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Acetylcholinesterase Reactivators Antagonize Epileptiform Bursting Induced by Paraoxon in Guinea Pig Hippocampal Slices

W. ENDRES,[1] A. SPULER and G. ten BRUGGENCATE

Department of Physiology, University of Munich, Munich, Federal Republic of Germany

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ABSTRACT

The electrophysiological actions of paraoxon, an irreversible blocker of acetylcholinesterase, and their antagonism by a series of organophosphate cholinesterase reactivators, were studied in area CA1 of the guinea pig hippocampus in vitro. To avoid indirect effects elicited by excitation of CA3 neurons, the CA2/3 regions were removed routinely before the recording of extracellular field potentials in CA1. Under these conditions, paraoxon (1 μM) induced regular burst activity (rate, 2–10/min; amplitude, 0.2–1 mV; duration, 100–500 msec). The antagonism of this burst activity by atropine (0.3–1.0 μM) and pirenzepine (1.0 μM) suggested the involvement of muscarinic cholinoceptors in the mediation of this response. The reduction in frequency of paraoxon-induced bursting by the cholinesterase reactivators was taken as an index of their efficacies. The four oxime compounds tested were all active in the low micromolar range (rank order of potencies: obidoxime > HGG 12 = HLö 7 > HI 6). In experiments without paraoxon, these oximes did not depress either evoked population spikes in normal artificial cerebrospinal fluid or bursts induced by superfusion with Mg++-free artificial cerebrospinal fluid. Thus, an unspecific inhibitory effect of oximes can be excluded. It is concluded that the in vitro hippocampus provides a suitable system for the quantitative electrophysiological evaluation of cholinesterase reactivators in the central nervous system.

Organophosphorus compounds, which irreversibly inhibit the enzyme AChE, induce a severe poisoning which is characterized by convulsions and paralysis, respiratory failure and, finally, death. It is not known, however, whether symptoms related to the CNS, like convulsions, are due solely to the accumulation of ACh in the brain (Stewart, 1952; Matin and Kar, 1973), or whether peripheral factors contribute to the central effects (Holmstedt, 1959). Thus, it would be important to study direct CNS effects of organophosphates by using isolated brain tissue.

The hippocampus has been shown to develop epileptiform activity after application of various convulsants (Klee et al., 1982) and is known to play an important role in the generation and conduction of seizures, also with respect to cholinergic systems (Turski et al., 1983). The presence of a neuroanatomically and histochemically defined cholinergic input to this brain structure (Lewis et al., 1967; Houser et al., 1983; Cuello and Sofroniew, 1984), together with the well-characterized muscarinic receptor-mediated actions of cholinoceptor agonists in this tissue (Halliwell and Adams, 1982; Müller and Misgeld, 1986), makes the hippocampus an ideal system in which to evaluate the electrophysiological actions of agents known to interact with AChE.

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[1] Present address: Beecham-Wülfing GmbH, 3212 Gronau, Federal Republic of Germany.

In the present study, therefore, we have examined in the hippocampal slice preparation the electrophysiological effects of paraoxon, an organophosphate which irreversibly blocks AChE. In the majority of slices studied, paraoxon evoked epileptiform discharges in area CA1 which were reduced or abolished by the muscarinic receptor antagonists, atropine and pirenzepine. The four oxime AChE reactivators tested were each effective in reversing the paraoxon-induced bursting. We conclude that the guinea pig hippocampal slice preparation is suitable for the investigation of the electrophysiological actions of organophosphate compounds in an in vitro CNS system.

Methods

Female guinea pigs (180–250 g) were decapitated under ether anesthesia. Both hippocampi were dissected from the removed brain and transverse slices (0.5 mm) were cut using a Vibroslice (Campden Instruments, London, UK). The slices were stored in a carbogen-gassed (95% O2–5% CO2) chamber filled with ACSF of the following composition (millimolar): NaCl, 118; KCl, 3.0; NaHCO3, 25; NaH2PO4, 1.2; MgCl2, 1.0; CaCl2, 1.5; and glucose, 10. After at least 1 hr, slices were transferred to an experimental chamber in which they were submerged and superfused continuously with gassed ACSF (pH 7.4). The bath temperature was maintained between 29 and 31°C. The exchange time of the bath was about 2 min. In most experiments, paraoxon was added to the ACSF to give a final concentration of 1 μM. AChE reactivators and other drugs were added to the paraoxon-containing superfusate in

ABBREVIATIONS: AChE, acetylcholinesterase; CNS, central nervous system; ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; fB, bursting frequency.

appropriate amounts of concentrated stock solutions (drugs dissolved in distilled water). In some experiments without paraoxon, Mg++ was removed from the ACSF. Spontaneous burst discharges were observed 5 to 10 min after the onset of the perfusion with Mg++-free ACSF.

As the CA3 area of the hippocampus under certain conditions is prone to epileptiform activity (Schwartzkroin and Prince, 1978; Miles et al., 1984), which can be conducted to CA1 neurons, the CA2/3 regions were removed (Hablitz, 1984) in paraoxon-experiments before the placement of electrodes. The remaining Schaffer collateral input to CA1 pyramidal neurons was stimulated via thin tungsten electrodes (constant current pulses, 100 μsec duration). Evoked field potentials and "spontaneous" discharges (i.e., burst discharges evoked by paraoxon, see "Results") were recorded extracellularly in the CA1 cell body layer using glass microelectrodes (ACSF filled, 5–10 megohms) connected to an Axoclamp-2 amplifier (Axon Instruments, Burlingame, CA). The amplifier output was fed to a digital oscilloscope (Nicolet 4094) and then to an analog plotter. For monitoring burst discharges, the amplifier output was filtered (high pass, 0.3–1 Hz cutoff), displayed on a chart recorder (Gould RS3400: frequency response, 50 Hz; or Kontron: frequency response, 3 Hz) and in parallel stored on a digital video tape system (Neurocorder, Neurodata Instruments, New York) for further analysis.

Burst discharges reached a stable reduced frequency level about 10 to 15 min after application of HI 6, HGG 12 and HLö 7. Therefore, the potencies of the oxime reactivators were estimated according to the relation:

$$\left(1 - \frac{\text{mean fB in the 16th-20th min of application}}{\text{mean fB during the 5 min before application}}\right) \times 100\%,$$

where fB is the frequency of bursting induced by paraoxon. In the case of obidoxime, the frequency became stable after 5 to 10 min. Therefore, the 11th to 15th min of application was taken to evaluate the frequency reduction. In experiments with Mg++-free ACSF, the 25th to 30th min of application was taken. Sometimes the frequency after washout of an antidote was increased compared to control burst frequency. In these cases, the sum of the mean fB before application and the mean between the 20th to 25th min of washout was taken and divided by two. The resultant value was then used as the denominator in the above relation. Only if the effect of a reactivator was reversible (fB after washout period at least within 90% of control value) were data included in concentration response curves.

Drugs sources were as follows: paraoxon, Dr. S. Ehrenstorfer (Augsburg, FRG); atropine methylnitrate, Sigma Chemical Co. (Munich, FRG); pirenzepine dihydrochloride, Dr. Karl Thomae GmbH (Biberach, FRG); and obidoxime chloride, E. Merck (Darmstadt, FRG). HGG 12 dichloride [pyridinium-1-(3-benzoylpyridinio)methylmethoxy-2-(hydroximino)methyl-dichloride] was synthetized by E. Merck (Darmstadt, FRG); HI 6-dichloride monohydrate [1-(4-aminocarbonylpyridinio)methylmethoxy-2-hydroximinomethyl-pyridinium-dichloride] was synthetized by Dr. P. Lockwood and kindly made available by Dr. J. G. Clement, Ralston (Alberta, Canada); HLö 7-diiodide [1-(4-aminocarbonylpyridinio) methylmethoxy-2,4-bis (hydroximino-methyl-pyridinium diiodide] was synthesized by the group of Professor J. Hagedorn (Freiburg, FRG).

Results

In hippocampal slices superfused with ACSF, Schaffer collateral stimulation evoked typical field potentials (Langmoen and Andersen, 1981). When recorded in the cell body layer of CA1, they consisted of positively directed excitatory synaptic fields (arising in the dendrites), upon which negative-going compound cell discharges ("population spikes") were superimposed (not illustrated). Upon addition of paraoxon (10 μM), the amplitude of the population spike was increased; at higher concentrations (100 μM) a second population spike appeared (not illustrated). A central finding of the present study was that paraoxon, in low concentration (1 μM), evoked spontaneous electrical activity in about 70% of the slices (15–60 min after the start of drug application). In extracellular recordings, the amplitude of these burst discharges was 0.2 to 1 mV, their duration 100 to 500 msec and their frequency 1 to 10/min. Both shape and amplitude of the burst discharges typically depended upon the position of the recording electrode (cf. Schwartzkroin and Prince, 1978). Often an initially negative potential deflection was followed by a positive one (examples of individual events are shown in fig. 4). Occasionally, monophasic negative or positive potentials occurred. The discharge frequency, however, was independent of the electrode position. Both frequency and amplitude usually stabilized within 30 min of paraoxon application and then remained constant for several hours. Typical effects produced by paraoxon are shown in figure 1.

To establish whether these discharges were cholinergic in origin, we examined the effects of two muscarinic receptor antagonists, atropine and pirenzepine. In all slices tested, atropine (0.1–1 μM; figs. 1, A and B) and pirenzepine (1.0 μM; fig. 1C) reduced or abolished the paraoxon-induced burst dis-

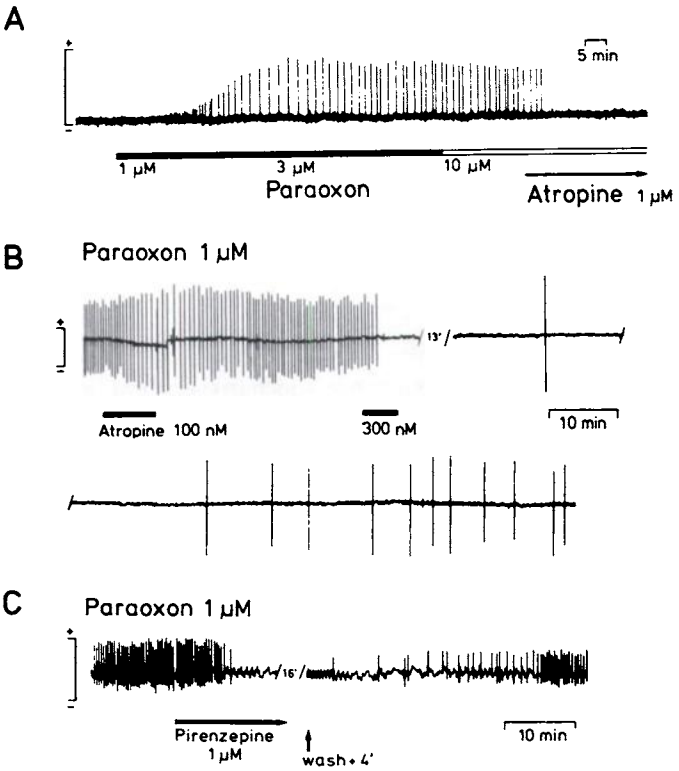

Fig. 1. Paraoxon-induced spontaneous burst discharges and actions of muscarinic antagonists. A, potentials recorded extracellularly in pyramidal cell layer of the hippocampal area CA1. Fifteen minutes after perfusion of ACSF containing 10 μM paraoxon, small spontaneous potentials developed that grew rapidly and reached an amplitude of 0.25 mV and a frequency of 3/min after 35 min. During 3 and 10 μM paraoxon a small increase in frequency and decrease in amplitude could be observed. Atropine (1 μM) blocked completely these events within 5 min. B, field discharges recorded as in A and evoked by paraoxon (1 μM). Atropine (100 nM) reversibly reduced the frequency of paraoxon-induced discharges by about 50%; 300 nM blocked completely the bursts after 3 min; a first burst occurred 30 min after the start of washout and the recovery remained incomplete within another 80 min. C, 1 μM pirenzepine blocked paraoxon-induced discharges after 8 min. Despite a relatively long application time (28 min), the burst discharges reappeared 8 min after washout of pirenzepine. After 30 min, the frequency equalled the control, although the amplitude of the burst discharges was still reduced. Voltage calibration is 0.5 mV in this and all subsequent figures.

charges, suggesting the involvement of muscarinic cholinoceptors in the mediation of this response.

We next investigated the abilities of a series of bispyridinium oxime compounds to reverse the bursting induced by paraoxon. All compounds reduced the frequency of the burst discharges without influencing their amplitude. As illustrated in figure 2, obidoxime had a clear and reversible dose-dependent action upon the paraoxon-induced discharges. A concentration-response curve derived from the results of 10 experiments is illustrated in figure 5 (IC50 between 200 and 300 nM).

In order to evaluate the model further, we tested three more oximes with established in vivo efficacy (HGG 12, HI 6 and HLö 7). The antidote HGG 12 also antagonized the bursts in a dose-dependent way (see fig. 5). HI 6 was found to be clearly less effective (fig. 3) as compared to HGG 12, whereas a newly developed oxime, HLö 7, appeared to be about equipotent to HGG 12. Figure 4 illustrates a representative experiment. Above the uppermost trace (fig. 4), specimen of paraoxon-induced burst discharges are shown on a fast time scale, illustrating that the amplitude of the individual discharges was not reduced. A summary of the effects of HGG 12 (IC50, 1–3 μM), HI 6 (IC50, 5–10 μM) and HLö 7 (IC50, 1–3 μM) in all slices studied is shown in figure 5.

Some control experiments were carried out to exclude an unspecific action of organophosphates. HGG 12 at 10 and 30 μM (five and six slices, respectively), HLö 7 at 10 and 30 μM (three and six slices, respectively) and obidoxime at 1 μM (two slices) did not reduce, but even slightly enhanced the amplitude of orthodromically evoked population spikes (up to 10%). Also, a small second population spike sometimes developed.

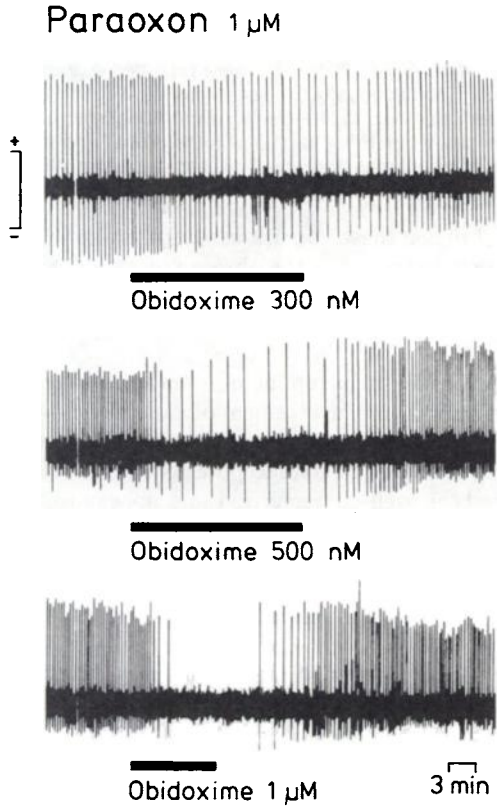

Fig. 2. Effects of obidoxime on paraoxon-induced discharges. Obidoxime blocked paraoxon-induced discharges dose-dependently. Application time was 20 min (300 or 500 nM) or 10 min (1 μM), respectively; 300 nM obidoxime (uppermost trace) and 500 nM obidoxime (middle trace) reduced the discharge frequency by about 50 and 80%, respectively; 1 μM obidoxime abolished completely the discharges. Notice reversibility of action at all doses tested.

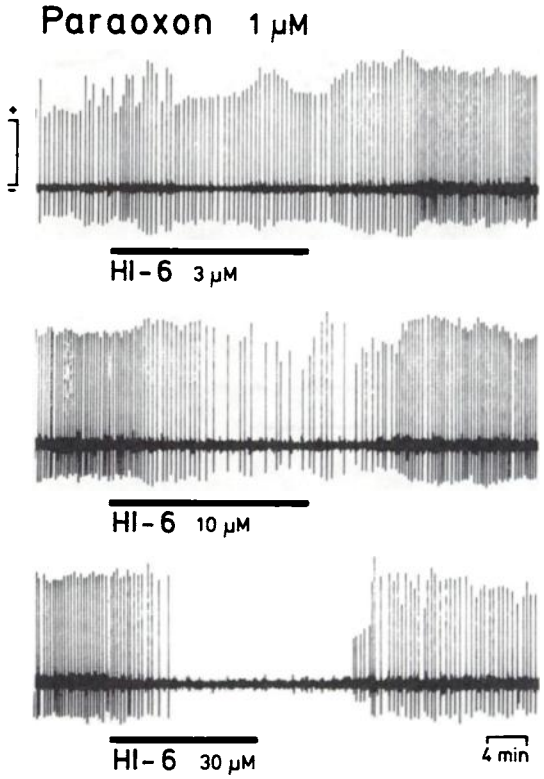

Fig. 3. Reduction of discharge frequency by HI 6. Same arrangement as in figure 2. HI 6 at 10 μM reduced the frequency by 70%, at 30 μM discharges were blocked totally.

In addition, the actions of organophosphates upon burst discharges evoked by omitting Mg++ from the ACSF were tested in 15 slices. Superfusion of ACSF without Mg ++ induced spontaneous bursts (1–15/min). Obidoxime (1 μM) or HI-6 (30 μM) added to Mg++-free ACSF for 30 min did not reduce, but increased the frequency of burst discharges marginally by 1.8 ± 5.2% (n = 5) and 6.2 ± 2.4% (n = 6), respectively (mean ± S.E.M.) (fig. 6). Under the same conditions, HGG 12 (10 μM) decreased the burst-frequency by 6.4 ± 2.1% (n = 8) (fig. 6).

Discussion

The spontaneous discharges induced by paraoxon in area CA1 of the hippocampus are comparable in form and duration to the extracellularly recorded field bursts induced by other epileptogenic agents. Such bursts have been described in the CA1 and CA3 area during superfusion with Mg++-free ACSF (Mody et al., 1987) and in the CA3 region under conditions of elevated potassium (Rutecki et al.,1985; Korn et al., 1987), or in the presence of a variety of convulsants (for example, penicillin; Schwartzkroin and Prince, 1977). In these earlier investigations it was also shown that the extracellularly observed field discharges occurred simultaneously with paraoxysmal depolarizing shifts recorded intracellularly. The epileptiform bursts observed in the present experiments with paraoxon were smaller in amplitude than synchronized discharges of other origin, which may indicate either that a smaller number of neurons were activated, or that the activation was less syn-

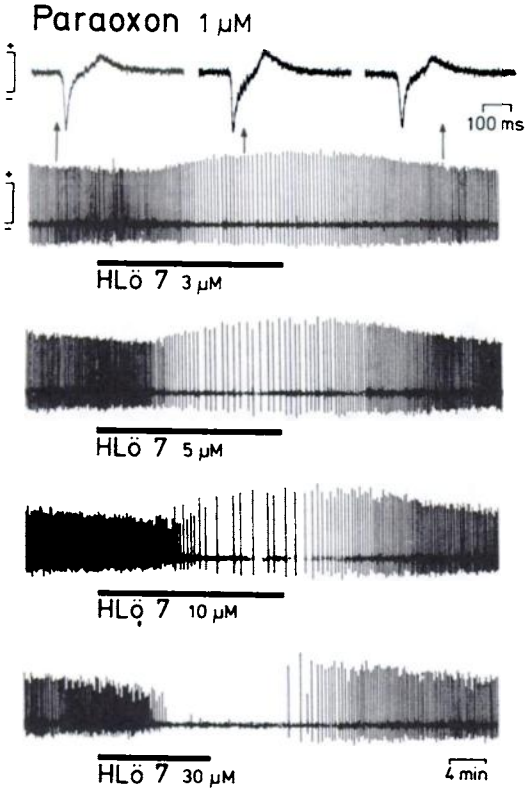

Fig. 4. Effects of HLö 7 upon paraoxon-induced discharges. HLö 7 blocked paraoxon-induced discharges in a dose-dependent manner. Application times were 20 (3, 5 and 10 μM) and 10 min (30 μM), respectively; 3, 5 and 10 μM HLö 7 reduced the discharge frequency by about 60, 75 and 85%, respectively; 30 μM HLö 7 abolished completely the bursts. The effects of HLö 7 were reversible at all concentrations used. Individual discharges before, during and after application of 3 μM HLö 7 are shown on top trace. Both 3 and 5 μM HLö 7 (latter concentration not illustrated) had only small effects upon amplitude and duration of the discharges.

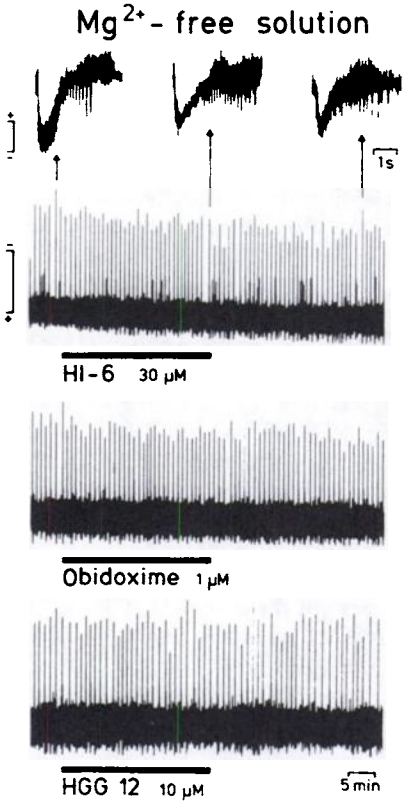

Fig. 6. Effects of HI 6, obidoxime and HGG 12 on spontaneous burst discharged induced by superfusion with Mg++-free ACSF. In contrast to paraoxon experiments, the area CA3 was not removed in experiments with Mg++-free ACSF. Potentials were recorded extracellularly in the CA1 region. Spontaneous burst discharges developed 10 min after the onset of perfusion with Mg++-free solution. Application of HI 6 (30 μM), obidoxime (1 μM) or HGG 12 (10 μM) for 30 min did not reduce the frequency of discharges. Individual discharges before, during and after application of 30 μM HI 6 are shown on top trace.

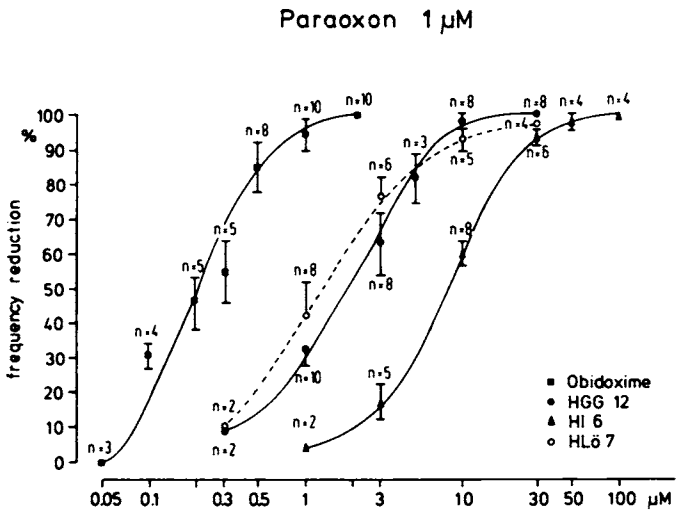

Fig. 5. Concentration-response curves derived from the frequency reduction of paraoxon-induced burst discharges. Curves show mean ± S.E.M. of the frequency reduction of the paraoxon-induced discharges for obidoxime, HGG 12, HI 6 and HLö 7. ED50 ranges are 200 to 300 nM for obidoxime, 1 to 3 μM for HGG 12 and HLö 7, 5 to 10 μM for Hi 6. For sample number less than 4, only the mean is given.

chronized under these conditions. Both interpretations, however, are consistent with the absence of area CA3 from these slices, in as much as this region seems to be more prone to synchronized epileptiform bursting than CA1 (Prince and Connors, 1986).

The blockade by atropine of paraoxon-induced bursting strongly supports the involvement of muscarinic cholinoceptors in the generation of these bursts. The excitatory actions of ACh and other muscarinic cholinoceptor agonists in the hippocampus have been well-characterized and appear to be mediated via blockade of a number of different potassium conductances (Halliwell and Adams, 1982; Müller and Misgeld, 1986; North, 1986). Furthermore, it has been demonstrated previously that epileptiform bursts can be induced by direct or indirect elevation of ACh. Thus, local application of ACh to CA1 neurons can transform a single orthodromically evoked population spike into a burst response (Benardo and Prince, 1982). These authors also observed persistent burst activity as well as epileptiform field potentials after prolonged exposure to ACh. Moreover, indirect elevation of ACh by application of the competitive AChE inhibitor, physostigmine, has been shown to induce epileptiform bursting in 20% of hippocampal slices (Cole and Nicoll, 1984). The induction by paraoxon in the present experiments of spontaneous bursting, together with the earlier findings with physostigmine, suggest that ACh is released under basal conditions. In untreated slices AChE presumably pre-

vents this cholinergic excitatory influence. The blockade of paraoxon-induced bursting by the selective M1-muscarinic receptor antagonist, pirenzepine (Hammer et al., 1980), may suggest the involvement of this receptor subtype in the response, although a complete analysis would be necessary to draw a firmer conclusion.

The cholinergic input subserving the above described effects of physostigmine and paraoxon probably arises from the septum in the intact animal (Lewis et al., 1967; Houser et al., 1983; Cuello and Sofroniew, 1984). Stimulation of the medial septum in vivo facilitates the CA1 population spike evoked by commissural fiber activation (Krnjevic and Ropert, 1982). Furthermore, this facilitation is potentiated by physostigmine and blocked by atropine.

All oximes tested showed neither a depressing effect on orthodromically evoked population spikes nor an inhibition of spontaneous bursts induced by removal of Mg++ from the ACSF. Thus, these results exclude a facilitation of inhibitory membrane conductances or a general, unspecific, block of burst discharges.

In contrast, all oxime tested showed consistent and concentration-dependent antagonistic actions on paraoxon-induced burst activity. The rank order of potencies of these compounds in reversing the effects of paraoxon was: obidoxime > HGG 12 = HLö 7 > HI 6. This rank order roughly corresponds to both the biochemical and the in vivo efficacy of the reactivators, although there are only few comparative data in the literature. For instance, obidoxime reactivates paraoxon-inhibited AChE quickly and has a reliable antidotal effect in paraoxon intoxication (Erdmann and von Clarmann, 1963). The concentrations used in our study were similar to those obtained in the cerebrospinal fluid of rats in which the toxic effects of paraoxon have been antagonized (Erdmann, 1965). Accordingly, the oxime HGG 12, which was less potent than obidoxime in reducing paraoxon-induced bursting, was also a less potent reactivator of AChE compared to obidoxime in paraoxon intoxication (Hauser et al., 1981). With respect to HI 6, in vivo studies (Clement, 1982; Ligtenstein and Kossen, 1983; Klimmek and Eyer, 1986) suggest that reactivation of AChE is achieved at cerebrospinal fluid concentrations of 10 to 20 μM. This concentration range is of the same order of magnitude as the IC50 of 5 to 10 μM obtained in our in vitro study.

In addition to the AChE-reactivating properties of the oximes, these compounds also possess affinity for the muscarinic cholinoceptor (Kuhnen-Clausen et al., 1983). It is conceivable, therefore, that some of the paraoxon blocking efficacy of the oximes is mediated by antagonism of the actions of endogenously released ACh at muscarinic receptors. Such a possibility, however, seems unlikely, in the case of obidoxime and HI 6 as the affinity constants of those oximes for the muscarinic receptor (Amitai et al., 1980) are 1 to 2 orders of magnitude higher than their IC50 values for reversing paraoxon-induced bursting. On the other hand, as a result of functional studies on guinea pig ileum, an apparent Ki of 1.6 μM has been derived for HGG 12 (Kuhnen-Clausen et al., 1983). In addition, CNS binding studies on muscarinic receptors yielded an apparent Ki of 3.3 μM (Kloog et al., 1985).

In conclusion, paraoxon-induced burst discharges in the guinea pig hippocampus in vitro appear suitable for evaluating cholinergic effects of organophosphates. First, they are stable for several hours and are amenable to blockade by cholinergic antagonists. Second, the discharge frequency can easily be recorded and can serve as a measure of antidote efficacy. Finally, the slice preparation allows a more quantitative pharmacological approach than that obtainable in vivo.

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Send reprint requests to: Dr. G. ten Bruggencate, Physiologisches Institut, Universität München, Pettenkoferstr. 12, 8000 München 2, Federal Republic of Germany.