Recent Advances in Nuclear Electrophysiology

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Symbols: **NE**, nuclear envelope; **INM**, inner nuclear membrane; **ONM**, outer nuclear membrane; **NPC**, nuclear pore complex; $\mathbf{R_{NE}}$, NE electrical resistance; $\mathbf{\Gamma_{NE}}$, NE electrical conductance; $\mathbf{R_{NPC}}$, NPC electrical resistance; $\mathbf{\gamma_{NPC}}$, NPC electrical conductance; $\mathbf{V_{NE}}$, voltage across the NE; $\mathbf{I_{NE}}$, electrical current across the NE; $\mathbf{V_{cytoplasm}}$, voltage at the cytoplasmic side of the NE; $\mathbf{V_{nucleoplasm}}$, voltage at the nucleoplasmic side of the NE; $\mathbf{P_{open}}$, probability of having a number of channels open in a channel population; $\mathbf{p_{open}}$, probability of finding a channel in the open state; $\mathbf{p_{closed}}$, probability of finding a channel in the closed state; **EM**, electron microscopy; **ER**, endoplasmic reticulum; **NHT**, nuclear hourglass technique

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PREFACE

The recent synergism of the Coulter counter principle with patch-clamp for the quantification of macromolecular translocation, as well as the application of nanopore-based electrical and atomic force microscopy techniques for the analysis and manipulation of molecular and supramolecular structures, hold the promise of producing significant contributions in the nanobiotechnology of gene control and expression, DNA and RNA sequencing, cellular cloning, etc. At the moment, two electrophysiological techniques are applied to the whole cell nucleus for the study of its function. One technique, patch-clamp, uses a microscopic approach to directly measure single channel activity at the nuclear envelope. From patch-clamp measurements one derives the macroscopic characteristics. The other technique, the nuclear hourglass, uses a macroscopic approach to indirectly measure the single channel activity at the envelope. From its measurements, the microscopic characteristics are derived. As efforts have just begun to develop microchips for patch-clamp at large scale, and as the post-genomic era has stimulated physiological genomics research, it seems timely to review the basic concepts on which these two leading electrophysiological techniques are based. This review is conceived to complement recent in-depth reviews in the area of nuclear electrophysiology. Finally, for sake of clarity and space, since published cell and molecular biology work in the field is copious (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?form=4&db=m&term=nuclear+pore+OR+nuclear+pores&dispmax=20), where possible, I have chosen the most relevant and/or the references with easiest internet access and set a cutoff date prior to the year 2000.

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INTRODUCTION

In the turbulent political decade of the 1960s, Loewenstein and colleagues published a series of papers that demonstrated the use of electrophysiological methods for the assessment of nuclear envelope (NE) permeability and, therefore, of nuclear pore complex (NPC) ion conductance (γ_{NPC}). The application of electrophysiological approaches to the analysis of nuclear function (i.e. nuclear electrophysiology) was practically discontinued because the measurement of NE resistance (R_{NE}) was carried out with microelectrodes and the concern prevailed with microelectrode plugging during their insertion into the cell and nucleus (recently reviewed in Mazzanti et al., 2001). The electrical approach for the assessment of NE permeability was then resumed when the Nobel prize-winning patch-clamp technique (e.g. Neher and Sakmann, 1991, 1992; Neher, 1992; Sakmann, 1992) was applied to the isolated whole nucleus (Matzke et al., 1990; Mazzanti et al., 1990 – reviewed in Mazzanti et al., 2001). Unfortunately, these patch-clamp investigations were carried out under saline solutions, without the necessary substrates for NPC-mediated macromolecular transport (see, for example, Adam, 2001; Fahrenkrog et al., 2001; Komeili and O´Shea, 2001; Macara, 2001; Ribbeck and Görlich, 2001; Fahrenkrog and Aebi, 2002; Smith et al., 2002). Fortunately, with the recent introduction of naturally occurring living syncytial nuclei (Bustamante, 2002), for patch-clamp and nucleocytoplasmic research, potential questions about lack of substrates are eliminated.

Since 1999, some of the patch-clamp data and their interpretation have been either confirmed, complemented or challenged by an indirect, macroscopic approach for the measurement of γ_{NPC}: the nuclear hourglass technique (NHT - Danker et al., 1999; 2001; Shahin et al., 2001). The NHT derives γ_{NPC} from a simple geometrical formula for electrical resistance, under the assumption of a high-quality seal between the nuclear surface and the tube within which it is placed (a concept reminiscent of the gigaseal that we will discuss later). Thus, due to the indirect nature of the NHT, one must address the same questions raised for the few techniques contemporaries to patch-clamp (e.g. Kostyuk, 1982, 1984; Kostyuk and Krishtal, 1984). Specifically, *is the electrolyte leakage or shunt sufficiently low to prevent proper extrapolation to the microscopic world of the single NPC?* Or, in electrical circuit terms, *is the shunt resistance (R_{shunt}) much higher than the NE resistance (R_{NE})?*

The potential benefits of electrical measurements of ion channel activity deriving from NPCs and non-NPCs are great and far-reaching (e.g. Kasianowicz et al., 1996; Matzke and Matzke, 1996; Hanss et al., 1998; Meller et al., 2000; Howorka et al., 2001; Marziali and Akeson, 2001; Matzke et al., 2001; Vercoutere et al., 2001; Wang and Branton, 2001; Fertig et al., 2002; Klemic et al., 2002; Sigworth and Klemic, 2002). Therefore, we must discuss the foundations and the current issues of nuclear electrophysiology. This is especially relevant to patch-clamp data obtained with pipettes placed on the cytoplasmic side of the outer membrane of the NE and to new techniques introduced to assess the macroscopic behavior of the NE. The ideas presented here are aimed at a wide audience (from biologists and clinicians to physicists and engineers). They are also intended to complement reviews presented elsewhere (e.g. Bustamante, 1996; DeFelice and Mazzanti, 1997; Bustamante and Varanda, 1998; Mazzanti et al., 2001).

EXPERIMENT AND THEORY

Structural Considerations

Most of a cell's electrical resistance is due to the resistance of the cell surface membrane: *the plasmalemma*. In the same manner, most of the electrical resistance of the nucleus is due to the permeability barrier offered by the NE to the movement of physiological ions. The NE, however, is more complex than the plasmalemma in several ways (see, for example, Fahrenkrog et al., 2001; Fahrenkrog and Aebi, 2002). The envelope consists of two membranes: *the inner and the outer nuclear membranes* (INM and ONM, respectively), that delimit a cisterna known as *the perinuclear space*. The cisterna seems to be a reservoir for important constituents and signaling molecules (e.g. Garner, 2001). Both NE membranes have been shown to contain receptors and pumps similar to those found in the plasmalemma and other organelles: (1) losartan-senstivity angiotensin II receptors (AT_1 – e.g. Booz et al., 1992; Tang et al., 1992; Eggena et al., 1993; Jímenez et al., 1994; Merjan et al., 2001), (2) IP_3 receptors (IP_3R - e.g. Guihard et al., 1997; Adebanjo et al., 2000; Jaimovich et al., 2000; Tovey et al., 2001), (3) ryanodine receptors (RyR – e.g. Adebanjo et al., 1999, 2000; Kapiloff et al., 2001), (4) Na^+-K^+-pumps (e.g. Garner, 2001), (5) Ca^{2+}-pumps (e.g. Rogue et al., 1998; Abrenica and Gilchrist, 2000), etc. (reviewed in Bustamante, 1994b). The ONM contains ribosomes and is continuous with the endoplasmic reticulum, ER. This has led to the idea that, when a patch-clamp pipette is placed at the cytoplasmic side of the NE, the ion channel activity derives only from ONM or ER sources (see Mazzanti et al., 2001). At discrete locations, the NPCs join these two membranes in a manner resembling dual-membrane channels such as gap junction and plasmodesmata ion channels. Under the electron microscope, EM, the large mass of NPCs (over 125 MDa in vertebrates) makes these supramolecular structures the only conspicuous channels in the NE (e.g. Mazzanti et al., 2001). The NPCs are decorated with filaments that protrude into the cytoplasm: *the cytoplasmic filaments* (e.g. Fahrenkrog et al., 2001; Fahrenkrog and Aebi, 2002). On the nucleoplasmic face, filaments emanating from the NPC form a basket: *the nuclear baskets* (e.g. Fahrenkrog et al., 2001; Fahrenkrog and Aebi, 2002). The NPC has a large central aqueous channel whose diameter for passive diffusion is in the order of 10 nm. This large nanochannel is present in all structural models of the NPC, whether or not they have a central plug. Some investigators have interpreted the plug as a transporter for macromolecular translocation. The most discriminating structural data on the central plug has been produced with atomic force microscopy (AFM), a technique that uses a molecular-sized tip to image the topology in chemically unfixed preparations (elasticity and other parameters can be imaged but this has yet to be reported for the NE). With AFM, Rakowska et al. (1998) imaged ATP-dependent changes in the central plug whereas Perez-Terzic et al. (1996, 1999) and Wang and Clapham (1999) imaged Ca^{2+}-dependent changes in the plug that they interpreted as conformational changes on a plug that forms part of the NPC. The central plug, however, was not seen under AFM when macromolecular transport substrates were provided to the preparation (Stoffler et al., 1999a; reviewed in Stoffler et al., 1999b and Fahrenkrog et al., 2001; Fahrenkrog and Aebi, 2002). Therefore, the controversy on whether the central plug is an integral part of the NPC appears to have reached its final conclusion: *the central plug is a macromolecule in transit through the NPC diffusional channel*. Accordingly, the Coulter counter principle can be used as a working patch-clamp paradigm, to characterize the dynamics of macromolecular translocation along the

NPC (Bezrukov, 2000). Diffusion through the large channel seems to operate via hydrophobic exclusion (Ribbeck and Görlich, 2002).

A decade ago, peripheral channels for ion diffusion were proposed based on EM reconstruction studies (Hinshaw et al., 1992 – see Fahrenkrog and Aebi, 2002). Corroborating data from other EM laboratories have not been forthcoming. Thus, most recent structural models of NPCs do not incorporate this feature (e.g. Rout and Aitchison, 2001; see review by Fahrenkrog and Aebi, 2002). Peripheral channels are tantamount to interpreting the background or leakage current obtained with the indirect, macroscopic approach of the NHT (Shahin et al., 2001) as the authors have recently interpreted this leakage as resulting from ion flow along these peripheral channels. In contrast, only a single diffusional channel was derived from direct EM observations (Felherr and Akin, 1997) and from high-resolution fluorescence microscopy (Keminer and Peters, 1999). Therefore, when taken together, both EM and fluorescence microscopy do not support the existence of these peripheral channels. Furthermore, since patch-clamp also does not support the existence of the peripheral channels (e.g., Bustamante et al., 1995a, 2000a; Bustamante, 2002), we are forced to analyze the assumptions on which NHT is based.

Basic Principles of Electrophysiological Approaches

As some of the disagreement between patch-clamp and NHT data (existence of peripheral channels, leak or shunt resistance, etc.) appears to originate from different interpretations of the electrical circuit theory, we need to discuss the principles that underlie these two leading techniques of nuclear electrophysiology. I shall, however, refer the reader to other publications that deal with a more in-depth analysis of the general methodology (e.g. DeFelice, 1997; Ypey and DeFelice, 1999). In the end, we shall learn what biologists know well: *that not all in nature is black and white* as formulas and equations would have us believe.

Seminal to the measurement of electrical resistance is Ohm's law which tells us that the movement of positive electrical charges per unit time (i.e. electrical current, I) between two points, say 1 and 2 (i.e. I_{12}), is given by the voltage drop across these two points (V_{12}) divided by the resistance between these two points (R_{12}):

$$I_{12} = V_{12} / R_{12} \qquad (1)$$

In terms of the NE, we have (see also DeFelice and Mazzanti, 1997; Bustamante and Varanda, 1998):

$$I_{NE} = V_{NE} / R_{NE} \qquad \text{or} \qquad I_{NE} = \Gamma_{NE} \times V_{NE} \qquad (2)$$

where I_{NE} is the electrical current (produced by the flow of ions, the major electrical charge carriers) between the outside and inside faces of the NE, V_{NE} is the voltage drop across these two faces and R_{NE} is the electrical resistance posed by the NE to the ion flow (the inverse of NE

conductance: Γ_{NE}). If $V_{cytoplasm}$ is the voltage on the cytoplasmic side of the NE and $V_{nucleoplasm}$ is the voltage at the nucleoplasmic side of the NE, then:

$$V_{NE} = V_{nucleoplasm} - V_{cytoplasm} \quad (3)$$

Under no nucleocytoplasmic concentration gradient for the electrical charges (e.g. if K^+ were the charge carriers, $[K^+]_{cytoplasm}=[K^+]_{nucleoplasm}$), when $V_{cytoplasm}>V_{nucleoplasm}$, positive charges will move toward the nucleoplasm. For negative charges (e.g. Cl^-, with $[Cl^-]_{cytoplasm}=[Cl^-_{nucleoplasm}$) their movement will be towards the nuclear exterior. That is, in both cases the electrical current is inward (inward movement of positive electrical charges) although the flow of ions may not be inward if the ions are negative. Thus, one must not confound the direction of the flow (defined as the movement of particles of any charge) with the direction of the ion current (defined as the movement of positive electrical charges). **Fig. 1** illustrates the flow of positive charges for $V_{cytoplasm}>V_{nucleoplasm}$. When $V_{cytoplasm}<V_{nucleoplasm}$, and there is no concentration gradient, the flow of ions is reversed and the currents are outward (negative ions will move inwardly). Finally, in the absence of gradients for both the chemical potential (e.g. $[K^+]_{cytoplasm}=[K^+]_{nucleoplasm}$) and the electrical potential (i.e. $V_{cytoplasm}=V_{nucleoplasm}$) there is no electrical driving force for the charge carriers is absent and there is no ion flow if there is no concentration gradient. Note that, as established in the classical ion channel theory (e.g. Hodgkin and Huxley, 1952 – see Huxley, 2002), the force determining the movement of ions is of electrochemical origin. For an NE patch, the total current due to an ion species X is given by Ohm's law:

$$I_X = \Gamma_X (V_{NE}-V_X) \quad (4)$$

where Γ_X is the patch conductance for the ionic species X and V_X is the equilibrium potential for this ionic species (also known as Nernst potential, Nernst-Planck potential, etc.). In its simplest form, commonly found in textbooks:

$$V_X = (RT/Fz_X) \ln ([X_{cytoplasm}]/[X_{nucleoplasm}]) \quad (5)$$

where R is the gas constant, T is the absolute temperature, z_X is the valence of the ion species X, F is the Faraday constant, and $[X_{cytoplasm}]$ and $[X_{nucleoplasm}]$ are, respectively, the concentration of ions on the cytoplasmic and nucleoplasmic sides of the NE. This equation will be relevant to our discussion on ion selectivity, later in this chapter. To date, there appears to be a general agreement that the equilibrium potential for the major electrical charge carriers (e.g. K^+) is negligible (e.g. Bustamante, 1992, 1993; Boehning et al., 2001).

Since ions in solution are the major electrical charge carriers in nuclear electrophysiology, for large preparations generating large currents, two pairs of half-cell electrodes (e.g. Ag-AgCl) are used to avoid effects of electrode polarization due to electrolysis and electrodeposition (e.g.

Sherman-Gold, 1993). For patch-clamp measurements of single ion channel gating, electrode polarization is not a concern due to the low values of the currents generated and, therefore, only two Ag-AgCl electrodes are used (e.g. Sherman-Gold, 1993). The two electrodes are connected to the probe of an instrumentation amplifier that operates like a virtual-ground (an operational amplifier connected in such a manner that forces the input to the ground potential). The electrode used to record current, the one inside the patch-clamp pipette, is forced to a known voltage (for this reason, it is not a virtual ground configuration). The bath electrode is used as reference ($V_{reference} = V_{bath} = 0$). Therefore, the potential difference applied to the NE is the difference between the potential inside the pipette, $V_{pipette}$, and $V_{reference}$. Note, however, the potential experienced by the NE will be influenced by the NE potential when there is no potential applied: the NE resting potential. As mentioned above, so far, the experiments have shown that this potential is negligible under steady-state conditions (when the nucleocytoplasmic concentration difference has had time to dissipate). Consequently, one is to expect that, in patch-clamp, the ion flow will be controlled by $V_{pipette}$ only because $V_{reference} = 0$.

A major problem with any electrophysiological technique is the presence of a leak or shunt resistance (R_{shunt}), in parallel to the target of measurement (the nucleus in our case), through which electrical charge carriers escape. Another problem, dealt with since the times of Hodgkin and Huxley (1952), is the presence of a series resistance (R_{series}) through which there will be a voltage drop proportional to the current. As a result of R_{series}, the resistance offered to the movement of electrical charges is increased by this amount and the resistance that is the target of our measurement (i.e. R_{NE}) is underestimated. In mathematical terms, the resistance measured ($R_{measured}$) is:

$$R_{measured} = R_{series} + [\ (R_{shunt})^{-1} + (R_{NE})^{-1}\ ]^{-1} \qquad (6)$$

As a consequence of the above relationship, there are two golden rules of electrical circuits. First, the measured resistance, $R_{measured}$, of a circuit of resistors in series will always be greater than the value of the largest resistor. Second, and most relevant to our case, *the measured resistance, $R_{measured}$, of a circuit of parallel resistors can never be greater than the smallest component resistance: R_{shunt} or R_{NE}.*

Finally, other components of the electrical circuit (e.g. access resistance), are of importance but not relevant to our central theme. For this reason, the interested reader should consult the excellent textbooks on the topic (e.g. Sakmann and Neher, 1995).

Patch-Clamp: The Microscopic Approach

The study of native, *in situ* single ion channel behavior was greatly facilitated with the introduction of the patch-clamp technique for the imposition of a voltage gradient across a membrane (i.e. voltage-clamp –see Sakmann & Neher, 1995). Patch-clamp succeeded when the possibility was discovered to attain R_{shunt} in the order of gigaohms (GΩ, 10^9 ohm). The gigaohm value indicated that the seal resistance, R_{seal}, between the tip of the glass pipette and the biological membrane was in the order of GΩ. This seal was henceforth coined *the gigaseal*. This breakthrough took patch-clamp to the spotlight, for the gigaseal was the last remaining requirement for the precise analysis of ion channel function (the other requirements, mainly

concerned with solid-state electronic devices, had been conquered). The gigaseal was not just a mere achievement of a high-valued R_{shunt} for it also dramatically reduced the baseline noise (see Neher's Nobel lecture in Neher and Sakmann, 1991 and Neher, 1992).

$$\sigma_{theoretical} = (4\ kT\ \Delta f)^{1/2} \times R_{system}^{-1/2} \quad \text{from where} \quad R_{system} = \text{constant} \times \sigma_{theoretical}^{-2} \qquad (7)$$

where $\sigma_{theoretical}$ is the theoretical current noise of the signal source, k is the Boltzmann constant, T is the absolute temperature (here assumed constant during an experiment), Δf is the frequency bandwidth (also assumed constant), and R_{system} is the resistance of the system (i.e. $R_{measured}$, determined by R_{NE} and R_{shunt} as discussed above). From this equation one reasons that for a bandwidth of 1 kHz, and a 1 pA of ion channel current one should have an R_{system}>2 GΩ to guarantee a 10% resolution of the signal (see Neher, 1991). This particular characteristic of the noise in patch-clamp recordings is useful to monitor whether a gigaseal has been attained, even when the NPCs of an NE patch are all simultaneously open and thus the R_{shunt} cannot be measured.

Patch-clamp, reliably measures R_{shunt} and this quantity gives the value of R_{seal} whenever there is no ion channel opening and the membrane is not leaky to ions. However, if we consider the contribution from the ion flow through the channels at the NE (NPCs and channels at the ONM and INM), R_{shunt} is derived from the parallel circuit formed by the seal resistance, R_{seal}, and the NE resistance, R_{NE}.

$$R_{measured} = R_{shunt} = [\ (R_{seal})^{-1} + (R_{NE})^{-1}\ ]^{-1} \qquad (8)$$

Thus, when all channels are closed (i.e. $R_{NE} >> 1$), if $R_{measured} > 1$ GΩ, then we can be sure that $R_{seal} > 1$ GΩ. That $R_{seal} > 1$ GΩ in patch-clamp investigations of the NE can be appreciated in the recordings of several patch-clamp publications and, therefore, should not be taken as a mystery (e.g. Bustamante, 1992, 2002). Thus, the patch-clamp researcher is neither required, nor should he/she expect, to have a gigaseal prior to measuring the patch ion conductance if the number of NPCs conducting ions is sufficiently high. One may have to wait for a long time before a simultaneous closing of all the channels is observed (e.g. Bustamante, 2002). For example, if the patch has 2 NPCs of γ_{NPC} = 500 pS simultaneously open, then the patch ion conductance (Γ_{patch}) would be 1 nS and $R_{measured} \leq 1$ GΩ, even if $R_{seal} \geq 10$ GΩ. Likewise, for 10 NPCs of 500 pS simultaneously open, Γ_{patch} = 5 nS and $R_{measured} \leq 0.2$ GΩ, even if $R_{seal} \geq 10$ GΩ. Thus, a general rule for patch-clamp of the NE is that, when all NPCs are open, say N, $R_{measured} \leq (N \times \gamma_{NPC})^{-1}$, even if $R_{seal} \geq 10$ GΩ.

Patch-clamp must be applied and interpreted with caution because the structure of the NE is more complicated than that of a single membrane. For example, for the nucleus-attached patch configuration (i.e. pipette tip touching the ONM of a whole nucleus), one has to check that the bath electrode makes a virtual connection with the INM through the nucleoplasm. As shown in **Fig. 2** this can be demonstrated with fluorescence microscopy by placing small fluorescent probes (e.g. 4-kD dextran, 5.4-nm dendrimer) on the cytoplasmic side of the nucleus (Fig. 2a,c) and showing their diffusion into the nucleus (Fig. 2b,d - see Bustamante et al., 2000b;

Bustamante, 2002). Under these conditions, V_{NE} is given by the voltage difference between the bath and pipette electrodes (see discussion above). It then follows that, in the nucleus-attached configuration, if the NPCs are closed then any recorded ion channel activity should derive from sources other than NPCs because these sources should be the pathway to close the circuit between the pipette and bath electrodes. Since I have never seen ion channel activity when the NPCs are closed or plugged (demonstrated by the lack of entry of small dextran and dendrimer particles upon macromolecular transport stimulation – e.g. Bustamante et al., 2000b, Bustamante, 2002), I cannot exclude NPCs as sources of ion channel activity, even when the ion conductance is below 100 pS. For this reason, the ion channel activity may be interpreted as a sub-state for ion conduction of the NPC (Bustamante, 1994a). Under this context, it can then be reasoned that a change in ion activity induced by hormones and other agonists (e.g. angiotensin II, IP_3, ryanodine, etc.) may be the result of either direct or indirect actions, mediated by their corresponding receptors and whatever coupling mechanisms there may be such as G-proteins and Ca^{2+} release feedback. For the excised patch configuration (i.e. pipette tip touching the ONM of a piece of excised NE), the requirement for demonstrating the status of NPC ion conduction remains. Only when we have proven that the NPCs are closed or plugged (e.g. with fluorescence microscopy) can we say that the ion channel activity derives from sources other than NPCs. The understanding of this requirement holds the key to understanding the relationship between NPC-mediated nucleocytoplasmic transport of macromolecules and ion channel activity recorded from the NE (i.e. *the Rosetta stone of nuclear electrophysiology*). Indeed, ever since the first usage of a medium that supported macromolecular transport along NPCs, as well as transcription and translation (e.g. TNT, Promega), it was observed that macromolecular transport along NPCs restricts ion diffusion (Fig. 1 in Bustamante, 1994b; see also Bustamante et al., 1995a-c 2000a,b; Bustamante, 2002). Fig. 2 also illustrates an experimental fluorescence microscopy protocol for the determination of the capacity of NPCs for macromolecular translocation and the role of this phenomenon on NPC ion channel activity (e.g. Bustamante et al., 1995a, 2000a,b; Bustamante, 2002). Briefly, a nuclear-targeted macromolecule (one that contains a nuclear localization signal or NLS) is placed on the cytoplasmic side (Fig. 2e). A convenient probe that I have used (e.g. Bustamante et al., 1995a, 2000a,b; Bustamante, 2002) is the high quantum efficiency B-phycoerythrin (240 kDa, Molecular Probes), conjugated to the NLS of the4 SV40 large T antigen (Sigma Chemical). If the NPCs are capable of macromolecular transport, then the macromolecular probe will go inside the nucleus (Fig. 2f). If not, it will remain outside (Fig. 2e).

The Nuclear Hourglass Technique, NHT: The Macroscopic Approach

When used in the radio frequency range, the same approach used by NHT can produce information on general cellular characteristics such as the nuclear-to-cytoplasmic volume ratio – a parameter relevant to various pathological states of cells (e.g. Coulter International, 1996; Beckmann-Coulter, 2002). The radio frequency currents are electrically transparent to the cell membranes and have the added advantage that they also appear to leave the cells undisturbed (e.g. Coulter International, 1996; Beckmann-Coulter, 2002).

In the NHT (Danker et al., 1999, 2001 – no frequency details given), a known value of current, $I_{injected}$, is injected to the lumen of the capillary tubing and the voltage drop between the two ends of the tubing measured, $V_{measured}$. From these values, the electrical resistance of the system is calculated, $R_{measured}$:

$$R_{measured} = V_{measured} / I_{injected} \quad (9)$$

If $I_{injected}$ were constant, then, by simple differentiation, it can be shown that the variation in $V_{measured}$, $\Delta V_{measured}$, caused by the introduction of the nuclei inside the capillary, can be used to calculate the variation in $R_{measured}$, $\Delta R_{measured}$ (ΔR in Danker et al., 1999, 2001):

$$\Delta R_{measured} = \Delta V_{measured} / I_{injected} \quad (10)$$

Note, however, that since $I_{injected}$ is alternating when not in the electrophoretic mode (see Danker et al., 2001), the above relationship does not necessarily hold. In setting the foundations for NHT, Danker et al. (1999) depart from the undisputable premise that the measured resistance, $R_{measured}$, is the result of the resistance of the cell nucleus ($R_{nucleus}$) in parallel with R_{shunt}. Although no explicit consideration of R_{series} was made, this seems of no major consequence as the analysis is made with changes in $R_{measured}$ (i.e. $\Delta R_{measured}$ or ΔR) and R_{series} should vary very little upon the introduction of the nucleus inside the tubing.

$$R_{measured} = R_{series} + [(R_{shunt})^{-1} + (R_{nucleus})^{-1}]^{-1} \quad (11)$$

$$R_{nucleus} = \{ [\Delta R_{measured}^{-1} - R_{shunt}^{-1} \}^{-1} \quad (12)$$

In the NHT R_{shunt} is estimated by using, instead of a nucleus, a stage I whole oocyte of *Xenopus L.* and by applying a direct (rather than alternating) current to increase the value of $R_{measured}$. With their most recent approach (Danker et al., 2001) they arrived at a value of about 650 pS rather than the 1,700 pS previously calculated (Danker et al., 1999). Since their original approach (Danker et al., 1999) has been replaced (Danker et al, 2001), I shall concentrate on their most recent data and their interpretations.

There are several problems with the assumptions made in the NHT. 1st The use of a whole oocyte is an approach that the investigators agreed is controversial (see bottom of page 13532 in Danker et al., 1999). 2nd The surface properties are probably not the same for the oocyte surface membrane and the NE. Therefore, it is likely that stickiness between the NHT tube and membrane surface of the oocyte and its NE are quite different. 3rd The responses to physical and chemical maneuvers (e.g. voltage and antibodies) cannot be assumed to be the same for both the oocyte plasmalemma and the NE. 4th The large body of accumulated experience with similar techniques (e.g. Fishman, 1975; Lopez-Barneo et al., 1981; Kostyuk, 1982, 1984; Kostyuk and Krishtal, 1984) lead us to conclude that the leak in the NHT must be of concern. My own experience (e.g. Bustamante, 1981, 1983; Bustamante and McDonald, 1983 – see **Fig. 3** in this paper) is that current injection alone can be used to increase R_{shunt} (a current of opposite polarity usually decreases R_{shunt}). This issue will be revisited later in this chapter. My patch-clamp

observations (e.g. Bustamante et al., 1991; Ruknudin et al., 1993), shared by the patch-clamp community (e.g. Sherman-Gold, 1993) include that current injection alone can be used to increase R_{shunt}, (opposite polarity in $I_{injection}$ usually decreases R_{shunt}). This phenomenon does not require NPC plugging. Therefore, the Aquilles' heel/tendon of the NHT is R_{Shunt}. For this reason and for the significance of the interpretations of NHT data, we must center our attention on NHT justification for the assumptions on R_{shunt}.

In the NHT, a good experiment is defined as one in which the lumen of the capillary neck (thus their terminology of hourglass) has a diameter smaller than the diameter of the nucleus (Danker et al., 2001). This produces two pieces of NE in unrestricted contact with the physiological saline. NHT assumed that the tubing and the nucleus were cylindrical, that the width of gap between the nucleus and the capillary was w, and that the surface of interest between the NE and the tubing wall (the one where they had macroscopic contact) had a length L (L_{gap}), and radii of r (r_{gap}) and r-w ($r_{nucleus}$), respectively. NHT investigators determined that the electrical conductivities of their saline (κ) and the nucleoplasm (κ') had the same value of 13.2 mS/cm^2 (I think that they meant to say mS/cm). Based on the geometry of their system, they arrived at the following formulas.

$$R_{shunt} = L\,\kappa^{-1}\,\pi^{-1}\,(r^2 - (r - w)^2)^{-1} \quad (13)$$

$$R_{nucleus} = R_{NE} + R_{intranuclear} \quad (14)$$

$$R_{intranuclear} = L\,\kappa^{-1}\,\pi^{-1}\,(r - w)^{-2} \quad (15)$$

They defined $R_{replaced}$ as the resistance of the space replaced by the nucleus and the shunt. Thus:

$$R_{replaced} = L\,\kappa^{-1}\,\pi^{-1}\,r^{-2} \quad (16)$$

From these formulas, they derived the formula for R_{NE}:

$$R_{NE} = ((\Delta R + R_{replaced})^{-1} - R_{shunt}^{-1})^{-1} - R_{intranuclear} \quad (17)$$

NHT also assumed that all measurement errors are negligible, except for w, because *"they can be easily measured with fair resolution"* (Danker et al., 2001 – but see Appendix in this paper). Note, however, that NHT proponents did not consider that in the intact NE, the NPCs have protrusions into the cytoplasm (the cytoplasmic filaments discussed above). These protrusions may have interfered with the surface scan of their AFM probe or may have collapsed during isolation of the oocyte nucleus. Furthermore, as these investigators previously showed with AFM (Danker et al., 1997), their *Xenopus L.* oocyte nucleus was contaminated with ER and

by other structural features. It must be added that transmission EM demonstrates that the NE surface is not a smooth surface (e.g. Feldherr and Akin, 1997; Panté and Kann, 2001). Therefore, it would appear that the gap width, w, used in the mathematical model of Danker et al. (2001) could have been better, and much more easily evaluated, with readily available fluorescent probes (e.g. calibration probes from Molecular Probes).

NHT investigators concluded that $R_{shunt}>50\ R_{nucleus}$ (i.e. $R_{shunt}>>R_{NE}$) and that their measurement error was as small as 3.1% (Danker et al., 2001 – but, again, see Appendix in this paper). Since the calculations carried out by them are the basis for their justification, they deserve a close scrutiny. From their calculations, with L=270 μm, r=135 μm, w=1 μm and κ=13.2 mS/cm^2 (not mS/cm as it should be) one has R_{shunt} = 24,000 Ω.m. When the correct units for conductivity (i.e. mS/cm) one obtains 2.4 MΩ.

Fig. 4a shows a curve drawn with the expected values from the contributions to NE resistance of the NE areas involved (the caps created by squeezing the nucleus inside the hourglass region of the tube). Please, note that this curve is not drawn according to Danker et al. (1999, 2001). Say that the surface density of ion conducting NPCs, σ_{NPC}, is 10 NPCs/μm^2 and that the single NPC channel conductance, γ_{NPC}, is 500 pS. These values are in the order of the most recent data from Oberleithner´s group: γ_{NPC} (Danker et al., 2001) and σ_{NPC} (Schäfer et al., 2002). The conductance of an NE segment, Γ_{NE}, is determined by σ_{NPC}, γ_{NPC} and by the area of the NE surface, A_{NE}:

$$\Gamma_{NE} = \sigma_{NPC} \times A_{NE} \times \gamma_{NPC} \qquad (18)$$

When A_{NE} is 10,000 μm^2, Γ_{NE} will be 10^{-5} S and R_{NE} will be 20 kΩ . If we now consider that there are two electrically resistive surfaces connected in series, then the contribution of these two segments of the NE will be 40 kΩ. Values of R_{NE} computed with this approach are given in Fig. 4a for radii of the NE caps (areas in unrestricted contact with the saline) between 5 and 100 μm. As shown in Fig. 4a, R_{NE} values ranged between 12.7 kΩ and 5.1 MΩ. That is, they never fell below 10 kΩ.

Fig. 4b shows curves predicted by NHT formula for R_{shunt} (Danker et al., 2001), using the conductivity of 10 mS.cm^{-1} and the geometry for patch-clamp and NHT. As seen from Fig. 4b, according to the NHT model, R_{shunt} should very high, never be below 1 MΩ. Contrasting their theoretical calculations are my unpublished observations on R_{shunt} made during the experiments I carried out between 1980 and 1990 with adult cardiac myocytes and with cultured neuroblastoma cells (e.g. Bustamante 1981, 1983, 1985, 1989; Bustamante and McDonald, 1983). It is very likely that contemporary investigators using pipettes and tubes for voltage-clamp had similar experience to mine but that, like me, considered them of no scientific relevance. The value of R_{shunt} that I obtained when I perfused the tubing with physiological saline was in the order of 1 MΩ while a cell was placed inside the small hole of the intracellular perfusion tubing (Fig. 3). When I used the NHT formula to calculate the R_{shunt} expected for my intracellular perfusion experiments with human heart atrial myocytes (e.g. Bustamante and McDonald, 1983), I obtained a value of 40.8 MΩ for L= 5 μm, r = 2 μm, and w=0.1 μm. As I used enzymatic digestion for the isolation of the myocytes (e.g. Bustamante et al., 1981, 1982a,b), the membrane coat or glycocalyx was greatly removed and, therefore, the gap width between the cell surface

and the tubing should certainly be in the nanometer scale (see EM images in Bustamante et al., 1981, 1982a). Perhaps, more telling about the correctness of the model is that when I applied the NHT formula for the calculation of R_{shunt} for cell-attached patch-clamp experiments, the mathematical prediction was that the indirect macroscopic approach of NHT had a significantly better seal than patch-clamp. Fig. 4b shows the expected values of R_{shunt} for patch-clamp and NHT, as a function of the gap width, w, gap lengths (L_{gap}) fixed to 100 and 0.1 μm, respectively, and gap radius (r_{gap}) fixed to 100 and 1 μm, respectively. The conductivity of the fluid was set to 10 $mS.cm^{-1}$ and, therefore, its resistivity (ρ_{fluid}) was fixed at 100 Ω.cm.

Fig. 5 shows how chemical and physical maneuvers can be used simultaneously to eliminate the current leak. In the specific experiment with adult human heart myocyte (Bustamante, 1980 - unpublished), R_{shunt} was dramatically increased upon replacement of Cl^- in the intracellular perfusion with F^- and of K^+ with Cs^+. This maneuver also facilitated the study of Na^+-channel currents by blocking other ion channel currents (e.g. Bustamante and McDonald, 1983).

The computations using the NHT formula demonstrate that there must be other physical and/or chemical factors that were not considered in the mathematical modeling of R_{shunt}. One simple explanation is that their simplifying formulas were originally conceived for systems of larger dimensions and/or for diluted solutions and, consequently, these formulas are valid when there is no molecular crowding and interactions, and other chemical and physical principles that take effect at the molecular level. Indeed, the conclusions presented here are supported by their very own measurements of R_{shunt} when they used the whole oocytes in stage I (Danker et al., 1999). Their reported values for R_{shunt} were greater than 30 kΩ, a value that contradicts my computations with their formula when the dimensions for conductivity are corrected (i.e. geometrical formula predicts values greater than 1 MΩ – see Danker et al., 2001). Other explanations may also be given. 1st Their injected currents produced voltage drops of opposite polarities at each of the two NE caps in unrestricted contact with the saline solution. According to their observations, the currents produced readily observable changes in the chromatin structure, suggesting NE alterations. 2nd The voltage drop caused by their currents could be substantial and could have caused irreversible damage of the NE, leading to an artificially leaky NE. Indeed, if their rationale that $R_{shunt} >> R_{NE}$, were correct, then most of injected current would flow through the NE and, with their minimal value of $I_{injected}$ of 100 μA (maximal of 1,000 μA in Danker et al., 1999; 200 μA in Danker et al., 2001), they would have caused a voltage drop with a lower limit of about 1,000 mV when R_{NE} was 10 kΩ (see calculations for R_{NE} in the following paragraph). In my experience, the NE from adult cardiac myocytes and cancer cells cannot withstand voltages greater than ±50 mV for tens of seconds (Danker et al., 2001 injected currents for several tens of seconds). This observation has prompted me to favor the use of 2-s voltage pulses in my experiments (e.g. Bustamante et al., 1995, 2000a,b). 3rd Their placement of the nucleus inside the tubing may have caused unnatural stretch-induced channel openings or closings (e.g. Bustamante et al., 1991; Ruknudin et al., 1993). This concept is supported by my unpublished observations of stretch-sensitive NE channels. 4th Their experimental system lacked control of the voltage across the NE, both spatially and temporally (e.g. Johnson and Lieberman, 1971) because some of the currents have an inactivation-like mechanism (e.g. Bustamane, 1992).

A conclusion made with NHT that deserves our attention is the data interpreted as supporting the existence of the diffusional channels peripheral to the large central channel of the NPC (Danker et al., 1999, 2001; Shahin et al., 2001; Schäfer et al., 2001 - see Mazzanti et al., 2001). If valid, the conclusion leads to the interpretation of the function of these putative channels as

alternate pathways for passive ion transport during macromolecular plugging of the NPC (see Mazzanti et al., 2001). However, the accumulated experimental data from patch-clamp as well as from pipette- and tubing-based voltage-clamp techniques suggest that the electrical leak in NHT is not negligible (see above discussion). Since the current NHT model does not fit the experimental observations (see Fig. 4), the interpretation of the leak current as originating from peripheral channels will require further experimental, and not theoretical, work. This conflict between experiment and theory does not arise when the patch-clamp technique is applied to the cell nucleus because no published patch-clamp data supports the existence of these channels. Indeed, since EM shows that NPCs has a preferred 8-fold geometry (see Fahrenkrog and Aebi, 2002), one is to expect that if there were 8 identical channels peripheral to the large central NPC channel, then one should observe a statistical probability function with 8 peaks corresponding to 8 identical channels per NPC (or to a multiple of 8) if these peripheral ion channels function independently of one another. Instead, such a probability function has never been observed (e.g. Bustamante, 1992). Let us see the statistical basis for this prediction.

For a steady-state system consisting of 8 identical units (the putative peripheral channels are identical) fluctuating or *gating* (in the biophysical sense) between 2 states (say fully open and fully closed) with two fixed or quantized values (say 0 and 500 pS, corresponding to the open and closed states, respectively), we have a distribution of peaks (each peak described by a normal distribution) that is described by the binomial distribution:

$$P_{open}(n) = [\ N! / n!\ (N-n)!\ ]\ p_{open}^{\ n}\ (1-p_{open})^{N-n} \qquad (19)$$

where P_{open} is the open probability of the channel population, p_{open} is the probability that a channel opens, N is the total number of functional ion channels (whether open or closed) and n is the number of ion conducting, open channels (see Bustamante, 1992). Excellent simulation tools for the discrete binomial distribution function, and for its analog continuous function (normal distribution), are currently available in the internet (e.g. Narasimham, 2002; Stark, 2002). For a two state channel system, the probability that a channel is open, p_{open}, plus its probability of being closed (p_{closed}) should always be one. Note that, as originally conceived (see Hinshaw et al, 1992), these 8 peripheral channels are permanently open. Therefore, a constant current (i.e. very similar to a technical artifact caused by electrical leak or shunt) should be recorded. This is also not observed in patch-clamp experiments.

Fig. 6a shows simulations of the probability function histograms for a population of 4, 8, 16 and 24 identical independent channels with 2 states: open and closed, and for conditions in which p_{open} is 50% and 75%. The 4, 8, 16 and 24 values of N would correspond to 0.5, 1, 2 and 3 times the number of peripheral channels assumed to exist in an NPC. Note that each bar in the histogram corresponds to a current level (here set to an arbitrary unity, say 400 pA) plus its variation (here half the unit value, say 200 pA). That is, the bar graphs correspond to superimposed normal distributions. Thus, for example, for the 4-channel histogram (half the population of peripheral channels of an NPC) we would have 4 normal, bell-shaped curves (see Bustamante, 1992). As shown by panel b of Fig. 6, the experimental data does not support the existence of these peripheral channels because one never records such a P_{open}.

As mentioned at the beginning of this chapter, an additional source of confusion is the fact that many patch-clamp recordings are carried out in the nucleus-attached mode, a procedure in which the tip of the patch-clamp pipette is placed against the outermost NE membrane, the ONM. Clearly, if the NPCs were permanently closed or plugged, the ion channel activity recorded in this mode would have to come necessarily from ion channels at the ONM. Instead, it has been shown with fluorescence microscopy that when nuclei are bathed in saline, small particles such as dextrans and dendrimers do enter the nucleus (Bustamante et al., 2000a,b; Bustamante, 2002). Therefore, due to the large contribution of NPCs to nucleocytoplasmic ion flow, electrophysiological measurements must be validated with an independent technique, such as fluorescence microscopy, to test the state of conduction of the NPC population. While one should not be skeptic about the existence of important ion channels at the ONM and INM (e.g. Rousseau et al., 1996; Franco-Obregon et al., 2000; Guihard et al., 2000; Valenzuela et al., 2000; Boehning et al., 2001; see Mazzanti et al., 2001), to avoid potential conflict with prevalent cell biology concepts, the ion channel investigator using whole nucleus must prove that NPCs are excluded as the source of ion channel activity. Note that this is not required for those using artificial systems such as lipid bilayers. **Fig. 7** illustrates both large and small conductance ion channel activity detected with patch-clamp from NE patches of Dunning G prostate cancer cells (Bustamante, unpublished – see also Bustamante et al., 2000a). In my experience, the small conductance ion channel activity is a rare event (in the order of 1 in 100 experiments). It is not clear whether the small conductance ion channel activity results from a partially occluded NPC or whether it is a genuine sub-state for ion conduction along the NPC. That it cannot correspond to the hypothetical peripheral channels is seen by the fact that the value of the small conductance activity is not an 8^{th} of the large conductance value. The small conductance activity may also be interpreted as a sub-state of the channel (Bustamante, 1994a). Finally, the low probability with which I observe these small ion channel openings may be due to the fact that my techniques produce isolated nuclei with NPCs capable of macromolecular transport. In fact, I recently showed in intact living syncytial nuclei that when the extranuclear environment is replaced with saline, the final state of the NPCs is one of 100% open probability (Bustamante, 2002).

Identifying Nuclear Pores As The Source For The Recorded Ion Channel Activity

Although all electrophysiological observations are of great value, perhaps the most relevant to current cell and molecular biology is the possibility of quantifying NPC gating and transport capacity with electrophysiological techniques. Since the techniques for the analysis of ion channels other than NPCs are standard to channels found in other cellular structures (recently reviewed in Mazzanti et al., 2001), it seems more productive for us to focus on the description of how can we tell that NPCs, and not other channels, are responsible for the observed ion channel behavior.

As discussed above, the fact that the recording patch-clamp pipette is placed on the cytoplasmic face of the NE may be taken to mean that the recorded ion channel activity derives from ion channels from the ONM. To determine if this is indeed the case, one must carry out the fluorescence microscopy tests described above (see Fig. 2) for determining the capability of NPCs for the passive diffusion of ions and for the active transport of macromolecules. It is important to note that NPCs appear to require the integrity of the NE for leak of its cisterna (i.e. the perinuclear space) will result in the impairment of NPC-mediated macromolecular transport (e.g. by Ca^{2+} loss – see Bustamante, 1994b, Bustamante et al., 1994). To further support that the

source of ion channel activity are the NPCs, one may apply an NPC monoclonal antibody that has been shown effective on blocking ion channel activity (Bustamante et al., 1995a; Prat and Cantiello, 1996). Finally, one may use the concept developed for other preparations that the organelle-targeted proteins block ion channel activity (e.g. Thieffry et al., 1992; Lohret and Kinnally, 1995; Pelleschi et al., 1997; Teter and Theg, 1998; Heins et al., 2002 – see reviews by Neuhaus and Wagner, 2000; Bölter and Soll, 2001) which, in our case, would be macromolecules known to contain nuclear localization signals (NLS) or artificially conjugated to contain an NLS (Bustamante et al., 1995a, 2000a,b; Bustamante, 2002 - see Fig. 2). To determine whether ER channels are present in the patch from which one is recording activity from fewer simultaneous channel openings than the NPC surface density (e.g. less than 5 channel openings when the NPC density is 10 per μm^2), one may use puromycin to clear out protein conducting channels of the ER from nascent proteins (Simon and Blobel, 1991). In my hands, however, the application of puromycin to NE patches has neither resulted in the appearance of new, added ion channels nor in the alteration of the recorded ion channel activity (e.g. Bustamante et al., 1995a – Bustamante, unpublished data). It is for these reasons that, under these conditions, it is possible to state without hesitation that the observed derives from NPCs and not from other structures. Therefore, patch-clamp is a useful tool to investigate and quantify NPC function. One should expect that NHT would also be very useful (like the loose patch-clamp technique has been) once independently supported with the fluorescence microscopy tests that I proposed above and illustrated in Fig. 2.

Identifying Ion Channel Selectivity for Nuclear Ion Channels

So far, it appears that only K^+, under normal conditions, is the major charge carrier for currents purportedly resulting from the NPC (e.g. Bustamante, 1992, 1993). IP_3R-operated channels have been assigned Ca^{2+} selectivity (Boehning et al., 2001). Selectivity for Cl^- has been assigned to channels from other preparations (e.g. Franco-Obregón et al., 2000; Valenzuela et al., 2000 – see Mazzanti et al., 2001 for a complete list). To estimate the selectivity of an ion channel one departs from the concept that, in a complex system, the equilibrium or resting potential is given by the relative contributions of each ion species diffusing through the channel:

$$V_{system} = \Sigma\, C_j V_j \qquad (20)$$

where C_j and V_j are, respectively, the relative conductance coefficient and the equilibrium potential for the j^{th} ion species. Note that the equilibrium potential of a system can be measured as the value of potential at which the current reverses direction. For this reason, this potential is also known as the reversal potential ($V_{reversal}$). The relative conductance coefficient, C_j, is given by:

$$C_j = G_j/G_{total} \quad \text{and} \quad G_{total} = \Sigma\, G_j \qquad (21)$$

where G_{total} is the sum of all the relative coefficients.

One next has to take into account the fact that the permeability is a quantity that refers to particles and not to charges (this is the reason why we have the valence of the ion in the Nernst equation). Therefore, the results have to be calibrated accordingly. For a system consisting of K^+ and Ca^{2+} ions, one has, according to the Goldman-Hodgkin-Katz equation (see Hille, 2001):

$$V_{reversal} = (RT/F)\ \ln\{(P_K[K^+]_{cytoplasm}+2P_{Ca}\ [Ca^{2+}]_{cytoplasm})/(P_K[K^+]_{nucleoplasm}+2P_{Ca}\ [Ca^{2+}]_{nucleoplasm})\} \quad (22)$$

where the sub-index for each parameter indicates the compartment to which one refers to. Note that if there were an electrogenic pump (an ATP-dependent mechanism that generates an electrical current during exchange of ions between the two sides of the membrane), an additional term would have to be added or subtracted to the factor containing the permeabilities. So far, no report has appeared on electrogenic pump of the NE.

To simplify the biophysical analysis, one uses bi-ionic conditions (e.g. Hille, 2001). Bi-ionic conditions mean that, at one side of the membrane one replaces the reference ion (e.g. K^+) with the tested ion (e.g. Ca^{2+}). Thus, bi-ionic conditions do not appear to be a friendly environment for NPCs. The reversal potential under bi-ionic conditions is then used with the equation to determine the permeability ratio for the two ion species tested. Thus, for K^+ and Ca^{2+}:

$$V_{reversal} = (RT/F)\ \ln\{(P_K[K^+]_{cytoplasm})/(2P_{Ca}\ [Ca^{2+}]_{nucleoplasm})\} \quad (23)$$

Using this approach, Boehning et al. (2001) determined that K^+ was 15 times more permeant than Cl^- and that Ca^{2+} was 4 times more permeant than K^+. Note, however, that the bi-ionic conditions used for the calculation of Ca^{2+} permeability relative to K^+, Boehning et al. (2001) used 50 mM Ca^{2+}. The use of this high level of Ca^{2+} may be too extreme for the NE to handle. Indeed, Stehno-Bittel et al. (1995) reported that Ca^{2+} levels higher than 1 mM destroyed the NE of *Xenopus L.* oocytes. Therefore, it would appear that a less stressful approach would be to still use the two ions but under less demanding conditions for the NE structure (i.e. using the more complex equation 22).

One must also be careful when making ion replacements because this maneuver may seriously affect the junction potentials caused by ion species difference (a phenomenon similar to the potentials developed at metal junctions such as those created when copper and aluminum are joined). Therefore, one has to correct for this with a proper equation (e.g., Neher, 1995; Barry, 2002). But variations of divalent ions like Ca^{2+} are known to affect the surface charge of the cell membranes and this effect alone can affect the voltage sensed by the ions, having greater effect with larger variations (e.g. Ehrenstein, 2001).

There is also a pharmacological approach to identifying the selectivity of an ion channel. This may be useful when considering the questions just mentioned. For example, drugs known to block Cl^--channels were used to identify the activity recorded from hepatocyte nuclei (e.g. Tabares et al., 1991) and heparin, a favorite probe for blocking IP_3R-operated channels, was also used to identify the ion channel activity (e.g. Boehning et al., 2001). The pharmacological

approach, like others, has also its drawbacks. Thus, for example, the once though specific Ca^{2+}-antagonists drugs were shown to be less than specific and the hunt has always been for specific drugs and/or definitions of channels according to the pharmacological blocker (e.g. Bustamante, 1983, 1985 – Bustamante, unpublished observations; Ren et al., 2001). Heparin is another example of a pharmacological tool that must be used with caution for it is used to extract fractions from the NE (e.g. Strambio-de-Castillia et al., 1995; Rout and Field, 2001) and has been shown to alter ion channel behavior (e.g. Knaus et al., 1990; Krasilnikov et al., 1999). Taken together, it appears that one should exercise extreme care when using pharmacological tools with the NE, and specially, when recording from whole nucleus.

CONCLUSIONS

Four decades have passed since the first publication on NE electrical resistance (e.g. Loewenstein et al., 1962). Since 1990, efforts in applying patch-clamp to the cell nucleus (Matzke et al., 1990; Mazzanti et al., 1990) have led us through an arduous path that promises to fuse electrophysiology with the cell/molecular biology of the nucleus. Despite the efforts, however, some questions remain to be solved before the electrophysiological observations can be applied to other fields (see Bustamante, 2001).

One vexing problem discussed here is that of the shunt resistance, R_{shunt}, another is the identification of the source for the recorded ion channel activity. Ever since the time that Nobel laureates Hodgkin and Huxley introduced the voltage-clamp technique, R_{shunt} has been a parameter to reckon with (see Hodgkin and Huxley, 1963). The application of glass capillaries and plastic tubes for the recording of ionic currents has met the challenge with certain preparations because the input impedance of the system (preparation together with the pipette/tubing) has been shown to be sufficiently high (e.g. Kostyuk, 1982, 1984; Kostyuk and Krishtal, 1984). The achievement of this high impedance has been possible, in many preparations, with the usage of un-physiological ions such as fluoride (e.g. Kostyuk, 1982, 1984; Kostyuk and Krishtal, 1984). It is for this reason that patch-clamp has succeeded in popularity over these alternate techniques. I have no doubt about the merit of the NHT approach (see introductions in Danker et al., 1999, 2001). However, the major vexing problem challenging the NHT is that there is no frame of reference but a leak current that can not be assumed to derive from channels peripheral to the NPC. A justification similar to that of the NHT was used for the loose patch-clamp (e.g. Almers et al., 1983; 1984; Anson and Roberts, 1998 – see review by Roberts and Almers, 1992). As originally conceived, this technique was applied to the study of voltage-dependent plasmalemmal Na^{+}-channels. The loose patch-clamp technique could be validated because the behavior of these Na^{+}-channels were well established. At difference from pipettes and tubing techniques used in the past for the recording of classical voltage-dependent ion currents (e.g. Bustamante and McDonald, 1983) the macroscopic ion currents through NPC populations look very much like leak currents (e.g. Bustamante, 1992, 1993, 1994a; Bustamante et al., 1995a; 2000a,b). As discussed here, a solution to this current limitation of both the NHT and of some of the patch-clamp data being produced is the incorporation of fluorescence microscopy, an approach not uncommon to many electrophysiological laboratories working with isolated cells (e.g. Bustamante et al., 1995a, 2000a,b; Bustamante, 2002). The major criticism on the artificiality of patch-clamp experiments due to the physiological solutions used without NPC-mediated macromolecular substrates appears to have found a remedy with the recent introduction of living syncytial nuclei in their native environment (Bustamante, 2002). In addition, the new

preparation demonstrates a high rate of macromolecular transport as well as of transcription and translation. This was shown by their transport of nuclear-targeted proteins and by their expression of foreign cDNA for the green fluorescent protein (GFP – Bustamante, 2002). It must be noted that, when the NPCs of this intact preparation are engaged in macromolecular transport, both patch-clamp and fluorescence microscopy (low-sensitivity CCD – Bustamante, 2002 and unpublished observations) show that the NE restricts the diffusion of monoatomic ions and of small fluorescent particles such as 4 kDa dextrans and 5.4 nm diameter dendrimers (Bustamante, 2002). This observation requires further testing with high-sensitive light detectors since it challenges the dominant thinking of NPCs freely permeable to monoatomic ions. This dominant thinking has been consolidated, in recent years, with data from permeabilized cells, from isolated nuclei in cell extract, and from cells and nuclei injected with micropipettes. Therefore, as done for patch-clamp experiments identifying NPC ion channel behavior (e.g. Bustamante, 2002; Bustamante et al., 1995a-c, 2000a,b), NHT experiments could greatly benefit from the integration of fluorescence microscopy approaches. The advanced level of progress of the fluorescence microscopy industry (e.g. Molecular Probes) should facilitate this integration.

The identification (i.e. inclusion or exclusion) of the NPC as the source of ion channel activity appears to be of great importance. The electrophysiological thinking, which has been mostly biophysical and pharmacological, must be complemented with the accumulated knowledge of nucleocytoplasmic experts in cell and molecular biology. If NPCs engaged in heavy macromolecular traffic do indeed block physiological ions (e.g. Bustamante et al., 1995a; Bustamante, 2002), then many of the phenomena observed on nucleocytoplasmic gradients of ions will no longer need to be considered a technical artifact and may receive undivided attention. The application of single-channel statistics and the molecular Coulter counter principle (e.g. Bezrukov, 2000) has conferred electrophysiological methods with high hopes for the study of not only functional but also structural genomics (e.g. Kasianowicz et al., 1996; Matzke and Matzke, 1996; Hanss et al., 1998; Meller et al., 2000; Howorka et al., 2001; Marziali and Akeson, 2001; Vercoutere et al., 2001; Wang and Branton, 2001; Fertig et al., 2002; Klemic et al., 2002; Sigworth and Klemic, 2002). Since several diseases have been reported to be connected to nuclear structures relevant to NPCs (e.g. Nagano and Arahata, 2000; Wilson, 2000; Berry et al., 2001; Burke et al., 2001; Hutchison et al., 2001; Invernizzi et al., 2001; Morris, 2001; Mounkes et al., 2001; Raharjo et al., 2001; Arbustini et al., 2002;), electrophysiological measurements may be also relevant to future tools for the diagnosis of cellular pathologies (see Bustamante, 2001).

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APPENDIX: Measurement Theory

Common to all measurements is the fact that errors add up, they never cancel each other (e.g. Barford, 1985; Baird, 1988). To illustrate this principle of physical measurements, let us take the simple example of the error made in deriving the area of a rectangle, $A_{rectangle}$, from its width, W, and length, L. That is,

$$A_{rectangle} = W \times L \tag{24}$$

In order to derive the relative error, we can apply the L'Hospital rule:

$$\log (A_{rectangle}) = \log (W \times L) = \log(W) + \log(L) \tag{25}$$

Note that the logarithm is taken due to the differential calculus properties of this function (see next equation).

$$(\Delta A_{rectangle}/A_{rectangle}) = | (\Delta W/W) | + | (\Delta L/L) | \tag{26}$$

Note also that the absolute values (indicated by the vertical bars) are taken because errors always add up; they do not cancel each other.

Likewise, the error made in calculating the area of a circle, A_{circle}, of radius r is

$$A_{circle} = \pi r^2 \tag{27}$$

applying the L'Hospital rule:

$$\log (A_{circle}) = \log (\pi r^2) = \log(\pi) + 2 \log(r) \tag{28}$$

$$(\Delta A_{circle}/A_{circle}) = 2 | (\Delta r/r) | \tag{29}$$

Thus, for the above formulas, the relative error in the calculated quantity equals the sum of the absolute values of the individual relative errors. From the above it is clear that with smaller quantities, the greater the relative resulting error. This concept must be used for proper assessment of the relative error in R_{shunt}, whether patch-clamp or NHT. In the end, we have to apply common sense to decide what the error is. For example, even if our statistical error in

A_{circle} is 0.000001%, (e.g. as a result of a large number of measurements) if our relative error is 10%, our final error (the one we must report to other scientists) cannot be better than 10%. In the particular case of patch-clamp or NHT, since we have to consider the complex geometry and forces for the molecular interactions between the NE and the glass of the pipette (patch-clamp) or tube (NHT), it seems easier to use fluorescent probes to directly assess the dimension of the leak or gap for the two techniques.

FIGURES

Fig. 1. Ion channels at the nuclear envelope. (a) Schematics of a nucleus-attached patch-clamp recording. A pipette is placed to the cytoplasmic surface of the nucleus and the ion current is recorded while a known voltage is applied between the electrodes inside the pipette and the bath (reference set to ground or zero potential). Under physiological saline, the NPCs (shown as large channels) outside the pipette tip facilitate the virtual grounding of the nucleoplasmic side of the NE. As the dominant pathway for ion flow is that of the NPCs, electrical signals must derive from NPCs. Only when all the NPCs are plugged or closed is that we can say that the recorded ion channel activity derives from ion channels other than NPCs. (b) Diagram illustrating the different channel types that may be found at the NE. The only conspicuous channel under EM and AFM is the NPC. Ion channels at the outer and inner nuclear membranes, ONM and INM, may also contribute to ion fluxes. The arrows show the inward direction of the electrical current (i.e. the movement of positive ions such as K^+) when the voltage in the cytoplasmic side of the NE is greater than the voltage at the nucleoplasmic side of the NE. When negative ions (e.g. Cl^-) are the charge carriers, the direction of the electrical current will remain inward, although the ions will move toward the nuclear exterior. When the voltage at the cytoplasmic side is smaller than that at the nucleoplasmic side, the electrical current is outward, the flow of positive ions is outward, but the flow of negative ions is inward. Finally, if there is no voltage gradient, the electrical driving force for the charge carriers is absent and there is no ion flow. As the force determining the movement of ions is electrochemical in origin, ion gradients must be taken into account, as done for classical ion channel currents. To date, patch-clamp data suggests that, under steady-state conditions, the chemical gradient is negligible (Bustamante, 1992; Boehning et al., 2001). Finally, references are made to receptors found at the NE such as for angiotensin II (AT_1), IP_3 (IP_3R), and ryanodine (RyR) as well as to pumps such as Na^+-K^+ and Ca^{2+}.

http://www.geocities.com/nuclear_electrophysiology/FIG_1_SMALLEST.htm

Fig. 2. Use of fluorescence microscopy to determine the state of ion and macromolecular conduction of nuclear pores. (a, b) Tests for NHT are performed with probes (e.g. Alexa Fluor-Dextrans, Molecular Probes) to determine the relevance of the leak or shunt current and the dimension of the gap. In a, the fluorescent probe passes through the gap between the nucleus and the tube wall but does not enter the nucleus. Therefore, the shunt is too great to assume it negligible (i.e. $R_{shunt} << R_{NE}$). In b, the gap is of small dimensions and the molecular interactions between the NE and the tube wall are strong enough to produce a tight seal. Therefore, the shunt is negligible (i.e. $R_{shunt} >> R_{NE}$). (c, d) Test for patch-clamp are performed with similar probes. When the NPCs are closed or plugged, panel c, the probes cannot enter the nucleus because the NPCs are the only direct route for nucleocytoplasmic transport. Therefore, any ion channel activity recorded derives from the ONM. When the NPCs are open and unplugged (i.e. there is no macromolecular translocation), panel d, the probes go inside the nucleus. Therefore, the ion channel activity derives from NPCs. Under these conditions, the NPCs outside the pipette provide a bypass route that makes the nucleoplasmic side of the NE virtually connected to the bath electrode. Panels e and f demonstrate the use of macromolecular probes (e.g. B-phycoerythrin, Molecular Probes, conjugated to the NLS of the SV40 large T antigen, Sigma Chemical). A macromolecular probe is placed outside the nucleus. Whether or not the molecules enter the nucleus, will indicate that the NPCs are or ar not capable of macromolecular transport.

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Fig. 3. Tubing system for simultaneous single cell voltage-clamp and intracellular perfusion. (a-d) Images taken with a custom inverted microscope (Bustamante, 1991). The images show the process of forming a seal between the hole in the plastic pipette (e.g. Bustamante & McDonald, 1983) and a neuroblastoma cell (about 20 µm in diameter). (a) Neuroblastoma cells. (b) Low magnification view of the tubing placed over the target cell. (c) High magnification view of the tubing placed just over the target cell, a few seconds prior applying a gentle suction to attract the cell towards the hole in the tube. (d) High magnification view of the cell in the hole ready for voltage-clamp and intracellular perfusion (achieved after a vacuum pulse). From Bustamante (1983). (e) Diagram showing the placement of the two pairs of half-cells, Ag/AgCl electrodes, for the simultaneous recording of current and voltage. The 4-electrode system is a requirement of experimental arrangements where large polarizing currents are used (e.g. Hodgkin and Huxley, 1952). In the voltage-clamp configuration, the detection system incorporates a circuit for the compensation of the resistance in series to the target, R_{series} (e.g. Bustamante and McDonald, 1983).

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Fig. 4. Computed values for the resistance contributed by the NE and for the shunt resistance using the NHT model. (a) Plot of expected values for the resistance of the NE caps, $R_{NE\text{-}caps}$, with NPC conductance, γ_{NPC}, of 500 pS and with density of ion conducting NPCs set to 10 NPC.µm^{-2}. (b) Plot of shunt resistance, R_{shunt}, due to the leak of electrolyte between the NE and the wall of the NHT tubing (gap length, L_{gap}, of 100 µm; gap radius, r_{gap}, 100 µm) and between the NE and the tip of the patch-clamp pipette (L_{gap} = 0.1 µm, r_{gap} =1 µm). The resistivity, ρ, was assumed to be 100 Ω.cm (conductivity= 10 mS.cm^{-1}). The values of gap width, w, used were between 1.0 and 10^{-4} µm. For NHT, R_{shunt} goes between 1.6 MΩ and 15.9 GΩ. For patch-clamp, R_{shunt} goes between 318.3 kΩ and 1.59 GΩ. Therefore, from these models one should expect that the NHT method provided better seal than patch-clamp. However, this is not supported by the experimental observations.

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Fig. 5. Reduction in shunt resistance induced by current injection simultaneous to intracellular perfusion with fluoride ions. (a, b) Human atrial myocyte placement prior and after its placement inside the hole of a perfusion tube with suction (see Fig. 3). (c) Superimposed storage-oscilloscope traces of membrane potential recorded during current injection of 100 nA and tubing perfusion with buffered 150 mM CsF (see Bustamante and McDonald, 1983). The uppermost and lowermost traces correspond, respectively, to the initial and final (steady-state) levels of the membrane potential of –12 and –102 mV. From these observations can be concluded that R_{shunt} was about 0.1 and 1 MΩ. Without F^- inside the tubing, the higher value of R_{shunt} was not attained (Bustamante, 1981). Experiment 81070401.

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Fig. 6. Theoretical and experimental amplitude histograms for patch-clamp recordings from the nuclear envelope. (a) Binomial distribution histograms (gray bars) for a population of N identical and independent ion conducting channels having two states: open and closed. N was set to 4, 8, 16 and 24 to predict the effect of the putative peripheral channels (thought to be 8 per NPC). The histograms on the left and right columns were generated for single channel open probability, p_{open}, of 50% and 70%, respectively. On the horizontal axis, the relative NE conductance amplitude is given. The number n represents the relative level of conductance (as ion channel opens and closes, their populations will display quantum jumps in the value of NE conductance). Therefore, n corresponds to the number of channels simultaneously open. On the vertical axis, the relative probability of finding the particular value of NE conductance is given. As the measured current is a continuous variable and is contaminated by noise, for each particular level, there is a normal distribution of values described by a Gaussian curve (black bell-shaped curves). The simulations were carried out with the binomial java applet given by Stark (2002). (b) Experimental histograms from 12 experiments. The histograms were generated with pClamp (Axon Instruments). As for our discussion, neither the height nor the width is relevant, to facilitate comparison, the peaks of each histogram were set to the same height. Although the widths of the histograms are the same, they do not necessarily correspond to the same conductance interval because not all channels have the same conductance (e.g. one may be 350, the other may be 450 pS). Finally, note that the NE conductance starts at zero conductance (leftmost point in the axis).

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Fig. 7. Patch-clamp detection of NE large and small conductance ion channels. (a) Ion current recordings for two experiments with Dunning G prostate cancer cell nuclei showing an NE patch containing 10 channels of 416 pS and a patch containing a channel of 362 pS and a channel of 91 pS. The current signals were generated with 2-s voltage pulses applied between the pipette and the bath electrodes. Note that the onset of the voltage pulse generates a capacitive current that is related to the dielectric and not the ion conducting properties of the NE. (b) Diagram illustrating the two-electrode approach of patch-clamp. One electrode is applied to the inside of the recording pipette and the other is applied to the bath. The electronic apparatus is based on the *virtual-ground* operational amplifier. The electrode inside the pipette receives a known clamp voltage (thus patch-clamp). The bath electrode is used as reference. The voltage difference between the two electrodes contributes to the driving force for the electrical charge carriers. Although the pipette is applied to the ONM, the large number of open NPCs outside the pipette short-circuit, and thus make transparent to the recording, the NE outside the NE patch. Only when all NPCs are closed or plugged (i.e. not allowing ion flow) can we be sure that the recorded ion channel activity derives from sources other than NPCs.

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