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THE INFLUENCE OF MOTOR NERVE TETANIZATION ON THE EFFECT OF ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS ON NEUROMUSCULAR TRANSMISSION

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1 The changes in the activity of synaptic cholinesterases (ChE) of the ant. tibial muscle in the anaesthetized cat were detected by recording the changes of the blocking activity of acetylcholine (ACh) and comparing them with the blocking activity of carbaminoylcholine (carbachol) injected intra-arterially or intravenously. After the administration of organophosphorus inhibitors (OPI) of ChE the ACh blocking dose diminished 500 to 2000-fold but the carbachol blocking dose did not change. In 4–6 h after the injection of OPI the ACh blocking dose increased again 8 to 15-fold, but the dose of carbachol still remained unchanged. The transmission of high frequency impulses improved after OPI in parallel with the decrease of the ACh blocking activity. Thus the synaptic ChE is partly restored in a few hours after its irreversible inhibition with OPI.

2 Tetanization of the motor nerve (50–60 Hz, 10 min), started simultaneously with the intravenous injection of OPI (armine, Gd–42), diminished the impairment of neuromuscular transmission. On the side of tetanization the ACh blocking action was less pronounced and the transmission of high frequency impulses better than on the control side. Thus the tetanization produced some protection of synaptic ChE against inhibition by OPI. The protective effect of tetanization was absent when the tetanization was performed before the injection of OPI or was started 10–20 min after the injection of OPI.

3 The protective effect of tetanization was also observed on the isolated phrenic nerve diaphragm preparation of the rat.

4 The possible mechanisms of the protective effect of tetanization are discussed.

Introduction

The irreversible organophosphorus inhibitors (OPI) of cholinesterases (ChE) can produce a reversible block of neuromuscular transmission. The reversibility of the block produced by OPI has been shown by many authors, but in most cases the restoration of neuromuscular transmission was not accompanied by a detectable rise in muscle ChE activity (Berry & Evans, 1951; McNamara, Murtha, Bergner, Robinson, Bender & Wills, 1954; Meeter & Wolthuis, 1968; Meeter, 1969). However, it is very difficult to evaluate quantitatively the activity of synaptic ChE in the muscle. The histochemical method is not quantitative. With homogenates of the whole muscle it is possible to determine biochemically only the total cholinesterase activity, synaptic and extrasynaptic, including the intracellular enzyme which does not play any functional role (Koelle, 1963). Barstad (1960) showed that only a slight increase of ChE activity was necessary to restore the neuromuscular transmission impaired by DFP. Such a minute degree of ChE reactivation may be undetectable with the usual biochemical methods.

Comparison of the neuromuscular blocking doses of acetylcholine (ACh) and carbaminoylcholine (carbachol) in the whole organism can give an idea of the activity of synaptic ChE. The drugs can be injected intra-arterially, that is, directly to the muscle (Danilov, 1967), or intravenously. With intravenous injection ACh is also hydrolysed in other tissue although the role of blood in the hydrolysis is not great (Michelson, Muske & Protas, 1974). Using our *in vivo* method (see methods section) we found that the synaptic ChE, having been almost totally inactivated by OPI, could be spontaneously restored to a certain degree during the first few hours after the administration of OPI.

It is well known that ACh can protect ChE from irreversible inactivation by OPI (see, for instance, Koelle, 1946; Augustinsson & Nachmansohn, 1949; Burgen, 1949; Aldridge, 1950; Brestkin, Volkova &

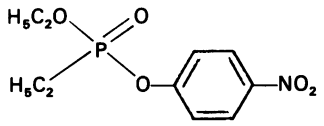
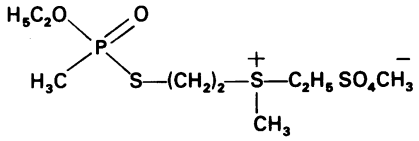
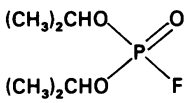
Rosengart, 1964, Volkova, 1965). The protective action of ACh was also shown on the isolated diaphragm of the rat (Barstad, 1960; Mittag, Ehrenpreis & Hehir, 1971) and on mouse diaphragm (Ostrowski & Barnard, 1961). It is possible that in the intact muscle the transmitter which accumulates in the synapse during the action of OPI can protect the synaptic ChE from irreversible inhibition by OPI. The accumulation of ACh in synapses during the action of OPI can be accelerated by tetanization of the motor nerve which increases the release of ACh from the nerve endings in a unit of time (Straughan, 1960, Krnjević & Mitchell, 1961; Bowman & Hemsworth, 1965). Thus, tetanization of the motor nerve should enhance the protective action of the synaptic ACh against the irreversible inactivation of synaptic ChE by OPI. This possibility was tested experimentally.

Elucidation of the protective properties of synaptic ACh may help to explain the reversibility of the transmission blockade produced by OPI. Some preliminary results of these experiments have been published (Ivanov, 1972; Danilov & Ivanov, 1972; Ivanov, Danilov, Zelixon & Michelson, 1972).

Methods

Cats (2 to 4 kg) of either sex were anaesthetized with urethane (500 mg/kp, i.p.) and chloralose (50 mg/kg, i.p.) and injected with atropine sulphate (7 mg/kg, i.m.). The trachea was cannulated and artificial respiration was given. The peroneal nerve(s) on one side or on both sides were stimulated, usually at a frequency of 0.13 Hz by rectangular pulses of 0.15 ms duration and supramaximal intensity. Sometimes high frequencies up to 200 Hz were used. The isotonic contractions of the ant. tibial muscle(s) were recorded on a smoked drum. OPI (armine or Gd–42, see Table 1), ACh and carbachol were injected through a cannula into the left external jugular vein in a volume of 0.3–0.4 ml. In another series of experiments ACh and carbachol were injected into a femoral artery retrogradely through the cannulated *a. circumflexa femoris lateralis*. The blocking activity of ACh and carbachol was estimated by finding the intra-arterial or intravenous doses that reduced the height of single twitches by 50–60%. To assess the changes in the activity of synaptic ChE, the blocking effects of ACh

Table 1 The ability of organophosphorus compounds to inhibit cholinesterases and to block neuromuscular transmission

Inhibitor	k_{II} † *1 M^{-1} min^{-1}*		*Blocking dose*‡
	AChE	*BuChE*	*(mol/kg i.v.)*
 Armine	*	2.6×10^5	$(2.6 \pm 0.2) \times 10^{-6}$ (4)
 Gd–42	2.5×10^8	3.7×10^6	$(3.0 \pm 0.1) \times 10^{-8}$ (5)
 DFP	*	6.7×10^6	

* Not assayed. † k_{II} -bimolecular constants of the rate of interaction of AChE or BuChE with OPI; the k_{II} values for armine and DFP are from Volkova (1965), and the k_{II} values for Gd–42 are from Brestkin, Volkova, Godovikov & Kabachnik (1968). ‡ The dose reducing by 80–90% the height of single twitches of cat ant. tibial muscle; the values are the means ± s.e. mean; the numbers of observations are given in parentheses after the appropriate doses.

and carbachol were compared before and after the administration of OPI. The ability to maintain a tetanus (for 5 s) induced by high frequency stimulation was also tested before and after OPI.

Spontaneous recovery of synaptic transmission impaired by OPI

The contractions of one tibial muscle stimulated at a frequency of 0.13 Hz were recorded. The intra-arterial blocking doses of ACh and carbachol and the ability of the muscle to maintain tetanic contractions were determined before the administration of OPI and at different intervals of time after its administration.

The influence of tetanization on the effect of OPI

The contractions of both tibial muscles stimulated at a frequency of 0.13 Hz were recorded simultaneously. One minute before the injection of OPI the stimulation of the two motor nerves was discontinued. Simultaneously with the intravenous injection of OPI the stimulation of the motor nerve on one side was started and continued for 10 minutes at a frequency of 50–60 Hz. Then, the stimulation of the motor nerves at a frequency of 0.13 Hz was resumed on both sides, and the effects of intravenous injections of ACh and carbachol and the ability to maintain a tetanus were compared in the two muscles.

The isolated phrenic nerve diaphragm preparation of the rat (Bülbring, 1946)

The right and left hemidiaphragms were mounted in two similar baths containing 40 ml Liley solution (Liley, 1956) at 30°C and pH 7.3–7.4 which was bubbled with a gas mixture (95% O_2 and 5% CO_2). The contractions of both hemidiaphragms were recorded in response to stimulation at 0.13 Hz. Drugs were added to the baths in a volume not exceeding 0.5 ml. Before the addition of OPI the stimulation of the two hemidiaphragms was stopped. Simultaneously with the addition of OPI to both baths tetanization of one muscle (50 Hz, for 9 min) was started. OPI remained in the two baths for 7 min and then was washed out three times at 2 min intervals. Tetanization was stopped after the second washing. The stimulation of both muscles at 0.13 Hz was resumed, and the blocking effects of ACh and carbachol (added to the bath for 3 or 5 min), as well as the ability to maintain a tetanus, were compared in the two hemidiaphragms.

Drugs

The following drugs were used: armine, Gd–42 and di*iso*propyl phosphorofluoridate (DFP) (see Table 1), atropine sulphate, acetylcholine chloride (ACh), and carbaminoylcholine chloride (carbachol).

Results

Experiments on cat ant. tibial muscle

The spontaneous recovery of synaptic transmission blocked by OPI is illustrated in Figure 1. Before the administration of OPI, ACh and carbachol given intra-arterially induced a partial block (a reduction of the height of single twitches at a frequency of 0.13 Hz) of about 50% in doses of 10.0 and 0.1 μmol/kg, respectively, and the stimulation of the motor nerve with high frequencies (100–200 Hz, for 5 s) resulted in a well maintained tetanus (Figure 1a). The intravenous injection of OPI (in this case Gd–42, 34 nmol/kg) induced a temporary increase in the height of single twitches and then a complete block of transmission for about 30 min (Figure 1b). After some spontaneous restoration of the contractions (about 70 min after the injection of OPI) the blocking dose of ACh was reduced 2000-fold (0.005 μmol/kg i.a.), but the blocking dose of carbachol remained unchanged (0.1 μmol/kg i.a.) (Figure 1c). At this stage the muscle was unable to maintain contractions even at frequencies of 10 Hz and 35 Hz. When 4 h had elapsed after the injection of OPI (Figure 1d), the blocking dose of ACh had increased 10-fold (0.05 μmol/kg) but the blocking dose of carbachol remained the same (0.1 μmol/kg); at this stage the muscle maintained a tetanus produced by stimulation at 35 Hz, though not at 100 Hz. Similar results were obtained in 17 out of 18 experiments with Gd–42 and in 24 out of 25 experiments with armine. In all cases the blocking dose of ACh was greatly reduced after the administration of OPI, usually 500 to 2000-fold. However, the blocking dose of ACh increased again with time, and when 4–6 h had elapsed after the administration of OPI the blocking dose increased 8 to 15-fold. The blocking dose of carbachol invariably remained the same. The increase with time of the blocking dose of ACh paralleled the increase in the ability of the muscle to maintain a tetanus. These results are consistent with the suggestion that the activity of synaptic ChE, severely inhibited after the injection of OPI, was partly restored in the subsequent few hours and that the gradual improvement of synaptic transmission was due to the recovery of the synaptic ChE activity.

The influence of tetanization of the motor nerve on the effect of OPIs. In previous experiments with armine and Gd–42, it had been shown that the minimal blocking doses of these drugs injected intravenously quickly disappear from the circulating blood, and within 10 min after injection, the OPI cannot be detected in the blood (Ivanov & Muske, 1974). Therefore it seemed reasonable to stimulate the motor nerve for at least 10 min after the injection of OPI to reveal the protective action of the transmitter. Figures 2 and 3 illustrate this form of experiment.

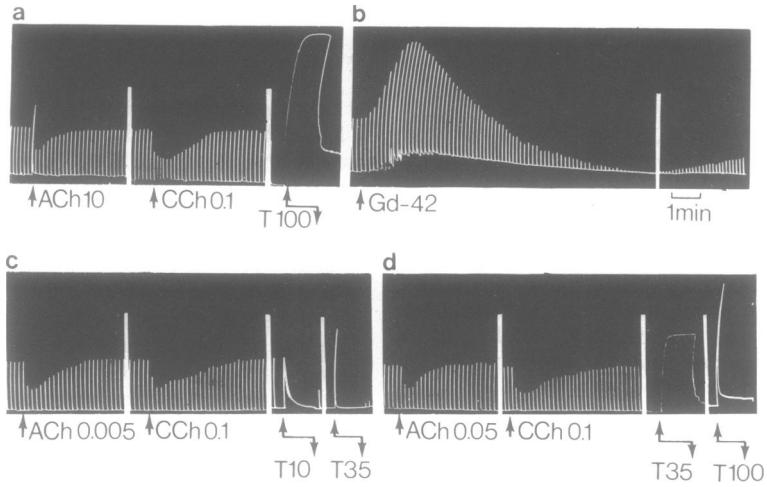


Figure 1 The spontaneous recovery of neuromuscular transmission impaired by organophosphorus cholinesterase inhibitor Gd–42 and the blocking action of intra-arterial injection of acetylcholine (ACh) and carbaminoylcholine (carbachol, CCh). Anaesthetized cat. Atropine 7 mg/kg given intramuscularly. Artificial respiration. Contractions of ant. tibial muscle stimulated indirectly at 0.13 Hz. During periods (5 s) of tetanization (T) at 10–100 Hz the speed of the drum was increased. White vertical lines indicate intervals of 10–15 minutes. (a) Before Gd–42 the blocking doses of ACh and carbachol are 10.0 and 0.1 μmol/kg, respectively. Stimulation at 100 Hz results in a well maintained tetanus. (b) The intravenous injection of Gd–42 34 nmol/kg produces first an increase in the height of contractions and then a complete block of transmission; 30 min later spontaneous restoration of responses begins. (c) Seventy min after the injection of Gd–42; the blocking dose of ACh is reduced 2000-fold (to 0.005 μmol/kg) but the blocking dose of carbachol is unchanged (0.1 μmol/kg). No tetani were obtained at 10 and 35 Hz. (d) Four hours after the injection of Gd–42; the blocking dose of ACh has increased 10-fold (to 0.05 μmol/kg) and the blocking dose of carbachol remains the same (0.1 μmol/kg); stimulation at 35 Hz but not at 100 Hz results in a well maintained tetanus.

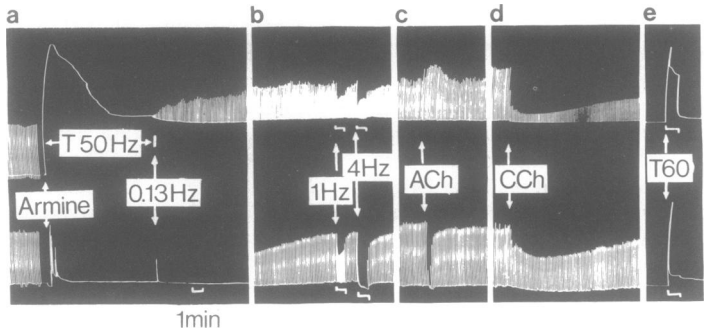


Figure 2 Tetanization of the motor nerve diminishes the impairment of neuromuscular transmission produced by armine. Anaesthetized cat. Atropine 7 mg/kg given intramuscularly. Artificial respiration. Isotonic responses of both ant. tibial muscles induced by indirect stimulation are recorded. White vertical lines—intervals of 20, 10, 20 and 60 min. (a) Stimulation at 0.13 Hz is stopped and armine 2.9 μmol/kg injected intravenously; simultaneously with the injection a 10 min tetanization (T) at 50 Hz of the motor nerve on the left side (upper tracing) is started. During this period the muscle on the control side (lower tracing) shows spontaneous fasciculations induced by armine; after the end of tetanization stimulation at 0.13 Hz is renewed on both sides but induces twitches only on the side of tetanization. (b) Transmission in response to stimuli at 0.13 Hz is restored on the control side but with more frequent stimulation (4 Hz) there is a block on the control side. (c) Acetylcholine (ACh, 0.1 μmol/kg i.v.) produces a blocking effect only on the control side, on the side of tetanization there is a slight increase in the height of contractions which is characteristic of the action of smaller doses of ACh. (d) Carbachol (CCh, 0.5 μmol/kg i.v.) produces a blocking effect on both sides, with that on the side of tetanization somewhat more pronounced. (e) Tetanization at 60 Hz for 5 s (during the tetanization the recording speed was increased); the diminished reaction is much more pronounced on the control side.

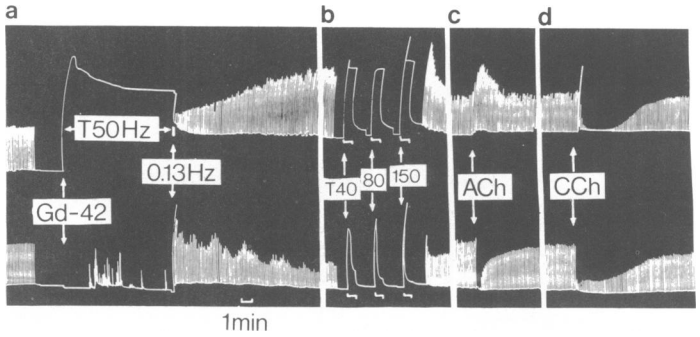


Figure 3 The influence of tetanization on the effect of Gd–42. Cat: Details as in Figure 2. White vertical lines—intervals of 15, 20 and 15 min respectively. (a) Gd–42 (24 nmol/kg i.v.) given simultaneously with the beginning of a 10 min tetanization (T) at 50 Hz on the left side (upper tracing). On the control side (lower tracing) fasciculations of the muscle are seen; after the end of tetanization the stimulation at 0.13 Hz is resumed on both sides causing contractions of increasing size on the side of tetanization and decreasing size on the control side. (b) Tetanization (T) of both motor nerves at 40 Hz, 80 Hz and 150 Hz for 5 s each; on the upper tracing the tetanus is well maintained, but on the control side there is a poor response. (c) Acetylcholine (ACh 0.3 μmol/kg i.v.) induces a complete block of the control muscle and only an increase in the height of the contractions on the side of tetanization. (d) Carbachol (CCh, 0.5 μmol/kg i.v.) induces a block of both muscles more pronounced on the side of tetanization.

Both anterior tibial muscles were stimulated at a frequency of 0.13 Hz. Then stimulation was stopped and armine was injected intravenously (Figure 2a). Simultaneously with the injection of OPI the tetanization of one motor nerve was started and continued for 10 minutes. After the end of tetanization, stimulation at 0.13 Hz was resumed on both sides, but it induced single twitches only on the side of previous tetanization; on the control side, transmission was completely blocked. After the twitches were restored on both sides, stimulation with higher frequencies revealed a marked difference: the muscle previously tetanized maintained a tetanus at 60 Hz but on the control side even 4 Hz caused a complete block (Figure 2e, 2b). The intravenous injection of ACh produced a blocking effect only on the control side, but on the side of tetanization a slight increase in the height of contractions was observed; such an increase is characteristic of the action of smaller (sub-blocking) doses of ACh (Figure 2c). Carbachol induced a block in both muscles and the block was even more pronounced on the side of tetanization (Figure 2d).

Figure 3 illustrates the action of Gd–42. The intravenous injection of ACh induced a complete block of transmission on the control side, but on the side of tetanization only an increase in the height of contractions was observed (Figure 3c). Carbachol induced a block in both muscles and the block was again more pronounced on the side of tetanization (Figure 3d). A tetanus with 40, 80 and 150 Hz was well maintained in the 'protected' muscle but on the control side a tetanus was not maintained (Figure 3b).

Similar results, that is, showing the protective action of tetanization, were obtained in 17 experiments with armine and 14 experiments with Gd–42.

In a control series without administration of OPI (5 experiments), it was shown that tetanization itself (50 Hz, 10 min) did not change either the effects of ACh and carbachol or the reaction of muscle to high frequency tetanization. The protective action of tetanization was also absent when the OPI was injected after the end of tetanization or 10 min before the beginning of tetanization.

The protective action of a tetanization starting simultaneously with the injection of OPI was most obvious when the OPI was injected in a sub-blocking dose (Figure 3a). With a minimal full blocking dose of OPI the protective effect of tetanization was also seen (for instance, Figure 2a), but was usually less pronounced and lasted only 1–1.5 h after the injection of OPI. If OPI was injected in a dose greater than the blocking one the protective effect was usually absent (Figure 4).

Experiments on the isolated rat phrenic nerve diaphragm preparation

Six experiments with Gd–42, 3 with armine and 4 with DFP were performed. Final concentrations of OPI were used that impaired the ability of the muscle to sustain a tetanus at 30–60 Hz after a 7–10 min exposure. These concentrations were 4×10^{-7} M, 1×10^{-6} M and 4×10^{-5} M for Gd–42, armine, and DFP, respectively. One experiment with Gd–42 is illustrated in Figure 5 which shows that the 'protected' muscle maintained a tetanus up to 200 Hz, whereas

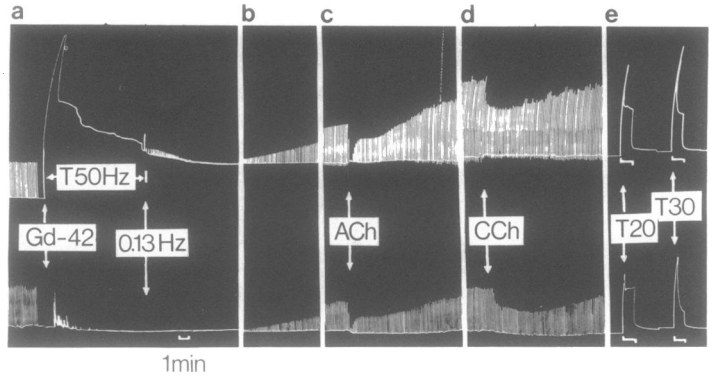


Figure 4 The lack of protective action of tetanization when a large dose of OPI is injected. Cat: Details as in Figure 2. White vertical lines—intervals of 30, 7, 15 and 15 min, respectively. In (a) Gd–42 (50 nmol/kg) is injected intravenously (the minimal full blocking dose is about 34 nmol/kg); a complete block of transmission of single impulses (0.13 Hz) develops both on the tetanized and the control sides. (b) The transmission of single impulses is gradually restored. (c) Acetylcholine (ACh, 40 nmol/kg i.v.) produces a similar blocking effect on the tetanized and on the control muscles. (d) Carbachol (CCh, 0.5 μmol/kg i.v.) also induces an equal degree of block of the two muscles. (e) The two muscles react similarly to the tetanization at 20 and 30 Hz (5 s each).

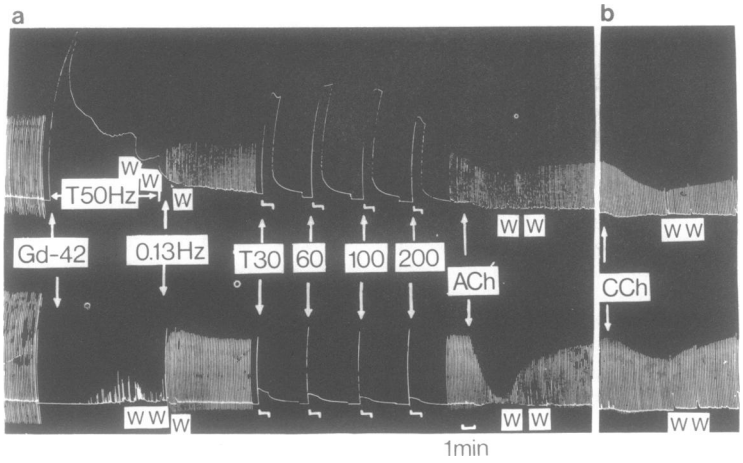


Figure 5 The protective effect of tetanization on the isolated phrenic nerve diaphragm preparation of the rat. The right and left hemidiaphragms are mounted in two similar baths; the isotonic responses to indirect stimulation are recorded. White vertical line—interval of 10 minutes. (a) Stimulation at 0.13 Hz is stopped and Gd–42 (final concentration 4×10^{-7} M) is added to both baths; simultaneously with the addition of OPI tetanization (T) (50 Hz, 9 min) of the left hemidiaphragm (upper tracing) is started; the unstimulated right hemidiaphragm (lower tracing) shows fasciculations. Exposure of both muscles to Gd–42 lasted 7 min, then the inhibitor is washed out 3 times with an interval of 2 min, the tetanization is stopped and the stimulation of the two muscles at 0.13 Hz resumed. With short (6 s) tetanizations (T) at 30, 60, 100 and 200 Hz the upper muscle maintains all contractions well whereas in the lower muscle no tetanus is maintained even with 30 Hz. Acetylcholine (ACh, 3×10^{-5} M, 3 min) produces a more pronounced block on the control (lower) muscle than on the tetanized muscle. (b) Carbachol (CCh, 4×10^{-5} M, 5 min) produces a similar effect on both muscles.

the control muscle did not maintain a tetanus with 30 Hz (Figure 5a). The addition of ACh (final concentration 4×10^{-5} M) produced a blocking effect on both muscles but on the control muscle the block was more pronounced (Figure 5a). With carbachol the block was nearly equal on both sides (Figure 5b). Similar results were obtained in other experiments. In two experiments with DFP the protective effect of tetanization was very weak. Generally the protective effect of tetanization on the rat diaphragm *in vitro* was less pronounced than in the cat ant. tibialis *in situ*.

Discussion

Tetanization of the motor nerve, started simultaneously with the intravenous injection of OPI, diminished the impairment of neuromuscular transmission and presumably the degree of synaptic ChE inhibition produced by OPI.

It has been shown in rats that a prolonged (1–2 h) tetanization of the motor nerve increased the activity of synaptic ChE (histochemical determination) but the total ChE activity of the muscle (biochemical determination) did not change (Gerebtzoff, Goffart & Dresse, 1963). The authors suggested that during the prolonged tetanization the enzyme moved from the sarcoplasm into the synapse. This suggested mechanism cannot explain our results because in the absence of OPI the 10 min tetanization did not change the blocking action of ACh.

Our results can be explained on the theory that ACh at the synapse can protect a part of the synaptic ChE from irreversible inhibition by OPI and that the increase in the liberation of ACh during tetanization enhances this protective effect. Unfortunately we do not know the concentration of transmitter that can be achieved in the synaptic cleft after the inactivation of synaptic ChE. Nevertheless this concentration is probably high enough for a protective effect. The ACh concentration in nerve terminal vesicles is very high, not less than 1.5×10^{-1} M in warm blooded animals. Even after dilution of this concentration by several magnitudes the ability to protect ChE from irreversible inhibition should still be retained (Koelle, 1946; Brestkin *et al.*, 1964; Volkova, 1965).

Biochemical determinations of residual ChE activity in cat muscle homogenates revealed that on the side of tetanization the inhibition was usually somewhat less pronounced than on the control side (Ivanov *et al.*, 1972). But the main proof of a protective effect of tetanization was obtained by comparison of the effects of ACh and carbachol on the two sides. By this method it was possible to reveal a protection of the synaptic ChE, which is the only portion that is important for synaptic transmission.

The protective action of ACh cannot be achieved when the enzyme is already phosphorylated by OPI. Thus tetanization started 10–20 min after the injection of OPI had no protective action. When tetanization was finished before the injection of OPI the impairment of transmission could even be increased, probably because of the increased supply to the muscle of OPI due to post-tetanic hyperaemia.

The protective effect of tetanization diminished with an increase in the dose of OPI, and was sometimes absent with very large doses. One possible reason is that with a large dose, OPI does not disappear from the blood during the period of tetanization (10 minutes). The protective effect also diminished with a minimal blocking dose if the tetanization lasted only 1–3 minutes. Furthermore, with high doses of OPI the irreversible inhibition of synaptic ChE may occur before the accumulation of ACh in a concentration sufficient for effective protection.

It has been shown that in rats the spontaneous restoration of neuromuscular transmission blocked with sarin is accelerated by raising the muscle temperature by 3–4°C (Meeter & Wolthuis, 1968). In our experiments on cats only a slight (0.6–1.6°C) rise of temperature in the tetanized muscle was observed. The temperature of the control muscle did not change significantly after the administration of OPI. However, the observed small rise of temperature cannot explain the protective effect of tetanization because such a rise produced by artificial heating of the leg (instead of by tetanization) did not influence the effect of OPI (Ivanov, 1973).

Data have been published (Khayutin, 1971) indicating that during a strong tetanic contraction the blood vessels can be constricted and the blood supply to the muscle impaired. If so, the reduction of the blood supply of the tetanized muscle would reduce the amount of OPI reaching the muscle and this could be the major reason for the protective effect of tetanization. But control experiments showed that the blood flow through the femoral artery was not reduced but markedly increased on the side of tetanization started simultaneously with the injection of OPI, and that it greatly surpassed the blood flow on the control side (Ivanov *et al.*, 1972). Thus, the protective effect of tetanization occurred in spite of the fact that the amount of OPI delivered to the side of tetanization was probably greater.

The protective effect of tetanization may be due not only to the action of transmitter ACh, as we have suggested earlier (Ivanov *et al.*, 1972). In experiments on the longitudinal muscle of the guinea-pig ileum, Mittag, Ehrenpreis & Patrick (1971) have shown that ACh added to the bath is hydrolysed 4 times less rapidly in isotonic conditions, when the muscle is shortened under the influence of ACh, than in isometric conditions when the muscle remains in the extended state. The authors suggested that the penetration of ACh to the muscle ChE is hampered when the muscle is contracted and shortened.

Michelson & Shelkovnikov (1976) have presented evidence that the isolated rectus abdominis muscle of

the frog also hydrolyses ACh more rapidly in an extended state (isometric conditions) than in a shortened state (isotonic recording).

Lancaster (1973) added a quaternary ammonium OPI to the bath containing a nerve-muscle preparation of rat diaphragm and observed that in the tetanized muscle the degree of ChE inhibition was less than in the control.

In the light of all these findings one can postulate that the shortening of the muscle contracting under isotonic conditions impedes the penetration to muscle ChE not only of substrates but also of anticholinesterases. We recorded the responses isotonically and the protective effect of tetanization could have been due partly to the hampered penetration of OPI to the ChE of the tetanized muscle in spite of its better blood supply. Indeed, the corresponding control experiments (5 trials) had shown that the protective effect of tetanization is less pronounced in isometric than in isotonic conditions. In another series (7 experiments) both muscles were tetanized but during the tetanization one muscle was under isotonic and the other under isometric conditions. In 4 cases the protectice effect of tetanization was more pronounced on the side of isotonic responses (showing the protective effect of shortening). In 3 other experiments the effect of OPI was similar on both muscles. Thus, in isotonic conditions the protective effect of tetanization is probably due not only to the increased protection of synaptic ChE by the transmitter, but also to the greater difficulty of penetration of the OPI in the tetanized (and shortened) muscle.

Our experiments have shown that the spontaneous restoration of neuromuscular transmission blocked by OPI is probably accompanied by an increase in the residual activity of synaptic ChE. This is indicated by the gradual decrease in the blocking action of ACh during the first hours after the injection of OPI (Figure 1). It is unlikely that the increase in the blocking doses of ACh is due to desensitization of cholinoreceptors because the blocking dose of carbachol did not change.

It is clear that ACh released in the synapse during the development of the ChE inhibition by OPI not only protects a part of the synaptic ChE from irreversible inhibition but also inhibits a part of the protected synaptic AChE by the mechanism of substrate inhibition. Substrate inhibition requires higher concentrations of ACh (about 10^{-2} M) than those required for the protective effect. Nevertheless there is some evidence that the local concentration of ACh can be sufficient for transient substrate inhibition even in the absence of OPI (Cohen & Hagen, 1964; Kerkut, Geoffrey, Rick & Walker, 1970; Whittaker & Dowdall, 1975). In this case the block of transmission must be due to heterogeneous inhibition of the synaptic enzyme: one part (probably the greater) will be irreversibly inhibited by OPI, and another part will be under reversible inhibition by ACh. Some decrease in the concentration of ACh in the synaptic cleft will be sufficient for reactivation of the latter portion of AChE. OPI quickly disappears from the blood (Ivanov & Muske, 1974) and the reactivated part of the enzyme will not be inactivated again by OPI. Even if only a small part of synaptic AChE was under substrate inhibition its reactivation can significantly improve transmission, an increase of a few percent may be enough (Barstad, 1960; Barnard, Wieckowski & Chin, 1971). However, the substrate inhibition probably disappears fairly quickly and therefore it seems more reasonable to ascribe the increase in synaptic AChE activity occurring in 4–6 h after the administration of OPI mainly to the synthesis of new enzyme or to partial reactivation of the phosphorylated enzyme.

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