Expression of skeletal muscle-type voltage-gated Na^+ channel in rat and human prostate cancer cell lines

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Abstract Previous electrophysiological work has demonstrated expression of a voltage-gated Na^+ channel (VGSC) specifically in two highly metastatic prostatic epithelial tumour cell lines: MAT-LyLu (rat) and PC-3 (human). However, the identity of the channel(s) present was uncertain. The present study used a combination of molecular biological techniques to demonstrate that full-length skeletal muscle type 1 (SkM1) VGSC mRNA is present in the mRNA pool of the MAT-LyLu cell line. mRNA for this particular channel type was also expressed in the PC-3 cells. In situ hybridisation data suggested that the level and pattern of rSkM1 mRNA expression were different in the Dunning cells of markedly different metastatic potential. Interestingly, the same type of mRNA was also detected in the weakly metastatic counterparts of the cells: AT-2 (rat) and LNCaP (human).

Key words: Metastasis; Na^+ channel; Primary sequence; Prostate cancer; rSkM1; hSkM1

1. Introduction

Voltage-gated Na^+ channels (VGSCs) are well known to play a crucial role in 'excitable' (nerve and muscle) cells where they mediate a variety of functions, especially regenerative conduction of impulses, and mechanisms of stimulation-secretion and excitation-contraction coupling [1]. Interestingly, increasing evidence suggests that VGSCs also occur in supposedly 'non-excitable' cell types such as glia [2], lymphocytes [3], osteoblasts [4], endothelia [5] and fibroblasts [6]. In the latter group of cells, however, the functional role of VGSCs is not so well understood.

Physiologically and pharmacologically different VGSCs are expressed differentially during development and among tissue types (reviewed in [7]). Nine different full-length α-subunit cDNAs have so far been identified from a variety of excitable tissue types in the rat alone [8–14]. Nevertheless, all cloned VGSCs possess the same basic structural features: four membrane-spanning pseudo-subunit domains containing multiple potential α-helical transmembrane segments. Most cloned VGSCs belong to the Na_v1 subfamily.

Recently, we have found that VGSC activity occurs in the highly metastatic prostatic carcinoma cell line (MAT-LyLu) of the rat; in contrast, no such currents were detected electrophysiologically in the related but weakly metastatic AT-2 cell line [15]. A positive correlation was also found between Na^+ channel expression and invasiveness in numerous rat and human cell lines and transfected cells [16]. Furthermore, blockage of VGSCs by tetrodotoxin (TTX) reduced the MAT-LyLu cells' invasiveness in vitro by up to 50% indicating strongly that VGSC activity could contribute to the metastatic process [15]. Broadly similar results were obtained from analogous human cell lines: highly metastatic PC-3 and lowly metastatic LNCaP cells [17]. Electrophysiological and pharmacological experiments on the MAT-LyLu cells suggested that the VGSC(s) expressed had properties consistent with those of Na_v1-type TTX-sensitive channels [18].

The main purpose of the present study was to use a molecular biological approach to investigate further VGSC expression in the two rat model cell lines, and extend this to the comparable human cells.

2. Materials and methods

2.1. Tissue culture

The MAT-LyLu and AT-2 cell lines were cultured using the protocol described in [15]. PC-3 and LNCaP cells were grown as in [17].

2.2. RNA extraction

For cloning and sequencing work, total RNA was extracted as described in [19]. For Northern blots, which were carried out for the rat tissues only, a different protocol was used. Briefly, cells were homogenised in a solution ('A'), using an IKA homogeniser, such that 1 ml of solution was used per 0.1 g of tissue. Solution A consisted of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.72% (v/v) β-mercaptoethanol. To this were added 2 M sodium acetate, pH 4.0 (10% volume of solution A), phenol (equal volume of solution A) and chloroform (20% volume of solution A). After mixing and centrifugation, two isopropanol precipitates were performed and the samples resuspended in sterile distilled water.

2.3. Cloning and sequencing

DNA was removed from all the extracts by digestion with DNase and 5 μg of the total RNA was used as the template for single-stranded cDNA (sscDNA) synthesis (Superscript II, Gibco BRL). For MAT-LyLu and AT-2 work, sscDNA synthesis was primed with an oligo dT primer/adaptor (dTAdap), as described in [20]. VGSC cDNA was then amplified from the cDNA pool by PCR (Taq DNA polymerase, Pharmacia Biotech) using four degenerate primers (NA1, NA2, NA3, NA4; designed specifically to amplify all currently known Na_v1-type VGSC cDNA sequences; Table 1) in conjunction with the anchored 3′ primer added during cDNA synthesis (the 20mer section, 'Adap'). PCR reactions were performed in a final volume of 20 μl under the following typical conditions: 94°C for 5 min; 1 U enzyme added; 94°C for 1–1.5 min; 54–56°C for 1 min (depending on the primer pair); 72°C for 1–1.5 min; final incubation at 72°C for 10 min with the main section repeated 25–30 times. Four independent NA1-NA3/NA2-NA3 clones from the MAT-LyLu and three independent NA2-NA3 clones from the AT-2 were sequenced

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Table 1
PCR primers used to clone and sequence rSkM1 and hSkM1 VGSC α-subunits from the prostate adenocarcinoma cell lines

Degenerate Primers

NA1 - 5' TT(T/C)ATGAC(A/C/G/T)GA(A/G)GA(A/G)CA(A/G)AA 3' (3910-3929)
NA2 - 5' AA(A/G)TA(T/C)TA(T/C)AA(T/C)GC(A/C/G/T)ATGAA 3' (3931-3950)
NA3 - 5' ACCATCAT(A/C/G/T)G(T/C)(A/C/G/T)ACCAT(A/G)TT 3' (4075-4094)
NA4 - 5' TG(T/C)GG(A/C/G/T)GA(A/G)TGGAT(T/C/A)GA(A/G)AC 3' (2257-2276)
NA10 - 5' TCT(C/G)CTGCTGAG(T/C)TCCTTCA 3' (2379-2398)
NA23 - 5' TGAGCCCAG(G/A)ATGC(G/A)AGGAT 3' (-18-2)

Specific Primers

dTAdap - 5' GCGAATTCGGATCCGATATCTTTTTTTTTTTTTTT 3'
Adap - 5' GCGAATTCGGATCCGATATC 3'
NA5 - 5' CGGCCTCAGAACAAGATCCA 3' (3988-4007)
NA6 - 5' TGGATCTTGTTCTGAGGCCG 3' (3988 - 4007)
NA7 - 5' CTGAGGACCTTCCGTGTGCT 3' (652-671)
NA8 - 5' GGCATGGTGTACGACTTCGT 3' (4009-4028)
NA9 - 5' GCCATCTAGACAAG AGACTC 3' (5509-5528)
NA11 - 5' TTATCTTCCGGTGGCGGCTC 3' (2617-2636)
NA12 - 5' GTACGCTGAGCAGAATGAGG 3' (1329-1348)
NA13 - 5' GGACTTGTTGTTGACCACCG 3' (3581-3600)
NA14 - 5' AACCTACAGATTGCCATCGG 3' (2443-2462)
NA15 - 5' CTGCGCAGTGTCCACCACAT 3' (3025-3044)
NA16 - 5' CCACAGAGGAGGAGACAGAC 3' (2786-2805)
NA17 - 5' GATCCTCATCTGCCTCAACA 3' (4059-4078)
NA18 - 5' AATGGCCAGCTCATCTCTGC 3' (-1-19)
NA19 - 5' TGCACACTTGTACCACCACG 3' (1619-1638)
NA20 - 5' TTGGCCAGCTTGAAGACACG 3' (2005-2024)
NA21 - 5' CTTCGGCATGTCTAACTTCG 3' (4473-4492)
NA22 - 5' TTGGTC AGGGCAAAGAGGAT 3' (5014-5033)
NA24 - 5' CCCATCTCAAAGCCCTTCCC 3' (5557-5576)
H1 - 5' ACTATGCCGAGACTCAGTGG 3' (hSkM1: 5609-5628)

Primer annealing positions are indicated in parentheses (numbering according to [10,23]).

and found to be identical to the rat skeletal muscle VGSC (rSkM1) sequence [10]. Additional PCR primers specific for rSkM1 cDNA were then designed (see Table 1) and sscDNA was synthesised (using NA6, NA9, NA11 and NA24 primers, specific for rSkM1) in order to obtain the full coding sequence of the channel. Combinations of the sense and anti-sense PCR primers produced a series of overlapping fragments for each of the cell lines.

All chosen fragments were gel purified, and either ligated directly into the pMOS (Amersham pMosBLue T-vector kit) or pGEM vector (Promega pGEM-T Easy vector system), or kinased and blunt-end ligated into phosphatased pUC18 at the *Sma*I site. These were then used to transform *E. coli* (pMOSBlue). The fragments were then subjected to automated DNA sequencing along both strands using either ΔTaq or Thermo Sequenase (Amersham) fluorescent cycle sequencing kits and the Vistra DNA 725 automated sequencer. Independent clones and overlapping DNA fragments were compared to identify PCR errors. The full MAT-LyLu/AT-2 rSkM1 sequence obtained has been submitted to GenBank.

2.4. Northern blots

Approximately 20 μg of mRNA extracted from both AT-2 and MAT-LyLu cell lines (Qiagen oligotex mRNA extraction kit) were run on a 1.0% agarose gel containing 10% 10×MOPS (pH 7.0) and 18% formaldehyde. Samples were loaded in a buffer containing 62.5% formamide, 25% formaldehyde, 12.5% 10×MOPS (pH 7.0). Transfer of RNA from the gel to a positively charged nylon membrane was then performed overnight, as described in [21]. Blots were cross-linked by automated exposure to a low dose of ultraviolet radiation (254 nm), and hybridised with a ^{32}P-labelled DNA probe (Promega 'Ready Go' kit) corresponding to the NA8-NA22 region of the AT-2/MAT-LyLu rSkM1 channel. Prehybridisation and hybridisation steps were performed at 65°C in a buffer solution (containing 0.25 M Na_2HPO_4, 0.25 M NaH_2PO_4, 1 mM EDTA (pH 8.0) and 7% SDS) for 30 min and overnight, respectively. The blots were washed twice for 15 min at 57°C with 2×SSC/0.1% SDS and twice with 0.2×SSC/0.1% SDS for 15 min at 61°C. Autoradiography was for 92 h at −70°C with an intensifying screen. The amount of mRNA required for gel loading was determined by trial and error.

2.5. In situ hybridisation

The RNA probes used would recognise nucleotide sequences 5970–6277, 7385–7820, 6807–7302, 6325–6822, 140–669 and 6461–6761 (GenBank numbering) of VGSC α-subunits SkM1, rat brain (RB) I, RB II, RB III, NaG and Na6, respectively. The construction of the digoxigenin-substituted riboprobes has already been described and these probes have previously been utilised to localise VGSC mRNAs in a variety of excitable and non-excitable cells [4,21,22]. Cells were processed and in situ hybridisation performed exactly as in [21], except that the hybridisation solution contained only 0.25 ng/μl of probe, and hybridisation was performed at 54°C.

Table 2
Nucleotide differences in full coding MAT-LyLu/AT-2 rSkM1 cDNA compared with the published rat sequence [10]

Variant name	Nucleotide position	Amino acid change	Structural region
R1	358 (G to A)	Val to Ile at 120	N terminus
R2	1194 (G to A)	No change (Thr 398)	D1: S5–S6
R3	2442 (C to T)	No change (Asn 814)	ID 2–3
R4	2748 (G to C)	Gln to His at 916	ID 2–3
R5	3132 (G to A)	No change (Glu 1044)	D3: S1
R6	3605 (C to G)	Ser to Cys at 1202	D3: S5–S6
R7	3769 (C to G)	His to Asp at 1257	D3: S5–S6
R8	3942 (T to C)	No change (Asn 1314)	ID 3–4
R9	4929 (A to G)	No change (Pro 1643)	C terminus
R10	5264 (A to G)	Asp to Gly at 1755	C terminus
R11	5407 (G to A)	Glu to Lys at 1803	C terminus

The first 19 and the last 15 nucleotides of the full coding sequences are derived from the NA18 and NA9 primers used in PCR reactions and consequently do not necessarily corrrespond to the original MAT-LyLu/AT-2 rSkM1 sequence.

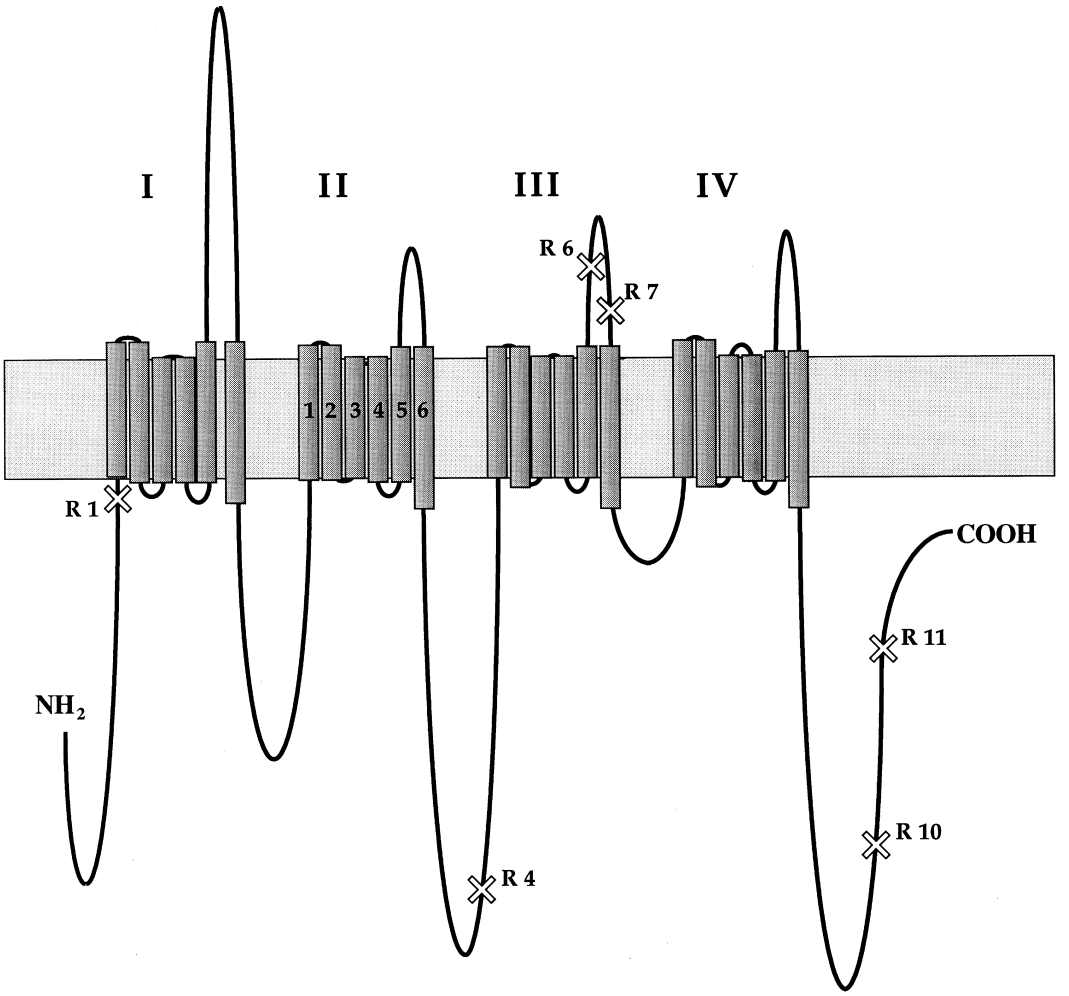

Fig. 1. Schematic representation of the rSkM1 α-subunit, drawn to approximate scale, with channel topology adopted from current VGSC models. Domains, each containing six transmembrane segments, are numbered I to IV. Amino acid changing nucleotide differences detected in the MAT-LyLu/AT-2 rSkM1 α-subunit are indicated by crosses and labelled according to Table 2.

3. Results

3.1. Full rSkM1 sequence

The full coding sequences of the VGSC mRNA transcripts detected in the MAT-LyLu and AT-2 cell lines are identical to each other. Furthermore, apart from 11 base positions, this is identical to the published sequence of the rSkM1 channel [10]. The differences found are summarised in Table 2 and illustrated in Fig. 1. Most of the sequence changes are located in the second half of the channel coding region (including all of the third and fourth transmembrane domains), and most substitute purine residues. Only one of these 11 nucleotides (R5) is located in a proposed transmembrane region. Six of the nucleotide differences (R1, R4, R6, R7, R10 and R11), all in non-transmembrane regions of the proposed channel structure, would cause amino acid sequence changes in the channel protein (Fig. 1 and Table 2). Four of these six substitutions (R4, R7, R10 and R11) result in charge differences. R7 and R11 substitute positive for negatively charged residues in the overall negatively charged channel pore and carboxyl tail (C-tail) regions, respectively. In contrast, R10 removes a negatively charged residue in the C-tail, and R4 introduces a positive amino acid in the cytoplasmic linker connecting the second and third transmembrane domains.

3.2. Detection of full-length rSkM1 mRNA

As shown in Fig. 2, ^{32}P-labelled probes, generated from a cDNA template corresponding to the NA8-NA22 region of the partial sequence, hybridised with an RNA species of approximately 9 kb in size in both AT-2 and MAT-LyLu mRNA extracts (lanes A and B), similar to the size of the signal from the total RNA extract of rat skeletal muscle (lane C). The size corresponding to all three of the bands agrees well with the 8.5 kb transcript size previously reported for rSkM1 [14]. This would indicate that full-length rSkM1 mRNA is present in both cell lines, possibly at a higher level in the MAT-LyLu cells (Fig. 2).

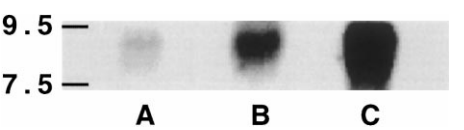

Fig. 2. Northern blots of AT-2 (lane A), MAT-LyLu (lane B) and rat skeletal (lane C) muscle extracts (20 μg of mRNA for A and B, and 5 μg of total RNA for C) exposed for 92 h after hybridisation with the ^{32}P-labelled rSkM1 DNA probe derived from NA8-NA22. Hybridisation with a ca. 9 kb transcript was seen in all three lanes. Positions of RNA molecular weight standards (in kb) are indicated on the left.

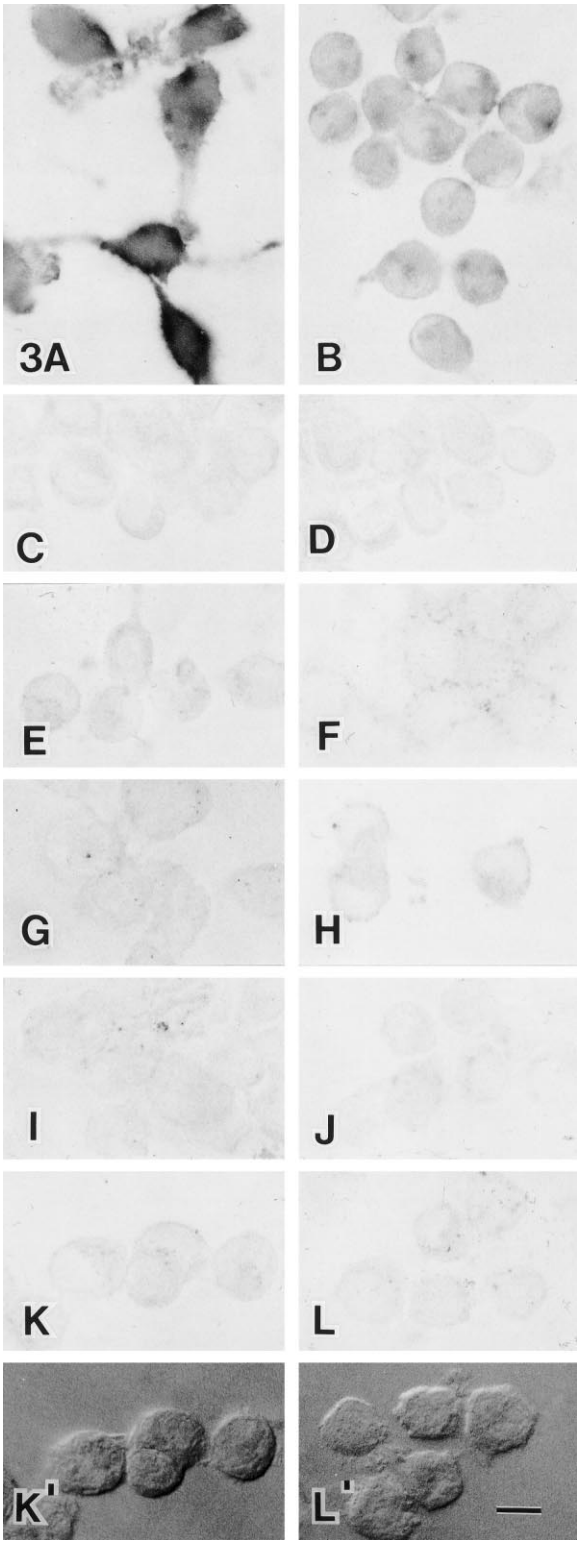

Fig. 3. Detection by in situ hybridisation of VGSC α-subunit mRNA expression in MAT-LyLu (left column) and AT-2 (right column) cells. MAT-LyLu cells (A, C, E, G, I, K) and AT-2 cells (B, D, F, H, J, L) were hybridised with riboprobes specific for VGSC mRNAs SkM1 (A, B), RB I (C, D), RB II (E, F), RB III (G, H), NaG/SCL-11 (I, J) and Na6 (K, L). SkM1 mRNA was expressed in both MAT-LyLu and AT-2 cells, but the hybridisation signal was substantially greater in MAT-LyLu cells. VGSC mRNAs RB I, RB II, RB III, NaG/SCL-11 and Na6 were not detected in either MAT-LyLu or AT-2 cells. K′ and L′ are Nomarski images of the bright fields shown in K and L, respectively. ×600. Bar, 10 μm.
←

3.3. Expression pattern of VGSC mRNA

The expression of rSkM1 mRNA was examined in MAT-LyLu and AT-2 cells by non-radioactive in situ hybridisation cytochemistry utilising isoform-specific riboprobes. Hybridisation signal for rSkM1 transcripts was detected in both cell lines, although greater signal was generally observed in the MAT-LyLu cells (Fig. 3A,B). However, the MAT-LyLu cells did not display a uniform level of rSkM1 hybridisation signal; rather, some cells were intensely labelled while other cells showed more moderate levels of expression. In the case of the AT-2 cells, the rSkM1 hybridisation signal was more uniform although most cells exhibited much lower levels of expression. On the other hand, hybridisation signal above background was inconsistent or not detected when the MAT-LyLu and AT-2 cells were treated with riboprobes specific for VGSC mRNAs RB I (Fig. 3C,D), RB II (Fig. 3E,F), RB III (Fig. 3G,H), NaG/SCL-11 (Fig. 3I,J) and Na6 (Fig. 3K,L).

3.4. Partial SkM1 sequence from human prostate cancer cell lines

A partial sequence corresponding to the whole of transmembrane domain four and the proposed intracellular C-tail of human skeletal muscle type 1 (hSkM1) VGSC was obtained in the human PC-3 and LNCaP cell line using PCR primers specific for SkM1 and a cloning strategy like that used for the 3′ end cloning of the rSkM1 channel, described above. The 3′ end of the channel was targeted specifically as this part of the SkM1 sequence crosses a known intron site [23] and is considerably different between rSkM1 and hSkM1 orthologs. The corresponding sequences in the two cell lines are not identical but display variations from the hSkM1 sequence as reported previously [24], being possible natural polymorphisms [25]. Thus, PC-3 hSkM1 possesses an adenine residue at bp 4616 (all numbering according to [36], and the LNCaP hSkM1 has an adenine residue at bp 4203, and an adenine or guanine residue (indicating, perhaps, that more than one allele of the hSkM1 channel was being expressed) at bp 4946.

4. Discussion

The main conclusions of the present study are three-fold. (1) Both MAT-LyLu and AT-2 cell lines express VGSC mRNA, although the channel appears functional only in the former. (2) In both cells, rSkM1 VGSC mRNA is present. (3) SkM1 mRNA is similarly expressed in analogous human prostate cancer cell lines. The present study is also the first to demonstrate SkM1 type VGSC mRNA expression in cells other than skeletal muscle.

4.1. Molecular identity of the rat VGSC(s)

The data obtained from cloning and sequencing, Northern blots and in situ hybridisation, taken together, are consistent with expression of full-length rSkM1-type VGSC mRNA in both the MAT-LyLu and the AT-2 cell lines.

The AT-2 and MAT-LyLu cell lines, both originally derived from the same prostatic tumour in a Copenhagen rat, possess VGSC mRNA with identical sequence. The published rSkM1 sequence was derived from an adult male Wistar rat. Differ-

ences between the determined MAT-LyLu/AT-2 rSkM1 channel and the published rSkM1 sequences may, therefore, result from the different sources of rat RNA. Alternatively, the sequence differences may (i) reflect natural polymorphisms, like those identified in hSkM1 in this study and by other workers (e.g. [25]), which are thought to have little effect on channel functionality; and/or (ii) be due to the pathophysiology of the disease and have functional significance.

Alignment of all previously cloned and sequenced VGSCs allows more reasoned speculation as to whether the amino acid substitutions observed would have functional consequences. Thus, four of the six amino acid substitutions, generated by nucleotide changes at R1, R4, R6 and R10 (Table 2 and Fig. 1), appear to be conservative in that the introduced residues are present at corresponding positions in the other SkM1 channels sequenced to date (either equine or human). Indeed, the change at R6 generates a cysteine residue at 1202 which is present at the corresponding position in all other presently cloned and sequenced vertebrate and invertebrate VGSCs, apart from *Drosophila para*. In contrast, changes at R7 and R11 are more likely to have some effect on rSkM1 properties. R7 introduces an acidic aspartate residue into the S5–S6 region of the third domain of the channel, 16 amino acids from the proposed SS2 region. This part of the channel is thought to act as the ion selectivity filter, and has also been indicated to be the site of binding of the guanidinium toxins, TTX and saxitoxin [26,27]. Interestingly, amongst other VGSCs, aspartate is only present in the corresponding position of Na6 and its mouse orthologue, Scn8a; channels which are proposed to play an essential role in the central nervous system [28]. R11 substitutes an acidic for a basic residue in the negatively charged rSkM1 C-tail. The C-tail of VGSCs has been indicated to play a role in channel activation and inactivation by virtue of its negativity [29]. Both activation and inactivation kinetics of the MAT-LyLu VGSC differ slightly from those measured for rSkM1 [30], which may be a reflection of this change in the primary channel sequence. However, the glutamate substituted here is not conserved in other sequenced SkM1 channels, which may indicate that this residue does not have a major effect upon channel kinetic properties. It should be noted, additionally, that activation and/or inactivation kinetics vary widely depending on the expression system used [31,32], and can be modulated by factors such as β-subunit coexpression, degree of post-translational glycosylation and different lipid domains [31,33].

Application of 1 μM μ-conotoxin (μ-CTX) has been found to have a limited (0–5%) blocking effect on the VGS currents in MAT-LyLu cells whilst 10 μM μ-CTX caused complete block ([18]; S.P. Fraser, unpublished observations). Only rat and human SkM1 and eel electroplax VGSCs are suppressed by μ-CTX, being blocked by nM concentrations [32]. It appears, therefore, that the functional VGSCs present in the MAT-LyLu cells display pharmacological properties that differ quantitatively from rSkM1. This may be because rSkM1 VGSC sensitivity to μ-CTX is reduced in the MAT-LyLu cells. Possibly the R6/R7 changes in D3 S5–6 alter the μ-CTX binding site in the channel pore directly, or these changes in the outer region of the pore indirectly reduce efficacy of μ-CTX blockage of channel current. Although the exact location of the μ-CTX binding site is unknown, mutations in the ion selectivity filter have been demonstrated to significantly affect μ-CTX binding [34,35]. Alternatively, novel folding or glycosylation of the rSkM1 channel in this particular cell type may account for the reduced toxin efficacy.

4.2. Expression of VGSC mRNA in both MAT-LyLu and AT-2 cells

MAT-LyLu cells displayed heterogeneity in channel mRNA expression, with some cells more intensely labelled (by rSkM1 riboprobes) than others (Fig. 3). We have reported previously that expression of functional VGSCs in MAT-LyLu cells shows strong heterogeneity, providing an electrophysiological parallel to these observations [15]. On the other hand, the AT-2 cells showed more uniform but much lower levels of mRNA expression.

No evidence for functional VGSC expression has been found by electrophysiological recording in lowly metastatic cell lines modelling prostate cancer (AT-2 from rat [15]; LNCaP from human [17]). It was surprising, therefore, that in the present study we have found expression of VGSC mRNA in the AT-2 cells, albeit at low levels (Fig. 3). This apparent contradiction could be due to the following. (a) Normal channel proteins may be present in the AT-2 cell line but at a sub-threshold density, below that required for electrophysiological detection. (b) The VGSC in the AT-2 cells may be non-functional. This could be due to several possible reasons, including (i) mutation(s) in non-coding regions of the AT-2 channel mRNA; and (ii) existence of post-translational mechanism(s) in the AT-2 cell line non-conducive to functional rSkM1 production. Several studies indeed have demonstrated that functional expression of VGSCs can be directly affected by post-translational modifications, such as glycosylation [36], phosphorylation [37], and β-subunit co-expression [38].

4.3. Relevance of the Dunning model system to human prostate cancer

The finding that SkM1 mRNA is expressed in both rat and human prostate cancer cell extends the evidence that the Dunning system of highly and lowly metastatic cell lines is a viable model of human prostate cancer. Importantly, species boundaries aside, such consistency is striking considering that the two human cell lines were obtained originally from unrelated sources: PC-3 cells were derived from a bone marrow metastasis of a prostatic adenocarcinoma from a 62-year-old Caucasian [39], whilst the LNCaP cell line was initiated from a supraclavicular lymph node from a 50-year-old Caucasian with hormone-refractory prostate cancer [40]. Further work is required to determine the possible functional significance of the conserved expression of skeletal muscle type VGSC in rat and human prostate cancer cells.

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