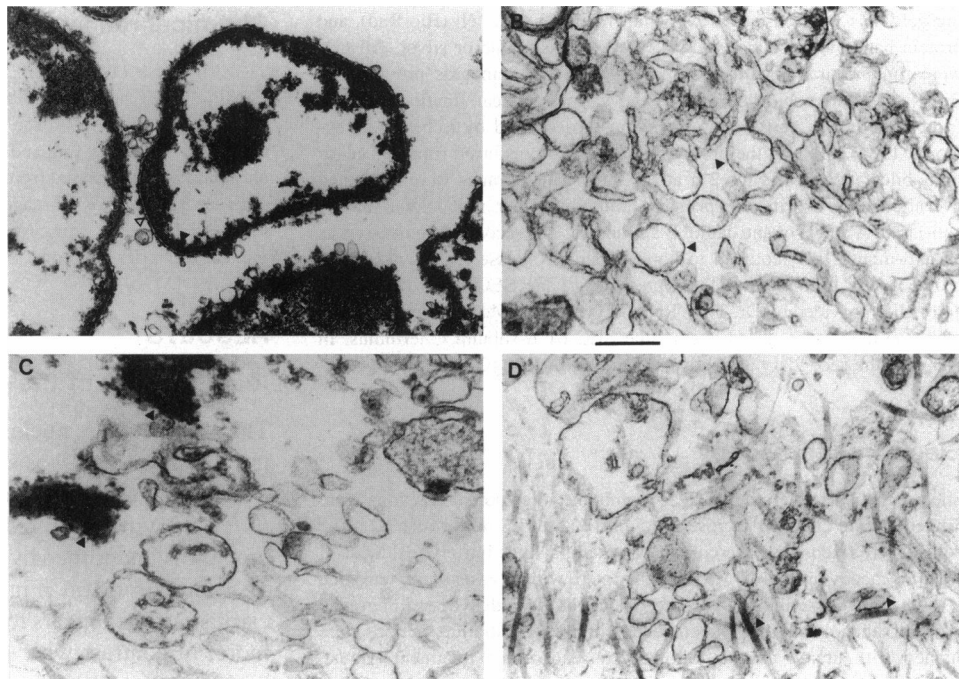
After chromatin digestion, the NE appeared as large membrane ghosts (data not shown), similar to those described previously for rat liver nuclei (Lebel and Raymond, 1984; Howell and Lefebvre, 1989). Electron microscopy was also used to determine the quality of the nuclear membrane subfractions separated by means of sucrose gradients. The subfractions (SF₂ to SF₄) are constituted mainly of single membrane vesicles. SF₂ is the most homogeneous vesicle population (Fig. 1 B), whereas SF₃ and SF₄ include additional aggregates of fragmented DNA (Fig. 1 C) as well as myosin filaments (Fig. 1 D). SF₄, which has the higher density, is characterized by smaller vesicles, most of them containing an internal matrix, supposedly derived from the perinuclear space.

Characterization of the nuclear microsomal preparations by SDS-PAGE

Fig. 2 shows the protein profiles of whole nuclei and NE membrane subfractions separated on step sucrose gradients (Fig. 2 A). We first demonstrate that the protein profile of the NE (Fig. 2 B, lane 1) is different from the typical profile of the heavy sarcoplasmic reticulum (SR) prepared from the same tissue (Fig. 2 B, lane 2). Moreover, the protein profile of NE was different from that of the RER (not shown).

SDS-PAGE analysis of the four NE subfractions (SF₁–SF₄) recovered from the sucrose gradient reveals qualitative and quantitative differences between the various fractions (Fig. 2 C), although all of the protein bands were present in the starting material (Fig. 2 B, lane 1). SF₁, which corresponds to the top of the gradient, contains essentially low-

FIGURE 1 Electron micrographs of the cardiac nuclei, SF₂, SF₃, and SF₄ fractions. Isolated nuclei and microsomal fractions recovered from the step sucrose gradient. Each sample was pelleted and fixed in 2.8% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2), postfixed in the same buffer plus 2% osmium tetroxide (OsO₄), and dehydrated. Ultrathin sections were stained with uranyl acetate followed by lead citrate treatment. (A) Intact nuclei. *Open arrow*, NE; *double arrow*, lamin network. Scale bar = 1.2 μ m. (B) SF₂ unilamellar vesicles (*arrows*). (C) SF₃ with residual DNA (*dark arrows*). (D) SF₄ myofilaments (*dark arrows*). Scale bar = 0.2 μ m for B, C, and D.



molecular-weight proteins of 14 kDa that might be histones (Gilchrist and Pierce, 1993). SF₂ corresponds to the first band on the sucrose gradient (see Fig. 2 A). It contains various proteins from the nuclear membrane. SF₄, which corresponds to the bottom of the gradient, looks qualitatively similar to SF₃ and contains several high-molecular-weight protein bands. One of these protein bands, migrating at the level of the 205 kDa standard, might correspond to the myosin band. The smears could also reveal the presence of small fragments of contaminating DNA in the SF₃ and SF₄ fractions (Fig. 2 C). The presence of significant amounts of residual DNA in SF₃ and SF₄ vesicle populations suggests that these fractions might be enriched in membrane vesicles capable of interacting with heterochromatin fragments. These observations correlate well with the relative DNA content of each fraction (see row labeled DNA in Table 1), demonstrating that upon limited chromatin digestion and fragmentation of NE, residual DNA was detected in the high-density microsomal fractions (namely SF₃ and SF₄). tRNA was detected in the homogenate and in the nuclei, but not in the subfractions (see row labeled tRNA in Table 1). Note that basically no rRNA was found in the cardiac nuclei before any high salt extraction, as well as in the microsomal fractions, which would indicate a low level of contamination by the RER.

Further characterization of the microsomal fractions recovered from the sucrose gradient was carried out before the reconstitution experiments as suggested by Woodbury (1993). Antibodies were raised against different lamins, which are known as polytopic integral proteins of the inner membrane of the NE (Georgatos and Blobel, 1987; Foisner and Gerace 1993; Smith and Blobel, 1993). As shown in Fig. 3 A, lamin A and C, as well as lamin B (Fig. 3 B)

monoclonal antibodies revealed that in the starting material, intact nuclei (Fig. 3, A and B, lane 2), the SF₃ and the SF₄ fractions were similarly enriched in lamins (Fig. 3, A and B). On the other hand, we have consistently observed that SF₂ was contaminated with approximately one-fourth of the lamins A and B present in the SF₃ and SF₄ fractions (Fig. 3, A and B). Finally, SF₁ was basically deprived of lamins and other high-molecular-weight proteins (Fig. 1 C). Control experiments were performed to assess the specificity of the lamin antibodies. Omission of primary antibodies or their denaturation (98°C for 30 min) yielded no protein band staining. Despite the fact that the protein profile of the nuclei and the nuclear vesicle populations were quite different from the profile of a typical RER fraction, and that the isolated cardiac nuclei were basically deprived of rRNA (Table 1), it was of interest to assess the putative contamination of the nuclear subfractions by RER membranes using a specific protein marker of this membrane system involved in protein synthesis and translocation (Gorlich et al., 1992), bearing in mind that the outer membrane of the NE might not be functionally different from the RER, the former often being presented as a specialized structural area of the latter.

Fig. 3 shows Western blot experiments performed either in the presence or the absence of anti-Sec 61 antibodies. The use of this specific antibody revealed the presence of a faint band in SF₁ and a consistent positive signal in SF₂ (Fig. 3 C; *first, second, and third lanes*), whereas no immunostaining was observed in SF₃ and SF₄ (Fig. 3 C, *upper row*). Neither type of control experiment performed to assess the specificity of the immunoreactions yielded protein band staining (Fig. 3 C, *middle and lower rows*). Consequently, it was assumed that RER membrane vesicles were a minor contaminant of our nuclear preparation and that they comi-

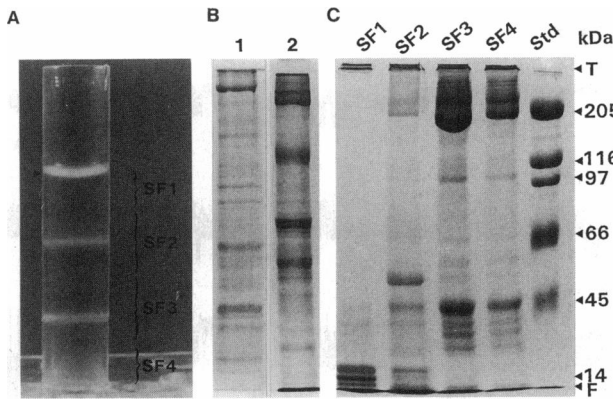


FIGURE 2 Separation of nuclear fractions on step sucrose gradients and SDS-PAGE analysis of their protein profile. (A) Macroscopic aspect of the step sucrose gradient upon centrifugation. The dark arrow on the left shows the position of the liquid meniscus. Two major bands of fluffy material are clearly visible. They were recovered as subfractions SF₂ and SF₃, respectively. SF₁ and SF₄ correspond to the upper and lower parts of the gradient, respectively. The SF₄ fraction contains high-density particles of DNA fragments and myofilaments in suspension in 51% sucrose (w/w). (B) Protein profiles of intact nuclei (lane 1) and cardiac heavy SR (lane 2). (C) Profiles of the four subfractions (SF₁ to SF₄). Note the relative differences in protein distribution between the various fractions. Smears in SF₃ and SF₄ fractions might be due to the presence of small DNA fragments. All fractions were analyzed on 10% SDS-PAGE in parallel with high-molecular-weight-standard from Biorad (lane 5) and revealed by Coomassie blue staining. Standard molecular weights are indicated on the right (T, top; F, front).

grated with the nuclear membrane vesicles recovered in the SF₂ population, to which they are closely related. To evaluate the putative contamination of our nuclear fractions by SR membranes, binding experiments involving [³H]ryanodine as a marker of the SR Ca²⁺ release channel were performed. We estimate, according to their relative contents in high-affinity ryanodine-binding sites, that the contamination of the nuclear pellet by heavy SR vesicles (HSR) did not exceed 2% (see Table 1). On the other hand, crude microsomal fractions, as well as HSR, derived from sheep myocardium, display residual [³H]IP₃ binding, whereas the

cardiac nuclei retained 12 pmol [³H]IP₃/mg protein, which is lower but on the same order as the 87 pmol/mg protein measured in liver nuclei, reported previously by Malviya et al. (1990). Together, these results suggest that it would be possible to prepare microsomal populations of various buoyant densities containing membrane vesicles derived from either the outer or the inner membranes of the NE.

Fusion and characterization of NIC into planar lipid bilayers

To assess the activity of either cation- or anion-selective channels from the NE, vesicles from the outer nuclear membrane-enriched fraction (SF₂) were fused into planar lipid bilayers (PLB) in an asymmetrical KCl buffer system. Fig. 4 A shows typical current traces recorded before, during, and after a single fusion event. The start of the upper trace illustrates the base line (zero current level) before fusion. Upon fusion of a single vesicle (arrowhead), a small negative leak current was observed at 0 mV (Fig. 4 A), followed by the activation of two channels, whose amplitude increased with negative voltages (subsequent traces). The unitary conductance of these channels was 115 pS. Because the current amplitude was negative at 0 mV and increased at more negative voltages (−10 and −30 mV), it was suspected that this electrical activity was supported by two Cl[−]-selective channels simultaneously fused into the bilayer.

To further characterize these channel activities, and to eliminate the putative contribution of cation-selective channels, subsequent reconstitution experiments were performed with an asymmetrical CsCl gradient. Fig. 4 B shows unitary current traces recorded at various holding potentials in 50 mM *trans*/250 mM *cis* CsCl. At 0 mV under steady-state conditions, cations and anions flow from the *cis* to the *trans* chamber, along their own concentration gradients. However, at negative voltage, Cl[−] ions generated a negative current. Long-lasting subconducting states, which are a hallmark of Cl[−] channels, were consistently observed at various

TABLE 1 Characterization of the various cardiac nuclei and microsomal fractions

	Homog.	Nuclei	SF1	SF2	SF3	SF4	Crude	HSR
Sucrose								
% (w/w)	8.5	63	<28	35	43	51	8.5	37
M	0.25	2.4	<0.9	1.17	1.5	1.8	0.25	1.25
[Protein] (mg/ml)	15.2	12.1	0.5	3.4	2.9	5.7	7.2	12.5
[³ H]Ryanodine binding (fmol/mg protein)	ND	20*	ND	ND	ND	ND	300*	1065*
[³ H]IP ₃ (fmol/mg protein)	ND	12*	ND	ND	ND	ND	1.3*	5*
DNA	+	+	−	±	+	+	ND	−
rRNA	+	−	−	−	−	−	ND	−
tRNA	+	+	ND	ND	ND	ND	ND	−

ND, not determined; Homog., cardiac homogenate; Crude, crude microsomal fraction; HSR, heavy sarcoplasmic reticulum.

**n* = 3.

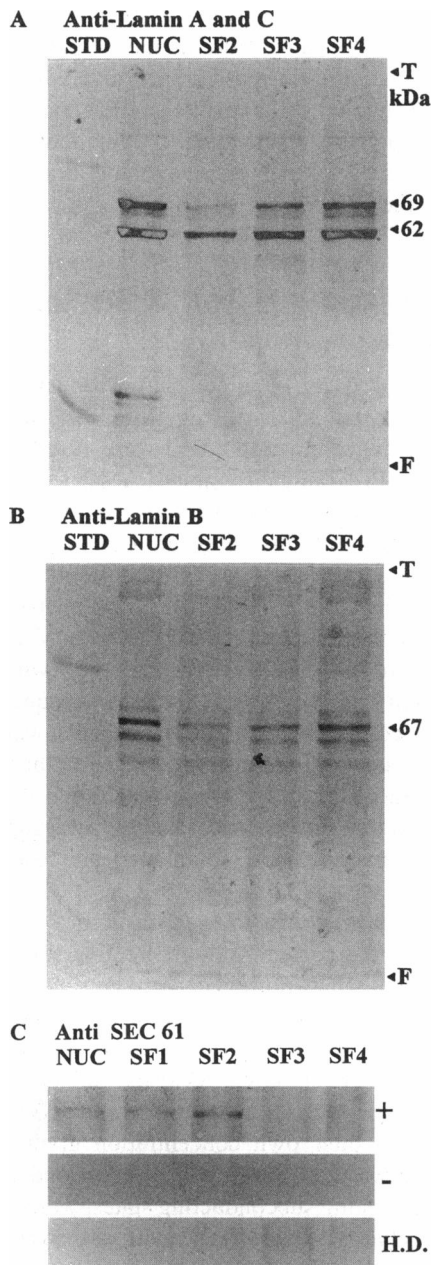


FIGURE 3 Western blot analysis of purified nuclei and vesicle populations derived from the NE. (A) After protein separation of the various nuclear membrane fractions on a 10% SDS-PAGE, the lamins A and C were revealed by a monoclonal anti-lamin A and C antibody. Their molecular weights were 69,000 and 62,000, respectively. (B) Western blot of the same fractions using a monoclonal anti-lamin B as primary antibody. The calculated molecular weight of lamin B was 67,000. Both Western blots attest that lamins A, B, and C were present in the purified nuclei and that stronger signals were observed in the SF₃ and SF₄ membrane fractions. Std, standard; Nuc, Nuclei; SF₂, SF₃, SF₄, microsomal fractions recovered from the step sucrose gradient; T, top; F, front. Note that the SF₁ fraction, which is recovered from the top of the sucrose gradient, was not shown because it was basically deprived of protein (see Fig. 2 C and Table 1). (C) Detection of the SEC 61 protein in the nuclei and the corresponding subfractions. This RER protein marker was probed with anti-SEC 61 antibodies after 10% SDS-PAGE separation. The upper row presents the results obtained with anti-SEC 61 (+) for the different membrane fractions. The middle and lower rows show the negative and positive control experiments obtained in the absence (-) of primary antibody and the presence of heat denatured (HD) antibody, respectively.

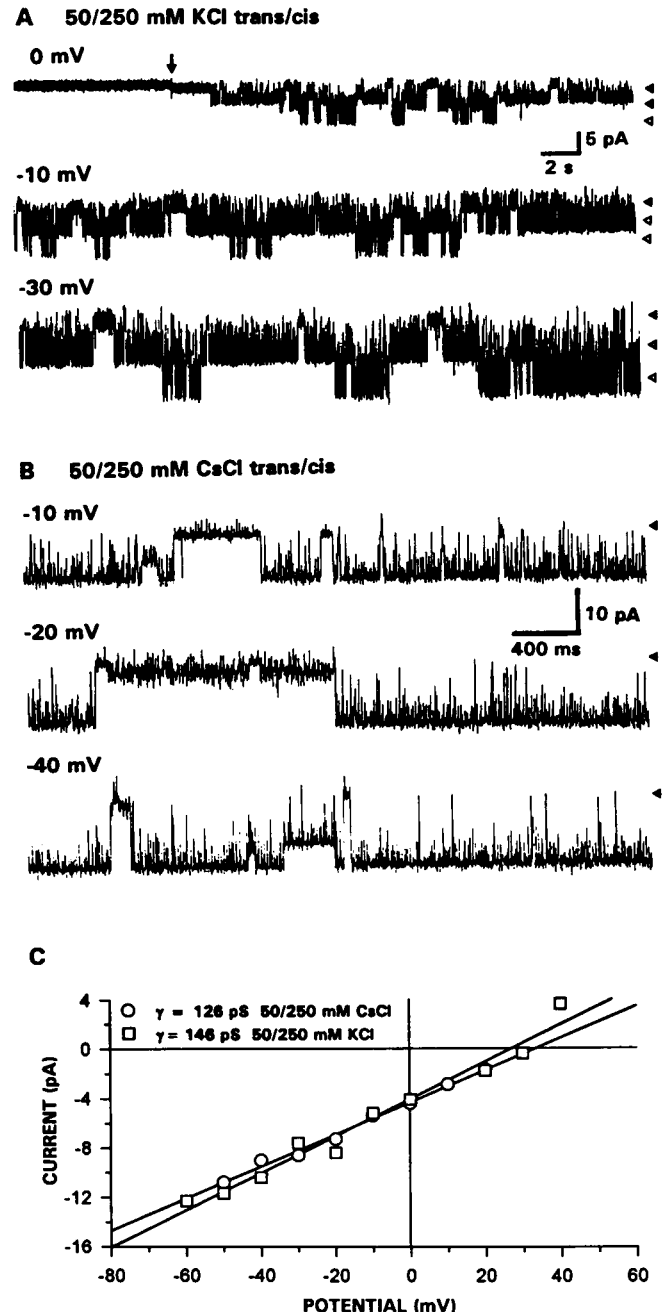


FIGURE 4 Multiple and single channel activities upon fusion of SF₂ vesicles in asymmetrical KCl and CsCl buffer solutions. (A) Unitary current fluctuations of two channels recorded in 50 mM *trans*/250 mM *cis* KCl plus 10 μ M free Ca²⁺ (100 μ M EGTA + 109 μ M CaCl₂) in 10 mM Tris-HEPES, pH 7.2. Channel activity displayed a bursting behavior and was poorly voltage dependent. Dark arrows on the left represent the closed state level, and open arrows the open states. (B) Current traces of a channel recorded in 50 mM *trans*/250 mM *cis* CsCl plus 10 μ M free Ca²⁺ in 10 mM Tris-HEPES, pH 7.2. Note the presence of subconducting states. Dark arrows on the left represent the closed state level. (C) Current/voltage relationships of the nuclear ionic channels recorded in A and B corresponding to conductance values of 126 pS ($n = 5$) and 146 pS ($n = 4$), respectively. In both cases, the value of the extrapolated zero-current potential (reversal potential) argues in favor of a Cl⁻ selectivity for these large conducting nuclear ionic channels.

potentials. The current-voltage relationship for the main and the subconducting states of this single-channel recording yield conductance values of 182 and 91 pS, respectively. The open probability (p_o) of the channel was high and relatively voltage insensitive at negative potentials (oscillating between 0.65 to 0.87), but p_o decreased with increasing positive potentials. The conductance and selectivity of these channels were assessed under various conditions. Fig. 4 C displays mean I/V curves obtained from several experiments performed in either CsCl or KCl buffer systems. The average conductances were 126 ± 10 pS ($n = 5$) and 146 ± 12 pS ($n = 4$), respectively. Extrapolated reversal potentials were close to or higher than +30 mV, regardless of the cation used. In asymmetrical 50 mM *trans*/450 mM *cis* CsCl, the conductance was 119 pS ($n = 5$) and the reversal potential was 43 mV, whereas in symmetrical 450 mM CsCl, the unitary conductance was 144 pS and the reversal potential was 0 mV (data not shown). Together, these observations argue in favor of the Cl^- selectivity of this pathway over monovalent cations. These large conductance values are close to the mean values of 150 pS determined in 140 mM Cl^- , as previously reported for nuclear Cl^- channels seen in patches on liver nuclei (Tabares et al., 1991). Of the 56 bilayers that possessed channel activity upon fusion of SF_2 vesicles in Cl^- buffer systems, 66% (24/36) contained characteristic large conducting Cl^- -selective channels. Because most single fusion events resulted in multiple Cl^- channel activities, the density of this type of channel has been estimated in the range of 2 to 4 per vesicle. Assuming a mean radius of $0.150 \mu\text{m}$ for a vesicle, this would correspond to a channel density of 10 channels/ μm^2 .

It was of interest to test if this kind of channel was expressed in cardiac nuclei from other mammalian species. Nuclear microsomal fractions were prepared from rabbit cardiomyocytes and fused into phospholipid bilayers. The channels reconstituted from rabbit nuclei share several features with the Cl^- channels reconstituted from sheep preparations. Its unit conductance was 170 ± 12 pS ($n = 3$), and subconducting states were observed at various potentials, but their contribution did not exceed 10% of the total p_o . Rabbit nuclear ionic channels were also slightly voltage sensitive at negative potentials, and their p_o decreased at potentials close to 0 mV (data not shown). Thus, the high Cl^- conducting channel is functionally expressed in the cardiac nuclei from various species. It was mainly reconstituted from the SF_2 fraction (24 times out of 36 recordings), but it was also detected upon fusion of SF_3 and SF_4 vesicles (7 times out of 24 recordings). This raises questions about their physiological role and the putative regulation of their gating mechanism along the cell cycle.

Modulation of the NIC activity

To identify putative ligands that might modulate the activity of the Cl^- channel, we have tested various agents such as Ca^{2+} , the corticosteroids thyroid hormone (T_3), glucocorti-

coids (dexamethasone), and retinoid (retinoic acid), all known to possess nuclear receptors (Howell and Lefebvre, 1989; Hudson et al., 1990). Neither the gating nor the conducting behavior of the high-conductance Cl^- -selective channel was directly affected by varying the Ca^{2+} concentrations from 0.1 μM to 2 mM, added either to the *cis* (cytoplasmic) or the *trans* (luminal) side of the channel protein. The thyroid hormone and dexamethasone had no effect on the high-conductance Cl^- -selective nuclear ionic channel, but retinoic acid was found to activate the channel. Fig. 5, A, B, and C, illustrates typical unitary current traces recorded at -30 mV, in 250 mM *cis*/50 mM *trans* CsCl, before and after the addition of 10^{-8} M retinoic acid. Channel activation started within 2 min, resulting in higher open probability (Fig. 5, A'-C'). Fig. 5 D shows that the stimulatory effect of retinoic acid on the channel lasted at least 4 min, increasing the p_o values from 0.27 to a mean value close to 0.90. Nevertheless, retinoic acid had no effect on

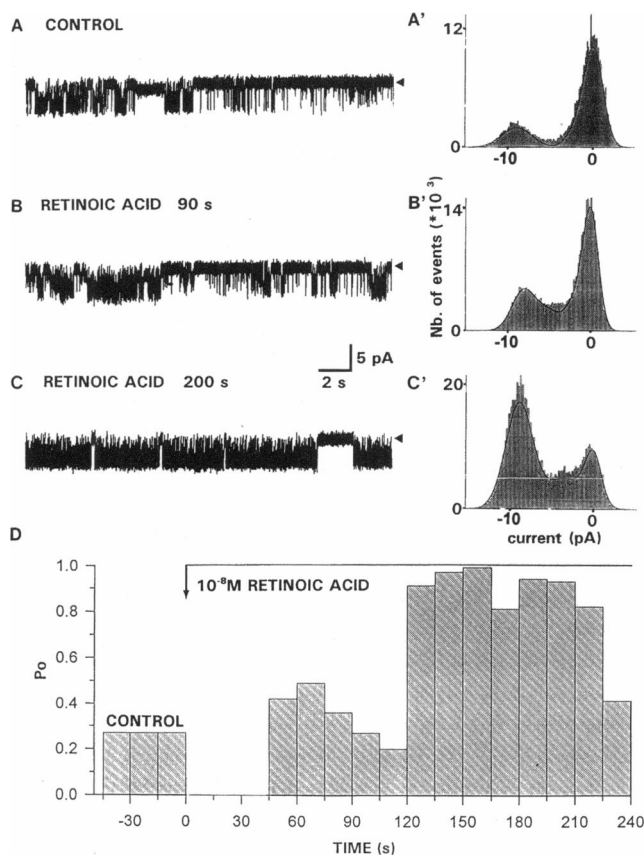


FIGURE 5 Effect of retinoic acid on the high-conductance Cl^- channel of the nuclear envelope outer membrane. Single channel activity recorded at -30 mV upon fusion of a vesicle derived from the SF_2 fraction, in the standard experimental conditions described in Fig. 4 B. (A) Current trace obtained in control conditions. (B and C) Single channel activities recorded 90 and 200 s after the addition of 10^{-8} M retinoic acid in the *cis* chamber. (A', B', C') Corresponding amplitude histograms demonstrating the stimulatory effect of retinoic acid on the open probability. (D) Time-dependent activation of the Cl^- channel after a single addition of retinoic acid as quantified on 15-s segments of steady state recording at the same holding potential.

the current amplitude and unitary conductance of the channel. The dose dependence and the specificity of this stimulating effect on the gating of NIC remains to be ascertained under various conditions. However, it represents the first example of the modulation of the gating behavior of a NIC by a morphogenic drug.

Reconstitution of a low-conductance, anion-selective channel

A low-conductance channel was detected in the SF₂ (Fig. 6 A) as well as in the SF₃ fraction. This channel displays a high p_o at all voltages applied (Fig. 6 A), with a low conductance of 30 pS and a positive reversal potential around +20 mV (Fig. 6 B), which argues in favor of its Cl⁻ selectivity. This channel activity was recorded less frequently in the SF₂ than in the SF₃ or SF₄ fractions taken together, with 33% compared to 53% of the total number of fusion events in Cl⁻ buffers. Because a similar NIC activity was reported to be ATP sensitive (Tabares et al., 1991), we have tested the effect of ATP on its gating behavior. Fig. 6, C and C' show the qualitative results obtained from a single channel experiment before and after the addition of 2 mM Mg-ATP to the *cis* chamber. ATP induced a flickering and a decrease of the overall p_o . This reduction in p_o was mainly related to a decrease in the mean open times (Figs. 6, D and D'). The increase in mean closed time induced by Mg-ATP was not significant. However, the number of closed events increased.

Reconstitution of cation channels in SF₂ and SF₃ populations

To test for the presence of cation-selective channels in the cardiac nuclear membranes, we have fused vesicles from the SF₂ or the SF₃ fraction in either KCl or K-gluconate solutions. As illustrated on Fig. 7, fusion of SF₂ vesicles yielded high-conductance channels in 7 of 9 experiments performed in KCl. This channel activity was voltage sensitive, displaying low p_o at negative potentials and high p_o at positive potentials. Conducting behavior and selectivity are reported in Fig. 7, B and C. This channel displayed a mean conductance of 181 ± 10 pS in 50 mM *trans*/450 mM *cis* KCl, with a reversal potential of -41 mV (Fig. 7 B). Its unit conductance was not affected by a reduction of the ionic gradient across the bilayer. However, the reversal potential, which was close to 0 mV in symmetrical 450 mM KCl, was shifted toward more negative values, -17 and -41 mV in asymmetrical 250 mM *trans*/450 mM *cis* and 50 mM *trans*/450 mM *cis* KCl, respectively, thus arguing for the cation selectivity of this channel. The *I/V* curve of the subconducting state yielded a conductance value at 56% of the main conductance level and an identical reversal potential (Fig. 7 C). In another series of experiments, cation channels of lower conductance values (75 pS) were observed in a small number of bilayers (8 of 24) in which we were able to fuse vesicles from the SF₃ and SF₄ populations, believed to be mainly derived from the inner membrane of the NE (Table 2).

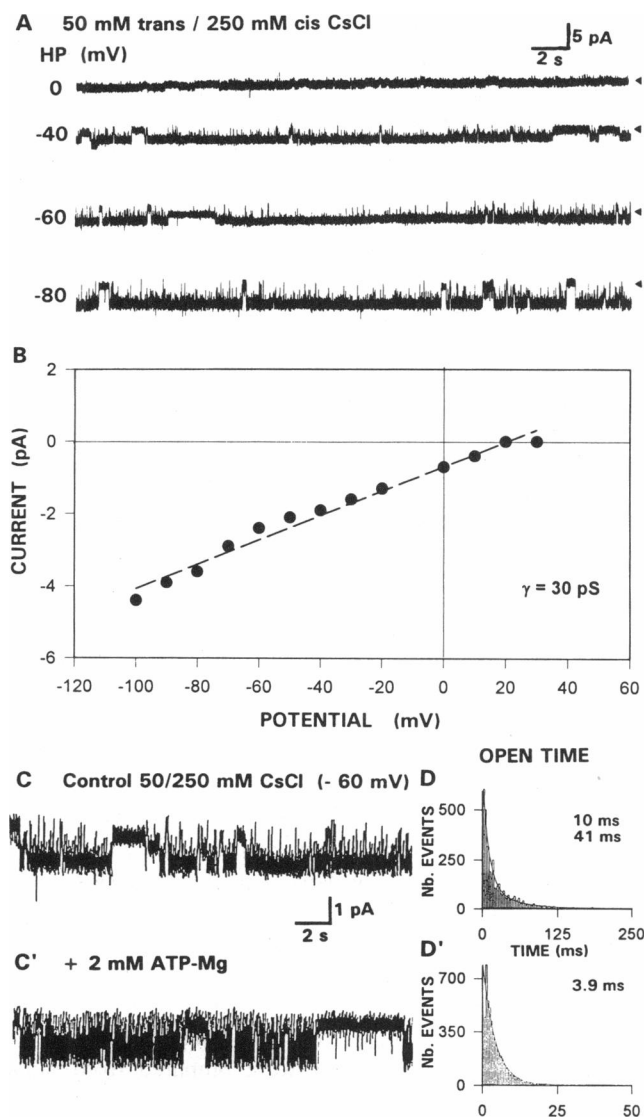


FIGURE 6 Biophysical properties and ATP sensitivity of the low-conductance anion-selective channel. (A) Unitary current traces recorded under steady-state conditions in 50 mM *trans*/250 mM *cis* CsCl, plus 10 μ M free Ca²⁺ (109 μ M CaCl₂ + 100 μ M EGTA) in 10 mM Tris-HEPES, upon fusion of a vesicle from the SF₂ fraction. Channel activity increased at more negative potentials. Dark arrows on the left represent the closed-state level. (B) This channel displayed a low unit-conductance value ($\gamma = 30$ pS) and a positive reversal potential, which reveals its Cl⁻ selectivity over Cs⁺ or HEPES. (C) Magnified current trace recorded in the same buffer system. Note the occurrence of subconductance states. (C') Addition of 2 mM ATP to the *cis* chamber induced a flickering behavior. (D) The open time distribution was fitted by the sum of two exponentials with time constant values of 10 and 41 ms, respectively. (D') In the presence of Mg-ATP, the open time distribution was fitted by a single exponential with a time constant of 3.9 ms. Note the different time scales: $T_{\max} = 250$ ms in D and 50 ms in D'.

DISCUSSION

This study demonstrates that it is possible to enrich vesicle populations in outer or inner membranes from the nuclear envelope of mammalian cardiomyocytes. Using the planar lipid bilayer fusion technique, we have recorded and char-

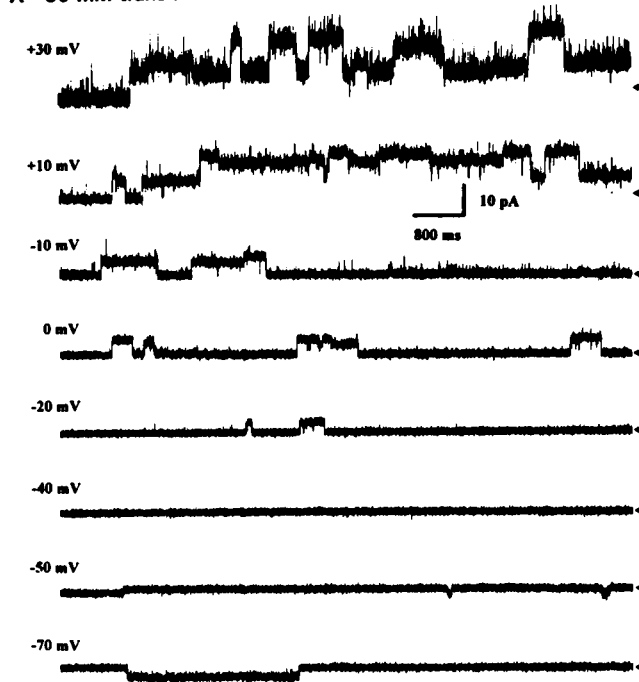
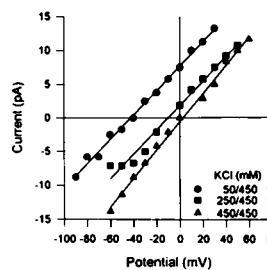
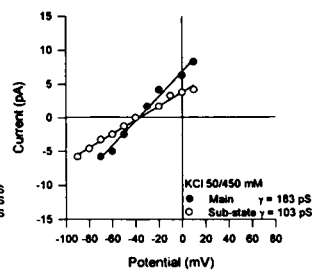
A 50 mM trans / 450 mM cis KCl**B****C**

FIGURE 7 Cation-selective channel reconstituted from the nuclear outer membrane. (A) Unitary channel activities recorded after fusion of SF₂ vesicles into bilayers made of PS/PE (70/30), in 50 mM *trans*/450 mM *cis* KCl, 2 mM CaCl₂, and 10 mM Tris-HEPES, pH 7.2. Because of their voltage sensitivity, the presence of two channels was revealed at positive voltages. Dark arrows on the left represent the closed-state level. (B) Current-voltage relationships determined in various experimental conditions 50/450–250/450 and 450/450 mM KCl (*trans/cis*). Note the shift in reversal potentials (zero-current voltage) toward 0 mV in symmetrical 450 mM KCl. (C) Corresponding *I/V* curves of the main (●) and subconducting states (○) in asymmetrical 50/450 mM KCl, showing identical reversal potentials around -40 mV.

acterized the ionic channels present in this membrane system, which delimits the perinuclear space. Moreover, we demonstrate that this methodological approach represents a useful and versatile alternative to the patch-clamp technique recently used to probe the outer membrane of the NE (Mazzanti et al., 1991; Dale et al., 1994; Bustamante, 1994a; Stehno-Bittel et al., 1995).

Characterization of the nuclear membrane fractions

As demonstrated in the Results by various methodological approaches, cardiac nuclei can easily be separated from

TABLE 2 Number of experiments performed with each subfraction of the NE and relative occurrence of the various channel activities

	No. of experiments	Channel occurrence			
		150 pS Cl ⁻ channel	30 pS Cl ⁻ channel	180 pS K ⁺ channel	Low-conductance X ⁺ channel
SF ₁	4	—	—	—	—
SF ₂	56	24/36	4/11	7/9	0/9
SF ₃	31	3/ 9	7/12	0	5/10
SF ₄	14	4/14	7/14	0	3/14
Total	105				

Note: Several reconstitution experiments yielded complex multiple channel activities coupled to a leak current. Consequently, it was difficult if not impossible to ascertain the number and the type of channel recorded.

other organelles such as mitochondria, SR, and RER. These separations were performed in the absence of detergent treatment to preserve the integrity of the NE and to prevent bilayer destabilization upon membrane reconstitution. The chromatin of isolated nuclei was digested after a short sonication step to prepare ghosts of NE, whose membranes were then vesiculated by sonications and separated into various membrane populations on sucrose gradients. Electron microscopy, gel electrophoresis, Western blot analysis, as well as biochemical and binding assays, all demonstrate qualitative and quantitative differences between the various subfractions (SF₁–SF₄). Data summarized in Table 1 show that according to [³H]ryanodine binding levels and DNA contents, the nuclei are basically not contaminated by the membrane vesicles derived from the SR, and vice versa. Furthermore, various observations indicate that our cardiac nuclei preparations displayed little contamination by RER membranes, as in the case of nuclei isolated from starfish oocytes (Dale et al., 1994). Together, these observations confirm that the cardiac nuclear preparations were of good quality, because they were only slightly contaminated by other intracellular materials.

Specific intranuclear markers

The lamins were shown to be associated with the inner membrane of the animal and fungal nuclear envelope (Lebel and Raymond, 1984; Georgatos and Blobel, 1987). Because they are preferentially immunolocalized within the SF₃ and SF₄ fractions recovered from sucrose or CsCl gradients, it was proposed that these two nuclear membrane populations were slightly enriched in vesicles derived from the inner membrane of the NE (see Results and Fig. 3, A and B). Evidence such as the low lamin and DNA contents suggests that the SF₂ population would contain vesicles mainly derived from the outer membrane of the NE, which was shown to be related to the RER by the presence of the SEC 61 protein (Fig. 3 C). These vesicle populations were then used for our proposal investigations involving membrane reconstitution.

Reconstitution of chloride conductances of the NE

Using the PLB fusion technique, single- and multiple-channel measurements indicate that it is possible to record the activity of anion-selective channels of the NE. These channels were spontaneously active for several minutes at various transmembrane potentials in the absence of cytoplasmic factors and ATP. We have characterized the properties of a high-conductance (150–180 pS) Cl^- -selective channel from the cardiac nuclei of two mammalian species, namely, sheep and rabbit. This type of channel was poorly voltage sensitive at negative voltages. However, the p_o increased with positive voltages. This high-conductance, anion-selective channel might be related to the 150 pS NIC initially described in the outer membrane of liver nuclei by Tabares et al. (1991), using the patch-clamp technique in the nucleus-attached configuration. Actually, our data complement the initial recordings shown by Tabares and co-workers. For instance, we have ascertained the presence of a subconducting state whose conductance occurred at 50% of the main conductance.

The exact physiological role of this type of nuclear Cl^- channel is not known. However, according to its density (10 channels/ μm^2), this channel must be highly expressed in the outer membrane of the NE, thus suggesting that the NE displays a large permeability to monovalent anions. Because a Ca^{2+} - Mg^{2+} ATPase (Lanini et al., 1992) and IP_3 receptors (Malviya et al., 1990; Nicotera et al., 1990; Stehno-Bittel et al., 1995) have been reported in the NE, one possibility is that these channels might play a role as counter-charge carriers during the Ca^{2+} uptake and release fluxes from the perinuclear space. As mentioned recently, counter charge might also be required during the transport of negatively charged macromolecules such as mRNA across nucleopores (Bustamante, 1994b). An alternative possibility to be considered is that these Cl^- channels, which are poorly voltage sensitive, might be activated during remodeling of the NE along the cell cycle, which also involves lamin network depolymerization and polymerization processes (Meier and Georgatos, 1994). The artificial fragmentation procedure used in our methodological approach might facilitate the activation of this type of channel in our reconstitution system. On the other hand, the gating of this channel protein might be controlled by biochemical agents that were lost during the isolation procedure and remain to be identified.

Ligand-modulated NIC

Various agents such as dexamethasone, thyroid hormone (T_3), and retinoic acid possess nuclear receptors (Howell and Lefebvre, 1989; Newcomer, 1995). However, their mode of action on transmembrane signaling remains to be clarified. In this work we have disclosed that retinoic acid has a stimulating effect on the activity of the 150 pS anion-selective channel. This original observation is of in-

terest, because biological effects of retinoic acid have been associated with binding of intranuclear heterodimeric receptor-ligand complex to specific hexanucleotide sequences (AGGTCA) (Newcomer, 1995). We cannot conclude that the effect of retinoic acid on the high-conductance Cl^- channel represents a new way to promote gene replication. But this observation might represent one mode of action of retinoids, which have a wide variety of effects in pharmacological concentrations. Moreover, the present observation opens the path to a new series of investigations to ascertain the role of the receptors of NE in regulating the ionic conductances of this membrane system.

The presence of a 30-pS Cl^- -conducting channel was also reported upon fusion of SF_2 , SF_3 , or SF_4 vesicles (Table 2). Consequently, it is difficult to ascribe a specific localization of this type of anion channel. ATP was found to modify its gating behavior, inducing flickering and a decrease in p_o . The inhibitory effect of ATP for this low-conductance anion-selective nuclear channel was also reported in patch-clamp experiments (Tabares et al., 1991).

Despite the fact that ATP was shown to increase the Cl^- conductance of the ER microsomes derived from pig pancreas (Bégault et al., 1993), we have never observed an effect of Na^+ -ATP or Mg^{2+} -ATP (up to 2 mM) on the activity of the large Cl^- channel, which is believed to be localized in the outer membrane of the NE (Figs. 4 and 5 and Table 2). It was recently shown that addition of ATP in patch pipettes increases the macroscopic currents recorded from *Xenopus* oocyte nuclei, in situ (Mazzanti et al., 1994). These authors also reported that ATP failed to activate macroscopic currents in isolated nuclei. Based on their electrophysiological measurements and morphological observations, they assumed that the ionic conductances that were activated by an ATP-dependent mechanism were associated with the proteins forming the nucleopore complexes. It could be hypothesized that the bilayer technique might help to resolve the issue of the putative presence of an ionic channel in the core of one of the protein subunits forming the NP.

Based on the patent radius of 43 Å (Paine et al., 1975), nucleopores (NPs) are expected to display very large conductances of 1000 pS (Bustamante, 1994a; Mazzanti et al., 1994). From the very beginning of this study, we have tried unsuccessfully to detect the incorporation of NP into PLBs. We have never observed such a large current event under steady-state conditions. However, we should mention that large electrical activities were often observed transiently (<1 s). It was impossible to tell if these activities resulted from NP incorporation or bilayer destabilization. It is reasonable to assume, however, that incorporation of NP complexes into bilayers would result in rapid destabilization of the film, because NP would tend to incorporate between the two hemi-leaflets of the phospholipid bilayers (Dessev, 1992). This view is based on the well-characterized topology and the structure of the NP within the NE in situ, where the hydrophobic regions of NP complexes were shown to interact with the luminal hemi-lipid-leaflet.

Cation channel segregation within the NE

The presence of cation channels in the membrane population derived from the NE were tested by using various buffers. The differential distribution of the cation channels between the outer and inner membranes of the NE seems easier to establish than the distribution of the Cl^- channels. We have shown that a low-conductance cation channel of poor selectivity is mainly found in the membrane vesicles of the SF_3 population, which would be enriched in vesicles derived from the inner membrane (see Table 2). The results from another series of experiments ($n = 9$) using KCl buffer systems indicate that a high-conductance (180–200 pS) K^+ -selective channel was consistently expressed in the vesicles of the SF_2 population, which was mainly derived from the NE outer membrane. The p_o of this K^+ channel is voltage sensitive (as illustrated in Fig. 7 A).

Similar high-conductance cation channels have already been detected at the surface of mouse cardiac nuclei by using patch-clamp measurements (Bustamante, 1994a,b). Although of higher conductance, this K^+ channel shares several similarities with the K^+ channel present in the SR membrane ($\gamma = 140$ pS) of cardiac cells (Rousseau et al., 1992), as both channels are insensitive to various blockers (Cs^+ , TEA^+) and typical inhibitors of surface membrane K^+ channels (Bustamante, 1994b).

Implication of NIC in stimuli-replication coupling

Stimuli affecting gene expression necessarily involve the communication of signals to the nucleus. Modulation of the permeabilities of the nuclear membranes might represent one of these signaling pathways that has not yet been considered (Jans, 1994). Although the exact role of the NICs has not yet been elucidated, they could be involved in the signaling processes that preceded replication, mitosis, and apoptosis. Recent studies suggest that DNA replication is preceded by a change in the permeability of the nuclear membrane induced by lysophosphatidyl choline (Leno and Munshi, 1994). Moreover, NICs are probably involved in electrolyte movements that might occur during reassembly of NEP-A and NEP-B particulate fractions of the NE at the end of mitosis (Vigiers and Lohka, 1991; Sullivan et al., 1993). This working hypothesis could be challenged in cell-free systems in which nuclear membrane reassembly was studied (Burke and Gerace, 1986). Alternatively, other metabolites and biological agents, like angiotensin, whose nuclear membrane receptors have been described (Eggena et al., 1993), might represent relevant ligands for NICs.

CONCLUSION

The present study represents a new step toward the elucidation of the electrophysiological properties of the outer and inner membranes of the nuclear envelope. Despite the fact that we could underestimate the role of cytoplasmic as well as nucleoplasmic and luminal (perinuclear space) factors,

our present results demonstrate that it is possible to record the activity of several types of NIC in an artificial reconstitution system. From this respect, the NIC does not differ from the channel proteins reconstituted from other intracellular SR membranes (Rousseau et al., 1992).

Further characterization of the former and newly identified NIC, such as the IP_3 -activated and heparin-inhibited channels (Mak and Foskett, 1994; Stehno-Bittel et al., 1995), may provide valuable information about the physiological significance of the ionic permeabilities of nuclear membranes in eucaryotic cells. Moreover, our experimental approach might be useful to test the putative effects of various drugs characterized as mitogenic, tumorigenic, or morphogenic agents whose nongenomic mode of action remains to be ascertained.

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