

T-lymphocyte invasiveness: control by voltage-gated Na⁺ channel activity

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Abstract Whole-cell patch-clamp recordings showed that a sub-population (10%) of Jurkat cells, a model of human T-cells, expressed a functional voltage-gated sodium channel, which was tetrodotoxin (TTX)-resistant. Expression of voltage-gated sodium channel protein was confirmed by western blots. Semi-quantitative PCR analysis revealed that mRNAs for the α -subunits of multiple voltage-gated sodium channel subtypes were present but indicated that Na_v1.5 was the predominant subtype, consistent with the TTX-resistant nature of the recorded currents. Importantly, 10 μ M TTX reduced the number of Jurkat cells invading a Matrigel basement membrane by $93.0 \pm 5.5\%$. Since similar sodium channels have also been detected in normal human T-lymphocytes, it is concluded that the activity of voltage-gated sodium channels could represent a novel mechanism potentiating the invasive capacity of these cells. © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

Efficient functioning of the immune system depends on the regulated trafficking of lymphocytes [1]. This ‘homing’ process eventually targets immune effector cells to tumours or to sites of infection. Circulating lymphocytes are round, ‘non-motile’ cells, which redistribute their cytoskeletal elements and organelles to acquire a constantly changing shape and polarised morphology during inflammatory response [2]. However, the mechanism(s) controlling this response is not fully understood. We have shown previously that cancer metastasis, a process that involves a series of basic cellular behaviours, including invasion, is controlled by voltage-gated Na⁺ channel (VGSC) activity [3–10]. Accordingly, functional expression of VGSCs could distinguish strongly and weakly metastatic prostate and breast cancer cells [3–5,9,10], and blocking VGSC activity in these cancer cells by application of tetrodotoxin (TTX) can significantly reduce their invasiveness in vitro [3–5,9,10].

Cells of the immune system traditionally have been categorised as ‘electrically inexcitable’, although most possess voltage-dependent ion channels in their membranes, e.g., [11].

In particular, human T-lymphocytes express VGSCs, but their molecular identity and functional role in these cells is not known, e.g., [12]. The human T-cell leukaemia cell line ‘Jurkat’ maintains functional characteristics of T-lymphocytes and has been used widely as a model system, e.g., [13,14].

Voltage-gated Na⁺ channels are glycosylated transmembrane proteins that form pores in the cell membrane, permitting influx of Na⁺ down its ionic gradient in response to membrane depolarisation. VGSCs are composed of a central α -subunit (~260 kDa) associated with accessory β -subunits (33–36 kDa) that can modify channel properties and interact with cytoskeletal and extracellular matrix proteins [15]. Most VGSCs show sensitivity to nanomolar concentrations of TTX and are thus classified as TTX-sensitive (TTX-S). However, a subset of VGSCs shows sensitivity to TTX in the micromolar range and is thus classified as TTX-resistant (TTX-R).

In the present study, using the Jurkat cell line, we investigated (1) the molecular identity of the VGSCs expressed and (2) whether VGSC activity could be involved in the cells’ invasive behaviour.

2. Materials and methods

2.1. Cell culture

Jurkat cells were grown and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies Ltd, Paisley, UK) supplemented with 4 mM L-glutamine, 100 μ g/ml penicillin, 100 μ g/ml streptomycin and 10% foetal bovine serum (FBS). Cells were seeded into 100 mm Falcon tissue culture dishes (Becton Dickinson Ltd, Bedford, UK) and grown in an incubator at 37 °C, 100% humidity and 5% CO₂.

2.2. Electrophysiology and pharmacology

The electrophysiological techniques used have been described in detail previously [3,4,16]. Two basic command voltage protocols were used with a holding potential of –100 mV: (1) *Current–voltage (I–V) protocol*. This was used to study the voltage-dependence of VGSC activation. Cells were pulsed to depolarising test potentials between –70 and +70 mV, in 5 mV steps. The test pulse duration was 40 ms and the interpulse period was 2 s. (2) *Repeat single-pulse protocol*. This was used to monitor the effects of TTX on current amplitude. Test pulses were to –10 mV. The test pulse duration was 40 ms, the interpulse duration was 10 s and there were 10 repeat pulses. TTX (Alomone Labs Ltd, Jerusalem, Israel), was applied using a gravity-fed Y-tube perfusion system. The effect of TTX on the inward current was calculated as the percentage block of current (*B*), as follows: $B (\%) = [1 - (I_a/I_b)] \times 100$, where *I*_b and *I*_a are the current amplitudes recorded before and after TTX application, respectively.

2.3. RNA extraction and cDNA synthesis

Total cellular RNA was isolated from three separate batches of the Jurkat cell line using a total RNA miniprep kit (Stratagene, La Jolla,

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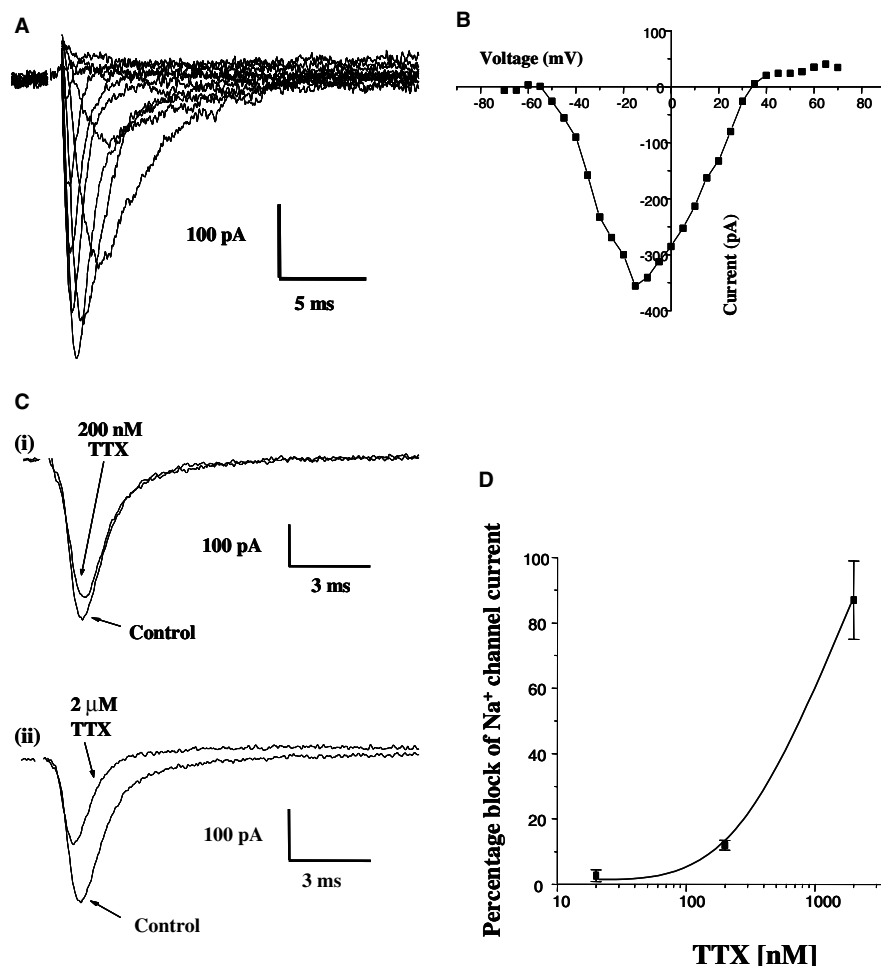


Fig. 1. Electrophysiological recordings from Jurkat cells. (A) Traces showing activation of an inward current by pulsing the cell from a holding potential of -100 mV in 5 mV steps from -70 to $+70$ mV with an interpulse interval of 2 s. Only every other trace is shown for clarity. (B) A plot of the current–voltage relationship for the inward currents recorded as detailed in (A). (C) Suppression of the inward current by (i) 200 nM and (ii) 2 μ M TTX. The cell was pulsed from a holding potential of -100 to -10 mV for 30 ms with a repeat interval of 10 s. The effect of TTX was recorded on the fifth pulse. (D) Partial dose–response curve to TTX over the concentration range 20 nM to 2 μ M. Data points denote mean \pm S.E. ($n \geq 3$). Cells were pulsed as outlined in part (C). The intracellular pipette solution contained Cs^+ to block outward K^+ currents in all recordings shown.

CA, USA) according to the manufacturer's instructions. RNA quality was assessed by gel electrophoresis and its quantity determined by spectrophotometric analysis. cDNA was synthesised as described previously [17].

2.4. Semi-quantitative PCR

cDNA was amplified by PCR using oligonucleotide primers directed against the TTX-R VGSCs $\text{Na}_v1.5$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ and two other VGSC subtypes, $\text{Na}_v1.6$, $\text{Na}_v1.7$, previously found to be expressed in cancer/invasive cells [9,10,17]. NADH-cytochrome b5 reductase (Hcytb₅R), which is expressed at very similar levels in cells derived from numerous tissue types [17,18], was used as the control amplicon. The primer sequences and PCR conditions used have been described previously [17]. To allow direct comparison of results obtained from each cell line, all comparable sscDNA and PCR reactions were performed simultaneously. 'Blanks', with no template added, were used as controls. A kinetic observation approach was adopted such that an aliquot of 5 μ l from the 60 μ l reaction was taken at the end of each amplification cycle, for eleven cycles, while reactions were held at 72 $^{\circ}\text{C}$. The amplification cycle at which aliquots were first taken differed depending on the VGSC α studied.

2.5. Western blotting

VGSC proteins were extracted and prepared according to the manufacturer's instructions (Upstate Biotechnology, Buckingham, UK) and further details have been described previously [4]. The pri-

mary antibody was a pan-VGSC (Upstate Biotechnology) and the secondary a peroxidase-conjugated swine anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark). The amount of protein loaded has been specified in the figure legend.

2.6. Matrigel invasion assay

Cell culture invasion chambers with an 8 μ m pore size precoated with Matrigel basement membrane matrix (BD Biosciences, Bedford MA, USA) were rehydrated according to the manufacturer's instructions. Jurkat cells were loaded at a density of 5×10^5 cells/ml and 100 μ g/ml SDF-1 α (Insight Biotechnology, Middlesex, UK) was used as a chemoattractant [19]. The experiment was done both in the presence and absence of 10 μ M TTX and was repeated three times. Invaded cells were quantified after 6 h by either (i) counting 20 fields of view (at $200\times$ magnification; Nikon TMS, Nikon Instruments Inc., Melville, NY, USA) or (ii) counting cells in a Neubauer hemocytometer.

2.7. Toxicity assay

The toxicity assay protocol has been described in detail previously, e.g., [6]. Briefly, the relative proportion of dead cells was determined by trypan blue exclusion following a 24 h incubation with either normal growth medium or with 10 μ M TTX. The number of live vs. dead cells was determined from 30 randomly chosen fields of view at $100\times$ under an inverted microscope (ID 03 Carl Zeiss Ltd, Welwyn Garden City, UK). The experiments were repeated three times.

2.8. Data analysis

All quantitative data are presented in the text as means \pm S.E. Statistical significance was determined by Student's *t*-test. Data were determined to be normally distributed except the toxicity data, which were transformed prior to statistical analysis.

3. Results

3.1. Electrophysiology

Jurkat cells showed a mean resting potential of -31.6 ± 1.9 mV ($n = 42$) and membrane capacitance of 11.2 ± 0.7 pF ($n = 60$). In 10% ($n = 13/126$) of recorded cells, membrane depolarisation evoked an inward current that reached a peak within a few milliseconds and then inactivated fully (Fig. 1A). This inward current was completely abolished when the extracellular Na^+ was replaced with choline $^+$ (not shown). The maximum size of the inward current was -83 ± 26 pA which corresponded to a current density of 7.0 ± 1.8 pA/pF ($n = 13$). A typical current–voltage (*I*–*V*) relationship is shown in Fig. 1B. The threshold for activation was around -50 mV. Currents activated more rapidly with successive depolarising steps, reaching a peak at around -20 mV. The inward currents were suppressed by TTX in a dose-dependent manner (Fig. 1C). The dose–response relationship suggested an IC_{50} of approximately 900 nM (Fig. 1D). Taken together, these results were consistent with 10% of Jurkat cells expressing functional TTX-R VGSCs.

3.2. Western blot and SQT-PCR

Western blots using a pan-specific VGSC antibody detected a band of ~ 200 kDa, similar to VGSC proteins found in

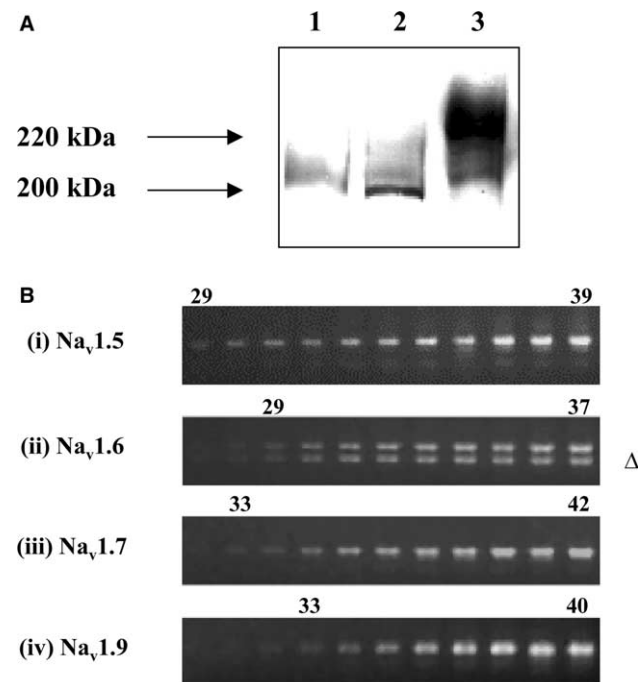


Fig. 2. (A) Expression of voltage-gated Na^+ channel α -subunits in Jurkat cells studied by Western blot using a pan-specific Na^+ channel antibody (Upstate, Milton Keynes, UK). Lane (1) 40 μg Jurkat cell membrane preparation; lane (2) 10 μg mouse heart; lane (3) 10 μg mouse brain. The data are representative of three separate experiments. (B) Semi-quantitative PCR gel images for (i) $\text{Na}_v1.5$, (ii) $\text{Na}_v1.6$, (iii) $\text{Na}_v1.7$ and (iv) $\text{Na}_v1.9$ products amplified from Jurkat cells. Representative PCR cycle numbers for given bands are indicated above the gels. Δ denotes the mis-transcribed/exon-skipped form of $\text{Na}_v1.6$.

mouse heart, that expresses mainly $\text{Na}_v1.5$, and brain (Fig. 2A). Semi-quantitative PCRs (SQT-PCR) indicated expression of $\text{Na}_v1.5$ (TTX-R) and $\text{Na}_v1.6$ (TTX-S), and to a much lesser extent, $\text{Na}_v1.7$ (TTX-S) and $\text{Na}_v1.9$ (TTX-R) (Fig. 2B). However, $\text{Na}_v1.8$ (TTX-R) expression could not be detected using PCR primers known to successfully amplify this VGSC type (not shown) [17]. $\text{Na}_v1.6$ mRNA was present only in neonatal and the mis-transcribed/exon-skipped truncated forms, denoted by Δ (Fig. 2B), neither of which would form functional $\text{Na}_v1.6$. It was concluded that $\text{Na}_v1.5$ was the main VGSC expressed, consistent with the TTX-R nature of the sodium currents recorded (Fig. 1D).

3.3. Invasion assays

To determine possible functional consequences of VGSC activity, Matrigel invasion assays were performed. Under control conditions, $\sim 8\%$ of Jurkat cells were invasive, close to the percentage of cells expressing electrophysiologically detectable VGSCs. Following treatment with 10 μM TTX, invasion decreased such that only $\sim 0.6\%$ of the cells invaded the Matrigel. Thus, TTX reduced Jurkat cell invasiveness by $93.0 \pm 5.5\%$ ($P < 0.001$; $n = 3$; Fig. 3). Equimolar TTX had no

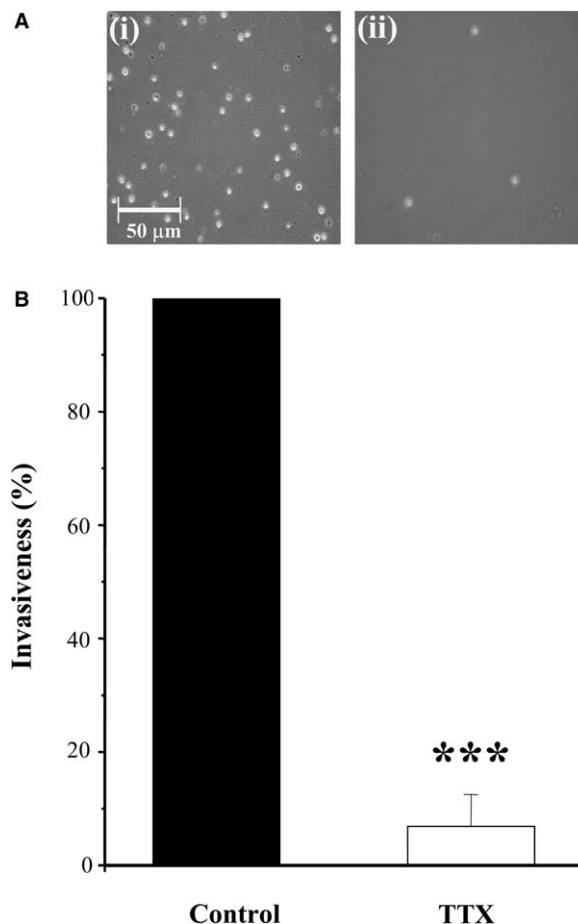


Fig. 3. (A) Pictures showing typical fields of view of Jurkat cells following invasion through a Matrigel-coated chamber under control conditions (i) and following treatment with 10 μM TTX (ii). (B) Matrigel invasiveness by Jurkat cells under control conditions (dark bar) and following treatment with 10 μM TTX (light bar). Data are presented normalised to control values of 100% and are the mean \pm S.E. of three independent experimental repeats. The asterisks (***) indicate that the difference was statistically significant ($P < 0.001$).

effect on cell viability ($99 \pm 0.1\%$ cf. control value of 100%; $P > 0.05$).

4. Discussion

In the present study, we have shown that Jurkat cells express TTX-R VGSCs that potentiate the cells' invasiveness. Of the three TTX-R VGSC α s (Na_v1.5, Na_v1.8 and Na_v1.9) SQT-PCR studies revealed that only Na_v1.5 was expressed at appreciable levels in the Jurkat cells. In contrast, Na_v1.8 was not detectable whilst Na_v1.9 was expressed at low levels. SQT-PCR studies also indicated that two TTX-S VGSC α (Na_v1.6 and Na_v1.7) mRNAs were expressed. However, rather than adult Na_v1.6, this subtype was present in two alternative splice forms, which would not form functional Na_v1.6 [20]. Thus, this channel would not contribute to any functional VGSC activity. Taken together, the SQT-PCR data with the TTX pharmacology ($IC_{50} \sim 900$ nM) and electrophysiology, would suggest that the inward current recorded was due mainly to Na_v1.5 and, to a much lesser extent, Na_v1.7 expression. Interestingly, since we detected functional VGSCs in only 10% of Jurkat cells, VGSC expression would appear to be under tight control. Indeed, VGSC expression is highly dynamic and regulated through numerous signal transduction pathways and transcription factors (reviewed in [21]).

The Matrigel assays demonstrated that TTX significantly blocked invasiveness of the Jurkat cells by about 93%. As TTX is a highly specific blocker of VGSCs, and invasion was reduced dramatically, it would follow that VGSC activity would normally potentiate Jurkat cell invasion. Although, the precise mechanism(s) through which the VGSCs could regulate cellular invasiveness is not yet known, several possibilities can be considered. Specifically, it has recently been reported that Na⁺ influx through VGSCs can regulate cell volume in Jurkat cells [22] and changes in ion flux and cell volume may be integral to the invasion process, e.g., [23]. Furthermore, VGSC activity is known to potentiate other cellular behaviours linked to invasion, including secretion [7], adhesion [24] and motility [6,8,25]. In turn, the subcellular/molecular mechanisms underlying these effects may involve the cytoskeleton (directly via β -subunit interaction and/or indirectly via local Ca²⁺ fluxes), protein kinase activity and gene expression [6,25].

In summary, our results demonstrated that Jurkat cells express a functional TTX-R VGSC, activity of which enhances the invasiveness of these cells. As the Jurkat cell is a widely accepted model for T-lymphocyte behaviour, it is tempting to speculate that VGSCs may play an important role in the invasive properties of normal human T-lymphocytes (which also express functional VGSCs [12,26]) necessary to their intra-/extra-vascular and inflammatory response. Other 'normal' cells (e.g., fibroblasts and endothelial cells) capable of invasive behaviour also have functional VGSCs [27,28]. Additionally, several different cancers (prostate, breast, small-cell lung carcinoma, neuronal tumours) express functional VGSCs [3,4,9,10,29,30] that have been shown to potentiate cell invasion in prostate and breast cancer cell lines [3–5,9,10]. Interestingly, parallels have been drawn between lymphocyte trafficking and cancer metastasis [31,32]. In overall conclusion, therefore, VGSC expression/activity may represent a general mechanism for potentiating cellular invasion under both normal and pathophysiological conditions.

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