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INTERACTION OF POLYMYXIN WITH VERTEBRATE PERIPHERAL NERVE AXONS

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Summary

The effect of the peptide antibiotic polymyxin on nerve membrane was investigated on the voltage clamped node of Ranvier. Low polymyxin concentrations ($<2\times10^{-5}$ M) had little effect. Polymyxin (2×10^{-5}–10^{-4}M) produced a large increase in leakage conductance accompanied by large inward current and loss of evoked sodium currents. In choline-substituted Ringer's solution the increase in leakage conductance was small and potassium currents remained near control levels. Tetrodotoxin (10^{-7}M) and calcium increased the threshold for the effect. Above 10^{-4}M polymyxin, leakage conductance increased to levels associated with loss of membrane integrity. The results suggest that polymyxin forms or opens Na and K-permeable channels in nerve membrane.

Introduction

Polymyxin-B, a cyclic polycationic peptide, strongly interacts with negatively charged phospholipids (1,2). A recently suggested model (3,4) proposes that polymyxin causes a phase separation in which domains of phospholipid are bound to peptide molecules. The association of polymyxin and phosphatidic acid is a cooperative process, caused by the elastic distortion of the lipid layer by the highly asymmetric peptide. In the model established by Hartmann et al (3) a cap is formed within the membrane that can be visualized by electron microscopy. The center of this cap, where two opposing orientations of the polymyxin/phosphatidic acid subunits touch each other, may act as a pore within the membrane. Moreover antibiotic-bound phospholipid domains are more fluid than the surrounding phospholipids, a difference possibly related to changes in membrane permeability (5). In mixed membranes containing uncharged and negatively charged phospholipids, polymyxin specifically binds to

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the negatively charged phospholipids, collects and concentrates them, and segregates them from the uncharged matrix (3).

Our present study was undertaken to examine the effects of polymyxin on a functionally active protein, the sodium channel of the vertebrate peripheral nerve axon. The existence of high concentrations of negatively charged phospholipids in nerve membrane (6) suggested that the polymyxin restructuring of phospholipid bilayers shown in model systems might have functional consequences on ion channel activity. In particular, it seemed likely that the reorganization of negatively-charged phospholipids might alter the surface charge distribution near the channel. Our previous studies on other agents which decrease phase transition temperatures, the general anesthetic agents, had shown that they exert specific effects on sodium channel function (7).

Methods

The node of Ranvier of amphibian sciatic nerve (Xenopus laevis) was arranged for voltage clamping as previously described (8). Single axons were dissected in Ringer's solution (NaCl 114 mM, KCl 2.4 mM, $CaCl_2$ 2 mM, buffered with HEPES 10 mM and pH adjusted to 7.4). In some experiments tetraethylammonium chloride (2.5 mM) was used to block potassium channels. In others, sodium channels were blocked by tetrodotoxin (10^{-7}M) or by substituting choline chloride for NaCl. The solution in contact with the cut ends of the internodes was 120 mM KCl, 5 mM NaCl, buffered with 20 mM HEPES, pH adjusted to 7.3. Temperature was maintained at 10^{o} C. Polymyxin obtained from Sigma, St. Louis, was dissolved in the Ringer's solution at concentrations between 10^{-6} and 10^{-3}M and applied by perfusion to the pool containing the node. Records of sodium currents were taken after 20-30 minutes in each solution.

Results

Polymyxin had little effect at low doses (10^{-6} to 10^{-5} M). There were no apparent changes in the current-voltage relationship and no consistant changes in the level of inactivation (Fig. 1). Our prediction that polymyxin would induce changes in channel function consequent to surface charge alteration was thus not born out. There was a small increase in the time constant of channel activation, which was not sufficient to alter sodium current amplitude (Fig. 1).

At the highest concentrations tested (10^{-4} to 10^{-3} M) there was a rapid, very large increase in the leakage conductance following polymyxin applica-

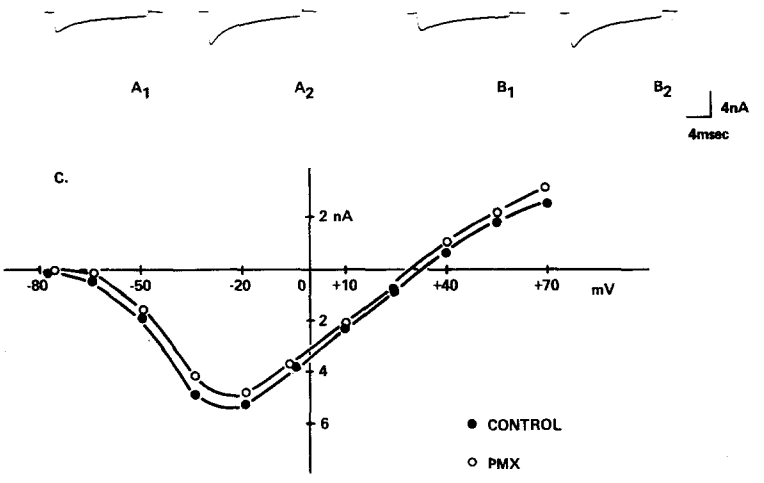

Figure 1. Polymyxin (10^{-5} M) exerts little effect on surface charge dependent properties of the sodium current. Potassium currents blocked by TEA. A_1, A_2: control sodium currents at normal (-82 mV) holding potential (A_1) and with inactivation maximally removed by a hyperpolarizing pulse to -135 mV (A_2). B_1, B_2: polymyxin does not change the level of channel activation at the holding potential. There is a slight increase in the time constant of channel opening. C: Current/voltage relationships are not altered by polymyxin at 10^{-5} M.

tion. In preparations in normal Ringer's solution without blocking agents, the membrane appeared to have been destroyed as a semiperimeable barrier. In all cases the increase in leakage conductance took place very rapidly, over the course of at most five minutes following drug application. Simultaneously a large inward current developed and the amplitude of the voltage-dependent sodium current decreased until it was no longer possible to evoke a current. Attempts to hold the membrane at a stable point within this progression of events or to reverse the effects by washing with drug-free solution were only temporarily successful; as soon as active perfusion with drug-free solution was stopped, the conductance began to increase again. The apparent irreversibility is probably due to the very high lipid solubility of the drug, with large concentrations in the myelin adjacent to the node; polymyxin incorporates quantitatively into membrane lipids (9; F. Sixl and H.J. Galla, unpublished data).

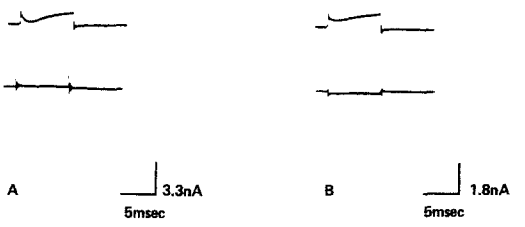

Figure 2. Polymyxin (5×10^{-5} M) on a membrane with potassium channels functioning. Choline substituted for external sodium. A: Control, upper trace, potassium current (upward deflection), lower trace, leakage conductance electronically compensated so that no additional current flows in response to a hyperpolarizing pulse (marked by artifacts at beginning and end). B: Polymyxin application is followed by an increase in leakage conductance which causes the potassium current to appear greater than it is.

The first presumption entertained was that the changes detected as a very large increase in membrane leakage corresponded to a catastrophic disruption of the cell membrane similar to that of antibiotic-treated bacterial cell walls (10). However, when choline was substituted for sodium in the Ringer's solution the effects of polymyxin were significantly modified. At concentrations of polymyxin (5×10^{-5} M to 10^{-4} M) which invariably caused apparent loss of the membrane in the presence of sodium, the potassium channels remained functioning (Fig. 2) in an apparently intact membrane. Leakage current increased during polymyxin application in sodium-free Ringer (Fig. 2).

At higher concentrations (10^{-4} - 10^{-3} M polymyxin) large leaks developed even in sodium-free Ringer and the membrane again appeared to be destroyed.

Since choline substitution prevented the large membrane leak associated with polymyxin in the presence of external sodium, it was of interest to test the effects of tetrodotoxin. Tetrodotoxin (10^{-7} M) abolishes almost all the sodium current in the node of Ranvier. The large increase in leakage conductance produced by polymyxin was not prevented by tetrodotoxin to the same extent as by choline substitution. However, there was a suggestion that tetrodotoxin raised the threshold for the effect, as two nodes out of five remained stable at 5×10^{-5} M polymyxin; at 10^{-4} M, the polymyxin-associated

increase in permeability developed more slowly (over 10 minutes) than was the case with nodes whose sodium channels were not blocked by tetrodotoxin.

In calcium-free Ringer's solution with normal sodium levels the threshold for the rapid permeability increase was lowered to 10^{-5} M polymyxin; otherwise the polymyxin effect was not altered by the presence of calcium.

Discussion

Polymyxin does not destroy the nodal membrane at concentrations as high as 10^{-4} M, the highest concentration at which nodes remained stable (but with increased leakage conductances) in choline-substituted Ringer's solution. In the presence of external sodium, a large increase in leakage conductance is brought about and evoked sodium currents disappear. In the absence of external sodium, there is a small, stable conductance increase. These results are consistent with the hypothesis that polymyxin forms or opens channels in the nodal membrane which are permeable to both sodium and potassium but not to choline. When external sodium concentration is high, the channels permit a large inward flow of current carried by sodium. In the absence of external sodium the channels are also formed; however since the membrane is already highly permeable to potassium and near the potassium equilibrium potential, the resulting changes in membrane properties are relatively small.

Tetrodotoxin binding raised the threshold for the polymyxin effect and slowed its development as did the presence of calcium. The present results are consistent with the hypothesis that polymyxin may organize negatively charged lipids in nerve, forming domains which act as pores permeable to small ions such as sodium and potassium. The polypeptide molecules compete for negatively charged membrane lipids with the normal sodium channel, which is believed to be surrounded by a higher density of negative charges than is characteristic of the rest of the membrane. Tetrodotoxin and calcium stabilize the charge density at the channel, raising the threshold for the polymyxin action. Interestingly, polymyxin exerted no surface-charge related effects at concentrations below those associated with conductance increases.

The concentrations at which polymyxin effects were observed on the nodal membrane are similar to those which produce neuromuscular block and other evidences of neurotoxicity (11,12). The effects of polymyxin reported here, which would be accompanied by a large membrane depolarization, may account for the drug's "local anesthetic-like" action on nerve and muscle (11). Depolarization would also account for the increase in spontaneous miniature end plate potential frequency and decrease in evoked end plate potential quantal content observed at the neuromuscular junction, for the incomplete reversibility of polymyxin neuromuscular block by calcium, and for failure of neostigmine to reverse the block (12).

At polymyxin concentrations above 10^{-4} M a large increase in membrane conductance occurs even without external sodium; this may correspond to a destructive loss of the nerve membrane similar to that observed in bacteria.

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