BRIEF COMMUNICATION

INTERACTION OF NONYLGUANIDINE WITH THE SODIUM CHANNEL

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ABSTRACT Alkyl and aromatic guanidines interact strongly with the tetrodotoxin (TTX)-receptor site in eel electroplaque membranes, showing competition with TTX. That these guanidines could be useful as highly reversible small molecular weight blockers of Na^+ currents is therefore suggested. We have investigated the mechanism of interaction of one of these derivatives, nonylguanidine, by studying its effects on Na^+ currents in squid giant axons using voltage clamp techniques. Although nonylguanidine competed with TTX for binding to eel electroplaque membrane fragments ($K_i = 1.8 \times 10^{-5}$ M), it reversibly blocked both inward and outward Na^+ currents in intact axons only if applied to the interior. In axons with the Na^+ inactivation removed by papain nonylguanidine produced a time-dependent block very similar to that reported for strychnine and pancuronium. The reduction of steady-state currents in these axons was also voltage-dependent, with increasing block observed with increasing step depolarization. These results suggest that nonylguanidine binds to a site accessible from the axoplasmic side of the channel, simulating Na^+ inactivation in papain-treated axons and competing with the normal inactivation process in untreated axons. The competition between internal nonylguanidine and external TTX may result from perturbation by the positively charged nonylguanidine of the TTX-binding site from within the channel itself.

It is well established that action potentials in nerve and muscle fibers result from the movement of cations across the cell membrane. Furthermore, these ion movements are believed to occur through discrete ion specific transmembrane channels controlled by voltage-sensitive mechanisms. Although electrical measurements on intact nerve axons have provided a remarkably detailed picture of many aspects of channel dynamics, the molecular events at the membrane level are still very obscure. It is not surprising, therefore, that there is now considerable interest in defining the chemical and physical properties of these ion conducting channels both in the native membrane state and in solubilized form. One of the most successful research approaches has been the application of channel specific toxins as chemical probes. Tetrodotoxin (TTX), a Na^+ channel specific neurotoxin, has been particularly valuable in this respect. By measuring the interactions of [^{3}H]TTX, it has been possible to infer indirectly many of the key properties of the Na^+ channel in a variety of nerve and muscle preparations (1). It is generally believed that the high binding affinity of TTX for the

channel receptor site is due, in part, to electrostatic interactions between the positively-charged guanidinium group of the toxin and a carboxylate near the channel opening (2–4).

In an attempt to characterize in more detail the chemistry of this binding site, we have synthesized a series of aromatic and alkyl guanidine derivatives and studied their interactions with the TTX-binding site in eel electroplaque membranes by competition experiments (5). Although all of these cations appeared to compete with [^{3}H]TTX, one of the alkyl derivatives, nonylguanidine, had a particularly high affinity. This observation raised the possibility that nonylguanidine could be of potential use as a convenient, highly reversible, small molecular weight blocker of Na^+ channels in electrophysiological studies. In this paper, we report on the equilibrium interactions of nonylguanidine with eel electroplaque membranes, and on the rather unexpected effects of this molecule on Na^+ currents in squid giant axons.

The equilibrium binding of [^{3}H]TTX to electroplaque membranes isolated from *Electrophorus electricus* has been shown to follow normal hyperbolic saturation with a dissociation constant for the [^{3}H]TTX receptor complex of $\sim 6 \times 10^{-9}$ M (6). Many of the toxin binding properties of this membrane preparation appear to be very similar, if not identical, to those reported for other nerve and muscle preparations (1), and there is no reason to believe at this time that the Na^+ channel-TTX receptor site differs significantly from one tissue to another.

Fig. 1 shows the effect of nonylguanidine on [^{3}H]TTX binding to the electroplaque membranes as measured by equilibrium dialysis. The binding data, as shown in the double-reciprocal form varying [^{3}H]TTX at fixed levels of nonylguanidine (Fig. 1 *A*), is consistent with apparent competition between the alkyl guanidine and TTX for a common receptor site. The inhibition constant, K_i calculated from Fig. 1 *B*, 1.8×10^{-5} M, is significantly higher than the K_d for TTX, but is at least three orders of magnitude lower than for simple guanidines (5).

To define the nature of this interaction, we studied the effects of nonylguanidine on Na^+ currents in squid giant axons using standard voltage clamp and internal perfusion techniques (7). Data from the clamp experiments were collected, stored, and analyzed, using a Nova 3 minicomputer (Data General Corp., Westboro, Mass.) with appropriate analog/digital interfacing.

When concentrations of up to 1×10^{-4} M nonylguanidine were applied externally to voltage-clamped giant axons, no effect was observed on either transient Na^+ currents or steady-state K^+ currents. Furthermore, with K^+ currents blocked with tetraethylammonium (TEA), external application of the guanidine did not alter significantly either peak or steady-state Na^+ currents. In addition, the ability of externally applied TTX to block Na^+ currents was unaffected at external nonylguanidine concentrations of up to 1 mM. Clearly, in squid axons, nonylguanidine did not bind to an externally accessible TTX-receptor site.

In contrast to these results, when nonylguanidine was applied internally, a marked reduction of both inward and outward Na^+ currents was observed. A substantially smaller reduction of K^+ currents was also noted. Fig. 2 *A–C* shows typical Na^+ current traces associated with step depolarizations from -69 to $+91$ mV in the absence (*A*) and presence (*B*) of 1.0×10^{-4} M nonylguanidine. The third panel (*C*) shows recovery of the Na^+ current after removing the compound, indicating that the effect of nonylguanidine is freely reversible.

An analysis of the records of Fig. 2 shows that the time constants for the decline of the

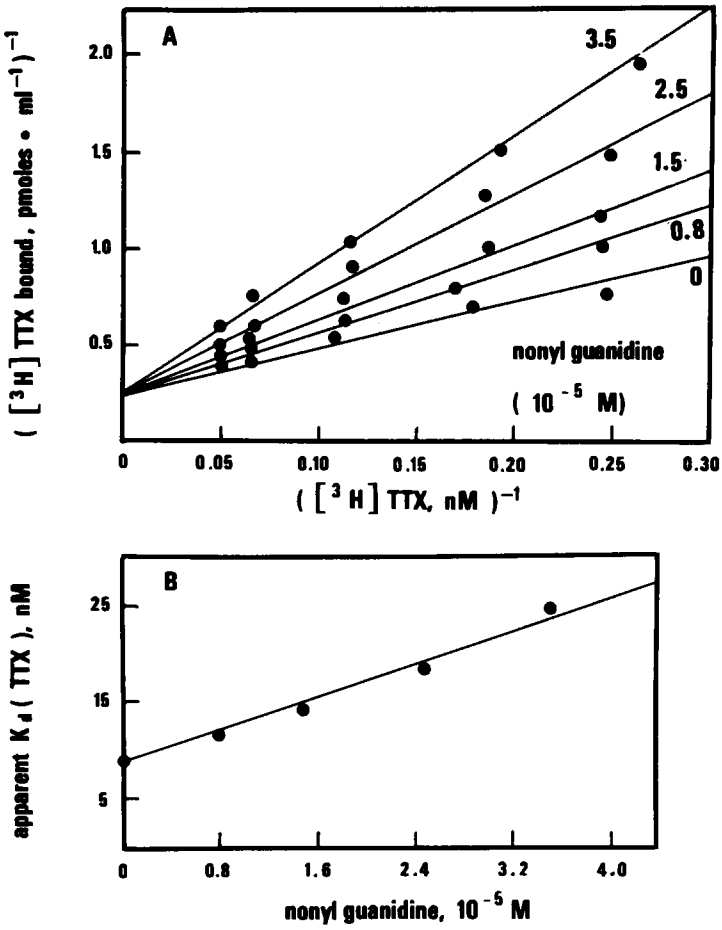

FIGURE 1 Competitive inhibition of [^{3}H]TTX binding to eel electroplaque membranes by nonylguanidine. (*A*) Double reciprocal plot varying [^{3}H]TTX at fixed concentrations of nonylguanidine. (*B*) Replot of the increase in "apparent" K_d for TTX (from *A*) with increasing concentrations of nonylguanidine. The inhibition constant K_i for nonylguanidine calculated from *B* is 1.8×10^{-5} M. [^{3}H]TTX binding was measured at 4°C under equilibrium conditions in 50 mM NaCl, 10 mM (K) phosphate buffer pH 7.0, and 0.25 M sucrose. Isolation of membranes, experimental conditions, and treatment of data were as described previously (5,6).

outward Na^+ currents are decreased by nonylguanidine. The time constant for the uppermost current record of Fig. 2 *A* is 0.54 ms, and after adding nonylguanidine (Fig. 2 *B*) the value is reduced to 0.45 ms. This effect on the time constant could be due to either an interaction of nonylguanidine with the Na^+ channel inactivation gate or a time-dependent block of the Na^+ channel by this compound. To distinguish between these two possibilities and to investigate the block of the steady-state Na^+ current, we removed most of the normal Na^+ inactivation with the proteolytic enzyme papain.

Fig. 3 shows the Na^+ currents recorded after internal perfusion of the axon with papain (1.5 mg/ml). For clarity, only the records for a 140-mV depolarization are displayed. The upper trace shows clearly that papain-treated axons show very little Na^+ inactivation, as has

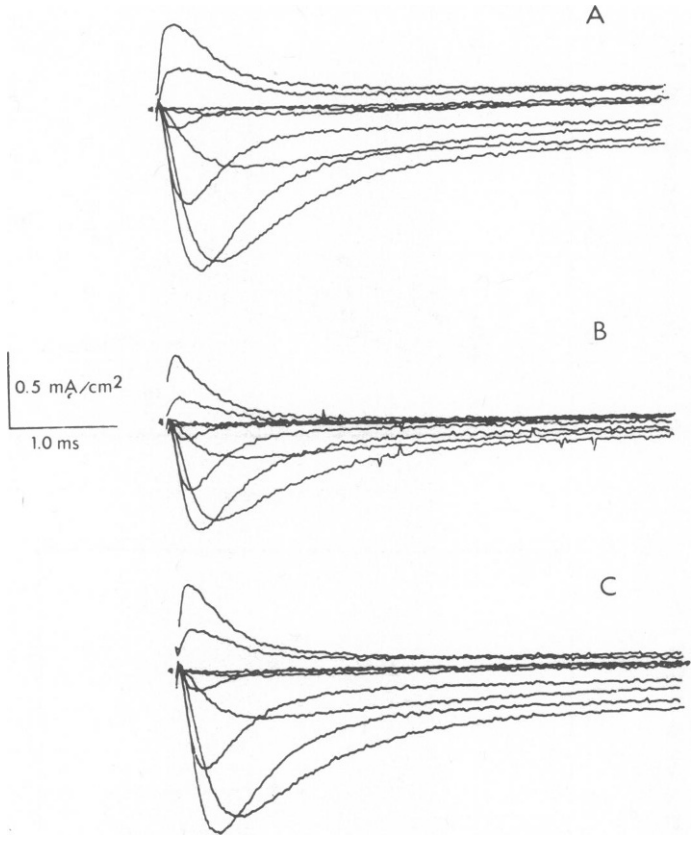

FIGURE 2 The effect of internal perfusion of nonylguanidine on Na^+ currents in a squid giant axon. Panels *A–C* show families of membrane currents corresponding to various step depolarizations from a resting potential of −69 to +91 mV in 20-mV increments in the absence (*A*) and presence (*B*) of 1.0×10^{-4} M nonylguanidine, and (*C*) 12 min after removal of the compound. The internal perfusion solution contained 300 mM K glutamate, 50 mM KF, 390 mM glycine, and 5 mM Tris-Hepes buffer, pH 7.6. 20 mM TEA was used to eliminate potassium currents. Artificial sea water (external perfusate) contained 440 mM NaCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, and 5 mM Tris-Hepes buffer, pH 7.6. Temperature was 10°C. Each test pulse was preceded by a 50-ms hyperpolarizing pulse of 40 mV. Leakage and capacity currents were subtracted using current records obtained in the presence of 300 nM TTX.

been reported for pronase-treated axons (8). In the presence of 1×10^{-5} M nonylguanidine, the Na^+ currents first increase, then decrease, to a steady-state level of approximately half that of the control. Leakage currents have been subtracted and therefore these currents represent a time-dependent block by nonylguanidine. These results are strikingly similar to the time-dependent reduction in Na^+ currents produced by a quaternary analogue of strychnine (9) and by pancuronium, a bisquaternary ammonium ion (10).

The reduction of the steady-state Na^+ currents by nonylguanidine is also voltage-dependent. Using the same papain-treated axon, the effectiveness of nonylguanidine to block the steady-state Na^+ currents was recorded for a series of step depolarizations. Table I shows clearly that the fractional block of the steady-state current increases with increasing depolarization.

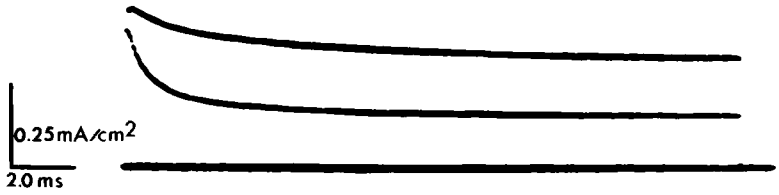

FIGURE 3 Effect of internally perfused nonylguanidine on Na^+ currents with inactivation removed by papain treatment. The axon was exposed to papain (1.5 mg/ml) for ~3 min. The current records correspond to a step depolarization of 140 mV from a resting potential of −64 mV. Control current appears as the uppermost trace. The lower trace is the current in the presence of internally perfused 1×10^{-5} M nonylguanidine. The rising phase of the current records was not captured by the film. Internal perfusion solution contained 20 mM Na glutamate, 675 mM glycine, 15 mM Na_2HPO_4, and 150 mM CsF. The pH was 7.2 and the temperature was maintained at 10°C. Artificial sea water was the same as in Fig. 2. Potassium currents were eliminated by internal Cs^+ while leakage currents were subtracted using an analog subtraction circuit.

That nonylguanidine appears to simulate inactivation in the papain-treated axon may not be altogether surprising. Recent studies have provided evidence to suggest that an arginyl residue may be part of the normal channel inactivation component (11). Nonylguanidine may therefore function as an inactivation "particle" with the nine-carbon alkyl chain sequestered in a hydrophobic region in or near the channel and the positively-charged guanidine competing with the normal inactivation mechanism. This is analogous to the model proposed by Rojas and Rudy for the effects of hydrophobic derivatives of TEA (8).

On the other hand, it is difficult to reconcile the effects of nonylguanidine on TTX binding. It is well established that TTX blocks Na^+ currents only from the external surface of the channel (12). Since nonylguanidine affects Na^+ currents only when applied to the axoplasmic surface, a common external binding site for TTX and nonylguanidine is clearly excluded. Indeed, a single binding site would be very unlikely in view of the obvious structural differences between the two molecules. Another possibility is that the apparent competitive effects observed in the electroplaque membrane studies may result from the presence of the positively charged guanidinium group of nonylguanidine in the channel so as to perturb the binding of TTX to its receptor site. If nonylguanidine does enter the Na^+ channel from the

TABLE I
VOLTAGE DEPENDENCE OF THE NONYLGUANIDINE BLOCK OF STEADY-STATE Na^+ CURRENTS

Membrane potential	Fractional block of the steady-state current by nonylguanidine*
(mV)	
−24	0.36
−4	0.37
16	0.44
36	0.51
57	0.50
76	0.53

Steady-state currents were recorded for a 20-ms step depolarization from a holding potential of −64 mV.
*1×10^{-5} M nonylguanidine was applied internally.

interior surface, then the blocking reaction would be expected to show voltage dependence, as has indeed been shown (Table I).

The electroplaque membrane preparation used in this study appears to consist of vesicular-like structures (6). To reconcile the apparent asymmetric interaction of TTX and nonylguanidine, we must assume that in the membrane fragments, nonylguanidine has free access to the interior surface of the channel. This is not an unreasonable assumption, particularly since long equilibration times of >12 h were routinely used in the binding studies. Furthermore, we obviously cannot rule out the possibility that the isolated electroplaque membranes may be intrinsically more permeable to small molecular weight compounds than the intact squid axon, a reflection perhaps of tissue differences. Indeed, other important pharmacological differences may exist between these two tissues, particularly with regard to regions of the channel other than the TTX-receptor site.

The results presented here suggest that nonylguanidine may be an extremely useful tool to study the properties of Na^+ channels relating to the inactivation process. Although its mode of action appears to be very similar to pancuronium and *N*-methylstrychnine, nonylguanidine is effective at significantly lower concentrations. It is a smaller molecule and its effects are reversible within minutes.

In addition, in view of the effects of nonylguanidine on TTX binding, this molecule may also prove to be a valuable probe in studying possible allosteric interactions between regions at the channel which mediate ion conduction and those associated with the inactivation mechanisms.

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