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Methanethiosulfonate-modification Alters Local Anesthetic Block in rNav1.4 Cysteine-substituted Mutants S1276C and L1280C

J.P. O'Reilly[1]*, S.Y. Wang[2], G.K. Wang[1]
[1]Department of Anesthesia Research, Brigham & Women's Hospital, Harvard Medical School, 75 Francis St, Boston, MA 02115, USA
[2]Department of Biological Sciences, State University of New York at Albany, Albany, NY 12222, USA

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Abstract. We previously showed that lysine substitutions at two residues in segment 6 of domain 3 in voltage-gated Na^+ channel rNav1.4 (S1276K, L1280K) reduced steady-state inactivated local anesthetic block. Here we studied cysteine substitutions at the same residues (S1276C, L1280C). We used whole-cell recordings to determine local anesthetic block (100 μM bupivacaine) before and after cysteine modification with 1.5 mM 2-aminoethyl methanethiosulfonate (MTSEA). Compared with rNav1.4, steady-state resting bupivacaine block at −180 mV was increased in S1276C, while inactivated block at −50 mV was not different in the mutants. After application of MTSEA at −160 mV, rNav1.4 showed enhanced bupivacaine block and a negative shift in $V_{1/2}$ of the bupivacaine affinity curve, while L1280C and S1276C showed a decrease in inactivated bupivacaine block after MTSEA. Application of MTSEA at 0 mV produced similar results in rNav1.4 and L1280C, but an opposite effect in S1276C, i.e., enhancement of bupivacaine block, with a large negative shift in $V_{1/2}$ of the bupivacaine affinity curve similar to that found in rNav1.4. We conclude that 1) MTSEA modification of 1276C or 1280C decreases inactivated bupivacaine block similar to that found in L1280K and S1276K, 2) residue 1276C is only accessible to MTS-modification in the resting state, and 3) MTSEA may modify a native cysteine in rNav1.4 that produces an allosteric, indirect effect on bupivacaine affinity.

Key words: Na^+ channels — Local anesthetic — Methanethiosulfonate (MTS) — Site-directed mutagenesis

Correspondence to: J.P. O'Reilly; email: joreilly@selu.edu

**Present address:* Department of Biological Sciences, Southeastern Louisiana University, Hammond, LA 70402, USA

Introduction

Voltage-gated Na^+ channels (Navs) play a critical role in the initiation and propagation of action potentials in excitable tissues (i.e., muscle, heart, nerve). In response to depolarization of the membrane potential, Navs open briefly and allow Na^+ ion influx that underlies the depolarization phase of the action potential. Navs close into a non-conducting inactivation state within milliseconds after opening, which contributes in part to the repolarization phase of the action potential. Our understanding of the molecular mechanisms involved in these processes has been greatly aided by the cloning and sequencing of a number of Nav isoforms. The functional expression of many of these clones has shown that the α subunit of Navs (*see* Fig. 1) has four homologous domains (D1–D4), each with six transmembrane segments (S1–S6), and that the α subunit appears to be sufficient for the critical Nav functions of activation, inactivation, and ion selectivity (Noda et al., 1984, 1986; Trimmer et al., 1989; Gellens et al., 1992). In addition, studies using site-directed mutagenesis have assigned these specific functions to specific regions of Navs (Stühmer et al., 1989; Heinemann et al., 1992; Smith & Goldin, 1997; McPhee et al., 1998).

Local anesthetics (LAs) are molecules that block Na^+ ion conduction through Navs. Blocking Nav conduction in excitable tissues reduces or abolishes action potential initiation and propagation, and hence LAs are used clinically to modulate or eliminate electrophysiologic function in these tissues. Although we know that LAs bind with Navs, the precise molecular events associated with this interaction are not yet fully understood. Specifically, the exact site of Navs where LAs bind and produce their physiological effect is still under active investigation. Studies using site-directed mutagenesis have begun to illu-

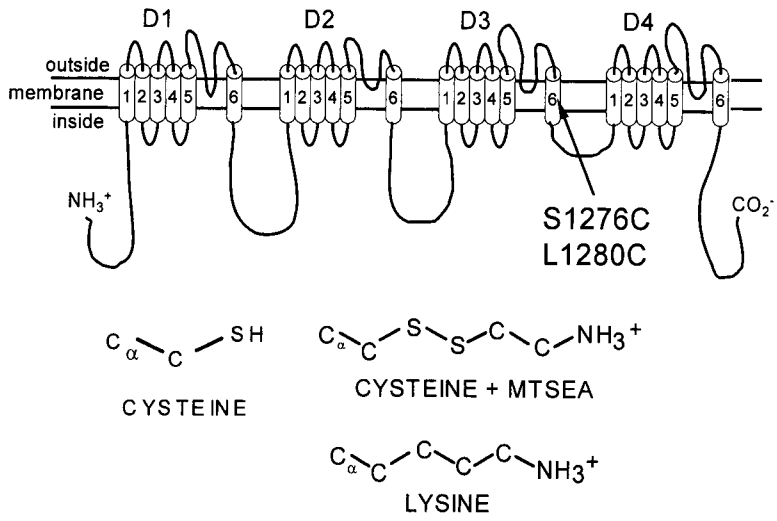


Fig. 1. The α subunit of the voltage-gated Na channel μ1 from rat skeletal muscle (rNav1.4). The α subunit contains four homologous domains (D1–D4) each with six transmembrane segments (S1–S6). Cysteines (*C*) were substituted for the native serine (*S*) at position 1276 and the native leucine (*L*) at position 1280. Both substitutions were in the S6 segment of domain D3. Shown below the α subunit are the side chain of cysteine, the molecular structure of the cysteine side chain after modification with MTSEA, and the side chain of lysine. C_α is the alpha carbon of the amino acid.

minate the molecular interaction of LAs and Navs. Several of these studies have suggested that the S6 segments in D1, D3, and D4 may form part of the LA binding site in Navs (Ragsdale et al., 1994; Wright, Wang, & Wang, 1998; Nau et al., 1999; Wang, Nau, & Wang, 2000).

Recently we have proposed that two specific residues (S1276 and L1280) in the S6 segment of domain 3 (D3–S6) play a critical role in forming the LA binding site in Navs (Wang et al., 2000). We based this conclusion on the reduced LA affinity in mutants with lysine (K) substitutions for the native serine (S) in S1276K or the native leucine (L) in L1280K. In the present study we substituted cysteine (C) at these same residues and looked at LA affinity (100 μM bupivacaine) before and after application of 1.5 mM 2-aminoethyl methanethiosulfonate (MTSEA). We used MTSEA because the chemical reaction between the side chain of cysteine and MTSEA results in a molecular structure that resembles the side chain of lysine (Fig. 1). Therefore, MTSEA modification of cysteine substitutions in S1276C and L1280C allowed us to test the hypothesis that the decreased bupivacaine block found in S1276K and L1280K is due to the specific molecular nature of the lysine side-chain.

We found that MTSEA modification of S1276C or L1280C produces a bupivacaine affinity profile that is similar to that found in the lysine-substituted mutants S1276K and L1280K, i.e., decreased steady-state inactivated bupivacaine affinity. Surprisingly, we also found that MTSEA application in wild-type rNav1.4 resulted in an enhancement of bupivacaine affinity, i.e., the opposite effect. The MTSEA effects found in rNav1.4 and L1280C were consistent whether MTSEA was applied when the channels were at rest (−160 mV) or inactivated (0 mV). However, in S1276C, MTSEA application at 0 mV produced an opposite effect, i.e., an increase in bupivacaine affinity, similar to that found in rNav1.4.

Our results suggest that MTSEA modifies the substituted cysteines in S1276C and L1280C resulting in a decrease in bupivacaine affinity. This effect may be mediated by the positive charge on MTSEA in a manner similar to lysine substitutions in S1276K and L1280K. We also propose that MTSEA may modify an as yet undetermined native cysteine in rNav1.4 and that this modification enhances bupivacaine affinity via an allosteric, indirect effect.

Materials and Methods

Site-Directed Mutagenesis and Transient Transfection of cDNA Clones

Mutants were constructed with cysteine substitutions in rat skeletal muscle Na^+ channel μ1 (rNav1.4) as described previously (Wang & Wang, 1997). cDNA clones of wild-type rNav1.4 and mutant Na^+ channels (Navs) were transiently transfected into HEK293t cells by the calcium, phosphate precipitation method (Graham & Eb, 1973) as previously described (O'Reilly et al., 1999). The transfection included 0.5 μg of CD8 (cell surface antigen) and 5–10 μg of Nav cDNA subcloned in the pcDNA1/amp vector (Invitrogen, San Diego, CA).

Electrophysiology

We used standard patch-clamp techniques (Hamill et al., 1981) to record whole-cell peak Na^+ current (I_{Na}) from transiently transfected HEK293t cells. Recordings were performed at room temperature (22 ± 2°C), and no correction was made for the liquid junction potential. Activation (conductance-voltage) and steady-state fast-inactivation (h_∞) curves were obtained ≈10 min after rupture of the membrane. Recording micropipettes (Drummond Scientific, Broomall, PA) were pulled on a Model P-87 Flaming-Brown puller (Sutter Instruments, Novato, CA). Pipette resistance ranged from 0.5 to 1.5 MΩ. The extracellular recording solution was (in mM): 65 NaCl, 85 choline-Cl, 2 $CaCl_2$, and 10 HEPES, titrated to pH 7.4 with TMA-OH. After establishing whole-cell conditions, the cells were continuously bathed in the extracellular solution with a gravity-fed superfusion system (≈0.1 mL/min). The pipette (intracellular) solution was (in mM): 100 NaF, 30 NaCl, 10

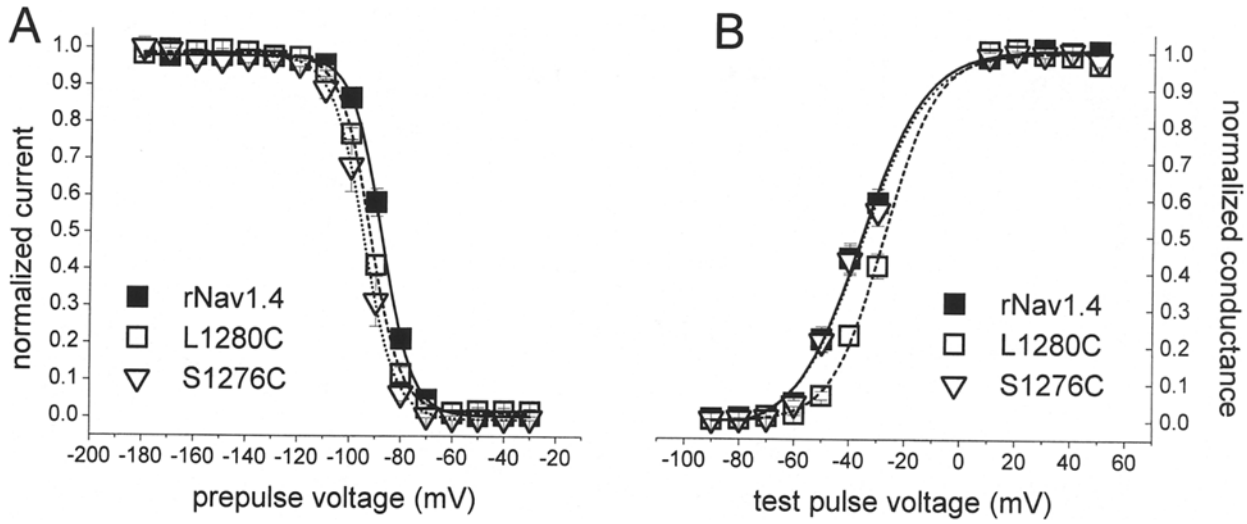


Fig. 2. Differences in steady-state fast inactivation and activation in L1280C and S1276C compared with wild-type rNav1.4. Protocols are described in Materials and Methods and data is presented in the text. (*A*) Steady-state inactivation curves for S1276C and L1280C showed a shift towards more negative voltage compared with rNav1.4 ($P < 0.01$). (*B*) The activation curve in L1280C was shifted in the depolarizing direction ($P < 0.01$) and was steeper ($P < 0.05$) compared with rNav1.4, while S1276C was not different.

Table 1. Steady-state resting bupivacaine affinity (K_R) and inactivated bupivacaine affinity (K_I) before and after application of 1.5 mM MTSEA at −160 mV or 0 mV in wild-type rNav1.4, L1280C, and S1276C

Channel [*n*]	Before MTSEA		After MTSEA	
	K_R (μM)	K_I (μM)	K_R (μM)	K_I (μM)
MTSEA at −160 mV				
rNav1.4 [6]	142.7 ± 12.0	8.3 ± 1.8	98.8 ± 2.3[b]	3.7 ± 0.9[a]
L1280C [8]	185.7 ± 14.6	12.0 ± 3.0	163.2 ± 19.3	65.0 ± 5.9[c]
S1276C [6]	111.8 ± 7.3[d]	6.6 ± 1.5	128.0 ± 21.3	25.2 ± 3.6[c]
MTSEA at 0 mV				
rNav1.4 [4]	154.0 ± 56.5	7.5 ± 1.6	105.8 ± 11.2[a]	2.8 ± 0.8[a]
L1280C [6]	177.8 ± 29.7	14.8 ± 1.2	212.5 ± 24.0	80.8 ± 19.2[b]
S1276C [6]	117.3 ± 9.5[d]	5.3 ± 0.6	82.5 ± 7.2[a]	3.3 ± 0.4[a]

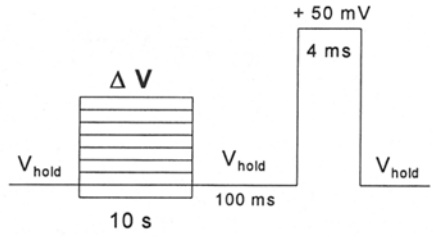
K_R and K_I were determined as described in Materials and Methods.
Different compared with rNav1.4: [d] = $P < 0.05$.
Different compared with values before MTSEA: [a] = $P < 0.05$, [b] = $P < 0.01$, [c] = $P < 0.001$.

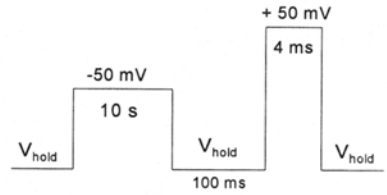
EGTA, and 10 HEPES, titrated to pH 7.2 with CsOH. These solutions create an outward Na^+ gradient and an outward Na^+ current at the test pulse of +50 mV, thus reducing potential problems associated with space clamp or series resistance errors (Cota & Armstrong, 1989). Series resistance was compensated at 80%, which resulted in voltage errors of < 5 mV. Linear leak subtraction based on five hyperpolarizing pulses was used for all recordings. Any endogenous K^+ currents were blocked with Cs^+ in the pipette, and HEK cells express no native Ca^{2+} current (Ukomadu et al., 1992). Cells were selected for recording on the basis of positive immunoreaction with CD8 Dynabeads (Dynal Biotech, Lake Success, New York).

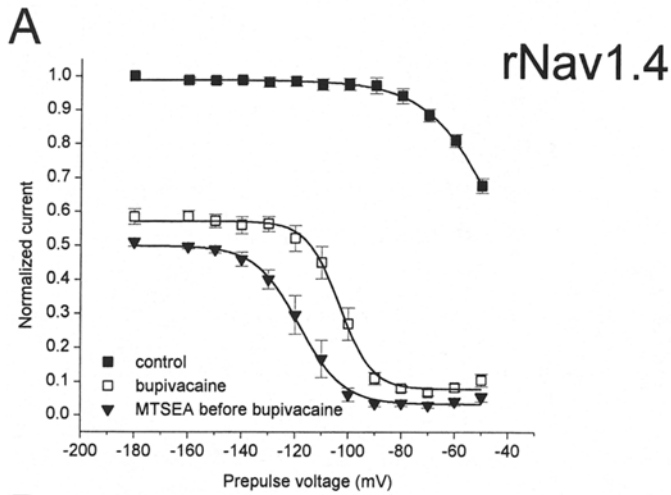
The holding potential (V_{hold}) for all experiments was −160 mV. A test pulse to +50 mV (4 ms) was used to record peak available Na^+ current (I_{Na}). Activation curves were obtained from the peak current recorded with pulses from V_{hold} to voltages over the range of −90 mV to +50 mV in 10-mV increments. $V_{1/2}$ of the curve and slope factor k were obtained from a fit of the mean data with a Boltzmann function $G/G_{max} = 1/(1 + \exp((V_{1/2} - V)/k))$, where $G = I_{Na}/(V - V_{reversal})$. Steady-state fast inactivation (h_∞) was determined with a test pulse to +50 mV to record I_{Na} following a conditioning prepulse (100 ms) from −180 mV to −30 mV in 10-mV increments. $V_{1/2}$ and slope factor k were obtained from a fit of the mean data obtained with a Boltzmann function, $I/I_{max} = 1/(1 + \exp((V - V_{1/2})/k))$, where $V_{1/2}$ is the mid-point of the curve and k is the slope factor.

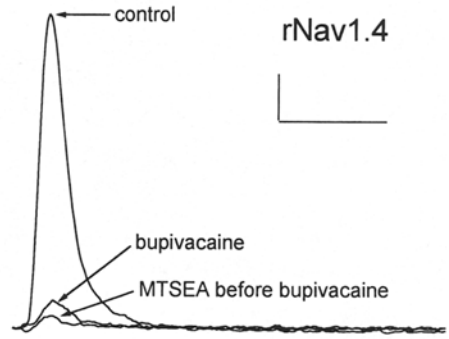
Pharmacology

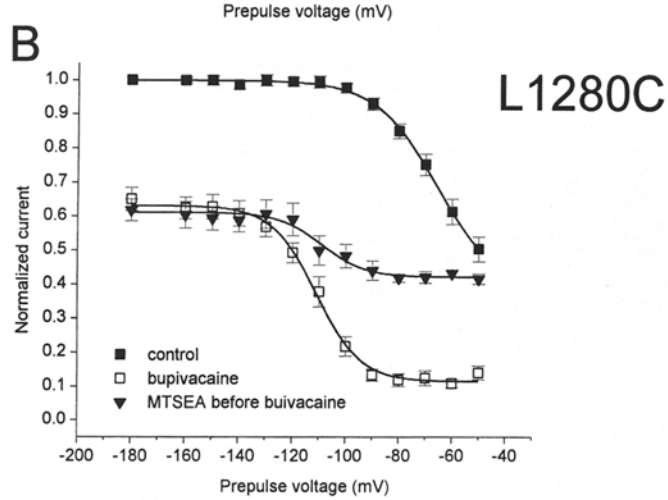
Bupivacaine was applied extracellularly by switching to a different pipette of the gravity-fed superfusion system. We used bupivacaine because it binds tightly with the channels and does not dissociate much during the 100 ms interpulse, which allows us to do voltage scanning (Nau et al., 1999). Bupivacaine was diluted into the extracellular solution from a stock solution in DMSO. The final concentration of DMSO was 0.1%, and control experiments with DMSO up to 1% had little to no effects on I_{Na} (*data not shown*). The protocol for bupivacaine affinity consisted of a 10-s prepulse at voltages from −180 to −50 mV (10-mV increments), a 100-ms step

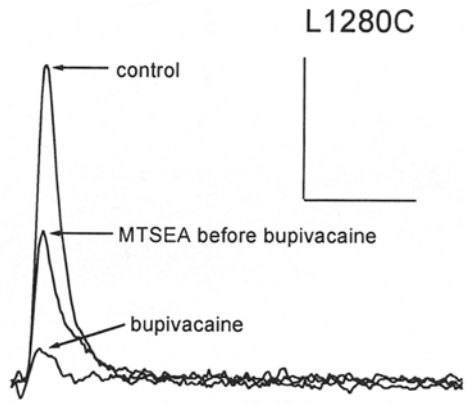

+ 50 mV
4 ms
Δ V
V hold
V hold
V hold
100 ms
10 s

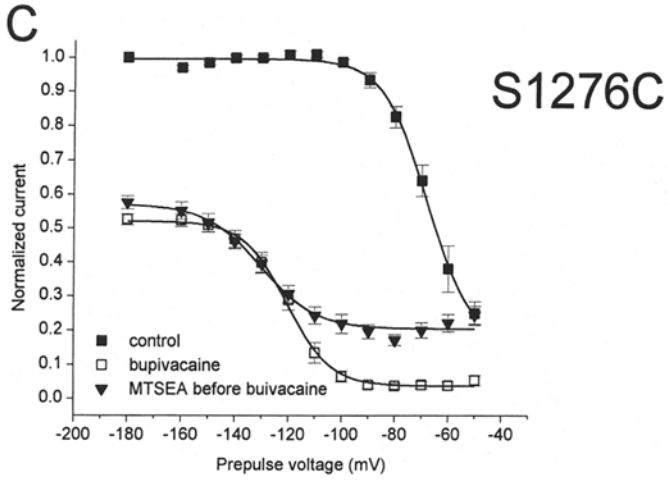

+ 50 mV
4 ms
-50 mV
10 s
V hold
V hold
V hold
100 ms


A
rNav1.4
Normalized current
control
bupivacaine
MTSEA before bupivacaine
Prepulse voltage (mV)


control
rNav1.4
bupivacaine
MTSEA before bupivacaine


B
L1280C
Normalized current
control
bupivacaine
MTSEA before buivacaine
Prepulse voltage (mV)


L1280C
control
MTSEA before bupivacaine
bupivacaine


C
S1276C
Normalized current
control
bupivacaine
MTSEA before buivacaine
Prepulse voltage (mV)

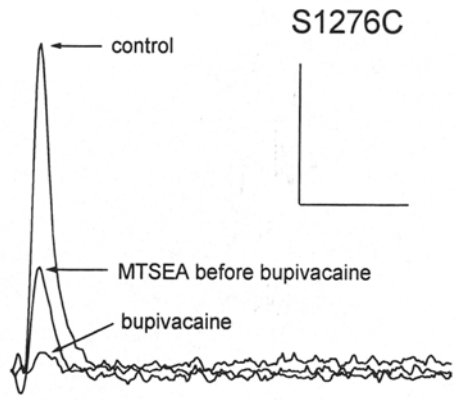

S1276C
control
MTSEA before bupivacaine
bupivacaine

Fig. 3.

Fig. 3. Application of 1.5 mM MTSEA at V_{hold} (−160 mV) increases bupivacaine affinity in rNav1.4, but decreases bupivacaine affinity in L1280C and S1276C. Data were collected using the pulse protocol in the *upper left* of the figure and fit with Boltzmann functions (*see* Materials and Methods for complete description). Control data (■) were collected before bupivacaine and MTSEA, bupivacaine data (□) were collected in the presence of 100 μM bupivacaine, and MTSEA before bupivacaine data (▼) were collected in the presence of 100 μM bupivacaine after 3–5 min exposure to 1.5 mM MTSEA. Resting (K_R) and inactivated (K_I) steady-state bupivacaine affinity are presented in Table 1. Representative traces from the test pulse recorded after 10 s at −50 mV (*see* pulse protocol at *upper right*) are shown on the right side of the figure. Scale bars: 1 nA, 1 ms. (*A*) Bupivacaine affinity is increased in rNav1.4 after MTSEA treatment, as demonstrated by an increased block of available Na^+ current (ordinate) at all voltages tested (abscissa). The $V_{1/2}$ of the bupivacaine affinity curve was shifted in the negative direction by −14 mV after MTSEA. (*B*) In L1280C the same experimental protocols resulted in reduction of bupivacaine affinity that was most pronounced at depolarized potentials above −100 mV. (*C*) In S1276C, MTSEA also reduced inactivated bupivacaine affinity.

to V_{hold}, then the 4-ms test pulse to +50 mV to record I_{Na} (Wright et al., 1997). This protocol was run during continuous exposure to bupivacaine. Control data were normalized to peak I_{Na} recorded with the test pulse from −180 mV before bupivacaine exposure. Data for bupivacaine affinity were normalized to individual control data at each voltage. Data were fit with a Boltzmann function as for the h_∞ curve (*see* above) to produce a bupivacaine affinity curve. The apparent steady-state bupivacaine affinity (K_{app}) was determined at −180 mV when the channels were at rest (K_R) and at −50 mV when the channels were inactivated (K_I) using the Langmuir isotherm: $I_{Na} = K_{app}/([\text{bupivacaine}] + K_{app})$, where I_{Na} is the normalized peak current in the presence of bupivacaine and [bupivacaine] is the concentration of drug (Hille, 2001).

Methanethiosulfonate Application

2-Aminoethyl methanethiosulfonate hydrobromide (MTSEA; Cat. #A609100, Toronto Research Chemicals, Toronto, Ontario, Canada) was applied in the extracellular superfusion system (O'Reilly, Wang, & Wang, 2001). On the day of the experiments, a concentrated (10×) working stock solution was made by dissolving the MTSEA in water. The solution was kept on ice in the dark and diluted (1.5 mM) into the extracellular solution immediately before application. Effects of MTSEA application on bupivacaine affinity were assessed with the same protocol as above. Post-MTSEA bupivacaine affinity data were normalized as above to control data collected after MTSEA exposure and fit with a Boltzmann function.

Data Collection and Analysis

Data were collected with an Axopatch 200A amplifier (filtered at 5 kHz) and pClamp software (pClamp 6; Axon Instruments, Foster City, CA). Curve fits and data analysis were performed with pClamp and Origin software (MICROCAL Software, Inc., Northampton, MA). Differences were considered significant at $P < 0.05$ (ANOVA). Grouped data are presented as means ± SEM.

Results

Cysteine Substitution in S1276C or L1280C has Small Effects on Steady-state Fast Inactivation and Activation

Steady-state fast inactivation (h_∞) and activation (conductance-voltage) curves were generated by fitting the data with a Boltzmann function (*see* Materials and Methods). The h_∞ curves for the mutants S1276C and L1280C were shifted in the hyperpolarized direction by several millivolts ($P < 0.01$) compared with wild-type rNav1.4 (S1276C: $V_{1/2} = -94.8 \pm 0.3$ mV, $k = 6.3 \pm 0.3$, $n = 7$; L1280C: $V_{1/2} = -92.4 \pm 0.1$ mV, $k = 6.1 \pm 0.1$, $n = 11$; rNav1.4: $V_{1/2} = -87.8 \pm 0.1$ mV, $k = 6.0 \pm 0.1$, $n = 9$; Fig. 2*A*). The conductance-voltage curve for L1280C was shifted in the depolarized direction ($P < 0.01$) and was steeper ($P < 0.05$) compared with rNav1.4, while the conductance-voltage curve for S1276C was not different from rNav1.4 (L1280C: $V_{1/2} = -27.6 \pm 0.8$ mV, $k = 9.2 \pm 0.9$, $n = 11$; rNav1.4: $V_{1/2} = -35.9 \pm 0.9$ mV, $k = 11.6 \pm 1.2$, $n = 9$; S1276C: $V_{1/2} = -35.5 \pm 1.1$ mV, $k = 11.8 \pm 1.4$, $n = 7$; Fig. 2*B*).

Cysteine Substitution in S1276C Increases Steady-state Resting Bupivacaine Block in S1276C Compared with rNav1.4

Compared with rNav1.4, steady-state resting bupivacaine block at −180 mV was increased in S1276C (Table 1; $P < 0.05$), but was not different in L1280C (Table 1). Steady-state inactivated bupivacaine block at −50 mV was not different in the mutants compared with rNav1.4 (Table 1).

2-Aminoethyl Methanethiosulfonate (MTSEA) Application at −160 mV Enhances Bupivacaine Block in rNav1.4

In rNav1.4, superfusion of 1.5 mM MTSEA at V_{hold} of −160 mV (i.e., when the channels are in the resting state) for ≈3 min prior to bupivacaine application enhanced bupivacaine block (Fig. 3*A*, Table 1). Steady-state resting bupivacaine block and steady-state inactivated block were increased and the $V_{1/2}$ of the bupivacaine affinity curve was shifted in the negative direction by ≈14 mV (Table 1; −118.0 ± 3.4 vs. −103.8 ± 2.4 mV; $P < 0.05$; Fig. 3*A*). Control experiments to determine any time-dependent shifts in the bupivacaine affinity curve showed no difference in K_R or K_I (*data not shown*) and only a modest non-significant shift in the $V_{1/2}$ over the time course of the protocols (≈−4 mV over 15 min; $n = 7$).

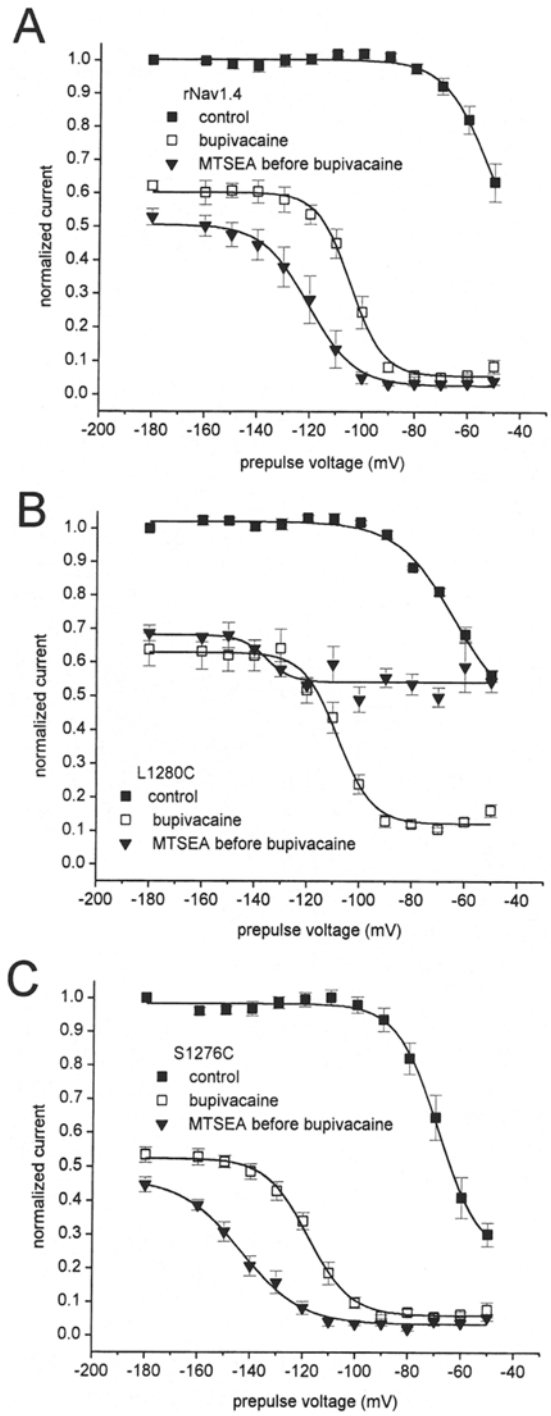


Fig. 4. Application of 1.5 mM MTSEA at 0 mV increases bupivacaine affinity in rNav1.4, decreases bupivacaine affinity in L1280C, but increases bupivacaine affinity S1276C. (Data were collected using the protocol in the upper left of Fig. 3). Control data (■), bupivacaine data (□), and MTSEA before bupivacaine data (▼) were collected as in Fig. 3. However, MTSEA was applied while the cells were held at 0 mV rather than −160 mV. (*A*) In rNav1.4, MTSEA application at 0 mV produced results similar to those found at −160 mV (*see* Fig. 3*A*). (*B*) In L1280C, similar results were also found whether MTSEA was applied at 0 mV or −160 mV (*see* Fig. 3*B*). (*C*) An opposite effect was found in S1276C when MTSEA was applied at 0 mV. MTSEA at 0 mV produced an increased bupivacaine affinity similar to rNav1.4 (*see* Fig. 3*A*) but in contrast to the decreased bupivacaine affinity seen when MTSEA was applied at −160 mV in S1276C (*see* Fig. 3*C*).

MTSEA Application Decreases Bupivacaine Block in S1276C and L1280C

In L1280C and S1276C, an opposite effect, i.e., decreased bupivacaine block, was found following MTSEA application at −160 mV (Table 1; $P < 0.001$; Fig. 3*B*, *C*). In L1280C, K_I was fivefold greater and in S1276C K_I was fourfold greater (Table 1). This resistance to bupivacaine block was similar to that found in L1280K and S1276K (Wang et al., 2000).

MTSEA Application at 0 mV Produces Similar Results in rNav1.4 and L1280C, but an Opposite Effect in S1276C

To determine if the MTSEA modification was state-dependent, we repeated the experiments at a holding potential of 0 mV (i.e., when channels were inactivated) during MTSEA application. In rNav1.4 and L1280C, superfusion of 1.5 mM MTSEA for ≈3 min at V_{hold} of 0 mV prior to bupivacaine application produced results similar to those found at −160 mV (Fig. 4*A*, *B*; Table 1), although the shift in $V_{1/2}$ of the bupivacaine affinity curve in rNav1.4 was greater after MTSEA at 0 mV compared to −160 mV (−30 mV *vs.* −14 mV, respectively; $P < 0.01$). However, in contrast to rNav1.4 and L1280C, a different result was found in S1276C. MTSEA application at 0 mV in S1276C enhanced bupivacaine affinity, similar to the effect found in rNav1.4. Application of MTSEA at 0 mV produced a decrease in K_R and K_I (Table 1) and a large negative shift in the $V_{1/2}$ of the bupivacaine affinity curve (Tabel 1; −142.8 ± 4.1 *vs.* −118.3 ± 1.8 mV; $P < 0.001$; Fig. 4*C*).

MTSEA Application in Mutants with Other Amino Acid Substitutions at L1280 (L1280A and L1280W) Produces Results Comparable to rNav1.4

We also asked the question: is the MTSEA modification specific to the substituted cysteines ? If so, then MTSEA application in mutants with amino-acid substitutions other than cysteine should not decrease bupivacaine affinity. That is, we would expect to see results in these mutants similar to those found in rNav1.4. To address this question, we examined two other mutants, L1280A (alanine substitution) and L1280W (tryptophan substitution). Before MTSEA, K_R in L1280A and L1280W was not different from rNav1.4 (*data not shown*). K_I was not different in L1280A (*data not shown*), but was decreased in L1280W (1.6 ± 0.4 μM; $P < 0.01$) compared with

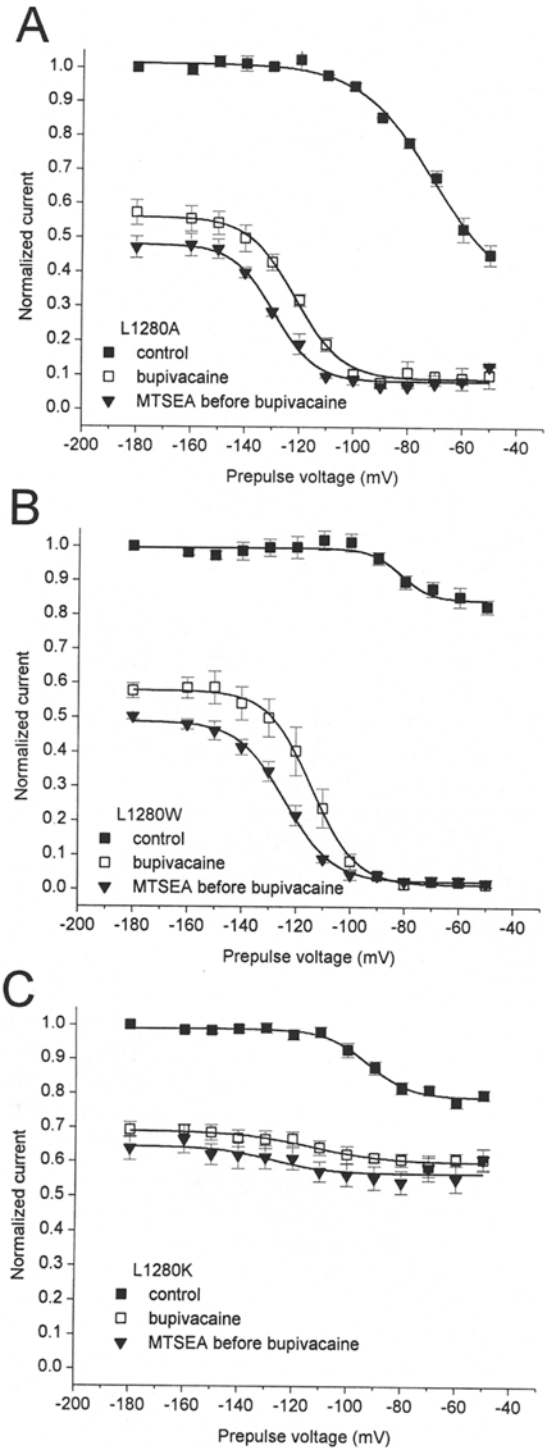


Fig. 5. MTSEA application at −160 mV in (*A*) L1280A or (*B*) L1280W produces results similar to rNav1.4. (*C*) MTSEA at −160 mV in L1280K produces no effect on bupivacaine affinity. Data were collected as in Fig. 3 and are presented in the text.

rNav1.4. We applied MTSEA at −160 mV in these mutants and found similar results as in rNav1.4. MTSEA application reduced K_R in L1280A (from 131.5 ± 18.5 to 90.7 ± 9.6 μM; $P < 0.05$; $n = 4$; Fig. 5*A*) and L1280W (from 149.4 ± 20.6 to 97.6 ± 3.5 μM; $P < 0.05$; $n = 5$; Fig. 5*B*), but with no significant change in K_I (*data not shown*). In addition, the $V_{1/2}$ of the bupivacaine affinity curve was shifted in the negative direction in L1280A (−128.7 ± 2.3 vs. −121.5 ± 1.6 mV; $P < 0.05$; Fig. 5*A*) and L1280W (−124.4 ± 2.2 vs. −115.2 ± 2.6 mV; $P < 0.05$; Fig. 5*B*).

MTSEA Application in L1280K has no Effect on Bupivacaine Affinity

We also hypothesized that if the MTSEA-mediated decrease in bupivacaine affinity in L1280C was the result of specific modification of the substituted cysteine at L1280, then MTSEA application in L1280K (lysine substitution) should produce no additional changes in bupivacaine affinity. This would be expected because the lysine (K) substitution at L1280 would have already produced the bupivacaine affinity phenotype that we observed in L1280C after MTSEA modification. Our experiments showed that MTSEA superfusion for up to 5 min produced no changes in bupivacaine affinity in L1280K (Fig. 5*C*; $n = 6$).

Discussion

The findings of this study are that 2-aminoethyl methanethiosulfonate (MTSEA) application in wild-type rNav1.4 enhances bupivacaine block, and that MTSEA application in L1280C and S1276C decreases steady-state inactivated bupivacaine block. However, in S1276C, the MTSEA-mediated change in inactivated bupivacaine block is only seen when MTSEA is applied at −160 mV.

We studied these particular mutants (S1276C and L1280C) because our previous study using lysine substitutions implicated these residues in forming part of the local anesthetic binding site (Wang et al., 2000). We used cysteine substitutions in this study because cysteine can be modified by methanethiosulfonate (MTS) reagents (Akabas et al., 1992). This technique is interesting for at least two reasons. First, application of MTS reagents can provide information about the location, specifically the "accessibility", of amino-acid residues in the protein of interest. This aspect of cysteine modification has been called the substituted-cysteine accessibility method (SCAM) (Karlin & Akabas, 1998). Second, MTS modification of cysteine can produce a molecular structure that is similar to other amino acids. For example, reaction of MTSEA with cysteine can produce a structure that is molecularly and physiologically similar to lysine (K) (Middleton, Pheasant & Miller, 1996). Using MTSEA modification of cysteine substitutions in S1276C and L1280C, we tested the hypothesis that our previous observations of decreased inactivated bupivacaine affinity in S1276K and L1280K

(Wang et al., 2000) may be due to the molecular and physiologic properties of the substituted lysine residue.

Some effects on baseline biophysical properties were observed with cysteine substitution in S1276C and L1280C. For example, the steady-state fast inactivation curve was left-shifted in both mutants, and the conductance-voltage curve was right-shifted in L1280C. Changes in these properties are not uncommon with site-directed mutagenesis and probably reflect subtle changes in the complex molecular interactions that underlie these processes.

Before application of MTSEA, neither mutant showed a difference (compared with rNav1.4) in steady-state inactivated bupivacaine block at −50 mV, although in S1276C there was a modest increase in steady-state resting bupivacaine block at −180 mV. Therefore, cysteine substitution alone has little effect on bupivacaine affinity in S1276C and L1280C.

Following application of MTSEA, bupivacaine affinity was altered in rNav1.4, S1276C, and L1280C. One surprising result from our studies was the effect of MTSEA modification on bupivacaine affinity in wild-type rNav1.4. MTSEA modification increased resting and inactivated bupivacaine block in rNav1.4, as well as increased bupivacaine block at intermediate voltages. We propose that this effect may be due to MTSEA modification of an as yet undetermined native cysteine in rNav1.4 (of which there are thirty-eight). We suggest that this modification produces an indirect, allosteric effect that results in increased bupivacaine affinity.

In contrast to rNav1.4, MTSEA application decreased inactivated bupivacaine block in L1280C and S1276C and did not enhance bupivacaine block at other voltages. In light of the results with rNav1.4, the effect may be even greater than that seen with our protocols. That is, because MTSEA in rNav1.4 *increases* bupivacaine block, and MTSEA in L1280C and S1276C *decreases* bupivacaine block, the relative change in bupivacaine affinity in L1280C and S1276C may be greater than the absolute change recorded here.

The MTSEA-mediated change in bupivacaine affinity in L1280C was evident whether MTSA was applied at −160 mV (resting) or at 0 mV (inactivated), i.e., MTSEA accessibility in L1280C *is not* state-dependent. Therefore, the 1280C residue remains "accessible" to MTSEA regardless of the voltage-dependent conformational state of L1280C. This may indicate that L1280 is a residue that lines the ion-conducting pore in rNav1.4.

In S1276C, MTSEA application at −160 mV also decreased bupivacaine block, similar to the result found in L1280C. However, in S1276C, the MTSEA-induced decrease in bupivacaine affinity was not observed when MTSEA was applied to the channels in the inactivated state (0 mV). This result demonstrates that the 1276C residue is only "accessible" to MTSEA when the channels are in the resting (closed) state, i.e., MTSEA accessibility in S1276C *is* state-dependent. Hence, we propose that there is a voltage-dependent change in the relative position of 1276C such that 1276C may face the ion-conducting pore when the channel is at rest, but is "buried" within the protein or otherwise inaccessible to MTSEA when the channel is inactivated.

Also of interest in S1276C is that MTSEA application at 0 mV results in bupivacaine affinity that is remarkably similar to wild-type rNav1.4. Our interpretation of this result is that 1276C is not accessible to MTSEA when the channels are in the inactivated state (as stated above), but that the same cysteine that is modified in rNav1.4 *is* accessible in inactivated S1276C channels. Therefore, when 1276C (or 1280C) *is* modified by MTSEA, the resulting change (decrease) in bupivacaine affinity "overrides" the opposing effect (enhanced bupivacaine affinity) that is found in wild-type rNav1.4. We propose that this is a direct effect of MTSEA modification at residues 1276C or 1280C (or lysine substitution in S1276K or L1280K) that disrupts the normal molecular interaction between bupivacaine and the Na^+ channel local anesthetic binding site.

Our results with other substitutions at L1280 support our conclusions that the changes in bupivacaine affinity are the result of modification of the substituted cysteines in the mutants S1276C and L1280C. When alanine (in L1280A) or tryptophan (in L1280W) was substituted at L1280, MTSEA application resulted in a bupivacaine phenotype similar to wild-type rNav1.4. This would be the expected result if MTSEA was specifically modifying the cysteine substitution in L1280C. Also, in L1280K there was no effect with MTSEA. This is also the expected result because the bupivacaine phenotype (decreased bupivacaine block) has already been established by the lysine substitution in this mutant. In addition, decreased steady-state inactivated bupivacaine affinity is only found in the lysine-substituted mutants (S1276K, L1280K) and the MTSEA-modified cysteine-substituted mutants (S1276C, L1280C). This effect is not found with alanine or tryptophan substitution, or with cysteine substitution alone. This supports our conclusion that the decrease in steady-state inactivated bupivacaine affinity is due to the specific nature of the lysine or MTSEA-modified cysteine side chain, probably due to the addition of a positive charge.

In conclusion, we found that MTSEA modification of S1276C and L1280C results in an increased steady-state inactivated bupivacaine affinity that is similar to that found in S1276K and L1280K. In S1276C, the MTSEA-mediated change in bupivacaine affinity only occurs when MTSEA is applied when the channels are at rest, suggesting a state-

dependent conformational change of this residue. In addition, MTSEA application in wild-type rNav1.4 produces an opposite effect, i.e., an enhancement of bupivacaine block, possibly due to modification of an unknown cysteine, producing an indirect, allosteric effect on bupivacaine affinity.

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