Modification of Cardiac Sodium Channels by Carboxyl Reagents

Trimethyloxonium and Water-soluble Carbodiimide

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ABSTRACT In TTX-sensitive nerve and skeletal muscle Na⁺ channels, selective modification of external carboxyl groups with trimethyloxonium (TMO) or water-soluble carbodiimide (WSC) prevents voltage-dependent Ca²⁺ block, reduces unitary conductance, and decreases guanidinium toxin affinity. In the case of TMO, it has been suggested that all three effects result from modification of a single carboxyl group, which causes a positive shift in the channel’s surface potential. We studied the effect of these reagents on Ca²⁺ block of adult rabbit ventricular Na⁺ channels in cell-attached patches. In unmodified channels, unitary conductance (γ_Na) was 18.6 ± 0.9 pS with 280 mM Na⁺ and 2 mM Ca²⁺ in the pipette and was reduced to 5.2 ± 0.8 pS by 10 mM Ca²⁺. In contrast to TTX-sensitive Na⁺ channels, Ca²⁺ block of cardiac Na⁺ channels was not prevented by TMO; after TMO pretreatment, γ_Na was 6.1 ± 1.0 pS in 10 mM Ca²⁺. Nevertheless, TMO altered cardiac Na⁺ channel properties. In 2 mM Ca²⁺, TMO-treated patches exhibited up to three discrete γ_Na levels: 15.3 ± 1.7, 11.3 ± 1.5, and 9.8 ± 1.8 pS. Patch-to-patch variation in which levels were present and the absence of transitions between levels suggests that at least two sites were modified by TMO. An abbreviation of mean open time (MOT) accompanied each decrease in γ_Na. The effects on channel gating of elevating external Ca²⁺ differed from those of TMO pretreatment. Increasing pipette Ca²⁺ from 2 to 10 mM prolonged the MOT at potentials positive to approximately −35 mV by decreasing the open to inactivated (O → I) transition rate constant. On the other hand, even in 10 mM Ca²⁺, TMO accelerated the O → I transition rate constant without a change in its voltage dependence. Ensemble averages after TMO showed a shortening of the time to peak current and an acceleration of the rate of current decay. Channel modification with WSC resulted in analogous effects to those of TMO in failing to show relief from block by 10 mM Ca²⁺. Further, WSC caused a decrease in γ_Na and an abbreviation of MOT at all potentials tested. We conclude that a change in surface potential caused by a single carboxyl modification is inadequate to explain the effects of TMO and WSC in heart. Failure of TMO and WSC to prevent Ca²⁺ block of the cardiac Na⁺ channel is a new distinction among isoforms in the Na⁺ channel multigene family.
INTRODUCTION

Besides its well-known effect on surface potential (McLaughlin, 1989), Ca\(^{2+}\) blocks open Na\(^{+}\) channels in a voltage-dependent manner by occluding the ion permeation pathway in nerve (Woodhull, 1973; Yamamoto, Yeh, and Narahashi, 1985), skeletal muscle (Weiss and Horn, 1986a, b), and cardiac muscle (Sheets, Scanley, Hanck, Makielski, and Fozzard, 1987; Nilius, 1988). This block is most obvious at negative potentials, where the macroscopic instantaneous inward current is reduced by Ca\(^{2+}\) and an outward-going rectification is observed. At the single channel level, the individual Ca\(^{2+}\) blocking and unblocking events are much too fast to resolve, and the measured unitary current, representing a time average of unresolved openings and closings, is decreased by Ca\(^{2+}\) block. If Ca\(^{2+}\) occludes the pore a smaller fraction of time, the measured unitary current will increase. Experiments with group-specific reagents suggest that a carboxyl group is involved with Ca\(^{2+}\) block. Exposure to the carboxyl-specific, O-methylation reagent trimethyloxonium (TMO) prevents voltage-dependent Ca\(^{2+}\) block of reconstituted nerve Na\(^{+}\) channels and increases the unitary current in the presence of Ca\(^{2+}\) (Worley, French, and Krueger, 1986). TMO and other carboxyl-specific reagents also decrease the affinity of nerve and skeletal muscle Na\(^{+}\) channels for guanidinium toxins, tetrodotoxin (TTX) and saxitoxin (STX) (Shrager and Profera, 1973; Baker and Rubinson, 1975; Reed and Raftery, 1976; Spalding, 1980; Guelden and Vogel, 1985; Krueger, Worley, and French, 1986; Worley et al., 1986), and in the absence of Ca\(^{2+}\) block, decrease the nerve Na\(^{+}\) channel unitary conductance, \(\gamma_{Na}\) (Sigworth and Spalding, 1980; Krueger et al., 1986; Worley et al., 1986).

Worley et al. (1986) observed that TMO always altered Ca\(^{2+}\) block, guanidinium toxin affinity, and \(\gamma_{Na}\) concurrently, and concluded that all three actions resulted from the modification of a single carboxyl group at the Ca\(^{2+}\) binding site. Methyl-

ation by TMO of a carboxyl residue accessible from the extracellular solution decreases the negative surface potential of the channel, and Worley et al. (1986) argued that such an alteration in the local surface potential could explain the diverse effects of TMO on Na\(^{+}\) channel properties. Other evidence also suggests that divalent cation block, guanidinium toxin affinity, and \(\gamma_{Na}\) may be linked. Cultured rat myoblast Na\(^{+}\) channels are TTX resistant (i.e., micromolar affinity), are only poorly blocked by external Ca\(^{2+}\), and possess a lower \(\gamma_{Na}\) than cultured rat myotube Na\(^{+}\) channels, which are TTX sensitive (i.e., nanomolar affinity) and significantly blocked by Ca\(^{2+}\) (Weiss and Horn, 1986a, b). That is to say, this naturally occurring TTX-resistant channel behaves like a TTX-sensitive Na\(^{+}\) channel after modification by TMO.

The cardiac Na\(^{+}\) channel deviates from this pattern, however. Despite the much lower affinity for guanidinium toxins (Moczydlowski, Uehara, Guo, and Heiney, 1986) and a lower \(\gamma_{Na}\) (Baumgarten, Dudley, Rogart, and Fozzard, 1991) of mammalian cardiac than nerve Na\(^{+}\) channels, the cardiac channel retains a relatively high affinity site for Ca\(^{2+}\) block (Sheets et al., 1987; Nilius, 1988). The divalent cation blocking site in heart has a different sequence of potency and a notably higher affinity for group IIb metals than the nerve Na\(^{+}\) channels (DiFrancesco, Ferroni, Visentin, and Zaza, 1985; Frelin, Cognard, Vigne, and Lazdunski, 1986; Sheets and
Hanck, 1992; Tanguy and Yeh, 1988; Baumgarten and Fozzard, 1989; Visentin, Zaza, Ferroni, Tromba, and DiFrancesco, 1990). These data suggest that the divalent cation blocking site may be different in heart and nerve.

We investigated whether treatment with carboxyl group reagents would deter block of adult rabbit ventricular Na⁺ channels by Ca²⁺. TMO and water-soluble carbodi-imide (WSC), two reagents that specifically modify carboxyl groups, failed to prevent block of cardiac Na⁺ channels by 10 mM Ca²⁺. The reagents significantly affected several other channel properties, however. When pipette Ca²⁺ was reduced to 2 mM, Na⁺ channels opened to three distinct unitary current levels. Rather than behaving as substates, each open level appeared to reflect a population of channels with a distinct modification. Consequently, it is necessary to postulate multiple sites for the action of TMO on the cardiac Na⁺ channel. Although carboxyl modification by TMO must modify surface potential, the data suggest that a change in surface potential alone is insufficient to explain all the actions of TMO. Differences in the response of cardiac and nerve Na⁺ channels to carboxyl modifying reagents may have important implications for models of the Na⁺ channel.

Preliminary results of these studies have been reported in abstract form (Dudley and Baumgarten, 1990a, b).

M E T H O D S

Cell Isolation Procedure

Ventricular myocytes were isolated from New Zealand white rabbits (1.5–2.5 kg) by a collagenase-protease digestion procedure modified from Poole, Halestrap, Price, and Levi (1989). A cannula was placed in the aorta, and hearts were perfused retrogradely for 4 min with a warm (37°C) Ca²⁺-containing modified Tyrode's solution consisting of (mM): 130 NaCl, 20 taurine, 10 creatine, 0.75 CaCl₂, 0.4 NaH₂PO₄, 5.4 KCl, 0.75 CaCl₂, 0.4 NaH₂PO₄, 3.5 MgCl₂, 5 HEPES, and 10 glucose (titrated to pH 7.25 with NaOH and bubbled with 100% O₂). After rinsing with a nominally Ca²⁺-free Tyrode's solution to which 100 μM EGTA was added, low Ca²⁺ Tyrode's solution containing 1 mg/ml collagenase (type II; Worthington Biochemical Corp., Freehold, NJ), 0.1 mg/ml protease (prospan E, type XIV; Sigma Chemical Co., St. Louis, MO), and 80 μM added Ca²⁺ (total Ca²⁺ ~ 200 μM) was recirculated for 15 min. The ventricles were then isolated, cut into small pieces (~5 × 5 mm), and divided between three flasks containing 3 ml of the enzyme solution with the addition of 10% bovine serum albumin. The flasks were gently shaken for up to 15 min in a Dubnoff metabolic shaking incubator (Precision Scientific, Chicago, IL) maintained at 37°C. Single myocytes were separated from the undigested material by filtration through nylon gauze (250 μm pore size). Isolated cells were washed twice and stored at room temperature in a Kraft-Bruhe solution containing (mM): 88 KOH, 80 glutamic acid, 11 glucose, 10 taurine, 10 KH₂PO₄, 10 HEPES, 0.5 EGTA, 2.5 KCl, and 1.8 MgSO₄ (titrated to pH 7.2 with 1 N KOH). This procedure consistently yielded >70% Ca²⁺-tolerant, rod-shaped cells.

Chemical Modification

Channels were modified by two nominally carboxyl-specific reagents (Brodwick and Eaton, 1982): trimethyloxonium tetrafluoroborate (TMO) and 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluenesulfonate in the presence of glycine methyl ester (WSC). TMO modifies carboxylic acid residues by creation of a methyl ester derivative, while carbodiimide activates the carboxyl for nucleophilic substitution. Glycine methyl ester was added as the
nucleophile and, in the presence of carbodiimide, should result in the formation of an
uncharged amide derivative of the original carboxylic acid. However, any nucleophile present
can react with a carboxyl group activated by carbodiimide, and protein crosslinking may occur.

**TMO protocol.** Isolated cardiac myocytes were placed in a solution containing (mM): 90
KCl and 100 HEPES (pH 8.0). To prevent hydrolytic breakdown, TMO was weighed
immediately before use and was added to the solution as a solid to yield a final concentration of
50 mM. The cells were stirred periodically and incubated for 10 min at ~0°C. The final pH of
the reaction solution was 7.36 ± 0.04 (n = 10). This acidification in the presence of 100 mM
HEPES buffer indicated that the TMO was active; a proton was released during the reaction of
TMO with a nucleophile. To remove TMO’s reaction byproducts, dimethylether and methanol,
the cells were washed twice by centrifugation in 10 times the volume of the cell pellet of the
K-aspartate extracellular solution used during patch clamp or the Kraft-Bruhe solution.

**WSC protocol.** Modification of myocytes with 50 mM carbodiimide and 50 mM of a
nucleophile, glycine methyl ester, was carried out in solutions containing (mM): 50 KCl and 50
2-(N-morpholino) ethanesulfonic acid (MES). The pH of the reaction medium was adjusted to
pH 5.5 with 1 N KOH to maximize the carboxyl selectivity of WSC (Baker and Rubinson, 1975).
Both reagents were added as solids to the reaction solution, which was gently agitated to insure
dissolution and mixing with the cells. Cells were incubated for 15 min at 25°C and then washed
by the same procedure used for TMO-treated cells.

Initial attempts to modify Na⁺ channels utilized 100 mM WSC and pretreated for 30 min.
These conditions resulted in difficulty in forming gigaohm seals, and extremely few channels
were functional upon seal formation. Even with a 50% reduction in both the concentration of
WSC and the treatment time, high resistance seals were difficult to obtain, and patches
exhibited a paucity of functional channels. The propensity for WSC to render tissues inexcitable
was also noted by Shrager and Profera (1973).

**Single Channel Recordings**

Unitary Na⁺ channel currents were measured with an EPC-7 patch clamp amplifier (List
Biological Laboratories, Inc., Campbell, CA) in the cell-attached mode (Hamill, Marty, Neher,
Sakmann, and Sigworth, 1981). Cells were affixed to the floor of a chamber with poly-L-lysine
(>300,000 mol wt; Sigma Chemical Co.). The membrane potential in series with the patch
potential was depolarized to near 0 mV by an extracellular solution containing (mM): 140
K-aspartate, 1 or 10 EGTA, and 5 HEPES (pH 7.4) at 10°C. The low temperature was used to
better resolve channel openings and kinetics. To increase the signal to noise ratio, the pipette
solution contained (mM): 280 NaCl, 10 HEPES (pH 7.4), and either 2 or 10 mM CaCl₂. The
patch electrodes (2–5 MΩ) were pulled from 7740 glass, coated with Sylgard 184 (Dow Corning
Corpor., Midland, MI) to improve their capacitive properties, and heat polished.

Pulse protocols, data acquisition, and analysis were directed by custom programs written in
ASYST (Keithley Asyst, Rochester, NY) and run on an Intel 80386-based computer. Holding
potential typically was ~130 mV, and step depolarizations 45 ms in duration were applied at 1
Hz. Current records were digitized at 10 kHz (12 bits) after filtering at 2 kHz (~3 dB, 8-pole
Bessel). Capacity transients were partially compensated for by analog circuitry, and the average
current in sweeps without openings was subtracted from the original records to eliminate the
residual capacity transient. In addition, the leak current was subtracted sweep-by-sweep so that
the averaged current baseline was 0 pA.

The half-amplitude threshold criterion was applied in assigning openings, and open channel
amplitude histograms were calculated after excluding the first and last points above threshold
which were affected by filtering (t₀.₅₋₉₀ = 0.08 ms). Two methods for calculating MOT were
used. After excluding the first bin to allow for missed openings, a single exponential was fitted
to the open time histogram using a least-squares criterion. The exponential time constant was
reported as the MOT. The MOT also was calculated by the formula (Neher and Steinbach, 1978):

$$\text{MOT} = \theta - t$$

where $\theta$ is the arithmetic mean of the open duration of all openings with a duration greater than $t$, and $t$ is the minimum open duration accepted for analysis, usually 250 $\mu$s. This method also assumes an exponential distribution of open times. The results of the two methods rarely diverged, and in those instances the MOT based on the arithmetic mean was usually accepted.

Statistical analyses of multiple, unpaired observations were carried out using a two-way analysis of variance. Measured values are reported as means ± SE. Nonlinear curves were fitted by the Marquardt method with PROC NLIN (SAS Institute, Cary, NC) or with ASYST. Other statistical methods are presented with the results.

**RESULTS**

TMO and WSC Fail to Prevent Ca$^{2+}$ Block of the Cardiac Na$^{+}$ Channel

Block of open cardiac Na$^{+}$ channels by Ca$^{2+}$ is illustrated in Fig. 1. Typical consecutive sweeps showing openings on depolarizing from −130 to −50 mV are shown. Fig. 1A is from a patch with 280 mM Na$^{+}$ and 2 mM Ca$^{2+}$ in the pipette solution, and Fig. 1B is from another patch exposed to 280 mM Na$^{+}$ and 10 mM Ca$^{2+}$. Increasing the pipette Ca$^{2+}$ from 2 to 10 mM decreased the amplitude of the unitary current. This Ca$^{2+}$-induced reduction of unitary Na$^{+}$ currents has been

![Figure 1](image-url)
reported previously in squid giant axon (Yamamoto et al., 1985), cultured rat skeletal muscle (Weiss and Horn, 1986a, b), rat brain (Worley et al., 1986), canine Purkinje fibers (Sheets et al., 1987), and guinea pig ventricle (Nilius, 1988). The reduced current has generally been attributed to a decrease in the time-averaged current caused by unresolved blocking and unblocking of the open channel by binding of Ca\(^{2+}\) to a site within the pore.

If methylation of carboxyl groups prevents Ca\(^{2+}\) block, as it does for reconstituted nerve Na\(^+\) channels (Worley et al., 1986), the unitary currents of cardiac Na\(^+\) channels pretreated with TMO should be greater in amplitude than those recorded from untreated cells because the modified channel would be occluded by Ca\(^{2+}\) for a much smaller fraction of the time. Fig. 1 C shows consecutive sweeps obtained from a cell that was pretreated with 50 mM TMO. The pipette and bath solutions were identical to those used to record the sweeps in Fig. 1 B. Contrary to the results

![Figure 2](image.png)

**Figure 2.** Open channel amplitude histograms at -60 mV and least-squares fits to single Gaussian functions. Amplitude histograms were constructed after excluding the points affected by filtering, the first and last points of each opening above the 50% threshold. In 2 mM Ca\(^{2+}\), unitary current amplitude was -2.13 pA (●; \(\sigma = 0.28\) pA) and was reduced to -1.30 pA (▲; \(\sigma = 0.23\) pA) by increasing pipette Ca\(^{2+}\) to 10 mM. The unitary current amplitude in 10 mM Ca\(^{2+}\) was unaffected by pretreatment with 50 mM TMO, -1.30 pA (■; \(\sigma = 0.23\) pA). The ordinate is the percentage of the maximum number of open channel data points for each data set.

obtained by Worley et al. (1986) in nerve, the amplitude of the openings in 10 mM Ca\(^{2+}\) appeared to be quite similar in TMO-treated and untreated cells. Since each panel represents a separate patch, inferences about the probability of channel opening cannot be drawn from these data.

Open channel amplitude histograms were used to quantitatively analyze the unitary Na\(^+\) currents recorded between -70 and 0 mV. Fig. 2 illustrates histograms constructed from currents recorded at -60 mV. This voltage was chosen for illustration because the sensitivity for detection of the relief of block should be high at more hyperpolarized potentials where voltage-dependent Ca\(^{2+}\) block is maximized. In the examples shown, increasing Ca\(^{2+}\) from 2 mM (●) to 10 mM (▲) decreased the unitary Na\(^+\) current from -2.13 to -1.30 pA, and an identical unitary current, -1.30 pA, was recorded in 10 mM Ca\(^{2+}\) from a cell that had been exposed to TMO (■). On average, the unitary Na\(^+\) current at -60 mV was -2.17 ± 0.03 pA in 2 mM
Ca\(^{2+}\) (n = 9), -1.30 ± 0.02 pA in 10 mM Ca\(^{2+}\) (n = 6), and -1.32 ± 0.02 pA in 10 mM Ca\(^{2+}\) after exposure to TMO (n = 5). Although increasing Ca\(^{2+}\) from 2 to 10 mM resulted in a significant reduction in unitary Na\(^+\) current in 10 mM Ca\(^{2+}\), currents recorded from TMO-treated cells were indistinguishable from those of control cells. This indicates that TMO failed to prevent Ca\(^{2+}\) block of open cardiac Na\(^+\) channels.

Current–voltage curves depicted in Fig. 3 show the effect of Ca\(^{2+}\) and TMO over the entire voltage range tested. Increasing pipette Ca\(^{2+}\) from 2 mM (●) to 10 mM (▲) reduced \(\gamma_{Na}\). The \(\gamma_{Na}\) after TMO pretreatment (■) resembled that of the unmodified channel (▲). The outward rectification of the current–voltage curve in 10 mM Ca\(^{2+}\) reflects the voltage-dependent Ca\(^{2+}\) block which is increased at more negative potentials. Based on a least-squares linear regression over the entire voltage range, \(\gamma_{Na}\) was 18.6 ± 0.9 pS in 2 mM Ca\(^{2+}\) (●), 5.2 ± 0.8 pS in 10 mM Ca\(^{2+}\) (▲), and 6.1 ± 1.0 pS in 10 mM Ca\(^{2+}\) after exposure to TMO (■). Because of rectification, these values overestimate \(\gamma_{Na}\) in 10 mM Ca\(^{2+}\) at negative potentials. Nevertheless, the unitary currents recorded under identical conditions with and without TMO pretreatment overlapped in 10 mM Ca\(^{2+}\) and demonstrated that TMO pretreatment was ineffective over the entire voltage range (P = 0.47).

To confirm the inability of carboxyl modifying reagents to relieve Ca\(^{2+}\) block of cardiac Na\(^+\) channels, we performed experiments with WSC, another carboxyl-
specific reagent. As shown in Fig. 4, pretreatment of cells with 50 mM WSC also failed to deter block of unitary Na\(^+\) current by 10 mM Ca\(^{2+}\). Fig. 4A shows consecutive records at -50 mV from control and WSC pretreated channels exposed to the same pipette and bath solutions. Open channel amplitude histograms (Fig. 4B) indicate that the unitary Na\(^+\) current was \(~20\%\) less in WSC pretreated channels (■) as compared with unmodified channels (○). Unitary Na\(^+\) currents in channels exposed to 10 mM Ca\(^{2+}\) after pretreatment with WSC were -1.05 ± 0.08 (n = 3), -0.98 ± 0.04 (n = 3), and -1.00 pA (n = 1) at -50, -40, and -30 mV, respectively. Under identical conditions, unitary currents from control channels were -1.28 ± 0.04 (n = 9), -1.25 ± 0.03 pA (n = 6), and -1.22 ± 0.01 pA (n = 4). These significant decreases in unitary Na\(^+\) current are antithetical to expectations for relief of Ca\(^{2+}\) block.

**The Effect of TMO on Single Channel Properties in Low Ca\(^{2+}\)**

Although TMO did not alter \(\gamma_{Na}\) when the external surface of the channel was exposed to 10 mM Ca\(^{2+}\), more complex behavior was observed with 280 mM Na\(^+\).
and 2 mM Ca^{2+} in the pipette. Fig. 5 demonstrates the results of TMO pretreatment on unitary Na^{+} currents in low external Ca^{2+}. Traces from patches pretreated with TMO and depolarized to −60 mV are shown, and the dotted line is the average unitary Na^{+} current in unmodified channels exposed to the same pipette and bath solutions (see Fig. 1). TMO-treated patches in 2 mM Ca^{2+} displayed openings to several distinct current levels. As illustrated, most openings were to a normal or slightly less than normal current amplitude, but others were much less than in controls. Transitions between the open channel current levels were never observed.

Open channel amplitude histograms were constructed at test potentials from −70 to −20 mV to quantify the multiple open current levels induced by TMO. Amplitude histograms from openings at −60 mV in one control and three TMO-treated patches are shown in Fig. 6. Fig. 6 A is a typical open channel amplitude histogram from an unmodified control channel. Control histograms showed only one peak. A second low amplitude opening was noted in < 10% of unmodified patches as previously reported in cardiac channels by others (Cachelin, dePeyer, Kokubun, and Reuter, 1983; Kunze, Lacerda, Wilson, and Brown, 1985; Scanley and Fozzard, 1987; Patlak, 1988). The amplitude was ~40% of the more common amplitude, and in one patch with sufficient low amplitude events to permit reliable analysis, the MOT of the high and low amplitude events were similar. Because low amplitude events typically were ~1% of openings when present in control patches, further analysis was not undertaken.

In 2 mM Ca^{2+}, three levels of unitary current were observed after TMO pretreatment. All three levels were simultaneously present in the patch analyzed in Fig. 6 B. In Fig. 6 C, only the largest and the smallest current levels are present. Fig. 6 D contains only the middle current level. All combinations of the three levels were seen except that the smallest current level was never seen in isolation.

Current–voltage curves with 2 mM Ca^{2+} and 280 mM Na^{+} in the patch pipette for each of the three conductance states found in TMO-treated and the single conductance state in control patches are shown in Fig. 7. To construct these curves, open channel amplitudes in a given patch were assigned to one of the three levels. The largest current level was easily distinguishable from the smallest level, especially if
both levels existed in a patch. Otherwise, current amplitude values were assigned to
the level with a mean value closest to that amplitude. Because the differences between
unitary current levels were large compared with the variance of the measurements,
the assignments were usually unambiguous. As estimated by a weighted least-squares
linear regression of the mean unitary currents, the $\gamma_{Na}$’s for the three current levels in
TMO-treated patches were $14.8 \pm 2.4$ pS (●), $11.2 \pm 2.2$ pS (▲), and $10.7 \pm 1.7$ pS

![Open channel amplitude histograms at -60 mV and Gaussian fits in one control and three TMO-treated channels (pipette solution, 280 mM Na\(^+\) and 2 mM Ca\(^+\) in all panels). The dotted lines represent the unitary current of $-2.08$ pA ($\sigma = 0.12$ pA) for the control patch (A). With TMO pretreatment, three levels of unitary current were observed. All three levels ($-2.03$ pA, $\sigma = 0.03$ pA; $-1.81$ pA, $\sigma = 0.29$ pA; $-0.88$ pA, $\sigma = 0.11$ pA) are present simultaneously in the patch analyzed in B. In C, only the largest and the smallest current levels ($-2.06$ pA, $\sigma = 0.10$ pA; $-1.13$ pA, $\sigma = 0.14$ pA) are present. D contains only the middle current level ($-1.78$ pA, $\sigma = 0.17$ pA). All combinations of the three levels were seen except that the smallest current level was never seen in isolation. To better resolve multiple current levels, the average unitary current of an opening was weighted for open time before constructing the histograms.](image)

(●) in 280 mM Na\(^+\) and 2 mM Ca\(^+\). Statistically indistinguishable estimates of $\gamma_{Na}$, $15.3 \pm 1.7$ pS ($n = 4$), $11.3 \pm 1.5$ pS ($n = 6$), and $9.8 \pm 1.8$ pS ($n = 5$), were obtained by averaging $\gamma_{Na}$’s calculated patch-by-patch. Although the largest conductance level in TMO-treated patches was $\sim 20\%$ smaller than that in control, $18.6 \pm 0.9$ pS, the difference was not statistically significant ($P = 0.17$). In contrast to the $\sim 10\%$ difference in $\gamma_{Na}$ for the smallest two levels, the unitary currents were
statistically distinct from each other and from the largest amplitude openings and control openings based on an analysis of variance. For example, at -50 mV the unitary currents were $95 \pm 2$, $86 \pm 1$, and $55 \pm 4\%$ of the control unitary current. The extrapolated reversal potential for the middle opening level, $107 \pm 26$ mV, is significantly more positive than that for the smallest opening level, $47 \pm 14$ mV. With the limited voltage range of the data, however, one cannot distinguish between differences in rectification and in the reversal potential.

**Effects of TMO and WSC on Mean Open Time**

When at least two different unitary current levels were observed in the same patch, analysis of the MOTs showed that a reduction in $\gamma_{Na}$ was always associated with a reduction in the MOT. This relationship is illustrated in Fig. 8. At each test potential, openings to different levels in the same patch are connected by a solid line. In 14 of 14 pairs, MOT was shorter for openings to a lower current level ($P < 0.01$). Thus, within the same patch there was a clear linkage between the amplitude of the unitary current and the dwell time in the open state. Analysis of the relationship between $\gamma_{Na}$ and MOT for channels within the same patch excludes any confounding effects of cell-to-cell variations in Na\textsuperscript{+} channel regulation and of $E_m$ in series with the patch.
Na⁺ channel MOT also was affected by elevating Ca²⁺ from 2 to 10 mM and by carboxyl modification in 10 mM Ca²⁺. Fig. 9 shows the voltage dependence of MOT under several conditions and fits to the kinetic model described below. At depolarized potentials, pretreatment with TMO effected the MOT–voltage relationship in an opposite manner from elevations in Ca²⁺ alone. However, neither changes in Ca²⁺ nor TMO treatment altered MOT at more negative potentials. By itself, increasing Ca²⁺ from 2 mM (Δ, solid line) to 10 mM (●, dotted line) resulted in a prolongation of the MOT at potentials positive to −30 mV. In high Ca²⁺, pretreatment with TMO (■, dashed line) resulted in a decreased MOT at potentials positive to approximately −35 mV. The decrease in MOT in 10 mM Ca²⁺ at positive potentials after TMO treatment bore some similarity to the previously described link between TMO modification and a decrease in MOT in low Ca²⁺. In 2 mM Ca²⁺, however, TMO pretreatment seemed to decrease MOT even at more negative potentials (see Fig. 8), while MOT at the higher Ca²⁺ was unaffected at potentials negative to −40 mV by TMO. In contrast to the lack of effect of TMO pretreatment or Ca²⁺ elevation on MOT at negative potentials, WSC altered channel gating near threshold. With 10 mM Ca²⁺ in the pipette, WSC exposure resulted in a decreased MOT at all potentials tested (●).

The time a channel remains open is determined by the kinetics of transitions away from the open state. We used the customary model:

\[ C \rightarrow O \rightarrow I \]

in which open to closed (O → C) and open to inactivated (O → I) transitions have opposite exponential dependencies on voltage (e.g., Scanley, Hanck, Chay, and
Fozzard, 1990) to estimate kinetic parameters. This is sufficient for analysis of channel open time in 2 and 10 mM Ca\(^{2+}\) and in 10 mM Ca\(^{2+}\) after TMO treatment because only one open state was observed under these conditions. Accordingly, MOT is given by:

\[
\text{MOT} = \frac{1}{[K_{oc} \exp(V/S_{oc}) + K_{oi} \exp(V/S_{oi})]}
\]

where \(K_{oc}\) and \(K_{oi}\) are the O \(\rightarrow\) C and O \(\rightarrow\) I transition rate constants at 0 mV, \(S_{oc}\) and \(S_{oi}\) are the slope factors reflecting the voltage sensitivities of the transitions, and \(V\) is the test potential. The MOT data were fitted numerically by the Marquardt method, and all the parameters of the nonlinear fits and asymptotic standard errors are listed in the legend of Fig. 9.

As previously noted, the major effect of both TMO pretreatment and Ca\(^{2+}\) elevation appeared to be in the voltage range positive to \(-35\) mV, where closings to the inactivated state predominate over those to the closed state. The kinetic analysis suggested that this results from a change in \(K_{oi}\) rather than the voltage dependence of the O \(\rightarrow\) I transition. Increasing Ca\(^{2+}\) from 2 mM (solid line) to 10 mM (dotted line) slowed \(K_{oi}\) from 1.19 \(\pm\) 0.07 to 0.79 \(\pm\) 0.13 ms\(^{-1}\) with little change in \(S_{oi}\) or in the rate constant and voltage dependence of O \(\rightarrow\) C transitions. In contrast, TMO pretreatment (dashed line) dramatically accelerated \(K_{oi}\) to 1.82 \(\pm\) 0.61 ms\(^{-1}\), but again \(S_{oi}\) and the parameters reflecting O \(\rightarrow\) C transitions were relatively unaffected.

The gating characteristics described here were strikingly similar to previous kinetic analyses. We found that MOT was a biphasic function of voltage with a peak at \(-40\) mV, a relationship seen by others (Grant and Starmer, 1987; Kirsch and Brown, 1989; Scanley et al., 1990). Also, our calculated rate constants and voltage sensitivities...
ties for the O → C and O → I transitions from multichannel patches were comparable to the rates reported for a single channel patch by Scanley et al. (1990). Extrapolating to 0 mV, their $K_{\infty}$ and $K_{oi}$ are $\sim 0.1$ and $1.50$ ms$^{-1}$, and the slope factors for these transitions are $-30 \pm 4$ and $27 \pm 4$ mV, respectively. Our results in 2 mM Ca$^{2+}$ indicate $K_{\infty}$ and $K_{oi}$ were $0.10 \pm 0.06$ and $1.19 \pm 0.07$ ms$^{-1}$, and the slope factors were $-27.3 \pm 7.1$ and $23.7 \pm 4.9$ mV. These data confirm that inactivation from the open state is slightly voltage dependent. Further, both TMO pretreatment and elevations in Ca$^{2+}$ should have altered the surface potential and the transmembrane voltage gradient, but neither intervention affected $K_{\infty}$. This suggests that $K_{\infty}$ may be only secondarily voltage sensitive and that a large part of the voltage sensitivity of Na$^{+}$ channel opening may reside at an earlier C → C transition.

![Figure 10](image-url)
Because the C → O transition and transitions between closed states also might have been altered by TMO, ensemble averages were evaluated at −30 mV. Comparing ensemble averages in low Ca^{2+} from patches with or without TMO pretreatment revealed changes in macroscopic kinetics with TMO (Fig. 10). As the decay of the ensemble averages were linear to the level of the noise on semilogarithmic plots, the macroscopic rate of current decay was estimated by a least-squares fit to a single exponential. In general, TMO-treated patches exhibited an earlier time to peak and a faster macroscopic inactivation. Before TMO, the time to peak current and the decay rate were 3.8 ± 0.5 and 4.7 ± 0.9 ms (n = 5), respectively. After pretreatment with TMO, the time to peak current and the decay rate were 2.2 ± 0.1 and 2.6 ± 0.2 ms (n = 3), respectively. A priori comparisons of means were performed by a Fisher's protected least significant difference test, and the reduction in both parameters was significant (P < 0.05). A decrease in MOT is expected to shorten the time to peak but by itself cannot simply explain the more rapid decay of macroscopic current (Aldrich, Corey, and Stevens, 1983; Scanley et al., 1990).

**DISCUSSION**

Na^{+} channels in nerve, heart, and skeletal muscle are blocked by a number of divalent cations including Ca^{2+} (Woodhull, 1973; Yamamoto et al., 1985; Weiss and Horn, 1986a, b; Sheets et al., 1987; Nilius, 1988). A carboxyl group is implicated in Ca^{2+} block of reconstituted rat brain channels because block is eliminated after carboxyl modification as evidenced by an all-or-none increase in unitary conductance in high Ca^{2+} media to its value in Ca^{2+}-free media (Worley et al., 1986). Contrary to these results on a neural isoform, block of cardiac Na^{+} channels by 10 mM extracellular Ca^{2+} was not prevented by preincubation of isolated myocytes with either 50 mM TMO or 50 mM WSC, reagents that covalently modify external carboxyl groups and eliminate their negative charge. Rather than increasing unitary Na^{+} currents in 10 mM Ca^{2+}, as would be expected with the relief of unresolved brief blocking episodes during a single channel opening, TMO had no effect and WSC decreased unitary current in 10 mM Ca^{2+}.

Previous studies indicate that TMO reduces γ_{Na} when Ca^{2+} is low and argue that this results from alteration of surface potential and a fall in Na^{+} concentration at the mouth of the channel (Sigworth and Spaulding, 1980; Worley et al., 1986). We confirmed that TMO reduced γ_{Na} under some conditions. In 2 mM Ca^{2+}, we observed three distinct current levels that were less than or equal to the unitary currents in unmodified channels, and MOT was decreased. The appearance of multiple open levels differs from the results of Worley et al. (1986), who observed only a single γ_{Na} at each level of Ca^{2+}, but is consistent with brief reports of multiple open levels in batrachotoxin (BTX)-treated nerve Na^{+} channels exposed to either TMO (Cherbavaz, 1990) or two forms of WSC (Chabala, Green, and Andersen, 1986).

One also might evaluate the effect of TMO on Ca^{2+} block by comparing the ratio of γ_{Na} in high and low Ca^{2+} before and after TMO treatment. For untreated cells, the γ_{Na} ratio was 5.2 pS/18.6 pS = 0.3. That is to say, increasing Ca^{2+} to 10 mM reduced γ_{Na} to ~30% of that in 2 mM Ca^{2+}. Similar calculations can be made for each of the three levels observed in 2 mM Ca^{2+} after TMO treatment. The ratios are: 6.1/15.3 = 0.4; 6.1/11.3 = 0.5; and 6.1/9.8 = 0.6. Assessed in this way, Ca^{2+} appeared to be
somewhat less effective as a Na⁺ channel blocker after TMO treatment, but critically, increasing Ca²⁺ still significantly reduced conductance. Thus, regardless of the method used to assess the effect of TMO on Ca²⁺ block, our results in heart differed from those of Worley et al. (1986) in nerve. It should be noted that more than the ability of Ca²⁺ to occlude the pore is evaluated here. Elimination of negative charge near the mouth of the pore in 2 mM Ca²⁺ is expected to reduce γNa by modifying the local Na⁺ concentration, as was argued for nerve (Sigworth and Spaulding, 1980; Worley et al., 1986). Surface potential effects should be smaller in high Ca²⁺ because of screening.

Why Are TMO and WSC Ineffective in Preventing Ca²⁺ Block of Cardiac Na⁺ Channels?

The failure of carboxyl reagents to prevent Ca²⁺ block of cardiac Na⁺ channels may arise from either of two possibilities. Modifiers could fail to react with the site that prevents Ca²⁺ block, or modifiers could continue to react but fail to induce the conformational change necessary to relieve Ca²⁺ block. Both possibilities require a difference in the primary amino acid sequence or in posttranslational modification of the two channels. Analysis of a neonatal rat cardiac Na⁺ channel clone (rat heart I) confirms that the cardiac and nerve Na⁺ channels are distinct isoforms (Rogart, Cribbs, Muglia, Kephart, and Kaiser, 1989), but little is known about differences in posttranslational modification. Changes in the cardiac channel that could prevent carboxyl modification by TMO and WSC include a deletion of, substitution for, or posttranslation modification of a reactive carboxyl. Alternatively, the reactivity of the carboxyl group could be reduced by alterations to adjacent amino acids that hinder access by occupying space or altering the local electric field.

One hypothesis is that differences in isoform reactivity to carboxyl modifiers arise from a single amino acid substitution or modification. In nerve Na⁺ channels, carboxyl-specific reagents not only prevent voltage-dependent Ca²⁺ block (Worley et al., 1986) but also reduce guanidinium toxin binding affinity (Srager and Profera, 1973; Baker and Rubinson, 1975; Reed and Raftery, 1976; Spalding, 1980; Gülden and Vogel, 1985; Krueger et al., 1986; Worley et al., 1986) and decrease unitary conductance when Ca²⁺ is omitted (Sigworth and Spalding, 1980; Krueger et al., 1986; Worley et al., 1986). Because these three effects occurred concurrently in an all-or-none fashion in their experiments, Worley et al. (1986) proposed the one hit hypothesis in which carboxyl reagents modify a single group that is responsible for all three observations. In the same way, a single, naturally occurring amino acid substitution or modification could explain the cardiac channel's lower affinity for guanidinium toxins (e.g., Moczydlowski et al., 1986) and the lower unitary conductance under identical conditions (Baumgarten et al., 1991).

Stühmer and co-workers (Noda, Suzuki, Numa, and Stühmer, 1989) found that point mutation of glutamate-387 to glutamine (E387Q) renders the rat brain II channel insensitive to TTX and STX and lowers macroscopic current. They suggest that glutamate-387, which is located in the putative SS1-SS2 pore-forming region of repeat I, may be the carboxyl group modified by TMO in nerve. As these authors point out, the corresponding glutamate in rat hcart I (amino acid 376) is conserved, but a positively charged arginine replaces a neutral asparagine as the adjacent
residue. Such a substitution might be expected to reduce the affinity of both guanidinium toxins and carboxyl modification reagents. Although this hypothesis is appealing, the situation is likely to be more complicated. Comparison of the primary sequences of rat heart I (Rogart et al., 1989) and rat brain II (Noda, Ikeda, Kayano, Suzuki, Takeshima, Kurasaki, Takahashi, and Numa, 1986) reveals a number of other potential sites for carboxyl modification in nerve that are not present in the cardiac channel or should have reduced reactivity because of the charge on an adjacent residue. One of these is in the pore-forming SS1-SS2 region of repeat IV: replacement of glutamate-1698 in nerve by glutamine-1697 in heart (for further discussion, see Dudley, 1991).

The sensitivity of cardiac Na⁺ channels to block by divalent transition metals also is relevant. High affinity for group IIb metals (DiFrancesco et al., 1985; Frelin et al., 1986; Sheets and Hanck, 1992; Tanguy and Yeh, 1988; Baumgarten and Fozzard, 1989; Visentin et al., 1990) cannot be explained readily by substitution of a positively charged amino acid in rat heart I adjacent to the putative TMO reaction site proposed by Noda et al. (1989). It is noteworthy that sensitivity to transition metals and TTX resistance occur together both in heart and in several atypical nerve Na⁺ channels (Bossu and Feltz, 1984; Ikeda and Schofield, 1987; Jones, 1987), and recently Schild and Moczydlowski (1991) provided evidence that in cardiac channels Zn²⁺ binds to a sulfhydryl group within the STX binding site. Furthermore, Satin, Kyle, Chen, Bell, Cribbs, Fozzard, and Rogart (1992) showed that replacement of cysteine-374 in rat heart I with tyrosine, the analogous residue in the “ITX-sensitive skeletal muscle (µI) channel isoform, increases TTX affinity by >700-fold and decreases Cd²⁺ affinity nearly 30-fold.

The idea that a single amino acid modification can explain the different behavior of nerve and cardiac isoforms appears, nevertheless, to be an oversimplification. Our identification of two lower γNa open states implies that TMO caused two distinct modifications of the cardiac Na⁺ channel rather than one as in nerve (Worley et al., 1986). Further, the Ca²⁺ binding site was unaltered by exposure to carboxyl modifiers, yet TTX and STX binding affinities are reduced upon exposure of neonatal rat heart homogenates to TMO (Doyle, Guo, Lustig, Satin, Rogart, and Fozzard, 1993). Taken together, these chemical modification data suggest that the carboxyl group responsible for modulating guanidinium toxin affinity is not responsible for Ca²⁺ block in heart.

Low Amplitude Openings

TMO-treated myocytes exhibited openings to three unitary current levels within a single patch exposed to 2 mM Ca²⁺. The levels were 55, 86, and 95% of the control unitary current at −50 mV. These data are consistent with brief reports on reconstituted, BTX-treated nerve Na⁺ channels which suggest that TMO (Cherbavaz, 1990) and WSC (Chabala et al., 1986) cause at least two reductions in γNa in the absence of Ca²⁺. It is unlikely that previously described substates (Cachelin et al., 1983; Kunze et al., 1985; Scanley and Fozzard, 1987; Patlak, 1988) were observed here. In our experiments, low amplitude events had a shorter MOT than control channels and were both much more common and different in amplitude than those reported previously. The results are also unlikely to be explained by an observation
of Patlak (1990), who noticed slowly developing multiple amplitude events after intracellular acidification to pH values < 6.0. Although TMO liberates protons on hydrolysis, the pH of the highly buffered reaction media was 7.3 after completion of the reaction. Furthermore, the cells were washed after exposure to TMO, resuspended in pH 7.4 media, and incubated for at least 1 h before use.

In contrast to the behavior in 2 mM Ca\(^{2+}\), multiple open amplitudes were not present when pipette Ca\(^{2+}\) was elevated to 10 mM. One possible explanation is that high Ca\(^{2+}\) preferentially reduced the rate of opening to the lower current levels or the duration of the open state. Elevated Ca\(^{2+}\) reduces the signal-to-noise ratio, making the detection of low amplitude events more difficult. If the ratio of current amplitudes remained the same in 10 mM as in 2 mM Ca\(^{2+}\), however, the signal-to-noise ratio should have been sufficient to allow detection of low amplitude events unless they were very brief. Alternatively, low amplitude events in 2 mM Ca\(^{2+}\) could occur because Ca\(^{2+}\) block is enhanced in modified channels, and unitary currents might be too small to detect in 10 mM Ca\(^{2+}\). Although the basis for observing multiple current levels only in 2 mM Ca\(^{2+}\) remains unresolved, this issue does not detract from the primary observation; contrary to findings in nerve, carboxyl modification does not increase unitary Na\(^{+}\) current in high Ca\(^{2+}\).

Are Multiple Unitary Current Levels Substates or Unique Populations of Modified Channels?

Because there was more than one channel in each patch studied, multiple open levels after carboxyl modification can be explained either by the opening of individual channels to multiple substates or by modifications that create several populations of channels, each opening to a distinct current level. We never detected transitions among the three open current levels in TMO-treated patches despite the fact that the mean dwell times in each open state were long enough to resolve clearly. Further, the proportion of openings to each current level varied greatly from patch to patch (see Fig. 6). Such behavior is inconsistent with transitions between multiple open states of a single channel and argues that the three levels of openings represent different populations of channels. The data suggest that TMO does not modify all of the available reaction sites on each Na\(^{+}\) channel. Rather, sequential modifications of the same channel or two entirely different modifications of separate channels may give rise to the lower conductance states.

Mechanism of Action of TMO and WSC

Chemical modification of carboxyl groups by TMO and WSC removes negative charges from membrane proteins and therefore must reduce the magnitude of the negative surface potential. In addition, WSC can crosslink proteins. Our results suggest that a change in surface potential alone cannot explain all of the effects of TMO and WSC in a simple way. First, TMO induced multiple levels of unitary current in 2 mM Ca\(^{2+}\), a phenomenon that is not reproduced by shifting the transmembrane voltage in untreated channels. Second, as compared with untreated cells, TMO shifted the MOT-voltage relationship in 10 mM Ca\(^{2+}\) in the hyperpolarizing direction between -30 and 0 mV. If TMO acted simply by decreasing the magnitude of negative surface potential, a shift in the opposite direction is expected. Third, the
effects of two carboxyl reagents on MOT and $\gamma_{Na}$ were qualitatively different. In view of these findings, it seems likely that TMO and WSC may induce conformational changes in the Na$^+$ channel in addition to altering surface potential. Conformational changes might result from the bulk of the added moieties, indirectly from local changes in surface potential, or in the case of WSC, from its ability to crosslink reactive residues (Baker and Rubinson, 1975). In a brief communication, Cherbavaz (1990) has also suggested that a reduction in surface potential cannot fully explain the TMO-induced decrease in $\gamma_{Na}$.

In summary, we observed that the effect of carboxyl modification reagents on cardiac Na$^+$ channels is different from that on nerve Na$^+$ channels. These data, along with the other known biophysical and sequence differences among channel isoforms, should help to refine structural models of the Na$^+$ channel.

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REFERENCES

Aldrich, R. W., D. P. Corey, and C. F. Stevens. 1983. A reinterpretation of mammalian sodium channel gating based on single channel recording. *Nature*. 306:436-441.

Baker, P. F., and K. A. Rubinson. 1975. Chemical modification of crab nerves can make them insensitive to the local anaesthetics tetrodotoxin and saxitoxin. *Nature*. 257:412-414.

Baumgarten, C. M., S. C. Dudley, R. B. Rogart, and H. A. Fozzard. 1991. Cardiac and neuroblastoma sodium channels have different unitary conductances and kinetics. *Biophysical Journal*. 59:69a. (Abstr.)

Baumgarten, C. M., and H. A. Fozzard. 1989. Cd$^{2+}$ and Zn$^{2+}$ block unitary Na$^+$ currents in Purkinje and ventricular cells. *Biophysical Journal*. 55:313a. (Abstr.)

Bossu, J.-L., and A. Feliz. 1984. Patch clamp study of tetrodotoxin-resistant sodium current in group C sensory neurones. *Neuroscience Letters*. 51:241-246.

Brodwick, M. S., and D. C. Eaton. 1982. Chemical modification of excitable membranes. In *Proteins in the Nervous System: Structure and Function*. B. Haber and R. Perez-Polo, editors. Alan R. Liss, Inc., New York. 51-72.

Cachelin, A. B., J. E. dePeyser, S. Kokubun, and H. Reuter. 1983. Sodium channels in cultured cardiac cells. *Journal of Physiology*. 340:389-402.

Chabala, L. D., W. N. Green, and O. S. Andersen. 1986. Covalent modification of external carboxyl groups of batrachotoxin-modified canine forebrain sodium channels. *Biophysical Journal*. 49:40a. (Abstr.)

Cherbavaz, D. B. 1990. TMO-modification of Na$^+$ channels does not remove surface charge near the conduction pathway. *Biophysical Journal*. 57:300a. (Abstr.)

DiFrancesco, D., A. Ferroni, S. Visentin, and A. Zaza. 1985. Cadmium-induced blockade of the fast Na$^+$ channels in calf Purkinje fibres. *Proceedings of the Royal Society of London, Series B*. 223:475-484.

Doyle, D. D., Y. Guo, S. L. Lustig, J. Satin, R. B. Rogart, and H. A. Fozzard. 1993. Divalent competition with [H]saxitoxin binding to tetrodotoxin-resistant and -sensitive sodium channels. *Journal of General Physiology*. 101:153-182.
Dudley, S. C. 1991. The effects of carboxyl-group specific modification and triodo-L-thyronine on cardiac sodium channels. Ph.D. dissertation. Virginia Commonwealth University, Richmond, VA. 49–51, 53–55.

Dudley, S. C., and C. M. Baumgarten. 1990a. Trimethyloxonium fails to prevent Ca\(^{2+}\) block of cardiac Na\(^{+}\) channels. *Biophysical Journal.* 57:106a. (Abstr.)

Dudley, S. C., and C. M. Baumgarten. 1990b. Trimethyloxonium (TMO) decreases unitary conductance and mean open time of cardiac Na\(^{+}\) channels. *Journal of Molecular and Cellular Cardiology.* 22(Suppl. I):P38. (Abstr.)

Frelin, C., C. Cognard, P. Vigne, and M. Lazdunski. 1986. Tetrodotoxin-sensitive and tetrodotoxin-resistant Na\(^{+}\) channels differ in their sensitivity to Cd\(^{2+}\) and Zn\(^{2+}\). *European Journal of Pharmacology.* 122:245–250.

Grant, A. O., and C. F. Starmer. 1987. Mechanisms of closure of cardiac sodium channels in rabbit ventricular myocytes: single channel analysis. *Circulation Research.* 60:897–913.

Gülßen, K. M., and W. Vogel. 1985. Three functions of sodium channels in the toad node of Ranvier are altered by trimethyloxonium ions. *Pflügers Archiv.* 403:13–20.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.* 391:85–100.

Ikeda, S. R., and G. G. Schofield. 1987. Tetrodotoxin-resistant sodium current of rat nodose neurones: monovalent cation selectivity and divalent cation block. *Journal of Physiology.* 389:255–270.

Jones, S. W. 1987. Sodium currents in dissociated bull-frog sympathetic neurones. *Journal of Physiology.* 389:605–627.

Kirsch, G. E., and A. M. Brown. 1989. Kinetic properties of sodium channels in rat heart and rat brain. *Journal of General Physiology.* 93:85–99.

Krueger, B. K., J. F. Worley, and R. J. French. 1986. Block of sodium channels in planar lipid bilayers by guanidinium toxins and calcium: Are the mechanisms of voltage dependence the same? *Annals of the New York Academy of Sciences.* 479:257–268.

Kunze, D. L., A. E. Lacerda, D. L. Wilson, and A. M. Brown. 1985. Cardiac Na\(^{+}\) currents and the inactivating, reopening, and waiting properties of single sodium channels. *Journal of General Physiology.* 86:691–720.

McLaughlin, S. 1989. The electrostatic properties of membranes. *Annual Review of Biophysics and Biophysical Chemistry.* 18:113–136.

Moczylowski, E., A. Uehara, X. Guo, and J. Heinéy. 1986. Isochannels and blocking modes of voltage-dependent sodium channels. *Annals of the New York Academy of Sciences.* 479:269–292.

Neher, E., and J. H. Steinbach. 1978. Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *Journal of Physiology.* 277:153–176.

Nilius, B. 1988. Calcium block of guinea-pig heart sodium channels with and without modification by the piperazinylindole DPI 201-106. *Journal of Physiology.* 399:537–558.

Noda, M., T. Ikeda, T. Kayano, H. Suzuki, H. Takeshima, M. Kurasaki, H. Takahashi, and S. Numa. 1986. Existence of distinct sodium channel messenger RNAs in rat brain. *Nature.* 320:188–192.

Noda, M., H. Suzuki, S. Numa, and W. Stühmer. 1989. A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II. *FEBS Letters.* 259:213–216.

Patlak, J. B. 1988. Sodium channel subconductance levels measured with a new variance-mean analysis. *Journal of General Physiology.* 92:413–430.

Patlak, J. B. 1990. The effect of pH on subconductance state frequency and duration in bursting Na\(^{+}\) channels of skeletal muscle. *Biophysical Journal.* 57:395a. (Abstr.)
Poole, R. C., A. P. Halestrap, S. J. Price, and A. J. Levi. 1989. The kinetics of transport of lactate and pyruvate into isolated cardiac myocytes from guinea pig. Biochemical Journal. 264:409–418.

Reed, J. K., and M. A. Rafiery. 1976. Properties of the tetrodotoxin binding component in plasma membranes isolated from Electrophorus electricus. Biochemistry. 15:944–953.

Rogart, R. B., L. L. Cribbs, L. K. Muglia, D. D. Kephart, and M. W. Kaiser. 1989. Molecular cloning of a putative tetrodotoxin-resistant rat heart Na⁺ channel isoform. Proceedings of the National Academy of Sciences, USA. 86:8170–8174.

Scanley, B. E., and H. A. Fozzard. 1987. Low conductance sodium channels in canine cardiac Purkinje cells. Biophysical Journal. 52:489–495.

Scanley, B. E., D. A. Hanck, T. Chay, and H. A. Fozzard. 1990. Kinetic analysis of single sodium channels from canine cardiac Purkinje cells. Journal of General Physiology. 95:411–437.

Shrager, P., and C. Profera. 1973. Inhibition of the receptor for tetrodotoxin in nerve membranes by reagents that modify carboxyl groups. Biochimica et Biophysica Acta. 318:141–146.

Visentin, S., A. Zaza, A. Ferroni, C. Tromba, and C. DiFrancesco. 1990. Sodium current block caused by group IIB cation in calf Purkinje fibres and in guinea-pig ventricular myocytes. Pflügers Archiv. 417:213–222.

Woodhull, A. M. 1973. Ionic blockade of the sodium channels in nerve. Journal of General Physiology. 61:687–708.

Yamamoto, D., J. Z. Yeh, and T. Narahashi. 1985. Interactions of permeant cations with sodium channels of squid axon membranes. Biophysical Journal. 48:361–368.