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Presence of Zinc and Calcium Permeant Channels in the Inner Membrane of the Nuclear Envelope

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The nuclear envelope possesses specific ion channels that regulate the ionic traffic between the cytoplasm or the perinuclear space and the nucleoplasm. Using the patch-clamp technique to isolated rat nuclei exhibiting only the inner membrane of the nuclear envelope, we report the existence of calcium and zinc permeant channels. These channels displayed similar characteristics (conductance : 8 and 11 pS respectively, open time constant (3.5 ms and 3.7 ms) and close time constant (5.1 ms and 4.8 ms)) and were insensitive to different types of calcium channels blockers and to calcium concentration in the bathing solution. The exact role of these channels remains to define, but they may contribute to the regulation of intranuclear Ca^{++} or Zn^{++} dependent processes as important as cell proliferation or programmed cell death. Moreover, this work demonstrates that our nuclei preparation provides a way to study the inner membrane of the nuclear envelope. © 1997 Academic Press

Various biological activity inside the nucleus are regulated by mineral ions and will depend upon the transfer from cytosol across the nuclear membrane. Nuclear envelope consists of an inner membrane and an outer membrane interrupted by nuclear pores. The outer nuclear membrane constitutes a continuum with the endoplasmic reticulum, whereas the inner membrane is lined by the nuclear lamina. Nuclear envelope contains pores for the selection of macromolecules, but these pores are considered to be too large to act as barriers against ions (1). Nevertheless, electrical potential across nuclear envelope and segregation of ions in the nucleoplasm have been reported, suggesting that there could exist ion-selective channels in the nuclear membrane (2). Patch clamp studies performed on the outer membrane of zygote pronuclei and erythrocytes, liver and pancreatic nuclei have demonstrated the presence of different types of ionic conductances, in particularly chloride, potassic and calcium channels (3, 4, 5, 6). However, only few studies have reported the presence of Ca^{2+} channels on the inner membrane of the nuclear envelope (6, 7, 8). Nevertheless, differences between the Ca^{2+} concentration in the nucleoplasm and the cytosol have been observed (9) and it has been proposed that the nucleus is largely insulated from large cytosolic Ca^{2+} changes (10). It is now recognized that the nuclear envelope itself functions as a Ca^{2+} storage pool and that the nucleus contains an endoplasmic reticulum-type Ca^{2+} pump (11), enzymatic equipment for inositol 1,4,5-trisphosphate (IP3) production (12-13) and functional receptors for the Ca^{2+} releasing messenger IP3 (13) and cyclic-ADP-Ribose (14). Altogether, these indications suggest that nuclear and cytosolic calcium signals are differentially regulated and are independent of each other. Less data exist concerning the mechanism of Zn^{++} incorporation in the nucleus. However, it is known that the zinc content in the nucleus is regulated. The level of free zinc in the nucleus is very low comparatively to the extranuclear level, suggesting a regulatory process. Incubation of nucleus with a zinc-rich medium fails to produce a net increase in the nuclear zinc level unless ionophores are added to the medium By contrast with calcium the accumulation of intranuclear zinc is not ATP dependent and is not inhibited by tharsigargin a calcium-pump inhibitor (15)

Calcium and zinc play both an important role in biological intranucleus processes, in particularly they both possess a wide variety of biological intranuclear activities. For example, Ca^{2+} contribute to the regulation of gene expression (16-17), to cell viability, the control of the cell cycle, apoptosis and to the growth of transformed cells. Zn is essential at several points of the mitotic cycle, where it may interact reversibly to acti-

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vate DNA polymerase (18). Zinc is an essential component in hundreds of nuclear proteins involved in the transcription of genes. The zinc finger family is a very large group of transcrition factors forming with zinc particular domains classified in 10 different structural groups (19). The removal of zinc increases the sensitivity to oxidation and the inactivation of factors as p53 or Sp1. It is important to notice that a nuclear zinc storage protein metallothionein is involved in activation of transcription factors with zinc finger domain as Sp1 (20). Calcium and zinc are involved in regulation of cell numbers by their roles in both proliferation and apoptosis (17, 21, 22).

Although it has been shown in different cells that zinc blocks voltage-activated calcium channels, zinc is able to induce excitation-transcription through voltage-dependent calcium channels (16). Nevertheless, if previous reports have already established the presence of calcium channels in the outer membrane of the nuclear envelope, zinc channels or transporters have never been reported. Taken together these observations lead to suspect the existence of functional structures on the inner membrane of nuclei, resulting in cytoplasmic/nucleoplasm (or nuclear envelope/nucleoplasm) Ca^{++} and Zn^{++} transfers. So we have applied the patch-clamp technique to the inner membrane of rat isolated nuclei. The present paper provides procedure to obtain nuclei exhibiting only inner membrane and demonstrate the presence of calcium and zinc channels on this membrane.

MATERIALS AND METHODS

Nuclei isolation. Hepatocyte nuclei were isolated using a non enzymatic extraction technique. All steps were performed at 4°C. Pieces of liver lobe (600 mg) were placed in a buffer (solution A) containing (mM) Tris-HCl, 10 (pH 7.4); $MgCl_2$, 10 ; sucrose, 250 ; phenylmethylsulfonyl fluoride, 0.1 ; dithiothreitol, 2.6 and homogenised using a Potter. The homogenate was centrifuged at 1000 × g for 10 min. The pellet was resuspended in 15 volumes, then filtered through a Nylon membrane with a mesh size of 100 mm, and centrifuged as before. The pellet was resuspended in the buffer containing 0.5% Triton X-100 to further lyse the cells, centrifuged as before, and finally resuspended in buffer containing 2.2 M (instead of 0.25M) of sucrose (solution A). Before use, nuclei were placed at 4 ¡C for 4 hours in solution A containing 2.2 M of sucrose to allow their adherence to the bottom of a Petri dish.

Patch-clamp single channel recording. We have used the patch-clamp technique in the "nucleus-attached" configuration. The Ag/AgCl electrodes were connected to a patch-clamp amplifier (RK300 Biologic, Claix, France). Data were stored on a digital tape recorder Biologic DTR 1200. All experiments were performed at room temperature (20–23°C).

Single channel current records were later digitised and analyzed with Biopatch (Biologic software - Claix, France). Unitary currents were determine by multigaussian adjustments of the amplitude distributions. The open probabilities were evaluated using an iterative process to minimise the chi square (X^2) calculated with a sufficiently large number of independent observations.

Solutions and chemicals. All solutions were buffered with 10 mM HEPES. pH of the pipette solution containing $Ba(CH_3COO)_2$ was adjusted to 7.4 with BaOH. However, for the pipette solution containing $Zn(CH_3COO)_2$, pH was equal to 6.9 to prevent precipitation. Solution bathing the nuclei, nominaly free calcium, contained (mM) : KCl, 145. Pipette contained (mM) : $Ba(CH_3COO)_2$, 90 or $Zn(CH_3COO)_2$, 90. All chemicals were obtained from Sigma.

Electron microscopy. Hepatocytes nuclei were fixed in 2.5% glutaraldehyde, post-fixed in OsO4 and embedded in Epon. Ultra-fine sections of this material were stained with uranyl acetate and lead citrate and examined under a CM10 Phillips electron microscope.

RESULTS

Nuclei. Isolated nuclei (98%) were devoided of outer membrane and are used for patch-clamp studies Only few of them (2%) exhibit an intact nuclear envelope but are Nuclei isolated as describe above were observed by electron microscopy, as shown in figure 1.

Calcium-channels. With $Ba(CH_3COO)_2$, 90 mM in the pipette, a 8-pS-conductance channel was observed in all experiments that was spontaneously active (n=78) (fig. 2). Its open time constant is about 3.5 ms, and its close time constant about 5.1 ms. different substances were used in order to activate or to inhibit the recorded current. Thus, this channel turned out to be insensitive to different calcium blockers (nifedipine, verapamil, diltiazem) and to the increase of calcium concentration (2mM) in the bathing medium. Furthermore, the recorded currents were insensitive to classical blockers of other channels such as DIDS - SITS (Cl^- blockers), TEA (K^+ blockers), TTX (Na^+ voltage sensitive blockers).

Zinc-channels. With $Zn(CH_3COO)_2$, 90 mM in the pipette, a 11-pS-conductance channels was observed in all experiments (n=51) (fig.3) Its open time constant is about 3.7 ms and its close time constant 4.8 ms. As for the Ca^{++} currents, the recorded currents were insensitive to calcium blockers and other classical blockers such as DIDS - SITS (Cl^- blockers), TEA (K^+ blockers), TTX (Na^+ voltage sensitive blockers) and to an increase of the Ca^{++} concentration (2mM) in the bathing medium.

DISCUSSION

The nuclear envelope exhibits a complex structure : the outer membrane has endoplasmic reticulum characteristics and a different protein composition from the inner membrane. The space between these two membranes could be considered as an extension of reticulum endoplasmic lumen. The previous observations that the outer membrane of the nuclear envelope contains ion channels rise the question of the presence of ionic channels on the inner membrane of the nuclear envelope since few studies have been performed on this membrane.

In this study, we describe a technique for nuclei preparation leading to 98% of nuclei devoided of outer mem-

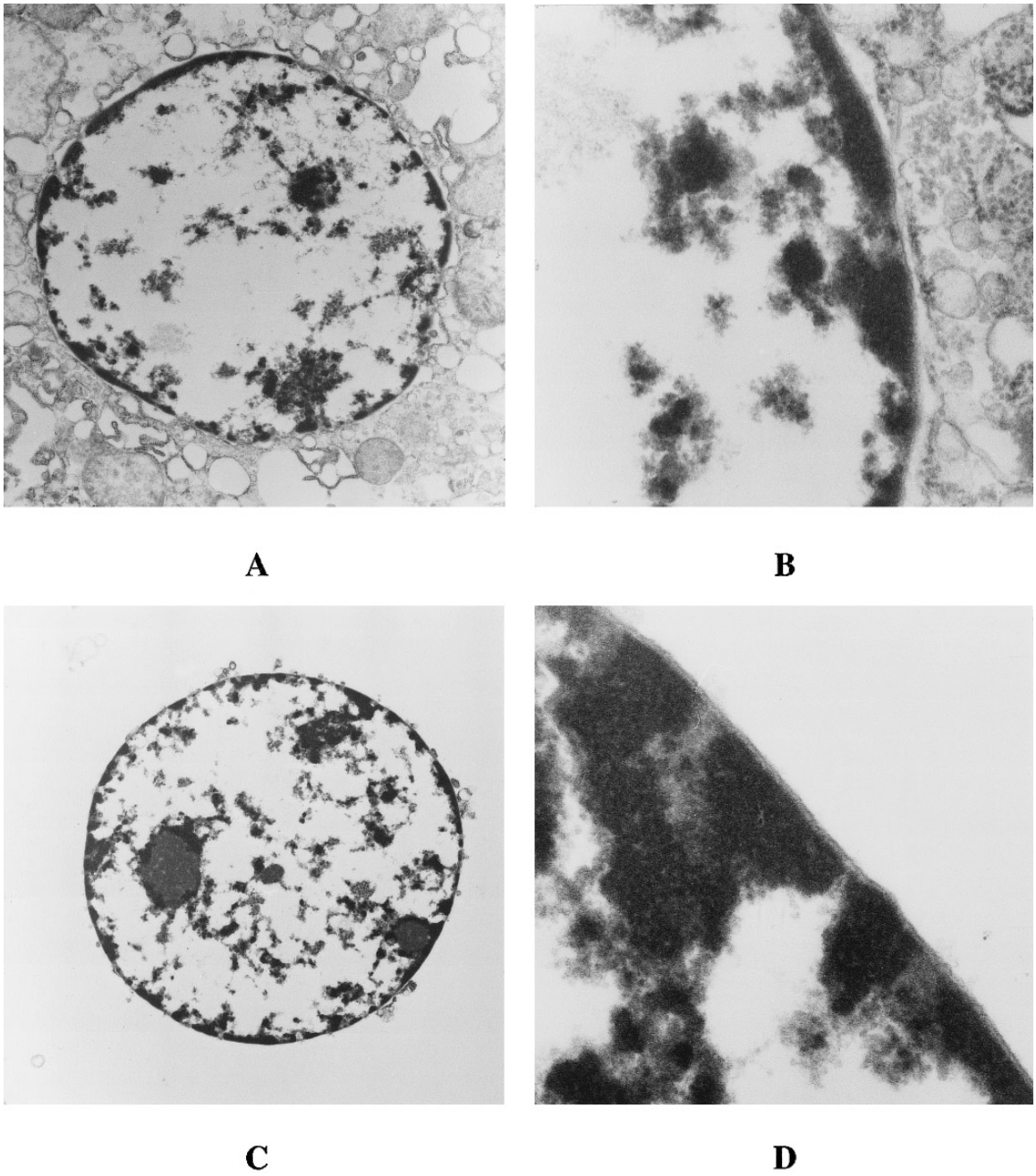

FIG. 1. Electron micrographs of hepatocytes nuclei A. Nucleus exhibiting and intact nuclear envelope (Magnification × 10 000). B. Internal and external membrane are clearly visible (Magnification : × 46 000). C. Nucleus devoided of external membrane (Magnification × 8 000). D. Only the inner membrane is visible (Magnification × 64 000).

brane and thus it provides a way to study the inner membrane of the nuclear envelope.

This inner membrane exhibits Ca^{++} and Zn^{++} channels, which have never been identified in the inner membrane of liver nuclei, with an unitary conductance of about 10 pA (respectively 8 pS and 11 pS). These channels are insensitive to calcium blockers and to the presence of Ca^{++} in the bathing solution. However a Ca^{++} channel of 100 pS has been shown on the outer membrane of nuclei from different types cells (7, 8). Since it is now recognized that the nuclear envelope functions as a Ca^{++} storage pool and that Ca^{++}-mobilizing cellular receptors and messengers has been identified in this membrane, it could be hypothesized that Ca^{++} inner channels could be implicated in the release of Ca^{++} from the lumen of nuclear envelope in the nucleoplasm.

Zn^{++} and Ca^{++} channels observed in our experimen-

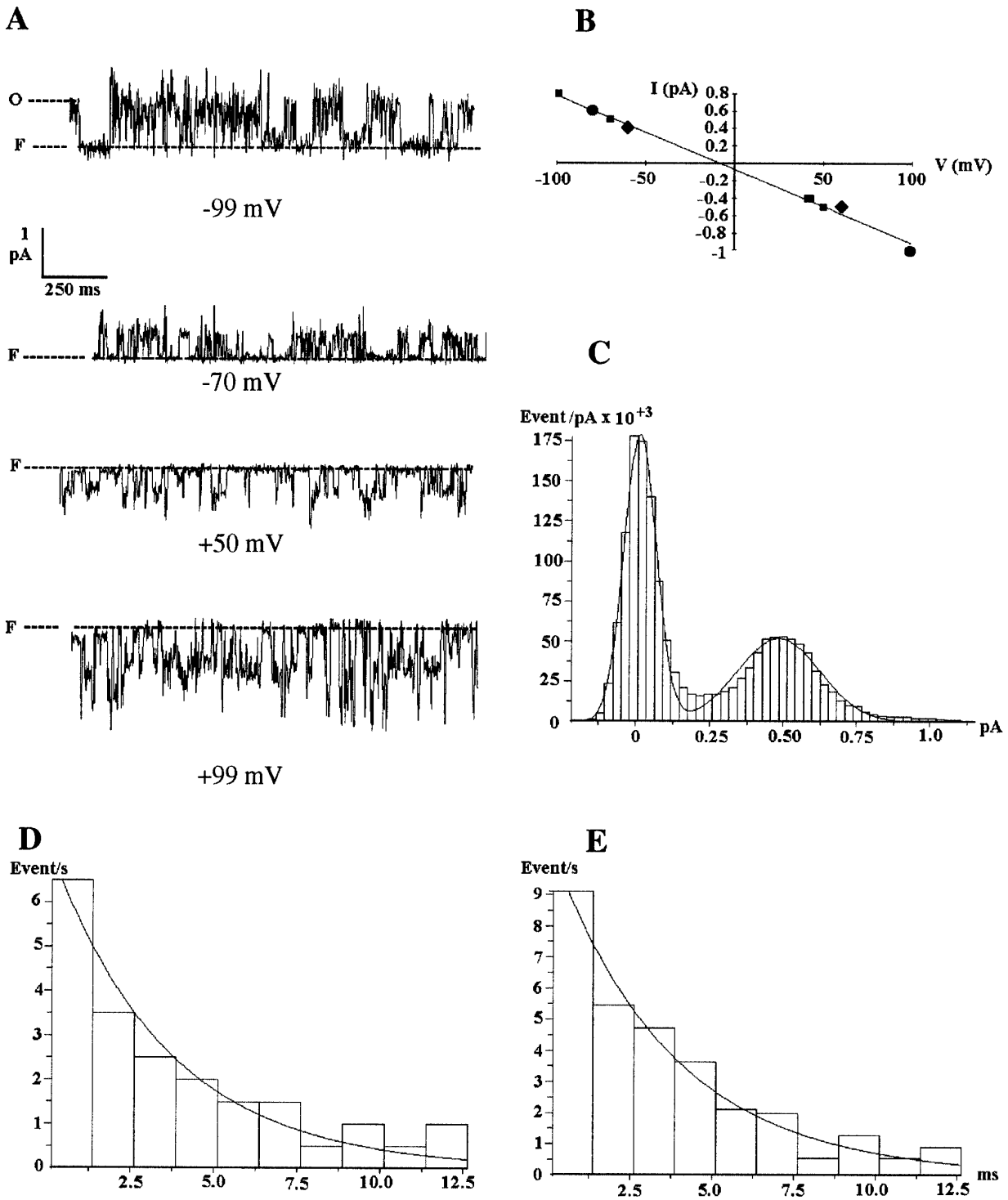

FIG. 2. Ca^{2+} permeant channel activity (n=78) A. Single channel currents in a patch held at various holding potentials. B. Current voltage relationship. The I/V relationship defines a 8-pS-channel (●, ■ and ♦ correspond to different patch experiments) C. Amplitude histogram realised for an holding potential of − 70 mV defines a 0.5 pA unitary current. D. Open time constant is 3.5 ms. E. Close time constant is 5.1 ms.

tal conditions appear to be very similar (conductances, open and close time constant). So we can not exclude the possibility that these channels are in fact the same channel, and that Zn^{++} and Ca^{++} move through the inner membrane of the nuclear envelope nucleus *via* the same channel This hypothesis is corroborated by

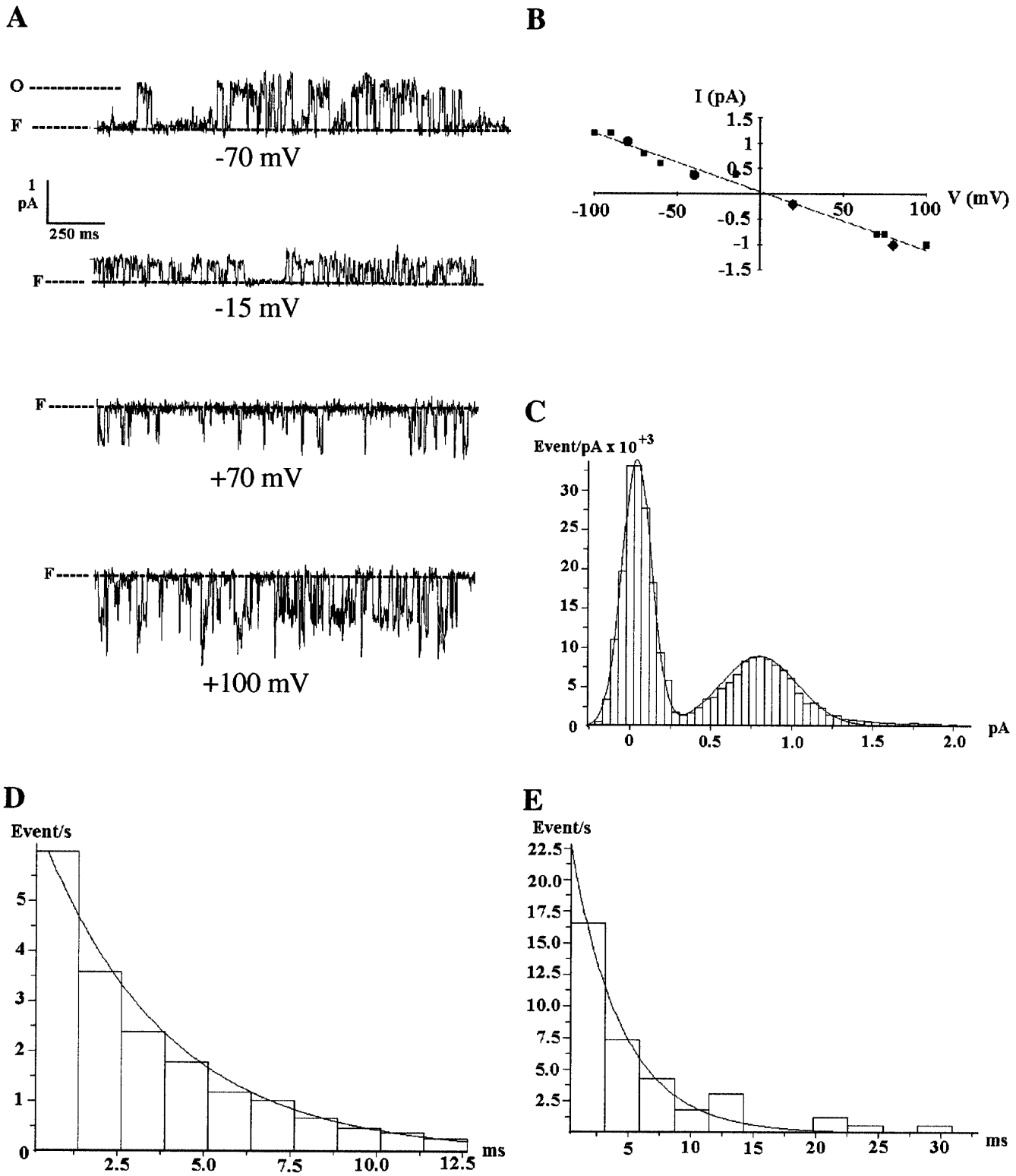

FIG. 3. Zn^{2+} permeant channel activity (n=51) A. Single channel currents in a patch held at various holding potentials. B. Current voltage relationship. The I/V relationship defines a 11-pS-channel (●, ■ and ♦ correspond to different patch experiments). C. Amplitude histogram realised for an holding potential of − 70 mV defines a 0.8 pA unitary current. D. Open time constant is 3.7 ms. E. Close time constant is 4.8 ms.

previous report showing that that Zn^{++} entry into heart cells depends upon electrical stimulation and occurs dihydropyridine-sensitive Ca^{++} channels (16). Finally, the regulation of this channels should modulate ion passage across the nuclear envelope and, consequently, should control indirectly intranuclear activities and may play a crucial role in nuclear processes such as transcription and in nuclear-dependent cell processes. Indeed, these channels situated on the inner membrane of the nuclear envelope could contribute to the regulation of the intranuclear free Ca^{++} and Zn^{++} concentrations. The control of these concentrations is a basic requirement for the modulation of Ca^{++} and Zn^{++} regulated processes in the nucleus. However, another role of these channels may be to balance the charge carried by macromolecules moving in and out of the nucleus, and therefore they could be involved in regulating gene expression and cell division (23). Furthermore, the demonstration of Ca^{2+} and Zn^{++} permeant channels is particularly important in regard to the antagonistic effect of zinc and calcium in the cascade of events leading to apoptosis. Many data demonstrate that the DNA cleavage during apoptosis is a calcium dependent process inhibited by zinc (24-25-26).

In particular, it has been shown that when entered inside the nucleus the two metals act mainly as opposite modulators of the Ca-Mg dependent endonuclease activity (27).

In conclusion, the results presented in this paper demonstrate directly the presence of a Ca^{++} channel and a Zn^{++} channel or functional cationic channel on the inner membrane of the nuclear envelope. As it could be speculated that these channels could control nuclear Ca2+ and Zn2+ influx and consequently gene expression or apoptosis, it will be important to identify the physiological events leading to their opening or closure.

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