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On the Development of Behavioral Tolerance to Organophosphates II: Neurophysiological Aspects

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MELCHERS, B. P. C. AND H. P. M. VAN HELDEN. *On the development of behavioral tolerance to organophosphates II: Neurophysiological aspects*. PHARMACOL BIOCHEM BEHAV **35**(2) 321–325, 1990.—In a recent study (9) it was found in rats that chronic treatment with the irreversible cholinesterase inhibitors DFP or soman led to behavioral tolerance in the case of DFP, but not in the case of soman. Biochemically, no explanation was found for this difference between these two inhibitors. Notably, chronic administration of each of these inhibitors did not affect the availability of the nicotinic receptors at the motor endplate, in spite of very low cholinesterase activity. In an attempt to explain the different effects of these inhibitors a neurophysiological approach seemed appropriate. The spontaneous quantal release of acetylcholine from diaphragm muscles in vitro from animals chronically treated with each inhibitor showed a similar trend; compared with controls the MEPP frequency was decreased, which was significant for DFP, and the MEPP amplitude was increased, which was significant for soman. Neuromuscular function of muscle strips obtained from both DFP- or soman-treated animals appeared significantly more sensitive to additional inhibitor added in vitro. This could simply be explained by the high preexisting level of cholinesterase inhibition, but seems in contrast with the phenomenon of tolerance.

Organophosphates Tolerance Neuromuscular transmission Miniature endplate potentials

IN a recent study of our group on behavioral tolerance following treatment with irreversible organophosphorus cholinesterase inhibitors, it was found that such a tolerance developed after DFP, but not after soman (9). A biochemical explanation for this difference between these two inhibitors was not found. Amongst others, chronic administration of each of these two inhibitors resulted in a down-regulation of muscarinic receptors in the central nervous system, but surprisingly caused no changes in the number of nicotinic receptors at the motor endplate in spite of a profound inhibition of acetylcholinesterase (AChE). Since the execution of normal or abnormal performance does not only depend on the intactness of the function of the central nervous system (CNS), but also on that of the neuromuscular apparatus, it was necessary to investigate if and in which (other) way neuromuscular transmission adapted to the cholinesterase inhibition and whether there were differences in the effects of DFP and soman. Furthermore, as has been shown in a number of studies, changes in the neuromuscular transmission may occur following chronic treatment with cholinesterase inhibitors, both at the pre- and postsynaptic level (1–3, 5–7). Therefore, it was decided to have a more detailed look at processes involved in the neuromuscular transmission of these chronically treated rats, pending a study on the CNS effects of these two inhibitors to be investigated in hippocampal slices. A neurophysiological approach seemed appropriate.

METHOD

General

For the present experiments rats were taken, at random, from a larger group of animals which were chronically treated with soman or DFP. The behavioral and biochemical data for this group of rats were reported in a recent study, in which the detailed procedures are described (9). In brief: male Small Wistar rats were used with an initial body weight of 150–170 g. They were bred and kept under SPF conditions in our laboratory, food was ad lib. The animals were chronically treated by subcutaneous injections of the two AChE inhibitors DFP and soman. They were injected every other day during a period of one month with sublethal doses of soman (7×65 μg/kg SC and 8×60 μg/kg SC) or DFP (15×600 μg/kg SC). After seven injections the soman dose was lowered since one animal died at that time. Animals injected with a physiological salt solution served as controls. During the course of the experiment the animals were tested in a shuttlebox for behavioral tolerance. Body weights were determined during the experiment. At the start of the experiment the body weight of all rats was similar. At the end of the experiment some differences were found in the body weights of the animals: the average weight of the saline-treated group was 264 g, that of the soman-treated group 253 g and that of the DFP-treated rats 246 g. All animals

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that were initially included in the chronic experiment were used, either for the biochemical (9), or for the neurophysiological experiments, except for the one soman-treated animal that died during the experiment. One day after the final injection of OP the animals were sacrificed by decapitation and the hemidiaphragms derived from these animals were divided into 3–5 mm wide strips. In these strips the AChE activity was determined and they were used to record the neuromuscular transmission, resting membrane potentials and the spontaneous quantal release.

Electrophysiology

For the electrophysiological experiments the strips were pinned down on Sylgard on the bottom of a petri dish (10 ml volume) in Ringer solution (in mM: NaCl: 114; KCl: 4.6; $NaHCO_3$: 25; NaH_2PO_4: 1; $MgSO_4$: 1; $CaCl_2$: 2; and glucose: 11.1) which was constantly gassed with 95% O_2/5% CO_2. The electrophysiological experiments were done at room temperature (18–22°C). Intracellular recordings were made with conventional recording techniques in the endplate region of the muscle strips. Endplates were only accepted for analysis when the resting membrane potential was at least 60 mV and did not decrease by more than 10% during the recording period, the number of endplates which had to be rejected was similar in all treatment groups. The data represent recordings from 4–8 endplates per animal. Microelectrodes were filled with 3 M KCl and had a resistance of 10–15 MΩ. Miniature endplate potentials (mepps) were amplified, displayed on an oscilloscope and stored on magnetic tape by means of a RACAL FM tape recorder. Mepp frequency was assessed from chart records made on a Siemens ink-jet recorder.

Neuromuscular Transmission (NMT) and Acetylcholinesterase (AChE) Determination

Both NMT and AChE activity were measured according to Van Dongen *et al.* (8). In brief, muscle strips were mounted in Krebs-Ringer solution at 37°C, which was continuously gassed with 95% O_2/5% CO_2. The capacity of the muscle strips to sustain tetanic contractions was tested and served as a criterion for NMT. The strips were supramaximally and indirectly stimulated by field stimulation (pulse duration 3 μsec, tetanus duration 3 seconds). Every 10 minutes a series of consecutive tetani of 25, 50 and 100 Hz were administered with an interval of 30 seconds. Between these tests the strips were stimulated at 0.2 Hz. For the determination of dose-response curves (Fig. 1), the failure of NMT was measured at increasing in vitro concentrations of DFP and soman in diaphragm preparations obtained from rats chronically treated with saline, DFP or soman. After testing for NMT at t = 0, either DFP (20 μg/100 ml) or soman (0.2 μg/100 ml) was administered to the bath. After an incubation period of 10 minutes the NMT was tested again. Administration of inhibitor and subsequent testing was repeated until a complete failure of NMT was reached. To study the relationship between the NMT and the AChE activity (Fig. 2), a single dose of soman (0.2 μg/100 ml) or DFP (20 μg/100 ml) was administered to the bath. The muscle strips were incubated with this dose of inhibitor until a desired partial inhibition of the NMT was reached (5–20 minutes). The strips were then washed and after another 10 minutes tested again in order to check whether the NMT had stabilized. Subsequently, the muscle strips were homogenized for the determination of AChE activity.

Chemicals

Soman (pinacolylmethylphosphonofluoridate) and DFP (diisopropylfluorophosphate) were synthesized at the Prins Maurits Laboratory TNO, Rijswijk. All other chemicals were commercial products of analytical grade.

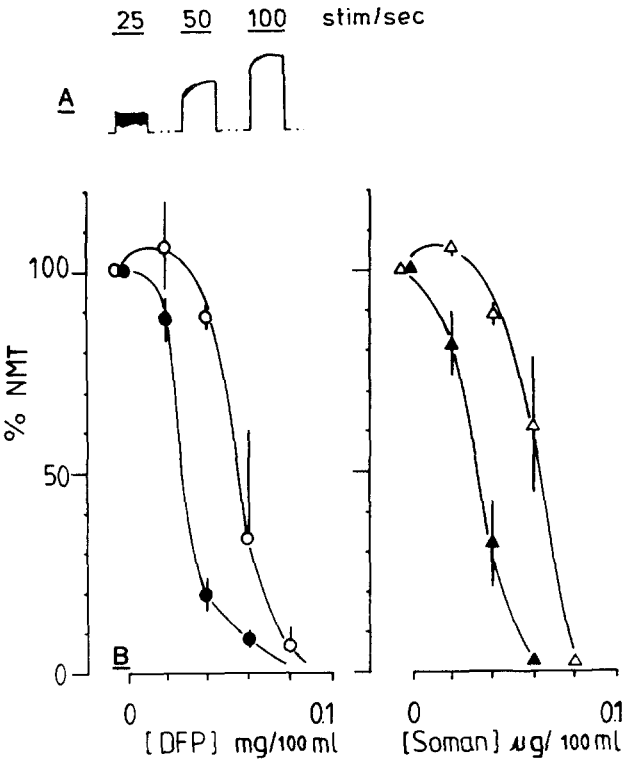

FIG. 1. (A) Recording of the neuromuscular transmission (NMT) in a normal control diaphragm preparation, stimulated indirectly at 25, 50 and 100 Hz. (B) Failure of the NMT at increasing in vitro concentrations of DFP (left) and soman (right), in diaphragm preparations derived from rats chronically treated with saline (open symbols, n = 5) and DFP (closed circles, n = 5) or soman (closed triangles, n = 5). The saline group was divided into two subgroups, one served as control for the chronic DFP group (n = 2), and was inhibited in vitro with DFP; the other was used as control for the soman group (n = 3) and was inhibited in vitro with soman. For each animal the dose-response curve was determined in two muscle strips. These results were averaged. For the quantification of the NMT, the area under the curve was calculated and expressed in proportion of that recorded just before in vitro addition of the inhibitor. This value was set at 100%. ED_{50} values for both DFP and soman were significantly lower in the diaphragms of chronically treated rats. Bars indicate S.E.M.

Statistics

A one-way ANOVA was performed to compare the electrophysiological data of the three groups. If the ANOVA showed significant differences, post hoc comparisons were made by means of the Student's *t*-test with a Bonferroni correction for multiple comparisons. An analysis of trends in the three groups was performed by means of the Jonckheere-Terpstra test (4). ED_{50} values of the sensitivity of the muscle strips to OP administration were compared using the Mann-Whitney U-test.

RESULTS

The results of the electrophysiological measurements are summarized in Table 1. A one-way ANOVA showed no significant differences in resting membrane potentials between the three groups of animals, $F(2,12) = 3.20$, $p = 0.08$. The one-way ANOVA revealed a significant difference between the three groups for the

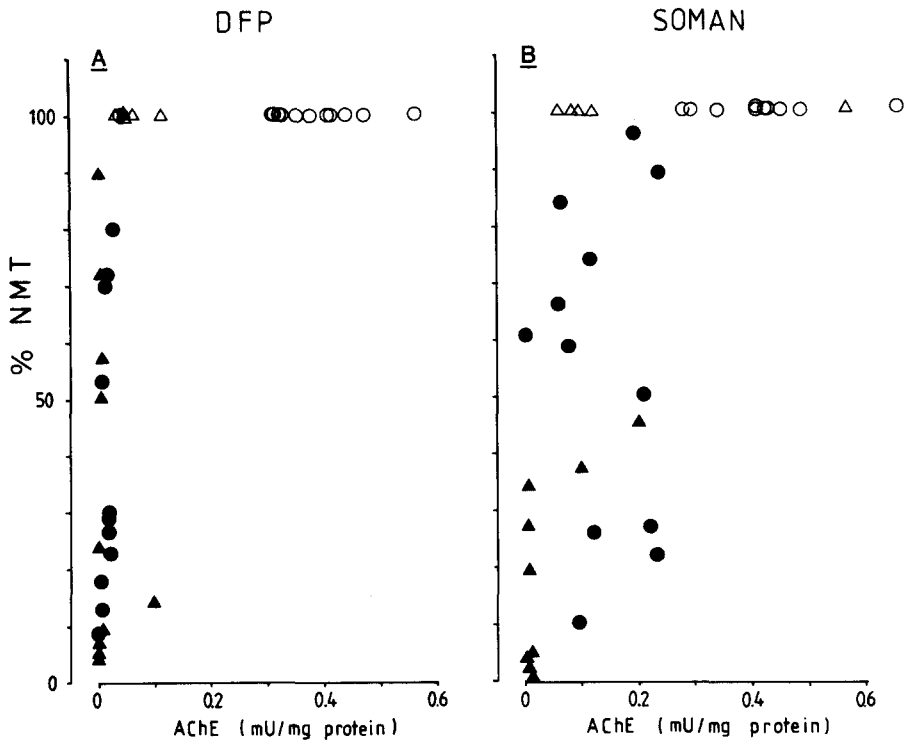

FIG. 2. Individual NMT values plotted against total AChE activity determined in diaphragm strips, obtained from chronically saline- (circles) or OP-treated (triangles) animals 24 hours after the last injection. NMT values were expressed in the same way as in Fig. 1B. The muscle strips were either not additionally inhibited in vitro (open symbols) or treated for different periods (5–20 minutes) with an in vitro dose of DFP (20 μg/100 ml) (A) or soman (0.2 μg/100 ml) (B) (closed symbols), leading to partial or total failure of NMT. Preparations from chronically DFP-treated animals were only inhibited in vitro with DFP (A); preparations from chronically soman-treated rats were only inhibited in vitro with soman (B). The muscle strips derived from chronically saline-treated rats were divided in two groups, one served as control for the soman group and was inhibited in vitro with soman, the other was used as a control for the DFP group and was inhibited in vitro with DFP.

mepp amplitude, $F(2,12)=5.05$, $p=0.026$, and for the mepp frequency, $F(2,12)=7.25$, $p=0.009$. In the soman-treated animals, the mepp amplitude was significantly enhanced in comparison with controls (Students t-test, $p<0.05$). No significant difference in mepp amplitude was encountered between the DFP-treated animals and controls and between DFP- and soman-treated animals. The mepp frequency in the DFP-treated animals was significantly decreased in comparison with controls ($p<0.05$). No significant differences in mepp frequency were found between soman-treated animals and controls and between DFP and soman-treated animals. A trend analysis showed a significant trend both in the mepp amplitude (control < DFP-treated < soman-treated, $p<0.05$) and in the mepp frequency (control > soman-treated > DFP-treated, $p<0.05$).

TABLE 1

RESTING MEMBRANE POTENTIALS, MEPP AMPLITUDE AND FREQUENCY DETERMINED IN DIAPHRAGM MUSCLE PREPARATIONS FROM RATS CHRONICALLY TREATED WITH SALINE, DFP OR SOMAN[a]

Treatment	Membrane Potential (mV)	mepp Frequency (sec^{-1})	mepp Amplitude (mV)[b]
Saline	−74 ± 1.5	0.81 ± 0.14	0.65 ± 0.09
DFP	−73 ± 1.2	0.27 ± 0.06*	0.73 ± 0.07
Soman	−77 ± 1.2	0.58 ± 0.08	1.00 ± 0.08*

[a]In each animal the parameters recorded in the individual 4–8 endplates were averaged and an overall average of 5 animals per treatment group was computed. These overall values are given ± S.E.M.

[b]Mepp amplitudes were corrected to a membrane potential of −75 mV.

*Significantly different from saline ($p<0.05$).

At the moment of sacrifice the NMT in the isolated diaphragm strips dissected from chronically OP-treated rats appeared to be recovered completely, i.e., the NMT was indistinguishable from the NMT in muscles from control animals. Tetanic contractions, as a result of indirect electrical stimulation with frequencies of 25, 50 and 100 Hz, were well sustained (Fig. 1A). After additional in vitro administration of soman or DFP, the NMT of the animals chronically treated with OPs started to fail at lower doses than the NMT in control diaphragm strips (Fig. 1B). The ED_{50} for the DFP-treated animals was 0.029 ± 0.002 mg/100 ml, compared with 0.057 ± 0.007 mg DFP/100 ml for the control group (Mann-Whitney, $p<0.05$). The ED_{50} for the soman-treated group was 0.30 ± 0.045 μg/100 ml compared with 0.57 ± 0.033 μg soman/100 ml for the controls (Mann-Whitney, $p<0.05$). There is a high preexisting level of cholinesterase inhibition in strips from chronically DFP- or soman-treated animals, which may obscure a possible decrease of the sensitivity of the NMT to AChE inhibition resulting from adaptive processes. At the moment of sacrifice, AChE activity was approximately 0.03–0.12 mU/mg protein and

0.05–0.12 mU/mg protein in the DFP- and soman-treated animals respectively, with one extreme value in the soman group (0.55 mU/mg protein). The values for the OP-treated groups were not significantly different. In control rats a much higher AChE activity (0.3–0.5 mU/mg protein) was found. To correct for the preexisting AChE inhibition in the OP-treated groups, the relationship between total AChE activity and NMT was investigated in the three groups of animals. Muscle strips were isolated and incubated in vitro with a dose of soman or DFP. After a desired partial or total inhibition of the NMT was reached, the excess of inhibitor was removed and the muscle strips were homogenized for the determination of AChE activity. For each muscle strip the NMT value was plotted against its AChE activity (Fig. 2). It appeared that a large portion of the total AChE, down to about 0.05–0.1 mU/mg protein, may be inhibited before neuromuscular function starts to decline. There was no clear difference in the relationship between the NMT and the AChE activity in strips from chronically OP-treated animals and strips obtained from control animals; in all groups the NMT failure occurred at similar levels of AChE inhibition.

DISCUSSION

Earlier studies in our group had shown that in animals chronically treated with saline, DFP or soman, tolerance developed to DFP, but not to soman (9). Since the functional integrity of the motor apparatus is an important factor in the ability of the animal to perform, the objective of this study was to investigate if and in which way neuromuscular function in the DFP- or soman-treated animals had adapted to the profound acetylcholinesterase inhibition, and whether any differences in this respect could be found between the diaphragms from DFP- or soman-treated rats.

The present electrophysiological findings (see Table 1) on mepp frequency in diaphragms from DFP-treated animals are comparable with the results of Thomsen and Wilson (5) and Carlson and Dettbarn (1) with paraoxon, those of Tiedt *et al.* with neostigmine (7) and with the recent results of Thomsen and Wilson with DFP (6). Together with the present results, these data suggest that changes take place presynaptically in response to the chronic treatment with cholinesterase inhibitors, which may imply adaptations in the regulation of transmitter release. The trend analysis showed that the decrease in mepp frequency was less profound in the soman-treated animals.

The mepp amplitude was significantly increased in the chronically soman-treated rats, in spite of the fact that no change in the number of nicotinic ACh receptors in the diaphragm could be demonstrated (9). At first glance this increase might be related to the lower AChE activity found in the diaphragms of the chronically OP-treated animals, as compared with controls. However, if this would be true, it does not explain why only an insignificant increase of the mepp amplitude was found in the DFP-treated animals, in which a similar or even larger decrease of AChE activity was encountered. The mepp amplitude is determined not only by AChE activity, but also by the sensitivity of the postsynaptic receptors and/or the amount of ACh per quantum. It is not possible to discern between these possibilities on basis of the present data. Changes of one or both of these two parameters in either of the OP-treated groups may account for the different effect of chronic soman and DFP treatment on the mepp amplitude. Experiments using ACh iontophoresis may give an answer in this respect. In any case, an increase of the mepp amplitude, as was found in the soman-treated group, will not be beneficial for the neuromuscular transmission under conditions of an inhibited ChE.

If NMT would have become "tolerant" after chronic OP-treatment, one would expect that higher doses of OP's would be required to obtain the same degree of NMT-failure than in diaphragms from saline-treated animals. In fact, lower doses sufficed (see Fig. 1), a finding which may be explained simply on basis of the preexisting level of AChE-inhibition in the OP-treated animals. However, this preexisting enzyme inhibition made a "fair" comparison between the sensitivities to OP's of control animals and OP-treated animals more difficult. If it were possible to decrease the ChE activity in control rats instantaneously to the levels found in chronically OP-treated animals, the NMT of diaphragm muscles of OP-treated animals might be less sensitive to OP's administered in vitro. In other words, the relationship between AChE activity and NMT may be a better measure for tolerance. However, no significant differences existed in this respect between saline- and OP-treated animals (Fig. 2), in all three groups the NMT started to fail at similar levels of AChE activity.

These data show that chronic treatment with DFP or soman does not lead to a tolerance to cholinesterase inhibition that is detectable with the presently used test of neuromuscular function. This seems to be in contrast with the behavioral tolerance which was induced by DFP (9). However, changes in spontaneous release parameters in the OP-treated groups were found. Since these changes appear to be different in chronic soman- and DFP-treated animals, it is tempting to relate the significant trends in the electrophysiological effects, i.e., a larger increase of the mepp amplitude (which may be seen as a negative effect) in the soman group and a larger decrease of the mepp frequency (which could be indicative for a positive effect on transmitter release) in the chronic DFP-treated animals, to the fact that behavioral tolerance was induced by DFP, but not by soman (9). It may be expected that the main adaptive processes underlying the development of behavioral tolerance for DFP reside in central cholinergic systems. If synaptic changes due to a chronic AChE inhibition are at least in part similar for all nicotinic synapses, the electrophysiological changes following a chronic DFP or soman treatment might be indicative of similar changes in central cholinergic function.

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