Trimethyloxonium Modification of Batrachotoxin-Activated Na Channels Alters Functionally Important Protein Residues

Diana B. Cherbavaz
Graduate Program in Biophysics, Brandeis University, Waltham, Massachusetts 02254 USA

ABSTRACT The extracellular side of single batrachotoxin-activated voltage-dependent Na channels isolated from rat skeletal muscle membranes incorporated into neutral planar lipid bilayers were treated in situ with the carboxyl methylating reagent, trimethyloxonium (TMO). These experiments were designed to determine whether TMO alters Na channel function by a general through-space electrostatic mechanism or by methylating specific carboxyl groups essential to channel function. TMO modification reduced single-channel conductance by decreasing the maximal turnover rate. Modification increased channel selectivity for sodium ions relative to potassium ions as measured under biionic conditions. TMO modification increased the μ-conotoxin (μCTX) off-rate by three orders of magnitude. Modification did not alter the μCTX on-rate at low ionic strength or Na channel voltage-dependent gating characteristics. These data demonstrate that TMO does not act via a general electrostatic mechanism. Instead, TMO targets protein residues specifically involved in ion conduction, ion selectivity, and μCTX binding. These data support the hypothesis that μCTX blocks open-channel current by physically obstructing the ion channel pore.

INTRODUCTION

Ion channels are integral membrane proteins that mediate passive transport of ions down electrochemical gradients. Voltage-dependent Na channels are responsible for the rising phase of action potentials in electrically excitable cells. They exhibit several hallmark characteristics of ion channel proteins: gating, ion selectivity, and high turnover rate. Many ion channels are natural targets of very high affinity toxins, which have proven to be valuable probes of protein function. Two classes of toxins of particular relevance to this study are the guanidinium toxins, tetrodotoxin (TTX) and saxitoxin (STX) and one of the peptide toxins, μ-conotoxin (μCTX), all of which block open-channel current. Biochemical studies show that μCTX is a competitive inhibitor for TTX binding (Cruz et al., 1985; Moczydlowski et al., 1986b; and Yanagawa et al., 1987), although there is only a partial overlap in their binding domains based on mutagenesis studies (Stephan et al., 1994). μCTX binds to Na channels with high affinity (K_d = 100 nM) under quasiphysiological conditions (Cruz et al., 1985).

In recent years, chemical modification of ion channels has been used to extract structural information from altered protein function. One such reagent is trimethyloxonium (TMO, $(CH_3)_3O^+$), a labile cationic methylating agent. TMO is thought to esterify carboxyl groups, as does a closely related compound, triethyloxonium, which preferentially esterifies catalytically reactive carboxyl groups (Parsons et al., 1969; Yonemitsu et al., 1969; Nakayama et al., 1970). A number of investigators have used TMO to study both native and batrachotoxin (BTX)-activated (although a misnomer, Na channels treated with BTX will be referred to as 'activated' for clarity.) Na channels at the whole-cell level as well as at the single-channel level. (Although a misnomer, Na channels treated with BTX will be referred to as activated for clarity.) They found that TMO modification decreased unitary channel conductance, made channels TTX resistant, did not alter channel activation or inactivation time constants, increased outward sodium current although the reversal potential remained unchanged relative to control channels, and did not alter the permeability ratios of a number of small guanidinium and ammonium ions (Shrager and Profera, 1973; Sigworth and Spalding, 1980; Spalding, 1980). Ca^{2+} block of inward current was reduced by TMO and attributed to a specific change in the conduction pathway (Worley et al., 1986; French et al., 1994). With the exception of the last observation, changes in Na channel function caused by TMO modification were attributed to a change in the surface charge density near the entrance of the ion channel pore resulting from esterification of ionized carboxyl groups. A reduction in the number of negatively charged groups on the surface of the channel protein would lower the local counterion concentration of sodium as well as that of cationic toxins (Bockris and Reddy, 1970; McLaughlin, 1977). It has since been shown that Na channel conductance data measured as a function of ionic strength are consistent with electrostatic models incorporating a low density of surface charge (Green et al., 1987a; Cai and Jordan, 1990; Cherbavaz, 1991; Latorre et al., 1992; Naranjo et al., 1994). How is it that surface charge influences protein function? Negative surface charge density on a protein arises from discrete ionized functional groups such as aspartate, glutamate, or sialic acid residues. Effective surface charge density refers to the charge density that attracts counterions (permeant ions or charged toxin molecules) resulting in an apparent increase in ion channel conductance or an enhancement in a charged toxin's activity. This enhancement is most pronounced at low ionic strength

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Address reprint requests to Dr. Diana B. Cherbavaz, Department of Biochemistry and Biophysics, University of California, San Francisco, 513 Parnassus Ave., San Francisco, CA 94143-0448. Tel.: 415-476-3937; Fax: 415-476-1902; E-mail: cherbavaz@msg.ucsf.edu.

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and does not influence protein function at high ionic strength as discrete charges are shielded or adsorb counterions. For a complete discussion of electrostatic double layer theory, see the excellent review of McLaughlin (1977).

In the present phenomenological study, changes in skeletal muscle BTX-activated voltage-dependent Na channel function caused by TMO treatment were examined by high resolution single-channel current-recording techniques. These experiments were designed to determine whether TMO modification altered channel function by a general through-space electric double layer mechanism or whether changes were mediated by alteration of carboxyl groups essential to channel function. My data show that TMO modification of the extracellular side of Na channels decreased unitary channel conductance, altered channel selectivity, and increased the μCTX off-rate. The effects of TMO modification are independent of large changes in ionic strength. These results indicate that TMO-mediated changes cannot not be explained by a general electrostatic mechanism that would decrease in the extracellular surface charge density. Instead, data can be explained by changes specific to the sodium conduction pathway and toxin-binding domain, indicating an overlap of these functional domains. Furthermore, these results strongly suggest that μCTX blocks open-channel current by physically occluding the ion channel pore. Some aspects of this work were previously presented in abstract form (Cherbavaz, 1990; Cherbavaz and Miller, 1991).

MATERIALS AND METHODS

Experimental system

Na-channel-rich transverse tubule vesicles from rat skeletal muscle were prepared as described previously (Moczydlowski and Latorre, 1983), with the following adaptations. All solutions included K-MOPS buffer (pH 7.4). The final membrane fractions were collected from above a 28% sucrose cushion (w/v) and resuspended with a 22-gauge syringe.

Most data were collected with a home-built two-piece Lucite chamber with replaceable Teflon film partitions (a gift from Alan Finkelstein, Albert Einstein College of Medicine, New York, NY). The chamber design minimized volumes that were poorly exchanged during perfusion. Planar lipid bilayers were cast from a 7:3 mixture of 1-palmitoyl-2-oleoylphosphatidylethanolamine and the corresponding choline (Avanti Polar Lipids, Birmingham, AL) at 20 mg/ml in *n*-decane (Gold Label, Aldrich Fine Chemicals, Milwaukee, WI). Ion channel fusion was favored by a large osmotic and electrochemical gradient. *Cis* was defined as the chamber to which vesicles were added, and *trans* was defined accordingly. Channel orientation was determined from voltage-dependent gating characteristics or by blockage of current by TTX. The extracellular solution was defined as ground and traces presented are in accordance with the standard convention, except for the recordings in Fig. 1. Exchange from fusion conditions to experimental conditions required extensive perfusion (at least 10-fold excess of the chamber volume).

Experimental conditions and toxins

The ambient laboratory temperature was maintained between 20 and 23°C at low relative humidity. Na solutions were prepared from a base solution of either 1, 3, 5, or 10 ml of fresh 1 N NaOH standard per 1 liter of ddH_2O, the acid form of 0.1 mM EDTA, and adjusted to pH 7.01 ± 0.03 with solid MOPS. Higher concentration Na solutions were prepared by addition of solid NaCl, and the pH was readjusted. Potassium solutions were prepared in a similar manner except that the potassium hydroxide solution was made from the solid and titrated against a fresh 1 N HCl standard to determine the equivalence point.

Lyophilized BTX (a generous gift from Dr. John Daly, National Institutes of Health, Bethesda, MD) was suspended in 95% EtOH to a final concentration of 500 μM, and stored at −70°C. Sodium citrate-free crystalline TTX (CalBiochem, La Jolla, CA) was prepared by acidification followed by suspension to a final concentration of 1 mM TTX in 100 mM sodium citrate and stored at −70°C. Synthetic μ-conotoxin GIIIA (a gift from Dr. Robert Gordon, Max-Planck Institute, Frankfurt, Germany) was suspended to a final concentration of 100 μM in ddH20. Lower concentrations were diluted into 100 mM NaCl and 0.5 μg/ml bovine serum albumin (BSA), which was included to prevent toxin loss due to nonspecific binding. BSA (30–45 μg/ml) was added to chamber solutions while stirring before the addition of μCTX.

TMO methodology

Single BTX-activated Na channels incorporated into planar bilayers were modified by TMO tetrafluoroborate in situ by one of two techniques (Fluka Chemical Co., Ronkonkoma, NY). Initially, the procedure used was analogous to those previously published (Worley et al., 1986; MacKinnon and Miller, 1989). Later, a substantial decrease in time between weighing and adding TMO to the experimental chamber (<1 min) required addition of less than 1 mM TMO (final concentration), significantly less then previously required (20–30 mM). The decrease in TMO concentration required to produce modification reduced the buffering capacity necessary to adsorb protons generated by the reaction to a single 20-ml perfusion of 50 mM MOPS, pH 7.5.

The latter method yielded a TMO modification success rate greater than 75% (in which success was defined as an experimentally viable membrane containing a TMO-modified channel); the overall success rate including both procedures was 60%. Modification was always observed when both channel and membrane survived chemical treatment irrespective of procedure used.

Data collection and analysis

A standard voltage-clamp circuit was used, followed by a home-made amplifier in series with a low pass, eight-pole active filter. Voltage was controlled by an on-line laboratory data acquisition computer (Indec, Sunnyvale, CA). All software driving hardware control, data acquisition, and data analysis routines were written in-house by Dr. Christopher Miller and other members of the Miller group.

On-line open-channel-current voltage ramps were computer assisted, stored, and analyzed (1341A HP display, Indec Laboratory computer system). A capacitive compensation circuit was used to subtract gross transients in membrane capacitance associated with changes in applied potential. The baseline membrane current was determined from toxin block of open-channel current, computer averaged, and digitally subtracted. Individual conductance measurements were averaged from 64 open-current voltage sweeps.

Reversal potentials were extracted from open-channel-current voltage ramps. After the appearance of one or two channels, solutions were perfused to 100 mM Na-MOPS external and 100 mM K-MOPS internal. An analogous procedure was performed for TMO-modified Na channels except a single channel per membrane was required. Chemical modification was performed before perfusion to experimental conditions, and modified channels were blocked with 100–400 nM μCTX.

Gating measurements were determined from control or modified channel open probabilities, *Po*, and were acquired from membranes containing one channel. Current data were recorded on VHS tape for subsequent computer analysis. Theoretical fit to the voltage-dependent gating equation was performed by a least squares routine based on program 11–5 Bevington (Data reduction and Error Analysis, 1969, McGraw-Hill, New York, 204–246) translated from FORTRAN into BASIC by Dr. Steven A. Sundberg. The μCTX block of control and TMO-modified channels was recorded at +50

mV with a cutoff frequency of 50 Hz. The minimum open and closed times applied during computer acquisition of data were 100 and 200 ms, respectively. An additional correction to subtract spontaneous channel closure was not applied (Moczydlowski et al., 1984). Block and unblock time constants were extracted from an exponential regression fit to data. Both analysis and generation of figures were performed with the Lotus Freelance program.

RESULTS

Single BTX-activated Na channel conductance data were recorded from open-channel-current voltage ramps, between −70 and +70 mV, under symmetric sodium concentrations between 10 mM and 1 M total Na. The average control conductance data used below are presented in Table 1.

TMO modification reduces single-channel conductance

The extracellular side of BTX-activated rat skeletal muscle Na channel proteins incorporated into neutral planar lipid bilayers were treated with TMO. Fig. 1 presents continuously recorded current traces from two different Na channels before, during, and after addition of TMO. The instantaneous decrease in channel current corresponds to the TMO reaction (indicated by arrows). Sixty percent of the 52 modifications attempted were successful (failure was defined as channel disappearance or an extreme increase in membrane current rendering interpretable data collection impossible). Altered channel behavior was observed for all channels that survived TMO treatment.

Experiments were designed to determine the mechanism by which TMO alters Na channel conductance. Does TMO change channel function via a general through-space electrostatic mechanism by methylating carboxyl groups that concentrate permeant sodium ions near the channel pore, or does it modify carboxyl groups specifically involved in ion transport? The appropriate experiments to distinguish between these two hypotheses required measuring the conductance of a single TMO-modified channel over a wide range of sodium concentrations. Fig. 2 presents the average conductance of control (*solid symbols*) as well as the conductance of a single TMO-modified Na channel (*open symbols*) plotted as a function of Na concentration. TMO-modified Na channel conductance paralleled that of the control population over a two-order-of-magnitude change in ionic strength (from 10 mM to 1 M Na).

TABLE 1 Mean slope conductance of control and TMO-modified BTX-activated Na channels measured at different sodium concentrations

[Na] (mM)	γ_s (pS)	n
10	10.4 ± 0.4	8
30	14.7 ± 0.3	7
100	17.3 ± 0.2	6
300	20.5 ± 0.2	6
1000	24.8 ± 0.2	4

The ratio of TMO-modified channel conductance (γ^{TMO}) to the average conductance of the control population (γ^{CNTL}) is presented as a function of Na concentration for four individual TMO-modified channels in Fig. 3. Although the conductance ratio, $\gamma^{TMO}/\gamma^{CNTL}$, was different for different modified channels, the ratio for a given modified channel remained nearly constant over a wide range of ionic strengths. Conductance ratios were measured for a number of different TMO-modified channels and range from 0.37 to 0.89. The variation in the observed conductance ratios suggests that different channel behavior results from modification of different active site residues. The most common ratio observed corresponded to approximately a 30% decrease in channel conductance.

TMO modification enhances Na selectivity

TMO-treated BTX-activated Na channels are more selective for sodium over potassium ions than are control channels.

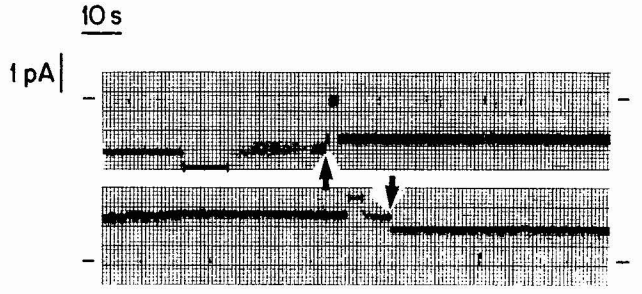

FIGURE 1 Continuous current recordings for two different BTX-activated Na channels before, during, and after addition of TMO. A decrease in channel current marks the moment at which TMO modification occurred (*arrows*) and the horizontal bars indicate the time required for TMO addition. Approximately 3 mg of solid TMO (<1 mM) was added to the chamber solution while stirring continuously. Traces are presented along with an antiphysiological current convention; hence, channel closures correspond to upward changes in current for the top trace recorded at a holding potential of 50 mV and to downward changes for the bottom trace recorded at −50 mV. Tick marks designate current level at zero applied voltage and the cutoff frequency was 50 Hz.

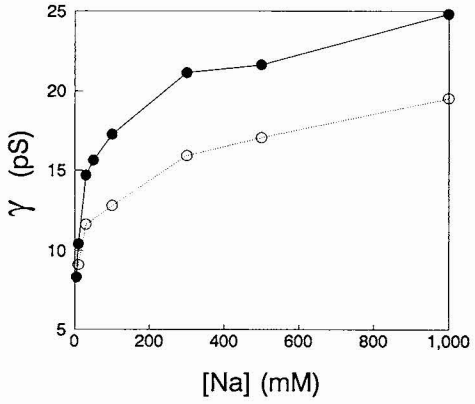

FIGURE 2 TMO modification decreases BTX-activated Na channel conductance. The conductance of a single TMO-modified Na channel (*open symbols*) was measured at a number of different sodium concentrations. Control data (*solid symbols*) represent an average of four or more independent measurements and are presented for comparison (Table 1). Symbols are greater than or equal to the standard error of the mean.

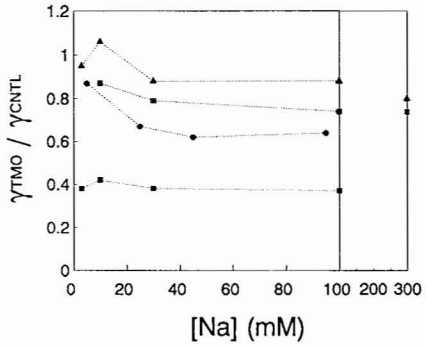

FIGURE 3 Conductance ratios for TMO-modified Na channels plotted as a function of sodium concentration. TMO-modified channel conductance data were collected for four individual channels. Although the maximal channel conductance was different for different modified channels, the conductance ratio ($\gamma^{TMO}/\gamma^{CNTL}$) remained flat or tended to decrease with increasing ionic strength.

Fig. 4 presents two superposed open-channel-current voltage ramps of the same Na channel before and after treatment with TMO. Data were recorded under biionic conditions with 100 mM Na-MOPS, pH 7.0, external and 100 mM K-MOPS, pH 7.0, internal, where external and internal correspond to the extracellular and intracellular sides of the ion channel protein, respectively. Two features are immediately noticeable: (1) the modified channel conductance decreased relative to the control channel conductance and (2) the reversal potential

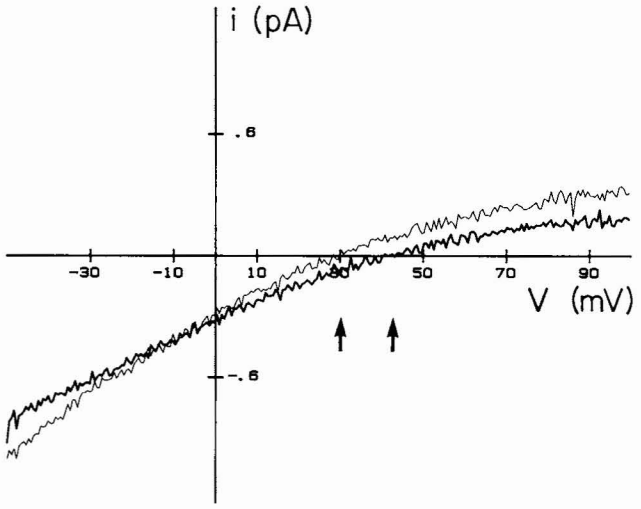

FIGURE 4 TMO modification alters Na channel selectivity as observed under biionic conditions. Open-channel-current voltage ramps of one BTX-activated Na channel before (control, *fine line*) and after (TMO, *bold line*) TMO modification. Data were recorded from asymmetric ion conditions: 100 mM Na-MOPS extracellular relative to 100 mM K-MOPS intracellular. Permeability ratios (P_{Na}/P_K) were determined from the Goldman-Hodgkin-Katz (GHK) relation applied to symmetric monovalent biionic conditions: $V_{REV} = RT/F^*\ln(P_{Na}/P_K)$, where R, T, and F are defined as usual, and P_{Na} and P_K are the sodium and potassium permeabilities (Hille, 1992). The reversal potentials, V_{REV}, are approximately 35 and 45 mV for control and modified channels, respectively, and are marked by arrows. The starred values in Table 2 correspond to the data presented. These data were recorded with a low-pass filter frequency of 50 Hz.

shifted to a more depolarized potential relative to unmodified channels. Table 2 presents the permeability ratios for five independent control channels and four independent TMO-modified channels, each recorded from a different membrane. Comparison of permeability ratios and conductance ratios shows no correlation between the change in selectivity and the decrease in channel conductance. Permeability ratios were calculated from the Goldman-Hodgkin-Katz equation (Hille, 1992) simplified for biionic conditions. Modification caused a shift in the reversal potential of greater than 10 mV, corresponding to a 60% increase in the permeability ratio from an average of 3.7 ± 0.1 for control channels to an average of 5.9 ± 0.5 for TMO-modified channels. The starred experimental values presented in Table 2 highlight the statistics that correspond to the channel recording presented in Fig. 4. Measurement of permeability ratios for control channels are consistent with previous observations (Garber, 1988; Fig. 4 and Table 2).

Voltage-dependent gating characteristics

No difference was observed between control and modified channel voltage-dependent gating behavior. Current traces recorded for a control Na channel and three independent TMO-modified Na channels at different holding potentials are illustrated in Fig. 5 *A*. The voltage-dependent gating equilibrium of ion channels is described by the Boltzmann distribution between the conducting and nonconducting states (Moczydlowski, 1986a). Two parameters were extracted from a Boltzmann fit to data (Fig. 5 *B*). They are V_o, the voltage at which channels are statistically open one-half of the time, and $z\delta$, the apparent gating charge that is determined from the slope of the curve. The averaged voltage dependence of BTX-activated Na channel open probability from a control population is presented in Table 3 ($z\delta = 6.2 \pm 1.1$ and $Vo = -89 \pm 7$ mV averaged from five independent channel measurements), as well as the open probabilities determined from three independent TMO-treated Na channels. The range of the TMO-modified channel gating parameters falls within the standard error determined from the control population (SE is presented in Table 3). Data were fit by a least squares fitting routine (control population, solid

TABLE 2 Permeability ratios for control and TMO-modified Na channels

Control	TMO-Modified	
P_{Na}/P_K	P_{Na}/P_K	γTMO/γCNTL
3.5	4.8	0.82
3.5	6.7	0.55
3.7	6.7	0.43
3.8*	5.5*	0.53*
3.8		

Each data entry was determined from an independent channel measurement. The mean permeability ratios and standard deviations for control and TMO-modified channel data are 3.7 ± 0.1 and 5.9 ± 0.5, respectively.
*Statistics that correspond to the channel recording presented in Fig. 4.

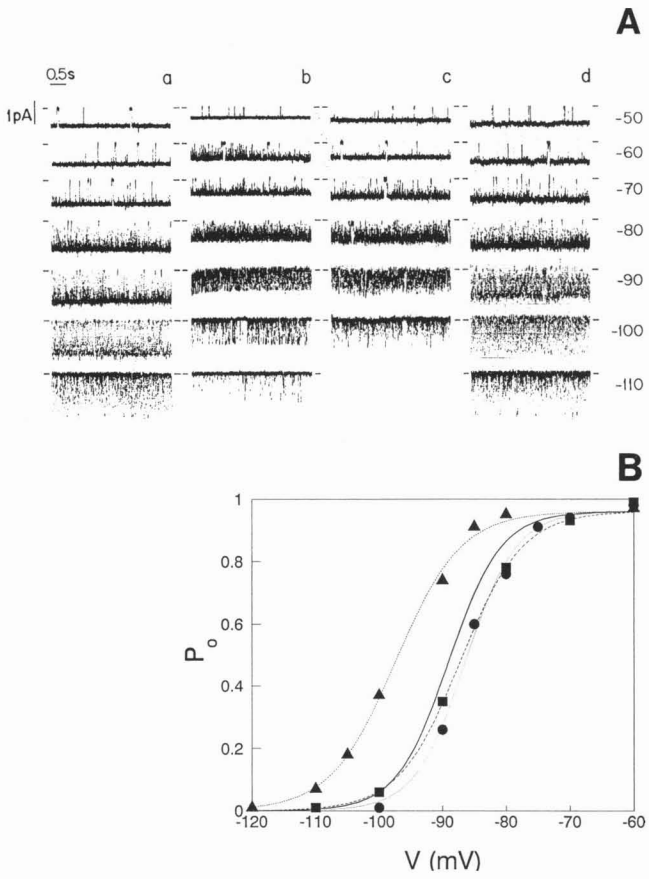

FIGURE 5 BTX-activated Na channel gating of control and TMO-modified Na channels. (*A*) Current traces for both control (column *a*) and three different channels modified by TMO (columns *b, c,* and *d*) are presented. Data were acquired under symmetric 100 mM sodium conditions and filtered using a 150-Hz cutoff frequency. Tick marks designate the current at zero applied voltage. (*B*) Gating curves for control and three independent TMO-modified Na channels. Gating parameters determined from a least squares fit to each of five control data sets were averaged and used to generate the solid curve. Open probabilities of the three TMO-modified channels are presented as solid symbols and interrupted curves. The gating relation used to fit the data is $\ln(P_o/(0.96 - P_o)) = z\delta F/RT(V - V_o)$, where P_o is the probability of a channel being open, $z\delta$ is the apparent gating charge, F, R, and T are defined as usual, V is the holding voltage, and V_o is the voltage at which $P_o = 0.5$ (Hille, 1992). The maximum open probability used was $P_{o,max} = 0.96$ (Moczydlowski et al., 1984). The corresponding gating parameters are presented in Table 3. These and all gating data were filtered at 150-Hz.

curve; individual TMO-modified channels, stippled curves). The maximum open probability, $P_{o,max}$, used was 0.96 (Moczydlowski et al., 1984).

BTX-activated Na channels blocked by μCTX GIIIA

μCTX GIIIA block of BTX-activated Na channel current is characterized by very long-lived block events. The toxin block events presented in Fig. 6 *A* were recorded at several different concentrations of bulk sodium (10, 30, and 100 mM Na-MOPS, 0.1 mM EDTA, pH 7.0) in the presence of 30–45 μg/ml BSA while stirring continuously. Toxin block and

TABLE 3 BTX-activated Na channel gating parameters for control and TMO-modified channel data

	$z\delta$	V_o (mV)
CNTL ± SE (n = 5)	6.2 ± 1	−89 ± 7
TMO (triangles)*	5.0	−97
TMO (circles)*	6.4	−86
TMO (squares)*	5.3	−87

*Corresponds to the three independent TMO-modified Na channels depicted in Fig. 5 *A*.

unblock durations were measured and the distributions are presented in Fig. 6, *B* and *C*, respectively. A semilogarithmic fit of the dwell-time histograms was used to extract mean block and unblock times and calculate toxin off-rate and on-rate constants. As expected for a reversible toxin-binding equilibrium, both block and unblock times were exponentially distributed. The histograms presented in Fig. 6, *B* and *C* were prepared from a single Na channel recording consisting of approximately 100 toxin-binding events; most other rate constants were extracted from data sets consisting of approximately 35 transitions.

μCTX on-rate varied greatly with increasing ionic strength. As the sodium concentration was increased, it was necessary to increase the μCTX concentration to maintain an equivalent open channel probability. Unlike the on-rate, the μCTX off-rate showed little change as the ionic strength was increased (Table 4).

μCTX blocks TMO-modified BTX-activated Na channels

After TMO modification, μCTX was still observed to block open channel current. Fig. 7 *A* presents several current traces each recorded with a higher concentration of μCTX present. As expected, the number of toxin blocking events increased with an increase in the toxin concentration. μCTX block and unblock durations were observed to be exponentially distributed for modified channels (histograms of Fig. 7, *B* and *C*), which is characteristic of pseudo-first-order binding reactions.

μCTX block of single-channel current was measured at several different symmetric sodium concentrations (10, 30, and 100 mM) for both control and TMO-treated channels (Fig. 8). The most striking difference between the control and modified channel behavior was the residence time of μCTX. Toxin block events of modified channels were short-lived (<1 s), whereas those observed for control channels are typically many tens of seconds. TMO modification decreased toxin residence time by up to three orders of magnitude. Different TMO-treated channels exhibited different μCTX residence times, although the decrease in channel conductance caused by modification was identical (Fig. 8; $\gamma^{TMO}/\gamma^{CNTL} = 0.69$ and 0.7 for traces *b* and *c*, respectively. μCTX off-rates were observed to be independent of toxin concentration for both control and TMO-modified channels. Table 4 presents μCTX off-rates for control channels and three independent TMO-treated channels (TMO-I, TMO-II, and TMO-III).

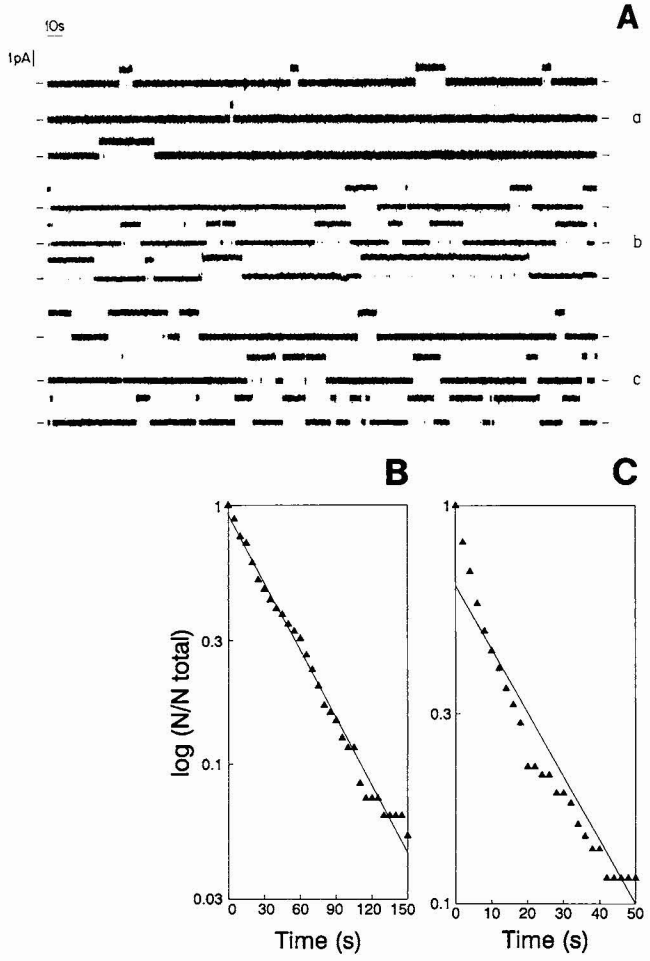

FIGURE 6 μCTX block of BTX-activated Na channels. (*A*) μCTX block of single Na channels was measured at 10, 30, and 100 mM sodium and 0.03, 0.3, and 30 nM μCTX (traces *a, b,* and *c*, top to bottom). Each triple set of current traces was prepared from a single continuous recording (top to bottom). BSA (30–45 μg/ml) was added to the external chamber solution before addition of μCTX, and data were recorded at 50 mV while the solution was stirred continuously. The cutoff frequency was 50 Hz. Channel unblock events correspond to upward changes in the current level. The tick marks designate the current level at zero applied voltage. (*B*) μCTX block time histogram and (*C*) μCTX unblock time histogram. The number of toxin block events, *N*, was determined for each time bin, normalized to the total number of events, N/N_{total}, and plotted as a function of time. A semilogarithmic fit of data by Lotus Freelance software package was used to determine the off-rate (k_{off}) and on-rate (k_{on}) constants, which are 2.0×10^{-2} s^{-1} and 1.5×10^{8} M^{-1} s^{-1}, respectively. These histograms were generated from 95 events acquired at 30 mM sodium and 0.3 nM μCTX and at an applied potential of 50 mV.

μCTX on-rates for control and TMO-modified channels were observed to be highly sensitive to ionic strength. At low ionic strength, on-rates for modified channels were comparable with those observed for control channels; at high ionic strength, on-rates for modified channels were less than those observed for control channels (Table 4). μCTX on-rate measurements for TMO-modified channels can be used only to determine the general trend relative to increasing ionic strength as the percentage of time μCTX blocked modified channels was much less than the percentage of time μCTX blocked control channels under comparable conditions. That is, toxin block events of modified channels at high ionic strength were few and infrequent although the μCTX concentration necessary to block those channels greatly exceeded (20–50 times) the amount necessary to block control channels one-half of the time. Observations were made using toxin concentrations that fell within the linear domain of the toxin dose-response regime.

TABLE 4 The μCTX on- and off-rate constants measured for control and TMO-modified BTX-activated Na channels measured as a function of symmetric [Na]

[Na] (mM)	CNTL k_{on} ± SE (10^6 M^{-1} s^{-1})	*n*	k_{on} (10^6 M^{-1} s^{-1}) TMO-I	TMO-II	TMO-III
10	1400 ± 1000	4	1200	920	
30	500 ± 400	3	220	24	
100	1.9 ± 1	3	0.2	0.1	
[Na] (mM)	k_{off} ± SE (10^{-3} s^{-1})	*n*		k_{off} (s^{-1})	
10	6 ± 1	6	0.50	3.8	1.01
30	18 ± 2	6	0.62	3.2	0.84
100	26 ± 3	4	0.73	3.1	1.19

DISCUSSION

Data presented in this study demonstrate that TMO modifies BTX-activated rat skeletal muscle voltage-dependent Na channels by targeting functionally important protein residues. This observation is not surprising in light of previous reports (Shrager and Profera, 1973; Sigworth and Spalding, 1980; Spalding, 1980; Worley et al., 1986). What is surprising is which channel functions were affected and how they were altered by TMO. Modification enhanced channel selectivity for Na ions relative to K ions at the expense of maximal channel conductance, and modification decreased μCTX block time. TMO modification did not alter the μCTX on-rate at low ionic strength or the voltage dependence of channel gating. The data are consistent with the notion that TMO modifies functional groups involved in ion conduction, ion selectivity, and toxin binding. These observations support the hypothesis that several protein residues are involved in multiple protein functions and suggest that μCTX blocks open-channel current by physically occluding the channel pore.

TMO is considered a carboxyl group modifier by analogy to the preference exhibited by a closely related compound, triethyloxonium (TEO). TEO reacts with carboxyl groups as well as the sulfur of methionine and the imidizole nitrogen of histidine in aqueous solution (Yonemitsu et al., 1969). TEO fails to react with hydroxyl, amine, or guanidinium groups. When compared with other carboxylate modifiers, carbodiimide and glycine ethyl ester, TEO preferentially alkylated the catalytically reactive carboxyl groups of trypsin (Nakayama et al., 1970). Furthermore, when lysozyme was

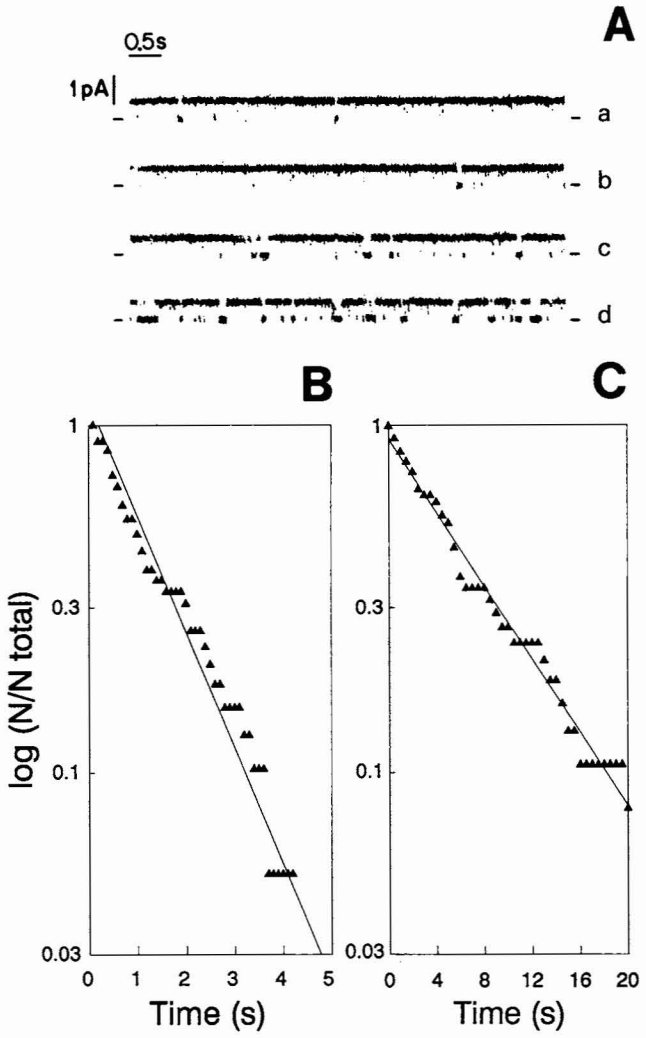

FIGURE 7 μCTX blocks TMO-modified BTX-activated Na channels. (*A*) μCTX block of a TMO-modified channel was recorded at different toxin concentrations: no toxin added, 0.3 nM, 3 nM, and 10 nM μCTX (traces *a*, *b*, *c*, and *d*, respectively). Although the block times are substantially shorter than those observed for control channels under identical conditions, the number of blocking events increased as the μCTX concentration was increased. The two closures in trace *a* (condition: no toxin added) were probably due to residual toxin from the previous data acquisition condition (measurement of μCTX block of this TMO-treated channel at 10 mM Na-MOPS) as complete removal of the highly charged toxin was not possible at low ionic strength. Data were recorded at 30 mM sodium, 40 μg/ml BSA, while the solution was continuously stirred, and the holding potential was 50 mV. (*B*) μCTX block time histogram of TMO-modified Na channel. Toxin block events still exhibit an exponential distribution although the μCTX block times were decreased by TMO modification. The corresponding off-rate is 0.8 s^{-1}. (*C*) μCTX unblock time histogram of a TMO-modified Na channel. μCTX unblock events of TMO-treated channels are also exponentially distributed. The on-rate is 1.2×10^7 M^{-1} s^{-1}. These histograms were prepared from 39 events recorded from a single TMO-modified channel at symmetric 30 mM sodium, in the presence of 5 nM μCTX and 40 μg/ml BSA, while continuously stirring the solution. The holding potential was 50 mV and the filter frequency was 50 Hz.

treated with TEO, carboxyl groups were exclusively methylated (Parsons et al., 1969). Esterification by TEO is pH dependent; its reactivity increases with increasing pH, indicating that negatively charged groups are more susceptible to attack. Both TEO and TMO are labile in aqueous conditions with half-lives reported to be 10 and 7 s, respectively (Nakayama et al., 1970; MacKinnon and Miller, 1989). It is likely that cationic TMO preferentially targets ionized catalytically reactive carboxyl groups of Na channels based on the precedent reviewed although the actual residues TMO alters cannot be ascertained without amino acid analysis.

The decrease in Na channel conductance caused by TMO modification has previously been attributed to a change in

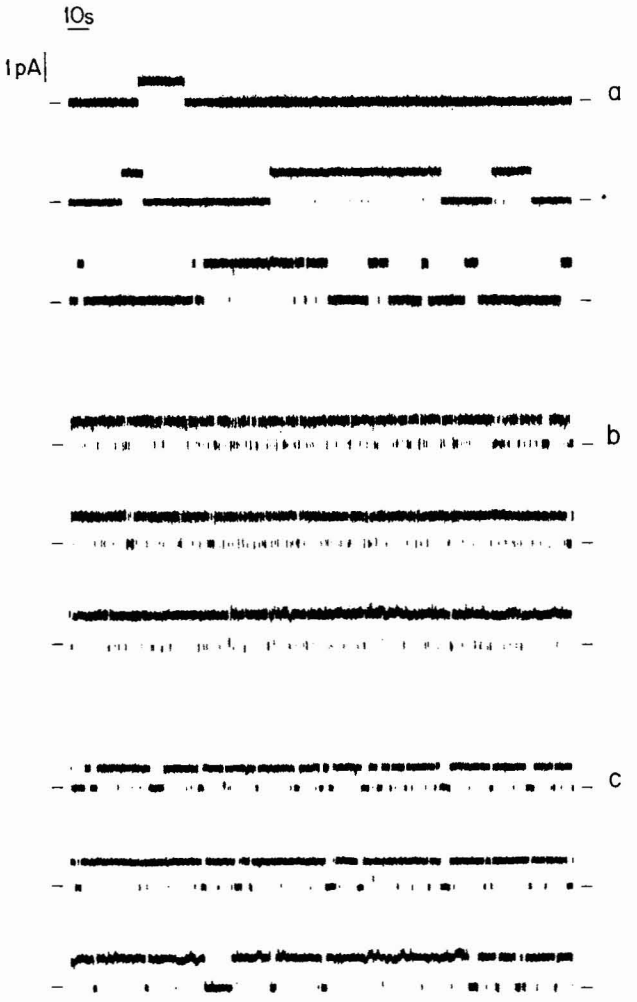

FIGURE 8 μCTX block of TMO-modified Na channels recorded at different sodium concentrations. μCTX block of a control (trace *a*) and two TMO-treated channels (traces *b* and *c*) were recorded at 10, 30, and 100 mM Na-MOPS (top to bottom within a given set of data traces). An experimental data set required recording data for each TMO-modified channel at several different sodium concentrations to determine the mechanism by which TMO alters μCTX block characteristics. Data were recorded, in the presence of 40 μg/ml BSA while the aqueous solution was continuously stirred (the source of excess noise), at 50 mV, and 50 Hz. The μCTX concentrations present during data acquisition were, from top to bottom within each set of traces, as follows: 0.03, 0.3, and 30 nM (trace *a*); 0.3, 10, and 1500 nM (trace *b*); and 0.1, 5, and 600 nM (trace *c*). Relatively high μCTX concentrations were necessary to block the two TMO-modified channels. Channel closures were defined as lasting longer than 0.2 s and channel openings were defined as lasting longer than 0.1 s and these bounds were applied during computer acquisition of data. No additional correction to adjust for spontaneous channel closures was applied.

surface charge density near the ion channel pore (Sigworth and Spalding, 1980; Spalding, 1980). The idea is a that cluster of negatively charged carboxyl groups near the pore influence channel conductance by increasing the local cation (permeant ion) concentration. TMO was thought to react with and neutralize those surface charges, thereby lowering the apparent channel conductance by lowering the local permeant ion concentration as predicted by electric double-layer theory. Double-layer effects were observed for TMO modification of Ca-activated K channels (K_{Ca} channels) (MacKinnon and Miller, 1989; MacKinnon et al., 1989). TMO modification decreased K_{Ca} channel conductance, but the conductance ratio increased to unity with increasing ionic strength. Two other sets of experiments showed that other channel functions were influenced by removal of surface charge. The voltage dependence of gating shifted to a more depolarized potential after treatment with TMO. This change was attributed to a less negative extracellular surface charge density. A less negative surface charge density would result in a less negative transmembrane potential and shift the gating curve to a more depolarized potential. The observation was consistent with the predicted effect. Furthermore, the highly charged charybdotoxin on-rates for TMO-modified K_{Ca} channels differed from control channels only at low ionic strengths at which surface charge effects are pronounced. The on-rates were identical at high ionic strength. Finally, there were no changes observed in charybdotoxin off-rates or ion selectivity. The differences observed between modified and control K_{Ca} channel function were pronounced at low ionic strength and negligible at high ionic strength. That trend is exactly predicted by double-layer theory. Direct comparison of K_{Ca} channel and Na channel data demonstrates that the effects of TMO modification on Na channel function are of a completely different nature from those changes that alter K_{Ca} channel function.

In the present experiments, assays that were expected to be influenced by a change in surface charge density remained unaffected by TMO modification whereas assays that should have remained independent of changes in surface charge density were altered. The voltage dependence of Na channel gating did not change after TMO modification, indicating no change in the transmembrane potential or net extracellular surface charge density. TMO modification did not alter μCTX on-rate at low ionic strength, suggesting that the effective surface charge density, thought to enhance charged guanidinium toxin on-rates at low ionic strength (Green et al., 1987b; Ravindran and Moczydlowski, 1989), was unaltered. In contrast, the conductance ratio remains constant with increasing ionic strength, Na channel selectivity was enhanced, and μCTX off-rate was significantly increased by modification. These observations are the antithesis of the K_{Ca} channel observations (MacKinnon and Miller, 1989 and MacKinnon et al., 1989).

The most significant and novel effect of TMO modification was alteration of Na channel selectivity. Modified channels were more selective for sodium ions relative to potassium ions. To achieve such an effect, TMO must react with residues that are part of the selectivity filter along the conduction pathway. It is a curious observation that esterification of carboxyl groups, i.e., addition of a methyl moiety to a hydrogen-bonding water replacement group, would enhance discrimination between two hydrated ions. As the unhydrated and partially hydrated atomic radii of sodium ions are smaller than those of potassium ions (Bockris and Reddy, 1970; Hille, 1992), the change in selectivity caused by TMO modification suggests that a physical constriction, or mechanical sieve, in the conduction pathway may be an important component in the selectivity mechanism for this type of Na channel. The turnover rate through the channel was significantly decreased and enhancement of ion specificity was purchased at the expense of maximal channel conductance. The second surprising effect of TMO modification was the enormous change in the μCTX off-rate, which increased by several orders of magnitude relative to the off-rate measured for control channels under identical conditions. The observed changes in the μCTX off-rate cannot be explained by a general electrostatic model because there is a complete absence of ionic strength dependence. Instead, these data suggest that the inherent binding stability resides in noncovalent interactions such as hydrogen bonding. Methylation of residues in the toxin-binding surface destabilizes attractive binding interactions resulting in rapid dissociation of the toxin molecule. Furthermore, as TMO modification changes both ion selectivity and μCTX off-rate, one may conclude that the same protein residues are involved in both functions, and this study supports the idea that μCTX blocks the pore of these Na channels as mounting evidence suggests (Becker et al., 1992; Lipkind and Fozzard, 1994).

Some of the observations presented here seem to contradict previous reports. Spalding (1980) investigated the effect of TMO modification on frog skeletal muscle Na channel currents permeability ratios of small ammonium and guanidinium cations and observed no significant change in channel selectivity relative to sodium ions. A direct comparison between those measurements made under a sodium Ringer solution and the permeability ratios determined here for two inorganic ions (sodium and potassium) under biionic conditions is at best difficult. Experimental conditions were completely different as well as the class of permeant ions under investigation. On the other hand, Worley et al. (1986) and French et al. (1994) concluded that the change in Ca block of TMO-modified Na channels could not be explained by a change in surface charge density but must represent a change in the Ca block site. Furthermore, the concomitant reduction of channel conductance and decrease in TTX/STX sensitivity as well as the decrease in Ca block pointed to modification of a specific site in the conduction pathway. They could not differentiate between a complete loss of toxin binding or a significant reduction in affinity of greater than two orders of magnitude. The interpretation of those observations are consistent with the data presented here, including the suggestion of a tremendous decrease in toxin affinity. In

this study, the affinity of a different class of toxin, μCTX, decreased by approximately three orders of magnitude after modification.

Worley et al. (1986) also observed that TMO modification reduced rat brain Na channel conductance by 37%. A previous study that used nonstationary fluctuation analysis of Na channel currents recorded from amphibian muscle Na channel currents under Na Ringer solution, showed TMO modification to reduce channel conductance by 70% (Sigworth and Spaulding, 1980). In this study, modification was observed to be variable for different channels treated. The most common change corresponded to a 30% decrease in channel conductance. These differences in the percent decrease in Na channel conductance observed in these three independent studies may reflect species-to-species differences in the number of residues that are susceptible to TMO attack.

TMO modification of rat skeletal muscle Na channels did not alter channel voltage-dependent gating characteristics. As the voltage-sensing apparatus of voltage-gated channels is very sensitive to changes in transmembrane potential, one can infer that TMO modification did not change the apparent surface charge density of these Na channels. How can a highly reactive reagent that preferentially donates a methyl moiety to negatively charged functional groups not alter the apparent surface charge density of the treated protein? One must consider the assumptions of electric double-layer models (Bockris and Reddy, 1970; McLaughlin, 1977). Although these models have proven very useful in understanding and explaining phenomena, they do approximate the surface potential created by discrete atomic charges by assuming a uniform smear of charge over a defined area. That assumption minimizes the contribution of specific charges as long as the total number of charges and charge density remain unchanged. Consider two examples of neutralizing carboxyl groups that would not alter the apparent charge density. (1) Approximately 30% of the molecular weight of mammalian voltage-dependent Na channel protein is contributed by sugar residues, approximately one-half of which are ionizable sialic acids (Barchi, 1983; Roberts and Barchi, 1987). The estimated number of sialic acid resides on electric eel Na channels is upward of 100, and they occur in tandem chains of 10 or more residues (James and Agnew, 1989). Ionization of these sialic acid residues is dependent on their pK_a as well as the electrostatic environment that governs the local pH by concentrating protons. A high density of sialic acid residues will consist of both ionized and protonated species in equilibrium. One may imagine TMO esterifying many sialic acid carboxylates and not significantly changing the overall charge density as another carboxyl group may be ionized for each one esterified in accordance with the local equilibrium. (2) The selectivity filter of an ion channel protein is thought to be located along the conduction pathway within the channel pore (Hille, 1992). One may imagine that TMO enters the conduction pathway and attacks catalytically reactive carboxylates. This hypothesis is supported by the fact that TMO changes Na channel selectivity. Neutralization of a sequestered charge would have little influence on the average charge density on the surface of the protein. In either case, methylation of an ionized carboxylate by TMO may indeed neutralize a charged group but would not necessarily alter the net surface charge density that influences protein function.

Finally, although TMO modification was always observed to decrease conductance, alter selectivity, and reduce toxin affinity, different channels modified with TMO exhibited different functional characteristics. For example, a different percent decrease in channel conductance was observed for different modified channels relative to control channels (Fig. 3 and $\gamma^{TMO}/\gamma^{CNTL}$ presented in Table 2). In fact, all functional characteristics that were altered by TMO modification exhibited some diversity in that trait after the modification procedure (permeability ratio, Table 2; μCTX off-rates, Fig. 8 and Table 4). These differences suggest that TMO modification can and does occur at a number of functionally distinct sites, as single modification reactions were observed to yield different conductance ratios, permeability ratios, and toxin block characteristics. This is not an unreasonable hypothesis in that mutagenesis studies of rat brain, cardiac, and muscle channels have revealed that replacement of a number of different carboxylates with uncharged residues (Noda et al., 1989; Stühmer at al., 1989; Terlau et al., 1991; Satin et al., 1992; Backx et al., 1992; Pusch et al., 1991; Kontis and Goldin, 1993; Lipkind and Fozzard, 1994) results in a decrease in TTX sensitivity. Alteration of two of those carboxyl groups also decreases channel conductance in rat brain channels (Terlau et al., 1991). These observations indicate that there are a number of different TMO-susceptible residues that are involved in ion selectivity and toxin binding.

CONCLUSIONS

Changes in skeletal muscle voltage-dependent BTX-activated Na channel function caused by TMO modification cannot be explained by a change in protein surface charge. Instead, the decrease in Na channel conductance caused by TMO treatment is a result of modification of specific protein residues involved in ion conduction. Modification enhanced Na channel discrimination between sodium and potassium ions such that sodium selectivity was increased relative to potassium when compared with control channels. The increase in ion specificity was gained at the expense of maximal channel conductance. TMO modification also increased the μCTX off-rate by 1000-fold. These data indicate that the ion selectivity apparatus and the μCTX binding domain share some carboxylate residues. Furthermore, these data support the hypothesis that μCTX blocks open channel current by physically occluding the channel pore.

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