Pathobiology of Prostate Cancer: Voltage-gated Sodium Channel Expression and Metastatic Potential

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Abstract

Prostate cancer (PCa) remains the most frequently diagnosed cancer and the second most common cause of death from malignancy in men in the Western world. Despite this, conceptual advances in the understanding of its pathobiology have remained elusive for over half a century. As with all cancers, it is metastasis (i.e. secondary cancer formation) and its consequences that present the most significant biomedical and clinical challenges to the host, carers and healthcare payors, as well as the researchers. Pre-metastatic or metastatic PCa is frequently treated by hormone-based therapies aiming at androgen-down-regulation. However, this classic method is only effective for a limited time and new approaches are necessary (a) to improve the understanding of the pathobiology of PCa and (b) to develop new therapies for it, as well as improving the existing ones. In this article, we present an overview of the pathobiology of PCa and evaluate the evidence for the role of functional voltage-gated sodium channel (VGSC) expression in disease progression. We also discuss the possibility that VGSC blockers could be effective as novel anti-PCa drugs for clinical management of the metastatic phenotype.

Keywords: Prostate Cancer, Voltage-gated Na^+ channel, metastasis
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Introduction

The prostate is a compound tubulo-alveolar exocrine gland, which is a part of the male reproductive system in mammals. It surrounds the urethra just below the urinary bladder. The main function of this gland is to produce a proportion (~30 %) of the ejaculate in admixture with seminal fluid which is rich in citrate and nourishes sperm (Mycielska et al. 2008). Prostate cancer (PCa), uncontrolled and abnormal growth of the prostate gland, continues to be a major cause of morbidity and mortality in ageing men worldwide. It is one of the most common cancers in the developed world and the second leading cause of male cancer related death (Foster et al. 1999; Jemal et al. 2006). The incidence of PCa increases with age, reaching ~50 % in men of 50 years of age and rising to ~80 % by age 80 years (Foster et al. 1999; Parkin et al. 1999).

The clinical management of PCa is determined by the stage and degree of differentiation of the disease at diagnosis. Localised PCa (confined to the gland) may be treated with active surveillance, radical excisional surgery (prostatectomy), radiotherapy (including brachytherapy), various locally ablative thermal energies (e.g. cryotherapy, thermotherapy), androgen down-

regulation or receptor blockade, or simple observation ("watchful waiting"). These treatment options are generally effective to varying degrees, but in as many as 50 % of men with disease, PCa will eventually progress and spread (metastasise) beyond the confines of the prostate gland (Shelley et al. 2007). Because the development / progression of PCa is dependent on circulating androgens, in particular testosterone, the standard treatment for metastatic PCa, introduced and popularized by Huggins (1942), has been androgen down-regulation therapy involving either blockage of androgen receptors or inhibition of androgen synthesis. Although this 'classic' therapy can control metastatic disease, it is not curative and hormone resistance sets in within 12-33 months, accelerating progression to death in the majority (Hellerstedt and Pienta 2002; Kageyama et al. 2007). Recent evidence suggests that androgen deprivation, in fact, may be no better than 'conservative' approach to localized PCa (Lu-Yao et al. 2008).

Metastasis is the spread of tumour cells from the primary site to other places (mainly bones, lymph nodes and lung in PCa) in the body and consists of a series of sequential interrelated steps (Bashyam 2002; Eccles and Welch 2007; Figure 1). There are 6 basic stages in metastasis: i) detachment of cells from their neighbours and local invasion of surrounding normal host tissue, facilitated by release of proteolytic enzymes; (ii) penetration into circulation (lymphatics or blood vasculature – "intravasation"); (iii) migration and survival in circulation; (iv) arrest in capillary beds of distant organs; (v) extravasation; and (vi) attachment and proliferation at the secondary site(s). Angiogenesis (formation of new blood vessels) occurs both early and late in the metastatic cascade, as primary and secondary tumours grow (Kerbel 2008).

Whilst metastasis continues to be the major problem in the treatment of PCa, for organ-confined clinical disease, it is not presently possible to reliably determine which cases of PCa will metastasize and which will not. This is crucial in the decision making process when offering local therapy with curative intent. Whilst radical local therapy may 'cure' PCa, it may also "over-treat" indolent disease and gives rise to significant side effects, such as urinary incontinence, erectile dysfunction, bowel dysfunction and bone loss (eg Smith 2007). Thus, radical curative local therapies should ideally be avoided or exercised only if the evidence is strong that the cancer has a high risk of spreading (assuming that this has not already occurred by the time of diagnosis). On the whole, therefore, there are two major problems in the clinical management of PCa:

1. How to determine effectively whether a given localized tumour of the prostate will metastasise or not; and

2. How to treat metastatic PCa with a long lasting, ideally non-toxic method.

2. A novel 'neuroscience' approach to understanding the pathobiology of metastatic PCa – basic principles

In seeking to understanding the pathobiology of PCa, we have adopted the fundamental view that ion channels could have a significant involvement, since the metastatic cascade can be considered as a series of basic cellular behaviour, including motility, invasion, secretion, adhesion etc, which are well known to be controlled by ionic activity. Ion channels are integral membrane proteins which mediate usually fast transport of ions in and out of cells, the direction of flux depending upon the electrochemical gradient of the ion(s) involved. These proteins are gated by three types of stimulus: membrane potential change, ligand binding and mechanical tension. Voltage-gated ion channels are activated mainly by membrane depolarization and exhibit varying degrees of selectivity for the permeant ion(s) - Na^+, K^+, Ca^{2+}, Cl^- etc. (Terlau and Stuhmer 1998). Voltage gated Na^+ channels (VGSCs) are members of the voltage-gated ion channel superfamily which share two major properties:

1. These channels are associated with membrane potentials that are equivalent to voltage gradients of some 10,000,000 V/m.

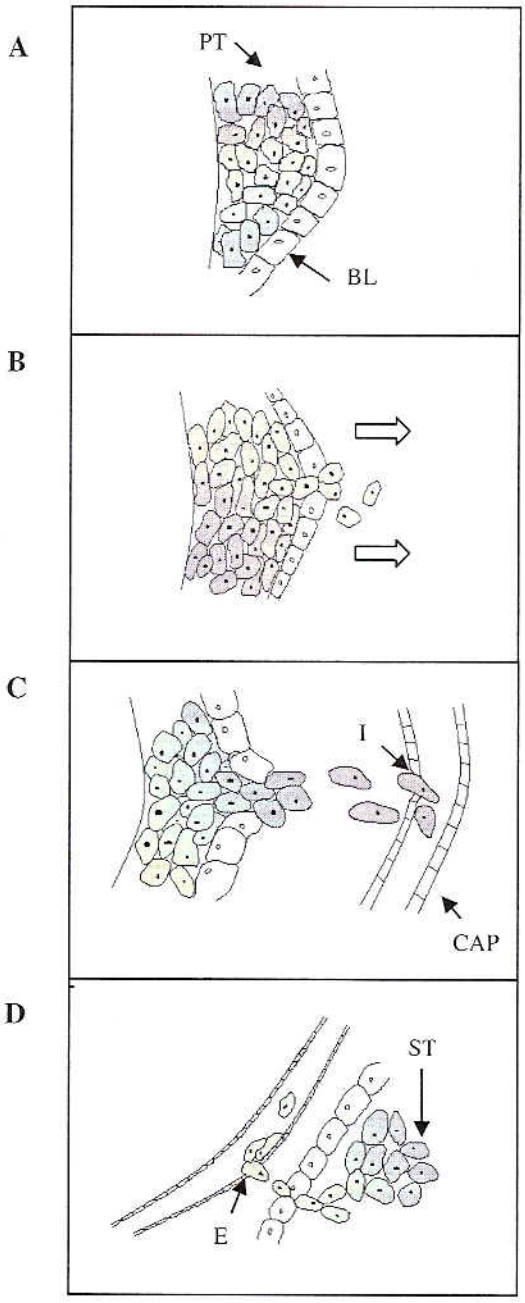

Figure 1. The main stages of the metastatic cascade, showing basic cellular behaviours. A: Primary tumour (PT) in epithelium surrounded by basal lamina (BL). B: Proliferation of primary tumour cells and invasion of BL (open arrow indicates direction of tumour progression). C: Intravasation (I); cells continue to migrate locally, adhesive properties change and cells detach to invade the capillary (CAP). D: Cells migrate in the bloodstream; adhesive properties are modulated to form attachments to the capillary wall. Extravasation (E); cells escape from the capillary and invade surrounding tissue to form a secondary tumour (ST). Please note that angiogenesis is not shown for simplicity. Modified from Williams and Djamgoz (2005).

2. When open, the channels can permeate at a rate of some 10,000 ions/msec.

It is probably because of these two remarkable characteristics that voltage-gated ion channels occur so widely in cells and subserve a range of cellular behaviours. VGSC's are well known to be responsible for initiation and non-decremental propagation of action potentials in 'excitable' cells, including nerve, muscle and neuroendocrine cells (Yu and Catterall 2003; Catterall et al. 2005). However, VGSC's have also been found to be expressed in 'non-excitable' cell types such as lymphocytes, glia and fibroblasts (Diss et al. 2004). Interestingly, transient expression or re-expression of VGSC's occurs during dynamic phases in cells, e.g. damage, regeneration, even (de)differentiation (Chiu 1988; Waxman et al. 2000; Waxman 2006; Wen et al. 1994; Yang et al. 1991). Here, we review the current knowledge on the functional expression and regulation of VGSCs in PCa *in vitro* and *in vivo* and outline the role of channel activity in metastatic cell behaviours in the context of possible clinical application.

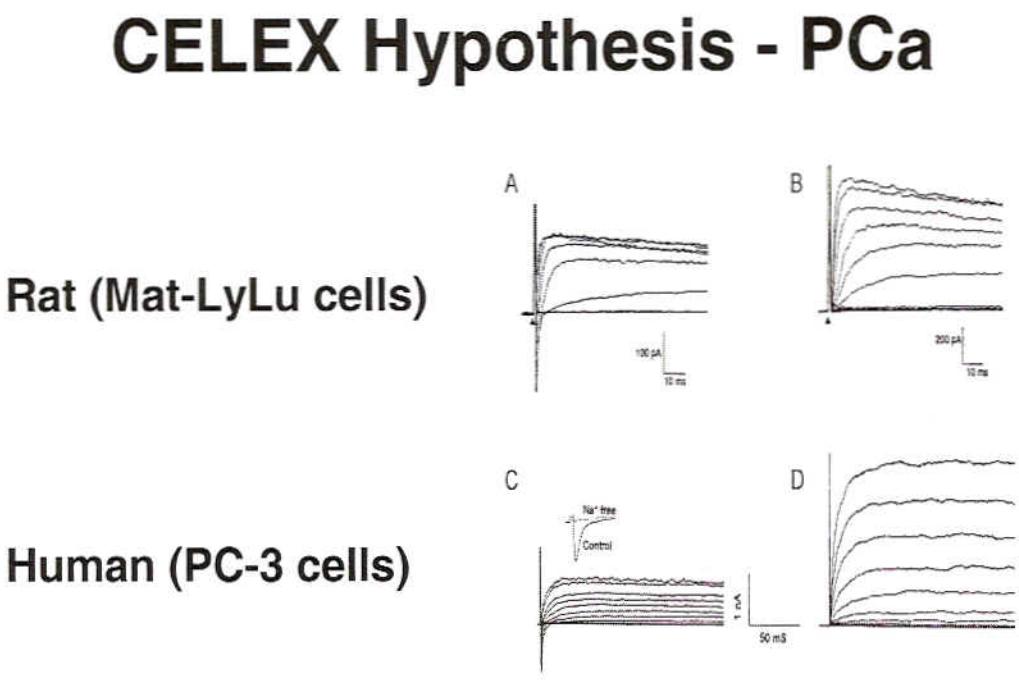

Figure 2. Electrophysiological basis of the CELEX hypothesis. Voltage-gated membrane current recordings from various PCa cell lines. A, Mat-LyLu. B, AT-2. These are isogenic rat prostate cancer cell lines of strong and weak metastatic potential, respectively. C, PC-3. D, LnCaP. These are human prostate cancer cell lines of strong and weak metastatic potential, respectively. In both pairs of recordings (A & B and C & D), the following two key features are apparent: The strongly metastatic cell lines are associated with (i) expression of a voltage-gated inward (Na^+) membrane current (sharp downward signal); and (ii) reduced sustained outward currents. The combination of (i) and (ii) would render these cell membranes 'excitable'. Note the difference in the respective amplitude scales in A & B, which underestimates the visual impact of the differences mentioned. Modified from Grimes et al. (1995) and Laniado et al. (1997).

3. Electrophysiology, pharmacology and molecular biology of VGSC expression in PCa *in vitro*

VGSC's have been found to be functionally up-regulated in metastatic PCa cells. In the original work of Grimes et al. (1995) on the Dunning rat model of PCa, whole-cell patch

clamp recordings showed that the strongly metastatic Mat-LyLu cells expressed functional VGSCs whilst the weakly metastatic AT-2 cells, derived from the same original tumour, did not (Figure 2A & B). In a similar manner, for human PCa, the weakly metastatic LNCaP cell line did not express functional VGSC's but the metastatic PC-3 cell line did (Figure 2C & D; Laniado et al. 1997). Interestingly, in both strongly metastatic cell lines (Mat-LyLu and PC-3), the outward currents (in part carried by Kv1.3, at least in the former; Fraser et al. 2003a), were significantly smaller, compared with the corresponding weakly metastatic cell lines. Taken together, this combination of a functional VGSC and reduced outward current would render the strongly metastatic cell membranes potentially excitable. A positive correlation between cell surface VGSC expression and metastatic potential of a range of rat and human PCa cell lines was demonstrated by Smith et al. (1998). Finally, Bennett et al. (2004) showed that direct transfection of a VGSC α-subunit alone into LNCaP cells was "necessary and sufficient" to increase the *in vitro* invasiveness of the cells.

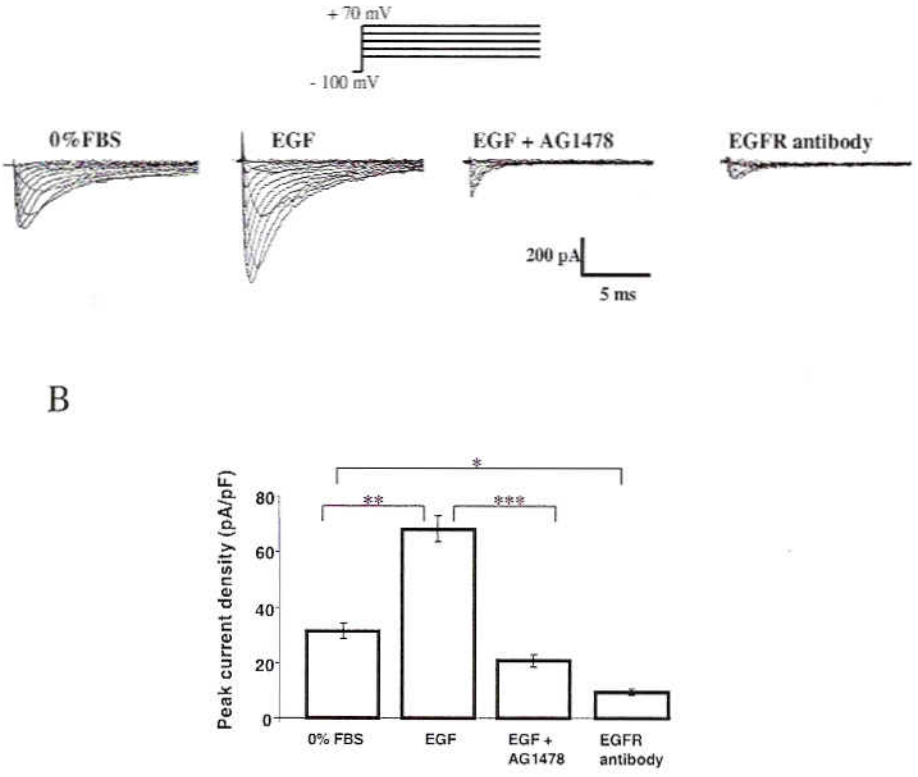

Figure 3. Upregulation of VGSC activity in Mat-LyLu cells by treatment for 24 h with exogenous epidermal growth factor (EGF). (A) Typical VGSC current traces recorded from Mat-LyLu cells in different culture conditions: 0 % foetal bovine serum (FBS), EGF (100 ng/ml), EGF + 1 μM AG1478 (EGF receptor kinase inhibitor), and anti-EGF receptor antibody (1 μg/ml). VGSC currents were recorded by pulsing membrane potentials from −50 to +70 mV in 10 mV increments, from a holding potential of −100 mV (indicated at the top). (B) Histograms showing mean values of peak VGSC current density recorded in different conditions (as in A). All data points shown are mean and SEM. Significance: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. Modified from Ding et al (2008).

From these studies, taken together, we proposed the "CELEX" (cellular excitability) hypothesis which proposes that metastatic cell membranes are 'excitable' and that this excitability underlies the cells' 'hyperactive' behaviour during metastasis (Djamgoz and Isbilen 2006).

The VGSC expressed in metastatic PCa cells activated at somewhat depolarized potentials (~ -40 mV) and had 'fast' inactivation kinetics. Pharmacologically, the current was highly sensitive to tetrodotoxin (TTX), with an IC_{50} of ~18 nM but was insensitive to 1 μM μ-conotoxin (a specific blocker of skeletal muscle-type VGSC) and 100 μM amiloride (a blocker of the epithelial-type Na^+ channels) (Grimes and Djamgoz 1998). Application of a 'ramp' voltage-clamp protocol showed that the membrane was capable of amplifying small currents (S.P. Fraser, unpublished observations), characteristic of neuronal Nav1.7 channels (Cummins et al. 1998). The VGSC currents in PC-3 cells were generally similar.

At the molecular level, semi-quantitative PCR analyses of Mat-LyLu and PC-3 cell lines showed that the predominant VGSC present was indeed Nav1.7, at an mRNA level that was >1000-fold higher than the corresponding weakly metastatic cells (Diss et al. 2001). Some minor mRNAs for other VGSC subtypes (Nav1.2, Nav1.4 and Nav1.6) were also present but their functional role, if any, is not known (Diss et al. 1998, 2001). Interestingly, in domain 1 (trans-membrane segment 3/4), a normally conserved negative aspartate residue (probably in the extracellular loop) was replaced with a neutral asparagine, consistent with the expressed Nav1.7 being a 'neonatal' splice variant, as found in other VGSC's (Diss et al. 2004). However, further work is required to test that this form of the channel is indeed developmentally regulated. If so, this would be another example of "oncofoetal" gene expression in cancer (e.g. Monk and Holding 2001; Boyerinas et al. 2008). Such a situation has been shown previously for the VGSC (Nav1.5) expressed in human breast cancer cells and tissues (Fraser et al. 2005). At present, it is not yet known why Nav1.7 specifically is upregulated in PCa (but see section 5; Bennett et al. 2004).

4. Regulation of functional VGSC expression in PCa

In 'excitable' cells generally, VGSC regulation is highly complex and dynamic, being spatiotemporal and hierarchical (Diss et al. 2004). Thus, specific VGSC subtypes are expressed in particular tissues, at suitable densities and at specific locations in the membrane, in order to carry out particular cellular and subcellular functions (e.g. Hille 1992). Hierarchically, regulation can occur at all levels, from transcription to post-translation, with feedback, by a variety of endogenous mechanisms (Diss et al. 2004; Shao et al. 2008). Some of the primary regulatory mechanisms are as follows:

Serum factors. Initial observations on the effects of altering serum level on VGSC expression gave insights into possible biochemical control mechanisms. For example, lowering the serum concentration from 1 to 0.1 % reduced the VGSC current density in Mat-LyLu cells (S.P. Fraser, unpublished observations). Interestingly, increasing serum concentration from 1 to 5 % also decreased functional VGSC activity (Ding and Djamgoz 2004). Thus, it would appear that VGSC expression/activity was under stimulatory and inhibitory control by substances (growth factors and hormones) present in the serum.

Growth factors. Nerve growth factor (NGF), a member of the neurotropin family of secreted proteins, is important for neurons, including as regards their plasticity, differentiation and functional maintenance (e.g. Lu et al. 2005). Importantly, the prostate gland, amongst peripheral organs, contains one of the highest levels of NGF (Murphy et al. 1984; MacGrogan et al. 1992). For Mat-LyLu cells, NGF application increased peak VGSC current density in a time- and dose-dependent manner (Brackenbury and Djamgoz 2007). NGF also shifted voltage to peak and the half-activation voltage to more positive potentials, and

produced currents with faster kinetics of activation. However, sensitivity to TTX was not affected. The effect of NGF appeared to be through a protein kinase A (PKA)-mediated pathway that did not affect the Nav1.7 mRNA level, but upregulated the total VGSC α-subunit protein level (Brackenbury and Djamgoz 2006, 2007). One possibility was that NGF induced local synthesis of VGSC protein by translating 'docked' mRNA.

Epidermal growth factor (EGF) is known to be associated with PCa progression, cellular invasiveness and androgen independence (Djakiew 2000; Montano and Djamgoz 2004). Importantly, prostatic fluid contains the highest levels of EGF in the human body (Russell et al. 1998; Gann et al. 1999). Exogenous EGF upregulated both VGSC current density in Mat-LyLu cells (Figure 3; Ding et al. 2008) and Nav1.7 expression in rat and human PCa cells *in vitro* (Uysal-Onganer and Djamgoz 2007), as had previously been found for PC12 cells (Toledo-Aral et al. 1995). Importantly, EGF also increased the migration of both the Mat-LyLu and PC-3 cells, and a significant proportion of these effects involved the enhanced VGSC activity (Ding et al. 2008; Uysal-Onganer and Djamgoz 2007). Comparable effects were obtained with AG1478, an inhibitor of EGF receptor tyrosine kinase. It was suggested, therefore, that endogenous EGF has a major role in the upregulation of Nav1.7 expression/activity and the two could represent an 'in-built' positive feedback mechanism accelerating the progression of PCa (Montana and Djamgoz 2004).

Importantly, one of the most aggressive forms of PCa is the so-called "neuroendocrine" type and this is often associated with androgen insensitivity (Musca et al. 1995). It is possible, therefore, that when PCa becomes hormone refractory, growth factors, like NGF and EGF, become significant and contribute to disease progression, at least in part, via functional VGSC expression/upregulation. This hypothesis is considered further in the following section.

Steroid hormones. Given the vital importance of androgens to both normal prostatic growth and function, and to the growth and metastasis of PCa, it is particularly striking that functional VGSC expression in PCa cell lines is inversely correlated with functional androgen receptor (AR) expression (both PC-3 and MAT-LyLu cells being AR-negative). This might suggest that functional VGSC expression is directly or indirectly suppressed in these cells by androgen signalling, and/or by the presence of a functional AR. Indeed, this would be entirely consistent with a previous study of the effects of androgen on VGSCs in mouse C2 muscle cells in which (i) VGSC current density was found to be reduced by ~50 % following long-term (7 days) treatment with the androgen dihydrotestosterone (and concomitant treatment with the anti-androgen hydroxyflutamide blocked the androgen-induced reduction), and (ii) stable over-expression of AR abolished VGSC currents (even in the absence of androgens) (Tabb et al. 1994). The precise mechanism (s) by which these distinct androgen-dependent and (androgen-independent) AR-mediated effects are brought about has not been fully investigated, so it remains unclear whether the effects are transcriptional upon the channel gene and/or post-transcriptional on the channel protein. It is also unknown whether androgens (and/or the AR) act specifically upon either the α- or β-subunit alone, or whether both VGSC subunits are simultaneously regulated. There is some evidence for this latter possibility in the literature. Long-term androgen treatment has been shown to significantly down-regulate the mRNA expression of the VGSC β1 subunit of fish electric organ (Liu et al. 2007). Furthermore, a recent study has reported that 24-48 hour treatment of cultured human bronchial smooth muscle cells with the synthetic steroid dexamethasone significantly reduced (more than 2-fold) both VGSC current density and Nav1.7 mRNA levels (Nakajima et al. 2008).

In order to begin to understand the effects of androgen signalling on VGSC expression in

PCa, we have measured the effect of dihydrotestosterone on the expression of the major VGSC β-subunit. Consistent with our hypothesis and the above studies, we found that β1 mRNA levels were significantly reduced (by ~40 %) in AR-positive weakly metastatic LNCaP cells following 24-48 hour treatment (Diss et al. 2008a). Furthermore, in preliminary experiments, following on from the work performed on mouse C2 muscle cells, we have found that stable over-expression of AR in otherwise AR-native PC-3 cells significantly reduced mRNA levels of both major VGSC subunits: Nav1.7 by ~50 % and β1 by ~80 % (J.K.J. Diss, unpublished observations). Both these androgen- and AR-mediated effects on VGSC α- and β-subunit expression were likely to be mediated by a classic AR-dependent genomic pathway acting directly upon the promoters of the genes encoding Nav1.7 and β1. Indeed, the recently identified promoter region driving Nav1.7 transcription possesses 4 putative highly specific androgen response elements close to the transcription initiation site (Diss et al. 2008b). We should note, however, that some of these effects may involve estrogen, given (i) that androgens exert their primary effect in some tissues (including bone and brain) via biochemical conversion to estrogens (by the aromatase enzyme) and (ii) that there is increasing evidence for the involvement of estrogens in PCa growth and progression (e.g. Prins and Korach 2008). Indeed, both VGSC mRNA and functional expression have been reported also to be altered by estradiol (Lin et al. 2004; Moller and Netzer 2006; Kow et al. 2006).

In conclusion, our results raise the possibility that the detrimental long-term effect of altered androgen signaling by anti-androgen therapy could involve the *release* of AR inhibition of VGSC expression, which might at least partially account for the characteristic fatal manifestation of androgen-insensitive metastatic disease 2-3 years after the onset of hormone therapy. Additionally, several studies suggest that prolonged androgen deprivation eventually results in tumour growth being suppressed rather than stimulated by testosterone, indicating that the effect of androgen signaling on target genes may be reversed (Kokontis et al. 1994; Chuu et al. 2006). If this is the case for Nav1.7 and β1, the inhibitory effect of AR would become a *potentiating* effect, leading to increased expression of functional VGSCs that, in turn, would increase metastatic potential. Consistent with this, and with androgens also having AR-independent effects (Foradori et al. 2008), we have observed that androgen treatment can significantly *increase* (2- to 3-fold) Nav1.7 and β1 mRNA expression in AR-negative PC-3 cells (Diss et al. 2008a).

Activity-dependent regulation (ADR). VGSC up regulation in both Mat-LyLu and PC-3 cells can finally also be controlled via an activity-dependent positive feedback mechanism (Brackenbury and Djamgoz 2006; Uysal-Onganer and Djamgoz 2007). Thus, long-term treatment with TTX led to down-regulation of Nav1.7 mRNA levels, and VGSC protein expression in plasma membrane in Mat-LyLu and PC-3 cells (Brackenbury and Djamgoz 2006; Uysal-Onganer and Djamgoz 2007). On the other hand, forskolin (an activator of adenylate cyclase) and monensin (Na^+ ionophore) both increased the peak VGSC current density (Brackenbury and Djamgoz 2006). Thus, the mechanism controlling the ADR was proposed to be via a Na^+ influx-dependent activation of PKA (Brackenbury and Djamgoz 2006).

VGSC expression / activity can also be modulated by several other mechanisms, although their direct relevance to PCa presently is unclear. Such mechanisms include the following: (a) Post-translation modification (including phosphorylation and N-linked glycosylation). Phosphorylation of VGSCs by protein kinases (e.g. PKA and PKC) has been shown to influence channel inactivation and peak sodium current density, amongst other characteristics (Shao et al. 2008). (b) Coupling with the accessory β-subunits can also modulate VGSC expression (via intracellular trafficking and) and kinetics (Brackenbury et al. 2008;

Shao et al. 2008). In addition, β-subunits may link VGSCs to extracellular matrix and cytoskeletal proteins and thus co-ordinate their spatial distribution and stability in plasma membrane (Brackenbury et al. 2008). Whether VGSC activity controls VGSC β-subunit expression and any possible consequent effect on metastatic cell behaviour have yet to be investigated. (c) Functioning of VGSCs may be influenced by their sub-cellular localisation. However, the mechanisms underlying the targeting and recycling of ion channels are largely unknown.

5. Enhancement of metastatic cell behaviours *in vitro* by VGSC activity

The role of VGSCs in metastatic potential has been investigated extensively in rat and human PCa. The highly specific VGSC blocker TTX (nM range – 1 μM) was used to suppress VGSC activity and the consequences of this on a range of metastatic cell behaviours were evaluated, as follows:

Process extension. The capacity of a cell to alter its morphology and migrate is inherent to cancer cell metastasis (Mohler 1993). Changes in cellular morphology generally involve sub-cellular mechanisms like changes in cell volume, cell-matrix interaction, cytoskeletal elements and ion channel activity. The possible involvement of VGSCs in the morphological change of rat PCa cells was investigated by Fraser et al. (1999). Incubation of both Mat-LyLu and AT-2 cell lines with TTX altered the morphology only of the Mat-LyLu cells by significantly decreasing process length and field diameter, and increasing cell body size and process thickness. Thus, the cells became more 'compact'. These results demonstrated that VGSC activity plays significant role in determining the morphological development of Mat-LyLu cells in such a way as to enhance their metastatic potential. However, the precise sub-cellular mechanism(s) regulating the cellular morphology in PCa cells is unclear. It was proposed that VGSC subunit(s) could interact directly and reciprocally with cytoskeletal elements to regulate cellular morphology (Fraser et al. 1999; also, Shao et al. 2008).

Motility. Motility, a complex process, considered as an indicator of the metastatic ability of cells, has been investigated in a number of ways. (i) *Lateral motility*. This is studied by "wound heal" assays and probably reflects the early/local invasion in the metastatic process. It was shown that the lateral motility of the Mat-LyLu cells was significantly reduced with TTX. In contrast, TTX had no significant effect on the AT-2 cell line (Fraser et al. 2003b). (ii) *Transverse migration*. This is studied by "Transwell" assays and may represent more intra/extravasation since cellular movement occurs via small spaces. Treatment with TTX, again, reduced transverse migration of Mat-LyLu and PC-3 cells (Ding et al. 2008; Uysal et al. 2007). Both lateral motility and transverse migration were reduced by 30-50 % by TTX, consistent with VGSC activity being a potentiating factor in metastasis. (iii)

Galvanotaxis. This is directional motility in a small d.c. electric field, thus taking into account the fact that local, trans-cellular voltage gradients occur commonly in epithelia. Mat-LyLu responded to such a field by migrating towards the cathode but AT-2 cells gave no such response. Importantly, TTX suppressed the galvanotactic response of the Mat-LyLu cells whereas veratridine, a VGSC opener, enhanced it (Djamgoz et al. 2001). These results showed that metastatic cells could be galvanotactic and VGSC activity could play a significant role in this process. Galvanotaxis may well occur *in vivo* since the lumen of (rat) prostatic epithelia is at ~-10 mV relative to stroma (Szatkowski et al. 2000) and also express VGSC protein (Diss et al. 2005). Such a trans-epithelial potential in human prostate could influence the local invasion process. Similarly, trans-endothelial potentials could also affect extra/intravasation of metastatic cells.

Endocytosis. This is retrieval of plasma membrane and can contribute to metastasis in two main independent ways: i) secretion (balancing vesicular release) and (ii) silencing

of signaling proteins by internalization. Cancer cells release growth factors and cytokines, as a part of tumour growth (e.g. Rhim et al. 2008), as well as proteolytic enzymes in order to 'digest' surrounding tissues during local invasion (Egeblad and Werb 2002). Furthermore, a variety of signalling proteins undergo dynamic endocytosis, including growth factor (eg EGF) receptors (Sorkin and Goh 2008) and VGSCs (Brackenbury and Djamgoz 2006). Using uptake and release of horseradish peroxidase (HRP) as a non-cytotoxic tracer, Mycielska et al. (2003) showed that Mat-LyLu cells were much more endocytic than the AT-2 cells. Importantly, TTX significantly reduced the uptake of HRP into the Mat-LyLu cells, without any effect on AT-2 cells (consistent with lack of expression of functional VGSCs in this cell line), and eliminated the endocytic difference between them. Similar results were obtained for exocytosis of HRP. Interestingly, VGSC activity also controlled the quality of endocytosis ('vesicular patterning') studied using fractal methods (Krasowska et al. 2004). Additionally, the possible role of VGSC activity in PCa cell secretion was studied in a clinical context by testing the effects of anticonvulsant drugs (VGSC blockers). Thus, Abdul and Hoosein (2001) showed that secretion of prostate specific antigen (PSA) and IL-6 from human PCa cell lines (LNCaP, DU145 and PC-3), measured by immunoanalyses, was inhibited by phenytoin and carbamazepine.

Adhesion. Changes in cell-cell and cell-matrix adhesion accompany the transition from benign to invasive cancer and subsequent dissemination of metastatic tumour cells (Christofori 2003). The effects of TTX on the adhesion of rat and human PCa cell lines of markedly different metastatic potential was investigated by Palmer et al. (2008). Pretreatment with TTX for 24 h increased the adhesion of Mat-LyLu and PC-3M cells. There was no effect on the weakly metastatic AT-2 cells and the normal human prostatic epithelial PNT2-C2 cell line. The TTX-induced increase in the adhesiveness of the strongly metastatic cells was consistent with the functional VGSC expression in these cells and the proposed role of VGSC activity in metastatic cell behaviour. However, the mode of VGSC action in controlling cellular adhesiveness is not known. One possibility VGSC activity controls expression of cell adhesion molecules (eg Itoh et al. 1995), including the auxillary β-subunit(s) of the VGSC itself which may behave as a cellular adhesion molecule (Isom 2002; Brackenbury et al. 2008). Since VGSC expression is under auto-control (Brackenbury and Djamgoz 2006), blocking its activity with TTX could affect β-subunit expression and thus influence adhesion.

Nitric oxide production. Nitric oxide (NO), as a pleiotropic molecule, has a complex involvement in cancer, including PCa (Williams and Djamgoz 2005). In the case of the Dunning rat model of PCa, a controlled series of experiments on the Mat-LyLu cells showed that inhibition of the VGSC activity with TTX increased NO release (Williams and Djamgoz, 2004). Further work is required, however, to determine the functional significance of this effect.

Invasion. Invasion, the ability of the cells to penetrate the basement membrane, is an important hallmark of malignancy and can be assessed *in vitro* using Boyden ("Matrigel") chamber invasion assays. Contribution of VGSC activity to invasiveness was investigated firstly in rat PCa cell lines by Grimes et al. (1995). In this comparative study, incubation of Mat-LyLu cells with TTX for 48 h reduced their invasive capacity by 33 %. In contrast, TTX had no significant effect on the invasiveness of AT-2 cells. Experiments performed on human PCa cell lines gave similar results (Laniado et al. 1997). A positive correlation was found between VGSC protein expression in plasma membrane and invasiveness of several rat and human PCa cell lines of a range of metastatic potentials (Smith et al. 1998). A more direct and extensive comparative study was performed on LNCaP and two increasingly tumourigenic daughter cell lines, C4 and C4-2 by Bennett et al. (2004).

This study showed (i) that VGSC α-subunit protein expression increased in line with invasive potential; (ii) that TTX eliminated the differences in the invasiveness; and (iii) that transient expression of an isoform (Nav1.4) of VGSC in all three cell lines increased their invasiveness and, again, TTX reduced to baseline. Whilst confirming the central role of functional VGSC expression in PCa cell invasiveness, the transfection experiments raised an important question: Does the subtype of VGSC expressed matter for increasing metastatic potential? This question arises from the fact that the predominant VGSC expressed in rat and human PCa cells is Nav1.7 (Diss et al. 2001, 2005) whilst Bennett et al. (2004) transfected Nav1.4. This is an important question that deserves further investigation.

6. VGSC expression in PCa *in vivo*

Elevated VGSC (Nav1.7) mRNA and protein have also been detected in PCa tissue biopsies (Diss et al. 2005). In this preliminary study involving a limited number of clinical samples, we have found that out of the mRNAs for various VGSC subtypes studied (Nav1.2, Nav1.3, Nav1.5, Nav1.6, Nav1.7 and Nav1.9), only Nav1.7 mRNA levels correlated with disease state; expression was strongly (~20-fold) upregulated in PCa compared with non-PCa prostate tissues. In addition, VGSC protein (as detected using a pan-specific VGSC antibody) was shown to be markedly upregulated in prostatic epithelial cells at the transition from low-grade to high-grade prostatic intraepithelial neoplasia (Diss et al. 2005). The latter is presently the earliest morphologically discernable disease stage used as a marker for prostatic adenocarcinoma (Bostwick et al. 1996). In a separate study, VGSCα protein expression in normal and tumour prostatic tissues was also studied using multitumour tissue arrays (Abdul and Hoosein, 2002). It was found, again, that the majority of tissue samples taken from PCa patients displayed greater levels of VGSCα proteins than 'normal' tissues. Diss et al. (2005) studied specifically Nav1.7 mRNA expression in low-grade vs high-grade cases of PCa (as determined by Gleason grading) and found that the mRNA level was significantly higher in high-grade PCa (Gleason sum ≥7.0). However, it remains to be determined whether it is Nav1.7 protein expression itself that differentiates between early and advanced stages of disease, since mRNA and protein may be regulated differently in cells (Orphanides and Reinberg 2002; Brackenbury and Djamgoz 2007). Interestingly, Abdul and Hoossein (2006) also studied voltage-gated K^+ channel expression in human PCa biopsies and showed that Kv1.3 expression was upregulated in at least a subset of cases, directly complementing the situation *in vitro* (Grimes et al. 1995; Laniado et al. 1997; Fraser et al. 2003a). Finally, Diss et al. (2005) performed "Receiver Operator Characteristics" (ROC) plot analysis and concluded that, even within the limited sample size studied, Nav1.7 mRNA expression was a highly sensitive and specific diagnostic marker of PCa.

7. Concluding remarks: Clinical aspects

The general conclusion from the 'neuroscience' approach to understanding the pathobiology of PCa that we adopted is that functional VGSC expression occurs as an integral part of the metastatic process in PCa and accelerates disease progression by potentiating a range of cellular behaviours implicit to metastasis. Thus, application of a new technique (electrophysiology) has generated both a new concept of metastatic disease in the form of the CELEX hypothesis and novel clinical possibilities for managing metastatic PCa. Accordingly, VGSC expression can serve as a solution to the two main clinical problems associated with PCa:

1. ***Definitive diagnosis.*** Since VGSC expression is functional and its pathobiology is consistent with expression early in progression of PCa to metastasis, it could act as a novel prognostic factor. Thus, if our hypothesis is correct, we would predict that PCa patients without VGSC should be metastasis-free for longer than those expressing VGSC.

2. ***Novel therapies.*** Assuming that VGSC activity potentiates cellular behaviors integral to the metastatic cascade *in vivo*, as *in vitro*, then VGSC blockers could be novel, non-toxic, non-hormonal drugs that could be useful to combat the metastatic PCa phenotype. Currently, clinically used VGSC blocker drugs include local anti-convulsants, anti-arrhythmics and local anaesthetics (Clare et al. 2000; Fiske et al. 2006; Roger et al. 2006; Le Guennec et al. 2007; Djamgoz 2007). The potential efficacy of these drugs against metastatic PCa remains to be tested.

Finally, we should note that such clinical potential, and the underlying CELEX hypothesis, may be applicable for other cancers where functional VGSC upregulation has been found *in vitro* and/or *in vivo*, including breast cancer (Roger et al. 2003; Fraser et al. 2005), small-cell lung cancer (Blandino et al. 1995; Onganer and Djamgoz, 2005), non-small-cell lung cancer (Roger et al. 2007), mesothelioma (Fulgenzi et al. 2006), melanoma (Allen et al. 1997) and cervical cancer (Diaz et al. 2007).

Acknowledgements

Our work on prostate cancer in the UK has been supported by the Medical Research Council, The Wellcome Trust, Prostate Cancer Charity, Prostate UK, and Pro Cancer Research Fund (PCRF). In Turkey, the research has been supported by TUBITAK. The work of SY at Imperial College London was supported by the Ömer Anıl Scholarship of Kanser Araştırma Vakfı (KAV, TRNC).

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