J. Membrane Biol. 131, 137–142 (1993)

The Journal of
Membrane Biology


# Identification of a Nucleo-Cytoplasmic Ionic Pathway by Osmotic Shock in Isolated Mouse Liver Nuclei

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**Summary.** The observation that the nuclear envelope outer membrane contains ion channels raises the question of whether these conductances communicate between the cytosol and the nuclear envelope cisternae or between the cytosol and the cytoplasm. Failure to detect large, nonselective holes using the patch-clamp technique has led to the speculation that ion channels and nuclear pores are in fact the same.

In this paper we present evidence that the ionic channel, recorded in isolated liver nuclei with the patch-clamp configuration of "nucleus-attached," spans the double membrane of the envelope, providing a direct contact between nucleoplasm and cytoplasm.

**Key Words** nuclear envelope permeability · ion channels · nuclear pores

## Introduction

The presence of ionic conductances in the nuclear membrane was first demonstrated in mouse zygote pronuclei (Mazzanti et al., 1990). Evidence that the nuclear envelope contains ion-selective channels in avian erythrocytes (Matzke et al., 1990), in mouse oocyte germinal vesicles, nuclei from two-cell embryos and liver nuclei (Mazzanti, De Felice & Smith, 1991) and rat liver nuclei (Tabares, Mazzanti & Clapham, 1991) could be sufficient to conclude that nuclear ionic conductances are widespread.

The interpretations about the function of these intracellular communication pathways are diverse and all are plausible. However, there are two main views that could coexist: the first speculates that the channel is located on the outer membrane of the envelope; the second associates the ionic conductance with the nuclear pore complex. Both these hypotheses have evidence in their support.

It is known that the external membrane of the nuclear envelope is continuous with the endoplasmic reticulum. It is therefore not surprising to find reticulum-like ionic channels (Coronado, Rosenberg & Miller, 1980; Tang, Wang & Eisenberg, 1989) also on the nuclear envelope. In addition, the association of ribosomes on the nuclear surface could justify the presence of a large ionic channel implicated in protein transport (Simon & Blobel 1991).

On the other hand, the presence of a nuclear resting potential in some nuclei (Kanno & Loewenstein, 1963; Loewenstein & Kanno, 1963; Matzke, Matzke & Neuhaus, 1988; Mazzanti, 1990) suggests the presence of an ion-selective nuclear envelope. The possibility that chromatin and large intranuclear proteins could generate a resting potential due to a Donnan equilibrium is unlikely: if this is the case, a nuclear resting potential would be present in all eukaryotic cells. Instead, it was experimentally demonstrated not to be true that, using different preparations, some nuclei present 10–15 mV hyperpolarization (Wiener et al., 1965). Moreover, applying the patch-clamp technique on isolated nuclei, the area of outer nuclear membrane isolated by the pipette in nucleus-attached configuration contains several nuclear pores (Mazzanti et al., 1991). If the pores are open it would be impossible to record any single-channel event. Therefore, we have to presume that either the pores are closed, or that parts of the pore complex function as a multiple-state conductance ionic channel.

The experiments presented in this paper are designed to elucidate if a direct nucleo-cytoplasmic ionic connection exists. Permeabilizing the envelope after reaching a "nucleus-attached" configuration would allow free access of test solutions into the nucleoplasmic side of the envelope, providing useful information towards an answer to this question.

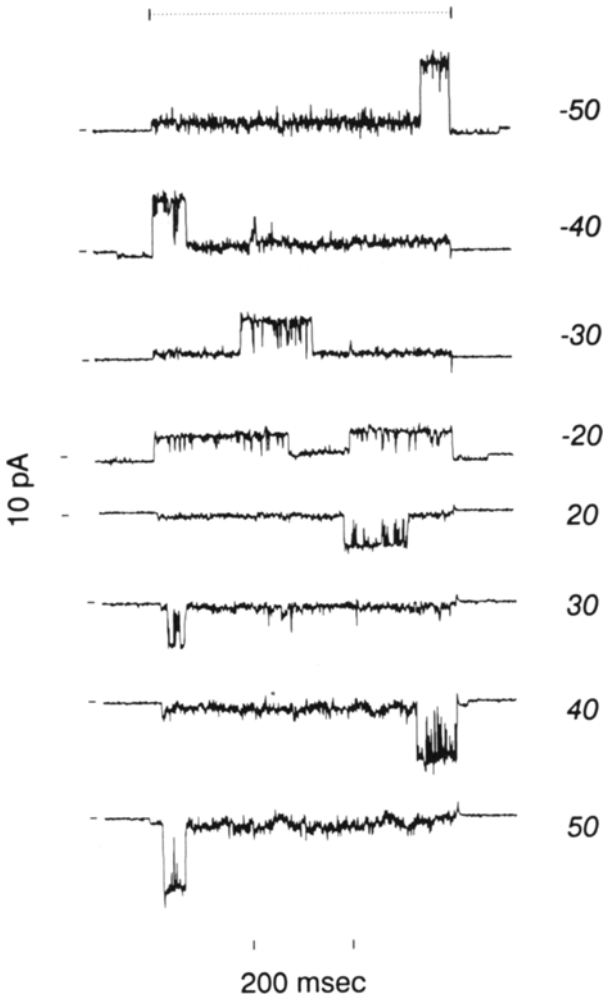


**Fig. 1.** Single-channel recordings from nucleus-attached patches on isolated mouse liver cell nuclei. The pipette potential (Vp) applied to the patch is indicated beside each trace. The voltage simulation was applied for 500 msec (dotted line above).

**Fig. 2.** Freeze-fracture image of a liver nucleus envelope. Horizontal bar: 500 nm. The area delimited by the patch-clamp pipette tip would have a diameter four times the bar, containing ≈30 nuclear pores.

## Materials and Methods

We isolated liver nuclei (Blobel & Potter, 1966) by shearing fresh liver tissue in a homogenizer with a Teflon pestle of 0.025 $\mu$m clearance, and centrifuging the homogenate on a sucrose pad (in mM: 50 Tris HCl, 25 KCl, 5 $MgCl_2$, 250 sucrose) at 30,000 rev/min in a Beckman SW41 rotor at 0–4°C. This procedure results in a pellet of pure nuclei, which we resuspend in the standard bath solution (in mM: 120 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1.1 EGTA, 10 HEPES, 5 glucose, pH 7.4) before use. The patch electrodes were pulled from hard borosilicate glass (Corning 7052) on a Sachs-Flaming P-84 puller (Sutter Instruments). The pipettes were coated with Sylgard (Dow Corning) and fire-polished to an external tip diameter of 1–2 $\mu$m. These electrodes had resistances of 7–10 M$\Omega$. We applied standard cell-attached patch-clamp techniques to obtain nucleus-attached patches between 20 and 50 G$\Omega$ in resistance and single-channel recordings. In all experiments the patch pipette contained 120 KCl solution (above). The ground connection in the recording chamber was agar bridge. All the records were made in steady-state conditions, preventing transient potential shifts due to perfusion of solutions with different ionic concentration. Single-channel currents were recorded with a List EPC-7 patch-clamp amplifier and were digitized and stored on a VCR. Data were analyzed off-line on an IBM-AT computer after filtering at 1,000 Hz.

The freeze-fracture pictures were produced by Hishashi C. Iida, Brenda K. Headrick and Denis DeFoe from the Department of Anatomy, Medical College of Georgia, Augusta, GA.

## Results

Figure 1 shows the current through a single channel isolated on the surface of a liver cell nucleus. Normally we record between two and five different conducting states with a conductance between 200 and 250 pS each (Mazzanti et al., 1991); a single-state transition is a selected rare occurrence. The surface of a liver nucleus presents a high density of nuclear pores (Maul, 1977) as is shown in Fig. 2. From freeze-fracture pictures of nuclear envelopes, it is possible to estimate a density of 14 ± 1.3 pores/$\mu m^2$. The membrane patch area is 2–3 $\mu^2$ (Mazzanti & DeFelice, 1987) and, as a consequence, either the

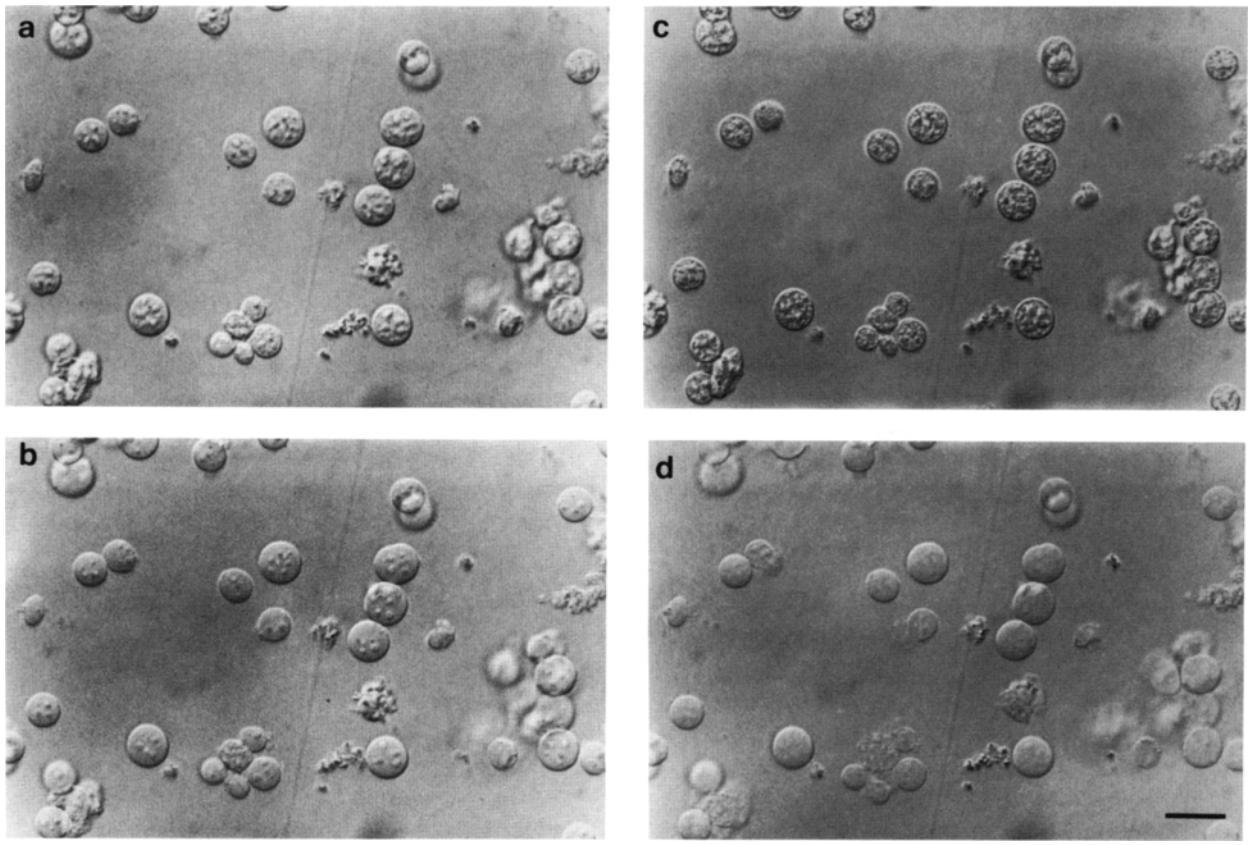


**Fig. 3.** Effect of perfusion solutions with different osmolarity on isolated liver nuclei. (*a*) nuclei are bathed in 120 mM KCl (the control solution). In (*b*) the ion concentration was lowered to 60 mM KCl. It is evident in this case that a swelling takes place. Replacing the bath with 240 mM KCl solution results in a shrinking of the nuclei (*c*). All these processes are reversible. The perfusion of 480 mM KCl, instead, produces an irreversible swelling of the nuclei (*d*). Horizontal bar in (*d*) is 20 $\mu$m.

ionic channels represent fewer pores opening in substate levels, or the pores are completely closed and the channels are different structures.

It is possible to test if the envelopes, after nuclei isolation, form a freely or a semipermeable barrier by superfusing them using solutions of different osmolarity. In the case of free nucleo-cytoplasmic ion diffusion, we should not see any morphological changes of the nuclei. This is not the case, as presented in Fig. 3. A nuclei dispersion is first bathed in 120 mM KCl (*a*). Then it is superfused with 60 (*b*), 180 (*c*) and finally with 480 mM KCl (*d*). The effect of hypo- and hyper-osmotic solutions on isolated nuclei was as expected for semipermeable membranes. The swelling of Fig. 3*b* and the shrinking of Fig. 3*c* is probably due to water fluxes in and out of the nucleus. These changes are fully reversible if the nuclei are then perfused with the control solution. When the nuclei are perfused with 480 mM KCl solution, at first they shrink until the solution in the bath reaches the final concentration and then they irreversibly swell. This phenomenon is expected because increasing the ionic strength of the bath above 400 mM KCl causes a conspicuous increase of the membrane border that delineates the array of pore complexes (Unwin & Milligan, 1982). The swelling that occurs is due to the equilibration of the 480 mM KCl solution with the nucleoplasm, which increases nuclear volume by changing the chromatin assembly (Delpire et al., 1985; Yarmola, Zarudyana & Lazurkin, 1985) and probably also by moving water into the nucleus. Distended nuclei appear stable for up to 30 min, suggesting that the envelope maintains its basic structure through the swelling in high salt.

We have found in this way a clean method to permeabilize the nucleus without destroying the envelope, changing the experimental configuration from "nucleus-attached" to, effectively, an "inside-out" configuration. The use of detergents would destroy the double membrane structure, causing confusion about the final configuration of the experiment.

Figure 4 presents the effect of perfusion on the channel in the patch. With the 120 mM KCl solution

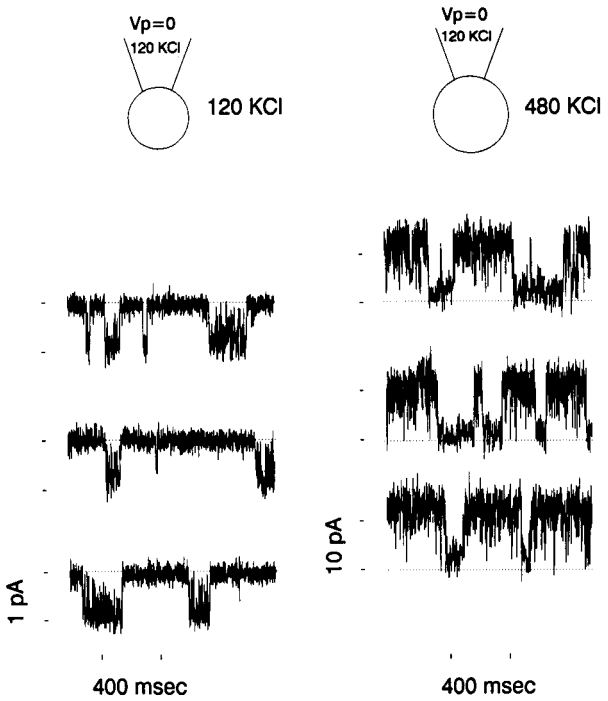


**Fig. 4.** Effect of high salt concentrations on single-channel activity. On the left are three examples of channel behavior at a pipette potential $Vp = 0$ mV in a bath solution containing 120 mM KCl. A relatively small inward current, noted by the downward deflections, flows through the channel isolated in the nuclear patch. On the right are three records from the same experiment, 5 min after the bath solution was changed to one containing 480 mM KCl. At the same pipette potential $Vp = 0$ mV but in 480 KCl, the current is larger (note the different scales in the left and right panels), and it is outward.

in both electrode and bath, and with a pipette voltage of 0 mV, an on-nucleus patch reveals a small inward current. Maintaining the patch electrode on-nucleus, 480 mM KCl solution was perfused. The right side of Fig. 4 depicts the change that occurs to the channel current at the same pipette potential. From inward, the ionic flow becomes outward and is greatly augmented (note the different vertical scale). In Fig. 5*A* the voltage in the pipette was manually changed to obtain a current/voltage relationship for the channel in high salt solution. Figure 5*B* summarizes six of such experiments at different voltages with the nuclei bathed in 120 mM KCl (diamonds) and in 480 mM KCl (squares). The conductance of a single channel in 120 mM KCl is 230 pS, as in the pronucleus. The conductance of the channel in 480 mM KCl is 510 pS, and its reversal potential shifts to the left by 35 mV. The size and direction of the shift indicates a K-selective channel, assuming that the K concentrations in the nucleus are 120 mM and 480 mM in the two cases. Subsequent bath perfusion with 120 mM KCl in three cases was able to reestablish 230 pS conductance but only for a few minutes. Examples of this return to base level are shown in Fig. 5*C*. Usually a change in ion concentration after perfusion with 480 mM KCl resulted in a disruption of the nucleus. In the few cases where we were able to record single-channel events, the experiments were interrupted by losing the G-seal configuration.

## Discussion

The main inconsistency that is found in patch-clamp experiments on the nuclear envelope is the structure-function relationship between the area of the external membrane under observation and the number of nuclear pores that such a membrane contains. In Fig. 2 the freeze-fracture picture shows that a large number of pores could be trapped inside the rim of a patch pipette tip. However, the electrical signals recorded in the nucleus-attached configuration are inconsistent with this morphological structure. The presence of many large aqueous pores with an approximate conductance of 1 nS each (Matzke et al., 1990; Mazzanti et al., 1990) would produce, under adequate stimulation, a large current. The inability to detect such a current, suggests a partial or total closure of the pores (Mazzanti et al., 1990). The idea that enucleation produces a closure of the nucleo-cytoplasmic pathways is supported directly and indirectly. In all the experiments on isolated liver nuclei (Mazzanti et al., 1990; Tabares et al., 1991) and on avian erythrocytes (Matzke et al., 1990), the limited number of events detected excludes the possibility that nuclear pores are fully open or even involved in the process of current transport. Furthermore, the results presented in Fig. 3, obtained by changing osmolarity in the external solution of isolated nuclei, show unequivocally that free diffusion between external (cytoplasmic side) and nucleoplasm does not exist in these conditions.

From these observations we can conclude: nuclear pores are capable of closing not only, as suggested by Akey (1990) to a minimum diameter of 90 Å, but also, in some conditions, of closing all the way, thus denying the passage of ions. The observation may partly explain the observed increased resistance of nuclear envelopes induced by ecdysone, which cannot be explained by a change in nucleoplasmic ion concentration (Ito & Loewenstein, 1965).

The perfusion of high salt solution is a good approach to permeabilize the envelope without destroying the nuclear architecture. Observing the swelling of the nucleus bathed in the high salt solution makes us confident that the nucleoplasm and the bath have reached essentially the same concentration during the perfusion. The shift in the channel

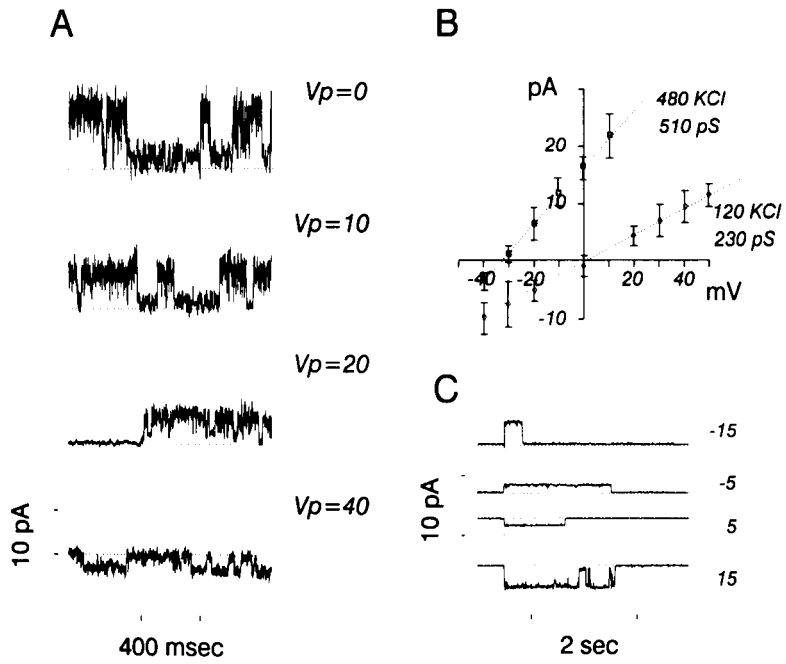


**Fig. 5.** Effect on the conductance of the nuclear channel after perfusion with high salt solution. The panel on the left (*A*) illustrates current traces at four pipette potentials. On the top right (*B*) current-voltage relationships generated from six separate experiments in which the solution was initially 120 mM KCl and subsequently replaced with 480 mM KCl (Fig. 4). The voltage axis in the $i(V)$ curves was calculated from the equation $V = 0 - Vp$. In (*C*) we plot current traces at four different $Vp$, representing the recovery in control solution (120 mM KCl).

reversal potential is predictable by the Nernst equation assuming a K-selective channel. The rise in the conductance is a consequence of increased concentrations facing the channel. Native conductance is resumed by perfusing a control solution (Fig. 5*C*).

These results led to further conclusions. High salt solution produced a continuity between the external solution and the nucleoplasm but appears not to involve the membrane structure in the patch during the recordings. The second observation is that the channel experiences a change in the ion concentration on the nucleoplasmic side of the patch. By the paradigm of the experiment, the ion channel thus spans the double membrane of the nuclear envelope. A different interpretation could be that the inner membrane of the envelope is extremely leaky. In this case even a channel on the outer membrane would have the same behavior, but would still implicate a total closure of nuclear pores.

In conclusion, we suggest that the ionic channel is part of the nuclear pore complex. The pores are, in the physiological condition, open. During enucleation, ionic similarity of "external" solution is not sufficient to maintain the pores fully open. Single-channel activity recorded with the patch-clamp technique is the result of partial functioning of the nucleo-cytoplasmic pathway.

The future challenge will be to find the cellular component(s) or the nucleo-cytoplasmic cooperative mechanism that is able to keep the nuclear pore open.

We thank Mr. G. Mostacciuolo for excellent technical assistance. We also thank Drs. F. Ferroni, L. Smith for critical reading of the manuscript and Hishashi C. Iida, Brenda K. Headrick and Denis DeFoe from the Medical College of Georgia, Augusta, GA, for the beautiful freeze-fracture pictures.

## References

Akey, C.W. 1990. Visualization of transport-related configuration of the nuclear pore transporter. *Biophys. J.* **58:**341–355

Blobel, G., Potter, V.R. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science* **154:**1662–1665

Coronado, R., Rosenberg, R.L., Miller, C. 1980. Ionic selectivity, saturation, and block in a $K^+$-selective channel from sarcoplasmic reticulum. *J. Gen. Physiol.* **76:**425–445

Delpire, E., Duchene, C., Goessens, G., Gilles, R. 1985. Effect of osmotic shocks on the ultrastructure of different tissues and cell types. *Exp. Cell Res.* **160:**106–116

Ito, S., Loewenstein, W.R. 1965. Permeability of a nuclear membrane: Changes during normal development and changes induced by growth hormone. *Science* **150:**909–910

Kanno, Y., Loewenstein, W.R. 1963. A study of the nucleus and cell membranes of oocytes with an intracellular electrode. *Exp. Cell Res.* **31:**149–166

Loewenstein, W.R., Kanno, Y. 1963. Some electrical properties of a nuclear membrane examined with a microelectrode. *J. Gen. Physiol.* **46:**1123–1140

Matzke, M.A., Matzke, A.J.M., Neuhaus, G. 1988. Cell age-related differences in the interaction of a potential-sensitive fluorescent dye with nuclear envelopes of *Acetabularia Mediterranea*. *Plant, Cell Environ.* **11:**157–163

Matzke, A.J.M., Weiger, T.M., Matzke, M.A. 1990. Detection

of a large cation-sensitive channel in nuclear envelopes of avian erythrocytes. *FEBS Lett.* **271(1,2):**161–164

Maul, G.G. 1977. The nuclear and cytoplasmic pore complex: structure, dynamics, distribution, and evolution. *Int. Rev. Cytol.* **6 (Suppl.):**75–186

Mazzanti, M., DeFelice, L.J. 1987. Na channel kinetics during the spontaneous heart beat in embryonic chick ventricle cells. *Biophys. J.* **52:**95–100

Mazzanti, M., DeFelice, L.J., Cohen, J., Malter, H. 1990. Ion channels in the nuclear envelope. *Nature* **343:**764–767

Mazzanti, M., DeFelice, L.J., Smith E.F. 1991. Ion channels in murine nuclei during early development and in fully differentiated adult cells. *J. Membrane Biol.* **121:**189–198

Simon, S.M., Blobel, G. 1991. A protein-conducting channel in the endoplasmic reticulum. *Cell* **65:**371–380

Tabares, L., Mazzanti, M., Clapham, D.E. 1991. Chloride channels in the nuclear membrane. *J. Membrane Biol.* **123:**49–54

Tang, J.M., Wang, J., Eisenberg, R.S. 1989. $K^+$-selective channel from sarcoplasmic reticulum of Split Lobster muscle fibers. *J. Gen. Physiol.* **94:**261–278

Unwin, P.N.T., Milligan, R.A. 1982. A large particle associated with the perimetre of the nuclear pore complex. *J. Cell Biol.* **93:**63–75

Wiener, J., Spiro D., Loewenstein, W.R. 1965. Ultrastructure and permeability of nuclear membranes. *J. Cell Biol.* **27:**107–117

Yarmola, E.G., Zarudnaya, M.I., Lazurkin, Yu. S. 1985. Osmotic pressure of DNA solutions and effective diameter of the double helix. *J. Biol. Structure & Dynamics* **2(5):**981–993

Received 16 April 1992; revised 9 July 1992