III. Rates of drug action at the node of Ranvier

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1. Fast exchange of solutions at the node of Ranvier

The amphibian myelinated nerve fibre is one of the most thoroughly studied preparations of excitable tissue (see e.g. recent reviews[7,8,20]). Such studies include many tests of drugs, often employed as tools to probe details in the function of ionic channels[6,21,22]. In fact, myelinated nerves are particularly suited for drug experiments since the nodal membrane appears to be exceptionally accessible to externally applied agents.

Exchange of solutions at the node is maximized in a nerve chamber whose basic design was given by Stämpfli[18]. A node is placed in a small slit across a thin polyethylene tube and is continuously superfused. A special stopcock of minimum dead space is located a few mm upstream and permits a rapid change of solutions. The effect of such a change from Ringer to isotonic KCl solution and back is illustrated by figure 1 wherein A and B show the concomitant changes in membrane current while the node is depolarized by 26 mV in the voltage clamp. The difference, ΔI, of current as defined in B is a good estimate of $\Delta[K^+]$, the change in K^+ concentration immediately outside the membrane. From ΔI and with the aid of an individual calibration curve, $\Delta I = f(\Delta[K^+])$, figure 1, C was constructed yielding symmetrical curves for application and washout of K^+ with a half time of 18 msec. This is very short indeed if one realizes that unimpeded diffusion of K^+ through a plane sheet of only 10 µm thickness fits about 85% of this exchange (see interrupted curve in C). Comparable results of Na^+ exchange at the node have been obtained with the maximum rate of rise of action potentials as a measure[24]. Increasing the rate of perfusion above a certain limit does not further speed up exchange at the membrane so that its maximum rate appears to be limited by diffusion through an unstirred layer covering the membrane. Since the excitable membrane allegedly lies at the bottom of the nodal gap, the unstirred layer must include the complicated structures filling the gap which defy a precise description as diffusion space. The disproportionately slow exchange of the last 10–15% (circles below interrupted curve in fig. 1, C) could point to a recessed portion of the axolemma to which access is more impeded.

It should be added here that the fast-exchange setup does not permit a high speed of voltage clamping but it is reliable when slower current phases are measured as in figures 1 and 5. Fast phases are preferably recorded in the standard setup[9] where, however, exchange of solutions is about 20 times slower though still fast enough for many drugs to achieve 'step' changes in concentration relative to their rates of action.

2. Drug-receptor kinetics on a step change in concentration

The drugs dealt with in this review are thought to exert their action after binding to some site ('receptor') at or in the ionic channels spanning the excitable membrane. The simplest form of interaction is a reversible 'one-to-one' reaction between a drug molecule, A, and a receptor, R, to form a drug-receptor complex, A.R,

$$A + R \underset{k_{2A}}{\overset{k_{1A}}{\rightleftharpoons}} A \cdot R$$

where k_{1A} and k_{2A} are the rate constants of association and dissociation. At equilibrium with a given drug

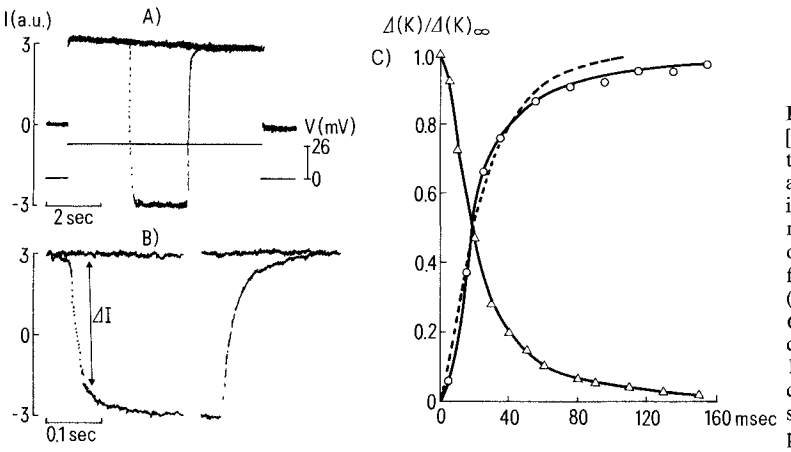

Figure 1. Rate of change of K^+ concentration, $[K^+]$, at the nodal membrane. *A* 2 superimposed traces of current (in arbitrary units) recorded during a 7-sec depolarizing pulse of 26 mV amplitude, once in Ringer solution throughout, then while 117 mMeK^+ was applied for 2 sec. *B* Expanded record of pertinent current details of A with ΔI = difference, at any instant, of current in 2.5 mM K^+ (Ringer solution) and on applying 117 mM K^+. *C* Normalized change of $[K^+]$ as obtained from ΔI, circles and triangles on applying and washing out 117 mM K^+. The interrupted curve gives the concentration change after diffusion through a plane sheet of 10 µm thickness. (From Ulbricht[22], with permission.)

concentration, [A], the fraction $y_A(\infty)=[A.R]/([R]+[A.R])$ of receptors occupied is given by

$$y_A(\infty)=[A]/([A]+K_A)=c_A/(1+c_A) \quad (1)$$

where $c_A=[A]/K_A$ with $K_A=k_{2A}/k_{1A}$, the equilibrium dissociation constant. On a step change in [A], y_A changes exponentially with a time constant, τ_A, whose reciprocal is

$$1/\tau_A=k_{1A}[A]+k_{2A}. \quad (2)$$

From eq. (2) it follows that the onset rate of receptor occupation increases with [A] and that the offset rate on washout ($[A]=0$), $1/\tau_A=k_{2A}$, is independent of previously applied [A].

To test these equations in a real experiment we first have to clarify the relation between receptor occupancy, y_A, and the electrically measurable effect. This seems least complicated in the case of tetrodotoxin (TTX), a low-molecular toxin (mol. w 320) from puffer fish which blocks sodium channels in nanomolar concentrations. It is assumed that block is due to plugging the external mouth of the channel[3] in an all-or-none fashion[17] so that binding can be equated with block. The maximum effect (sodium current $I_{Na}=0$) is achieved when $y_A(\infty)=1$. A Hill plot of log $\{y_A(\infty)/[1-y_A(\infty)]\}$ vs log [A] yields a slope of 0.97, close enough to the theoretical slope = 1 for a one-to-one reaction[16].

Block by TTX proceeds rather slowly so that it can reliably be followed in the standard voltage clamp setup by recording, every sec, peak I_{Na} during a constant test pulse. Block on applying 3.1 and 15.5 nM TTX and the respective washout curves are presented in figure 2. The curves through the points are calculated with a single set of rate constants, $k_{1A}=2.9\times10^6\ M^{-1}sec^{-1}$ and $k_{2A}=1.3\times10^{-2}\ sec^{-1}$ as predicted by eq. (2). Although k_{1A} and k_{2A} appear to be true rate constants of the reaction, the good fit may be deceptive since certain types of limited diffusion mimick this kinetic behavior[2]. Such limitations, however, can be excluded for several reasons, the most compelling being the high temperature coefficient of the rates[16] (Q_{10} of $k_{2A}=3.4$).

Saxitoxin (STX), a toxin produced by dinoflagellates, is of similar molecular size as TTX but chemically quite different. It blocks sodium channels very much like TTX and the reaction scheme for this toxin also applies to STX for which we write 'B'. Equilibrium occupancy is then given by

$$y_B(\infty)=c_B/(1+c_B) \quad (3)$$

with $c_B=k_{1B}\,[B]/k_{2B}$. If A (=TTX) and B (=STX) bind to a common receptor equilibrium block in a mixture of [A]+[B] will be proportional to

$$y_{A+B}(\infty)=y'_A(\infty)+y'_B(\infty)=\frac{c_A}{1+c_A+c_B}+\frac{c_B}{1+c_A+c_B} \quad (4)$$

where $y'_A(\infty)$ and $y'_B(\infty)$ are the fractions of channels (actually receptors) occupied by the respective toxin in the presence of the other. $y'_A(\infty)$ and $y'_B(\infty)$ are of course indistinguishable by electrical measurements but since STX binds and dissociates faster than TTX[25] ($k_{1B}=2.3\ k_{1A}$, $k_{2B}=1.7\ k_{2A}$), taking out STX after equilibration in a TTX-STX mixture leads to a non-monotonic partial recovery from block as shown by the triangles in figure 3. The reason is that in the mixture fewer channels are occupied by TTX than in the single-toxin solution of the same

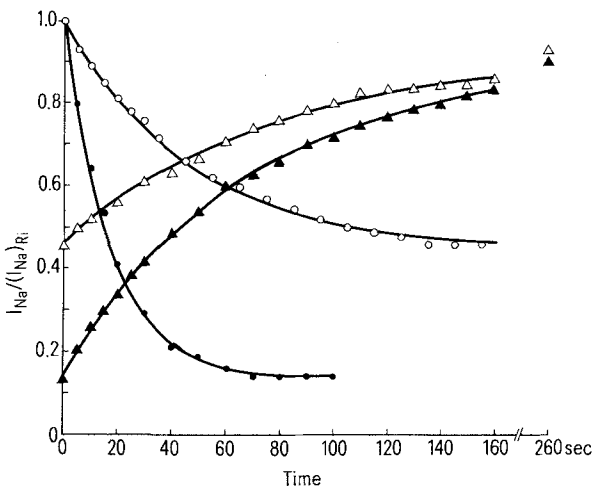

Figure 2. Rate of block of sodium channels by tetrodotoxin (TTX). Ordinate, peak I_{Na}, normalized to the value in Ringer solution; abscissa, time after solution change. Open circles refer to onset in 3.1 nM TTX (time constant $\tau_{on}=45$ sec), open triangles to the subsequent washout ($\tau_{off}=77$ sec). Filled symbols denote washin and washout of 15.5 nM TTX ($\tau_{on}=17$ sec, $\tau_{off}=78$ sec). Curves were computed as described in text. (From Schwarz et al.[16], with permission).

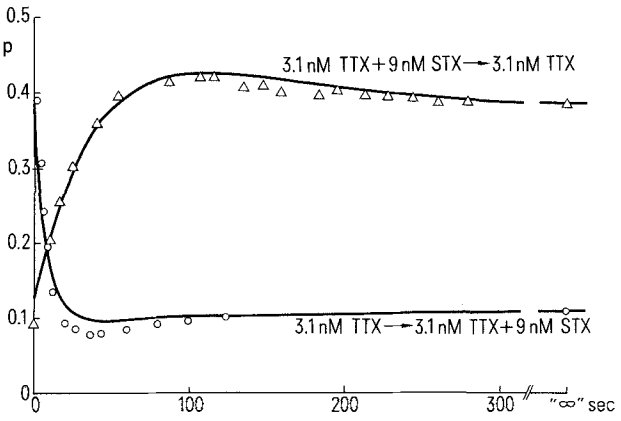

Figure 3. Non-monotonic time course of block when TTX and STX compete for the receptor. Ordinate, fraction p of channels left unblocked. Abscissa, time after solution change. Circles refer to adding 9 nM STX (B) after equilibration in 3.1 nM TTX (A), triangles to a change back to 3.1 nM TTX after equilibration in the mixture. The curves were computed from the mean results, $k_{2B}=1.4\times10^{-2}\cdot sec^{-1}$, $k_{2B}/k_{2A}=1.7$, $c_A=1.6$, $c_B=0$, or 6.3; 14.8 °C. (From Wagner and Ulbricht[25], with permission.)

[A], $y'_A(\infty) < y_A(\infty)$; on reverting to the pure TTX solution, STX will come off faster than TTX can claim its share of freed receptors which leads to an overshooting recovery. By a similar argument, adding STX after equilibration in TTX produces a non-monotonic additional block (circles). Since the overshoot increases with the ratio k_{2B}/k_{2A} the combination of the local anesthetic benzocaine and the indole alkaloid ervatamine for which the ratio is about 6 leads to a particularly large transient during recovery[10]. Block by these drugs is, in contrast to that by TTX or STX, sensitive to membrane potential which complicates the issue (see next section). In either case, however, the non-monotonic time course of block is evidence for a common receptor or at least for mutually exclusive binding.

3. Two ways to block a sodium channel

Classical local anesthetics, LA, reversibly block sodium channels like TTX or STX but their action differs from that of the toxins in several respects. LA block very rapidly and on a sudden application one half of their reduction of the maximum rate of rise of the action potential (an approximate though not linear measure of conducting sodium channels) is 0.27 sec for 1 mM procaine at pH 7.2 and only 0.12 sec for the equally effective 0.5 mM benzocaine[11]. There is evidence that the underlying reaction, especially that with benzocaine, is so fast that it cannot be resolved even with the fast-exchange method. Also, the uncharged form of LA readily permeates the lipid matrix of the membrane which makes it difficult to estimate the rate of change of drug concentration at the receptor. This receptor is thought to reside within the channel and access to it depends on the configuration of LA, cationic forms only through the axoplasmic mouth and past the open gate, uncharged forms also directly from the lipid matrix[4,5]. Finally, as

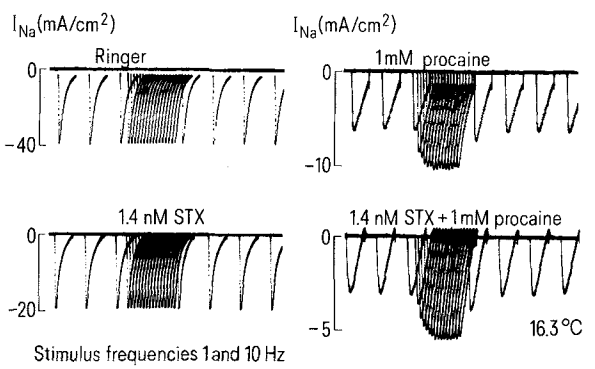

Figure 4. Effect of changing the pulse frequency from 1 to 10 Hz and back on peak I_{Na} observed with constant depolarizing pulses (60 mV amplitude) preceded by 50-msec hyperpolarizing (by 40 mV) prepulses. Standing pictures recorded on moving film. Note that the lower records were taken at twice the gain of the respective upper record to compensate for the 50% reduction by 1.4 nM STX; 16.3 °C. (From Wagner and Ulbricht[26], with permission.)

mentioned before, block is influenced by membrane potential (directly or via the gates), hyperpolarizing prepulses relieving it partially. With LA such as procaine ($pK_a = 8.9$) which mostly exist as cations at neutral pH, relief from block requires several pairs of such prepulse-test pulse combinations as shown in figure 4 where increasing the pulse frequency from 1 to 10 Hz leads to a substantial recovery of I_{Na}. Neither in Ringer solution nor in STX is such a re-increase of peak current observed. If STX is added at a concentration that itself halves I_{Na}, relief from procaine block is unaffected as revealed in figure 4 by the almost identical traces after doubling the gain of the amplifier. Such behavior suggests independence of block by the 2 agents. Let us assume that each channel bears 2 receptors, 1 for procaine (= A) and 1 for STX (= B) and that a channel is blocked if either site is occupied. In an A-B mixture the fraction of sites occupied by the respective drugs are y_A and y_B. The fraction, Y, of blocked *channels* is then given as

$$Y = y_A + y_B - y_A y_B \tag{5}$$

and for $y_B = \text{const.}$ we derive

$$dY/dt = dy_A/dt\,(1 - y_B). \tag{6}$$

We [26] observed in 1 mM procaine alone $y_A(\infty) = 0.81$ and in 1.4 nM STX alone $y_B(\infty) = 0.49$ with which eq. (5) yields $y(\infty) = 0.90$ as was indeed found in the mixture. Eq. (6) predicts that after equilibration in this mixture a change in occupancy, y_A, of the procaine site is linearly reflected in a change in block, Y, simply scaled down by $1 - y_B$, i.e. approximately halved in our example as indeed shown in figure 4. The results thus indicate separate receptors for STX and procaine to which the drugs must have independent access; this seems possible since the toxin site's location is at the external entrance of the channel.

Proof of a common receptor for cationic LA (e.g. procaine or lidocaine) or uncharged LA (benzocaine) as postulated by Hille[5] cannot directly be obtained by changing solutions as with TTX-STX (fig. 3) because the reactions are not rate-limiting; the very slowly blocking alkaloid ervatamine (see above) is an exception. Hence modulation of block must be achieved with an adequate pulse protocol. The one used in figure 4 does not change I_{Na} in benzocaine. In 0.25 mM lidocaine, however, as in procaine, a gradual relief from block is observed on increasing the pulse frequency. In a benzocaine-lidocaine mixture this gradual relief is almost suppressed as if benzocaine dominates. This and other results suggest that the 2 LA compete for the same site[15].

4. Rates of action of gate modifiers

Gate modifiers are drugs which solely or predominantly affect the rates at which channels open or close

as a function of membrane potential. Many such agents (which are of diverse chemical structure) retard inactivation of sodium channels, i.e. the rate of closing on sustained depolarization. Inactivation (in contrast to deactivation on repolarization) leaves the channels temporarily refractory to a depolarizing step in membrane potential. In frog nerve fibers internally applied iodate[19] or externally applied glutaraldehyde[13] cause inactivation to slow and become incomplete so that after 15 msec (at about 15 °C) when in the control almost no I_{Na} is flowing, a substantial persistent current component is observed. In contrast, sea anemone toxin ATX II achieves this very effect[12] in a reversible fashion[1,23]. Figure 5 illustrates this by the rise and fall of ΔI_{15msec}, the increment in persistent current (I_{15msec}) over the often negligible value before the application of toxin. The abscissa gives the time after the solution change corrected, with the effect of a step change in $[Na^+]$ on peak I_{Na}, for the arrival at the nodal membrane. Nevertheless, onset and offset of action are distinctly sigmoid. Also, a short (1.3 sec) application of 5 µM ATX II leads to a clear overshooting effect on washing[14] (filled circles). Sigmoid time course and overshoot could be due either to a diffusional delay of the relatively large toxin molecule (mol. wt ~ 5000) as compared with Na^+ or to a 'silent' intermediate in a 2-step reaction

$$A + R \underset{k_{2A}}{\overset{k_{1A}}{\rightleftharpoons}} A \cdot R_1 \underset{k_{4A}}{\overset{k_{3A}}{\rightleftharpoons}} A \cdot R_2$$

if only $A.R_2$ expresses neurotoxicity and is formed after a delay following a step increase in [A]. The rate of ATX II action increases with temperature with a Q_{10} of 2 which seems too large for a rate solely determined by diffusion. The kinetic results of figure 5 are mimicked by the time course of $[A.R_2]$ computed for a step change in [A] with a single set of rate constants. The computations, however, yield less delay and overshoot than the real experiments. Possibly diffusion of the large toxin molecule through the unstirred layer prevents [A] from rising in negligible time at the receptor. This receptor appears to be located at or near the external side of the membrane. ATX II does not seem to penetrate the membrane and is ineffective if applied to its axoplasmic side[1]. Ca^{2+} rapidly and reversibly suppresses I_{15msec} during voltage clamp pulses of 60–80 mV amplitude[23], an effect that cannot easily be explained (as can other Ca-induced changes in sodium permeability) by a change in surface potential. Rather, Ca^{2+} seems to interfere directly with toxin binding which, too, suggests a superficial site. This is a puzzling result since inactivation is believed to be connected to structures of the channel near its axoplasmic mouth. Incidentally iodate inhibits inactivation only from inside the axon and has no such effects if applied externally[19]. Solving this puzzle would clearly improve our understanding of inactivation in native nerve fibers and kinetic studies of drug action promise to contribute valuable data.

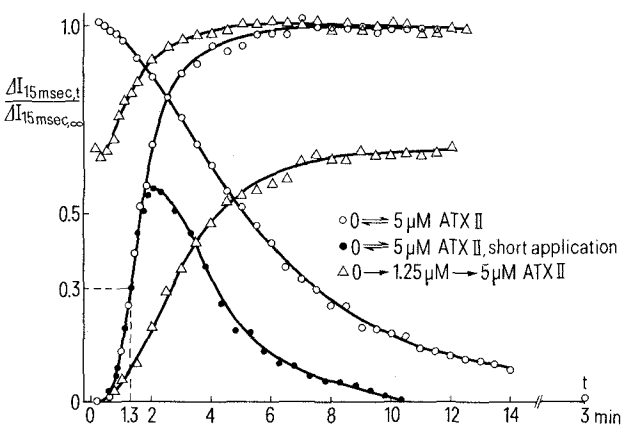

Figure 5. Onset and offset of ATX II effect on the increment, ΔI_{15msec}, of I_{Na} recorded at the end of 15-msec pulses of 60 mV amplitude, normalized to the value after equilibration in 5 µM ATX II. Abscissa, time after change of perfusate . Note the sigmoid time courses and the large overshoot following a short (1.3 sec) application. Symbols as explained by inset, curves drawn by eye; 15.4 °C. (From Schmidtmayer et al.[14], with permission.)

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IV. Nodal function of pathological nerve fibers

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The understanding of the disease mechanisms in nerves is linked to the identification of structural changes in human nerve biopsies. Much of the nervous dysfunction that can be identified clinically or as a decrease in nerve conduction velocity has an obvious relation to e.g. axonal degeneration or demyelination. The more detailed quantitative analysis in various clinical studies and animal models has, however, led to the conclusion that in addition there must exist functional changes in the nodes of Ranvier or the myelin sheaths that play an important role in the disease mechanism. For example in both human and animal (spontaneous) diabetes there are changes in nerve conduction velocity that are difficult to relate to morphological changes only[19,25,38,43]. Multiple sclerosis is another important example of sometimes poor clinicopathological correlation (for review, see Waxman[44]). Areas of focal demyelination can be identified postmortally, and the optic nerve conduction velocity may be decreased (increased latency of visually evoked response), without associated clinical symptoms.

The axonal impulse propagation is generally regarded as the strongest link in the chain of nervous signalling. The safety factor for conduction is great, consequently large alterations can appear in nodal function before it causes decreased conduction velocity or propagation block. There is a large uninvestigated field of nerve pathophysiology, where recordings from the single fiber and potential clamp analysis of its membrane properties probably will be necessary to reveal the nervous dysfunction. Squid axons and frog myelinated fibers are invaluable for the exploration of the basic membrane function, but for the understanding of disease mechanisms it seems necessary to turn to mammalian nerve and use the pathological models that have been established. It is, however, more difficult to dissect mammalian nerve fibers than nerve fibers of frog because of the more prominent interneural septa and collagen strands. The experiments must be performed at higher temperatures, which puts higher demands on the feed-back circuit since the membrane current changes are then more rapid.

The work with pathological fibers is also coupled to some new methodological considerations. When a demyelinated fiber is selected it is necessary to see that the fiber has the main parts of its internodes intact so it can be mounted in the recording chamber. It can be difficult to find a fiber with the right structure that can be isolated without too much work. The electric feed-back circuit for the potential clamp also sets limits for the changes in leak and capacitive properties that can be tolerated. This is relevant for the studies of demyelinated fibers, which have been restricted to fibers with paranodal demyelination. In the potential clamp work of *normal* fibers, like in other physiological work, experiments are discarded if they show that the preparation in some way is in poor condition. By experience from the changes during long experiments, an increased sodium inactivation and decreased specific permeabilities can be related to ‹run down› of the fiber[16]. Since the threshold for excitation in the intact isolated fiber and in the fiber after it has been cut off were the same when the