Expression of the Voltage-Gated Sodium Channel NaV1.5 in the Macrophage Late Endosome Regulates Endosomal Acidification

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Expression of the Voltage-Gated Sodium Channel NaV1.5 in the Macrophage Late Endosome Regulates Endosomal Acidification


Voltage-gated sodium channels expressed on the plasma membrane activate rapidly in response to changes in membrane potential in cells with excitable membranes such as muscle and neurons. Macrophages also require rapid signaling mechanisms as the first line of defense against invasion by microorganisms. In this study, our goal was to examine the role of intracellular voltage-gated sodium channels in macrophage function. We demonstrate that the cardiac voltage-gated sodium channel, NaV1.5, is expressed on the late endosome, but not the plasma membrane, in a human monocytic cell line, THP-1, and primary human monocyte-derived macrophages. Although the neuronal channel, NaV1.6, is also expressed intracellularly, it has a distinct subcellular localization. In primed cells, NaV1.5 regulates phagocytosis and endosomal pH during LPS-mediated endosomal acidification. Activation of the endosomal channel causes sodium efflux and decreased intraendosomal pH. These results demonstrate a functionally relevant intracellular voltage-gated sodium channel and reveal a novel mechanism to regulate macrophage endosomal acidification. *The Journal of Immunology, 2007, 178: 7822–7832.

Phagocytosis by macrophages and other bone marrow-derived cells represents one of the most primitive mechanisms of innate immune host defense (1–4). This mechanism requires rapid engulfment and subsequent processing of infectious and noninfectious particles through the endosomal pathway. Endosomal maturation from the early phagosome to the phagolysosome occurs through complex and highly regulated pathways that are dependent on host and exogenous factors. Processing of susceptible bacteria into progressively acidic endosomal compartments contributes to their efficient killing by the host (5). Priming of macrophages by IFN-γ and LPS increases their antimicrobial activity (6) and may enhance phagolysosome maturation (7–9).

We reasoned that a rapid, high-capacity biologic pathway such as phagocytosis could be regulated through voltage-gated sodium channels (10). These channels could act rapidly to transduce signals from the plasma membrane to intracellular compartments. Consistent with this hypothesis, prior studies demonstrated that the macrophage plasma membrane depolarizes during endocytosis and cellular activation (11, 12). Additional evidence suggests that endosomal acidification also has an electrogenic component coupled to cation flux that may be independent of the vesicular proton ATPase. This earlier study demonstrated that the endosome is permeant to not only protons but to a variety of anions and cations (13). This ionic flux in isolated endosomes correlates temporally with potential changes in the endosomal membrane. More recent work (14) on the electrogenic chloride-proton transporters, chloride-proton exchangers (CICs)3 C4 and CIC-5, has clarified some of these mechanisms. However, it remains unclear how membrane depolarization in endosomes is initiated and how cation flux in this organelle is regulated. It is also not clear what role voltage-gated proton channels (Hv) channels may play in endosomal acidification in immune cells (15, 16).

Although there has been no prior investigation of the role of voltage-gated sodium channels in bone marrow-derived phagocytes, a recent study (17) of microglia and macrophages within the mouse CNS demonstrated the presence of NaV1.6 sodium channels. These data suggested that prolonged inhibition of sodium channels could inhibit phagocytosis of noninfectious particles and improve clinical outcome in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, consistent with other studies of sodium channel blockers that show neuroprotection in experimental autoimmune encephalomyelitis (18, 19).

In this study, our goal was to characterize the functional role of voltage-gated sodium channels in human monocyte-macrophages. To our surprise, not only is the neuronal channel NaV1.6 expressed in differentiated THP-1 cells, a monocyctic human cell line, and primary human monocyte-derived macrophages, but the cardiac channel, NaV1.5, is as well. In contrast to other cell types such as neurons and muscle, these channels are expressed only on

3 Abbreviations used in this paper: CIC, electrogenic chloride transporter; DiSBAC2-3, bis-(1,3-diethylthiobarbituric acid) trimethine oxonol; EEA-1, early endosomal Ag-1; LAMP-1, lysosomal-associated membrane protein-1; MOI, multiplicity of infection; NaV1.5 and NaV1.6, voltage-gated sodium channels; SBFI, sodium-binding benzofuran isothiouronium; SCN, sodium channel gene designation (SCN5A, NaV1.5) (SCN8A, NaV1.6); TPA, 12-O-tetradecanoylphorbol-13-acetate; TTX, tetrodotoxin.

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intracellular structures and not the plasma membrane. NaV1.5 has a distinct subcellular localization to the late endosome and regulates macrophase phagocytosis and endosomal acidification through channel activation coupled to sodium efflux and subsequent proton influx.

Materials and Methods

Cells

THP-1 cells, a human premyelomonocytic leukemic cell line, were maintained in RPMI 1640 medium supplemented with 10% FBS, sodium pyruvate, and nonessential amino acids. Differentiation to macrophages was induced by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) (10 ng/ml, 72 h). Human CD14+ peripheral blood monocytes were commercially obtained (Cambrex) and were grown in the same culture medium as the THP-1 cells supplemented with 10 ng/ml M-CSF. Differentiated THP-1 cells and day 5 monocyte-derived macrophages were primed sequentially with IFN-γ (10 ng/ml, 18 h) in 1% FBS/RPMI 1640 and LP5 (Salmonella minnesota, 100 ng/ml, 1 h; Sigma-Aldrich) in serum-free medium. Fully primed cells were defined as TPA-differentiated THP-1 cells or day 5 primary macrophages that were treated with both IFN-γ and LP5.

RT-PCR, restriction analysis, and quantitative PCR

Total RNA was isolated from cells by guanidine isothiocyanate lysis and solubilization, followed by column purification and DNase treatment (RNeasy; Qiagen). Reverse transcription was performed with Superscript III (Invitrogen Life Technologies). Parallel samples were run in the absence of enzyme as controls for subsequent PCR.

A multiplex RT-PCR/restriction enzyme polymorphism assay was used to amplify products from sodium channels templates that might have been present in the cDNA pool as described previously (20). Briefly, generic primers (F1-R1) designed against highly conserved sequences in transmembrane segment 5 of domain III and the cytoplasmic loop (L3) joining domains 3 and 4. The amplified fragments encode the polypeptide sequence delineated by the sequences LVCLI (L is residue 1339 of human Nav1.1/Nav1.2/Nav1.7; F2 CTSGTCTGYCTCAT CT). The amplification products from Nav1.6 and Nav1.7. Restriction products were run on 1.8% agarose gels. All (K = G or T; S = C or G; Y = T or C; M = A or C; W = A or T).

The predicted lengths of amplified products range in length from 462 to 483 bp. The combination of length and restriction enzyme polymorphism permits the identification of the channel templates that are amplified by this assay.

PCR amplification was performed in a 60-μl volume using 1–2 μl of first-strand cDNA, 0.8 μM of each primer, and 1.75 U of Expand Long Template DNA polymerase enzyme mixture (Roche). Amplification was conducted in two stages using a programmable thermal cycler. First, a denaturation step at 94°C for 2 min, an annealing step at 55°C for 2 min, and an elongation step at 68°C for 2 min. Second, a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s, and an elongation step at 68°C for 30 s. The next two cycles were repeated 33 times, with a final elongation step of 10 min.

Restriction enzyme analysis of amplicon used 1/10th volume of the PCR products from sodium channels templates that might have been present in the cDNA pool as described previously. Briefly, generic primers (F1-R1) designed against highly conserved sequences in transmembrane segment 5 of domain III and the cytoplasmic loop (L3) joining domains 3 and 4. The amplified fragments encode the polypeptide sequence delineated by the sequences LVCLI (L is residue 1339 of human Nav1.1/Nav1.2/Nav1.7; F2 CTSGTCTGYCTCATCT). The amplification products from Nav1.6 and Nav1.7. Restriction products were run on 1.8% agarose gels. All (K = G or T; S = C or G; Y = T or C; M = A or C; W = A or T).

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Restriction enzyme analysis of amplicon used 1/10th volume of the PCR product in a final volume of 10 μl. Restriction enzymes that are used in the assay (Table I) are specific to individual templates or produce a unique

Phagocytosis assays

For immunohistochemistry, cells primed with LP5 were challenged with freeze-dried Escherichia coli labeled with Texas Red (Molecular Probes/Invitrogen Life Technologies) for 30–60 min at 37°C (multiplicity of infection (MOI) = 10:1). Cells were washed with PBS and processed as described above. For quantitative assays, IFN-γ-primed cells were plated on 96-well plates in serum-free medium (with or without LP5 100 ng/ml for 60 min) and challenged with freeze-dried E. coli labeled with FITC (Molecular Probes/Invitrogen Life Technologies) for 30–60 min at 37°C in HBSS (MOI = 100:1). During bacterial challenge, cells were treated with 0, 0.3, or 0 μM tetrodotoxin (TTX). Fluorescence was quenched with trypan blue, and fluorescent intensity per well was measured on a Wallac Victor3 (PerkinElmer).

Cell concentration per well was determined subsequently by a Hoechst assay, and FITC intensity values were normalized statistical differences between groups were determined by a two-tailed Student’s t test with a p < 0.05 considered significant.

Small hairpin RNA

The following lentiviral clones specific for human SCN5A were obtained from Sigma-Aldrich: SHVRSRC-TRC00000438-68, 69, 70, 71, and 72. These clones are identified as 68, 69, 70, 71, and 72 in the text. Differentiated THP-1 cells in complete medium were spin infected with lentiviruses particles at a MOI of 2:1 (~80,000 viral particles per 40,000 THP-1 cells in a single well of a 96-well plate). Two days later, cells were split 1:3, differentiated with TPA, and primed as described above. Differentiated and primed cells were characterized by real-time PCR for the percentage of SCN5As knockdown and in phagocytosis assays as described above.

Flow cytometry

Endosomes were purified by a modification of the method of Bananis et al. (22). Differentiated and primed THP-1 cells were homogenized, and a post-nuclear supernatant was prepared. Pooled supernatants were centrifuged (200,000 X g for 135 min) on a sucrose step gradient (62, 41, and 8.5%). Endosomes were collected from the 8.5/41% interface and stained with primary Abs to Rab-7 and Nav1.5, followed by secondary Abs donkey anti-rabbit Alexa 488 and donkey anti-goat Alexa 633 (Molecular Probes/Invitrogen Life Technologies).

For functional assays, purified endosomes were either kept in endosome purification buffer (35 mM KCl, 10 mM NaCl, 5 mM MgCl2, and 1 mM EGTA (pH 7.4)) or dialyzed overnight against an intracellular buffer (140 mM CsF, 10 mM NaCl, 1 mM EGTA, 10 mM HEPES, and 1% PBS (pH 7.0)). Endosomes were then loaded with lysosensor Green DND-189 (1 μM) or Sodium Green (4 μM) for 30 min. Stimulation was initiated by addition of the voltage-gated sodium channel agonist, veratridine, to a final concentration of 100 μM. Samples were run on a MoFlo Cell Sorter (DakoCytomation) and analyzed by Summit software.

To calculate intraendosomal pH and [Na], purified endosomes, maintained in endosome purification buffer, were treated with either 0.5 mM nigericin (pH) or 10 μM gramicidin [Na] and equilibrated in standard solutions before flow cytometry assays (see Fluorometry section below for standardization procedures).

Immunohistochemistry and Western blot analysis

Cells grown on multichambered glass coverslips were washed with PBS, fixed with 4% paraformaldehyde in PBS (10 min), and blocked in PBS containing 5% serum (goat or donkey, dependent on secondary Ab), 0.1% Triton X-100, 1% BSA and 40 μg/ml normal human IgG. Primary and secondary Abs were diluted in blocking solution. For staining of samples, including bacteria, primary and secondary Abs were cleared by preincubation with bacteria in blocking solution. The following primary Abs were used: rabbit anti-NaV1.6 and rabbit anti-human NaV1.5 (Alomone Laboratories); mouse anti-lysoosomal-associated membrane protein-1 (LAMP-1) and mouse anti-early endosomal Ag-1 (AEA-1) (BD Biosciences); mouse anti-vimentin, mouse anti-kinesin, and mouse anti-dynein H chain (Chemicon International); and goat anti-Rab-7 and goat anti-dynein L chain (Santa Cruz Biotechnology). Secondary goat and donkey Abs conjugated to Alexa fluorophores and phalloidin-Alexa 546 were obtained from Invitrogen (Molecular Probes). 4′,6-Diamidino-2-phenylindole was from Vector Laboratories. Images were obtained on a Zeiss Axiotver 200 fluorescent microscope. Immuno-EM was performed as described previously (21). Western blot analysis for NaV1.5 was performed according to the Ab manufacturer’s protocol (Alomone Laboratories).

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Intracellular voltage was assessed by a modification of the method of Dall’Asta et al. (23). Cells were primed with IFN-γ as described and then loaded for 60 min (25°C) with bis-(1,3-diethylthiobarbituric acid) trieth- thione oxonol (DiSBAC2-3) (1 μM) in HBSS supplemented with 2% FBS. Cells were washed with HBSS and then activated with LPS (S. minnesota, 100 ng/ml) for an additional 60 min at 25°C. During LPS activation, cells were treated with 0, 0.3, or 10 μM TTX. Images were rapidly acquired from multiple microscopic fields for each condition and analyzed using Axiovision Automeasure software (Zeiss). Intracellular organellar pH was determined with Lysosensor Blue DND-167 (pKa 5.1) (Molecular Probes/Invitrogen Life Technologies). Cells were loaded with 1 μM dye for 30 min, washed with HBSS, and activated with LPS for an additional 60 min. For each experiment, the average densitometric intensity and particle number per cell were calculated for each condition. Statistical differences between groups were determined by a Student’s t test with a p < 0.05 considered significant.

**Fluorometry**

For time resolved fluorescence analysis of isolated endosomes, purified endosomes were plated on poly-l-lysine-coated coverslips in endosomal purification buffer for 60 min. The coverslips were washed with the same buffer, and the endosomes were then labeled with Lysosensor Green DND-189 for 30 min. Coverslips were washed with buffer and mounted in a cuvette. Fluorescence during ATP (2.5 mM) and veratridine stimulation (100 μM) were determined in a LS-50b spectrophotometer (PerkinElmer). pH standard curves were determined subsequently following treatment of plated endosomes with nigericin (0.5 μM). Phosphate citrate buffers of varying pH (4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) were used for titration (24). Similar results were obtained with MES-based buffers.

For sodium flux measurements, plated endosomes were labeled with the ratiometric sodium indicator sodium-binding benzoﬁran isophthalate (SBFI)-acetoxymethyl ester (4 mM) for 40 min. The ratio of fluorescence intensities excited at 340/380 nm were monitored at an emission wavelength of 505 nm. Following collection of experimental data, endosomes were treated with 10 μM gramicidin, and the cuvette was purged with solutions of increasing sodium concentration to generate a standard curve (10–140 mM NaCl in 10 mM HEPES buffer, and 1 mM EGTA (pH 7.0), with varying amounts of KCl so that [Na⁺] = 150 mM). At least five points were used for the generation of each standard curve (10, 20, 40, 80, and 140 mM NaCl).

For ratiometric analysis of intraendosomal pH in whole cells, differentiated and IFN-γ-primed THP-1 cells plated on coverslips were loaded with 1 mg/ml FITC-dextran (70 kDa) for 30 min in HBSS, washed extensively in HBSS, and stimulated in the presence or absence of LPS (100 ng/ml) and TTX (0, 0.3, and 10 μM) for an additional 60 min. Emission at 520 nm was determined at excitation of 450 and 490 nm in a LS-50b spectrophotometer. Cells were permeabilized with HBSS and 0.1% Triton X-100 and then analyzed in phosphate citrate buffers to obtain a standard pH curve (24).

For ratiometric analysis of intraendosomal [Na⁺], differentiated and IFN-γ/LPS-primed THP-1 cells were loaded with 1 mg/ml unlabeled dextran (80 kDa) and cell impermeant SBFI (5 μM) for 30 min, washed with HBSS buffer, and incubated in HBSS buffer for an additional 30 min. Fluorometric readings for SBFI were taken as described above, and standardization was performed as described for SBFI above, except that HBSS and 0.1% Triton X-100 was used as the permeabilizing agent rather than gramicidin.

**Results**

*NaV1.5 and NaV1.6, but not other voltage-gated sodium channel subtypes, are expressed in differentiated THP-1 cells and primary human monocyte-derived macrophages*

We first examined mRNA expression of voltage-gated sodium channels in differentiated and primed THP-1 cells, a human monocytic cell line, and primary human macrophages derived from peripheral blood monocytes. Following activation with IFN-γ alone or with LPS, NaV1.5 (SCN5A) and NaV1.6 (SCN8A) mRNA were expressed at low but detectable levels in differentiated THP-1 cells and primary monocyte-derived macrophages as determined by quantitative RT-PCR using channel subtype-specific TaqMan primers (Table II). Transcripts for other voltage-gated sodium channel genes were not present. We confirmed these results using multiplex RT-PCR, followed by restriction digest of sodium channel-specific fragments generated with gene-specific primers (20). Using these

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**Table I.** Multiplex RT-PCR and restriction enzyme analysis of PCR products from domain III of human Nav1 family of sodium channels

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**Table II.** Real-time PCR analysis of voltage-gated sodium channel expression in differentiated THP-1 cells and primary human monocyte-derived macrophages

<table>
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<tr>
<th>Cell Type (treatment)</th>
<th>SCN5a (NaV1.5) (Ct ± SE)</th>
<th>SCN8a (NaV1.6) (Ct ± SE)</th>
<th>IL-12 p40 (Ct ± SE)</th>
<th>GAPDH (Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1 (IFN-γ)</td>
<td>34.53 ± 0.24 (n = 4)</td>
<td>34.68 ± 0.47 (n = 7)</td>
<td>35.17 ± 0.53 (n = 6)</td>
<td>17.0</td>
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<td>THP-1 (IFN-γ plus LPS)</td>
<td>34.49 ± 0.36 (n = 4)</td>
<td>34.29 ± 0.51 (n = 5)</td>
<td>28.82 ± 1.17 (n = 4)</td>
<td>17.0</td>
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<tr>
<td>Primary (IFN-γ)</td>
<td>32.76 ± 0.64 (n = 3)</td>
<td>34.25 ± 1.60 (n = 4)</td>
<td>35.54 ± 1.41 (n = 4)</td>
<td>17.0</td>
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<tr>
<td>Primary (IFN-γ plus LPS)</td>
<td>34.58 ± 0.38 (n = 3)</td>
<td>33.71 ± 1.44 (n = 4)</td>
<td>26.06 ± 0.91 (n = 4)</td>
<td>17.0</td>
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aTTPA-differentiated THP-1 cells and primary human monocyte-derived macrophages were primed for 18 h with IFN-γ (10 ng/ml) with or without subsequent priming with LPS (100 ng/ml) for an additional 1 h. mRNA was isolated, and cDNA was synthesized as described in Materials and Methods. Samples were amplified in a SmartCycler using FAM-labeled TaqMan primers for 40 cycles. Samples were normalized to a cycle threshold (Ct) score of 17.0 for GAPDH controls run simultaneously for each condition. Transcripts for SCN1a (Nav1.1), SCN2A (Nav1.2), SCN3A (Nav1.3), SCN4A (Nav1.4), SCN9A (Nav1.7), SCN10A (Nav1.8), and SCN11A (Nav1.9) were not present.
primers (Table I), only NaV1.5 and NaV1.6 sodium channel templates were observed in the cDNA pool, and other voltage-gated sodium channels were absent (data not shown). The multiplex RT-PCR was repeated using primers F1 and F2 only and the restriction enzymes \textit{Kpn}I and \textit{Hin}dIII, which cuts the products from NaV1.5, NaV1.6, and NaV1.7 amplicons (Table I). Products in the expected size range of 462–483 bp, which were amplified from both samples (Fig. 1A, lanes 1 and 4, not seen). Amplicons from THP-1 (lane 2) and primary macrophage (lane 5) samples were cut with \textit{Kpn}I-producing fragments that are in agreement with the expected size of 213 and 267 bp, suggesting the amplification of the cardiac channel NaV1.5 (Fig. 1A). \textit{Hin}dIII also cut both amplicons (lanes 3 and 6) producing fragments that are in agreement with the expected sizes 139 and 345 bp, consistent with the amplification of the neuronal sodium channel NaV1.6.

Immunofluorescence staining, using isotype-specific Abs for human NaV1.5 and 1.6, demonstrated strong staining for both sub-types that showed distinct staining patterns. Staining for NaV1.5 revealed a primarily vesicular pattern (Fig. 1B) that required cell activation with IFN-\(\gamma\) and LPS, whereas NaV1.6 staining showed a more filamentous pattern that was independent of the activation state (Fig. 1C). Neither channel appeared to localize to the plasma membrane.
NaV1.5 localizes to the late endosome and phagosomes but NaV1.6 associates with cytoskeletal filaments and the endoplasmic reticulum

To determine the intracellular localization of the channels, we performed colocalization studies using immunofluorescence. NaV1.5 colocalized with markers of the late endosome, Rab-7, and dynein L and H chains (Fig. 2, A–C). However, it did not colocalize with markers of the early endosome (EEA-1 and kinesin) (Fig. 2, D and E). Staining for the lysosomal and late endosome marker, LAMP-1, demonstrated an intermediate level of colocalization (Fig. 2F). In contrast, NaV1.6 did not localize to endosomes (data not shown) but was associated with cytoskeletal structures such as actin stress fibers and the intermediate filament vimentin (Fig. 3).

Triple staining for EEA-1, Rab-7, and NaV1.5 was performed and confirmed results from double staining (Fig. 4). There was \(~77.6\%\) pixel overlap between the late endosomal marker, Rab-7, and NaV1.5, whereas the overlap between EEA-1 and NaV1.5 was 13.2\% and between EEA-1 and Rab-7, 9.5\% (Metamorph software). These results were confirmed by immunogold-cryoelectron microscopy (Fig. 5). As with fluorescent immunofluorescence, gold particle labeling of NaV1.5 was observed in multivesiculated endosomes (Fig. 5C), consistent with localization to the late endosome. Staining was also seen in the Golgi and Golgi-associated vesicles (Fig. 5A). There was little or no staining of early and recycling endocytic vesicles (Fig. 5B). Intense staining for NaV1.6 was associated with filamentous structures (Fig. 5E). Less intense staining was seen in the rough and smooth endoplasmic reticulum (Fig. 5D). Neither channel was expressed on the plasma membrane.

Following a challenge with heat-killed fluorescent E. coli (Fig. 6), NaV1.5-positive staining was identified in phagosomes and in vesicles adjacent to phagocytosed particles. Thirty minutes following challenge with E. coli, cells positive for bacteria showed polarization with NaV1.5-positive vesicles concentrated near phagocytosed particles (Fig. 6A). Sixty minutes following challenge, phagosomes were more centrally located near the nucleus, and NaV1.5 staining was seen in phagosomes and in adjacent vesicles (Fig. 6B).

TTX blockade of NaV1.5, but not NaV1.6, inhibits phagocytosis in LPS-stimulated differentiated THP-1 cells and human monocyte-derived primary macrophages

Because NaV1.5 and NaV1.6 can be blocked by high-dose (10 \(\mu\)M) and low-dose (0.3 \(\mu\)M) TTX, respectively (10, 25, 26), the specific functional role of these channels in macrophage phagocytosis can be tested. At these concentrations, TTX is highly specific for voltage-gated sodium channels and does not block the function of other voltage-gated channels. At 10 \(\mu\)M TTX, both channels are blocked, whereas at 0.3 \(\mu\)M TTX, NaV1.6 (EC\(_{50} = 1–6\) nM) is completely blocked with only a modest effect on NaV1.5 (TTX EC\(_{50} = 1–5\) \(\mu\)M) (10, 20).
Phagocytes were primed sequentially with IFN-γ (10 ng/ml; 18 h) and LPS (100 ng/ml; 1 h) and then challenged with fluorescent E. coli for 30 or 60 min (Fig. 7, A–C). TTX (10 μM) inhibited phagocytosis by ~50–70% with either no effect or a modest effect at 0.3 μM TTX. In THP-1 cells not primed with either IFN-γ or LPS, in which NaV1.6 but not NaV1.5 is expressed, there was no effect of either high- or low-dose TTX on phagocytosis (Fig. 7D). Consistent with our results from microscopy, these results suggest that endosomal NaV1.5 regulates phagocytosis of bacteria by human macrophages.

**Gene knockdown of NaV1.5 in differentiated and primed THP-1 cells also inhibits bacterial phagocytosis**

As described in Materials and Methods, we characterized the knockdown of SCNSA mRNA, which encodes NaV1.5, by five different lentiviral clones, identified as clones 68–72. Clone 68 infection resulted in a knockdown of ~88% (12% of baseline expression as determined by real-time PCR) as compared with clone 72, which did not demonstrate any appreciable knockdown as compared with wild type. Clones 69–71 showed intermediate levels of knockdown ranging from 40 to 70% (Fig. 8A). Phagocytosis by cells infected with clone 72 demonstrated similar levels of bacterial ingestion as compared with wild-type cells (Fig. 8B as compared with Fig. 7C). Clones 69–71 showed a modest trend (~25% inhibition) toward reduced phagocytosis, but clone 68 demonstrated slightly >70% inhibition compared with clone 72 (n = 6, p < 0.02).

**Stimulation of purified endosomes with veratridine, a voltage-gated sodium channel activator, reduces intraendosomal pH and [Na]**

To further characterize endosomal NaV1.5, we isolated endosomes from fully primed and differentiated THP-1 cells. Flow cytometry of permeabilized endosomes demonstrated that ~43% of the isolated endosomes were late endosomes as determined by positive staining for Rab-7, and all of these expressed NaV1.5 (Fig. 9A, left panel). These NaV1.5-positive endosomes separated into Rab-7 dim (7.3%) and Rab-7 bright populations (35.4%). The presence of NaV1.5 in purified endosomes was confirmed further by Western blot analysis, which demonstrated a single band of 200 kDa molecular mass (Fig. 9A, right panel).

We next determined intraendosomal pH and [Na] in the presence and absence of veratridine, a selective activator of voltage-gated sodium channels. To measure endosomal pH by flow cytometry, endosomes were loaded with Lysosensor Green DND-189 (pK_a = 5.2) and subsequently analyzed with and without 100 μM veratridine in endosomal buffer (35 mM KPiPES, 10 mM NaCl, 5 mM MgCl_2, and 1 mM EGTA (pH 7.4)). In the unstimulated condition, the intraendosomal pH was 6.87 ± 0.16 and with stimulation the pH decreased to 6.05 ± 0.85 (p = 0.01, n = 6) (Fig. 9B). Intraendosomal [Na] was measured with the ion indicator Sodium Green and was 129 ± 9.5 mM in the unstimulated condition and 80.3 ± 9.6 mM with veratridine (p = 0.02, n = 3) (Fig. 9C).

We also measured similar relative changes in K^+-free buffer (140 mM CsF, 10 mM NaCl, 1 mM EGTA, and 10 mM HEPES
Primary Macrophages
LPS then E. coli 30 min

THP-1
LPS then E. coli 30 min

THP-1
LPS then E. coli 60 min

THP-1
No Priming

FIGURE 7. High-dose, but not low-dose, TTX decreases the amount of bacterial phagocytosis. A, IFN-γ- and LPS-primed human monocyte-derived macrophages were challenged with FITC-labeled E. coli for 30 min. Extracellular fluorescence was quenched with trypan blue, and the amount of internalized bacteria was determined on a microplate fluorometer. TTX (10 μM), which blocks both NaV1.5 and NaV1.6, but not 0.3 μM, which blocks NaV1.6 but not NaV1.5, inhibited bacterial uptake. A value of \( p = 0.0001 \) for 10 μM TTX compared with untreated cells. B, Similar results were seen for primed and differentiated THP-1 cells; \( p = 0.0001 \). The inhibitory effect of 10 μM TTX was seen also at 60 min following bacterial challenge; \( p = 0.004 \). C, In THP-1 cells not primed with either IFN-γ or LPS, there was much less phagocytosis overall and no effect of either low- or high-dose TTX.

High-dose TTX blocks LPS-stimulated acidification of endosomal compartments and endosomal membrane depolarization in whole cells

To determine whether voltage-gated sodium channel activity can regulate endosomal pH in live cells, we measured the effect of loaded with Sodium Green demonstrated a decrease in fluorescence when stimulated with veratridine, consistent with sodium efflux from the endosome during enhanced proton influx (Fig. 9E). We confirmed this veratridine-mediated acidification in purified endosomes using time resolved fluorometry (Fig. 9F).

We also used time resolved fluorometry to determine the acute effects of veratridine in the presence of high physiologic concentrations of ATP. We reasoned that even in endosomes acidified by ATP that sodium channel activation would lead to an additional hyperacidification. Lysosensor Green DND-189 was used for pH measurements, and the sodium indicator SBFI was used for sodium measurements. Full activation of the endosomal membrane with ATP (2.5 mM) resulted in rapid acidification of the endosome (Fig. 10A) associated with a small degree of sodium influx (Fig. 10B). The intraendosomal pH changed from ~6.6 to 5.4, and the intraendosomal [Na] changed from ~100 to 120 mM. Despite this acidification by high-dose ATP, subsequent addition of veratridine (100 μM) caused a further decline in pH to ~5.1 in the representative experiment shown in Fig. 10A (4.86 ± 0.12, \( n = 5 \)). This change was associated with a very large outward sodium flux from ~120 to 10 mM intraendosomal [Na], followed by an inward flux and a longer term re-equilibration to ~80 mM (Fig. 10B) (82 ± 8.2 mM, \( n = 4 \)).

Amiloride, a blocker of the Na/H exchanger, did not have any effect on ATP or veratridine-stimulated sodium flux in isolated endosomes. As expected, bafilomycin, a specific inhibitor of V-type ATPase, prevented ATP-dependent acidification but not the acute effect of veratridine stimulation. However, the pH effect of veratridine was not maintained as well in the presence of bafilomycin (data not shown).

In a separate set of experiments, we calculated intraendosomal [Na] by loading fully primed and differentiated THP-1 cells with dextran (80 kDa) associated with cell impermeant SBFI and analyzed the cells by time-resolved fluorometry. The calculated intraendosomal sodium was 74.4 ± 5.2 mM (\( n = 5 \)), which correlates well with the post-ATP and veratridine values seen in Fig. 10B. Similar calculations were made in whole cells for pH changes (see below; Fig. 12).

FIGURE 8. Small hairpin RNA-mediated gene knockdown of SCN5A (NaV1.5) also decreases the amount of bacterial phagocytosis. A, Lentiviral clones 68–72, specific for human SCN5A, were transduced into THP-1 cells. Following 2 days in culture in the undifferentiated state, cells were differentiated and primed with IFN-γ and LPS. mRNA expression of SCN5A was determined by real-time PCR. Clone 68-transduced cells demonstrated >75% gene knockdown (<25% of wild-type expression), whereas clone 72-infected cells showed essentially no decrease in expression. The other clones demonstrated intermediate levels on knockdown. B, Phagocytosis in transduced clone 68 cells was reduced by >70% as compared with clone 72-transduced cells (\( p = 0.019, n = 6 \)).

(\( \text{pH} 7.0 \)). Using flow cytometry, the mean fluorescent intensity of Lysosensor Green DND-189-loaded endosomes increased by ~30% in the veratridine-stimulated condition (Fig. 9D), demonstrating a decrease in intraendosomal pH. In contrast, endosomes

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were equilibrated in K/H11001 standard solutions. Treated with either nigericin (pH) or gramicidin [Na] and equilibrated in indicated dye for 30 min. Standard curves were generated in endosomes divided into a Rab-7 bright (R1, 35.4%) and dim (R2, 7.3%) populations. Total counts were 10,000 events. Presence of NaV1.5 in the endosomes was confirmed further by Western blot analysis of isolated endosomes. (Lysosensor Green DND 189–loaded endosomes in the presence or absence of TTX (Fig. 11A). As predicted, LPS stimulation for 30 min resulted in a mean fluorescent shift from a fluorescent intensity of 608 (unstimulated) to 1540 (stimulated, light diagonal lines), consistent with decreased intraendosomal [Na] that re-equilibrated to 80 mM (82 ± 8.2 mM, n = 4). Sodium channel blockade on LPS-induced endosomal and lysosomal acidification. For determination of pH, Lysosensor Blue was used because of its low pKa (5.1) and stable signal during live cell imaging. 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Intracellular voltage measurements were made using the slow voltage-sensitive dye, DiSBAC$_2$(3), a bis-oxonol. The negatively charged intracellular dye associates with the positively charged side of organelle membranes, concentrates in membranes with enhanced electrical activity, and provides a prolonged measurement of intracellular structures that show charge movement during the stimulation period (23). As with Lysosensor Blue, 10 μM TTX, but not low-dose (0.3 μM), TTX inhibited LPS-mediated intracellular acidification as shown by the micrographs (top) and quantitative analysis (table, bottom). B. Cells were primed and treated as in A, except they were loaded with the voltage sensitive dye, DiSBAC$_2$(3) (1 μM), for 1 h, rather than with Lysosensor Blue. Similar results were obtained and showed that high-dose, but not low-dose, TTX blocked LPS-mediated intracellular depolarization.

We confirmed these results using ratiometric fluorometry in FITC-dextran (70 kDa)-loaded THP-1 cells (Fig. 12) (24). Differentiated and IFN-γ-primed cells were loaded with dextran, washed, and then stimulated with LPS in the presence or absence of TTX. This pulse-chase approach selectively labels the late endosomal and lysosomal compartments with FITC-dextran (24). In the absence of LPS stimulation and any TTX treatment, the calculated pH of the acidic endosomal-lysosomal compartment was 5.26 ± 0.22. With LPS treatment for 60 min, the pH dropped to 4.98 ± 0.16, and with simultaneous high-dose TTX treatment (10 μM), the pH was 5.62 ± 0.04 (p = 0.007, n = 4). The calculated pH following LPS treatment correlates well with the pH observed in isolated endosomes following stimulation with ATP and veratridine (Fig. 10A).

**FIGURE 11.** A TTX-insensitive voltage-gated sodium channel mediates endosomal acidification and depolarization in LPS-stimulated cells and veratridine-stimulated endosomes. A. Differentiated THP-1 cells were primed with IFN-γ (10 ng/ml for 18 h) and loaded with Lysosensor Blue (1 μM) for 30 min. Cells were washed, primed for an additional 1 h with LPS (100 ng/ml) in the presence and absence of TTX (untreated, 0.3 and 10 μM), and then analyzed for densitometric intensity per cell and number of labeled acidic particles per cell. High-dose (10 μM), but not low-dose (0.3 μM), TTX inhibited LPS-mediated intracellular acidification as shown by the micrographs (top) and quantitative analysis (table, bottom). B. Cells were primed and treated as in A, except they were loaded with the voltage sensitive dye, DiSBAC$_2$(3) (1 μM), for 1 h, rather than with Lysosensor Blue. Similar results were obtained and showed that high-dose, but not low-dose, TTX blocked LPS-mediated intracellular depolarization.

**FIGURE 12.** High-dose, but not low-dose, TTX blocks LPS-dependent endosomal acidification in THP-1 cells loaded with FITC-dextran in the endosomal-lysosomal compartment. Live microscopy analysis (Fig. 11) was confirmed by quantitative fluorometric analysis of FITC-dextran-loaded THP-1 cells. Following a 30-min loading of the cells with FITC-dextran, the cells were stimulated with LPS (100 ng/ml) in the presence or absence of TTX (24). LPS treatment decreased the pH of the endosomal-lysosomal compartment, and high-dose TTX (10 μM), but not low-dose TTX (0.3 μM), prevented LPS-dependent acidification (pH = 4.98 ± 0.16 with LPS treatment vs 5.62 ± 0.04 with LPS and high dose TTX; p = 0.007). In the absence of LPS stimulation and any TTX treatment, the calculated pH of the acidic endosomal-lysosomal compartment was 5.26 ± 0.22.

**Discussion**

These results suggest a novel mechanism to regulate monocyte-macrophage endosomal acidification through expression of the
cardiac voltage-gated sodium channel, NaV1.5, in the late endosome. Activation of the channel in intact cells or by the channel agonist veratridine in isolated endosomes enhances proton flux into the late endosome. This proton influx is balanced by a concomitant sodium efflux. To our knowledge, this is the first demonstration of a functional role for voltage-gated sodium channels on an intracellular organelle. Although the neuronal sodium channel, NaV1.6, is also expressed in THP-1 cells and primary human macrophages, it does not appear to facilitate phagocytosis, although it may regulate cytoskeletal function, given its colocalization with F-actin and vimentin.

In intact cells, our results suggest a novel NaV1.5 mechanism to regulate macrophage endosomal acidification and phagocytosis following activation by LPS. A similar LPS-dependent acidification has been observed in lysosomes from dendritic cells (24). This ATP-dependent mechanism in dendritic cells is hypothesized to be mediated by enhanced function of the proton vesicular ATPase. However, endosomes purified from our macrophage cultures could be acidified further even after treatment with exogenous ATP by veratridine-mediated activation of voltage-gated sodium channels. In whole cells, LPS stimulation was associated with electrical activity of endosomal membranes, and the degree of acidification correlated best with intraendosomal pH observed in isolated endosomes activated by ATP and veratridine as opposed to ATP alone. These results suggest at least some dependence on ionic flux through specific channels as opposed to a purely vesicular-ATPase-dependent mechanism.

This mode of priming of the macrophage by LPS may permit more efficient phagocytosis and couple phagocytosis rate to channel activity. Acidified intracellular late endosomes and lysosomes would be prepared to process early phagosomes before contact with their bacterial target. In addition, because channel activity is required for optimal phagocytic capacity, the pathway can be more tightly regulated. For example, if the channel activity is blocked following LPS activation, then the cell will take up less bacteria. This switch may prevent macrophage cell death from ingesting excessive bacteria and also may create a timing mechanism to turn off active phagocytosis. Additional studies are required to determine how LPS and possibly other TLR ligands regulate these channels. Our results are consistent with prior studies of tissue macrophages that demonstrated an electrogenic component of endosomal acidification (13).

We speculate that NaV1.5 acts as a charge sink to move positive charge of the interior of the endosome to the cytoplasm. This charge movement may permit additional entry of protons into the endosome through other voltage-gated channels such as vesicular CIC or the vesicular ATPase proton pump. In other words, we hypothesize that NaV1.5 mediates physiologic endosomal hyperacidification in the macrophage that is comparable to pathologic endosomal hyperacidification seen in epithelial cells from patients with cystic fibrosis (27). In cystic fibrosis, hyperacidification of epithelial endosomes seems to occur through a lack of inhibition of the endosomal sodium exchanger, similar to the actions of NaV1.5 seen here, that moves sodium of the endosome to permit enhanced entry of protons.

Given its localization to the late macrophage endosome, the specific function of NaV1.5 seems to be to facilitate the decrease in pH seen as the endosome matures from the early state (pH $\approx 6.8$) to the lysosomal stage (pH $\approx 5.0$). As compared with other voltage-gated sodium channels, the subcellular localization and pH dependence of NaV1.5 make it ideally suited to the task. In cardiac myocytes, NaV1.5 undergoes complex subcellular trafficking to its ultimate localization in the intercalated discs (28). Its subcellular localization appears to be dependent on its interaction with a series of regulatory proteins, the absence of which can arrest the channel in a subcellular organelle. In addition, pH tightly regulates the current of NaV1.5, and it lacks a pH-independent current (29). As the intraendosomal pH decreases in the late endosome to that seen in the lysosome, the sodium current would become inactive, limiting the contribution of NaV1.5 to any further acidification once the lysosomal stage is reached. This current inactivation would be voltage independent.

Although NaV1.5 is the principal sodium channel in cardiac myocytes, it is also present in a number of noncardiac cells, including cell types that do not display electrical excitability (30). Our results demonstrate that NaV1.5 is present intracellularly in a differentiuated monocytic cell line, THP-1, and in monocyte-derived macrophages and suggest that this sodium channel regulates endosomal acidification. This mechanism of enhanced acidification by macrophages may be protective to the host during acute infections because it could lead to enhanced killing of infectious agents. However, this same mechanism also may lead to enhanced tissue injury in a variety of pathologic states where bone marrow-derived and tissue macrophages may play a role such as chronic infections, autoimmune diseases, and degenerative processes such as Alzheimer’s and Parkinson’s Disease.

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Disclosures

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References


