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Penetration of VX into Nerve Cells, and Effects on Electrical Function

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The organophosphorus ChE[3] inhibitors include research tools such as DFP, insecticides such as parathion to name only one, and potential military weapons such as Sarin and VX (HACKETT 1978, WADE 1975). The storage and disposal of these compounds present serious problems which have sometimes received attention in the popular press.

The discovery of an enzyme in cephalopod nerve and hepatopancreas capable of hydrolyzing several of these compounds (HOSKIN et al. 1966, GAY and HOSKIN 1979) has implications favorable to their disposal in the ocean. On the other hand there is reason to think that VX might not be so readily detoxified (HOSKIN and ROSENBERG 1967, HOSKIN et al. 1969, DETTBARN and HOSKIN 1975). It seemed possible that VX might nevertheless occupy the active site of (and thus inhibit) squid type DFPase, as we term the detoxifying enzyme (GARDEN et al. 1975). This would permit the use of VX to explore the physiological role of DFPase in the squid giant axon where the enzyme is found in remarkably high concentration.

While the application of VX to this problem has not yet proven fruitful, results have been obtained that may be pertinent to questions of its neurotoxicity. Generally the powerful ChE inhibitors have had little effect on axonal conduction, nor perhaps is there a fundamental reason to expect any (HOSKIN 1970, NACHMANSOHN 1970). However, effects have been observed on the electroplaque of the electric eel (BULLOCK et al. 1977) in which both conducting and synaptic membranes are present in a single cell. This report describes the use of the squid giant axon as a single cell model for studying the uptake of VX, and single electroplaques of Electrophorus for studying the effects of VX on the electrical properties of membranes. In the course of these studies we have explored the relationship of VX to the detoxifying

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[3]Abbreviations or trivial names: AChE and ChE, acetylcholinesterase and cholinesterase; 217A0, 0,0-diethyl S-(2-dimethylaminoethyl) phosphorothioate; ATCh, acetylthiocholine; DFP, diisopropylphosphorofluoridate; Mes, Hepes, Tris, 2-(N-morpholino)ethanesulfonate, N-2-hydroxyethylpiperazine-N'-ethanesulfonate, tris(hydroxymethyl)aminomethane; parathion, diethyl-p-nitrophenylthionophosphate; Sarin, isopropyl methylphosphonofluoridate; Soman, 1,2,2-trimethylpropyl methylphosphonofluoridate; VX, 0-ethyl S-(2-diisopropylaminoethyl) methylphosphonofluoridate.

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enzyme DFPase, have determined the inhibition of AChE by VX, and its spontaneous reactivation, and have made related observations that are reported here.

MATERIALS AND METHODS

Squids (Loligo pealei) were used at the Marine Biological Laboratory, Woods Hole, Mass. Electric eels (Electrophorus electricus) were purchased from World Wide Scientific Animals, Ardsley, N.Y., and were held at the Shedd Aquarium, Chicago, before use. VX was synthesized according to the method of TAMMELIN (1957). Squid type DFPase was purified in this laboratory (GARDEN et al. 1975). Other reagents were obtained commercially.

AChE activity was determined by the method of ELLMAN et al. (1961), and as modified for measurements on intact electroplaques (BULLOCK et al. 1977). When ChE inhibition by VX was being determined, either on AChE in solution as a means of assaying for VX, or on intact electroplaques, the VX solution was combined with buffer and AChE, or with eel Ringer (WEBB et al. 1972, BULLOCK et al. 1977) and cells, for exactly 30 min before either addition of substrate, ATCh, or commencement of washing of electroplaques and periodic addition of single electroplaques to ATCh. When the possible detoxication of VX was being determined, VX was combined either with purified DFPase or with freshly homogenized squid nerve so as to attain a final VX concentration of 10^{-4} M. At the end of the incubation period an aliquot of the VX-containing solution or suspension, suitably diluted, was assayed for VX as previously described. When the possible inhibition of squid type DFPase by VX was being examined, the enzymatic hydrolysis of DFP was measured with the fluoride-sensitive electrode as previously described (HOSKIN 1976).

Dissection of squid giant axons, bathing in test solutions, extrusion of axoplasm, electrical measurements on single electroplaques, and toxicity determinations on squids were carried out as previously described (BULLOCK et al. 1977, DETTBARN and HOSKIN 1975, HOSKIN and ROSENBERG 1965).

RESULTS

The second order rate constant (ALDRIDGE 1950) for the inhibition of AChE by VX (16 determinations) was found to be 3.43 ($\pm$ 0.27) X 10^{7} $l \cdot mole^{-1} \cdot min^{-1}$ (std. dev.), in close agreement with the value of 3 X 10^{7} reported by MICHEL et al. (1973). Although a comparable rate constant calculation is not justified for reporting the inhibition of ChE in intact electroplaques, in more practical terms electroplaques bathed in 10^{-4} M VX for 30 min showed 94% inhibition. For comparison, there was no measurable activity in a solution of AChE made 10^{-8} M in VX for 30 min. The spontaneous recovery of ChE activity on washing electroplaques previously bathed in 10^{-4} M VX is shown in Fig. 1. The recovery rate, 9 or 10% per hr, is intermediate between that observed with DFP and unreactivatable Soman-inhibited cells (BULLOCK et al. 1977).

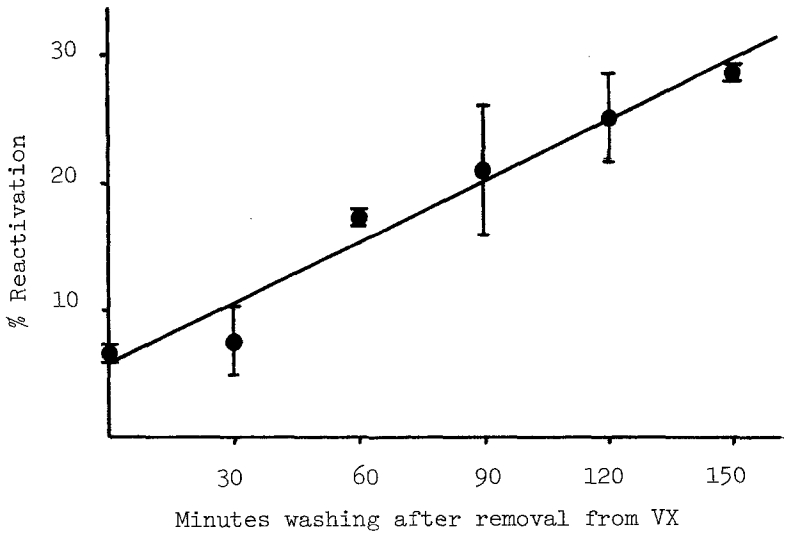

Figure 1. Spontaneous recovery of ChE activity on washing electroplaques previously bathed in 10^{-4} M VX for 30 min. Control electroplaques (100%) were also washed but were not exposed to VX. Vertical bars, std. dev.

When VX was incubated with homogenized squid axons, or homogenized optic ganglia, or with DFPase purified from these sources, under conditions which would have caused 98% hydrolysis of DFP, no loss of VX was detectable. VX also showed no inhibition of squid type DFPase, even when VX was present at a concentration 10 times greater than the substrate, DFP.

The penetration of VX into the squid giant axon as a function of the external pH is shown in Fig. 2. The pK_a' of VX was determined to be 7.9 by titrating a 5 X 10^{-4} M solution of VX in CO_2-free distilled water with standardized 10^{-2} M HCl.

TABLE 1

Toxicity of VX to squids. VX was dissolved in buffered seawater and injected into the optic orbit (DETTBARN and HOSKIN 1975).

Dosage, μmoles/kg	No. dead/No. injected
0.9	1/7
1.7	5/7
3.4	3/6
5.0	6/9
6.8	5/5

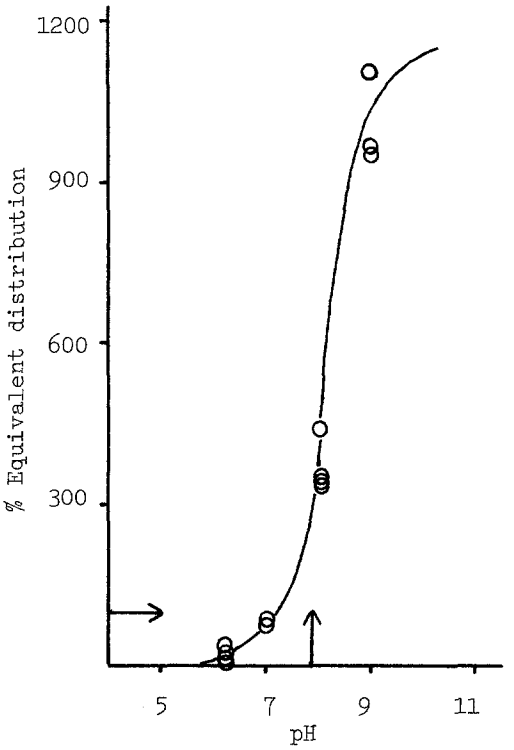

Figure 2. Penetration of VX into squid giant axon as a function of pH of external medium. VX, 10^{-4} M; penetration time, 1 hr; pH maintained with 10^{-3} M Mes, Hepes, or Tris over the pH range 6 to 9. Horizontal arrow, equivalent distribution, i.e., 100%; vertical arrow, pK_a' of VX, 7.9.

The toxicity of VX to squids is given in Table 1. The LD_{50}, estimated at approximately 2-4 μmoles/kg, is rather close to the value of 3-6 μmoles/kg for an organophosphorus compound of similar structure, 217A0 (DETTBARN and HOSKIN 1975). In that publication the opinion was expressed that squids reacted less dramatically to 217A0 than to DFP, although the differences were not clear-cut. It is now clear that although VX is the most toxic of the organophosphorus compounds tested so far, the signs of VX poisoning are the least dramatic. Squids receiving lethal or near-lethal doses of VX either sank to the bottom of the tank or continued to swim, with no ink discharges or leaps as described for DFP.

Because it was assumed that VX would have little effect on the action potential of the squid giant axon (HOSKIN et al. 1969), electrical observations were made on the single electroplaque of the electric eel. As expected, 2 X 10^{-3} M VX had no effects on the electroplaque resting potential or directly evoked action potential. In contrast, the indirect action potential, elicited through presynaptic stimulation and thus requiring synaptic transmission, was completely blocked after 10-20 min in 2 X 10^{-4} M VX. This was completely reversed by washing the electroplaque with 20 ml eel Ringer flowing across the innervated surface for 2 min.

The amplitude of the subthreshold end-plate potential (EPP) and the half-time of the falling phase of the EPP were both reduced by 2 X 10^{-4} M VX. These effects were reversible, and the commencement of the effects and of their reversal were essentially immediate.

The effect of VX on the end-plate currents of single electroplaques has been examined by the technique of the voltage clamp (BULLOCK et al. 1977, NAKAMURA et al. 1965). In these experiments Ba^{++} was present at 3 X 10^{-3} M in order to block rectifying K^{+} channels in the associated conducting membrane. This allowed the end-plate currents, at voltages clamped more negative than the resting potential, to be observed free from conducting membrane contributions (RUIZ-MANRESA et al. 1970). Two clamp experiments chosen to illustrate a large effect of VX are shown in Fig. 3A; in Fig. 3B all the clamp data from a control and VX-treated cell are plotted to show the changes in peak inward current as a function of the clamping voltage. The results show that the inward current and the conductance are reduced by VX.

DISCUSSION

The penetration of VX into cells, exemplified here by the squid giant axon, suggests that VX penetrates connective, Schwann, and basement layers, and finally the axonal membrane itself, in the unprotonated form, and accumulates inside in the protonated form. Making some assumptions about equilibrium of the influx-efflux process, the pH at equivalent distribution should be the intracellular pH of the squid giant axon. From Fig. 2 this is approximately 7.3, in close agreement with the value determined by BORON and DE WEER (1976). Although penetration far exceeds equivalent distribution at and above the pK_a' , entrapment of a hydrolysis product is not involved with VX as it was with DFP (HOSKIN et al. 1966), since squid type DFPase does not hydrolyze VX. Indeed, VX does not even appear to fit the active site of the hydrolytic enzyme.

While VX is a potent inhibitor of AChE in solution, with intact eel electroplaques a significant and easily measurable amount of esterase remains even at a VX concentration four orders of magnitude higher than that which causes complete inhibition of purified eel AChE in solution. In view of the ionic nature of the substrate and the penetrating properties of VX, this is probably due to other esterases (ROSENBERG and DETTBARN 1963) on the outer surface of the electroplaque which are relatively insensitive to VX. Even if AChE were essential to the electrical properties of the electroplaque, the rate of spontaneous reactivation of VX-inhibited AChE on intact electroplaques would be too slow to account for the prompt reversal of the effects of VX on these properties. The lack of effect on conduction, and the more easily reversible effects on the synaptic inward current are more compatible with an interaction of VX with the cholinergic receptor similar to that found with DFP (KUBA et al. 1973). Whether the effects are similar to those found with Soman (BULLOCK et al. 1977), or whether the phosphonylation of receptor is interfering

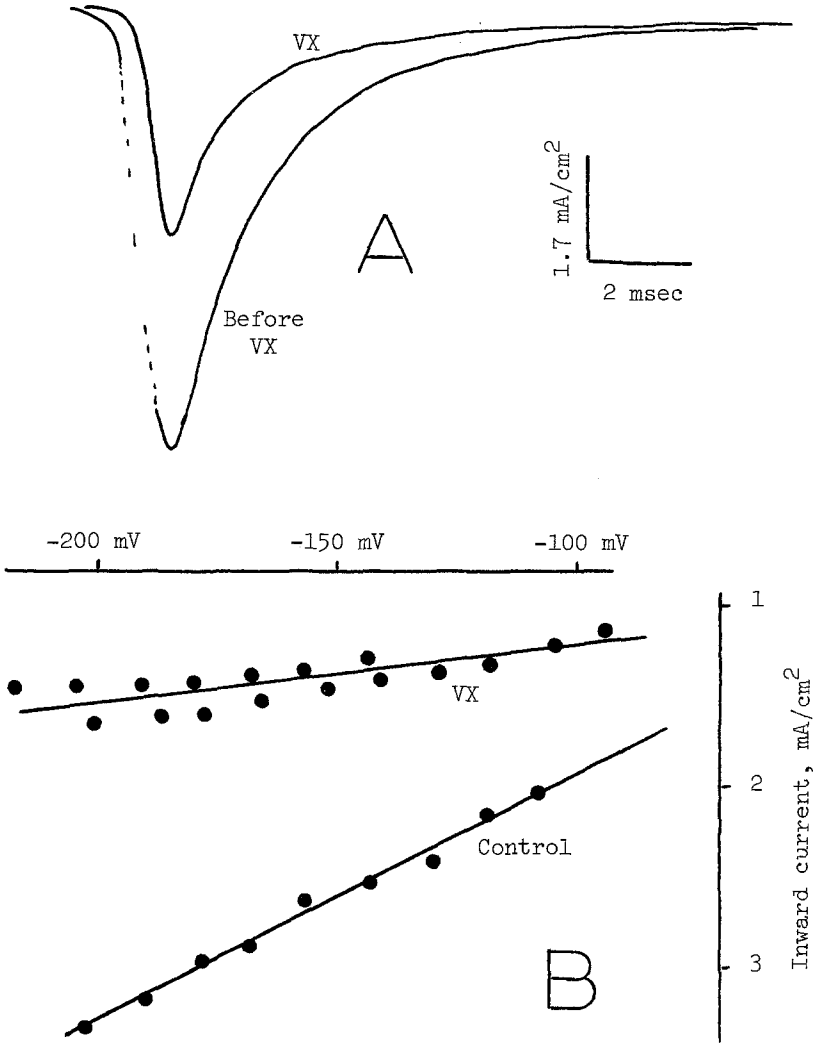

Figure 3. A, an Electrophorus electroplaque clamped at -204 mV before and after VX at 2 X 10^{-4} M; downward deflection, inward current. B, peak inward currents from control electroplaque, and the electroplaque treated with VX for two different times and clamped at various voltages.

with a natural cycle of phosphorylation (GORDON et al. 1977, TEICHBERG et al. 1977) is the subject of continuing research in this laboratory. Although our examination of the toxicity of VX to squids was more for the purpose of ruling out rapid detoxication reactions such as with DFPase, it should be noted that the signs of VX toxicity are what might be expected with receptor inhibition, as well as with the more demonstrable AChE inhibition.

These results suggest that the handling and disposal of VX

and similar phosphonothiolo compounds may be complicated by their ability to accumulate within cells, by their stability, and by their ability to interact with the cholinergic receptor as well as by their inhibition of AChE. The stability of VX in sea-water (MICHEL et al. 1973) and its resistance to enzymatic hydrolysis make ocean disposal less advisable than with Sarin, for example (HOSKIN 1971). On the other hand its more rapid disappearance in soil (VERWEIJ and BOTER 1976) may be related to the presence of microorganisms capable of metabolizing VX (HOLWERDA 1978).

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