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Identification and functional characterization of voltage-gated sodium channels in lymphocytes

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ABSTRACT

A variety of ion channels has been discovered in lymphocytes. RT-PCR and real-time PCR analysis revealed that ALL (acute lymphocytic leukemia) cell lines and human peripheral blood mononuclear cells mainly expressed TTX (tetrodotoxin)-sensitive voltage-gated sodium channels (VGSCs). Expression of VGSC protein was confirmed by western blots and Immunofluorescence. Whole-cell patch-clamp recordings showed that a sub-population (20%) of MOLT-4 cells expressed functional VGSCs, which were TTX-sensitive. Importantly, 2 μM TTX decreased the invasion of Jurkat and MOLT-4 cells ~90%. These results indicate that the activity of VGSCs could represent a novel mechanism potentiating the invasive capacity of these cells.

1. Introduction

A variety of ion channels has been discovered in lymphocytes, including voltage-gated potassium channels ($K_V1.3$) [1–3], calcium-activated potassium channels (K_{Ca}) [4,5], calcium release-activated calcium channels (CRAC) [6,7], and swelling-activated chloride channels (Cl_{swell}) [8]. Expression levels of ion channels in human T cells vary dramatically during thymic development, activation, and differentiation to effector cells [9].

To mount an effective immune response, T and B lymphocytes must migrate into tissue, which depends on the regulated trafficking of lymphocytes [10]. This 'homing' process eventually leads to the recruitment of immune effector cells to sites of antigenic or microbial invasion. Circulating lymphocytes are round, 'non-motile' cells, which redistribute their cytoskeletal elements and organelles to acquire a constantly changing shape and polarized morphology during inflammatory response [11]. However, the mechanism(s) underlying this response is not yet clear. There is increasing evidence suggesting that cancer metastasis is controlled by voltage-gated sodium channel (VGSC) activity [12–18]. Accordingly, the specific VGSC blocker TTX suppresses a variety of metastatic cell behaviors in vitro [13–18]. Interestingly, parallels have been drawn between lymphocyte trafficking and cancer metastasis [19,20]. Therefore, investigating the expression and function of VGSC in lymphocytes is necessary for better understanding the roles of lymphocytes during immune responses.

Cells of the immune system traditionally have been categorized as 'electrically inexcitable'. However, after activation, they pose some electrophysiological properties of "electrically excitable cells" owning to changes in expression patterns of ion channels and intracellular Ca^{2+} mobilization. Human T lymphocytes express VGSCs under tight control [15], but their molecular identity and functional roles in these cells are not yet well known. The VGSC consists of a major pore-forming α-subunit (250–260 kDa) and a variable number of auxiliary β-subunits (30–40 kDa) [21]. The mammalian VGSCα gene family contains at least 9 functional members ($Na_v1.1$-$Na_v1.9$, coded by genes SCN1A-SCN11A) identified to date [21]. Each isoform exhibits a specific sensitivity to the selective blocker TTX and two groups of VGSC isoforms are described, the TTX-sensitive (TTX-S: $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$, $Na_v1.6$ and $Na_v1.7$) and the TTX-resistant (TTX-R: $Na_v1.5$, $Na_v1.8$ and $Na_v1.9$) sodium channels, which are blocked by TTX at nM and μM levels, respectively.

Although VGSCs are already known to occur in lymphocytes, their molecular identity and isoforms are not yet well known. In addition, there are little reports about the functional roles of VGSCs in lymphocytes. The main aims of the present study were 2-fold: (i) to investigate the molecular identity of the VGSCα in lymphocyte

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cells; and (ii) to explore the role of VGSCs in the lymphocyte invasion. In the present investigation, we carefully identified the expression isoforms of VGSC in lymphocytes using RT-PCR, qPCR, western blots, Immunofluorescence (IF) and patch-clamp recordings. Our results suggest a novel mechanism potentiating the invasive capacity of lymphocytes.

2. Materials and methods

2.1. Cell culture

The human lymphocyte cell leukemia line MOLT-4, Jurkat and Ball were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA) maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco by Life Technologies, Carlsbad, CA, USA), 1% pen/strep (MP Biomedicals, Solon, OH, USA) and 2 mM L-glutamine at 37 °C in 95% air/5% CO_2 with 95% humidity. Culture media was replaced every 2–3 days.

2.2. Isolation of human peripheral blood mononuclear cells

Whole blood (5–10 ml) was collected from healthy human male and female donors (n = 9 each), according to The Code of Ethics of the World Medical Association. Mononuclear cells were isolated with human lymphocyte separation medium (Tbdscience, Tianjin, China) according to manufacturer's instructions. Briefly, human peripheral blood mononuclear cells (PBMCs) were separated by centrifugation at 900 × g for 30 min at 18–20 °C over a Ficoll–Paque PLUS gradient. The resulting PBMC layer was washed twice with nuclease-free 0.9% NaCl solution and prepared for RNA isolation.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from exponentially growing cells and human PBMCs using RNAsimple Total RNA Kit (TIANGEN Biotech, Beijing, China). Messenger RNA was reverse-transcribed (RT) to cDNA using oligo$(dT)_{15}$ primers and GoScript reverse transcriptase (Promega, Madison, WI, USA). The cDNA product was used as a template for subsequent PCR amplifications for VGSCα subunit, using sequence-specific primers. Primer sequences and product sizes are summarized in Table 1. The temperature profile was 2 min at 94 °C followed by amplification for 38 cycles which consisted of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C and a final extension for 7 min at 72 °C. PCR analysis was repeated at least three times with the same samples to confirm reproducibility of the results.

2.4. Quantitative PCR

Total RNA 1 μg was used to generate cDNA with GoScript reverse transcriptase as above. A 1-μl aliquot of each synthesized cDNA was analyzed by Quantitative Real-Time PCR (CFX96 Real-Time System, Bio-Rad, Singapore) using SYBR Green PCR Master Mix (Takara, Dalian, China) according to manufacturer's protocols and message level was determined using the $\triangle C_t$ method. Samples were assayed in triplicate for each gene, and the mean expression was used during subsequent analysis. QRT-PCR was carried out under the following reaction conditions: stage 1, 95 °C for 30 s (Rep 1); stage 2, 95 °C for 5 s then 60 °C for 1 min (Reps 40).

2.5. Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then resuspended in an RIPA lysis buffer (BOSTER, Wuhan, China) supplemented with 1% PMSF (Beyotime, Nantong, China). The samples were kept on ice for 1 h and then sonicated for 15 s. They were then centrifuged at 13,000 × g for 10 min at 4 °C. Total protein concentrations were determined in triplicate by BCA protein assay kit (Beyotime, Nantong, China). Supernatants were denatured by heat shock at 95 °C for 5 min in SDS buffer before being loaded at a total protein concentration of 100 μg per lane and run on a 4–15% gradient gel (Willget, Shanghai, China). Protein samples were then transferred to PVDF membranes (Millipore, Bedford, MA, USA). Non-specific binding was blocked by incubation with TBST (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4) plus 5% non-fat milk for 1 h at room temperature. Then the membrane was incubated overnight at 4 °C with gentle shaking with a pan-specific Na_v rabbit polyclonal antibody (1:200 dilution, Alomone Labs, Israel). Immunoblots were developed with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) incubated for 1 h at room temperature. Immunoblots were visualized with the ECL immunodetection system (Advansta, Menlo Park, CA, USA).

2.6. Immunofluorescence

Growth medium was removed and rinsed with PBS. Cells were fixed in 4% paraformaldehyde for 20 min, rinsed, then were plated on poly-L-lysine coated glass coverslips for 2 h. Then the cells were permeabilized with 0.5% Triton-X100-PBS for 10 min. After blocking with 5% BSA, the cells were incubated with a pan-specific Na_v rabbit polyclonal antibody (1:50 dilution, Alomone Labs) overnight at 4 °C. The cells were then rinsed and incubated with goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) conjugated to Oregon green (FITC) at a 1:100 dilution for 1 h in dark. The cells were also counterstained with the nuclear stain, DAPI. Then cells were rinsed and subjected to microscopy. The glass coverslips were examined under a Nikon Eclipse Ti–U microscope (Nikon, Tokyo, Japan) equipped with a 20× objective.

2.7. Whole-cell patch-clamp recording

Cells were grown on poly-L-lysine-coated glass coverslips. The electrophysiological techniques used have been described in detail previously [22]. For Na^+ current recordings, low-pass filtered at

Table 1
Oligonucleotides used to amplify transcripts of VGSCα subunits.

Gene name	Gene product	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Predicted size (bp)
SCN1A	$Na_v1.1$	TTCATGGCTTCCAATCCTTC	TAGCCCCACCTTTGATTTTG	178
SCN2A	$Na_v1.2$	GCCAGCTTATCAATCCCAAA	TCTTCTGCAATGCGTTGTTC	192
SCN3A	$Na_v1.3$	CAAAGGGAAGATCTGGTGGA	AAAGGCCAATGCACCACTAC	115
SCN4A	$Na_v1.4$	TCAACAACCCCTACCTGACC	ACGGACGAGTTCCCATCATA	148
SCN5A	$Na_v1.5$	CACGCGTTCACTTTCCTTC	CATCAGCCAGCTTCTTCACA	208
SCN8A	$Na_v1.6$	CGCCTTATGACCCAGGACTA	GTGCCTCTTCCTGTTGCTTC	247
SCN9A	$Na_v1.7$	GGCTCCTTGTTTTCTGCAAG	TGGCTTGGCTGATGTTACTG	196
SCN10A	$Na_v1.8$	ACCTGGTGGTGCTTAACCTG	TGCTGAAGAAGCTGCAAAGA	168
SCN11A	$Na_v1.9$	CTGTGGTCCTGGTCATTGTG	TGCATTCGCTTCTTGCATAC	233