

Kinetic Analysis of Pancuronium Interaction with Sodium Channels in Squid Axon Membranes

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ABSTRACT The interaction of pancuronium with sodium channels was investigated in squid axons. Sodium current turns on normally but turns off more quickly than the control with pancuronium 0.1–1 mM present internally. The sodium tail current associated with repolarization exhibits an initial hook and then decays more slowly than the control. Pancuronium induces inactivation after the sodium inactivation has been removed by internal perfusion of pronase. Such pancuronium-induced sodium inactivation follows a single exponential time course, suggesting first order kinetics which represents the interaction of the pancuronium molecule with the open sodium channel. The rate constant of association k with the binding site is independent of the membrane potential ranging from 0 to 80 mV, but increases with increasing internal concentration of pancuronium. However, the rate constant of dissociation l is independent of internal concentration of pancuronium but decreases with increasing the membrane potential. The voltage dependence of l is not affected by changing external sodium concentration, suggesting a current-independent conductance block. The steady-state block depends on the membrane potential, being more pronounced with increasing depolarization, and is accounted for in terms of the voltage dependence of l . A kinetic model, based on the experimental observations and the assumption on binding kinetics of pancuronium with the open sodium channel, successfully simulates many features of sodium current in the presence of pancuronium.

INTRODUCTION

Membrane ionic channels can be subject to pharmacological, chemical, and enzymatic modifications. The inactivation process of the Na-conducting system is selectively modified by the enzyme pronase (Armstrong et al., 1973) or by the specific protein reagent *N*-bromoacetamide (Oxford et al., 1976), yet the activation process is not affected by either of them. This suggests different identities of the macromolecules responsible for activation and inactivation mechanisms.

The direct interaction of chemicals with open sodium channels has been proposed to explain the voltage-dependent block of sodium conductance by H⁺ and Ca⁺⁺ ions (Woodhull, 1973), the frequency- and voltage-dependent block of sodium conductance by lidocaine derivatives (Strichartz, 1973; Courtney, 1975; Hille et al., 1975) 9-aminoacridine (Yeh and Narahashi, 1975*b*) and strychnine (Cahalan and Shapiro, 1976), and the time- and voltage-dependent block by

strychnine (Shapiro, 1975). However, detailed analysis of blocking kinetics has yet to be carried out owing to the complexity of the sodium inactivation mechanism.

Taking advantage of the selective removal of the sodium inactivation mechanism by pronase, we have been able to analyze the kinetics of pancuronium blocking of the sodium channels. Pancuronium is a bisquaternary ammonium compound with a rigid steroidal structure (Fig. 1) and is clinically used as a muscle relaxant (Baird and Reid, 1967; Savage et al., 1971). Pancuronium at internal concentrations lower than 1 mM selectively affects the sodium channels without any effect on the potassium channels. The sodium current rises normally but decays faster than the control. In the pronase-treated axons, pancuronium is still capable of inducing sodium inactivation, suggesting that pancuronium-induced acceleration of the decay of sodium conductance is not due to the acceleration of the Hodgkin-Huxley inactivation mechanism.

On the basis of this analysis, a kinetic model is proposed to account for the pancuronium-induced inactivation and other related observations. The steady-state block of sodium conductance is dependent on voltage but independent of current. The voltage-dependent block suggests the site of interaction's being inside the channel, and the current-independent block suggests the sodium channel's probably being one ion pore. A preliminary account of this investigation has appeared (Yeh and Narahashi, 1975*a*).

MATERIALS AND METHODS

Material

Giant axons from the squid *Loligo pealei* available at the Marine Biological Laboratory, Woods Hole, Mass., were used throughout the experiments.

Intact Axons

The giant axon was isolated, cleaned of connective tissues and thin nerve fibers, and mounted in a nerve chamber. It was continuously perfused externally with artificial seawater with or without a test compound.

Internally Perfused Axons

The isolated giant axon was cannulated with a glass capillary at the caudal end and transferred onto a rubber plate, and the axoplasm was squeezed out with the aid of a roller according to the method originally developed by Baker et al. (1961) and modified by Narahashi and Anderson (1967). The axon was then reinflated with standard internal solution (SIS). The reinflated axon was tied off and transferred to the nerve chamber. Hydrostatic pressure of SIS was adjusted after a slit had been made in the far end of the cannulated axon, and the axon was continuously perfused at a rate of 50–80 $\mu\text{l}/\text{min}$. The slit permitted the internal perfusate to run out of the axon and also served as an entry for the longitudinal internal electrode.

Electrophysiological Measurement

The electrodes and the method of voltage clamping were essentially the same as those described previously (Wu and Narahashi, 1973) with the exception that the series resistance compensation was incorporated into the voltage clamp circuit. The series resistance was determined by measurement of a potential jump caused by a brief current step and

was compensated as described by Armstrong (1969). Junction potentials were corrected for the internally perfused axons but not for intact axons. In voltage clamp experiments, the membrane potential was held at -70 mV in intact axons and -80 mV in internally perfused axons, and depolarizing or hyperpolarizing pulses of 8 ms duration were applied. The membrane currents associated with these step depolarizations or hyperpolarizations were recorded on film. In experiments described in Figs. 8 and 9, the leakage current and the capacitive current were electronically subtracted.

Solutions

Normal artificial seawater (ASW) had the following composition: 450 mM Na^+ , 10 mM K^+ , 50 mM Ca^{++} , 576 mM Cl^- , and 30 mM tris (hydroxymethyl)aminomethane (Tris) or 5 mM HEPES buffer at final pH 8.0. Tetramethylammonium chloride (TMA) was substituted for sodium on an equimolar basis in low Na^+ external solutions. An ASW containing 150 mM Na^+ is referred to as 150 mM Na ASW.

The standard internal solution (SIS) had the following composition: 350 mM K^+ , 50 mM Na^+ , 320 mM glutamate $^-$, 50 mM F^- , 333 mM sucrose, and 15 mM phosphate buffer at final pH 7.3. In some experiments, the concentration of sodium was adjusted to 0, 50,

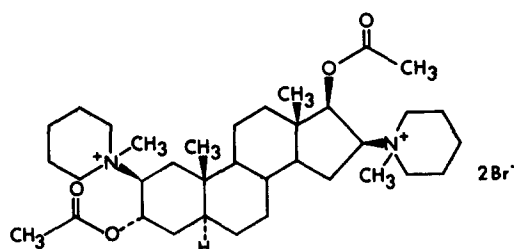


FIGURE 1. Chemical structure of pancuronium bromide (3 α ,17 β -diacetoxy-2 β ,16 β -dipiperidino-5 α -androstane dimethobromide).

and 150 mM by varying the concentration of TMA to 150, 100, and 0 mM, respectively, and by varying the sucrose concentration to maintain a constant osmolarity. Potassium-free internal solution (K-free SIS) was used in some experiments. This solution had the following composition: 275 mM Cs^+ , 50 mM Na^+ , 35 mM glutamate $^-$, 275 mM F^- , 400 mM sucrose, and 15 mM phosphate buffer at final pH 7.3.

The osmolarity was measured by vapor pressure osmometer (Wescor, Inc., Logan, Utah). The osmolarities of the external and internal solutions were kept at 1,040 and 1,060 mosM, respectively.

Data Analysis and Simulation by Kinetic Model

The membrane currents and potential recorded on 35-mm film were projected on graph paper and measurements were made manually.

The Moore-Cox kinetic model (Moore and Cox, 1976) or eight-compartment model of Hodgkin-Huxley formulation was modified to simulate the sodium current under the present voltage clamp conditions. In addition, Hodgkin-Huxley formulations for the potassium conductance system (Hodgkin and Huxley, 1952) were combined with the Moore-Cox kinetic model to simulate action potentials under current clamp conditions. The Palti version (1971) of voltage dependence of the rate constants α_m , β_m , α_n , and β_n was used in the Hodgkin-Huxley equation and Moore-Cox model. All simulations were performed either on an HP9821 programmable calculator with HP9864 digital plotter

output (Hewlett-Packard Co., Cupertino, Calif.) or a PDP-15 computer. The Euler method for numerical integration was employed, usually with steps of 10 μ s.

Statistical Analysis

The data are given as mean \pm SEM, and the difference between the means before and after application of drug was evaluated by the paired *t* test or Student's *t* test. The difference is regarded as significant when the *P* value is smaller than 0.05.

RESULTS

Effects on Membrane Potential and Action Potential

The resting membrane potential of perfused axons before and during internal application of pancuronium at concentrations ranging from 1×10^{-4} to 1×10^{-2} M was estimated to be -55.6 ± 1.2 mV ($n = 10$), and -55.1 ± 1.4 mV ($n = 10$), respectively. The difference is not statistically significant ($P > 0.05$). The resting membrane potential of intact axons varied from -50 to -58 mV, and was not affected by external application of 2.1×10^{-3} M pancuronium. The amplitude of the action potential of the internally perfused axons was decreased from 99.5 ± 5.7 mV ($n = 6$) to 89.3 ± 4.7 mV ($n = 6$) and 76.0 ± 6.3 mV ($n = 4$) by internal application of 1×10^{-3} M and 3×10^{-3} M pancuronium, respectively. The rising phase of the action potential was slightly affected, whereas the falling phase was accelerated initially and then slowed down, forming a hump (Fig. 2). At higher concentrations, the action potential was greatly suppressed or totally blocked. The action potential could be restored almost to the original shape upon washing internally with drug-free medium for 10–15 min for low concentrations and about 30 min for higher concentrations of pancuronium.

Membrane Currents in Voltage Clamp Experiments

The ionic mechanism underlying the change in the action potential caused by internal application of pancuronium was examined with voltage clamp techniques. The membrane currents associated with step depolarizations are illustrated in Fig. 3. Pancuronium at 1×10^{-3} M suppressed the peak transient sodium currents, whereas it had no effect on the steady-state potassium currents. Both inward and outward sodium currents were reduced by pancuronium. The effect of pancuronium on the sodium channel was studied mostly in axons internally perfused with SIS containing 20 mM tetraethylammonium (TEA) which preferentially blocks the potassium channel.

Internal Application of Pancuronium on Sodium Current

Sodium currents were measured with the axons perfused with standard internal solution containing 20 mM TEA or with K-free internal solution. Both the onset and decay kinetics of sodium current could be estimated directly by using the graphic technique. Before application of pancuronium, the turning-on of sodium current in internally perfused axons had the normal activation kinetics as intact axons, whereas the inactivation kinetics, measured from the decay of sodium current, was almost twice as slow as that of intact axons. With greater depolarizations, the sodium inactivation was not complete (Fig. 4 A). Pancuronium affected the sodium channel in a peculiar way (Fig. 4 B and C). The rising

phase of sodium current was not affected at all, indicating that the activation mechanism remains intact. However, the decay of sodium current was greatly accelerated. Fig. 5 shows that the falling phase of sodium current in the control axon decays quasiexponentially with a single time constant down to a steady-state level of 10%–15% of the peak value. After application of pancuronium, the

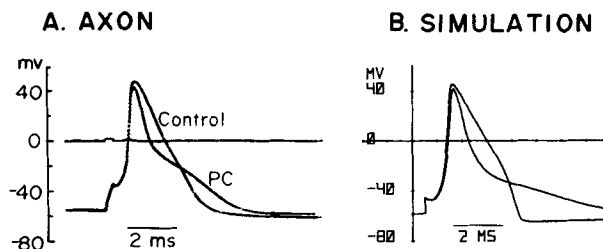


FIGURE 2. Effect of internal application of 1×10^{-3} M pancuronium (PC) on membrane action potential. A, axon; B, simulation by kinetic model. Note that the falling phase of the action potential is accelerated initially and then slowed down, forming a hump, and that the rising phase is only negligibly affected. Temperature 8°C .

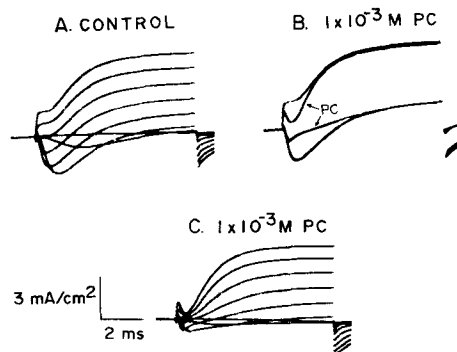


FIGURE 3. Specific effect of internal application of 1×10^{-3} M pancuronium (PC) on sodium current. A, C, Families of membrane currents associated with depolarizations in 20 mV steps (20–150 mV) from the holding potential of -80 mV. B, The upper traces are superimposed currents associated with 160 mV depolarizing pulses before and during application of PC, and the lower traces are those with 80 mV pulses. Temperature 6°C .

sodium current decayed more rapidly and consisted of multiple phases. As a result of acceleration of the decay of sodium current, the time to peak sodium current was greatly shortened, and the amplitude of peak sodium current was decreased.

Current-Voltage Relation of Peak Sodium Current

The current-voltage curve for peak sodium current of control axons was almost linear for the positive potential range. The linearity of the curve was improved after application of pancuronium because the block was less at higher depolari-

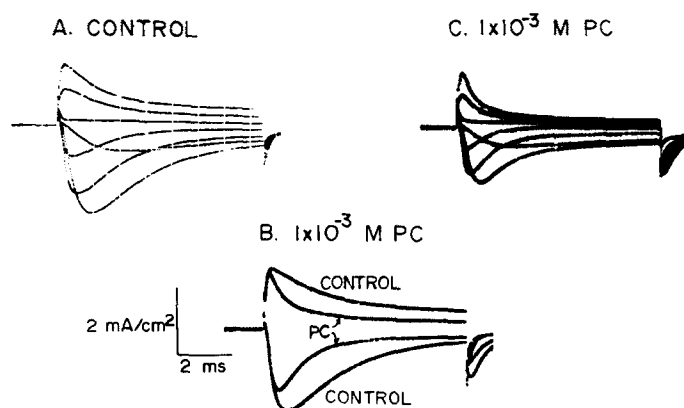


FIGURE 4. Effect of internal application of 1×10^{-3} M pancuronium (PC) on sodium currents in an axon internally perfused with 20 mM tetraethylammonium. A, C, families of sodium currents associated with 20 mV step depolarizing pulses (40–160 mV) from the holding potential of -80 mV. B, the upper traces are superimposed sodium currents associated with 160 mV depolarizing pulses before and during application of PC, and the lower traces are those with 80 mV pulses. Note that the rising phase of sodium current is not affected, while its falling phase is accelerated. Temperature 6.3°C .

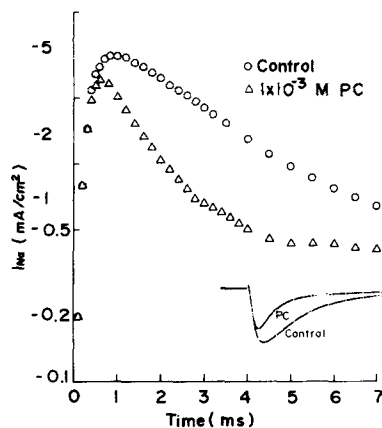


FIGURE 5. Semilogarithmic plot of the falling phase of the sodium current at 0 mV before and during internal application of 1×10^{-3} M pancuronium in the presence of 20 mM tetraethylammonium (TEA) inside. The apparent sodium currents are corrected for leakage and residual potassium current (measured by subtracting the current in 300 nM tetrodotoxin outside and 20 mM TEA inside). In the control, the sodium current decays quasiexponentially with a major fast phase falling to 10–15% of the peak value of the sodium current. During internal application of pancuronium, the sodium current decays with multiple phases.

zation. The reversal potential for sodium current was shifted from 45 ± 1.5 mV ($n = 10$) to 42 ± 1.0 mV ($n = 10$) by 1×10^{-3} M pancuronium. However, part of the shift could be due to the error in measurement of the outward sodium current in the presence of pancuronium. Pancuronium decreased the sodium conductance more significantly at 0 mV than at more positive potentials (Table

I). It is also interesting to note that the decrease in conductance was more pronounced at low temperature (5°–6°C). The temperature- and voltage-dependent block expected from the kinetic model is to be discussed later.

Sodium Ions Do not Compete with Pancuronium for Sodium Channels

Internal sodium concentration was varied, keeping the ionic strength constant with TMA, and the effect of pancuronium on sodium conductance was evaluated to see if there was any competition between Na⁺ ions and pancuronium molecules for sodium channels. Table II shows that there is no statistically

TABLE I
PERCENTAGE OF DECREASE IN PEAK SODIUM
CONDUCTANCE (G_{Na}) BY 1×10^{-3} M PANCURONIUM AT
DIFFERENT MEMBRANE POTENTIALS AND TEMPERATURES

Temperature °C	G_{Na} decrease	
	0 mV %	80 mV %
5–6*	41.7±0.93‡	29.3±1.98‡
8–9*	31.1±1.28§	21.7±1.23§

Data are given as mean ± SEM ($n = 6$).

* The mean values at 5–6°C and 8–9°C are significantly different at both 0 mV and 80 mV ($P < 0.005$).

‡ The mean values at 0 mV and 80 mV are significantly different ($P < 0.005$).

§ The mean values at 0 mV and 80 mV are significantly different ($P < 0.005$).

TABLE II
DECREASE IN PEAK SODIUM CONDUCTANCE (G_{Na}) BY 1×10^{-3}
M PANCURONIUM AT DIFFERENT MEMBRANE POTENTIALS
AND INTERNAL SODIUM CONCENTRATIONS ($[Na]_i$)

$[Na]_i$ mM	G_{Na} decrease		n
	0 mV %	80 mV %	
0	28.5±2.19	19.1±2.05	2
50	31.1±1.28*	21.7±1.23‡	6
150	28.8±1.99*	20.1±1.46‡	5

Data are given as mean ± SEM.

* The mean values in 50 mM and 150 mM Na are not significantly different ($P > 0.2$).

‡ The mean values in 50 mM and 150 mM Na are not significantly different ($P > 0.2$).

significant difference in the effect of pancuronium between 50 mM and 150 mM Na⁺ SIS at 0 mV. The pancuronium effects at these sodium concentrations are probably not significantly different when compared to those in Na-free SIS, although the number of experiments is too small to make a statistical test. Similarly, there are no significant differences among three Na⁺ concentrations when compared at 80 mV. These results indicate that there is no direct competition between Na⁺ ions and pancuronium molecules for the binding site in the sodium channel.

Effect of Pancuronium on Sodium Tail Current

Upon step repolarization, the sodium current is turned off with a fast time course. This current is referred to as a sodium tail current. The tail current of the control axon decayed with a single exponential function. The time constant was dependent on the membrane potential as given in Table III. In the presence of pancuronium, however, the shape and amplitude of the tail current depended on the duration as well as the magnitude of step depolarization. If the sodium current was terminated before or at the peak, the tail current had the normal time course of decay (Fig. 6 A and B; Table III). However, if the sodium current was terminated later, the tail current developed a hook (Fig. 6 D), that is, it initially increased in amplitude, then decayed slowly. The initial phase of tail current is complicated by capacitive and leakage currents. The time constants of

TABLE III
TIME CONSTANTS OF SODIUM TAIL CURRENTS UPON
REPOLARIZATION FROM 0 mV TO VARIOUS MEMBRANE
POTENTIALS (E_m) AT THE PEAK OF TRANSIENT SODIUM
CURRENT AND AT THE END OF 8-MS PULSE BEFORE AND DURING
INTERNAL PERFUSION OF 1×10^{-3} M PANCURONIUM

E_m mV	At peak		At 8 ms	
	Control μ s	Pancuronium μ s	Control μ s	Pancuronium μ s
-80	237 ± 18*	258 ± 20	237 ± 18*	427 ± 13
-70	368 ± 10	373 ± 22		
-60	587 ± 16	565 ± 29		

Data are given as mean ± SEM. $n = 17$ for measurements at -80 mV and $n = 4$ at -70 mV and -60 mV.

* Since the time constant of the tail current upon repolarization to -80 mV is independent of the duration of depolarization, the values at the peak and at the end of the 8-ms pulse are pooled together to give $237 \pm 18 \mu$ s.

tail current associated with the -80 mV membrane potential after an 8-ms step depolarization were 0.24 ± 0.018 ms and 0.43 ± 0.013 ms ($n = 17$) for the control and pancuronium-treated axons, respectively.

In a manner like that of the transient inward sodium current during step depolarization, the hooked tail current in pancuronium-treated axons disappeared upon elimination of sodium from the external medium, and was blocked by ASW containing 300 nM TTX (Fig. 6 E). These results show that the tail current in pancuronium is due to sodium ions flowing through the sodium channel. The small residual current in TTX is due to a potassium current because it is absent in K-free external and internal media.

The kinetics of the tail currents in the presence of pancuronium upon repolarization to various negative potentials was very much different from that of the control axon. It is clearly seen in records A and B of Fig. 7 that the pancuronium-induced tail currents associated with -40 and -80 mV decay more slowly than the control tail currents. The simulation by the kinetic model, which will be described later, could reproduce many features of the tail current as shown in parts C and D of Fig. 7.

Tail Current in Pancuronium

In normal axons, the tail current associated with step repolarization gradually decreased in amplitude as the step depolarization increased in duration beyond the time when the sodium current reached its peak, because the sodium channel is inactivated by maintained depolarization. After application of pancuronium, the tail current initially decreased in amplitude with increasing pulse duration, but it reached a steady-state level after a few milliseconds. The amplitude of tail

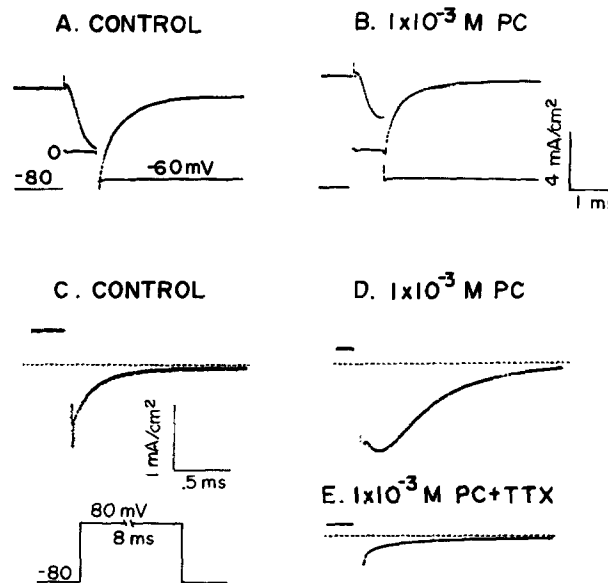


FIGURE 6. Effect of internal application of 1×10^{-3} M pancuronium (PC) on the sodium tail current in axons internally perfused with SIS containing 20 mM tetraethylammonium. A, B, the tail current associated with repolarization to -60 mV after a 0.7 ms depolarizing pulse to 0 mV in control (A) and in pancuronium (B). Pancuronium does not affect time course of turning-off of the tail current. C–E, the depolarizing pulse is terminated at a later phase (at 8 ms) and the membrane is repolarized to -80 mV. The tail current in the presence of PC (D) is very different from the control (C), exhibiting a pronounced hook and a slow decaying phase. The pancuronium-induced tail current is blocked by 300 nM TTX (E). The dotted lines in C, D, and E are absolute zero current line. Temperature 6°C .

current at a steady state is dependent on the conditioning potential as shown in Figs. 9 and 11. This will be discussed in more detail later.

THE HOOKED TAIL CURRENT IS PROPORTIONAL TO THE "OPENNESS" OF THE SODIUM CHANNEL Two observations suggest that the pancuronium block of the sodium channels requires the opening of the channel. First, during step depolarizations which open sodium channels, the rising phase of sodium current of the control and that of the pancuronium-treated axon are superimposable (see Fig. 4B). Second, the slow tail current with a hook develops a few milliseconds later than the peak of transient sodium current. This delay suggests a

possible precursor-product relationship (see model in Discussion) between the peak transient and the tail slow components. This relationship will be examined further in the next section.

If the development of pancuronium block depends upon the "openness" of

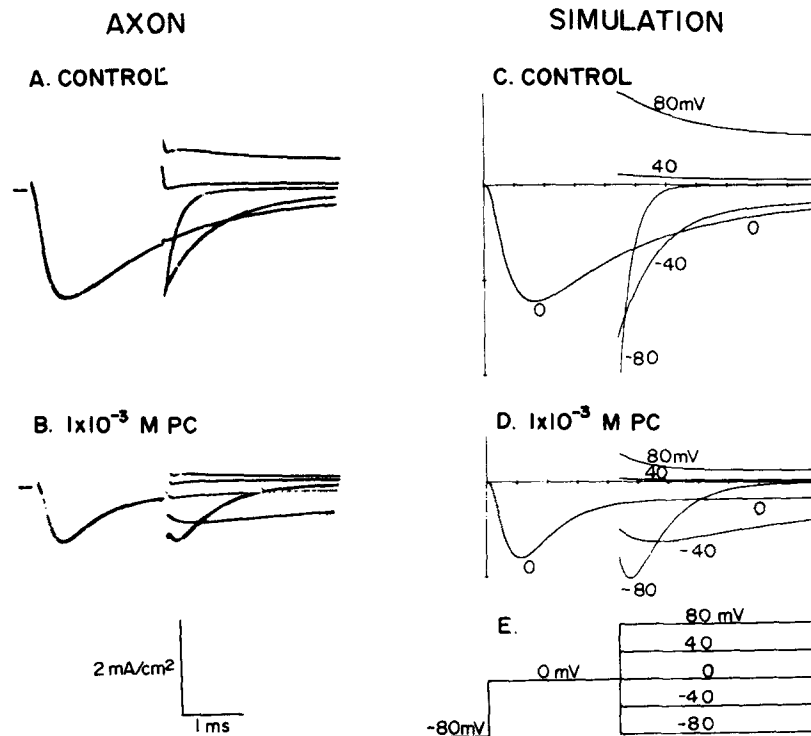


FIGURE 7. The sodium tail currents observed before (A) and during (B) internal perfusion of 1×10^{-3} M pancuronium (PC), and those simulated in the corresponding conditions (C and D). The membrane was depolarized to 0 mV for a period of 2.2 ms, and then step repolarized to 80, 40, 0, -40, and -80 mV (E). In the control, the tail currents are turned off very quickly at -80 mV, and more slowly at -40 mV. In PC, the tail currents are turned off very slowly especially at -80 and -40 mV. The rate constant of α_m and β_m are calculated from the Hodgkin-Huxley equation. The values of the rate constants, α_h , β_h , k , and l used in simulation are experimentally determined (in ms^{-1}): at 80 mV, $\alpha_h = 0.17$, $\beta_h = 0.707$, $k = 1.5$, $l = 0.075$; at 40 mV, $\alpha_h = 0.17$, $\beta_h = 0.707$, $k = 1.5$, $l = 0.15$; at 0 mV, $\alpha_h = 0.05$, $\beta_h = 0.50$, $k = 1.5$, $l = 0.32$; at -40 mV, $\alpha_h = 0.026$, $\beta_h = 0.27$, $k = 0.5$, $l = 0.65$; at -80 mV, $\alpha_h = 0.19$, $\beta_h = 0.097$, $k = 0.25$, $l = 2.0$. The rate constant from N to P route in Moore-Cox model (Scheme III) is $0.3 \beta_m$ for 80, 40, 0, -80 mV and $0.63 \beta_m$ for -40 mV. $E_{\text{Na}} = 35$ mV. Temperature 8°C .

the sodium channel, then the conductance during the tail current should be proportional to the sodium conductance during the peak current. Such a dependence could be examined only in experiments in which the openness of the sodium channel was varied by applying various prepulses which do not appreciably activate channels. The instantaneous amplitude of the tail current

could not be graphically estimated because of the short duration of the initial rising phase. Therefore, the amplitude of the tail current was measured at the peak that occurred at about 0.3 ms after repolarization (Fig. 6 D). The amplitude of the peak transient sodium current associated with a constant test pulse (0 mV) that followed various prepulses and the tail current associated with a constant repolarization pulse to -80 mV after the test pulse were both measured and plotted as a function of the prepulse potential in Fig. 8. These two currents show

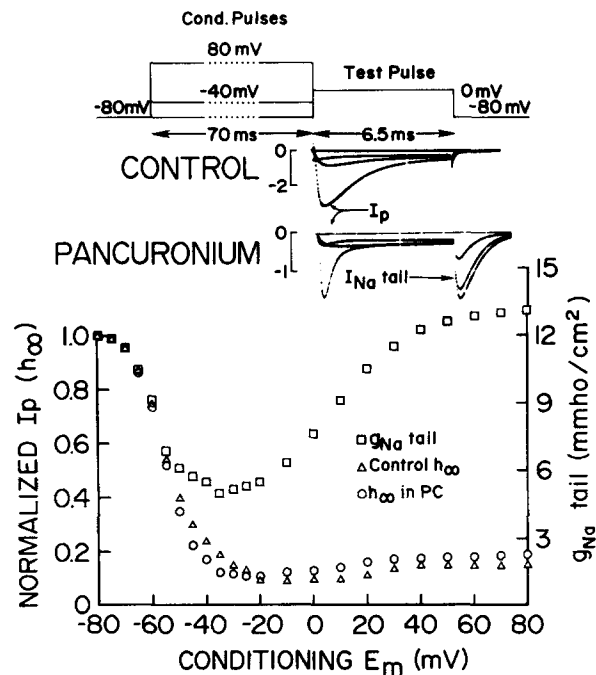


FIGURE 8. The amplitude of normalized peak sodium current associated with step depolarization to 0 mV (Δ h_{∞} in control and \circ h_{∞} in pancuronium) and the amplitude of tail conductance associated with step repolarization to -80 mV were plotted as a function of the membrane potential of the 70 ms conditioning pulse. The ordinate on the right represents the amplitude of the sodium tail conductance in the presence of 1×10^{-3} M pancuronium. Temperature 8°C .

the same potential dependence in the membrane potential range of -80 mV to -55 mV. The tail current increases with depolarization beyond -20 mV despite the fact that the steady-state inactivation remains almost constant. The potential dependence of the tail current will be analyzed in detail in the following section.

THE TAIL CURRENT IN PANCURONIUM IS DEPENDENT UPON MEMBRANE POTENTIAL The magnitude and rate of development of the tail current in the presence of pancuronium were dependent upon the membrane potential. The membrane was step depolarized to various levels and for various durations, and then was repolarized to -80 mV. The left-hand column of Fig. 9 depicts examples of membrane currents recorded under such conditions. The mem-

brane was depolarized to +80 mV, +45 mV, and 0 mV in records A1, B1, and C1, respectively. The tail current with a hook developed with a lengthening depolarizing step. The development of the tail current was faster at large depolarization than at small depolarization. It should be noted that the hooked tail current does not require net current flowing through sodium channels, for it is developed after depolarization to the reversal potential for sodium channels

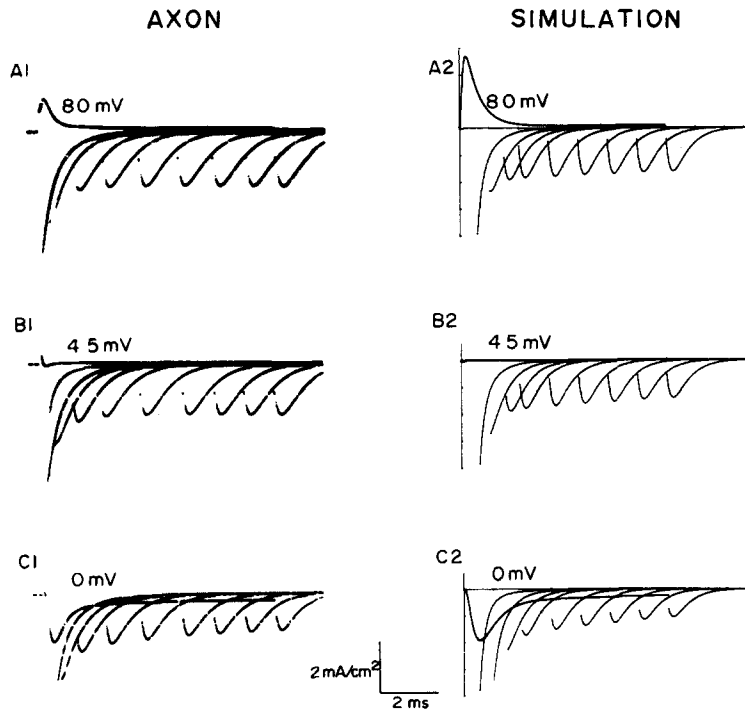


FIGURE 9. Time course of development of tail currents in the presence of 1×10^{-3} M pancuronium and its dependence on the conditioning pulse. A1-C1, observed; A2-C2, simulated. The tail currents were recorded at -80 mV after conditioning pulses of various durations and potentials (A, 80 mV; B, 45 mV; C, 0 mV). The values of the rate constants k and l used in simulation by the kinetic model are as follows (in ms^{-1}): at 80 mV, $k = 1.5$, $l = 0.075$; at 45 mV, $k = 1.5$, $l = 0.14$; at 0 mV, $k = 1.5$, $l = 0.25$; and at -80 mV, $k = 0.25$, $l = 2.0$. The rate constant in the Moore-Cox kinetic model for the N to P route is $0.3\beta_m$ at -80 mV. Temperature 8°C .

(Fig. 9B1). The simulation from the kinetic model gave a satisfactory fit as shown in the right-hand column of Fig. 9 (A2, B2, and C2).

Fig. 10A illustrates a family of the pancuronium-induced tail currents associated with repolarizations to -80 mV from various depolarizations of 8 ms duration. The amplitude of tail current increased with increasing step depolarization. The kinetic model simulation gives a satisfactory fit (Fig. 10B).

Sodium conductances (G_{Na}) were calculated from the tail currents upon repolarization to -80 mV after 8-ms depolarizing conditioning pulses of various amplitudes, and are plotted as a function of the conditioning membrane poten-

tial (E_m) in Fig. 11 (open triangles). The peak G_{Na} - E_m curves for the control (open circles) and the pancuronium-treated (closed circles) axons are also shown in Fig. 11 for comparison. The peak sodium conductance of the control and pancuronium-treated axons reached a maximum at membrane potentials more positive than 0 mV, indicating that the maximum number of sodium channels is open in this potential range. In contrast, the tail G_{Na} continuously increased with increasing depolarization beyond 0 mV, representing the voltage-dependent component for development of the tail current. However, the " m^3 " gate dependence of the development of pancuronium tail current is difficult to assess

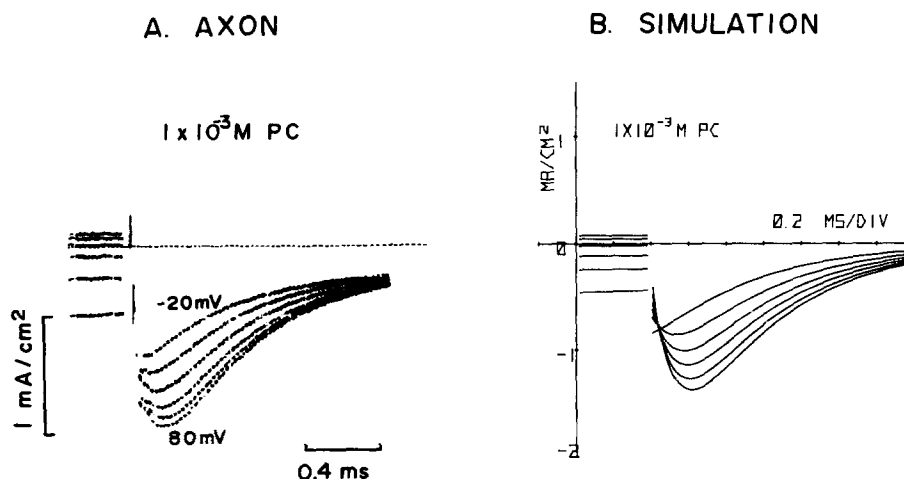


FIGURE 10. Voltage-dependence of the amplitude of tail sodium current in the presence of 1×10^{-3} M pancuronium (PC). A, observed; B, simulation by the kinetic model. The tail currents were produced by repolarizations to -80 mV after step depolarizations to -20 , 0 , 20 , 40 , 60 , and 80 mV for 8 ms. The potentials on the figure refer to the step depolarizations. The values of the rate constants k and l used in simulation at different membrane potentials are (in ms^{-1}): -20 mV, $k = 1.5$, $l = 0.45$; 0 mV, $k = 1.5$, $l = 0.32$; 20 mV, $k = 1.5$, $l = 0.22$; 40 mV, $k = 1.5$, $l = 0.16$; 60 mV, $k = 1.5$, $l = 0.11$; 80 mV, $k = 1.5$, $l = 0.075$, and -80 mV, $k = 0.25$ and $l = 2$. Temperature $8^\circ C$.

because there is no measurable tail current after smaller depolarizations where the parameter m has the steepest voltage-dependence.

Pancuronium Does not Change Inactivation of Unbound Sodium Channel

TIME CONSTANT OF SODIUM INACTIVATION Sodium channels can be inactivated by a membrane potential which by itself does not activate the channel. To obtain the time constant of inactivation, the membrane potential was clamped at -80 mV, and conditioning pulses to various membrane potentials ranging from -60 to -30 mV were applied for various durations and were followed by a test depolarizing pulse to 0 mV. The time course of the decrease in peak sodium current associated with the test pulse is indicative of the sodium inactivation kinetics at the conditioning membrane potential. To obtain the time constant of

recovery from inactivation, the membrane potential was clamped at -60 mV, and conditioning pulses to -100 mV were applied for various durations and followed by a test depolarizing pulse to 0 mV. The time course of the recovery in peak sodium current associated with the test pulse is also indicative of the sodium inactivation kinetics at the conditioning membrane potential.

The values of the time constant (τ_c) obtained by these two methods are given in Table IV. Pancuronium did not significantly alter the time constant, indicating that inactivation of the closed sodium channel is not affected by the drug.

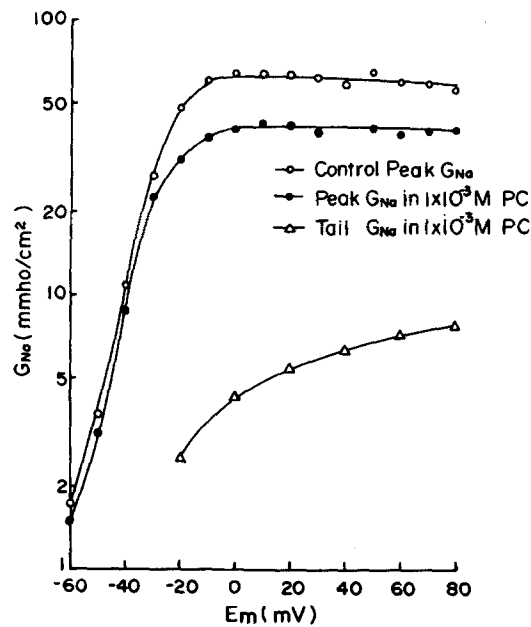


FIGURE 11. Semilogarithmic plot of peak sodium conductance (G_{Na}) before and during internal perfusion of 1×10^{-3} M pancuronium (PC) and of tail sodium conductance in PC as a function of the membrane potential (E_m). The tail G_{Na} values were estimated from the tail currents as illustrated in Fig. 9. The currents were measured at 0.3 ms after the beginning of repolarization to -80 mV. Note that peak G_{Na} attains a maximum around 0 mV, and that tail G_{Na} continues to increase with depolarization beyond 0 mV. Temperature 8°C .

STEADY-STATE SODIUM INACTIVATION (h_∞). Conditioning pulses of various amplitudes with a 70-ms duration were given before a constant test pulse to 0 mV, and the inward sodium current associated with the test pulse was measured and normalized to the maximum value. The normalized value is plotted against the membrane potential of the conditioning pulse in Fig. 8, and denoted as the h_∞ curve. The control h_∞ curve does not become zero with depolarization, and a foot appears at membrane potentials ranging from -20 mV to 80 mV.¹ We have

¹ Chandler and Meves (1970) observed that non-zero steady-state values of h_∞ in the squid axon perfused internally with 300 mM NaF were much greater at 52 mV than at -29 mV. Our internal perfusate contained only 50 mM Na^+ , and preliminary observations with varying internal sodium concentrations did not reveal the sodium dependence of the foot of the h_∞ curve.

not attempted to fit the values of steady-state inactivation of internally perfused axons by any model, and further experiments are needed to clarify whether this is unique in internally perfused axons or is due to ionic composition of internal solution.

According to the kinetic model described in section 4 of the Discussion, an open sodium channel (N state) is either inactivated (O) or occluded by pancuronium (Q state). The decrease in sodium current during a prolonged depolarization in pancuronium or the apparent sodium inactivation is a sum of the true inactivation (closing of h gate) and the pancuronium block. The relative importance of these two routes, one being the true inactivation through h gate and the other through pancuronium block, depends on the membrane potential of prepulse. At the membrane potentials more negative than -50 mV where the m^3 gate is largely in a closed state, the true sodium inactivation predominates since the prerequisite for pancuronium block is the activation of the sodium channel.

TABLE IV
TIME CONSTANTS (τ_c) OF SODIUM INACTIVATION
MEASURED BY DOUBLE PULSE TECHNIQUE BEFORE AND
DURING INTERNAL PERFUSION OF 1×10^{-3} M PANCURONIUM

Membrane potential mV	τ_c		n
	Control ms	Pancuronium ms	
-100	2.05 ± 0.10	2.16 ± 0.71	2
-60	15.33 ± 1.76	19.50 ± 2.84	3
-50	15.70 ± 3.21	14.67 ± 1.69	3
-40	9.48 ± 1.34	9.65 ± 1.59	4
-30	5.55 ± 0.73	4.97 ± 0.03	4

Data are given as mean ± SEM.

The values at -100 mV are the time constants for recovery of sodium current, and those at other potentials are the time constants for onset of inactivation.

The steady-state inactivation curve was not affected by pancuronium at membrane potentials more negative than -50 mV (Fig. 8). The membrane potentials at which h_∞ was equal to 0.5 were -50 ± 1.6 mV and -51 ± 2.0 mV ($n = 9$) in the control and pancuronium-treated axons, respectively. The route for pancuronium block becomes increasingly important as the conditioning membrane potential becomes more positive. This assertion was tested in the following manner. Long conditioning pulses (70 ms) of various amplitudes were immediately followed by a short test pulse (6.5 ms) to 0 mV, and the tail current associated with the repolarization of the test pulse to -80 mV was measured. The tail current peaks at about 0.3 ms after the end of the test pulse. The peak amplitude of tail current should reflect the population of pancuronium-bound sodium channel at the membrane potential more positive than -20 mV. Fig. 8 clearly shows that the amplitude of the sodium tail conductance increases with depolarization beyond -20 mV.

*Estimation of Direct Interaction Between Pancuronium
and the Open Sodium Channel*

The kinetics of the interaction between pancuronium and the open sodium

channel is difficult to study with axons that have an intact inactivation mechanism. The direct interaction could be investigated with the axons in which sodium inactivation had been removed by use of pronase. When the axon was internally perfused with 0.2 mg/ml pronase for about 10 min at 8°C, the sodium inactivation disappeared. The sodium channel had normal activation kinetics but failed to inactivate during maintained depolarization (Fig. 12). If pancuron-

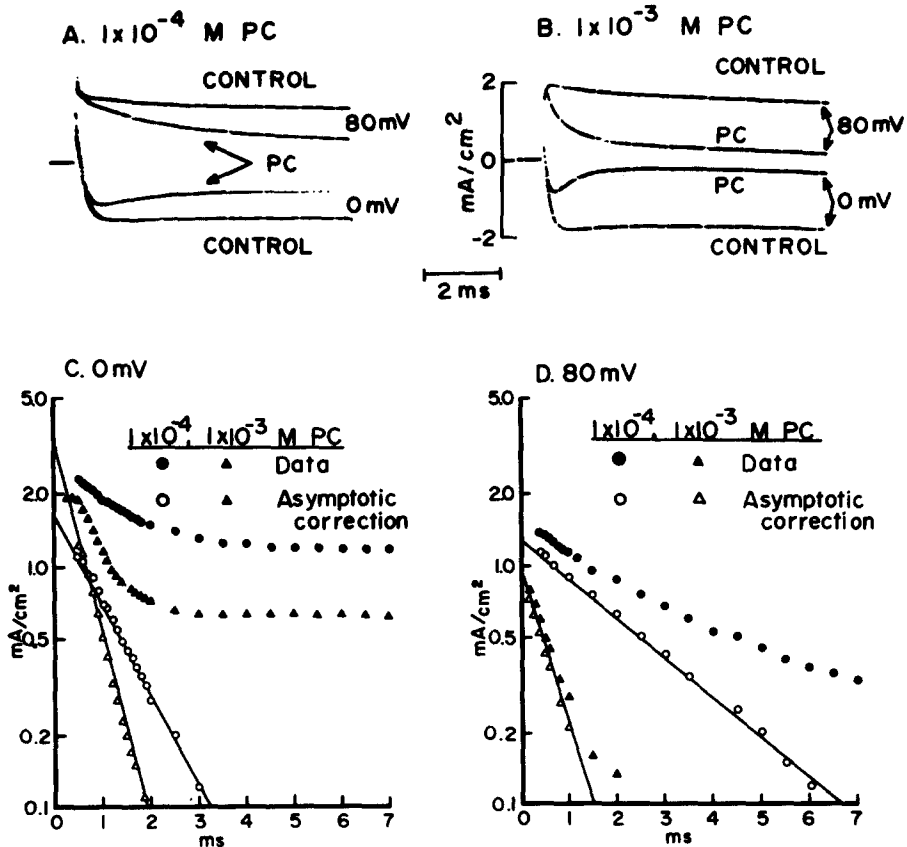


FIGURE 12. Time course of pancuronium (PC)-induced decay of sodium current in axons internally treated with 20 mM tetraethylammonium after internal pronase treatment. A, control and 1×10^{-4} M PC; B, control and 1×10^{-3} M PC; C and D, semilogarithmic plot of the falling phase of sodium current in the presence of PC at 0 mV and 80 mV, respectively. The time constants of PC-induced decay of sodium currents are estimated after asymptotic correction. Temperature 10°C.

ium binds to the open sodium channel from the axoplasmic side and blocks the passage of sodium ions through the channels, then the sodium current in the pronase-treated axons should decline during maintained depolarization in the presence of pancuronium. This was in fact the case, as is shown in Fig. 12 A, B. The sodium current decayed with a single exponential time course, reaching a steady-state value. The time constant was estimated graphically from the semilogarithmic plot of the falling phase of the sodium current as shown in parts C

and D of Fig. 12. The data are given in Table V which shows that the time constant is dependent on the concentration of pancuronium, becoming shorter with increasing concentration. However, the time constant did not exhibit a clear-cut membrane potential dependence. The steady-state value of the sodium conductance was dependent on both the pancuronium concentration and membrane potential. The voltage dependence of the pancuronium block suggests that the binding site is located somewhere within the membrane along the sodium channels.

Rate Constant for the Pancuronium Block

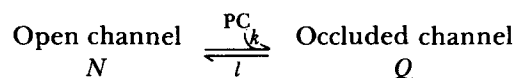
Pancuronium induced an inactivation with a single exponential time course in the axon in which sodium inactivation had been removed by pronase. This

TABLE V
TIME CONSTANTS OF THE PANCURONIUM-INDUCED
SODIUM INACTIVATION IN PRONASE-TREATED AXONS

Membrane potential mV	Pancuronium (mM)		
	0.1	0.5	1.0
-20	2.05±0.60	1.25	0.90
-10	1.33±0.17	1.07	0.60
0	1.42±0.22	1.03	0.54
10	1.42±0.06	1.00	0.48
20	1.47±0.31	1.11	0.53
30	1.85		
40	1.95±0.45		
50	2.07±0.247		
60	2.31±0.193	1.21	0.69
70	2.37±0.073	1.18	0.72
80	2.41±0.047	1.20	0.70

Data are given as mean ± SEM ($n = 3$) or as the average of two experiments.

suggests that the interaction of pancuronium with the sodium channel obeys first order kinetics. We assume that in the first order process a pancuronium molecule has to traverse a single energy barrier in order to bind to a site (energy well) within the sodium channel. The energy barrier model for drug-induced block of sodium conductance will be described elsewhere. The time constant of the pancuronium-induced inactivation is longer than the time constant for the sodium channel to open. Therefore, the following scheme can be used to represent the pancuronium-induced inactivation:



where PC refers to pancuronium, k is the forward rate constant indicative of pancuronium entering the open sodium channel with a concentration-dependent component, and l is the backward rate constant representing pancuronium leaving the channel and should be independent of pancuronium concentration. The time constant τ measured from the falling phase of sodium current is related to k and l :

$$\tau = \frac{1}{k + l}. \quad (1)$$

The change in sodium conductance after all sodium channels are open is given by

$$\frac{dN}{dt} = -kN + lQ, \quad (2)$$

where at a steady state $\frac{dN}{dt} = 0$. It follows from Eq. (2) that

$$\frac{N}{N + Q} = \frac{l}{k + l} = l\tau. \quad (3)$$

The term $N/(N + Q)$ equals the ratio of the sodium current in pancuronium to that of the control. Therefore, l can be estimated from Eq. (3) and k from Eq. (1). The values of k and l thus estimated are plotted as a function of the membrane potential in Fig. 13. The k value increased with the concentration of pancuronium as expected from the model but did not seem to be a linear function of pancuronium concentration. It did not exhibit a clear-cut voltage dependence at the membrane potentials more positive than -10 mV. At more negative potentials, k seems to show a tendency of voltage dependence, although the data scatter. The backward rate constant l exhibited a voltage dependence, whereas it is independent of the pancuronium concentration or external sodium concentration. The voltage dependence of l is expressed by the equation

$$l = l_0 e^{-z\delta E_m F/RT}, \quad (4)$$

where l_0 is the rate constant at zero potential, z is $+2$ for the charge of pancuronium, δ is the fraction of the distance along the potential field from the peak of energy barrier to the energy well, F , R , and T have their conventional meanings. This equation will be used for computation of action potential and sodium current of the pancuronium-treated axons.

Recovery from Time-Dependent Pancuronium Block

In the pancuronium-treated axon in which sodium inactivation has been removed by internal perfusion of pronase, sodium conductance during step depolarization declines in a time-dependent manner (Fig. 12 A and B). Recovery from the time-dependent pancuronium block of sodium conductance was studied by the double pulse method. Twin pulses separated by various intervals are given to activate the m^3 gate, and the amplitude of sodium current associated with the second pulse is a measure of the degree of recovery from pancuronium block occurring with the first pulse. As shown in Fig. 14 B, the sodium current associated with the first pulse in the pronase-treated axon rises quickly to a steady-state level and does not inactivate during the depolarization to 80 mV. Upon repolarization to -80 mV, the tail current turns off quickly as the m^3 gate is closing. However, the amplitude of the sodium current associated with the second pulse is the same as that associated with the first pulse. In contrast, the

sodium current associated with the first pulse in the presence of pancuronium rises normally but decays during a maintained depolarization to 80 mV, and the tail current turns off slowly upon repolarization to -80 mV (Fig. 14c). The slowness of the falling phase of tail current suggests that the m^3 gate of the pancuronium-bound sodium channel cannot be closed by repolarization to -80 mV. Rather, the sodium channel has to be free from pancuronium before the m^3 gate can be closed. The dissociation of pancuronium from the sodium channel is slow when compared to the closing of the m^3 gate. Consequently, the former

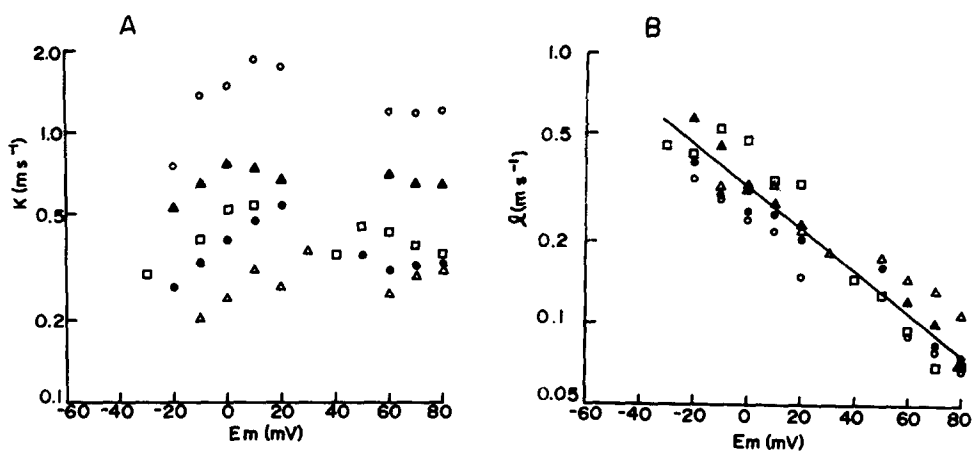


FIGURE 13. Relationship between the rate constants k and l and the membrane potential (E_m) at various concentrations of pancuronium (PC). The pancuronium concentration and external sodium concentration are 1×10^{-3} M PC and 450 mM Na (O); 5×10^{-4} M PC and 450 mM Na (Δ); 1×10^{-4} M PC and 450 mM Na (\square); 1×10^{-4} M PC and 150 mM Na (\bullet) and (Δ). A, the forward rate constant k is dependent on the concentration of pancuronium, but independent of membrane potential at $E_m > \sim 0$ mV. B, the backward rate constant l is dependent on the membrane potential but independent of the concentration of pancuronium. The pooled data were fitted by a linear regression line with correlation coefficient $r = 0.94$ ($n = 41$). The line is represented by Eq. (4), with $l_0 = 0.32 \text{ ms}^{-1}$, $\delta = 0.21$ and $z = 2$ for the charge of pancuronium. Temperature 8–10°C.

process becomes the rate-limiting step for the decay of the tail current. Recovery from pancuronium block is measured from the double pulse technique as shown in Fig. 14c. The sodium current associated with the second pulse reaches the same level as the first only when the second pulse is separated from the first by several milliseconds. The time course of recovery from pancuronium block is a single exponential function, as shown in Fig. 14a. The time constant of recovery measured at -80 mV by the double pulse technique was 0.65 ms which was the same as that measured from the falling phase of tail current in the same axon. Reducing the external sodium concentration to 25% of the normal value did not change the time constant of recovery from the time-dependent pancuronium block.

Effect on Potassium Current

Pancuronium had no appreciable effect on potassium current at concentrations up to 1 mM. At higher concentrations pancuronium exhibited some inhibitory effect on potassium current. The apparent dissociation constant estimated from the decrease in potassium current by pancuronium is around 12 mM, which is almost two orders of magnitude greater than the dissociation constant for sodium channel measured at zero membrane potential in pronase-treated axons.

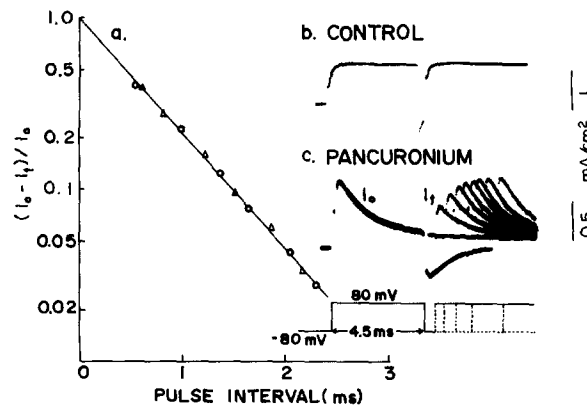


FIGURE 14. Time course of recovery from pancuronium block of the sodium channel. Two consecutive pulses to +80 mV separated by a variable pulse interval were applied to an axon in which the sodium inactivation mechanism had been removed by pronase treatment. The axon was bathed in external K-free normal Na or 25% Na ASW and internal K-free SIS. In the control pronase-axon, the amplitude of the sodium current associated with the second pulse was not affected by the first pulse (see *b* of this figure). In the presence of 3×10^{-4} M pancuronium, both sodium currents (labeled I_0 and I_1) were depressed, but the second one was more so than the first if the interval between the two pulses was very short. The ratio $(I_0 - I_1)/I_0$ is plotted as a function of pulse interval and is linear on semilogarithmic plot. The time constant measured in 25% Na ASW (\circ) is the same as in normal Na ASW (\triangle), being 0.65 ms.

External Application of Pancuronium

When applied externally to the intact or perfused axon, pancuronium had no effect on the resting and action potentials and on sodium and potassium conductances at concentrations up to 2.1 mM.

DISCUSSION

When perfused internally, pancuronium selectively affects the sodium-conducting system without changing the potassium-conducting system. The effect of pancuronium on the sodium current as measured in the presence of TEA inside is characterized by an acceleration of the falling phase. It resembles, at a glance, acceleration of the sodium inactivation process. Upon repolarization after several milliseconds of step depolarization, the sodium tail current is not turned off

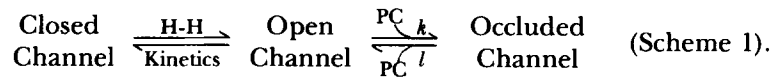
with a single exponential function. Instead, it rises initially then decays slowly, exhibiting a pronounced hook.

Acceleration of Decay of Sodium Current Is not Due to Speeding up of the Sodium Inactivation

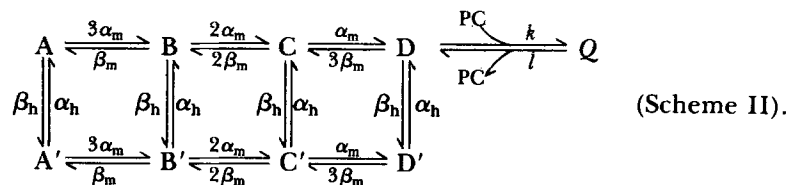
The steady-state sodium inactivation curve and the time constant of sodium inactivation are not affected by pancuronium. Thus the sodium inactivation mechanism is not impaired by pancuronium. Stronger evidence for this notion is provided by the pronase experiment. Internal perfusion of pronase removes the sodium inactivation, leaving the activation process intact. However, pancuronium causes the sodium current to decay in the pronase-treated axon. This observation strongly supports the hypothesis that pancuronium interacts with the open sodium channels and that the interaction results in a decrease in the number of conducting sodium channels.

Conceptual Model for Pancuronium Block of Sodium Channel

The most important feature of the pancuronium-induced sodium inactivation is that the inactivation is coupled with activation. This concept can be expressed by the following reaction scheme:



The first step can be described by the Hodgkin-Huxley gating mechanism and the second step represents the pancuronium interaction only with the open sodium channel. The pancuronium-containing sodium channel is occluded and incapable of conducting sodium ions. Both *h* and *m*³ gates of the sodium channel are still in the open state yet are occluded by pancuronium. In contrast, the *h* gate of the normal inactivated sodium channel is in the closed state. It is convenient to expand this general scheme to a compartment model incorporating the Hodgkin-Huxley gating kinetics. The rigorous test of the above scheme would be to represent the Hodgkin-Huxley equation by the conventional eight-compartment kinetic scheme (cited in Moore and Cox, 1976) with an addition of the ninth compartment representing the pancuronium-occluded channels.



Compartment D corresponds to the *m*³*h* kinetics as described by the Hodgkin-Huxley formulation.

The turn-on process (activation) of the sodium channel of the axons internally perfused with SIS containing TEA or K-free SIS is the same as that of intact

axons, and is very similar to that calculated from the Hodgkin-Huxley equation. However, the inactivation process of the sodium channel is slowed by a factor of about two in the perfused axon. A similar observation has been reported by Adelman (1972). Moreover, a larger portion of sodium current failed to inactivate at the potential more positive than 0 mV after a long pulse lasting for 70 ms. Whether these changes in the inactivation process are due to the internal perfusion per se or due to TEA interacting with the open sodium channel remains to be determined. In the simulation of sodium currents of internally perfused axons, normal m values were used for both normal and pronase-treated axons. The inactivation parameter h was assumed to be unity in the pronase-treated axon, since sodium inactivation was removed by this treatment. To fit the sodium inactivation, α_h and β_h were calculated from h_∞ and τ_h values which were determined experimentally.

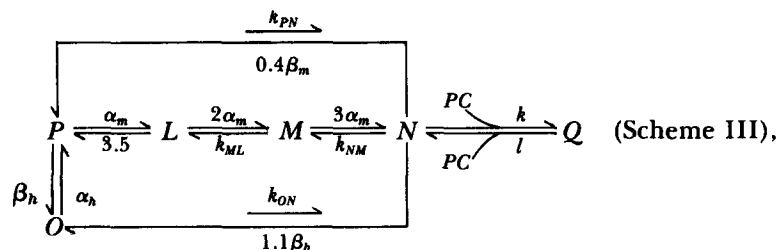
In the simulation of sodium currents in the presence of pancuronium, the kinetics of the gating process is assumed to be unaffected by pancuronium, since the initial rising phase of sodium current and the tail current associated with repolarization before the sodium current reaches its peak are not changed by pancuronium (see Figs. 4 B and 6 B). The sodium currents thus calculated are shown in Fig. 15. The currents in both control and pronase-treated axons resemble those observed experimentally (compare Fig. 15 A with Fig. 4 B for control, and Fig. 15 B with Fig. 12 B for pronase). Pancuronium is assumed to interact with the open sodium channels with rate constants k and l which can be determined experimentally.

The Moore-Cox kinetic model (Moore and Cox, 1976) was also used for simulation and fit as well as the simulation based on the modified nine-compartment model (Scheme II). This implies that our kinetic model for pancuronium block of sodium channel is independent of the formulation used to describe normal channel gating. Because of its simplicity, the Moore-Cox model was used in the following simulation.

Modified Moore-Cox Kinetic Model for Sodium Channel

A kinetic reaction sequence for the sodium conducting system in the squid axon has recently been proposed by Moore and Cox (1976). Their kinetic model not only matches the computation of the original Hodgkin-Huxley formulation but also simulates the experimental observations of Frankenhaeuser and Hodgkin (1957) on the effects of altered external calcium concentration on the kinetics of opening and closing of the channels. One of the important features of the Moore-Cox model is that inactivation is not totally independent of activation. The two are coupled to some degree. Since the calcium concentration in the present experiment was not varied, the calcium factor was not incorporated in this model. The time constants of tail currents measured at -60 , -70 , and -80 mV in the present study were eight times slower than those predicted by the Hodgkin-Huxley equation and by the Moore-Cox model. Therefore, for tail current simulations, the rate constant for the direct route from the activated state N to the resting state P was taken as $0.4\beta_m$ instead of $3\beta_m$. For larger depolarizations, this route is not very important because β_m becomes very small.

The modified Moore-Cox² for the sodium conducting system is as follows:



where *O* represents the inactivated state, *L* and *M* represent intermediate states from *P* to *N*, and *Q* represents the activated but occluded state when pancuron-

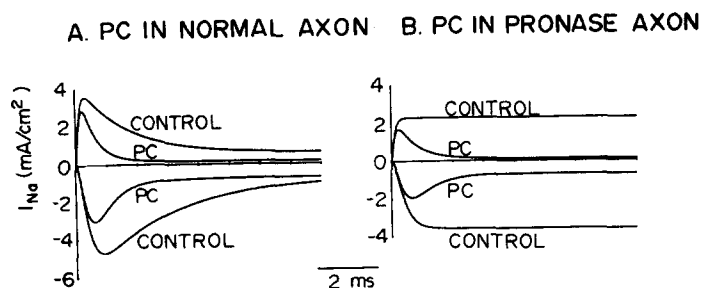


FIGURE 15. Simulation by the kinetic model of Scheme II in which the Hodgkin-Huxley formulation is incorporated for the sodium currents in normal and pronase-treated perfused axons with and without pancuronium (PC). Inward currents are associated with step depolarizations to 0 mV, and outward currents to 80 mV. The method of simulation is described in the text. The rate constants for α_h , β_h , k and l are determined experimentally. The constants used to calculate curves are $\alpha_h = 0.088 \text{ ms}^{-1}$, $\beta_h = 0.572 \text{ ms}^{-1}$, $k = 1.5 \text{ ms}^{-1}$, $l = 0.075 \text{ ms}^{-1}$ at 80 mV; $\alpha_h = 0.04 \text{ ms}^{-1}$, $\beta_h = 0.36 \text{ ms}^{-1}$, $k = 1.5 \text{ ms}^{-1}$, $l = 0.32 \text{ ms}^{-1}$ at 0 mV. The rate constants α_m and β_m are calculated from the Hodgkin-Huxley formulation. Temperature 6.3°C. The scale factor \bar{g}_{Na} in the pronase-treated axon is set at half of that of the normal internally perfused axon.

ium is internally applied to the axon. On the basis of experimental observations and model simulations of tail currents, the pancuronium-bound sodium channels (*Q* state) are assumed not to return directly to *P* or *O* state during maintained depolarization or upon repolarization. In other words, both m^3 gate and h gate of the pancuronium-bound channel cannot be closed.

Sodium Current in Pronase-Treated Axon and Pancuronium-Induced Inactivation

In order to fit the sodium current in the pronase-treated axon, both the coupled inactivation route (*N* to *O*) and the direct inactivation route (*P* to *O*) are

² In actual simulation, the following rate constants K_{PN} , K_{ML} , K_{NM} , and K_{ON} are made zero. A source of energy has to be injected into this system at some point. Moore and Cox (1976) considered the electrical field as an energy source for their kinetic model. Hille (1976) also considered the electric field as source of energy for the gating mechanism.

eliminated from the original scheme. Since the maximum sodium conductance in the pronase-treated axon is about 30–70% of the normal internally perfused axon, the precursor P is scaled down 30–70% of the control value. Other rate constants remain the same as the original values for normal axons. An additional step Q , which is added to incorporate the interaction of pancuronium with the open channel, represents the pancuronium-bound sodium channels which do not conduct sodium ions. The transient solution of the kinetic scheme at 80 mV is shown in Fig. 16. It is clear that the computed current curves fit the observed (circles) satisfactorily. As a further refinement, reduction of the precursor P (or \bar{G}_{Na}) by 10–20% in pancuronium gives a perfect fit (compare b and c in each

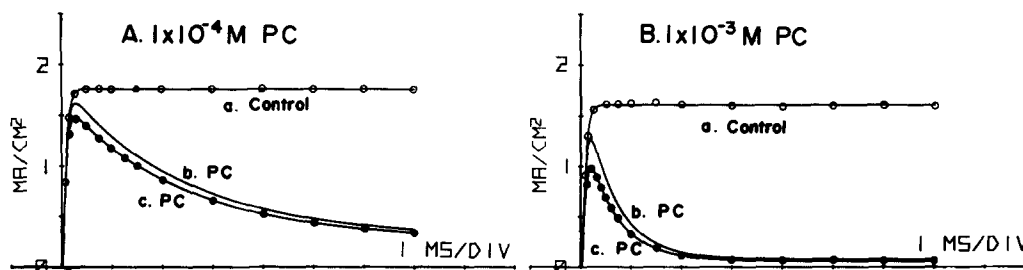


FIGURE 16. Simulation by the kinetic model to fit the sodium currents in the pronase-treated axon before (○) and during (●) internal application of pancuronium (PC) at concentrations of 1×10^{-4} M (A) and 1×10^{-3} M (B). The sodium currents were obtained by subtracting the currents with 20 mM tetraethylammonium inside and 300 nM tetrodotoxin outside from those without tetrodotoxin. The constants used to calculate curves a and b in A are: normal Hodgkin-Huxley α_m and β_m at 80 mV, $E_{Na} = 33$ mV, $k = 0.352$ ms $^{-1}$, $l = 0.07$ ms $^{-1}$. Scale factor (equivalent to \bar{G}_{Na}) = 40 mmho/cm 2 . The best fit of data was obtained by reducing the scale factor from 40 to 37 mmho/cm 2 (curve c). The constants used to calculate curves a and b in B are: the same α_m and β_m as for A above, $E_{Na} = 40$ mV, $k = 1.5$ ms $^{-1}$, $l = 0.075$ ms $^{-1}$, scale factor = 44 mmho/cm 2 . The best fit was obtained by reducing the scale factor from 44 to 35 mmho/cm 2 without changing other parameters (curve c). Temperature 10°C.

graph). It should be emphasized that the rate constants k and l are both determined experimentally. The simulations by the kinetic models based on both Moore-Cox and Hodgkin-Huxley formulations give essentially the same results.

Pancuronium-Induced Acceleration of Decay of Sodium Current in Normal Perfused Axons

Normal values for α_m and β_m , and half-values for α_h and β_h in the Hodgkin-Huxley equation were used in the simulation of sodium current in the perfused axon by the Moore-Cox kinetic model. The simulation could reproduce the sodium current very satisfactorily, as shown in Fig. 17. This simulation is very similar to that illustrated in Fig. 15 A which is made by the kinetic model based on Hodgkin-Huxley formulation. No attempt is made to simulate quantitatively the sodium currents associated with larger depolarizations in which a steady-state noninactivated portion of sodium current is observed.

The main features of pancuronium-induced inactivation of sodium current could be reproduced semiquantitatively without changing the rate constants α_m , β_m , α_h , and β_h . The acceleration in the decay of sodium current is due to the interaction of pancuronium with open sodium channels that results in blocking of the channel.

Tail Current

The initial rising phase of the tail current upon repolarization is produced as a result of pancuronium ions leaving the occluded sodium channels at a rate faster than that at which the open channels close. The rate of occluding the open channel population by pancuronium is proportional to the fraction of the channels that are open, and the rate at which pancuronium ions leave the

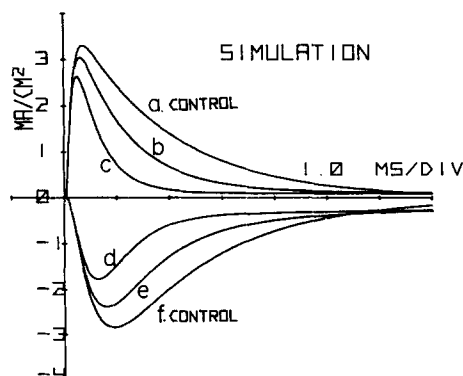


FIGURE 17. Simulation by the kinetic model of the sodium currents in normal axon before (curves *a* and *f*) and during (curves *b*–*e*) internal application of pancuronium. The rate constants α_m , β_m , α_h , and β_h used to calculate sodium currents associated with the membrane potentials 80 mV (curves *a*–*c*) and 0 mV (curves *d*–*f*) are described in the text. The rate constants used to simulate the effect of pancuronium are: $k = 1.5 \text{ ms}^{-1}$, $l = 0.075 \text{ ms}^{-1}$ for 80 mV (curve *c*) and $k = 1.5 \text{ ms}^{-1}$, $l = 0.26 \text{ ms}^{-1}$ for 0 mV (curve *d*), and for $1 \times 10^{-4} \text{ M}$, $k = 0.45 \text{ ms}^{-1}$, $l = 0.075 \text{ ms}^{-1}$ for 80 mV (curve *b*) and $k = 0.45 \text{ ms}^{-1}$, $l = 0.26 \text{ ms}^{-1}$ for 0 mV (curve *e*).

occluded channels is proportional to the fraction of the channels that are occluded. Therefore, the population of sodium channels shift after repolarization from the occluded state to the open state and then to the closed state. Thus, the m^3 gate of the occluded sodium channel (Q state) cannot be closed by abrupt repolarization until the channel is free of pancuronium. The channel population in the open state is buffered by the population of occluded channels that conduct sodium ions as pancuronium ions leave the channels. Because of this interaction, the tail current exhibits an initial rising phase which is followed by a delayed fall. The time constant of the falling phase is probably limited by the rate at which pancuronium molecules leave the channels. In simulation of tail current by the kinetic model, the time constant of the decay of tail current is indeed determined by the rate constant l . The rate constant k has very little effect on the fall of the tail current. Many other features of the pancuronium-induced tail current can be reproduced from the kinetic model. The time course of the

development of the tail current with a hook and slow decay during maintained depolarization (Fig. 9), and the voltage dependence of the amplitude and decay of tail current (Figs. 7 and 10) are also satisfactorily simulated by the model.

A physical interpretation of pancuronium interactions with sodium channels may be given from the kinetic model. Pancuronium enters the open sodium channel but cannot go through the channel because of its bulky size ($7 \times 11 \times 18$ Å by CPK model). Pancuronium binds to the channel more firmly with depolarization as a result of a decrease in the dissociation constant l . Therefore, more channels are bound by pancuronium. Since the pancuronium-bound channel is not inactivated but simply occluded, repolarization accelerates dissociation of pancuronium molecules from the channel, producing a hooked tail current. Thus the amplitude of the tail current increases with the increasing depolarizing step during prepulse.

Action Potential

The two essential features of the action potential of the pancuronium-treated axon are shown in Fig. 2. First, the initial falling phase is accelerated while the later phase is slowed down. Second, the rising phase of the action potential is only slightly affected. For the sodium-conducting system the Moore-Cox kinetic model is used in place of the Hodgkin-Huxley formulation. For the potassium conducting system the Hodgkin-Huxley formulation, $g_k = \bar{g}_k n^4 (E_m - E_K)$, is used in which g_k refers to the potassium conductance, \bar{g}_k is the maximum potassium conductance (constant), E_K the potassium equilibrium potential, and n is the dimensionless gating parameter for potassium. The leakage conductance of 0.3 mmho/cm² is also incorporated. The voltage dependence of rate constants in the Moore-Cox model and Hodgkin-Huxley formulation is calculated according to Palti's version of the Hodgkin-Huxley equation (Palti, 1971) with one modification, namely, α_h and β_h are scaled down to half of the original value based on the voltage clamp data with internally perfused axons, in which τ_h is two times longer than that predicted from the Hodgkin-Huxley equation. The simulation does reproduce the shape of the observed action potential of perfused axons. It is interesting to note that the hump in the falling phase of the action potential computed by the Hodgkin-Huxley equations occurs in the action potential of internally perfused squid axons.

In the presence of 1 mM pancuronium, only the sodium-conducting system is affected. In simulation of action potentials, therefore, only the sodium-conducting system is modified in such a manner that its gating mechanism is not affected, but pancuronium interacts with open sodium channels to decrease the conductance with the rate constants k and l which are estimated from voltage clamp experiments. In Eq. (4) which describes the backward rate constant l , l_0 and δ are estimated to be 0.32 ms⁻¹ and 0.21 , respectively, at zero membrane potential. The forward rate constant k is dependent on the concentration of pancuronium. However, the voltage dependency of k is not known. Using a constant k value it was possible to simulate many features of the pancuronium-induced action potential. However, if the voltage dependence as described in equation $k = k_0 \exp(zE_m F \delta / RT)$ where k_0 is the forward rate constant for zero

membrane potentials, z is 2, and δ is 0.21, is incorporated for an E_m less negative than -10 mV, a better fit is obtained as shown in Fig. 2. The voltage dependence in this voltage range is only tentatively formulated, because there were only a few measurable rate constants at this potential range. The effect of pancuronium concentration on the action potential could be reproduced by changing the forward rate constant k in the kinetic model which depends on the concentration, as seen in voltage clamp experiments.

Potential- and Time-Dependent Block

In the presence of pancuronium, the number of sodium-conducting channels decreases as the membrane potential is made more positive. This pancuronium block is also time dependent and occurs with some delay (usually measured at 8 ms after the beginning of a pulse). In the kinetic model, this block results from the increase in the occluded state Q as E_m becomes more positive. Since the rate constants k and l determine the fraction of the channels in the state Q at a steady state, the time course of channel block is measurable if k and l are small. Thus the delay in developing a steady-state block is expected. This is the case for pancuronium. The forward rate constant k is independent of the membrane potential ($E_m > -10$ mV) whereas the backward rate constant l is highly potential dependent. The rate constant l decreases as membrane potential increases. This indicates that pancuronium molecules leave the sodium channels more slowly at more positive membrane potentials, and accounts for the more pronounced block by pancuronium at higher membrane potentials. Voltage- and time-dependent block of sodium channels by strychnine has also been reported with frog nodes of Ranvier (Shapiro, 1975) and squid giant axons (Cahalan and Shapiro, 1976). Rojas and Rudy (1976) also observed a time-dependent block of sodium channels by internally applied quaternary ammonium compounds.

Since the pancuronium block is time-dependent, it is important to bear in mind that the equilibrium constant for drug binding should be evaluated at a steady state. For example, if the decrease in G_{Na} by pancuronium is evaluated at the peak of the transient sodium current as shown in Table I, the voltage-dependent block turns out to be opposite to that measured at a steady state. This is expected from the present kinetic model. First of all, the rate constant for opening the channel is highly dependent on the membrane potential, being greater at large depolarization than at small depolarization, whereas the rate constant for pancuronium to enter the open sodium channel is virtually independent of the membrane potential for values less negative than -10 mV. Therefore, we would expect that more sodium channels are in a conducting state at larger depolarization during the peak of sodium current. This would manifest to a lesser extent of block at 80 mV than at 0 mV. The temperature-dependent block as shown in Table I is also expected from the kinetic model if the rate constant for pancuronium to enter and block the channel has a lower temperature dependence than the rate constant for opening and closing of the sodium channel. Then we would expect that more sodium channels are in the conducting state during peak current at high temperature than at low temperature.

Comparison with TEA Derivatives for Potassium Channel Block

The modified Moore-Cox kinetic model for sodium channels fits the observations on the pancuronium block of the channels quite satisfactorily. Armstrong (1969, 1971) had previously proposed a similar kinetic model which could elegantly explain the interaction of TEA and its derivatives with potassium channels. Although the kinetic scheme of the present model at the macroscopic level is very similar to the Armstrong model, there are some important differences in assumption and observation which may reveal fundamental differences in the mechanisms at the microscopic level.

The similarities between the two cases may be summarized as follows.

- (a) Both drugs block channels only from the axoplasmic side.
- (b) Both drug blocks require the opening of channels.
- (c) Both drugs block channels at a rate that varies with their axoplasmic concentrations.
- (d) In both cases, the forward rate constant k is independent of the membrane potential. A possible explanation is that the entry rate of blocking ions may be limited by the rate of diffusion of blocking ions to the channel mouths, as suggested by Armstrong (1969).
- (e) In the presence of the drugs, the tail currents associated with repolarizations to large negative potentials exhibit a hook increasing initially and then decreasing.

The obvious differences between the two cases are summarized as follows.

- (a) TEA and its derivatives cause anomalous or ingoing rectification. Armstrong (1969, 1971, 1975 *a, b*) interpreted this as TEA being cleared from the occluded K channels by inward-moving K^+ ions. The ingoing rectification is probably related to the direction of current flow which is in turn related to the membrane potential. The pancuronium-induced voltage-dependent block is augmented at more positive membrane potentials.
- (b) The backward rate constant l , which probably represents the rate at which TEA ions leave K channels, is not a continuous function of the membrane potential. Over the potential range studied (see Table I of Armstrong, 1969), l changed very little, which fails to explain the anomalous rectifying block of K channels by TEA derivatives. However, a four to fivefold increase in l is required to reproduce the effect of hyperpolarization on recovery rate. The rate constant l for pancuronium, seems to be a continuous function of the membrane potential as seen in Fig. 13 B. The voltage-dependent l explains the more pronounced block of Na channels by pancuronium at higher depolarization.

The fundamental difference between potassium channels and sodium channels may be ascribed to the fact that potassium channels are single file pores (Hodgkin and Keynes, 1955), whereas sodium channels are one ion pores. The block produced by TEA suggests a single file mechanism in which several ions are lined up in a channel at the same time.

Difference in the Mechanisms of Voltage-Dependent Inhibition of Sodium and Potassium Channels

A voltage-dependent inhibition of potassium current by Na^+ , Cs^+ , and TEA

derivatives has been studied in detail (Armstrong, 1969, 1971; Armstrong and Binstock, 1965; Bezanilla and Armstrong, 1972). Two limiting possibilities are suggested for this voltage-dependent inhibition. The first limiting case is that Na^+ (or Cs^+) can reach a site in the potassium channel that lies halfway through the membrane field. The other limiting case is that the blocking ion is cleared by K^+ ions. A potassium channel is cleared of Na^+ (or Cs^+) by a K^+ ion that enters from outside and approaches the blocking ion close enough to repel it from the pore. The experiments with high external potassium concentration or with repolarization to more negative values shows that external K^+ ions have a clearing effect on Na^+ , Cs^+ , or TEA ions which block channels, thus suggesting the second possibility occurring to some extent. This result may rule out the first limiting possibility which calls for voltage-dependent block (Bezanilla and Armstrong, 1972).

The direct estimate of the rate constants for pancuronium to interact with sodium channels reveals two important points: first, only the backward rate constant l is dependent on the membrane potential, and its potential dependence is sufficient to account for the potential-dependent inhibition; second, the rate constant l is not appreciably influenced by external sodium concentration at a given membrane potential. These results strongly suggest that the first reaction mechanism calling for pancuronium molecules entering the sodium channels occurs and that the second alternative mechanism calling for a Na^+ ion repelling pancuronium molecules from the binding site is not important.

The voltage-dependent nature of sodium channel inhibition and the current-dependent nature of potassium channel inhibition may be due to fundamental differences in the ion transport mechanisms in these channels. It has been proposed that the potassium channel operates as a single-file pore whereas the sodium channel functions as a one ion pore. More experiments along this line are needed to define more precisely the architecture of the channels. However, recent observations on the current-dependent block of sodium channels by strychnine (Cahalan and Shapiro, 1976) are not consistent with the hypothesis that the sodium channel is a single ion pore.

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