VOLTAGE-DEPENDENT BLOCK BY TETRODOTOXIN OF THE SODIUM CHANNEL IN RABBIT CARDIAC PURKINJE FIBERS

E. CARMELIET
Laboratory of Physiology, University of Leuven, Belgium

ABSTRACT The two-microelectrode, voltage-clamp technique was applied to rabbit cardiac Purkinje fibers to study the interaction of tetrodotoxin (TTX) with the slowly inactivating Na current. Binding of TTX to rested, inactivated, and activated channels was estimated by measuring the relative decrease of current at the beginning (rested and inactivated channels) and the end (activated channels) of a 1 s depolarizing clamp to −45 mV. The accelerated decline of the Na current in the presence of a submaximal dose of TTX was interpreted as an increase in blocking efficiency upon depolarization. The experiments show that activated as well as inactivated channels are more sensitive to TTX than are rested channels. The dissociation equilibrium constants for the three states are 3.5×10^{-6} M for the rested, 0.94×10^{-6} M for the activated, and 0.75×10^{-6} M for the inactivated channels. The time course of activation block was dependent on TTX concentration. Rate constants for association and dissociation of the activated state are 1.3×10^{6} $M^{-1} \cdot s^{-1}$ and 1.5 s^{-1}, respectively.

INTRODUCTION

In nerve, block of the fast Na channel by tetrodotoxin (TTX) occurs by interference of the drug with the channel from the outer side of the membrane. When applied to the intracellular side, TTX is without effect (1). In nerve (2, 3) and skeletal muscle (4), block of the Na channel is not accompanied by any change in the kinetics of activation and inactivation; it is voltage-independent and not affected by changes in the frequency of applied depolarizations. In heart tissue evidence for a frequency-dependent block by TTX has been obtained either by measurements of maximal rate of depolarization ($\dot{V}_{max}$) (5–7) or by a direct measurement of the Na current under voltage clamp conditions (8). According to Cohen et al. (8), this finding is not the consequence of a change in affinity of the activated or inactivated channel vs. the rested channel, but can be explained by a slowing of the recovery from inactivation of the blocked channels. More recently a frequency-dependent block by TTX has also been described in cultured skeletal muscle cells (9). There is controversy concerning the existence of a voltage-dependent block by TTX in heart (5–8, 10). A shift of the inactivation curve has been found in experiments on mammalian heart tissue in which the Na conductance was estimated by $\dot{V}_{max}$ measurements (5, 6; but see reference 7 for absence of the effect in avian heart). However, the conclusions reached by these authors have been criticized because $\dot{V}_{max}$ is a highly nonlinear estimate of the Na conductance (11, 12).

In this paper, the effect of TTX is reported using the slow component of the Na current (13–15) in cardiac Purkinje fibers. This current, which is quite important and fundamental in the genesis of the plateau of the action potential, can be measured using the two-microelectrode, voltage-clamp method. The experiments show that activated as well as inactivated channels are more sensitive to TTX than are rested channels. Some of these results have been communicated to the Society of General Physiologists (16).

METHODS

Rabbit Purkinje fibers were dissected from both ventricles. Short segments (0.8–0.9 mm) were obtained by crushing the isolated fibers on the bottom of the perfusion chamber, using a wire grid.

The fibers were superfused with modified Tyrode's solution at a rate of 3 ml/min; temperature was 37°C. The composition was (in mM): Na, 150; K, 5.4; Ca, 1.8; Mg, 0.5; Cl, 137; HCO_3, 23; glucose, 5. 20 mM Cs was added to block the inward rectifying K channel. This made it easier to clamp the membrane, at very negative potentials, during seconds or minutes without causing K depletion. TTX (Sankyo Co., Ltd., Tokyo) was used in concentrations that varied between 3×10^{-7} M and 6×10^{-5} M.

The two-microelectrode technique was used to measure currents under voltage-clamp conditions. The current-passing electrode was inserted midway in the preparation; the second, potential-measuring electrode was positioned 250 μm from the end of a segment. The membrane potential was amplified and led to the input of the voltage clamp amplifier which also received rectangular command pulses. Transmembrane current was measured by an operational amplifier circuit that held the bath potential at virtual ground. Signals were displayed on an oscilloscope or a pen recorder (Brush 220, Gould Inc., Cleveland, OH) or were stored on magnetic tape (3964A, Hewlett-Packard Co., Palo Alto, CA). According to a careful analysis by Colatsky and Tsien (17), the rabbit Purkinje fiber allows the analysis of ionic currents with little interference from radial nonuniformities in membrane potential or ion concentration. The advan-

tage of the rabbit Purkinje preparation compared with the ungulate Purkinje preparation is related to the existence of wide intercellular clefts (18). However, the two-microelectrode technique is insufficient to measure under normal conditions the fast component of the Na current responsible for the upstroke of the action potential but allows an analysis of the slow component, which flows during the plateau of the cardiac action potential. Currents during the first 10 ms were not measured. The time course of TTX-sensitive current after the first 10 ms will be described by one exponential and a constant. The amplitude of the current corresponding to the sum of the exponential and the constant will be called initial slow current.

RESULTS

Block of Open or Activated Na Channels

When a voltage-clamp depolarization is applied from a holding potential of −100 mV to −45 mV, the only time-dependent current flowing through the membrane after the initial 5–10 ms is a slow Na current. Indeed, in the presence of 6×10^{-5} M TTX, the current record is entirely flat. The time course of the total TTX sensitive current up to 1–2 s can be described by one exponential and a constant (Fig. 1). The component considered a constant is still time-dependent, but time constants are at least one order of magnitude greater than the first one.

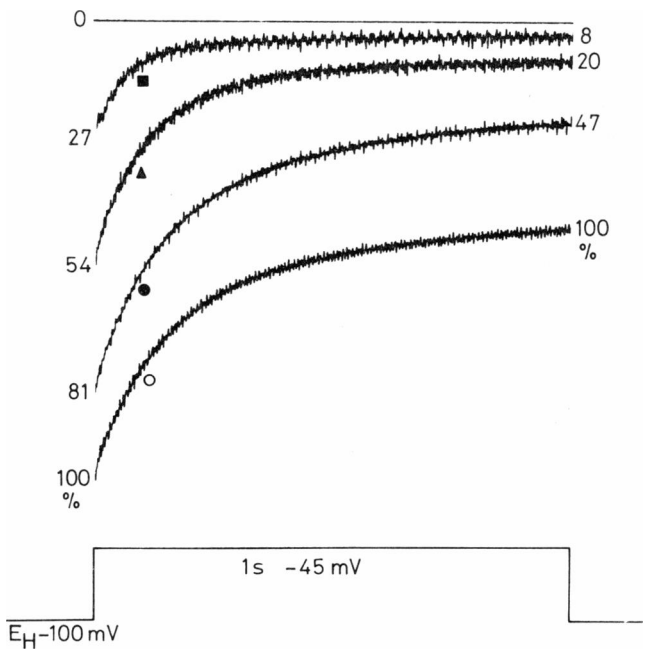

FIGURE 1 Examples of the slow Na current in the absence (*open circle*) and presence of different TTX concentrations: 10^{-6} M (*solid circle*); 3×10^{-6} M (*triangle*); 10^{-5} M (*square*). The reference current (100%) corresponds to the total TTX-sensitive current, measured by adding 6×10^{-5} M, and is equal to 47.3 nA at the beginning and 21.7 nA at the end of a 1 s depolarizing clamp to −45 mV. The time course under control conditions can be described by one exponential and a constant. In the presence of a submaximal dose of TTX the current is decreased but the relative decrease is more pronounced at the end of the pulse than at the beginning: in 10^{-6} M TTX, for example, the initial current is reduced to 81% of the control, whereas only 47% is left at the end of the 1 s pulse. Numbers indicate relative currents at the beginning and the end of the depolarizing pulse. This result can be interpreted as additional block of open or activated channels. Current during the first 10 ms of the depolarization is not shown. Holding current was −7.5 nA.

Addition of submaximal doses of TTX resulted in the following changes: the initial slow current, as well as the current at the end of the pulse, was decreased but the decrease at the end was more pronounced; the decay of the current was faster as if the inactivation process was accelerated.

The decrease of the initial slow current can be taken as a measure of the decrease in the number of Na channels available for activation, because of block by TTX of rested Na channels. This presupposes that the time of 5–10 ms, when the clamp was not under control, is small compared with the time constant for binding to activated channels (see Fig. 4). Similarly the additional decrease of the current after 1 s depolarization is a measure of the block by TTX of activated Na channels, assuming steady state conditions for block. The dose–effect relationship for TTX block of the rested and activated Na channels is shown in Fig. 2. The Hill plot of the same data was linear with a slope of 1.05, which suggests a first order reaction. The half maximum value was 3.5×10^{-6} M for the rested (K_R) and 0.94×10^{-6} M for the activated block (K_A), indicating higher affinity for TTX of activated channels.

Because of additional block of open channels, the current during a depolarizing clamp also decayed faster. By computing the relative decrease in current at every moment during the depolarizing pulse, the time course of this additional block can be obtained. It represents the time course of TTX binding to open channels insofar as the dissociation of TTX from the rested channels and the

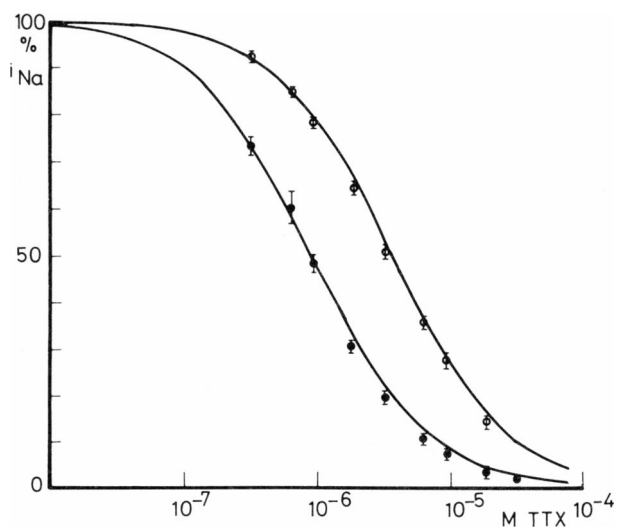

FIGURE 2 Na current as a function of TTX concentration. *Open circles*, initial current; *closed circles*, current at the end of a 1 s clamp. The change of initial and 1 s currents represents block of rested channels and of activated channels, respectively. Mean values ± standard error for eight preparations. The lines are theoretical lines calculated for a 1:1 reaction (Hill slope was 1.05); the dissociation constant for rested channels, K_R, was 3.5×10^{-6} M and for activated channels, K_A, 0.94×10^{-6} M.

conversion of blocked rested and blocked inactivated channels to blocked activated channels is slow and thus does not interfere.

Block of open channels by TTX was found to follow an exponential time course (Fig. 3) with time constants directly proportional to the concentration of TTX, in accord with the existence of a first order reaction. From this relationship the association (k_1) and dissociation constants (k_2) of TTX reacting with the receptor of the activated channel can be calculated and were found to be $1.3 \times 10^6\ s^{-1}\ M^{-1}$ and $1.5\ s^{-1}$ (Fig. 4). The ratio of the rate constants was 1.15×10^{-6} M, which provides an independent estimation of the dissociation constant of activated channels. In comparison with data on nerve (19, 20), the value for k_1 is similar, but k_2 in heart is two to three orders of magnitude faster.

Block of Inactivated Na Channels

The preceding experiments have shown a preferential blocking of open or activated channels. The question was asked whether a similar preferential binding occurs to inactivated channels. Two types of experiments were performed. In the first type, the effect of TTX was studied in preparations in which part of the channels was inactivated by lowering the holding potential. The effect was estimated by measuring the relative change of initial slow current for a depolarizing clamp to −45 mV (Fig. 5). From the normalized dose–effect relationship it is clear that TTX was more effective when part of the Na channels was already inactivated before the test pulse, suggesting that inactivated channels are more sensitive to TTX blockade than are rested channels. A complete dose–effect relationship at two holding potentials was performed in three

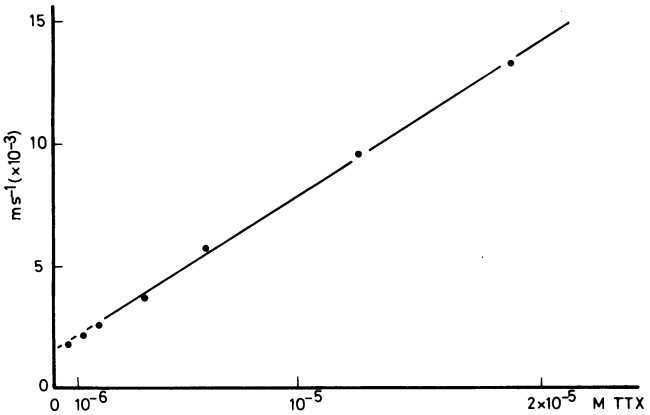

FIGURE 4 Rate constants for the development of block of activated channels as a function of TTX concentration can be described by a linear function $1/\tau = k_1$ [TTX] + k_2, indicating that the complex reactions occurring between rested and activated channels can be represented by a simple 1:1 reaction. From the relationship, k_1 and k_2 were estimated to be $1.3 \times 10^6\ s^{-1}\ M^{-1}$ and $1.5\ s^{-1}$, respectively.

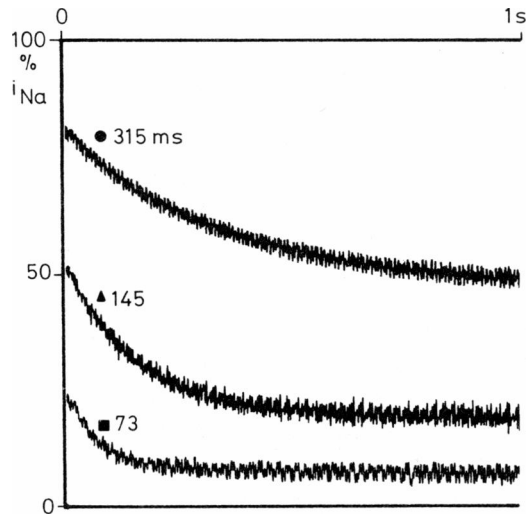

FIGURE 3 Time course of relative block during a depolarizing pulse. Same experiment as illustrated in Fig. 1. The curves represent calculated values for the ratio of the current in TTX over the current in control Tyrode and illustrates binding of TTX to the activated channel according to the reaction

$$R + \text{TTX} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R \cdot \text{TTX}.$$

The time course was fit by one exponential and a constant. Time constants varied from 561 ms ± 56 at 3×10^{-7} M TTX to 26.7 ms ± 4 at 3×10^{-5} M (n = 8). TTX concentration was 10^{-6} M (*circle*), 3×10^{-6} M (*triangle*), and 10^{-5} M (*square*). Numbers indicate computed time constants. Holding current is −7.5 nA.

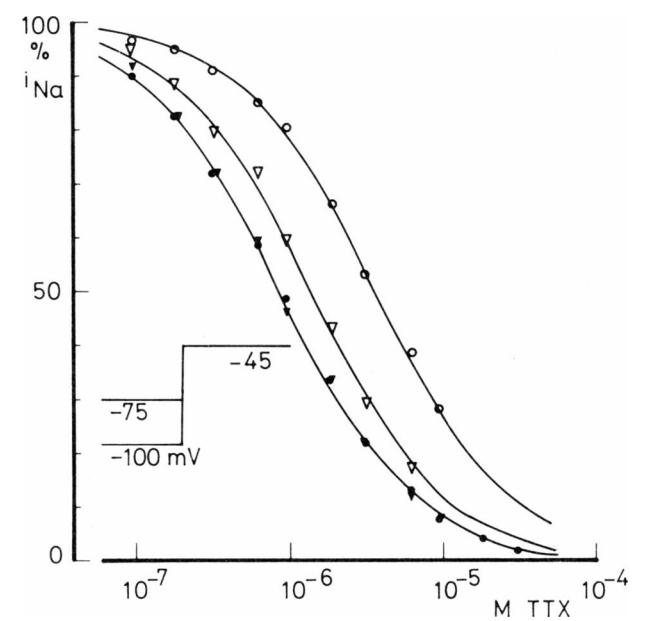

FIGURE 5 The effect of TTX concentration on the slow Na current activated during a depolarization to −45 mV from two different holding potentials, −100 mV and −75 mV. Mean values for three preparations. Depolarization to −75 mV resulted in 37, 46, and 49% inactivation. *Open symbols*, initial current; holding potential was −100 mV (*circles*) or −75 mV (*triangles*). *Closed symbols*, current measured at the end of a 1 s depolarizing clamp. The results show that TTX binds more efficiently to inactivated channels. From these data a dissociation constant for inactivated channels K_I was calculated to be 0.80×10^{-6} M.

preparations. The change of the holding potential resulted in 44% inactivation as a mean ($h = 0.56$). For a concentration of 3.7×10^{-6} M, which is the dissociation constant K_R for rested channels in these three preparations, the initial slow Na current, when the holding potential was changed to −75 mV, decreased to 15.7% instead of the 28% expected from simple inactivation. R, the relative number of available channels for activation, was then 0.157. According to the receptor modulated hypothesis (21, 22), the dissociation constant for inactivated channels, K_I, can be calculated if K_R, h, and R are known; this was found to be 0.80×10^{-6} M.

In a second type of experiment, the effect of TTX on the potential-dependency of the inactivation process was tested (Fig. 6). According to the receptor-modulated hypothesis (21, 22), a higher affinity for the inactivated than for the rested state predicts a shift of the inactivation curve to more negative potentials. In total, experiments on four preparations were performed; two with 10^{-6} M and two with 3×10^{-6} M TTX. In control conditions the potential for 50% inactivation (V_h) was −76.3 mV and the slope factor was 7.3 mV^{-1}. With 10^{-6} M TTX the shift was −3.8 mV and −6.5 mV and with 3×10^{-6} M it was −6.9 mV and −9.4 mV. From these values a K_I was calculated using the formula (8)

$$V_h = k \ln \{(1 + [TTX]/K_R)/(1 + [TTX]/K_I)\}.$$

Its mean value 0.69×10^{-6} M (range $0.42–0.94 \times 10^{-6}$ M) is not significantly different from the estimation in the first type of experiment.

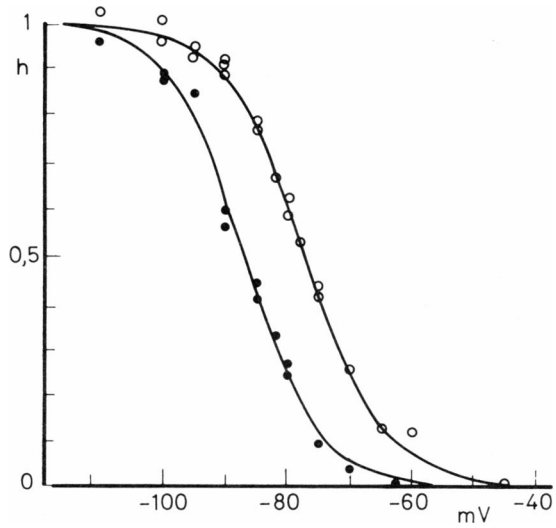

FIGURE 6 An example of the shift in the inactivation curve by addition of 3×10^{-6} M TTX. The two curves can be described by the equation $i_{Na} = \{1 + \exp [(V - V_h)/k]\}^{-1}$, in which k is 6.2 mV^{-1} and V_h is −77.0 mV in control conditions and −86.9 mV in the presence of TTX. This result is in accordance with the hypothesis of a more efficient block of inactivated channels. The dissociation constant, K_I, was calculated using the equation $V_h = k \cdot \ln \{(1 + [TTX]/K_R)/(1 + [TTX]/K_I)\}$ and showed a mean value of 0.69×10^{-6} M with a range between 0.42 and 0.94×10^{-6} M (four preparations).

Control Experiments

The analysis of the preceding experiments relies on the assumption that the voltage escape during the first few milliseconds of the clamp step did not affect the current recorded in different TTX concentrations. However, it might be argued that the trajectory of the voltage escape was different for each concentration of TTX, the voltage control being improved the higher the TTX concentration. Different currents would thus be activated, eventually distorting the current records.

To rule out this artifact, voltage escapes of different degrees were simulated by clamping the membrane to different potential levels for 10 ms. The experiments were done in the presence of a high concentration of TTX (3×10^{-5} M). This allowed for a faster voltage clamp control (within 3 ms; see Fig. 7 *B*) and a better evaluation of the possible interference of non-sodium currents. The results of such an experiment are shown in Fig. 7. The voltage records of Fig. 7 *B* indicate that a reasonably constant voltage level was obtained after the first 3 ms. During these short prepulses, currents were activated to a different degree, depending on the voltage. Fig. 7 *C* shows superimposed currents obtained for the voltage levels of −45 mV, −20 mV, and 0 mV. Only a small inward current was seen at −45 mV; outward currents were obtained in the two other cases, the larger one for the prepulses to 0 mV. Analysis of the records in Fig. 7 *A* reveals that the tail currents after the prepulses (records 3–6) were very small in comparison to the large TTX-sensitive current (difference between record 1 and 2). The small amplitude of these tails may at first seem unexpected, since during the conditioning prepulse rather large currents were activated. A possible explanation for this paradox can be given as follows. The current contributing most to the currents during the prepulse is the transient outward current. According to some authors (see reference 23), this current reverses at potentials around −30 mV. According to Coraboeuf and Carmeliet (24), the current is a K current but its deactivation is so fast that it cannot be recorded at potentials negative to −30 mV. TTX-insensitive currents activated during the voltage escape thus will contribute little to the current recorded at −45 mV.

Na current activated during a voltage escape is not expected to interfere because (*a*) the fast component, which is responsible for the upstroke of the action potential, completely inactivates, and (*b*) the slow components have no time to inactivate. In accordance with this reasoning, experiments similar to the one described in Fig. 7, but in the absence of TTX and with prepulses as long as 20 ms to different voltage levels did not show any effect on the time course of the current at −45 mV.

The conclusion from these control experiments is that currents generated during voltage escapes have a negligible effect on the time course of the current recorded at −45 mV. The different current pattern observed in the presence of various concentrations of TTX is thus not artifactual,

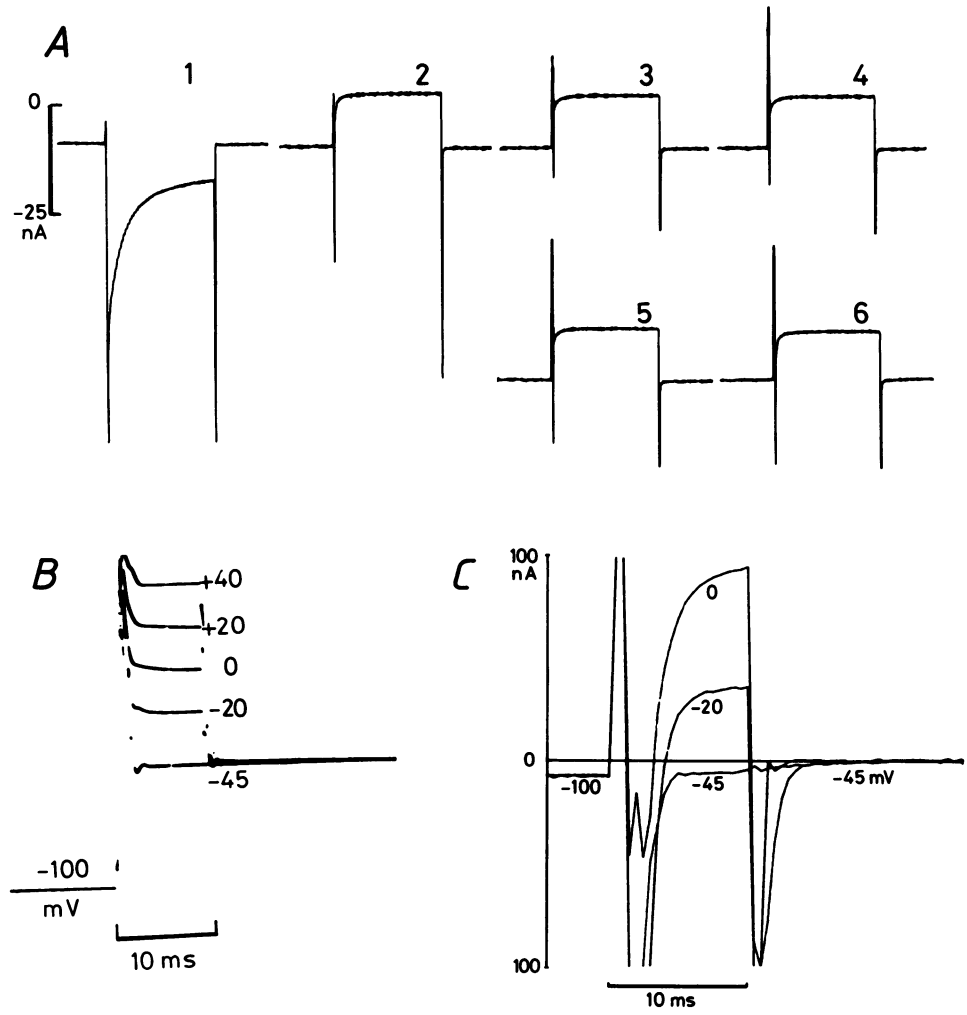

FIGURE 7 (*A*) Current records for a voltage clamp from −100 mV to −45 mV in the absence (*A1*) and in the presence of 3×10^{-5} M TTX (*A2*). In *A3–A6*, the clamp to −45 mV was preceded by a 10 ms prepulse to −20, 0, +20, and +40 mV; TTX was present. Duration of total voltage step is 1 s. (*B*) Examples of the voltage records during the clamp protocol with and without prepulse. Voltage levels are indicated by figures. (*C*) Superimposed records of currents activated during the 10 ms prepulses to 0 mV, −20 mV, and −45 mV.

but can be explained in terms of increased block of activated and inactivated Na channels.

DISCUSSION AND CONCLUSION

The main conclusion from this paper is that TTX blocks preferentially activated and inactivated Na channels in heart, at least in those channels responsible for the slow Na current. Interaction of TTX with the Na channels is therefore more complex than a simple plugging of the channel's mouth, as proposed for nerve, but presupposes an allosteric interaction with the activation and inactivation gate.

One can ask why this higher affinity of activated and inactivated channels escaped the attention of previous authors. A higher affinity of the activated vs. rested Na channels was derived from the observation of a faster decline of the Na current during a depolarizing clamp. The time constants for TTX binding were found to be rather long; they varied between 561 ms at 3×10^{-7} M TTX and 26.7 ms at 3×10^{-5} M (Fig. 4). These values are much too long to allow sufficient binding during the very short duration (1 ms) of the fast Na current responsible for the upstroke of the action potential. It is thus understandable that measurements of the fast Na current in heart (8, 25, 26) did not reveal changes in the time course of the Na current by submaximal concentrations of TTX. The fact that binding to activated channels is so slow further explains why the action potential duration of cardiac Purkinje fibers is much more sensitive than $\dot{V}_{max}$ of the upstroke to low concentrations of TTX (27).

This kind of reasoning, however, cannot explain why the present experiments demonstrated a shift of the inactivation curve and of the dose–effect relationship for inactivated channels, while such an effect was absent in the experiments of Cohen et al. (8). Another way to solve the problem is to assume that Na channels with slow inactivation differ from Na channels with fast inactivation in their interaction with TTX; according to this hypothesis only the

slowly inactivating Na channels would show the change in affinity with activation and inactivation. No direct information is available on this issue.

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REFERENCES

1. Narahashi, T., N. C. Anderson, and J. W. Moore. 1966. Tetrodotoxin does not block excitation from inside the nerve membrane. *Science (Wash. DC)*. 153:765–767.
2. Narahashi, T., J. W. Moore, and W. R. Scott. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axon. *J. Gen. Physiol.* 47:965–974.
3. Hille, B. 1968. Pharmacological modifications of the sodium channels of frog nerve. *J. Gen. Physiol.* 51:199–219.
4. Almers, W., and S. R. Levinson. 1975. Tetrodotoxin binding to normal and depolarized frog muscle and the conductance of a single sodium channel. *J. Physiol. (Lond.)*. 247:483–509.
5. Baer, M., P. M. Best, and H. Reuter. 1976. Voltage-dependent action of tetrodotoxin in mammalian cardiac muscle. *Nature (Lond.)*. 263:344–345.
6. Reuter, H., M. Baer, and P. M. Best. 1978. Voltage dependence of TTX action in mammalian cardiac muscle. *In* Biophysical Aspects of Cardiac Muscle. M. Morad, editor. Academic Press, Inc., New York. 129–142.
7. Inoue, D., and A. J. Pappano. 1984. Block of avian cardiac fast sodium channels by tetrodotoxin is enhanced by repetitive depolarization but not by steady depolarization. *J. Mol. Cell. Cardiol.* 16:943–952.
8. Cohen, C. J., B. P. Bean, T. J. Colatsky, and R. W. Tsien. 1981. Tetrodotoxin block of sodium channels in rabbit Purkinje fibers. *J. Gen. Physiol.* 78:383–411.
9. Gonoi, T., J. Sherman, and W. A. Catterall. 1985. Voltage clamp analysis of tetrodotoxin-sensitive and -insensitive sodium channels in rat muscle cells developing in vitro. *J. Neurosci.* 5:2559–2564.
10. Colatsky, T. J., and D. C. Gadsby. 1980. Is tetrodotoxin block of background sodium channels in cardiac Purkinje fibres voltage-dependent? *J. Physiol. (Lond.)*. 306:20P.
11. Cohen, I. S., and G. R. Strichartz. 1977. On the voltage dependent action of tetrodotoxin. *Biophys. J.* 17:275–279.
12. Cohen, C. J., B. P. Bean, and R. W. Tsien.. 1984. Maximal upstroke velocity as an index of available sodium conductance. *Circ. Res.* 54:636–651.
13. Gintant, G. A., N. B. Datyner, and I. S. Cohen. 1984. Slow inactivation in canine cardiac Purkinje fibers. *Biophys. J.* 45:509–512.
14. Carmeliet, E. 1984. Slow inactivation of the sodium current in rabbit cardiac Purkinje fibres. *J. Physiol. (Lond.)*. 353:125P.
15. Carmeliet, E. 1985. Slow decay of sodium current and effect of quinidine in rabbit cardiac Purkinje fibers. *In* Cardiac Electrophysiology and Arrhythmias. D. P. Zipes and J. Jalife, editors. Grune & Stratton, Inc., Orlando. 207–215.
16. Carmeliet, E. 1985. Voltage-dependent block of the sodium channel by tetrodotoxin in rabbit cardiac Purkinje fibers. *J. Gen. Physiol.* 86:13a. (Abstr.)
17. Colatsky, T. J., and R. W. Tsien. 1979. Electrical properties associated with wide intercellular clefts in rabbit Purkinje fibres. *J. Physiol. (Lond.)*. 290:227–252.
18. Sommer, J. R., and E. A. Johnson. 1968. Cardiac muscle. A comparative study in Purkinje fibers and ventricular fibers. *J. Cell. Biol.* 36:497–526.
19. Cuervo, L. A., and W. J. Adelman. 1970. Equilibrium and kinetic properties of the interaction between tetrodotoxin and the excitable membrane of the squid giant axon. *J. Gen. Physiol.* 55:309–335.
20. Schwarz, J. R., W. Ulbricht, and H.-H. Wagner. 1973. The rate of action of tetrodotoxin on myelinated nerve fibres of Xenopus laevis and Rana esculenta. *J. Physiol. (Lond.)*. 233:167–194.
21. Hille, B. J. 1977. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* 69:497–515.
22. Hondeghem, L. M., and B. G. Katzung. 1977. Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim. Biophys. Acta.* 472:373–398.
23. Kenyon, J. L., and W. R. Gibbons. 1979. Influence of chloride, potassium, and tetraethylammonium on the early outward current of sheep cardiac Purkinje fibers. *J. Gen. Physiol.* 73:117–138.
24. Coraboeuf, E., and E. Carmeliet. 1982. Existence of two transient outward currents in sheep cardiac Purkinje fibers. *Pfluegers Arch. Eur. J. Physiol.* 392:352–359.
25. Brown, A. M., K. S. Lee, and T. Powell. 1981. Voltage clamp and internal perfusion of single rat heart muscle cells. *J. Physiol. (Lond.)*. 318:455–477.
26. Benndorf, K., W. Boldt, and B. Nilius. 1985. Sodium current in single myocardial mouse cells. *Pfluegers Arch. Eur. J. Physiol.* 404:190–196.
27. Coraboeuf, E., E. Deroubaix, and A. Coulombe. 1979. Effect of tetrodotoxin on action potentials of the conducting system in the dog heart. *Am. J. Physiol.* 237:H561–567.