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**VOLTAGE-GATED SODIUM CHANNELS: NEW TARGETS IN CANCER
THERAPY ?**

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ABSTRACT

Early detection and treatment of cancers have increased survival and improved clinical outcome. The development of metastases is often associated with a poor prognostic of survival. Finding early markers of metastasis and developing new therapies against their development is a great challenge. Since a few years, there is more evidence that ionic channels are involved in the oncogenic process. Among these, voltage-gated sodium channels expressed in non-nervous or non-muscular organs are often associated with the metastatic behaviour of different cancers. The aim of this review is to describe the current knowledge on the functional expression of voltage-gated sodium channels and their biological roles in different cancers such as prostate, breast, lung (small cells and non-small cells) and leukaemia. In the conclusion, we develop conceptual approaches to understand how such channels can be involved in the metastatic process and conclude that blockers targeted toward these channels are promising new therapeutic solutions against metastatic cancers.

Keywords: Cancers, voltage-gated sodium channels, TTX, metastasis, invasivity, motility, galvanotaxis

Abbreviations: VGNaC, voltage-gated sodium channel; TTX, tetrodotoxin; TTX-S, tetrodotoxin-sensitive channel; TTX-R, tetrodotoxin-resistant channel; PCa, prostate cancer; MMP, matrix metalloproteases; I-V, current-voltage

Introduction

Cancers are among the leading causes of death in the world and as such represent a major problem of public health. This is the reason why governments, pharmaceutical companies, industrials and academics are all so intensely involved in a better understanding of the etiologies of the diseases. Such efforts aim at the improvement in the early detection of tumours in order to propose more efficient treatments. These could be effective in replacement of or in association with other therapies used presently. One of the new promising fields of investigation is the pharmacology of ionic channels which are known to be involved in different aspects of the carcinogenic process. Among ionic channels, voltage-gated sodium channels (VGNaC) make the topic of this review. These channels consist of a highly processed α subunit, which is approximately 260 kDa, associated with at least two auxiliary β subunits as displayed in figure 1 [1]. Sodium channels in the adult central nervous system contain β_2 plus either β_1 or β_3 subunits, while sodium channels in adult skeletal muscle have only the β_1 subunit. The pore-forming α subunit is sufficient for functional expression, but the channel and current density, kinetics and voltage-dependence of gating are modified by the β subunits [2]. A new β_2 -related subunit called β_4 was recently discovered but its possible interaction with the pore-forming α protein is not described yet [3]. Ten genes encoding VGNaC α subunits have been identified and nine of these have been functionally studied in expression systems. Among these potent ten isoforms, nine constitute a single family named Na_v1 according to their phylogeny and are designated $\text{Na}_v1.1$ to $\text{Na}_v1.9$ [4,5]. The remaining isoform, Na_x , shows a structure diverging from the Na_v1 family and seems to be gated by sodium concentration and not by voltage. Based on their tetrodotoxin (TTX) sensitivity, VGNaC are classified as TTX-sensitive or TTX-S ($\text{Na}_v1.1$ to $\text{Na}_v1.4$, $\text{Na}_v1.6$ and $\text{Na}_v1.7$) and TTX-resistant or TTX-R ($\text{Na}_v1.5$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$). VGNaC are classically described as critical elements of action potential initiation and propagation in excitable cells because they are responsible for the initial depolarisation of the membrane. However, these channels are also known to be expressed in non excitable cells such as T-lymphocytes [6].

To investigate the expression of proteins like ionic channels, techniques such as RT-PCR and immunoblot allow to determine the presence of mRNA and their translation into proteins. However, one state-of-the-art technique exists, namely the patch-clamp technique [7], which allows to quantify the activity

of these proteins. Displacement of ions from one compartment to another leads to the generation of a current due to the electric charges borne by the ions. The patch-clamp technique uses a glass pipette as an electrode to measure the electric current flowing through the ionic channels. Since the ionic flux depends mainly on passive diffusion, changes in current amplitude directly reflect the changes in channel conformation and thus activity. The two major configurations to measure currents flowing through all the channels of an isolated cell, and thus called whole cell recording, are the "ruptured patch" (the membrane patch at the tip of the pipette is broken and the intracellular content is replaced by the medium present into the pipette) and the "perforated patch" (antibiotics such as amphotericin B present in the pipette medium form pores, into the membrane trapped at the tip of the pipette, to get an electrical access to the interior of the cell while reducing the dilution of intracellular soluble molecules).

To study some aspects of cancers, cultured cell lines are generally used. Different aspects of their biology can refer to different aspect of the disease. For example, cell proliferation *in vitro* can be an index of the kinetics of tumour proliferation *in vivo*. The facilities with which the cells migrate through filters, covered or not with Matrigel® (mimicking the extracellular matrix) are considered as indexes of invasion and migration, respectively. These parameters are both involved in the metastatic properties of the cells. Even if metastatic properties do not depend on cell invasiveness only, extracellular matrix invasion is a prerequisite to get metastases. The evaluation of the capacity of cells to produce metastases can be assessed by injecting the cells into nude mice. Understanding this aspect of the disease is very important because it is the main cause of death by cancer. Metastasis can be summarised as a process whereby cells escaping from a primary tumour enter circulation (blood or lymph), migrate and become lodged at tissue-specific or non-specific sites where they proliferate to form secondary tumours. To escape the primary tumour and to set in a distant site, tumour cells need to produce, or induce host cells to produce, proteases (e.g. matrix metalloproteases, MMP) to degrade the extracellular matrix.

Prostate cancer

We will first review the involvement of VGNaC in prostate cancer (PCa) because, thanks mainly to

the group of Mustafa Djamgoz in London, it is the best understood one in term of mechanisms description.

PCa is the most frequently diagnosed male cancer and the second most common cause of death of men from cancer in Western societies [8]. The first study of a sodium current measured in patch-clamp conditions was made in a rat prostate cancer cell line [9]. In this paper, the authors describe the activity of sodium channels only in a highly metastatic cell line, Mat-Ly-Lu, but not in the weakly metastatic one, AT-2. This current is fully blocked by 1 μ M TTX suggesting that it is a TTX-S. Proliferation was not affected by TTX. Since the currents were recorded in the metastatic cell line, the authors investigated its possible involvement in the metastatic process using an *in vitro* Matrigel® invasion chamber assay. Incubation of the Mat-Ly-Lu cells with 600 nM TTX for 48h reduced their invasive capacity by 33 %. In contrast, TTX had no significant effect on the invasiveness of the AT-2 cell line. Later on, the same group performed similar experiments in human prostate cancer cell lines, PC-3 and LNCaP, and found similar results [10]. It must be underlined that in both studies [9, 10], the authors report that not all cells investigated in patch-clamp possess functional sodium channels. This led these authors to further investigate the relationship between protein expression and invasiveness [11]. This study was performed in both rat and human cell lines in which the expression of VGNaC was assessed by flow cytometry using a fluorescein-labelled polyclonal antibody. Weakly metastatic cell lines such as AT-2 and LNCaP displayed almost no expression of VGNaC. On the opposite, highly invasive cell lines such as PC3 and Mat-Ly-Lu displayed bimodal frequency distribution profiles with a subpopulation of cells expressing high levels of sodium channel protein. Comparison of the degree of sodium channels expression with the capacity of the different cell lines to invade Matrigel® shows a significant positive correlation. In addition, two transfected cell lines, obtained by transfecting genomic DNA from rat prostatic tumour cells into a recipient benign cell line (non invasive), were found to express significantly elevated levels of sodium channel protein compared to the original cell line. This enhanced expression was correlated with increased invasiveness *in vitro*. The only uncorrelated observation in this study is that PNT2 cell line, which is reported to be benign, contains numerous sodium channels. This expression could be subsequent to the transfection with the SV40 genome. It also could mean that cells need more channels than the amount expressed in PNT2 to become invasive [11]. It has also been speculated that the presence of the channels gives the potential for invasion but this is prevented by local

factors or the need for other essential criteria such as accumulation of other mutations [11]. Whatever the reason, it must be noticed that, to our knowledge, there was no attempt to determine the functional activity of the channels in PNT2 cells.

Bennet et al (2004) compared a weakly metastatic human cell line, LNCaP, and two increasingly tumorigenic daughter cell lines, C4 and C4-2. They showed that the increasing invasiveness of the C4 and C4-2 cell lines was related with an increasing level of VGNaC proteins measured by immunoblotting [12]. TTX at 1 μ M completely reversed the difference in invasiveness between C4, C4-2 and LNCaP cell lines. Moreover the transient expression of the skeletal isoform (Nav1.4) of the channel in the 3 cell lines increased their invasive capacity in a TTX-sensitive manner. From their work, it can be concluded that sodium channels expressed are sufficient for the invasivity of prostate cancer cells.

TTX sensitivity of VGNaC

In a very elegant study, the electrophysiological and pharmacological properties of Mat-Ly-Lu cells current have been characterised [13]. The only powerful blocker found was TTX with an IC₅₀ of about 18 nM (see table I). Such sensitivity makes this channel a TTX-S.

Isoform(s) of the channel(s)

The question of the isoforms expressed was addressed both in Mat-Ly-Lu (rat) and PC3 (human) cell lines. These cells express mRNA coding for the skeletal isoform SkM1 now named Nav1.4 [14]. *In situ* hybridation experiments indicated that the levels of mRNA expression were very variable from cell to cell in Mat-Ly-Lu cells. In contrast, this signal was more uniform in AT-2 cells although at much lower levels. In the same vein, LNCaP cells also expressed Nav1.4 mRNA. In that study, they did not find any mRNA expression for Nav1.1 (also called RB I), Nav1.2 (RB II), Nav1.3 (RB III), Nax (NaG/SCL-11) and Nav1.6 (Na6) in any of the cell lines tested. It was concluded that all cell lines expressed VGNaC mRNA although the channel was functional only in highly metastatic cell lines [14]. However, the authors noticed that the pharmacological properties of the functional protein were particular since 10 μ M μ -conotoxin was needed to completely block the current while nM concentrations are usually enough to block the skeletal sodium

current (see also [13]). This surprising result led the authors to re-investigate this point using other molecular biology methods (degenerate primer screening and semi-quantitative PCR) on the same cell lines [15]. They found that the main mRNA isoform expressed in Mat-Ly-Lu and PC3 cells was Nav1.7 and this was thus likely to be the main source of the functional VGNaC detected. However, other isoforms are observed such as Nav1.1, Nav1.2, Nav1.4 (see table I and figure 2A).

Interestingly, Nav1.4 has been transiently expressed in LNCaP, C4 and C4-2 cells lines [12]. In each cell line, transient expression of the channel induced a large increase in invasiveness which was completely reversed by 1 μ M TTX. This result confirms the role played by sodium channel in the invasion process. Above all, this result indicates that, even if Nav1.7 seems to be the channel primarily involved in invasive properties of rat and human prostate cancer cell lines, other isoforms of the channel can render cells invasive.

Biological consequences of sodium channels activity

To address this important question, the group of Mustafa Djamgoz tested three different cellular processes important to PCa metastasis: galvanotaxis, motility and invasion.

I - Galvanotaxis is the ability of cells to respond to an electric field by moving directionally. Such a property is known to be involved in a number of basic biological process such as embryonic development [16] but also in other physiological conditions such as wound healing [17]. To evaluate this properties, Mat-Ly-Lu and AT-2 cells were exposed to exogenous direct-current electric fields of physiological strength [18,19]. In these conditions, it has been shown that highly metastatic Mat-Ly-Lu cells responded to the application of an electric field by migrating towards the cathode. AT-2 cells gave no such response. The migration of Mat-Ly-Lu cells was blocked by 1 μ M TTX while 10 μ M veratridine, a sodium channel opener, enhanced it. This study thus showed that galvanotaxis of metastatic cells is under the control of the VGNaC activity. The precise mechanisms involved in this particular case are not understood but some hypotheses have been put forward based on other cases (see [20]).

II - Motility is an integral component of the metastatic cascade and involves sub-cellular mechanisms like changes in cell volume, cell-matrix interaction, cytoskeletal elements and ion channels

activity. In 1999, Fraser et al. [21] found that VGNaC activity played a significant role in determining the morphological development of Mat-Ly-Lu cells. Later on, the same group completed this observation by showing that lateral motility of these cells, assessed using a "wound-heal" assay, was significantly reduced with 1 μM TTX [22]. Quite surprisingly, 20 μM veratridine did not affect motility of these cells while aconitine (100 μM) and ATXII (25 pM), two other "sodium-openers", increased it. At this concentration, veratridine did not affect cell proliferation while at 50 μM , proliferation was reduced [23]. The lack of effect of veratridine could be due to its interaction with other ionic channels [22].

III – Before cells migrate into the local circulation and then interact with other particular tissues to form a secondary tumour, the extracellular matrix has to be digested. This is done by proteases such as matrix metalloproteases (MMPs) (see [24] for review). *In vitro*, this property, called invasiveness, is evaluated through the potency of cells to migrate through filters coated with a film of Matrigel®. An indirect way to evaluate the capacity of cells to release proteases by exocytosis is to measure the secretory membrane activity. This was done by Mycielska et al. who measured the uptake of horseradish peroxidase (HRP) [25]. Since the recycling of membrane constituents requires close matching between the levels of endocytosis and exocytosis, HRP uptake is a marker of secretory membrane activity. Mat-Ly-Lu cells have a higher activity than AT-2 cells [25]. This difference is abolished in presence of 1 μM TTX while veratridine did not increase the activity in Mat-Ly-Lu cells. To explain this surprising result, it has been proposed that the VGNaC would be working at maximal efficiency [25]. From these results, different hypotheses were proposed to link the VGNaC activity to the increased membrane trafficking as illustrated on figure 2B. One hypothesis was that Na^+ influx could disrupt intracellular pH which would lead to a release of Ca^{2+} from intracellular stores. This Ca^{2+} could then activate protein kinase C and/or CaM kinase II and lead to phosphorylation of the actin cytoskeleton thereby allowing secretion and endocytosis to occur. An alternative hypothesis is that a direct action of Na^+ on adenylate cyclase could alter levels of cAMP which activates protein kinase A. The changes in actin cytoskeleton phosphorylation would similarly allow secretion and endocytosis.

Since veratridine affects VGNaC dependent parameters such as galvanotaxis without affecting others like motility and membrane secretory activity, it appears that the relationship between the channels activity

and the final effects is rather complex as is the dependence of membrane secretory activity when analysed by fractal methods [26]. This might mean that motility and secretory activities are already maximal and thus cannot be enhanced by an increase in VGNaC activity.

One of the major functions of normal prostate gland is the synthesis, accumulation and secretion of large amounts of citrate [27]. During the progression of malignancy, the intermediate metabolism of citrate is altered. This can have important implications on cellular bioenergetics, cell growth and apoptosis, angiogenesis [27]. The mechanism of citrate release by human normal prostatic cells, PNT2-C2, has been studied [28]. They found that these cells functionally express an electrogenic citrate transporter which mediates the cotransport of 1 citrate (trivalent anion) alongside 4 K⁺ out of the cells. A similar study was also performed in a highly metastatic cell line, PC-3M [29]. The K⁺/citrate cotransporter found in PNT2-C2 cells was also found in PC-3M cells. However, PC-3M cells also expressed a novel Na⁺-dependent citrate transporter. Interestingly, the expression of this transporter was controlled by the VGNaC activity: treatment of the cells with 1 μM TTX induced a reduction of the activity of the Na⁺-dependent transporter while the K⁺-dependent transporter activity increased. The contribution of the citrate transporters to metastatic disease has to be understood to determine their interplay with VGNaC activity.

The diagram shown on figure 2B underlines a very important unresolved question: how does sodium enter the cells through the channel ? Usually, VGNaC are opened following a depolarisation leading to the triggering of an action potential. In the case of cancer cells, it has been proposed that metastatic prostate cancer cells membrane are "potentially electrically excitable" since they express both sodium and potassium voltage-gated channels [30]. However, such action potentials have never been observed, spontaneously or triggered, weakening such a hypothesis. An explanation might arise in view of some electrophysiological properties of the channel (see the Breast cancer section).

Regulation of prostate cancer VGNaC

Cancer cells are very sensitive to the chemical conditions of the *in vivo* environments encountered during tumorigenesis and metastatic spread (see in [30]). Among them Nerve Growth Factor (NGF) and

Epidermal Growth Factor (EGF) have been associated with prostate cancer progression and have also been found to up-regulate VGNaC expression (see [30]). Such factors can be found in the serum used to grow cells in culture. Using Mat-Ly-Lu cells, it was shown that, depending on the serum concentration used to grow cells, the electrophysiological properties of the current varied, suggesting both transcriptional and post-transcriptional regulations of the channel. Interestingly, the sensitivity of the current to TTX also varied [30]. This has been interpreted by the authors as a possible regulation of the expression of the different mRNA isoforms. This finding is of importance since weakly metastatic cells often express mRNA for VGNaC (see table I). It is thus possible that changes in the surrounding environment, such as an increase in growth factors, can amplify the functional expression of VGNaC rendering the cells more invasive.

Expression of VGNaC in patients

Up-regulations of VGNaC in PCa have been shown in two studies. In the first one, an antibody directed against an intracellular epitope of VGNaC was used on tissue microarray slides of 80 clinical PCa specimens and 4 normal prostate specimens [31]. Out of 80 PCa specimens, 44 showed higher levels of VGNaC compared to normal, with 14 (of 44) showing increased focal staining in scattered individual cells or small group of cells. It thus appears that the observations obtained in prostate cancer cell lines are pertinent with those made in PCa tumour specimens: in prostate cancer, an increased level of expression of the VGNaC is observed. Very recently, another study went further by showing that the level of expression of the mRNA coding for $\text{Na}_v1.7$ is related with the grade of PCa: the higher the grade, the higher the expression of $\text{Na}_v1.7$ mRNA [32]. Thus, the expression of $\text{Na}_v1.7$ has been proposed to be a potential novel marker for human prostate cancer [32].

These results led some research groups to develop new molecules targeted against VGNaC in order to block their activity (see [33] for review). This underlines the interest these channels might have as new therapeutic targets. The problems with these studies are that: (i) the VGNaC isoform tested ($\text{Na}_v1.2$) has not been shown to participate in invasion [34] and (ii) the cellular function evaluated (proliferation) is not affected by the functional expression of VGNaC [33, 34].

Breast cancer

Among women, breast cancer is the most common cancer and the first cause of death. Death occurs primarily after the development of metastasis. Until the beginning of the XXIst century, the studies on ionic channels and breast cancer focused mainly on potassium channels and their involvement in proliferation [35-39]. Also, the role of the Ca²⁺-activated chloride channel, coded by the CLCA2 gene, was also studied with regard to tumour metastasis [40]. It was found that it was a tumour repressor. All these studies showed that ionic channels can be involved in breast cancer genesis.

In the beginning of the 2000's, two different research groups, our group in Tours and the one of Mustafa Djamgoz in London, showed at the same time that VGNaCs were expressed in a highly metastatic breast cancer cell line, MDA-MB-231 [41,42]. As shown on figure 3A, the electrophysiological characteristics of the current are not particular except for the voltage activating threshold (voltage at which the channel starts opening) which is more positive than in the case of the cardiac current. This might be related either to the foetal *vs* adult isoform (see below) or to a particular arrangement with auxiliary β subunits. For example it is known that co-expression of β_1 with $\alpha_{\text{NaV}1.5}$ in HEK293 cells induces a positive shift of the activation and inactivation curves, associated with a slowing of the reactivation [43].

The current recorded in MDA-MB-231 cells was not observed in two other cell lines, MCF-7 and MDA-MB-468. MCF-7 cells are weakly invasive *in vitro* and non-metastatic in nude mice. MDA-MB-468 cells have an invasive capacity similar to that of MDA-MB-231 cells [41] but are less metastatic [44]. Like in PCa, it seems that VGNaC participates to invasion *in vivo* leading to higher metastatic potentials.

Pharmacological profile of VGNaC

The pharmacology of the sodium channel expressed in MDA-MB-231 cells is rather particular [41, 45]. While its TTX sensitivity is consistent with a TTX-R isoform of the channel (see Table I), the channel is surprisingly also sensitive to some calcium channel blockers with IC₅₀ of $53.2 \pm 3.6 \mu\text{M}$ for diltiazem and $37.6 \pm 2.5 \mu\text{M}$ for verapamil, which are concentrations classically used to block calcium channels [45]. The VGNaC mainly expressed in these cells is the foetal isoform of Na_v1.5 [46]. This could explain the

particular pharmacology of the channel. This finding could also bring another explanation why verapamil was beneficially used along with conventional chemotherapy [47]. In that study, verapamil was used as a chemoresistance inhibitor. We propose that it could also have worked as a VGNaC blocker.

Isoform(s) of the channel(s)

Semi-quantitative RT-PCR showed that $\text{Na}_v1.5$, $\text{Na}_v1.6$ and $\text{Na}_v1.7$ mRNA are expressed in MDA-MB-231 cells [46, 48]. The low TTX sensitivity of the channel ($\text{IC}_{50} \approx 2 \mu\text{M}$ [41]) strongly suggests that the main isoform functionally expressed is $\text{Na}_v1.5$. However, a minor expression of a functional TTX-S isoform has been also suggested [46].

Biological consequences of sodium channels activity ?

To evaluate the role of this channel, the specific blocker of the VGNaC, TTX was used. Since the $\text{Na}_v1.5$ is a TTX-R channel, $30 \mu\text{M}$ of TTX was used to block all the channels [41]. This elevated concentration of TTX has never been shown to affect other proteins activity, or more generally, other biological activity than VGNaC. Neither cell proliferation nor migration was affected by the blockade of VGNaC. On the other hand, *in vitro* invasion was reduced by about 30% in MDA-MB-231 cells and it was not changed in MCF-7 and MDA-MB-468 cells, which do not express such functional channels (figure 3B). Similar results were obtained in a more recent study using $10 \mu\text{M}$ TTX [46]. Conversely, invasion was increased by the "sodium-opener" veratridine (unpublished data) reinforcing the potential role of VGNaC in invasion. It thus seems that the activity of the channels participates in the degradation of the Matrigel®.

Other properties involved in the metastatic process such as galvanotaxis, exocytosis and lateral motility have been shown to be under the control of the VGNaC activity [46]. Regarding cell migration through filters with calibrated pores, results are discrepant. In one study, no effect of $30 \mu\text{M}$ TTX on transwell migration through $8 \mu\text{M}$ diameter pores was reported [41]. Moreover, we performed wound-healing assays with MDA-MB-231 cells and found no effect of TTX (unpublished data). In contrast, the group Djamgoz found that transwell migration through $12 \mu\text{M}$ diameter pores was inhibited partially by $10 \mu\text{M}$ TTX [46]. Since a similar reduction was observed in an *in vitro* invasion assay, the results obtained by

Fraser et al. [46] suggest that the main effects of VGNaC activity are exerted through modifications of cell motility while in the case of Roger et al. [41], the only effect is through the modulation of the capacity of cells to degrade Matrigel®. This apparent discrepancy has no obvious explanation.

Regulation of breast cancer VGNaC

The sodium channel expressed in MDA-MB-231 cells can be regulated but the precise mechanisms are still unknown. The configuration of the patch-clamp used to study the channel was reported to affect some electrophysiological characteristics of the sodium current (figure 3C) [47]. The ascending limb of the I-V curve (from -60 to -10 mV) was shifted leftward in ruptured patch compared to perforated patch conditions and this was associated with slower kinetics of recovery of the channel from inactivation. Interfering effects on signalling pathways with the patch-clamp configuration have been observed in cardiac cells and have been attributed to intracellular content dilution [48]. The observations made in the breast cancer cell line were thus interpreted as a consequence of the dilution of a factor involved in a signalling pathway such as the PKC pathway, induced by the intracellular perfusion in ruptured patch-clamp. Other dedicated experiments have to be performed to evaluate how the expression of the channel is regulated by environmental factors such as growth factors.

Expression of VGNaC in patients

The relationship between the expression of the channel mRNA with lymph node invasion has been studied [46]. It was found that patients with lymph node invasion, determined using anatomic-pathological techniques, expressed mRNA for Na_v1.5 while those who were negative for lymph node invasion did not express mRNA for this VGNaC. This correlation was not observed for Na_v1.6 and Na_v1.7. The immunohistochemical staining of human breast tissues for the Na_v1.5 indicates no staining in normal tissues while a strong heterogeneous staining was observed in cancerous tissues [46]. These findings suggest that the expression of neonatal Na_v1.5 can be used as a prognostic factor of metastatic development. The development of a neonatal specific antibody directed against the neonatal isoform of Na_v1.5 has been undertaken [49].

These results strongly suggest that the observation made in a highly metastatic cancer cell line, MDA-MB-231 are not restricted to a cell line but can be transposed to the evolution of the disease. The particular isoform of the channel expressed, associated with its unusual pharmacology suggests that a particular treatment targeted toward this channel, without side effects on cardiac function, can be developed.

The unanswered question is still: how are VGNaC involved in the invasive phenotype of these cells ? We proposed that, since it has never been possible to record an action potential, the membrane potential of MDA-MB-231 cells would have to be in a particular range of the sodium channel activity. Indeed, at that potential (-29 ± 2 mV, n= 65 cells), the sodium channel is partially activated and not fully inactivated [41]. The resulting ionic current is called a window current. What is important is that, even without any action potential, the partial opening of the channel leads to a continuous entry of sodium into the cell. This entry might be responsible for the increased intracellular sodium concentration observed in biopsies [52] which in turn can modulate other homeostases such as calcium or proton. After those changes, the consequences on cell physiology can be the same as the one presented on figure 2B which concerns prostate cancer cells.

Lung cancers

Lung cancer is the most common cancer over the world, with more than one million cases of death a year. It is divided in two major histopathological groups: non-small cell lung cancer and small cell lung cancer which occur with frequencies of 80% and 20% respectively [53]. Small cell lung cancer affects mainly non smokers and is characterised by rapidly proliferating tumours and poor prognosis. This cancer is often associated with a neuromuscular autoimmune disease characterised by the insufficient release of acetylcholine from the motor nerve terminal [54] and known as the Lambert-Eaton myasthenic syndrome. Non-small cell lung cancer is the very widespread kind of lung cancer which is associated with smoking. The occurrence of metastases, initially appearing in lymph nodes and then in other organs such as lung itself, brain, bone, bone marrow, liver, adrenal glands, is always a marker of poor prognosis.

Small cells

Small-cell lung cancer cells have been shown to be neuroendocrine-like cells capable of producing action potentials [55]. This discovery has been reinforced by others who suggested that small-cell lung cancer cells derived from primitive endodermal cells which differentiate into neuroendocrine cells [56]. Later, the ionic channels expressed in different cell lines (H128, H69 and H146) were characterised and VGNaC were found to be expressed in all of them [57]. These VGNaC and a calcium current also present were able to elicit action potentials [58]. Both channels were blocked by autoimmun antibodies responsible for the Lambert-Eaton syndrome.

The sodium channels expressed in H146 cells were weakly sensitive to TTX ($K_i=215$ nM) (see figure 4A) [58]. From their analysis of electrophysiological and pharmacological characteristics, they determined that only one population of TTX-resistant sodium channels was expressed. Since these cells are neuroendocrine-like and since they are able to produce action potentials, it is tempting to assume that they are involved in excitation-secretion coupling function. However, as noticed by Blandino et al. [58], the resting membrane potential of the H146 cells was approximately -44 mV, a level sufficient to inactivate most of the Na^+ channels (see figure 4B). Either the resting membrane potential of this kind of cells is actually more hyperpolarised in tumours than it was measured in H146 or these sodium channels are not involved in the elicitation of action potentials [58]. In that latter case, we propose an alternative hypothesis described in the conclusion section.

In a recent study, it has been observed that in H69 and other small-cell lung cancer cell lines (H209 and H510), 100 nM TTX reduced Horse Radish Peroxidase uptake suggesting a role for VGNaC in the exocytic activity [59]. However, the presence of functional VGNaC has not been undertaken in H209 and H510 cells and other properties involved in the metastatic phenotype have not been evaluated.

Non small cells

To our knowledge, no electrophysiological study ever evaluated the presence of VGNaC in this kind of cells except for a paper we recently submitted [59]. Some of the results presented in this paper are summarised here. In this study, 4 cancer cell lines (H23, H460, A549, Calu-1) and 2 non-cancerous epithelial cell lines (NL-20, BEAS-2B) were tested. While the non-cancerous cells did not have functional

channels as measured in patch-clamp conditions, three cancer cell lines (H23, H460 and Calu-1) expressed functional channels with different electrophysiological and pharmacological properties (see Table I).

TTX sensitivity of VGNaC

The most striking difference between the cell lines concerns the isoforms expressed functionally, as determined by their TTX sensitivity. This sensitivity was in the nanomolar range in H23 and H460 cells. Thus the protein isoforms belonged to TTX-S in these cell lines. For Calu-1 cells, there were clearly two kinds of channels expressed: TTX-R and TTX-S. In contrast with the other cancers described in this review, the heterogeneous sensitivity to TTX indicates heterogeneity in isoforms expression between cell lines.

Isoforms of the channels

Semi-quantitative RT-PCR experiments indicated that all the studied cell lines (cancerous and non-cancerous) expressed mRNA for the $Na_v1.6$ and $Na_v1.7$ isoforms which are both TTX-S. In the case of H23 and H460 cells, $Na_v1.7$ mRNA was more abundant and were possibly responsible for the expressed functional channels. However, a possible expression of $Na_v1.6$ cannot be rejected. These cells express mRNA for the $Na_v1.5$ isoform but the TTX sensitivity of the current suggests that it was not functionally expressed. Calu-1 cells expressed mRNA for all the isoforms except for $Na_v1.4$. The most abundant mRNA was, in descending order, those of $Na_v1.7$, $Na_v1.6$, $Na_v1.5$, $Na_v1.1$ and $Na_v1.3$. The particular pharmacology of the current, which is clearly both TTX-R and TTX-S, indicates that the $Na_v1.5$ is functionally expressed. With regard to TTX-S, the situation is more complicated since mRNA for $Na_v1.6$, $Na_v1.7$ and $Na_v1.1$ are comparably expressed and a possible participation of $Na_v1.3$ cannot be rejected.

Knowing that non-cancerous cells, NL-20 and BEAS-2B, express mRNA for $Na_v1.6$ and $Na_v1.7$, plus $Na_v1.5$ for the latter, like the cancerous cell lines, but did not have functional proteins, it is tempting to speculate that these cells have the potential to functionally express these VGNaC but they miss something to do it.

Biological control of sodium channels activity

The effect of μM TTX on proliferation, migration and Matrigel® invasion was tested on the 6 different cell lines [56]. We followed the same protocols that we previously used on breast cancer cell lines [41]. In all cell lines, TTX had no effect on proliferation and migration. An effect was only observed when TTX was applied and Matrigel® invasion evaluated. In that case, a reduction of invasion between 40 to 50% was observed in H23, H460 and Calu-1 cells which functionally express the VGNaC. Calu-1 cells express functional TTX-S and TTX-R VGNaC. Application of different concentrations of TTX blocking TTX-S or TTX-S + TTX-R VGNaC led to a gradual reduction of *in vitro* invasion. This indicates that both kinds of channels are involved in the invasion properties of the cells.

Expression of VGNaC in patients

In an unpublished study, we performed semi-quantitative RT-PCRs on 10 cancerous and associated non-cancerous lung biopsies collected from the same patients. The mRNA coding for the 9 isoforms of Na_v were tested. In contrast to what could be expected from the cell line experiments, we did not observe an up-regulation of Na_v mRNA expression. Rather, there is a tendency for a down-regulation. These results indicate that mRNA expression was modified during the disease. Nothing is known about their translation in proteins and VGNaC activity. This is of importance since we found that only VGNaC activity is important in the invasion process. Cross-talks have been described between the functioning of VGNaC and mRNA production. For example, the application of 1 μM TTX to rat prostatic cancerous cells Mat-Ly-Lu increased the production of mRNA coding for the VGNaC [29]. Thus, we can hypothesise that a reduction in mRNA can result in a decreased channel activity but also that it is a consequence of an increased channel activity. Additional experiments at the functional level, using patch-clamp, are needed to elucidate this question.

Leukaemia

There are few studies about the role of VGNaC in leukaemia. The first one was a brief description showing that the leukaemia cancer cell line K562 expressed a VGNaC [61]. During the same period of time, another research group, using the same cell line, did not find this current except in particular conditions in

which cells became resistant to anticancerous drugs (MDR phenotype) [62]. This current was blocked by nM TTX ($K_i < 100$ nM). While the functional expression of the sodium current was clearly associated with the MDR phenotype, it was not involved in the resistance to chemotherapy since 1 μ M TTX had no effect on vincristine (an anticancerous drug) uptake.

Following this work, leukaemia cell lines from T-cell origin were studied: CCRF-CEM and a MDR variant CEM/VLB₁₀₀ [63]. Contrarily to the work of Yamashita et al. [62], they found that a sodium current was observed in both cell lines and as such was not associated with the MDR phenotype. Here again, the current was sensitive to TTX ($K_i < 150$ nM).

In conclusions of these three studies, there is no clear link between VGNaC activity and the MDR phenotype. Moreover, the role of VGNaC, if there is one, is not described in these studies. Interestingly, a recent study showed that 8-9 % of Jurkat cells, a cell line derived from normal T-lymphocytes, expressed a sodium current [64]. This study confirmed the presence of a sodium current on T-lymphocytes and T-lymphocytes-derived cell lines [6]. This current is blocked by TTX with a K_i around 900 nM and as such can be classified as TTX-resistant. RT-PCR analysis indicates the presence of mRNA for at least 4 isoforms of VGNaC, two of them being TTX-R, $Na_v1.5$ and $Na_v1.9$. $Na_v1.8$ was not detected and for the authors, the protein which is most likely expressed is $Na_v1.5$. The mRNA coding for $Na_v1.6$ and $Na_v1.7$ were also observed but to a lesser extent. Interestingly, Matrigel® invasion assays on Jurkat cells were performed [64]. In control conditions, about 8% of the cells were invasive, close to the percentage of cells expressing an electrophysiologically detectable sodium current. Following treatment with 10 μ M TTX, invasiveness was decreased by approximately 93% (see figure 5). This suggests that the observation of a sodium current in leukaemia cells might not be due to a neo-synthesis of VGNaC but to an up-regulation of already expressed channels (see conclusions for development).

General conclusions

As shown on Table I, functional expression of VGNaC (mainly $Na_v1.7$, $Na_v1.6$ and $Na_v1.5$) is often associated with invasive properties of some cancer cell lines. VGNaC are also expressed in biopsies from

metastatic patients. VGNaC are classically described as being responsible of the excitability and propagation of the action potential. This is why the presence of VGNaC is often interpreted as an indication of possible excitability whatever the tissue in which it is found. However, in cancer cells, the membrane potential is less negative than it is in excitable cells. This strongly suggests that the channels must be partially inactivated. The membrane potential of cancer cells is often, if not always, located in a window of voltage where there is a continuous entry of sodium owing to a partial activation and incomplete inactivation of VGNaC (see figure 4). This led us to hypothesise that such an entry of sodium could be responsible for an increase in intracellular calcium which can then enhance the release of proteases [41], in accordance with results obtained on prostatic cancer cells [25]. This hypothesis, which needs further experiments to be strengthened, reconciles a lot of data in the literature in which the window current at the cellular membrane potential, although not determined, can be inferred from the illustrations. Another hypothesis can be proposed such as changes in intracellular pH (pHi). Intracellular sodium regulates pHi through the activity of the sodium-proton exchanger and the sodium-bicarbonate exchanger. Indeed, it is known that many proteins involved in the metastatic process are tightly regulated by the surrounding pH [25]. In the case of PCa, VGNaC activity regulates a specific sodium-dependent citrate transport [29]. However, this is one aspect of the physiology which is particular to the prostate functioning. The regulation of invasion by VGNaC activity strongly suggests that there is a regulation of proteases or growth factors release and/or activity which is common to all the cancers described.

Beside its effect on motility and invasion, the sodium channels can have other roles which are important in the process of metastatic tumour development. Indeed it is often reported that among highly invasive cell lines (as determined *in vitro*), those which express the VGNaC are more metastatic when injected in nude mice than are those which lack the VGNaC. This feature might be brought by the adherence properties of auxiliary β -subunits such as the β_2 [65]. Indeed, β_2 can help migrating cells to settle in specific metastatic site (e.g. lung, bones) and then invade this tissue to develop the secondary tumour.

A question arises considering that VGNaC can be expressed in different cancer cells and in very few "normal" cells. Does this expression represent dedifferentiation towards a more embryonic phenotype? It has been proposed that embryonic genes, which are silent in the cells of the mature organ, are re-expressed

in cancer cells [66]. In line with this hypothesis, the splice variant of VGNaC expressed in prostate and breast cancers are neonatal (see [67]). This hypothesis is also reinforced by the observation that cultured normal cells (smooth muscle cells, retinal pigment epithelial cells) express VGNaC while freshly isolated cells do not [68-72]. From the literature regarding the role of VGNaC in cancer cells, it seems that its main role is to participate in the invasive capacity of the cells. We can thus assume that VGNaC are expressed in cells during particular events of embryogenesis. For example, during the embryonic development, cells need to migrate and invade to find the nest where the organ can develop. The invasion before nesting can be due to the production of proteases which are, at least partially, under the control of a VGNaC. After the nesting of the cells, the no longer necessary genes encoding the VGNaC can be switched off. Mutations during carcinogenesis might lead to the switch on of the silent genes. It is known that some regions of the chromosomes are more sensitive to mutations like deletions. For example, it has been reported that in renal, breast and lung carcinomas, there are frequent deletions in chromosome 3p21.3 [73-74]. The 3p21 region contains numerous genes including $Na_v1.5$ and $Na_v1.9$ (see [4]). It is thus possible that the probability to have mutations leading to the oncogenic process is higher in chromosome regions corresponding to genes active during the foetal period. If this hypothesis is true, it could explain the foetal-splice form expressed in some cancer cells and the overall specificity of the isoform expressed in some cancers (for example $Na_v1.5$ in breast cancer and $Na_v1.7$ in prostate cancers).

In conclusion, VGNaC are found in several metastatic cancers and their functional expression may represent a more generic involvement in physiological and pathophysiological invasive processes. These ionic channels probably interfere with calcium homeostasis which in turn modifies signalling pathways involved in invasiveness, a prerequisite to metastasis formation. As such, these channels, as foetal isoforms, represent a promising new field of investigations to develop new specific blockers to fight some metastatic cancers.

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Figure 1

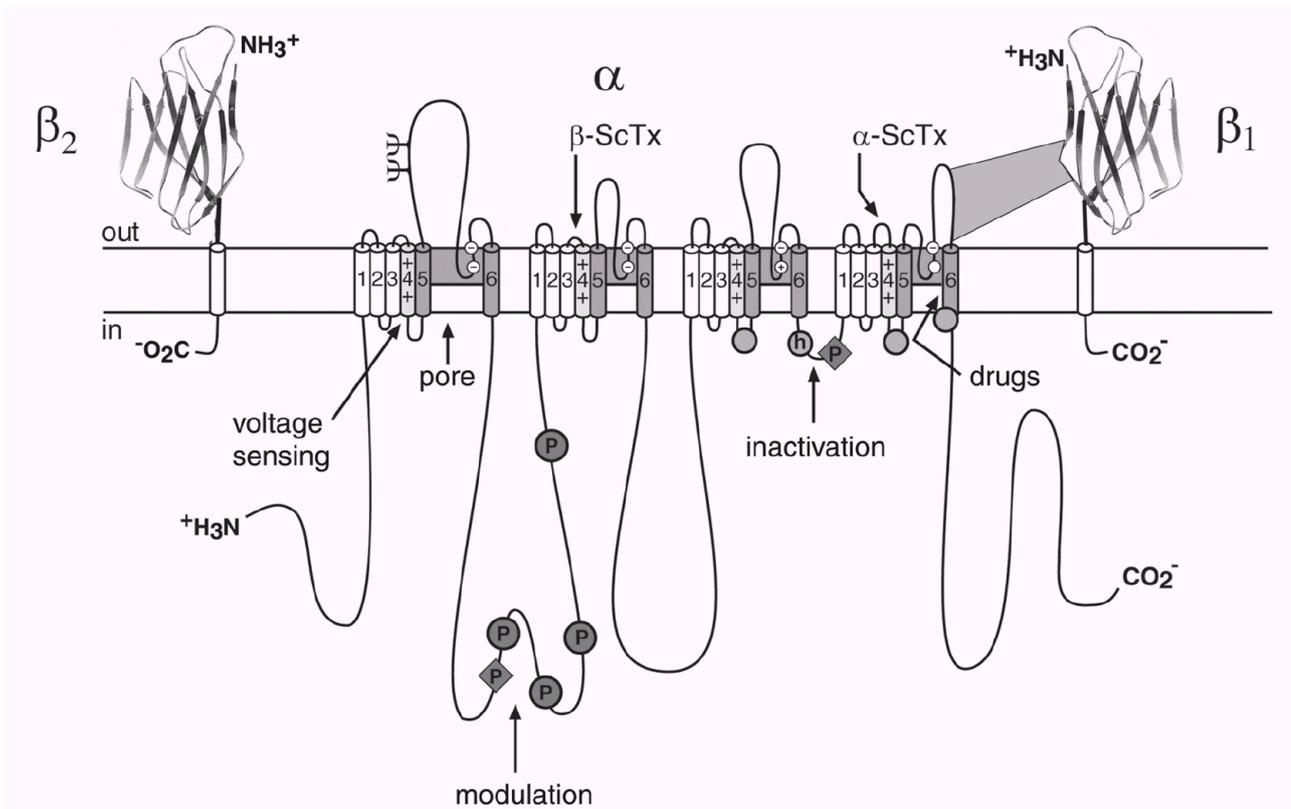
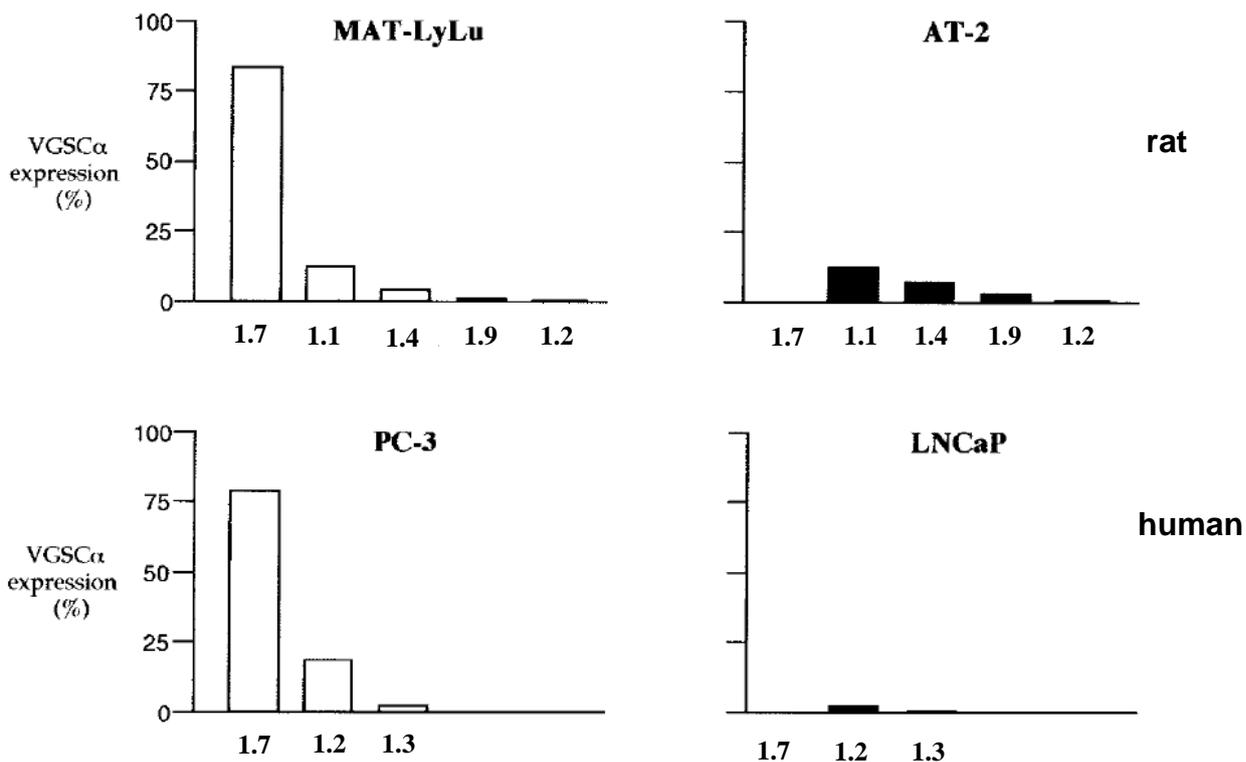


Figure 1: Subunit structure of the voltage-gated sodium channels. The secondary structures of the α and β subunits of the voltage-gated sodium channels are illustrated as transmembrane folding diagram. Cylinders represent probable alpha helical segments. The different important parts of the channel are indicated (voltage-sensing, pore, modulation, inactivation and interaction with drugs). P indicates sites of demonstrated protein phosphorylation by protein kinase A (circles) and protein kinase C (diamond). Sites of binding of α - and β -scorpion toxins are shown. Reprinted from [1] with permission from Elsevier.

highly metastatic

weakly metastatic

Figure 2 A



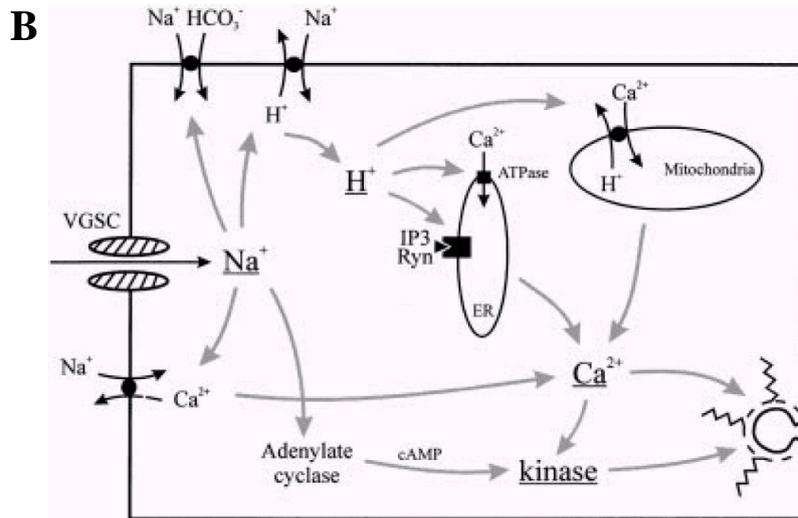


Figure 2: VGNaC in prostatic cancer cells. A, expression levels of the various VGNaC α subunits indicated below the bars. In each case, the vertical axis denotes the approximate level of expression with respect to mRNA levels in the highly metastatic counterpart. B, Schematic diagram of the possible mechanisms modulating membrane vesicle recycling involving Na^+ influx resulting from VGNaC (VGSC) activity. The scheme illustrates possible down-stream and microdomain effects of rising intracellular Na^+ . Intracellular sodium can affect intracellular calcium and pH. Both modulations of homeostasis could in turn modify the activity of kinases involved in exocytoses. Abbreviations: ER, endoplasmic reticulum; IP_3/Ryn , inositol triphosphate and/or ryanodine receptor. The "kinase" may be protein kinase A, C or CaM kinase II. A from [15] and B from [25]. Reprinted with permission of Wiley-Liss Inc, a subsidiary of John Wiley & Sons, Inc.

Figure 3

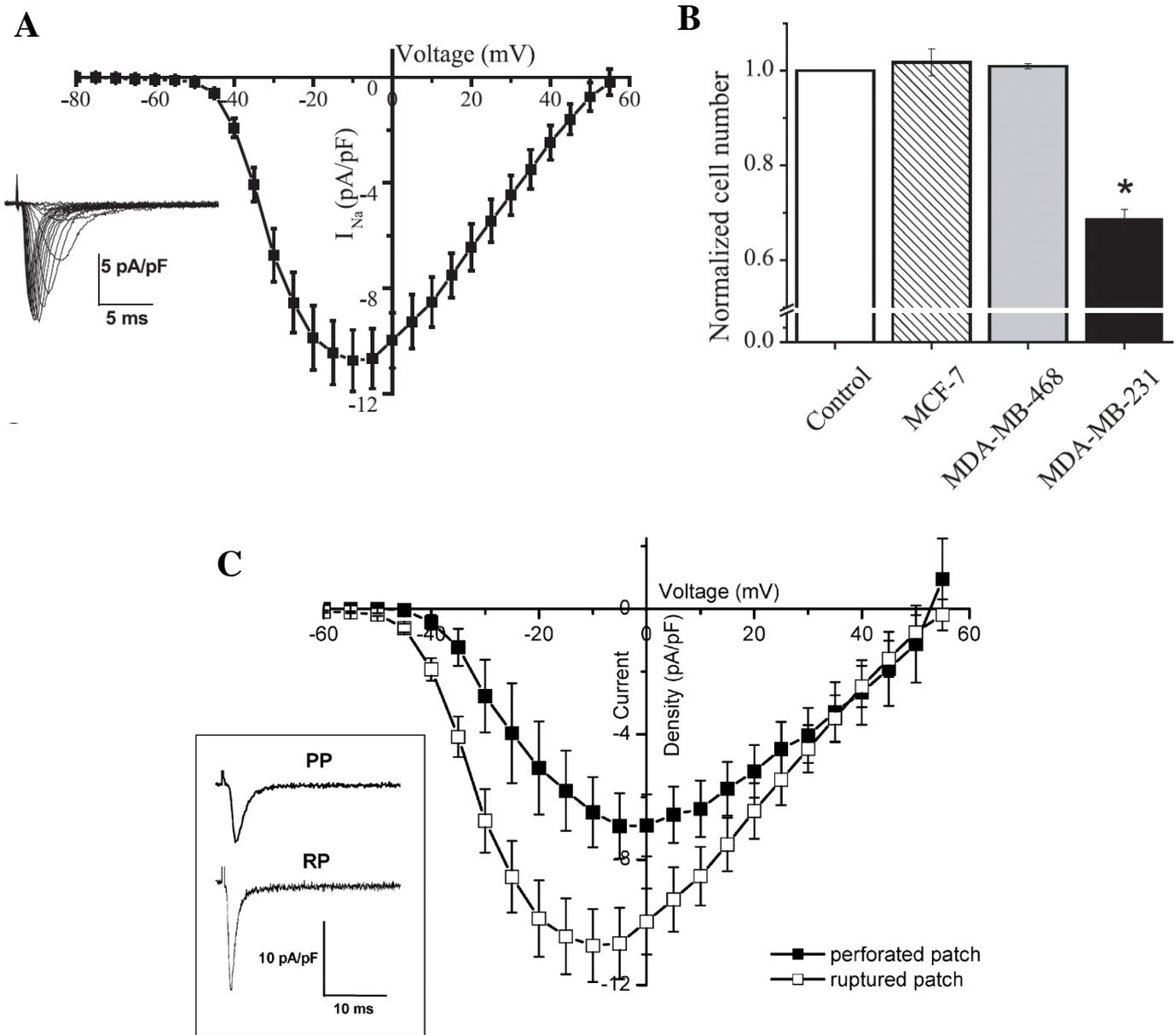


Figure 3: VGNaC in breast cancer cells. A, sodium current-voltage relationship of MDA-MB-231 cells. The inset on the left shows a family of currents recorded from a cell depolarised, from a holding potential of -100 mV, from -50 to $+20$ mV by 5 mV increments. B, relative effects of $30\mu\text{M}$ TTX on Matrigel® invasion of three different breast cancer cell lines as compared to control. C, influence of the patch-clamp configuration on sodium current-voltage relationship. The inset shows an example of currents recorded from cells depolarised at -10 mV from a holding potential of -100 mV in perforated patch (PP) and ruptured patch (RP). A and B from [41] reprinted with permission from Elsevier; C from [50] with kind permission of Springer Science and Business Media.

Figure 4

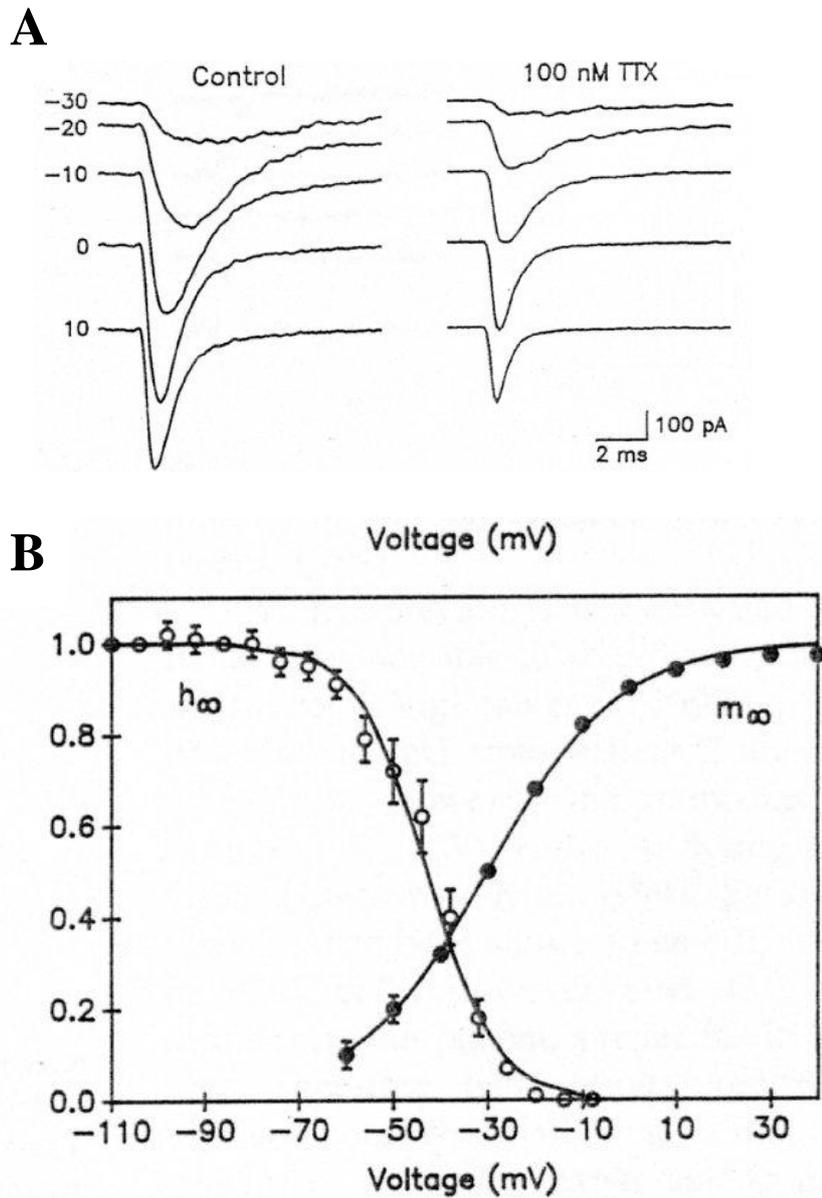


Figure 4: VGNaC in small cell lung cancer cells. Voltage dependent sodium currents recorded from H146 cells. A, whole-cell currents were evoked from depolarizing test potentials of 40 ms duration varying from -30 to $+10$ mV with 10 mV increments. The holding potential was -80 mV. Left, current observed in control conditions and the right panel illustrates current traces obtained in presence of 100 nM TTX. B, Pseudo-steady-state activation (m_{∞}) and inactivation (h_{∞}) parameters of I_{Na} . From [58] with kind permission of Springer Science and Business Media .

Figure 5

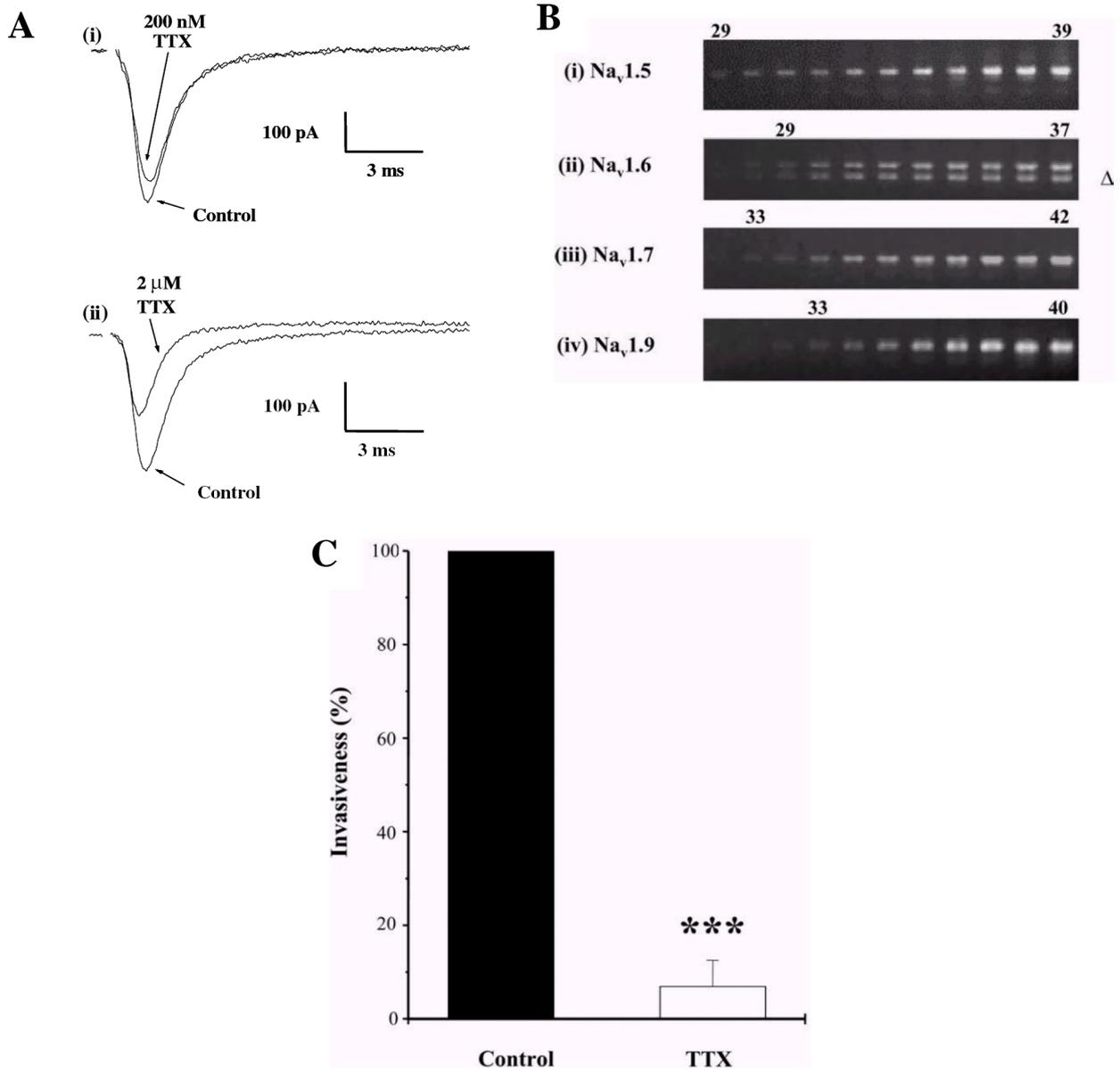


Figure 5: VGNaC in T-lymphocytes (Jurkat cells). **A**, From a holding potential of -100 mV, a 30 msec test pulse to -10 mV every 10 sec elicits a rapid inward current which is not very sensitive to 200 nM (i) and 2 μ M (ii) TTX. **B**, Semi-quantitative PCR gel images for (i) Nav_v1.5, (ii) Nav_v1.6, (iii) Nav_v1.7 and (iv) Nav_v1.9 products amplified from Jurkat cells. PCR cycle numbers for given bands are indicated above the gels. Δ denotes the mis-transcribed/exon-skipped form of Nav_v1.6. **C**, Matrigel® invasiveness by Jurkat cells under control conditions (dark bar) and following treatment with 10 μ M TTX (light bar). The asterisks (***) indicate that the difference was statistically significant ($p < 0.001$). From [63] with the permission of the authors.

Cancer type	Cell lines	mRNA expression	TTX sensitivity	Functional expression	VGNaC Role	VGNaC in Biopsies	References
Prostate	PC-3	Nav1.7 Nav1.2 Nav1.3	8.6 nM	fNav1.7	Invasion Motility (G) Motility (WH)	Yes (IH)	[10, 15, 31]
	LnCaP	Nav1.2		No current			[10, 15]
	Mat-Ly-Lu (rat)	Nav1.7 Nav1.1 Nav1.4	18 nM	fNav1.7	Invasion Motility (G) Motility (WH)	?	[9, 13, 15]
	AT-2 (rat)	Nav1.1 Nav1.4 Nav1.9		No current	---	---	[9, 15]
	Normal (immortalised) PNT-2	?		No current	---	---	[28]
Breast	MDA-MB-231	Nav1.7 Nav1.5 Nav1.6	≈2 μM	fNav1.5	Invasion	Yes (RT-PCR)	[41, 46]
	MCF-7	Nav1.5 Nav1.6 Nav1.7		No current	---	---	[41]
	MDA-MB-468	?		No current			[41]
	Normal (long term primoculture) HMEC	Nav1.6 Nav1.7 Nav1.2	?	Tiny current	Unknown (non invasive cells)		Personal data (not shown)
Small-Cell Lung Cancer	H146	?	215 nM	Current	?	?	[57, 58]
	H128	?	?	Current	?	?	[57]
	H69	?	?	Current	Endocytosis	?	[57,59]

Non-Small Lung Cancer	H460	Nav1.7 Nav1.6 Nav1.5 Nav1.3	≈10 nM	Nav1.7 Nav1.6	Invasion	Yes (RT-PCR)	[60]	
	Calu-1	Nav1.7 Nav1.6 Nav1.5 Nav1.1 Nav1.2 Nav1.3 Nav1.9 Nav1.8	≈5 nM + ≈1 μM	Nav1.5 Nav1.7 Nav1.6 Nav1.1 Nav1.2 Nav1.3	Invasion		[60]	
	H23	Nav1.7 Nav1.6 Nav1.5	≈10 nM	Nav1.7 Nav1.6	Invasion		[60]	
	A549	Nav1.6 Nav1.7		No current	---	---	[60]	
	Normal (immortalised)	NL20	Nav1.7 Nav1.6 Nav1.2 Nav1.3		No current	---	---	[60]
	Normal (immortalised)	BEAS-2B	Nav1.5 Nav1.7 Nav1.1 Nav1.6		No current	---	---	[60]
Leukaemia	K562	?	?	Current	?	?	[61]	
	K562			No current	---	---	[62]	
	K562/ADM	?	<100 nM	Current	?	?	[62]	
	CCRF-CEM	?	<150 nM	Current	?	?	[63]	
	CEM/VLB ₁₀₀	?	<150 nM	Current	?	?	[63]	
	Normal (immortalised)	Jurkat	Nav1.5 Nav1.7 Nav1.9	≈ 900 nM	Nav1.5	Nav1.5		[64]

Table I: List of the non-central nervous system cancers in which VGNaC have been studied. *Cell lines* indicates the human cancer cell lines used (except when "rat" is indicated in brackets); *mRNA expression* indicates the mRNA coding for the channels sorted in order of decreasing intensity as determined by semi-quantitative RT-PCR; *TTX sensitivity* indicates the constant of inhibition of the current by TTX; *functional expression* indicates the isoform of the channel which likely bears the current measured in patch-clamp. If a current was recorded but the isoform not determined then it is indicated "Current"; *VGNaC role* indicates the role of the channel *in vitro* as determined after inhibition by TTX. G indicates motility assessed by galvanotaxis; WH indicates motility assessed by wound-healing assay; *VGNaC in biopsies* indicates the presence or not of the mRNA (RT-PCR) or protein (immuno-histochemistry, IH) in biopsies obtained from patients. "?": not determined or unknown.