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VOLTAGE-GATED SODIUM CHANNELS POTENTIATE THE INVASIVE CAPACITIES OF HUMAN NON-SMALL-CELL LUNG CANCER CELL LINES

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ABSTRACT

Ionic channel activity is involved in fundamental cellular behaviour and participates in cancerous features such as proliferation, migration and invasion which in turn contribute to the metastatic process. In this study, we investigated the expression and role of voltage-gated sodium channels in non-small-cell lung cancer cell lines. Functional voltage-gated sodium channels expression was investigated in normal and non-small-cell lung cancer cell lines. The measurement, in patch clamp conditions, of tetrodotoxin-inhibitable sodium currents indicated that the strongly metastatic cancerous cell lines H23, H460 and Calu-1 possess functional sodium channels while normal and weakly metastatic cell lines do not. While all the cell lines expressed mRNA for numerous sodium channel isoforms, only H23, H460 and Calu-1 cells had a 250 kDa protein corresponding to the functional channel. The other cell lines also had another protein of 230 kDa which is not addressed to the membrane and might act as a dominant negative isoform to prevent channel activation. At the membrane potential of these cells, channels are partially open. This leads to a continuous entry of sodium, disrupting sodium homeostasis and down-stream signaling pathways. Inhibition of the channels by tetrodotoxin was responsible for a 40-50% reduction of *in vitro* invasion. These experiments suggest that the functional expression of voltage-gated sodium channels might be an integral component of the metastatic process in non-small-cell lung cancer cells probably through its involvement in the regulation of intracellular sodium homeostasis. These channels could serve both as novel markers of the metastatic phenotype and as potential new therapeutic targets.

INTRODUCTION

Lung cancer is the most common cancer over the world (Parker et al., 1997). This malignancy is responsible for more than one million deaths each year. It is classified into non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) which occur with a frequency of 80% and 20%, respectively. The occurrence of metastases is always a marker of a poor prognosis. While the preferred organs of metastasis seem to be mainly determined by surface adhesion molecules, numerous cellular mechanisms or properties are involved in the metastatic potential of cancer cells. Among those properties are the acquisition of an enhanced migration, that requires activation of different signaling pathways, modifications of the cytoskeleton, expression of specific surface and secreted proteins. Besides, a very critical property of metastatic cancer cells is the acquisition of invading abilities, i.e. the capacity to degrade their extracellular matrix requiring the secretion and activation of proteases. Therefore, the exploration of new signaling pathways and mechanisms by which cancer cells acquire the ability to degrade their surrounding environment, to migrate from the primary site to reach the lymph or blood circulation, and finally to produce secondary tumors, would allow the identification of new targets for anti-metastasis chemotherapy.

Voltage-gated sodium channels (Na_v) are large glycoproteins composed of an α -subunit (250-260 kDa) that constitutes the central pore of the channel (Catterall, 1986) and at least two auxiliary β -subunits (30-40 kDa) that modify the channel function and participate in the targeting to the membrane (Catterall, 2000; Isom, 2001). Until now, at least ten genes encoding α -subunits have been identified. Nine of these constitute a single family and are noted $\text{Na}_v1.1$ to $\text{Na}_v1.9$ (Goldin et al., 2000; Goldin, 2001). Each isoform exhibits a specific sensitivity to the specific blocker tetrodotoxin (TTX) and two groups of Na_v isoforms are described, the TTX-sensitive (TTX-S : $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$, $\text{Na}_v1.4$, $\text{Na}_v1.6$ and $\text{Na}_v1.7$) and the TTX-resistant (TTX-R : $\text{Na}_v1.5$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$, $\text{Na}_v1.8$ being the more resistant isoform with an IC_{50} of 100 μM) sodium channels, which are blocked by nM and μM concentrations of TTX, respectively. Four isoforms of auxiliary β -subunits have been described and named $\beta1$ to $\beta4$ (Isom, 2001; Yu et al., 2003).

Sodium channels are well described in excitable cells, where they are responsible for the rising phase and the propagation of action potentials. Interestingly, different Na_v isoforms have been found in different cancers (Roger et al., 2006). In breast and prostate metastatic cancer cells, the activity of sodium channels has been shown to be linked to the invasive properties of these cells (Smith et al., 1998; Roger et al., 2003; Fraser et al., 2005). We and others previously reported that a sodium current was expressed in the highly invasive breast cancer cell line MDA-MB-231, while it was missing in two weakly invasive cell lines (Roger et al., 2003; Fraser et al., 2005). This sodium current is due to the activity of the foetal isoform of $\text{Na}_v1.5$ (Fraser et al., 2005), even though these cells express mRNA for numerous Na_v isoforms (Jude et al., 2006), and is involved in the *in vitro* invasion of MDA-MB-231 cells (Roger et al., 2003; Fraser et al., 2005). Interestingly, this isoform is also expressed in biopsies of metastatic breast cancer (Fraser et al., 2005).

In the current study, we investigated the presence of Na_v currents in four NSCLC cell lines and compared these results to those obtained in two normal lung epithelial cell lines. Electrophysiological and pharmacological properties as well as molecular identity of the functional channels were determined. We found that functional sodium channels participate in the invasiveness of cancer cells through changes in sodium homeostasis.

MATERIALS AND METHODS

Cell culture

All the cell lines studied are from human origin, normal (NL-20 and BEAS-2B) and cancerous (H23, H460, A549 and Calu-1). They were purchased from the ATCC (Rockville, MD, USA) and cultured according to the depositors recommendations. The cancerous cell lines used in this study are deriving from epithelial origins and are representatives of the four main histological groups of cancer cells in NSCLC. Indeed, cells coming from H23, H460, Calu-1 and A549 cell lines are respectively lung adenocarcinomal cells (Gazdar et al., 1980), large cell lung cancer cells (Banks-Schlegel et al., 1985; Brower et al., 1986), lung epidermoid carcinomal cells (Fogh et al., 1977) and bronchioloalveolar cancer cells (Giard et al., 1973; Lieber et al., 1976). In addition clinical features observed from patient with pure bronchioloalveolar tumour demonstrated no or weak distant metastasis (Laskin et al., 2005) indicating a weak invasive potential of this cancer subtype *in vivo*.

Electrophysiology

Patch-clamp experiments and sodium current analysis were performed as already described (Roger et al., 2003). Current amplitudes were normalized to cell capacitance and expressed as current density (pA/pF).

Solutions

The Physiological Saline Solution (PSS) had the following composition (in mM): NaCl 140, KCl 4, MgCl₂ 1, CaCl₂ 2, D-Glucose 11.1, and HEPES 10, adjusted to pH 7.4 with 1 M NaOH. N-methyl-D-glucamine was substituted for sodium to create Na⁺-free external solution, and MgCl₂ was substituted for CaCl₂ to create a Ca²⁺-free external solution. The intrapipette solution had the following composition (in mM) : K-Glutamate 125, KCl 20, CaCl₂ 0.37, MgCl₂ 1, Mg-ATP 1, EGTA 1, HEPES 10, adjusted to pH 7.2. Percentage block or reduction of I_{Na} by tetrodotoxin (TTX) was calculated from the difference in peak current generated by a depolarization to -5 mV with and without the drug. The drug dose-response curves were fitted to a sigmoid logistic function (Roger et al.,

2003). Drugs and chemicals were purchased from Sigma-Aldrich (St Quentin, France) and Latoxan (France).

Fluorescence measurement of intracellular sodium ions

Intracellular levels of Na^+ were monitored using the ratiometric fluorescent dye SBFI. Cells were cultured in WillCo-dish® glass bottom dishes (WillCo Wells, The Netherlands). Cells were incubated for 180 min at room temperature in PSS containing 10 μM SBFI-AM. Excess dye was removed by rinsing the cells twice with PSS. The cells were then incubated for an additional 30-45 min in PSS. The dish was placed on the stage of a Nikon Eclipse TE2000-S inverted epi-illumination microscope (Nikon, France). Excitation light (75W Xenon arc lamp) at the two excitation wavelength maxima of SBFI (340/380 nm) was chopped by a monochromator (Cairn Optoscan, UK). The excitation protocol was a 50 ms excitation at each wavelength every 4 s. Excitation light was directed through a 60x oil immersion objective with a numerical aperture of 1.4 (Nikon Plan Apo, France). Fluorescence emission at 510 ± 20 nm was detected by a photomultiplier tube (PMT). The analogical signal of the PMT was digitized at a sampling frequency of 2 kHz. Autofluorescence was negligible. Background fluorescence, determined at 340 and 380 nm from a cell-free area of the coverslip after the loading period and wash, was subtracted. Calibration of intracellular sodium concentration was performed in each studied cell. This was done by perfusing two calibration solutions on the cells: 10 and 20 mM Na^+ . These solutions had the following compositions (in mM): EGTA 10; HEPES 5; NaCl + KCl 150; gramicidin D 0.005. SBFI was purchased from Molecular Probes (The Netherlands).

Confocal microscopy

NL20, A549 and H460 cells were cultured on glass coverslips for 48 h and were then fixed for 10 min in 4% paraformaldehyde in PBS at room temperature. In some experiments the cells were permeabilized by incubation for 15 min in 0.1% triton-X-100 in PBS. Unspecific sites were saturated by incubating for 30 min with 3% bovine serum albumin and 3% normal goat serum in PBS. $\text{Na}_v1.7$ channels were detected by incubating the cells for 60 min with a rabbit polyclonal anti- $\text{Na}_v1.7$ diluted 1:70 in PBS. The Ab anti-

Nav1.7 was prepared as described previously using the following peptide sequence: NH₃-CLRWP EENENETLHNRT-OH as an external epitope (Xu et al., 2005). This antibody recognizes a sequence of the third extracellular region (E3) of Nav1.7 channels and can also be used as a specific inhibitor for this isoform. Cells were then washed and incubated for 60 min with an Alexa fluor 488-coupled secondary antibody (Molecular probes) diluted 1:1000 in PBS. Confocal microscopy was performed with an Olympus Fluoview 500 instrument.

Cell survival and proliferation

Cells were seeded at $4 \cdot 10^4$ cells per well in 12 wells of a 24-well plate for a given condition on two separate experiments and were grown for a total of 5 days. The culture medium and TTX were changed every other day. Growth and viability of cells were measured as a whole by the tetrazolium salt assay (Mosmann, 1983) as previously described (Roger et al., 2003). Cell proliferation was expressed as formazan 570 nm absorbance and expressed as a ratio by comparison to a control condition, without TTX, for each cell line (on the same day of the experiment).

Migration and in vitro Invasion Assay

Migration was analyzed in 24-well plates receiving 8 μ m pore size polyethylene terephthalate membrane cell culture inserts (Becton Dickinson, France). The upper compartment was seeded with $4 \cdot 10^4$ viable cells in their basal culture medium. The lower compartment was filled with their culture medium supplemented with 20% FBS, instead of 10%, as a chemoattractant. After 48 h at 37 °C, remaining cells were removed from the upper side of the membrane, and cells that had migrated and were attached to the lower side were stained with haematoxylin and counted in the whole insert, using a light microscope at x200 magnification. *In vitro* invasion was assessed using the same inserts and the same protocol as above but with the membrane covered with a film of Matrigel® (Collaborative Research), an extracellular-mimicking matrix. Migration and invasion assays were performed in triplicate in 8 separate experiments. For easier comparison between cells lines, results obtained for migration and invasion were normalized to the control condition.

Molecular biology

Cell line RNA extraction and RT-PCR - Total RNA extraction was performed from all the cell lines. RNA yield and purity were determined by spectrophotometry and only samples with an A_{260}/A_{280} ratio above 1.6 were kept for further experiments. Two micrograms of total RNA were then reverse-transcribed. Standard Polymerase Chain Reaction (PCR) was performed, using cDNA obtained from 100 ng of total RNA. Primers used for PCR were specific for each Na_v α - and β -subunits isoform (see Table 1). The efficacy of these primers was checked in human tissues where the isoforms were previously described to be mainly expressed, i.e. in the central nervous system for $Na_v1.1$ to 1.3, the peripheral nervous system for $Na_v1.6$ to 1.9, skeletal muscle for $Na_v1.4$ and cardiac muscle for $Na_v1.5$. The temperature profile was 2 min at 95°C followed by amplification for 40 cycles which consisted of 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C and a final extension for 2 min at 72°C. PCR products were then analysed by electrophoresis in 2% agarose gel containing ethidium bromide, and visualized by UV trans-illumination (Gel Doc 1000 system, Bio Rad).

Western Blot Studies

Total proteins were extracted from the different cell lines studied. Total protein concentrations were determined in triplicate by the bicinchoninic acid (BCA) method. For the study of Na_v proteins, samples were incubated for 30 minutes in a SDS sample buffer at 37°C before being loaded at a total protein concentration of 100 μ g per lane and run on a 6% acrylamide gel. For the study of beta-actin proteins, samples were boiled for 5 minutes in a SDS sample buffer and loaded at the total protein concentration of 10 μ g per lane on a 10% acrylamid gel. In both cases, protein samples were then transferred to PVDF membranes. After saturating for 2 h in 5% non-fat milk TTBS solution (containing 0.5% Tween 20), the membrane was incubated overnight at 4°C with a pan-specific Na_v rabbit primary antibody (1:500 dilution, SP19, Alomone Labs, Jerusalem) or with a rabbit polyclonal anti-beta-actin antibody (1:2000 dilution, Abcam, UK) in a 2% non-fat milk TTBS solution. The membrane was then incubated for 1 h at room temperature, with a goat

anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, USA). Immunoblots were visualized with the ECL immunodetection system (Amersham Biosciences, UK). Specific bands for Na_v were evaluated by preincubating the antibody for 1 h at room temperature with the control antigen. In this case, only the specific band disappeared.

Statistics

Data are described as mean \pm standard error of the mean (n = number of cells). Two-way repeated measures ANOVA followed by a Student–Newmann–Keuls test were used to compare the sodium current densities between each cell line. One-way ANOVA on ranks followed by a Student-Newmann-Keuls test were used to compare cellular membrane potentials, cell proliferation, migration and invasion.

RESULTS

Properties of sodium channels and consequences of their activity on intracellular sodium homeostasis

Under voltage clamp conditions, a membrane depolarization triggered a rapid inward current in all H23, H460 and Calu-1 cells tested (see the arrows in Fig. 1A). No such current was observed in the normal NL-20 (11 cells studied) and BEAS-2B (9 cells studied) lung epithelial cells, nor in the A549 cancer cell line (11 cells studied). Removal of external sodium ions (“ Na^+ -free solution”) induced the total and reversible (“Wash out”) disappearance of the fast inward current triggered in H460 (Fig. 1Ba), H23 and Calu-1 cells (data not shown), while removal of external calcium ions did not have any effect on the inward current (Fig. 1Bb). Application of the specific inhibitor TTX (Fig. 1Bc) completely blocked this inward current without unmasking any other voltage-gated inward current. Indeed no other voltage-gated inward current such as calcium current was observed. Figure 1C shows the sodium current-voltage relationships for H23, H460 and Calu-1 cell lines. These currents activated above -55 mV for the three cell lines. The maximal currents were

obtained above 0 mV and the reversion potentials were close to +60 mV, accordingly to the sodium equilibrium potential in our conditions. The membrane potentials of all cell lines, both normal and cancerous, were approximately -30 mV and not significantly different (see Table 2):

It is interesting to note, in Figure 1, that the four cancerous cell lines showed an apparent down-regulation in the outward currents as compared to normal cell lines (NL-20 and BEAS-2B).

The conductance-voltage curves indicate that the inward current starts to activate above -55 mV for the three cell lines H23, H460 and Calu-1 (Fig. 2A), and that the activation is maximal above 0 mV. The availability-voltage curves show that the current starts to inactivate above -60 mV for H23 cells, -80 mV for H460 cells and -90 mV for Calu-1 cells (Fig. 2A), and that the inactivation is complete for voltages higher than 0 mV for all cell lines. In all three cell lines, the availability-voltage relationships are hardly fitted by a single Boltzmann relation, suggesting the presence of more than one functional Nav isoform in these cells. For each cell line, H23, H460 and Calu-1, the superposition of the availability-voltage curve with the conductance-voltage curve on the same graph shows that there is a window of voltage between -55 and 0 mV in which the channels are partially activated and not fully inactivated (Fig. 2A). This is more clearly seen on figure 2B in which a persistent sodium current was observed when the cell was depolarized from a holding potential of -100 mV to -30 mV, which is the mean membrane potential of the cells. Indeed, the continuous entry of sodium ions through the not-inactivated channels at the normal membrane potential of cancer cells must lead to a modification of the intracellular sodium homeostasis. Therefore we can reasonably expect that cancer cells displaying these sodium channels must have a higher internal sodium concentration than non cancerous cells. This hypothesis was confirmed by the measurement performed using the sodium specific fluorescent probe SBFI (Fig. 2C), showing that H460 cancer cells have an internal sodium concentration twice higher than the normal NL-20 cells (15.3 ± 2.2 mM and 7.8 ± 1.3 mM, respectively). Indeed, cancer cells having sodium currents might show an increased intracellular sodium concentration, higher than in normal cells.

Characterization of Na_v channels isoforms in lung cell lines

The gene expression pattern for all isoforms of Na_v channels was investigated in lung cancer cell lines by RT-PCR. All cell lines expressed numerous mRNA for α and β subunits, even those which do not express functional channels. Na_v1.6 and Na_v1.7 mRNA were always found in normal and cancerous cells. No specific pattern of mRNA expression could be distinguished between normal and cancerous cell lines (Fig. 3A).

H23, H460 and Calu-1 cells expressed mRNA for both TTX-S and TTX-R isoforms. We thus checked the relative sensitivity of Na_v channels to the specific blocker TTX (Fig. 3B). In all three cell lines, the sodium current was totally and reversibly blocked by TTX. The logistic fits give an IC₅₀ of 11.5 ± 1.3 nM for H23 cells, 13.0 ± 1.3 nM for H460 cells. For Calu-1 cells, the IC₅₀ is approximately 1 μ M if we consider the set of measures, but the dose-effect curve can be fitted by two logistic fits that distinguish TTX-S sodium currents with an IC₅₀ of 6.1 ± 1.7 nM (dotted line) and TTX-R sodium currents with an IC₅₀ of 0.72 ± 0.18 μ M (solid line). No other voltage-gated inward current was unmasked (data not shown). H23 and H460 cells only had TTX-S functional channels and the current was fully blocked by 1 μ M TTX. In the Calu-1 cell line, both TTX-S and TTX-R were functionally expressed and I_{Na} was fully blocked by 30 μ M TTX.

The presence of the Na_v protein was studied in all cell lines. Western blots using a pan-specific Na_v channel antibody detected a band of approximately 250 kDa, corresponding to the normal α -subunit protein, in all the cancerous and normal cell lines (Fig. 3C). A549, NL-20 and BEAS-2B cells also had another protein approximately 20 kDa smaller than the normal Na_v. The quantity of beta-actin was approximately the same in all the samples tested. The addressing of the protein to the membrane was evaluated in H460, A549 and NL-20 cells using immunolabeling with a Na_v1.7 antibody recognizing an extracellular epitope. As shown on fig. 3D, only H460 cells have a membrane labeling while both cell lines have Na_v1.7 channels in the cytoplasm.

Involvement of voltage-gated sodium channels in the oncogenic properties of cancerous cells

The role of Na_v was assessed using the specific blocker TTX. The TTX concentrations were chosen according to the concentration fully blocking the sodium current in the different cell lines: i.e. 1 μM TTX for H23 and H460 cells, 30 μM TTX for Calu-1 cells. For the cell lines which did not exhibit a functional voltage-gated sodium current (A549, NL-20 and BEAS-2B) a maximal concentration of 30 μM TTX was tested. As shown in Fig. 4A, cell proliferation and migration were not sensitive to the presence of TTX in the culture medium in all cell lines. Interestingly, *in vitro* invasion of the lung cancer cells H23, H460 and Calu-1 was sensitive to TTX. Indeed, the invasion was reduced by 40% to 50%. This effect was not observed in cell lines devoid of functional sodium channels (Fig. 4B).

Since Calu-1 cells express both TTX-S and TTX-R isoforms, we evaluated the effect of different concentrations of TTX on invasion, to discriminate the relative importance of these two classes of sodium channels. Three concentrations of TTX were used, 5 nM (blocking about 50% TTX-S sodium channels), 1 μM (blocking 100% of the TTX-S and approximately 50% of the TTX-R sodium channels) and 30 μM (blocking all the TTX-S and all the TTX-R sodium channels). As shown in Fig. 4C, TTX dose-dependently reduced the *in vitro* invasion of Calu-1 cells. The effect of each concentration of TTX on the sodium current was strictly correlated to its effect on the matrigel invasion by Calu-1 cells, indicating that both TTX-S and TTX-R sodium channels are involved to the same extent in regulating the invasion process. It can thus be inferred that the regulation of invasion can not be attributed to a particular Na_v isoform but rather to the global Na_v activity.

DISCUSSION

We report for the first time that several epithelial non-small-cell lung cancer cell lines express Na_v channels which are associated with their metastatic phenotype. We demonstrate with functional studies using the patch clamp technique that the sodium currents observed in the cancerous H23, H460 and Calu-1 cells are due to the activity of different isoforms of α - and β -subunits forming both TTX-S and TTX-R sodium channels.

In the breast cancer cell line MDA-MB-231 we (Roger et al., 2003; Jude et al., 2006) and others (Fraser et al., 2005) previously found the functional expression of the Nav1.5 isoform. In these cells the activation threshold of sodium channels is around -40 mV and is more depolarized than expected in other cells (Roger et al., 2003; Diss et al., 2004). These sodium channels can therefore be defined as “high-threshold voltage gated”. This property was proposed to be due to the expression of foetal isoforms of sodium channels which are known to be activated for more depolarized potentials as compared to the adult isoforms (Schmid & Guenther, 1998). This has been shown in breast and prostate human cell lines and cancer biopsies with the isoforms fNav1.5 (Fraser et al., 2005; Brackenbury et al., 2006) and fNav1.7 isoforms (Diss et al., 2005). These findings are consistent with the fact that human embryonic genes can be re-expressed in cancer cells (Monk & Holding, 2001). Indeed we can reasonably postulate that the sodium channels expressed here in lung cancer cells are foetal isoforms as well.

All the cancerous and normal cell lines studied express mRNA for numerous α - and β -subunits isoforms. They have different expression profiles, but all express the mRNA coding for the nervous Nav1.6 and Nav1.7 isoforms. More importantly, there is no obvious relationship between the mRNA expression and the translation into functional channels. For example, H23 and H460 cells express mRNA for the TTX-R Nav1.5 isoform, but we observed only TTX-S sodium currents. Calu-1 cells express both TTX-S and TTX-R sodium channel isoforms. Since Nav1.8 is the most resistant TTX-R isoform ($IC_{50} > 100$ μ M), and since Nav1.9 isoform is known for its very slow kinetics, the TTX-resistant component of the current is likely due to the activity of the Nav1.5 isoform. The conductance- and availability-voltage relationships clearly indicate the presence of different isoforms. In the cancerous A549, and normal NL-20 and BEAS-2B cell lines, which do not have the sodium current, numerous mRNA for α - and β -subunits are expressed. In these three cell lines, mRNA coding for Nav channels are translated into proteins of two molecular weights, the larger one (about 250 kDa) corresponding to the expected normal size of the glycosylated Nav protein and a smaller band (about 230 kDa), which could correspond to a non functional protein (truncated or misglycosylated). In cells that have the sodium current, only the 250 kDa protein is observed. Immunolabeling of the ubiquitously

expressed isoform, Nav1.7, indicates that in cancerous A549 and normal NL-20 cells the protein stay localized inside the cytoplasm. Therefore, the absence of current in these cells might not be due to a defective translation of mRNA into proteins but rather to a defective addressing of the protein to the membrane. The 230 kDa protein could exert a dominant negative effect. This result could also suggest that the aggressive phenotype can be due to the addressing of the Nav channels to the membrane and not only to a *de novo* expression. Beta subunits, mainly $\beta 1$, are known to be involved in the addressing of functional channels (Isom, 2001), but no obvious relation between mRNA expression for $\beta 1$ (or $\beta 3$ which is quite similar to $\beta 1$ (Morgan et al., 2000; Qu et al., 2001)) and the functional expression of the channel was seen in the present study. However, all that aspect of the work is rather preliminary.

In all the cell lines, blocking Nav channels with TTX did not interfere with cell proliferation or cell migration. However, the effect of TTX on cell invasiveness is about the same in H23, H460 and Calu-1 cells. TTX reduced the invasive capacity of the cells by 40 to 50% while it had no effect in A549, NL-20 and BEAS-2B cells, in which this current is lacking. This implies that the activity of the channel, and not only its presence, is required for cell invasion. The activity of such channels seems not to be essential to cancer cell invasive capacity but rather to be a feature of high metastatic ability. Indeed, A549 cells which originate from a bronchioloalveolar tumor (Lieber et al., 1976), known to be weakly metastatic (Laskin et al., 2005), do not possess functional Nav channels but degrade Matrigel® before migrating through the filter. In prostate and breast cancer, the presence of functional sodium channels can be related to their metastatic properties, which are determined *in vivo* (Diss et al., 2005; Fraser et al., 2005) or when cells are injected in nude mice (Zhang et al., 1991), and not only to their invasive properties *in vitro* (Grimes & Djamgoz, 1998; Roger et al., 2003). It is thus possible to infer that the presence of functional sodium channels is sufficient for lung cancer to become metastatic.

In the case of Calu-1 cells, the use of three concentrations of TTX to differentially block the two populations of functionally expressed channels (TTX-S and TTX-R), indicates that there is no particular link between a channel isoform and the invasiveness. These results are in line with those obtained by others in prostate cancer cell lines (Diss et

al., 1998; Diss et al., 2001; Bennett et al., 2004). All these results strongly suggest that the precise Na_v isoform of the sodium channel is not important for regulating invasion, provided that there is a sodium current and a continuous sodium influx.

The cellular mechanisms by which the activity of sodium channels participates in the modulation of invasiveness remain unknown and have to be determined. Since TTX suppressed invasion but not migration, it might be inferred that the activity of Na_v controlled more secretion of yet non identified proteases which are necessary to digest the extracellular matrix (mimicked here by the Matrigel®). As we already postulated for breast cancer cell line MDA-MB-231 (Roger et al., 2003), the invasive properties of non-small-cell lung cancer cells are under the control of proteolytic enzymes such as matrix metalloproteases (MMPs) (Egeblad & Werb, 2002). Interestingly, in metastatic prostatic cancer cell lines (Mycielska et al., 2003), or in small-cell lung cancer cells (Onganer & Djamgoz, 2005) sodium currents have been related to the endocytosis / secretion cycle activity of the cells. Numerous cell lines from small-cell lung cancers exhibit sodium currents and it has been postulated that these currents are involved in secretory activity (Blandino et al., 1995; Onganer & Djamgoz, 2005).

We have shown in H23, H460 and Calu-1 cells, that the sodium current exhibits a window of voltage between -55 and 0 mV. Therefore, there is a persistent inward sodium current at the mean membrane potential of these cells (approximately -30 mV). Indeed there must be a small, but continuous, influx of sodium ions into the cells which leads to an increased intracellular sodium concentration. We propose that this sodium influx regulates the intracellular sodium homeostasis, and could thereby regulate the expression, secretion or activation of matrix proteases. Interestingly, this hypothesis is coherent with the report that some tumours have an increased internal sodium content (Tamano et al., 2002) and with the findings supporting that intracellular sodium homeostasis is important in the development of cancer (Cameron et al., 1980), which would be complementary to the data of Fig. 2C and worthwhile mentioning. Such a continuous sodium influx into the cells might also affect the homeostasis of other ions, such as the internal calcium homeostasis (possibly through the activity of Na^+ - Ca^{2+} exchangers, NCX or NCKX) or the internal pH

(through the activity of $\text{Na}^+\text{-H}^+$ antiporters, NHE). Variations of the cell volume can also not be excluded.

In figure 1 we reported an apparent down-regulation of outward currents in lung cancer cell lines and especially in highly invasive cells (H23, H460 and Calu-1) as compared to non cancerous cell lines NL-20 and BEAS-2B. These differences in outward currents might be due to the regulation of potassium channels and are similar to observations made by the group of M. Djamgoz in prostate (Laniado et al., 1997) and breast cancers (Fraser et al., 2005). It has been proposed that the expression of Na_v concomitant to the reduced levels of voltage-gated K^+ channels could lead to the acquisition of neuronal characteristics and metastatic properties by cancer cells. However in all these three cancer types, cells are non-excitabile and no action potential has been recorded (Roger et al., 2003). Moreover, these differences in outward currents are observed for very depolarized membrane potentials which are never reach under normal conditions. Indeed, the question about the involvement of these outward currents in the invasion ability is not solved. Are the down regulations of outward currents hallmarks of invasive cancer cells or only cell lines particularities ? We can not conclude on these sole observations.

To conclude this study, we found that functional Na_v expression is associated with an increased invasiveness capacity of NSCLC cells which participates in their highly metastatic phenotype. This abnormal and surprising expression open new therapeutic strategy to specifically inhibit these channels in order to prevent the development of metastasis.

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FIGURE LEGENDS

Figure 1

Electrophysiological characterization of the normal (NL-20, BEAS-2B) and cancerous lung epithelial cell lines (H23, H460, Calu-1 and A549). *A*, Total currents recorded from representative normal NL-20 and BEAS-2B, and cancerous H23, H460, Calu-1 and A549 cells, from a holding potential of -100mV to $+70\text{ mV}$ with 10 mV voltage increments. The arrows indicate the inward current when it is present. *B*, Ionic characterization of the fast inward current recorded in the H460 cell line. (Ba) In presence of PSS, containing 140 mM Na^+ and 2 mM Ca^{2+} , the current triggered by a depolarizing step of -5mV from a holding potential of -100 mV was recorded, while when external Na^+ was replaced by equimolar N-Methyl D-Glucamine, it disappeared. This effect is reversible (“wash out”). (Bb) This current was not modified in absence of external calcium ions (“ Ca^{2+} -free”). (Bc) This current was completely blocked by $3\text{ }\mu\text{M TTX}$ without unmasking any other voltage-gated inward current. *C*, Mean sodium current-voltage ($I_{\text{Na}}\text{-V}$) relationships from -90 to $+60\text{ mV}$ recorded from 11 H23 cells, 21 H460 cells and 17 Calu-1 cells. The traces on the left part of each $I_{\text{Na}}\text{-V}$ relationship show typical recording of the sodium currents triggered from a holding potential of -100 mV to 15 ms -long depolarizing steps at -50 to $+30\text{ mV}$ (10 mV increments).

Figure 2

Influence of the sodium channel activity on intracellular sodium homeostasis. *A*, Superposition of availability-voltage (closed squares) and conductance-voltage (closed circles) relationships of H23 cells, H460 cells and Calu-1 cells ($n=6$ to 14). Notice the overlap of the two curves between potentials around -55 mV to 0 mV , indicating a partial activation of the channels and their incomplete inactivation. *B*, Persistence of a window sodium current recorded in a H460 cell depolarized to -30 mV from a holding potential of -100 mV . The current activates upon the depolarization and inactivates partially leading to a persistent window current indicated by the arrow. *C*, Intracellular sodium concentrations

in normal lung cells NL-20 (6 cells studied) and non-small-cell lung cancer cells H460 (7 cells studied), monitored using the fluorescent dye SBFI, are statistically different.

Figure 3

Isoform characterization of voltage-gated sodium channel in normal and cancerous lung cell lines. *A*, Expression profile of Na_v α -subunits (Na_v1.1 to 1.9 as indicated by the digits 1 to 9 above the gel) and β -subunits (β 1 to β 4) in human NSCLC (H23, H460, Calu-1 and A549) and normal (NL-20 and BEAS-2B) cell lines. *B*, Tetrodotoxin sensitivity of the Na_v currents found in H23 cells, H460 cells and Calu-1 cells. Dose-response curves of TTX on I_{Na}. Numbers above each point give the number of cells assessed at the given TTX concentration. The logistic fits give an IC₅₀ of 11.5 \pm 1.3 nM for H23 cells, 13.0 \pm 1.3 nM for H460 cells. For Calu-1 cells, the IC₅₀ is approximately 1 μ M if we consider the set of measures, but the dose-effect curve can be fitted by two logistic fits that distinguish TTX-S sodium currents with an IC₅₀ of 6.1 \pm 1.7 nM (dotted line) and TTX-R sodium currents with an IC₅₀ of 0.72 \pm 0.18 μ M (solid line). *C*, Western blot experiments showing the expression of Na_v α -subunits (upper) and of beta-actin (lower) proteins in human normal and cancerous cell lines. The presence of the protein of interest was studied in Western blot using a pan-specific Na⁺ channel antibody or a polyclonal anti-beta-actin antibody. There is the presence of Na_v α -subunits proteins (250-260 kDa) in all the cell lines. The arrow indicates an additional smaller α -subunit in A549 cells and in the normal epithelial lung cell lines NL-20 and BEAS-2B. The data are representative of three separate experiments. *D*, Cellular localization of Na_v1.7 channels in NL-20, A549 and H460 cells using a specific antibody recognizing an extracellular epitope. No labeling was observed in intact ("Non-permeabilized") NL-20 and A549 cells while a membrane labeling was observed for H460 cells. After permeabilization ("Permeabilized"), a cytoplasmic labeling is observed in all cell lines.

Figure 4

Involvement of Na_v currents in the proliferation, migration and invasion, monitored by inhibiting the channel with TTX. *A*, Relative effect of the TTX concentration fully

blocking the sodium current in each cell line on proliferation and survival (Aa) and migration (Ab) as compared to control without TTX. (Ac) Relative effect of the TTX concentration fully blocking the sodium current in each cell line on the *in vitro* invasion as compared to control without TTX. *** statistically different comparing control condition to TTX conditions at $p < 0.001$. B, Dose-effect of TTX on the *in vitro* invasion of Calu-1 cells as compared to a control condition without TTX. A representative normalized I_{Na} trace obtained in one Calu-1 cell in presence of corresponding TTX concentration is shown above each bar.