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This report describes continued studies on organophosphorus (OP) inhibitors of acetylcholinesterase (AChE) in the mammalian spinal cord. The mechanism and site of action of the potentiation and/or depression of synaptic transmission by the OPs are being revealed with the use of selective agonists and antagonists of putative neurotransmitters. The spinal cords were isolated from neonatal rats 5- to 9-days old, hemisected, and placed in experimental chambers. Electrodes were attached to dorsal and ventral root pairs, and recordings of the monosynaptic reflex (MSR) were made from ventral roots under varying stimulation and recording paradigms to examine the actions of these agents.

The ability of pralidoxime, trimedoxime or TMB-4, and diethyxime to reverse sarin-induced depression of the MSR in the isolated spinal cord from neonatal rats was examined. The oximes (0.1-10μM) did not affect the MSR but completely or partially reversed the depression caused by sarin in a concentration-dependent manner. The reversal of sarin-induced depression was not accompanied by a regeneration of AChE activity in the spinal cord, suggesting that the reversal of sarin-induced depression of synaptic transmission in the spina cord by these oximes was unrelated to an effect on AChE or the accumulation of acetylcholine. It is more likely that the reversal resulted from blockade of cholinergic (muscarinic) receptors in the spinal cord.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).

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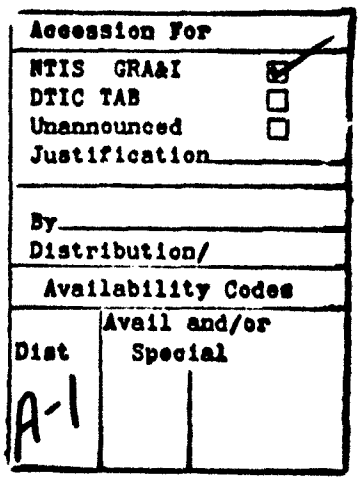

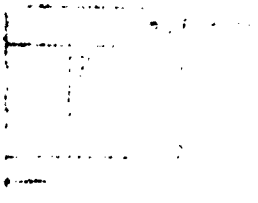

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SUMMARY

This report describes continued studies on organophosphorus (OP) inhibitors of acetylcholinesterase (AChE) in the mammalian spinal cord. The mechanism and site of action of the potentiation and/or depression of synaptic transmission by the OPs are being revealed with the use of selective agonists and antagonists of putative neurotransmitters. The spinal cords were isolated from neonatal rats 5- to 9-days old, hemisected, and placed in experimental chambers. Electrodes were attached to dorsal and ventral root pairs, and recordings of the monosynaptic reflex (MSR) were made from ventral roots under varying stimulation and recording paradigms to examine the actions of these agents.

The ability of pralidoxime, trimedoxime or TMB-4, and diethyxime to reverse sarin-induced depression of the MSR in the isolated spinal cord from neonatal rats was examined. The oximes (0.1-10 μM) did not affect the MSR but completely or partially reversed the depression caused by sarin in a concentration-dependent manner. The reversal of sarin-induced depression was not accompanied by a regeneration of AChE activity in the spinal cord suggesting that the reversal of sarin-induced depression of synaptic transmission in the spinal cord by these oximes was unrelated to an effect on AChE or the accumulation of acetylcholine. It is more likely that the reversal resulted from blockade of cholinergic (muscarinic) receptors in the spinal cord.

The effect of sarin on strychnine- and bicuculline-sensitive inhibitions was examined in spinal cords of neonatal rats, *in vitro*. An early (strychnine-sensitive) phase was evoked at conditioning-test (C-T) intervals of 1-15 msec with peak inhibition occurring at a C-T interval of 7 msec, whereas a late (bicuculline-sensitive) phase occurred at C-T intervals of 20-70 msec and was half the magnitude of the early phase. Low concentrations of sarin (3-20 nM), which facilitated the MSR, reduced the late (bicuculline-sensitive) phase of inhibition but had no effect on the early (strychnine-sensitive) phase of inhibition. At concentrations of sarin ($\geq$ 30 nM) which depressed the MSR, the late phase of inhibition was either blocked to a lesser extent or enhanced. Although the depression of the MSR has been attributed to activation of a muscarinic receptor and is unrelated to inhibition of AChE, the cause of the facilitation has heretofore remained unknown. It appears that the facilitation of the MSR by sarin may be caused by a blockade of a bicuculline-sensitive inhibition which is most likely mediated by gamma-aminobutyric acid.

To further understand the action of the OPs in the spinal cord, it was first necessary to characterize polysynaptic transmission in the isolated spinal cord, a study not previously reported. When Mg^{2+}, a known antagonist of the *N*-methyl-D-aspartate (NMDA) receptor-channel, was withdrawn from the physiological solution, the MSR increased in magnitude in a dose-dependent manner and was followed by a second potential, the polysynaptic reflex (PSR). Whereas the MSR was relatively resistant to NMDA antagonists [2-amino-5-phosphonovalerate (APV) and 2-amino-7-phosphonoheptanoate (AP7)], the PSR was markedly reduced in a dose-dependent manner. The magnitude of the MSR maximally decreased only 20-30% at concentrations of Mg^{2+} (1.3 mM), APV (10 μM) and AP7 (10 μM) which completely depressed the PSR. These results suggest that the MSR is subserved mostly (70%) by non-NMDA (kainate and quisqualate) receptors and to a small extent (30%) by NMDA receptors, the extent of which is governed by the local Mg^{2+} concentration. The PSR appears to be wholly subserved by NMDA receptors.

The characterization of polysynaptic transmission will be completed in the extension period along with an examination of the action of OPs on NMDA-mediated transmission and second messenger systems. The present results suggest 1) that oximes reverse OP-induced depression by cholinergic antagonism, unrelated to regeneration of AChE; 2) that muscarinic, but not nicotinic antagonists or adrenergic agonists, are effective in preventing or reversing OP-induced depression; 3) that blockade of inhibition subserves the excitatory action of OPs; 4) and that the PSR in the isolated spinal cord can be revealed by withdrawing Mg^{2+}, and that it is sensitive to known NMDA antagonist.

I. STATEMENT OF PROBLEM UNDER STUDY

The overall goal of this research is to determine the mechanism by which various inhibitors of acetylcholinesterase (AChE) affect synaptic transmission in the mammalian spinal cord and to establish modes by which these effects can be prevented and/or reversed. The study focuses on the ability of these agents to potentiate and/or depress mono- and polysynaptic transmission in the neonatal rat spinal cord. To pursue this course of study, the actions of selected organophosphorus (OP) and carbamate inhibitors of AChE on the isolated cord were first characterized on spinal monosynaptic transmission with full dose-response curves. Similarities between the OP inhibitors of AChE and that of the carbamates have been made through observations of spinal monosynaptic transmission. The ability of sarin to alter pre- and/or postsynaptic inhibition in the cord as a mechanism for facilitation has been pursued. Various mechanisms which might explain the observed depression of spinal monosynaptic transmission by the OPs are being followed with the use of anticholinergics (*viz.*, muscarinic and nicotinic antagonists), diazepam, thyrotropin-releasing hormone, carbamates (physostigmine and pyridostigmine), and oximes. The ability of cholinesterase (ChE) regenerators (*i.e.*, oximes) to restore monosynaptic transmission in cords exposed to OPs has been examined and correlated with the levels of ChE activity. The noted protective effect of carbamates on the lethal action of the OPs and their ability to hasten recovery from OP toxicity was assessed in the spinal cord. A study on the ability of thyrotropin-releasing hormone to reverse OP-induced toxicity has now shed light on the role of peptidergic and glutamatergic transmission in OP-induced toxicity. Additional studies are aimed at clarifying the action of OPs on N-methyl-D-aspartate (NMDA)- and non-NMDA-mediated spinal activity.

II. RATIONALE

The mechanism and site of action of the AChE inhibitors are being studied in spinal cords maintained *in vitro* in a nearly physiological state. This preparation is most suited to the studies because the external milieu is easily controlled with respect to O_2-CO_2 levels and electrolyte and drug concentrations, and because stable recordings can be made without interference from respiratory movements or vascular pulsations engendered *in vivo*. Unlike central neurons studied in tissue culture, neurons in isolated cord preparations maintain synaptic connections with other segmental and suprasegmental neurons (excitatory and inhibitory) such that the influence of various catecholaminergic, cholinergic, peptidergic, and glutamatergic neurons on the actions of the AChE inhibitors can be more easily assessed.

III. GENERAL METHODS AND MATERIALS

The housing of the animals and preparation of the isolated spinal cord from neonatal rats were similar to those already described.[1-3] Briefly, pregnant dams of the Wistar strain (Charles River Breeding Laboratories, Kingston, NY) were obtained at 12-16 days gestation, housed individually in polycarbonate cages with Betachips as the bedding, and allowed food (Purina Chow) and water *ad libitum*. The room was environmentally controlled with a 12-hr light/dark cycle at 22-23 °C and constant humidity. The rat pups were allowed to remain with the dam from the day of birth (day 0) until the day of the experiment (days 6 to 9). Thereupon, the pups were anesthetized with diethyl ether, and their spinal columns were removed and placed in a

petri dish containing oxygenated physiological solution. The spinal cord from the mid-thoracic to the mid-sacral level was removed along with the corresponding dorsal and ventral roots, hemisected in the longitudinal plane, and the dura was removed. The longitudinally hemisected cord was then transferred to a small Plexiglas bath (total volume, approximately 1 ml) through which oxygenated physiological solution maintained at 25 ± 0.5 °C was constantly perfused at 2 to 3 ml/min. Cords from either male or female rat pups were used in the experiments.

A. *Stimulation and Recording* A L_{3-5} dorsal root was stimulated supramaximally (duration, 0.2 msec; frequency, 0.1 Hz) through a suction electrode attached to a stimulus isolation unit which was controlled by a Digitimer (Medical Systems, model D4030). The monosynaptic reflexes (MSRs) evoked in the corresponding ventral root were amplified (WP Instruments, New Haven, CT, model M707; Axoclamp-2, Axon Instruments, Burlingame, CA), displayed on an oscilloscope (Tektronix, Beaverton, OR) and either stored on videotape using a video cassette recorder (SONY, model SL-HF900) via a digital pulse code modulator (SONY, model PCM501ES), or on FM tape using an FM tape recorder (Racal, Model 4 store DS, Sarasota, FL), or acquired by an on-line computer (IBM-AT) utilizing pCLAMP (version 4.2; Axon Instruments, Burlingame, CA) and stored on floppy diskettes for off-line analysis. The reflex evoked in this manner in cords from rats 6- to 9-days old is a stable wave form and similar in latency and time course to that seen *in situ*. In addition, at the optimum temperature of 25 °C and with adequate oxygenation, little decrement (< 10%) in the reflex is seen over a 10 hour period once the suction electrodes have sealed to the roots.

B. *Inhibition of Reflex Transmission* Two adjacent dorsal roots and a ventral root between the L_3 and L_5 segments were attached to suction electrodes. Supramaximal stimulation of a dorsal root elicited a response in the corresponding ventral root after a latency of 3-5 msec. The control amplitude of the MSR was first determined from an average of 3-5 reflexes elicited at 0.1 Hz prior to instituting any conditioning protocol. At this frequency of stimulation, there was neither potentiation nor depression of the reflex. Conditioning stimuli of supramaximal strength were delivered to an adjacent dorsal root at intervals of 1, 3, 5, 7, 10, 15, 20, 30, 50, and 70 msec) before the test stimulus was applied to elicit the MSR in the ventral root.[33] The conditioning stimulus by itself did not evoke any potential in the ventral root. A test MSR without a conditioning stimulus was also obtained just before and again after a MSR associated with a conditioning stimulus to confirm the stability of the MSR throughout the experiment. The experimental protocol thus involved recording 3-5 control responses followed by 10 or more pairs of conditioning-test (C-T) responses, each of which was preceded and followed by a single unconditioned MSR.

C. *Measurement of AChE Activity* Spinal cords without their roots were isolated from 8- to 10-day old rat pups of either sex, weighed, and homogenized in ice-cold 0.05 M phosphate buffer at pH 7.4 using a microhomogenizer. Control AChE activity was measured by Ellman's method[4] at 25 ± 1 °C, using an ultraviolet-visible spectrophotometer (LKB-4050, Ultrospec II, Princeton, NJ). In a parallel set of experiments, the effect of the oximes (10 μM) alone, sarin alone, and sarin + oxime (after sarin pretreatment) on AChE activity was determined. In the experiments with sarin, the spinal cords were exposed to the OP for 2 hr, after which the cords were superfused with each oxime (10 μM) + sarin for an additional 30 min. The residual AChE activity was then determined as above.

D. *Solutions and Drugs* The normal physiological solution had the following composition (mM): NaCl, 124; KCl, 5; $MgSO_4$, 1.3; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 15; and glucose 11. The solution was bubbled with 95% O_2-5% CO_2 and had a pH of 7.3 irrespective of the Mg^{2+} concentration. Mg^{2+} as the sulfate salt was added at various concentrations, as necessary, without adjusting for the small change in ion content or osmolarity. In those experiments in which the polysynaptic reflex (PSR) was also recorded, the spinal cords were prepared and maintained in a physiological solution free of Mg^{2+} and then subjected to each experimental paradigm. A stock solution of 1 M diisopropylphosphorofluoridate (DFP) (Sigma Chemical Co., St. Louis, MO) was prepared in propylene glycol (Sigma). Thereafter, all subsequent dilutions were made in physiological solution. Sarin, soman, tabun (2 mg/ml in physiological saline), and VX (1 mg/ml in physiological saline) were obtained from the U.S. Army Medical Research and Development Command and kept frozen (-80 °C). Stock solutions of the OPs (10^{-4} M) were prepared in physiological saline at the time of the experiment. Atropine sulfate, benactyzine, pirenzepine, pralidoxime, trimedoxime, physostigmine, DL-APV and DL-AP7, thyrotropin-releasing hormone (Sigma), diazepam, pyridostigmine (Hoffmann-LaRoche), and diethyxime (gift of Dr. Das Gupta) were prepared as stock solutions (10^{-2} M) in distilled water, refrigerated, and diluted as required in physiological solution. For the enzymatic assay of ChE, acetylthiocholine iodide and 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma) were prepared in 0.05 M phosphate buffer at pH 7.4, refrigerated, and used within the week.

E. *Analysis* In early experiments, taped responses were amplified, digitized, and stored on removable hard disks and then analyzed using a MINC\DECLAB 23 computer (Digital Equipment Corp., Maynard, MA). In later experiments, acquisition and analysis of signals were performed using an IBM computer in which the amplified signals were digitized, averaged, stored, and analyzed using pCLAMP (Axon Instruments, Burlingame, CA). Reflex activity was quantitated by measuring the area under the reflex curve. Details of the computer-assisted analysis can be found elsewhere.[2] The recovery of the MSR area (A) from sarin-induced depression was calculated according to the formula: A_o-A_i/A_c-A_i x 100 where A_o = Area after treatment with an oxime, A_i = Area after inhibition, and A_c = Area of the control (*i.e.*, 100%). Data were statistically analyzed using analysis of variance (ANOVA) followed by an appropriate test such at Dunnett's test and, where appropriate, Student's t test, and are presented as the mean ± SEM. A p value of 0.05 or less was considered statistically significant.

IV. THE REVERSAL OF SARIN-INDUCED DEPRESSION BY OXIMES

A. *Introduction*

The efficacy of quaternary bispyridinium oximes in treating OP toxicity has been attributed to their ability to reactivate peripheral AChE.[5-8] These compounds were therefore suggested as adjuncts along with a cholinolytic drug in counteracting OP toxicity.[6,7] However, the ability of the quaternary oximes to reactivate brain AChE as a mechanism for antidotal action in OP intoxication is controversial. On the one hand, quaternary reactivators such as pralidoxime (pyridine-2-aldoxime methiodide) exhibit low lipophilicity and do not easily penetrate the blood-brain barrier, although one study has shown results to the contrary.[9] But even pro-PAM, the lipophilic precursor of pralidoxime, provided only marginally greater

survival against DFP than did pralidoxime and provided no protection against sarin[10] or paraoxon in mice[11], even though it regenerated brain AChE.[11,12] Unlike most oximes, diethyxime {S-[2-(diethylamino)ethyl]4-bromobenzothiohydroximate hydrochloride} is a tertiary reactivator of AChE and a reportedly universal antidote against OP intoxication at both central and peripheral sites.[13] The availability of a lipophilic oxime has therefore aroused some interest in one's ability to antagonize the central effects of OP compounds.

That the antidotal action of the oximes might be attributed to a direct antagonism of ACh at nicotinic receptors as well as reactivation of phosphorylated AChE was suggested by the structural similarity between the oximes and nicotine, and the nicotinic conformation of ACh suggests[14] and findings confirm that oximes possessed antagonist activities at both nicotinic and muscarinic receptors.[15-17] In fact, both pralidoxime and HI-6, another AChE reactivator, can modify the functional properties of the ion channel of the nicotinic ACh receptor.[18]

The present study was therefore performed to determine whether tertiary (diethyxime) and quaternary {pralidoxime, trimedoxime [1,1'-trimethylene bis(4-formyl-pyridinium bromide) dioxime]} AChE reactivators could reverse OP-induced depression of the MSR in spinal cords of neonatal rats *in vitro* and whether their effects could be attributed to the regeneration of AChE.

B. *Additional Materials and Methods*

Control reflex activity was recorded after a stabilization period of 1.5-2.0 hr. The preparations were then exposed for 30 min to each oxime at increasing concentrations and recordings were again obtained. In a separate set of experiments, preparations were first exposed to sarin for 2 hr, and the MSR was then recorded. This period allowed the preparation to reach a steady-state depression. Thereafter, the preparations were simultaneously exposed to different concentrations of each oxime together with sarin for an additional 30 min, after which the MSR was recorded.

C. *Results*

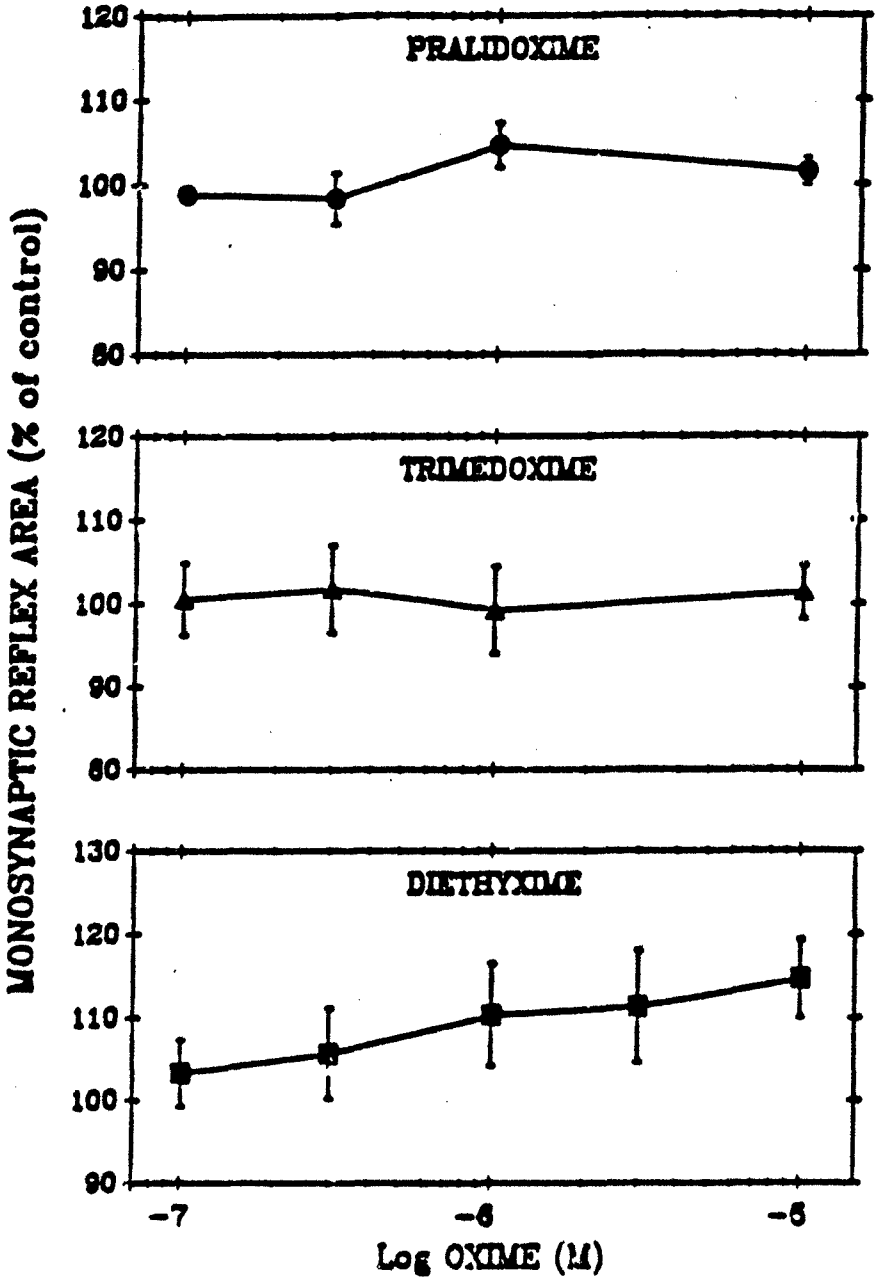
Neither oxime by itself had a significant effect ($p > 0.05$; ANOVA) on the MSR at concentrations from 0.1 to 10 μM (Figs. 1-3). Each of the oximes was, however, effective in reversing the depression caused by sarin (Figs 4-6). At 0.1 μM, sarin depressed the MSR to about 36% of control ($n = 10$) after exposure for 2 hr (Fig. 1). Subsequent exposure to sarin + varying concentrations of an oxime (0.1-10 μM) resulted in a dose-dependent reversal of OP-induced depression. At 10 μM pralidoxime + 0.1 μM sarin, the reflex had returned to the control value (97% recovery of the reflex) (compare Fig. 1 and Fig. 4). With trimedoxime (10 μM), the recovery was 111% (compare Fig. 2 and Fig. 5) and with diethyxime, 77% (compare Fig. 3 and Fig. 6).

Neither pralidoxime, trimedoxime, nor diethyxime (10 μM) had a significant ($p > 0.05$) effect on spinal cord AChE activity (109.8 $\pm$ 7.7, 107.3 $\pm$ 1.8, 114 $\pm$ 6.2% of control, respectively; $n = 3$ for each). When the spinal cord was exposed to sarin (0.1 μM) for 2 hr, the AChE activity was reduced ($p < 0.001$) to 52.6 $\pm$ 1.0% of control ($n = 9$). Spinal cord AChE activity in the presence of sarin (0.1 μM) + pralidoxime, trimedoxime, or diethyxime

FIG. 1

FIG. 2

FIG. 3

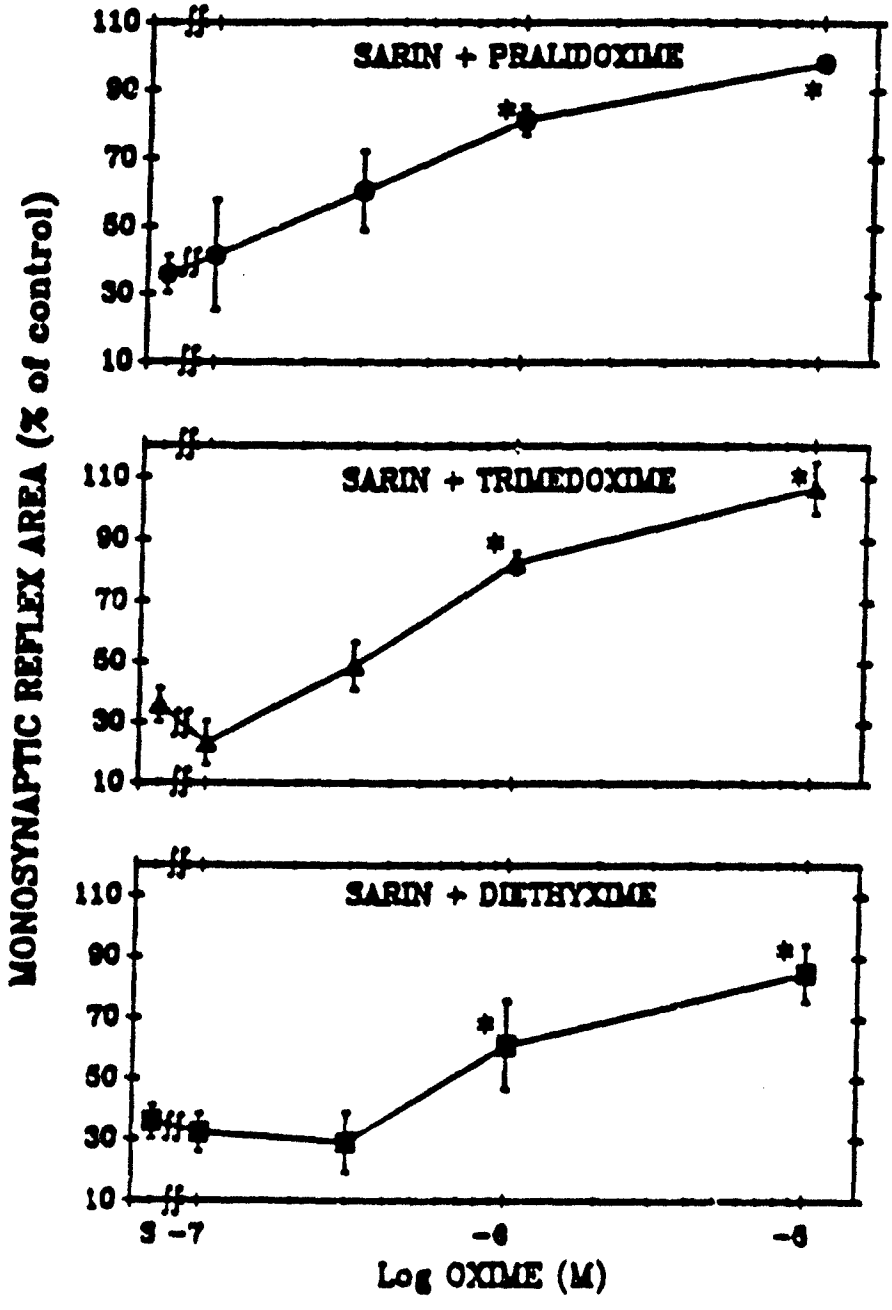


FIGs. 1-3. Effect of oximes on the monosynaptic reflex. In control experiments, each concentration of oxime superfused a cord for 30 min before recordings were made. The values shown are the mean ± SEM (n = 3-5 experiments at each concentration of oxime). There was no significant effect of the oximes on the reflex ($p > 0.05$; ANOVA).

FIG. 4

FIG. 5

FIG. 6



FIGs 4-6. Reversal of sarin-induced depression of the MSR by oximes in cords from neonatal rats. Recordings were made from cords before and after exposure to sarin (S; 0.1 μM) for 2 hr. Cords were then exposed to different concentrations of each oxime + sarin (0.1 μM) for an additional 30 min and recordings were again made. The values shown are the mean ± SE (n = 3-5 experiments at each concentration of oxime + sarin; n = 10 for sarin alone). An asterisk indicates a significant difference ($p < 0.05$; ANOVA) between the effect of sarin alone and in the presence of an oxime + sarin.

(10 μM) for 30 min was 55.5 ± 1.8%, 48.3 ± 1.5% and 45.2 ± 1.7% of control (n = 3 each), respectively, values which were not significantly different ($p > 0.05$) from sarin alone.

D. *Discussion and Conclusions*

In this study, the depression of spinal segmental transmission by sarin was reversed by tertiary and quaternary oximes without regeneration of spinal AChE. The order of potency of the three oximes in reversing sarin-induced inhibition in this study was: trimedoxime ≥ pralidoxime > diethyxime. A comparison of the efficacy of pralidoxime, trimedoxime, obidoxime, and diethyxime revealed that the latter agent was ineffective in acute intoxication by DFP[19], but effective against the peripheral effects of weaker inhibitors of AChE.[20] In the present experiments, however, diethyxime exhibited significant reversal of the segmental depression caused by sarin which approached that of the other oximes, while none of the oximes examined effectively regenerated AChE.

Although the therapeutic efficacy of oximes has been commonly related to their ability to dephosphorylate inactivated AChE,[6] the possibility exists that they may act on the ACh receptor. The structural similarities of oximes with ACh,[14] their nicotinic and muscarinic antagonist activities at central and peripheral sites[15,16,21,22], and their ability to block the nicotinic ion channel in frog skeletal muscle[18] all suggest that the oximes are cholinergic antagonists. Furthermore, pralidoxime can both facilitate and depress transmission in rat diaphragm and frog sartorius muscle, suggesting a dual action at the neuromuscular junction through its weak anti-ChE and channel-blocking action.[18,23]

Our previous studies demonstrated that depression of the MSR in neonatal rat spinal cord by sarin, soman, and DFP could be reversed by muscarinic, but not by nicotinic, antagonists.[24,25] Furthermore, the magnitude and time course of OP-induced alterations of synaptic transmission in both isolated autonomic ganglia and spinal cords persisted against a background of AChE inhibition.[24-26] That regeneration of AChE is therefore not a prerequisite to the reversal of OP-induced central depression was also apparent in the ability of thyrotropin-releasing hormone to reverse DFP-induced depression.[27]

It is evident from this study that reactivation of AChE is not a factor in reversing the sarin-induced depression of MSR in neonatal rat spinal cord. Instead, the reversal of OP-induced depression by oximes most likely results from an antimuscarinic action.

V. SARIN ON SPINAL INHIBITION

A. *Introduction*

We recently examined the action of several OP and carbamate inhibitors of AChE on the MSR in neonatal rats, *in vitro*. These studies revealed that sarin (isopropyl methylphosphonofluoridate) facilitated the MSR by 50% at low concentrations (2-20 nM), while it depressed the reflex at higher concentrations.[24,25,28] In similar studies with tabun (ethyl N-dimethylphosphoramidocyanidate), the degree of facilitation was much reduced in comparison with sarin[29], while

soman (pinacolyl methylphosphonofluoridate), DFP, physostigmine, and pyridostigmine did not cause potentiation.[27,28,30,31] In addition, both oxotremorine and carbamylcholine caused a facilitation and depression similar in magnitude to tabun, but at much higher concentrations.[29]

The depression of the MSR by these AChE inhibitors and muscarinic agonists could be either reversed or prevented by muscarinic antagonists but not by nicotinic antagonists. Furthermore, the depression of the reflex by the OP compounds persisted against a background of AChE inhibition.[25,32] While the depression by sarin could also be reversed by bis-pyridinium oximes, AChE was not regenerated simultaneously.[30] Of particular interest was our observation that the OPs and carbamates could also cause a paradoxical facilitation of the reflex at depressant concentrations in the presence of atropine, which by itself had no effect.[24,27,28,31] These observations led us to propose that the OP and carbamate inhibitors of AChE affect segmental transmission by activation of a muscarinic receptor most likely coupled to dopamine release,[30,34-36] that protective carbamylation of ChE is ineffective against OP-induced segmental depression, and that inhibition of ChE is unrelated to both carbamate- and OP-induced depression of the MSR.

Although the probable site and mechanism of OP- and carbamate-induced depression of segmental transmission has been examined, the mechanism of facilitation has hitherto eluded us. Obvious possibilities include a direct effect on the motoneuron (*e.g.*, depolarization), the release of a putative excitatory transmitter (*e.g.*, glutamate), and blockade of inhibition. Recently, we described the existence of inhibition in the spinal cord of neonatal rats, which consisted of an early and a late phase of inhibition, which could be blocked by strychnine and bicuculline, respectively. The early phase of inhibition may be of postsynaptic origin and mediated by glycine, while the late component of inhibition may be presynaptic in origin and mediated by gamma-aminobutyric acid (GABA).[33] We therefore examined the possibility that the facilitation induced by sarin could be related to an effect on inhibitory mechanisms in the spinal cord. The results indicate that sarin blocked the late, bicuculline-sensitive component of inhibition.

B. *Additional Materials and Methods*

Statistics and analysis. These reflexes were recorded on a video cassette recorder (SONY, Model SL-HF900) and were analyzed by using a MINC/DEC (Digital Equipment Company) computer.[2] The average amplitude of the MSRs obtained under the conditioning-test protocol at each interval were computed in relation to the mean unconditioned response to obtain the percent inhibition as recently described.[33] A combined analysis of variance (ANOVA) was performed on data relating the amplitude of the reflex to the conditioning-test interval (C-T interval). A p value less than 0.05 was taken as statistically significant.

C. *Results*

Test stimuli applied to a dorsal root evoked a MSR in the corresponding ventral root with a latency of about 4 msec and a rise time of less than 1 msec (Fig. 7). These values were in agreement with previously published results.[1,2,33] Maximal inhibition occurred at C-T intervals of 7 msec when the reflex was reduced to about 40% of control (Figs. 7 and 8). As the C-T interval was lengthened, the amplitude of the reflex increased such that at C-T intervals of 20-70 msec, the reflex had reached a plateau at about 70% of control. The early (7 msec) and late

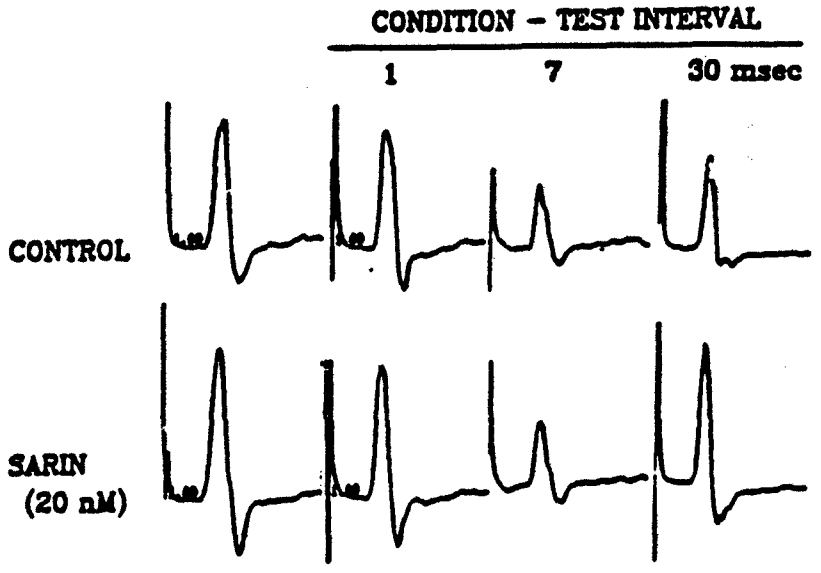


FIG. 7. Digitized records of single MSRs obtained under control conditions and at varying conditioning-test intervals before and after a facilitating concentration of sarin. The traces illustrate the early phase of inhibition occurring at 7 msec (strychnine-sensitive component) and the later phase of inhibition occurring at 30 msec. Note the increase in magnitude of the MSR in the presence of sarin and reduction of the later phase of inhibition. Calibration, 1 mV; 1 msec.

(20-70 msec) phases of inhibition correspond to the strychnine-sensitive, glycine-mediated, and bicuculline-sensitive, GABA-mediated inhibitions in the neonatal cord, respectively.[33]

When the cords were exposed to concentrations of sarin (3-20 nM) which are known to increase the amplitude of the reflex, the magnitude of the reflex increased, but the early phase of inhibition was unaffected (Figs. 7-9). The late phase of inhibition was, however, progressively reduced in magnitude such that at 20 nM sarin, the late phase of inhibition was completely absent (Fig. 8). As the concentration of sarin was increased above 20 nM (*i.e.*, to depressant concentrations), the amplitude of the MSR decreased concomitant with a loss and then enhancement of the bicuculline-sensitive phase of inhibition. There appeared to be no effect on the early, strychnine-sensitive phase of inhibition.

The concentration dependence of the block of inhibition is shown in Fig. 10 at a C-T interval of 50 msec. At 3-20 nM sarin, the bicuculline-sensitive inhibition was progressively blocked, but at higher concentrations inhibition was at first reduced and then enhanced.

D. *Discussion and Conclusions* The MSR obtained in the hemisected spinal cord preparation can be inhibited by stimulation applied to an adjacent dorsal root. The latter inhibition reaches a maximum when conditioning-test intervals were 3-5 msec, and inhibition was still apparent, yet significantly reduced, when these intervals were of the order of 25-30 msec. The inhibition is apparently chloride-dependent and can be blocked by strychnine, which leads to the suggestion that it is of postsynaptic origin.[1,33] We have examined this inhibitory pathway as a potential site for drugs which potentiate the MSR, and we have found pharmacological evidence for the existence of two types of inhibition in the spinal cord of neonatal rats, which have usually been associated with either pre- or postsynaptic inhibitory mechanisms.[33]

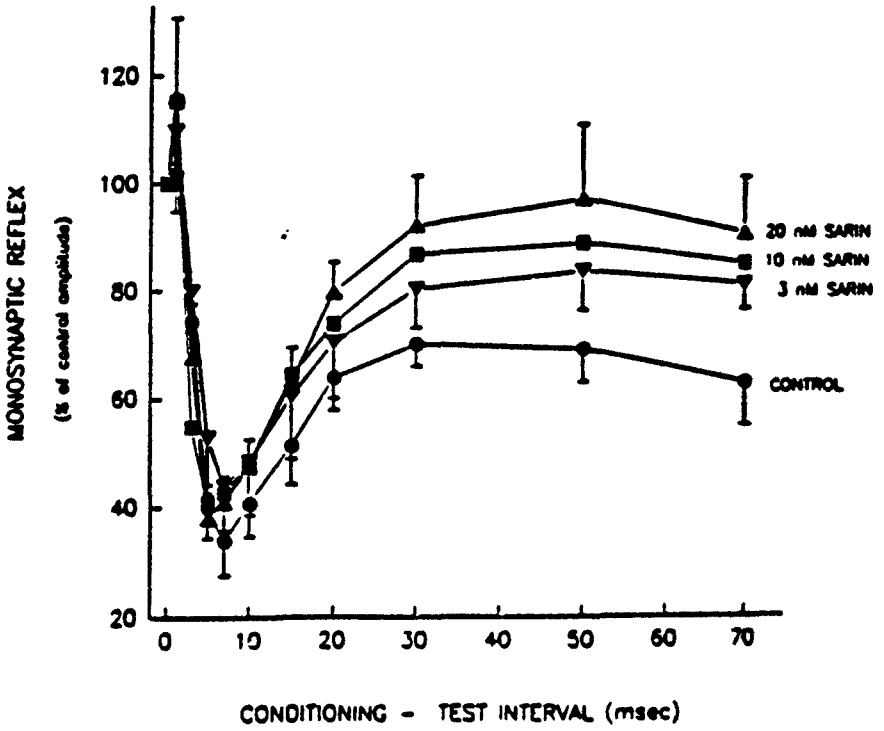


FIG. 8. Effect of sarin (3-20 nM) on the time course of inhibition of the MSR. The graph shows the mean response (± S.E.M.) of 4 control and 4 experiments with sarin at each concentration. The values in the presence of sarin at conditioning-test intervals of 20-70 msec were significantly different ($p < 0.05$, ANOVA) than control. The error bars at 10 nM were removed for clarity of presentation but did not exceed those at 3 nM sarin.

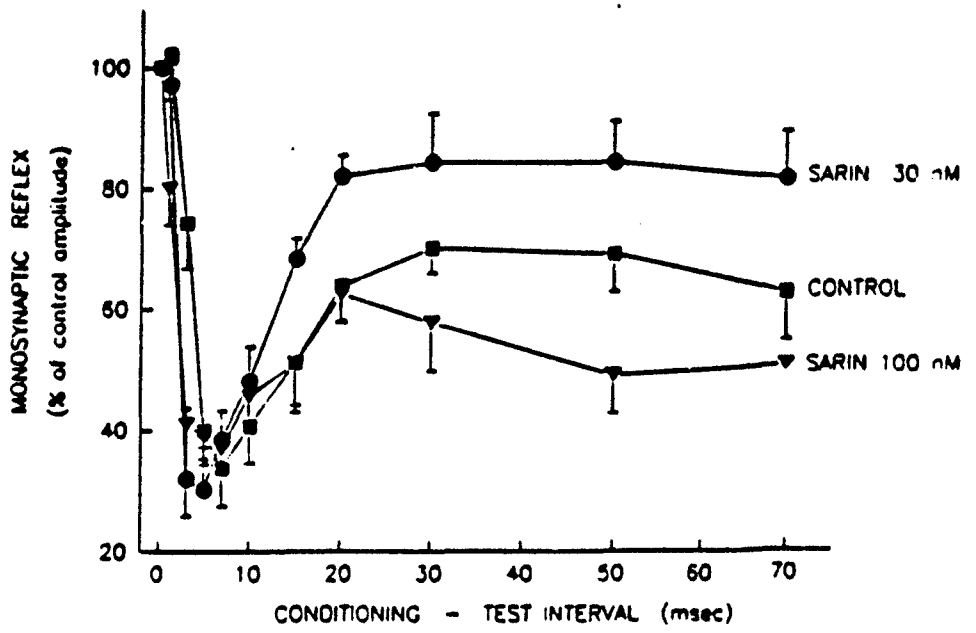


FIG. 9. Effect of sarin (30 and 100 nM) on the time course of inhibition of the MSR. The graph shows the mean response (± S.E.M.) of 4 control and 4 experiments with sarin at each concentration. The values in the presence of 30 nM sarin at conditioning-test intervals of 20-70 msec were significantly greater ($p < 0.05$, ANOVA) than control, while those at 100 nM sarin were significantly lower ($p < 0.05$, ANOVA) than control. The control curve is the same as in Figure 8.

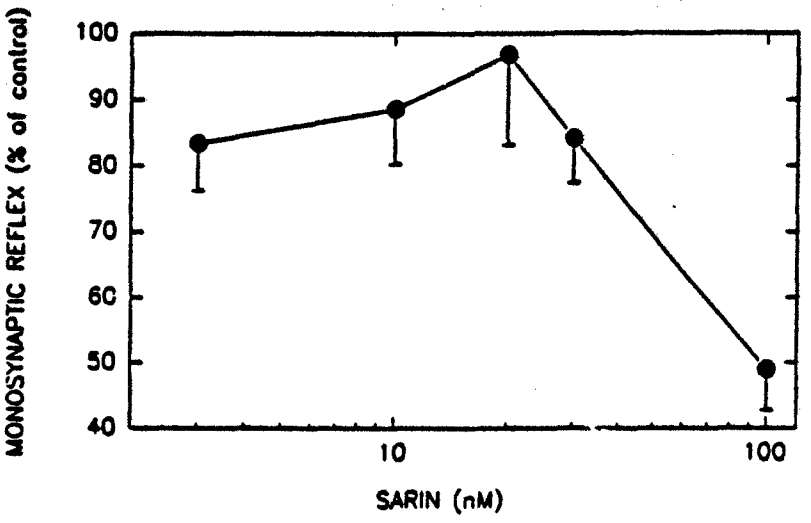


FIG. 10. Concentration-dependent block of bicuculline-sensitive inhibition by sarin. The degree of block decreased progressively from 3-20 nM sarin but was greater higher concentrations. The effect was determined at condition-test intervals of 50 msec. Each point is the mean of 3-5 observations.

In contrast to earlier reports on this process in neonatal cords, we have found that this inhibition appears to have two distinct phases: a fast-rising early phase and a more delayed, long-lasting late phase.[33] The early phase of inhibition, which is strychnine-sensitive, corresponds to the time course of postsynaptic inhibition in cats,[37] appears to reflect the time course of inhibitory postsynaptic potentials,[38] can be evoked synaptically by activation of glycinergic interneurons[39], and exhibits conductance changes which can be mimicked by microiontophoretically applied glycine. The inhibitory potentials are typically of short latency, having an onset of 1-2 msec, with peak effects at 5-6 msec, and lasting 20-25 msec.[38] In both cases, the inhibitory potentials and glycine-induced changes on α-motoneurons are blocked in a competitive manner by strychnine.[38,40] These results suggest that the neurotransmitter involved in the pathway for this early phase of inhibition in the neonatal cord is, most likely, glycine.

The later phase of inhibition in the neonatal cord exhibits similarities in time course to that of presynaptic inhibition[41,42] and it too is blocked by the GABA antagonist bicuculline. In comparison with postsynaptic inhibition, presynaptic inhibition has a longer latency (2-4 msec), its peak effect occurs at 20-30 msec, and lasts for 150-200 msec.[41] Both bicuculline and picrotoxin block this type of inhibition by interacting with GABA receptors[39,43], while semicarbazide reduces the magnitude of presynaptic inhibition by depleting the amount of presynaptic transmitter.[44] Thus, it would appear that this late inhibition in the neonatal cord is a presynaptic inhibition mediated by GABA-ergic neurons.

A decrease in extracellular Cl^- blocks both pre- and postsynaptic inhibitions in mammalian spinal cord[45,46] and postsynaptic inhibition in the neonatal cord.[1] The time course of both glycine- and GABA-mediated changes of Cl^- ion conductance determined in cultured mouse spinal neurons using voltage clamp is similar to the time course that we have observed here, *i.e.*, an early phase of glycine-mediated Cl^- ion conductance with a half-decay time of 60 msec and late phase of conductance mediated by GABA with a half-decay time of 330 msec.[47] Therefore, it is interesting to note that both pre- and postsynaptic inhibition in the neonatal rat spinal cord, apparently mediated by glycine and GABA, respectively, can be mediated via a similar -- yet temporally dispersed -- pathway.

The reduction of the late (bicuculline-sensitive) phase of inhibition by low concentrations of sarin (3-20 nM) which facilitated the MSR, but had no effect on the early (strychnine-sensitive) phase of inhibition, is indicative of a presynaptic mechanism. At concentrations of sarin ($\geq$ 30 nM) which depressed the MSR, the late phase of inhibition was either blocked to a lesser extent or enhanced. Although the depression of the MSR has been attributed to activation of a muscarinic receptor and is unrelated to inhibition of AChE, the cause of the facilitation has heretofore remained unknown. It appears that the facilitation of the MSR by sarin may be caused by a blockade of a bicuculline-sensitive inhibition which is most likely mediated by gamma-aminobutyric acid.

VI. CHARACTERIZATION OF SPINAL POLYSYNAPTIC ACTIVITY

A. *Introduction*

Relatively low concentrations (< 2 mM) of Mg^{2+} interrupt or mask the NMDA-mediated responses in central nervous system preparations *in vitro* (including the spinal cord).[48-51] Thus, the removal of Mg^{2+} from the physiological solution should allow the appearance of the PSR, which is apparently mediated via NMDA-modulated receptors and should thereby allow an examination of the action of OPs on polysynaptic *vs.* monosynaptic transmission, which is purportedly mediated via non-NMDA (quisqualate and kainate) receptors.[48,52,53] The actions of NMDA-specific antagonists were also studied to confirm the assertion that the transmission through mono- and polysynaptic pathways was indeed receptor-specific. Spinal cords in this part of the study were therefore usually maintained in a Mg^{2+}-free physiological solution to obtain the maximal PSR. Our goal was to delineate further the action of OPs on spinal reflex transmission in the absence of Mg^{2+} and to compare the pharmacological characteristics of the MSRs and PSRs.

B. *Results*

1. Effect of Mg^{2+} ions on the monosynaptic and polysynaptic reflexes. Stimulation of an L_{3-5} dorsal root in the absence of Mg^{2+} ions evoked two temporally distinct reflex potentials, with short and long latencies, in the corresponding ventral root which are apparently of monosynaptic and polysynaptic origin, respectively. The mean amplitude and latency of the MSR was 4.5 $\pm$ 0.3 mV and 5.6 $\pm$ 0.1 msec, and the amplitude and latency for the PSR was 1.5 $\pm$ 0.1 mV and 14.1 $\pm$ 0.5 msec (n = 36), respectively. The MSR was relatively resistant to an increase in the $[Mg^{2+}]_o$ while the PSR was markedly depressed when

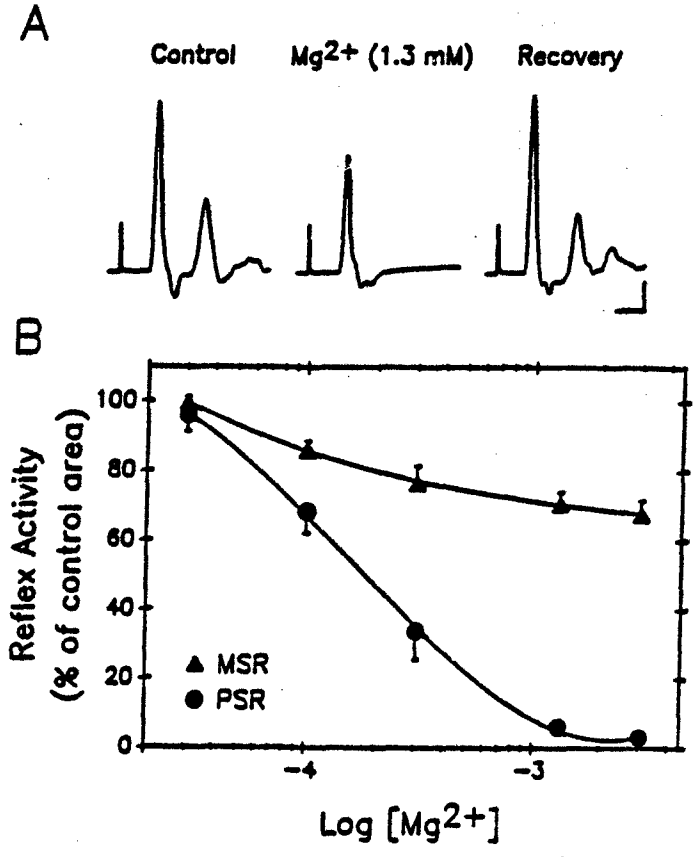


FIG. 11. Differential sensitivities of monosynaptic and polysynaptic reflexes to magnesium ions in spinal cords of neonatal rats *in vitro*. The spinal cords were isolated in and initially superfused with a Mg^{2+}-free physiological solution. The traces in (A) are signal-averaged records of 10 successive responses in a Mg^{2+}-free solution (Control), 20 min after raising the Mg^{2+} concentration to 1.3 mM and 30 min after rinsing in Mg^{2+}-free solution (Recovery). The calibrations are 1 mV and 5 msec. The concentration-response curves for the depression of the monosynaptic (MSR) and polysynaptic (PSR) reflexes by Mg^{2+} are shown in (B). Each point is the mean ± S.E.M. of 3 to 6 separate experiments in different cord preparations.

Mg^{2+} was added to the superfusate (Fig. 11A). The onset of the reflexes' depression after raising the $[Mg^{2+}]_o$ from zero to 1.3 mM occurred in 2-5 min, reached a plateau in 10-15 min, and usually returned to the control level 15-30 min after rinsing in a Mg^{2+}-free solution. The decrease in magnitude of the MSR by Mg^{2+} occurred initially in a concentration-dependent manner, but with a maximum reduction of about 30% at 1.3-3 mM (Fig. 11). On the other hand, the depression of the PSR by Mg^{2+} was concentration-dependent with 50% inhibition (IC_{50}) at 185 μM, and complete inhibition occurred at 1.3 mM Mg^{2+} (Fig. 11B).

2. Effects of NMDA antagonists on reflex activity. The MSR and PSR were depressed by the competitive NMDA receptor antagonists APV and AP7 in a concentration-dependent manner (range 0.3-10 μM) (Figs. 12 and 13). While the time course of depression of both reflexes by APV or AP7 was similar (onset, 3-8 min; peak effect, 10-20 min; recovery, within 30 min), these agents selectively depressed the PSR (IC_{50} = 2.6 μM for APV and 1.7 μM for AP7, respectively), while causing a small depression of the MSR (Figs. 12 and 13). For

example, during the exposure to 10 μM APV or AP7, the MSR only decreased by about 20%, while the PSR was completely blocked. No further decrease in the MSR was observed, even when the concentration of APV or AP7 was increased. At 30 μM APV, the MSR was 76.8 $\pm$ 2.8% and the PSR was 0.7 $\pm$ 0.7% of control (n = 3); at 30 μM AP7, the MSR was 76.0 $\pm$ 7.7% and the PSR was 2.0 $\pm$ 2.0% of control (n = 3).

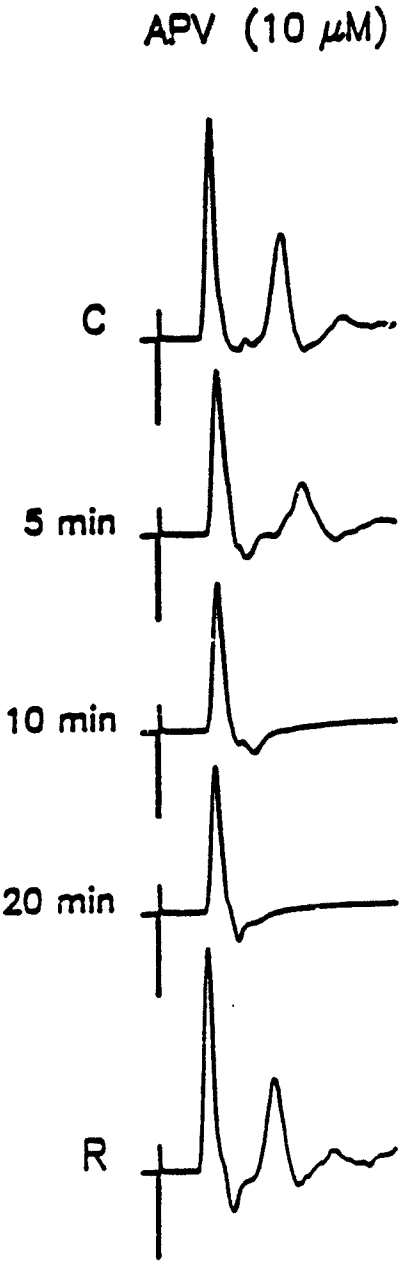


FIG. 12. Effects of the NMDA receptor antagonist APV on monosynaptic and polysynaptic reflexes in spinal cords of neonatal rats *in vitro*. The spinal cords were superfused with a Mg^{2+}-free physiological solution throughout the experiment. After the control (C) recordings were obtained, the spinal cords were exposed to APV (10 μM) for 20 min and then washed in drug-free physiological solution for 30 min, to allow recovery (R). The traces are signal-averaged records of 10 successive responses obtained in the same preparation. The calibrations are 1 mV and 5 msec.

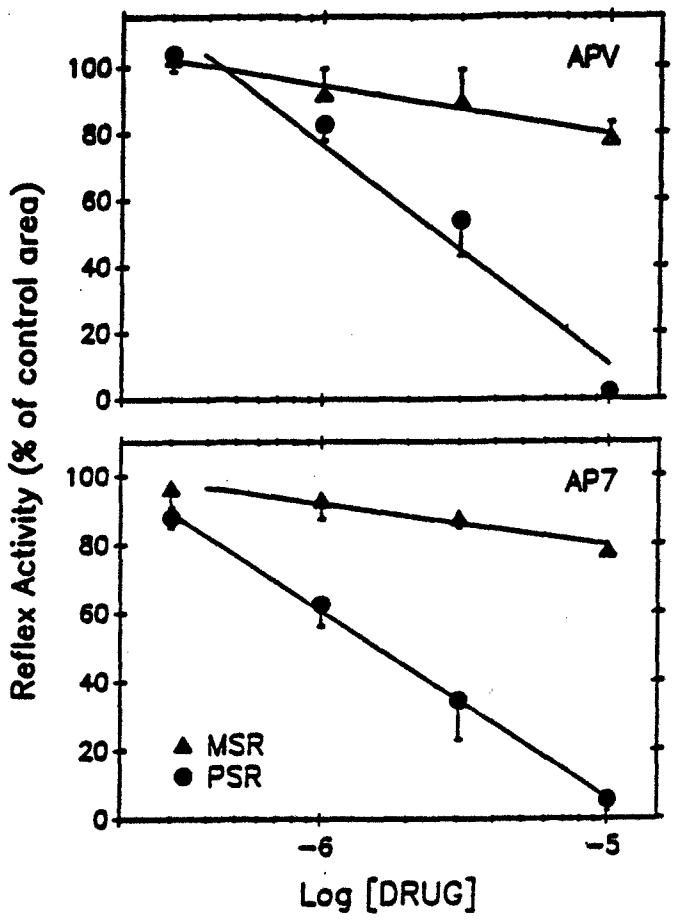


FIG. 13. Differential depression of the monosynaptic (MSR) and polysynaptic (PSR) reflexes by the NMDA receptor antagonists APV and AP7 in spinal cords of neonatal rats *in vitro*. The spinal cords were superfused with a Mg^{2+}-free physiological solution throughout the experiment. Each concentration of APV or AP7 was applied for 20 min. The points indicate the mean ± S.E.M. of 3 or 4 separate experiments in different cord preparations.

Experiments thus far have revealed that the PSR and a fraction (≈30%) of the MSR are comparably sensitive to the $[Mg^{2+}]_o$ and to NMDA antagonists. Therefore, we were interested in directly comparing the effect of Mg^{2+} removal and APV on the magnitude of the reflexes. Spinal cords were prepared and stabilized in a normal physiological solution which contained 1.3 mM Mg^{2+}. After recordings were obtained, the preparations were superfused in a Mg^{2+}-free physiological solution for 45 min and subsequently exposed to 10 μM APV in the absence of Mg^{2+} for 20 min. In the presence of a normal (1.3 mM) concentration of Mg^{2+}, only the MSR was clearly apparent -- the PSR was only of negligible magnitude (table 1; see also Ref[55]). During superfusion with the Mg^{2+}-free physiological solution, there was a small increase (18%) in the magnitude of the MSR concomitant with the appearance of the PSR (table 1). The augmentation of both reflexes disappeared when these cords were subsequently exposed to APV in the absence of $[Mg^{2+}]_o$ (table 1) or when the original level of Mg^{2+} (1.3 mM) was restored (data not shown, but see for example Fig. 11A).

TABLE 1

Augmentation of the monosynaptic and polysynaptic reflexes by removal of Mg^{2+} and its reversal by 2-amino-5-phosphonovalerate (APV) in isolated spinal cords from neonatal rats *in vitro*

The spinal cords were prepared and stabilized in a normal physiological solution containing 1.3 mM Mg^{2+} (control), then exposed to Mg^{2+}-free bathing medium for 45 min and subsequently to APV (10 μM) in the absence of Mg^{2+} for 20 min.

Treatment	Reflex Area (mV·msec)	
	Monosynaptic	Polysynaptic
Control (1.3 mM Mg^{2+})	4.9 ± 0.4	0.2 ± 0.1
Mg^{2+}-free	5.8 ± 0.5*	1.9 ± 0.3**
Mg^{2+}-free + APV (10 μM)	4.5 ± 0.2	0.2 ± 0.1

[a] Values presented are means ± S.E.M. of three separate experiments in different spinal cord preparations.

* $p < .05$, statistically significant as compared to control (paired Student's *t*-test).

** $p < .01$, statistically significant as compared to control (one-way ANOVA and Dunnett's test).

C. *Discussion and Conclusions*

In the present study, the MSR and PSR were found to be sensitive to alterations in the $[Mg^{2+}]$ of the physiological solution. The removal of Mg^{2+} from the superfusate resulted in an increase in the magnitude of the MSR, while allowing the generation of a PSR which is not normally seen in the presence of Mg^{2+}. In addition, the PSR was more sensitive than the MSR to depression by NMDA antagonists or by phencyclidine (PCP) and its analogues. In particular, the MSR was only decreased by less than 15% at concentrations of APV (≈2.5 μM), AP7 (≈1.8 μM), PCP (≈0.85 μM), and its analogues which reduced the PSR by 50%.

Low $[Mg^{2+}]_o$ (< 2 mM) selectively blocks the postsynaptic responses mediated by NMDA receptors, but not those by quisqualate or kainate receptors in the central nervous system.[48,52] In the isolated spinal cord, a MSR was consistently observed in the absence of a PSR when the physiological solution contained 1.3 mM Mg^{2+}.[53-55] In the present study, where the spinal cords were superfused in a Mg^{2+}-free solution, a relatively large PSR followed the MSR in all preparations tested. The magnitude of the MSR was significantly larger in the absence of Mg^{2+} than in its presence but was never decreased by more than 35% at the concentration range examined (0.3-3 mM). The PSR, on the other hand, was markedly sensitive to the $[Mg^{2+}]_o$ such that it was depressed in a concentration-dependent manner by Mg^{2+} and was also blocked by APV and AP7. The augmentation of the reflexes which followed withdrawal of Mg^{2+} from the superfusate was completely reversed by restoring Mg^{2+} or by application of APV. The range of concentrations at which Mg^{2+}, APV, or AP7 depressed the PSR in this study was comparable to that for the antagonism of NMDA-induced excitation of central neurons observed in previous studies.[48,51,56,57] Our results are therefore consistent with

previous studies on spinal reflexes[57-60] and indicate that the PSR is mediated by NMDA receptors. In addition, our results with Mg^{2+}, APV, and AP7 suggest that the fraction of the MSR (about 20-30%) which is sensitive to blockade by Mg^{2+} is mediated by NMDA receptors. The remaining fraction of the MSR is insensitive to Mg^{2+} and appears to be mediated by a non-NMDA-type receptor.[59-61] In fact, both APV and AP7 (up to 100 μM) failed to depress the MSR in the presence of Mg^{2+}.[55]

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