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Pre-junctional effects of oximes on [^{3}H]-acetylcholine release in rat hippocampal slices during soman intoxication

Ole Kristian Øydvin, Rita Tansø, Pål Aas*

Norwegian Defence Research Establishment, Protection Division, Postbox 25, NO-2027 Kjeller, Norway

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Abstract

In this study, the non-reactivating effects of oximes in the hippocampus of the rat are investigated. The potassium (51 mM) evoked release of [^{3}H]-acetylcholine and the liberation of [^{3}H]-choline were determined in hippocampal slices following in vitro exposure to soman and five oximes (toxogonin, HI-6, HLö-7, P2S and 2-PAM) in separate experiments by superfusion. In the absence of soman, toxogonin and HLö-7 in particular induced a concentration dependent significant increase in the evoked release of [^{3}H]-acetylcholine. There was also a significant effect of HI-6, but the effect was much smaller. Two pralidoxime salts, P2S (methanesulfonate salt) and 2-PAM (methiodide salt), had similar but lower effects that were only observed at relatively high concentrations. Experiments performed following complete inhibition of the acetylcholinesterase activity by soman (1.0 μM) showed that HI-6 and HLö-7 induced a significant decrease in the potassium-evoked release of [^{3}H]-acetylcholine, while the liberation of [^{3}H]-choline increased. Toxogonin, P2S and 2-PAM did not reduce significantly the evoked release of [^{3}H]-acetylcholine. Only limited reactivation of the acetylcholinesterase activity was observed in superfusion experiments with toxogonin, HI-6, P2S and 2-PAM following exposure of hippocampal slices to soman. However, HLö-7 was proved to be relatively more effective in reactivating the acetylcholinesterase activity at high concentrations (50 and 200 μM). The acetylcholinesterase activity was reactivated to approximately 12% and 40% of control, respectively. It is concluded that HI-6 and HLö-7 have important non-acetylcholinesterase reactivating properties following soman poisoning, as may be seen by the significant reduction in the evoked release of [^{3}H]-acetylcholine effected by these oximes. HLö-7 is of particular interest in view of its ability to additionally improve reactivation of the acetylcholinesterase activity.

Keywords: Acetylcholine; HI-6; HLö-7; Oxime; Pralidoxime; Toxogonin; Soman

1. Introduction

Acute intoxications by organophosphorus insecticides or nerve agents such as tabun, sarin, *cyclo*-sarin, soman and VX are medically treated with the cholinergic muscarinc antagonist atropine. One of the oximes HI-6, 2-PAM, P2S (methanesulphonate salt of 2-PAM (methiodide salt)) or toxogonin (obidoxime) and benzodiazepines like diazepam should also be used (Dawson, 1994; Lallement et al., 1998; Aas, 2003). Nucleophilic compounds, such as oximes, has a primary function as reactivators of phosphorylated cholinesterase enzymes if they are administered before the irreversible phenomenon named "aging" (dealkylation) occurs. Medical treatment using atropine and oxime is designed to attenuate the effect of enhanced cholinergic activity caused by excessive concentration of acetylcholine in the cholinergic synapses.

Several recently developed bispyridinium aldoximes such as HI-6 and HLö-7 have been shown to have good antidotal properties and to be more effective than the monopyridinium oximes 2-PAM, P2S and the bispyridinium compound toxogonin against intoxication by some organophosphorus compounds, particularly following poisoning by soman (Clement, 1981; Bošković et al., 1984; Lundy et al., 1992; Eyer et al., 1992; Van Helden et al., 1992; Walday et al., 1993; for review see Dawson, 1994;

* Corresponding author. Tel.: +47 63807843; fax: +47 63807509.
E-mail address: pal.aas@ffi.no (P. Aas).

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Worek et al., 1998). Furthermore, HI-6 was shown to be the most efficient reactivator of AChE against a new class of organophosphorus compounds, described in general as 2-dialkylaminoalkyl-(dialkylamido)-phosphonofluoridates (GV), which are extremely toxic compared to several other known chemical warfare agents (Kassa, 1995). The most recently developed oxime is HLö-7 (Löffler, 1986), and it structurally resembles HI-6. HLö-7 has been shown to be effective against soman, tabun and *cyclo*-sarin in guinea pigs. On the other hand, the oxime HI-6 has shown to be more effective than HLö-7 against soman in the same animal species (Lundy et al., 1992). Overall, HI-6 and HLö-7 are regarded as the first broad spectrum oxime reactivators in the medical treatment of nerve agent poisoning (Clement et al., 1992).

In experiments involving adult rhesus macaques (Hamilton and Lundy, 1989) or marmoset monkeys (Van Helden et al., 1992), only limited or no reactivation of soman-inhibited cholinesterase activity occurred when HI-6 was administered in conjunction with atropine and diazepam within 15 s of intoxication. Therefore, it has been suggested that some other mechanism of action of HI-6 other than cholinesterase reactivation is partly responsible for this enhanced protective activity of HI-6 (Hamilton and Lundy, 1989; Van Helden et al., 1992). A number of earlier studies have suggested that several oximes may have other effects in addition to cholinesterase reactivation (Oldiges and Schoene, 1970; Schoene et al., 1976; Smith and Muir, 1977; Clement, 1981; Van Helden et al., 1983). More recent studies have shown that the oxime HI-6 might have direct pharmacological effects in the cholinergic nervous system in skeletal muscles. It has been observed that HI-6 reduces the miniature endplate potentials and increases the quantal content by a dose-dependent decrease in the miniature endplate potential amplitude (Melchers et al., 1991). Furthermore, HI-6 may have effects on the γ-aminobutyric acid (GABA-ergic) neurotransmission in the central nervous system (CNS) (Melchers et al., 1994). Other possible explanations have been suggested for oximes at other targets in the nervous system, such as inhibition of synthesis of neurotransmitters (Clement, 1979), alteration of the release of neurotransmitters (Clement, 1979; Kloog et al., 1986), interaction at post-synaptic receptors (see review by Van Helden et al., 1996), and by interaction with presynaptic cholinergic nerve terminals (Aas, 1996). In addition to HI-6, several other oximes such as HLö-7, 1-(3-phenylcarbonylpyridinio)methoxymethyl-2-hydroxyiminomethyl-pyridinium dichloridemonohydrate (HGG-12), 1-(3-cyclohexyl-carbonylpyridinio)-methoxymethyl-2-hydroxyiminomethyl-pyridinium dichloride (HGG-42) and toxogonin have been shown to have molecular effects, which are not yet defined and are probably ascribable to other effects than reactivation of cholinesterases (Van Helden et al., 1994).

HI-6 has been used beneficially in the medical treatment of humans following organophosphate poisoning (Kušić et al., 1991). In these studies the general improvement of patients was often more rapid than the reactivation of blood acetylcholinesterase, pointing to direct pharmacological effects of HI-6. The potential of inherent non-reactivating effects of oximes has prompted the study of several oximes already in use, as well as the potential new oxime, HLö-7, against organophosphorus intoxication. The aim of the study is to investigate whether toxogonin, HI-6, 2-PAM, P2S and HLö-7 influence the evoked release of [^{3}H]-acetylcholine and spontaneous efflux of [^{3}H]-choline from cholinergic nerves in brain hippocampal slices, both in the presence and absence of the cholinesterase inhibitor soman. This is in contrast to the work of a previous study where the total release of [^{3}H] from cholinergic nerves in the lung was used to quantify the release of acetylcholine (Aas, 1996).

2. Materials and methods

2.1. Animals

The experiments were carried out using male Wistar rats (200–250 g; from Møllegaard, Copenhagen, Denmark). The rats were given a standard laboratory diet and water ad libitum. The animals were kept in standard laboratory cages, six in each, for approximately 2 weeks before the start of the experiments. The climatised vivarium (21°C, 60% relative humidity) was illuminated from 07.00 to 19.00 h. The sawdust bedding was replaced daily. The experiments were carried out according to the guidelines of the National Animal Research Authority.

2.2. Chemicals

The HLö-7 (1-[[[4-(aminocarbonyl)pyridinio]methoxy]-methyl]-2,4-bis[(hydroxyimino)-methyl] pyridinium dimethanesulfonate) used in the experiments was a gift from professor P. Eyer, Ludwig-Maximillians Universität, Germany and HI-6 ([[[(4-aminocarbonyl)pyridinio]-methoxy]-methyl]-2[(hydroxyimino) methyl]pyridinium-dichloride was a gift from Dr. C. Boulet, DRES, Canada. P2S (pralidoxime) (pyridine-2-aldoxime methanesulfonate), 2-PAM (pralidoxime) (pyridine-2-aldoxime methiodide), ethopropazine, choline kinase from bakers yeast and hemicholinium-3 were from Sigma Chemical Company, MA, USA. Toxogonin (obidoxime) (1,1[oxybis(methylene)]bis[4-[(hydroxyimino)methyl]-pyridinium]-dichloride and sodium tetraphenyl borate were from Merck, Darmstadt, Germany. Vinylacetonitrile (allyl cyanide) was provided by Aldrich, Norway. [^{3}H]-choline chloride came from New England Nuclear, Boston, USA. [^{14}C]acetylcholine chloride came from Amersham, Norway. The opti-fluor was from Packard Instruments, The Netherlands. Soman (*O*-[1,2,2-trimethylpropyl]-methyl-phosphono-fluoridate was synthesised at the Norwegian Defence Research Establishment. All chemicals used in the study were of laboratory analytical reagent grade.

2.3. Determination of [^{3}H]-acetylcholine release

Following decapitation, the two hippocampi obtained from the rat brain, were immediately put on ice in 0.32 M sucrose, sliced

transversely into 400 μm thick slices and stored in buffer (solution A). Before being used in the experiments, the slices were incubated for 60 min at 25 °C with 1.1 μM [^{3}H]-choline ([^{3}H]-choline) (370 GBq/mmol) in a shaking water-bath and then washed three times with 2 ml of buffer A. The tissue slices were placed in separate superfusion chambers (one slice in each chamber). The amount of [^{3}H]-acetylcholine or [^{3}H]-choline released was determined after stimulation of the slices with K^+ (51 mM). This was done for 5 min both in presence and absence of oximes using a superfusion system consisting of a peristaltic pump with a flow rate of 200 μl/min. During the experiments with [^{3}H]-choline, the superfusion media contained hemicholinium-3 (10 μM) to inhibit the high affinity uptake of choline (Yamamura and Snyder, 1973). The superfusion buffer (buffer A) was composed of the following (in mM): NaCl 140.0, KCl 5.1, $CaCl_2$ 2.0, $MgSO_4$ 1.0, Na_2HPO_4 1.2, Tris–HCl 15.0, glucose 5.0. The depolarisation buffer (buffer B) was largely the same as buffer A, except that it contained 51 mM KCl, and the concentration of NaCl was reduced accordingly to keep the ionic strength constant. The media were kept in a thermostatically controlled water bath of 25 °C during the experiments. It was observed that KCl (51 mM) induced approximately 50% of the maximal obtainable release of [^{3}H]-acetylcholine. A total of two (S1 and S3) or three (S1, S2 and S3) K^+ stimulations (51 mM) lasting 5 min each were made, with each separated by a 35 min superfusion with buffer A. The oximes were present 5 min prior to and during the second stimulation (S2), which also lasted 5 min. In some experiments soman (1.0 μM) was present for 5 min in the superfusion buffer before oximes were added. Each experiment with oximes was accompanied by its own control experiment.

The [^{3}H]-acetylcholine that was formed was separated from [^{3}H]-choline (and other [^{3}H]-choline derivatives) according to Goldberg and McCaman's (1973) method, as used by Alberts et al. (1982). To covert choline into phosphorylcholine the samples were lyophilized immediately after the 5 min collection period. Following lyophilization, 0.25 ml dd H_2O was added and the samples were incubated for 30 min at 37 °C with 10 mM Na_2 ATP, 0.06 U/ml choline kinase (Alberts and Ögren, 1988) with the 80 mM glycylglycine dissolved in sodium hydroxide (the final volume was 0.31 ml, pH=8.5). The pH was kept slightly below the optimum of the enzymatic reaction (pH 9.0–10.5, Haubrich, 1973) in order to avoid alkaline hydrolysis of the acetylcholine. The reaction was halted by placing the tubes on ice and adding 1.2 ml of ice-cold dd H_2O. After adding 1.5 ml of ice-cold sodium tetraphelylboron (15 mM) in vinylacetonitrile (Fonnum, 1969), the contents of the tubes were then carefully mixed. The difficulties of isolating small amounts of acetylcholine from ringer solutions could be overcome by using vinylacetonitrile as an extraction solvent (Fonnum, 1969). According to Fonnum (1969), extraction is not critically dependent on pH in this range. The aqueous and organic phases can be separated by a 5 min centrifugation at 1000 *g*. The amount of [^{3}H]-acetylcholine and [^{3}H]-phosphorylcholine was determined in 1 ml of the upper organic and lower aqueous phases, respectively. The recovery of added [^{14}C]-acetylcholine chloride (2.05 GBq/mmol) in the organic phase was 91.5±0.6% (n=4), while the recovery of added [^{3}H]-choline chloride (2997 GBq/mmol) was in the aqueous phase 90.8±1.9% (n=4). The collected fractions of the superfusion media were counted in a opti-fluor scintillation cocktail for aqueous and non-aqueous samples.

2.4. Determination of acetylcholinesterase activity in rat hippocampi

The rat hippocampi were homogenised (10% w/v) in a pH 7.4 buffer of 20 mM Na^+/K^+-phosphate. The slices were placed ice cold in glass homogenisers and processed for 20 strokes at 720 r.p.m. prior to determination of the acetylcholinesterase activity. The acetylcholinesterase activity was measured after inhibition of the pseudocholinesterase activity with ethopropazine (Todrik, 1954). The acetylcholinesterase activity was determined at a temperature of 30 °C using the radiochemical micromethod of Sterri and Fonnum (1978).

2.5. Statistics

Statistical analyses were done with Student's t-test (two-tailed). The fractional rate of the evoked release of [^{3}H]-acetylcholine and [^{3}H]-choline, their peak areas as well as the basal release before and after the depolarisation period, and the ratios between peak areas were all calculated. The potassium-evoked [^{3}H]-acetylcholine release was calculated by subtracting the basal release from the evoked release. The potassium-evoked release of [^{3}H]-acetylcholine was calculated as a percentage of that released in the first stimulation in each experiment. Mean and standard error of the mean (S.E.M.) were calculated for all data whereby n equals the number of experiments. The significance of differences between control and experimental groups was calculated by Student's t-test and a one-way analysis of variance (one-way ANOVA), with Dunnet's Multiple Comparison test as post test. The experiments included a control group and an experimental group and they were always performed at the same time in parallel. [a]$P>0.05$ (non-significant); [b]$P<0.05$; [c]$P<0.02$;[d]$P<0.01$.

3. Results

3.1. Effects of oximes on the potassium-evoked (51 mM) release of [^{3}H]-acetylcholine in the absence of soman

In this study it was observed that in the absence of soman, toxogonin (10–200 μM) and HLö-7 (5–200 μM) enhanced the potassium-evoked (51 mM) release of [^{3}H]-acetylcholine in a concentration-dependent manner (Fig. 1). There was an approximately three fold increase in the release of [^{3}H]-acetylcholine following exposure to the highest concentrations of toxogonin and HLö-7. The two oximes had no significant effects on the spontaneous release of [^{3}H]-acetylcholine from the cholinergic nerves in the absence of soman (not shown).

On the other hand, it was observed that HI-6 (10–200 μM), enhanced the potassium-evoked (51 mM) release of [^{3}H]-acetylcholine to a much lower extent than toxogonin and HLö-7 (Fig. 1). Similar to the results with toxogonin and HLö-7, HI-6 had no effect on the spontaneous release of [^{3}H]-acetylcholine (not shown).

The two pralidoximes, P2S (50–200 μM) and 2-PAM (50–200 μM), enhanced the potassium-evoked (51 mM) release of [^{3}H]-acetylcholine only to a limited degree (Fig. 1). Some effects on the potassium-evoked release were observed with 150–200 μM of the oximes, with the effect in a similar range as that observed for HI-6 (Fig. 1). The two oximes had no significant effects on the spontaneous release of [^{3}H]-acetylcholine from the cholinergic nerves in the absence of soman (not shown).

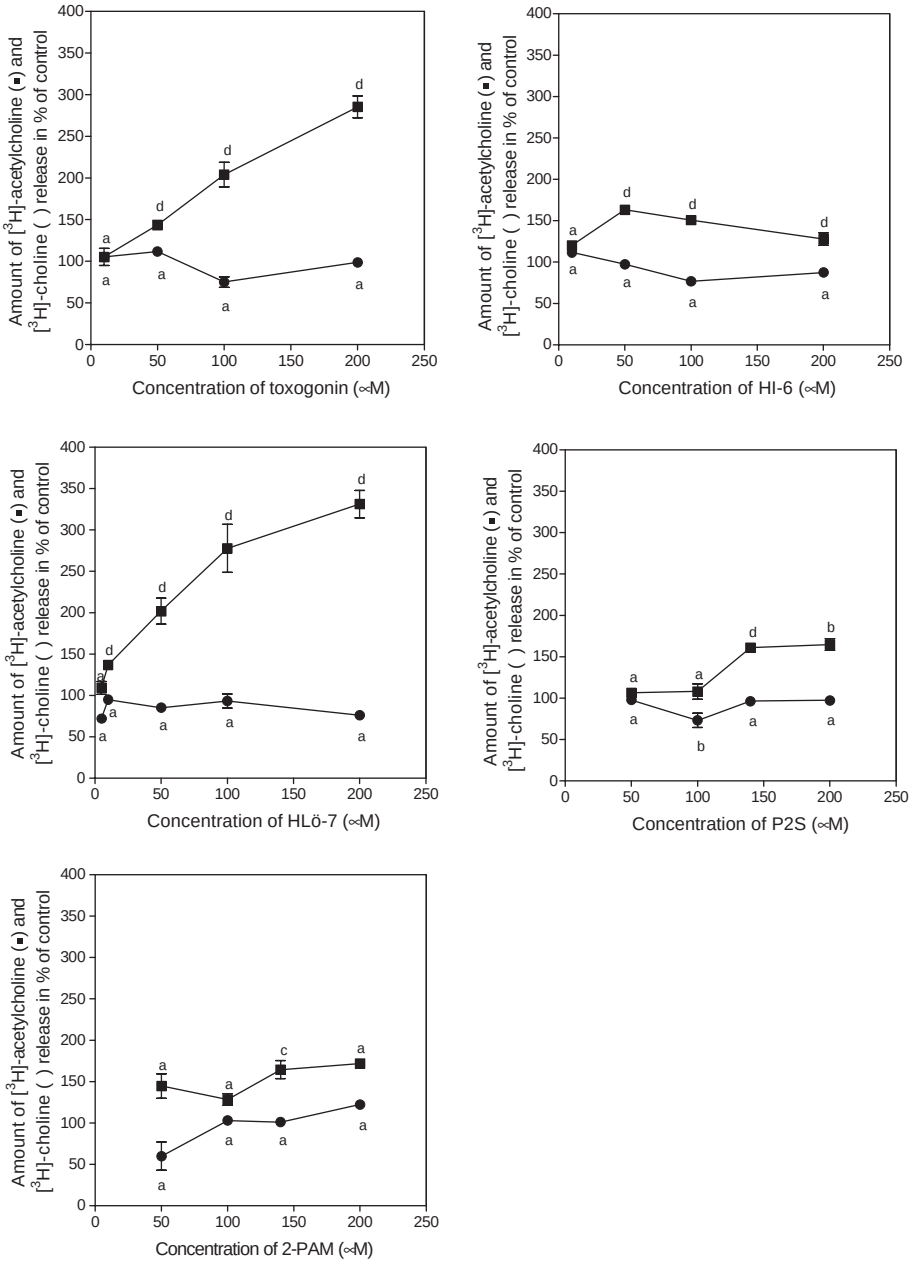

Fig. 1. The effect of toxogonin, HI-6, HLö-7, P2S and 2-PAM on the potassium (51 mM) evoked release of [^{3}H]-acetylcholine (■) and on the spontaneous liberation of [^{3}H]-choline (●) from rat hippocampal slices in the absence of soman. The results are expressed in percent of control without oxime present. The potassium (51 mM) evoked release of [^{3}H]-acetylcholine (control, 100%) in the absence of soman was 4.2±1.4 fmol/ml perfusion buffer (n=6). The spontaneous liberation of [^{3}H]-choline was 54.6±14.3 fmol/ml perfusion buffer (n=6). Significance of difference from control: $^aP>0.05$, $^bP<0.05$, $^cP<0.02$, $^dP<0.01$.

Neither of the oximes had any significant effects on the spontaneous liberation of [^{3}H]-choline in concentrations up to 200 μM in the presence of high potassium (51 mM) (Fig. 1) or in the absence of high potassium (not shown).

3.2. Effects of oximes on the potassium-evoked (51 mM) release of [^{3}H]-acetylcholine in the presence of soman

In the presence of soman (1.0 μM), toxogonin (10–200 μM) had no significant effect on the potassium-evoked release of [^{3}H]-acetylcholine (Fig. 2). Nor was there any significant effect of toxogonin on the spontaneous release of [^{3}H]-acetylcholine from the cholinergic nerves in the presence of soman (not shown). The spontaneous liberation of [^{3}H]-choline was however slightly increased at 200 μM of toxogonin (Fig. 2).

In the presence of soman (1.0 μM) and HI-6 (10–200 μM), or of HLö-7 (10–200 μM), there was a concentration-dependent significant reduction in the potassium-evoked release of [^{3}H]-acetylcholine (Fig. 2). However, there were no significant effects of HI-6 or HLö-7 on the spontaneous release of [^{3}H]-acetylcholine from the cholinergic nerves in the presence of soman (not shown).

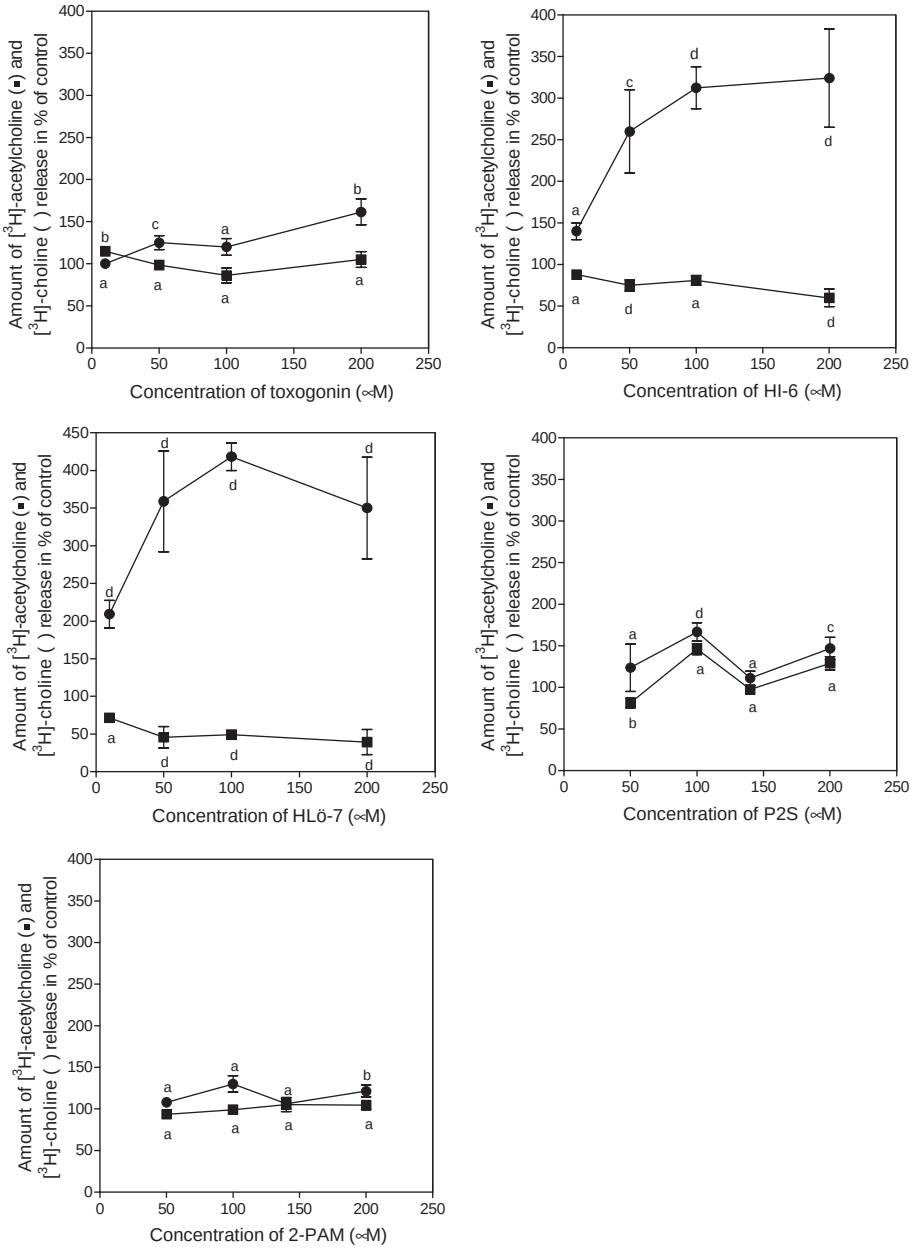

Fig. 2. The effect of toxogonin, HI-6, HLö-7, P2S and 2-PAM on the potassium (51 mM) evoked release of [^{3}H]-acetylcholine (■) and on the spontaneous liberation of [^{3}H]-choline (●) from rat hippocampal slices in the presence of soman (1.0 μM). The results are expressed in percent of control without oxime present. The potassium (51 mM) evoked release of [^{3}H]-acetylcholine (control, 100%) in the presence of soman was 50.9±13.0 fmol/ml perfusion buffer (n=6). The spontaneous liberation of [^{3}H]-choline was 9.8±1.1 fmol/ml perfusion buffer (n=6). Significance of difference from control: $^aP>0.05$, $^bP<0.05$, $^cP<0.02$, $^dP<0.01$.

Moreover, the liberation of [^{3}H]-choline from the hippocampal slices was significantly enhanced in the presence of HI-6 and HLö-7, although the concentration of [^{3}H]-choline was relatively low compared to the concentration of [^{3}H]-acetylcholine. The potassium (51 mM) evoked release of [^{3}H]-acetylcholine (control, 100%) in the presence of soman was 50.9±13.0 fmol/ml perfusion buffer (n=6). The spontaneous liberation of [^{3}H]-choline was 9.8±1.1 fmol/ml perfusion buffer (n=6). In contrast, in the absence of soman, the potassium (51 mM) evoked release of [^{3}H]-acetylcholine (control, 100%) was 4.2±1.4 fmol/ml perfusion buffer$^-$(n=6), while the spontaneous liberation of [^{3}H]-choline was 54.6±14.3 fmol/ml perfusion buffer (n=6).

In the presence of soman (1.0 μM), the effects of 2-PAM (50–200 μM) or P2S (50–200 μM) on the release of [^{3}H]-acetylcholine were small and non-significant (Fig. 2). There were no effects on the spontaneous release of [^{3}H]-acetylcholine from the cholinergic nerves in the presence of soman (not shown). There were only minor but significant effects on the spontaneous liberation of [^{3}H]-choline from the hippocampal slice (Fig. 2).

Table 1
Acetylcholinesterase activity of hippocampus-slices was measured in the presence and absence of soman and different concentrations of oximes (toxogonin, HI-6, HLö-7, 2-PAM and P2S)

	Acetylcholinesterase activity (control), %	Acetylcholinesterase activity with soman (1.0 μM), %	n
Control	100	8.3±0.8[a]	10
10 μM toxogonin	90.4±3.9[a]	7.5±1.5[a]	4
50 μM toxogonin	92.5±3.8[a]	3.8±2.6[a]	4
100 μM toxogonin	101.1±10.2[a]	10.1±1.2[a]	4
200 μM toxogonin	83.1±7.9[a]	8.6±0.7[a]	4
10 μM HI-6	74.8±12.9[a]	7.3±0.8[a]	4
50 μM HI-6	64.0±7.6[a]	3.1±1.5[a]	4
100 μM HI-6	91.8±9.9[a]	8.4±0.6[a]	4
200 μM HI-6	82.2±4.8[a]	9.6±0.7[a]	4
10 μM HLö-7	101.7±11.7[a]	8.5±0.5[a]	4
50 μM HLö-7	112.5±17.2[a]	12.4±7.4[b]	4
200 μM HLö-7	77.3±8.2[a]	39.1±5.5[d]	4
50 μM P2S	104.4±9.6[a]	10.3±1.2[a]	4
100 μM P2S	77.7±5.3[a]	9.0±0.5[a]	4
150 μM P2S	102.5±6.5[a]	4.5±0.6[a]	4
200 μM P2S	96.1±12.4[a]	3.9±0.5[d]	4
50 μM 2-PAM	105.7±6.3[a]	14.7±1.9[a]	4
100 μM 2-PAM	87.2±18.6[a]	17.5±3.2[a]	4
150 μM 2-PAM	104.9±3.5[a]	20.4±4.1[a]	4
200 μM 2-PAM	54.0±2.9[b]	6.4±0.4[b]	4

The values are calculated in percent of respective control in the absence of soman (100% acetylcholinesterase activity=208.1±18.6 μmol hydrolysed acetylcholine/min mg protein). The measurements are the mean values of n experiments±S.E.M. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.02$, [d]$P<0.01$.

3.3. Effects of oximes on acetylcholinesterase activity in the hippocampus in the presence of soman

Following exposure to soman (1.0 μM) there was only limited reactivation of the acetylcholinesterase activity in the hippocampus slice preparation by the oximes toxogonin (10–200 μM), HI-6 (10–200 μM), P2S (50–200 μM) and 2-PAM (50–200 μM) (Table 1). Some reactivation of acetylcholinesterase activity was observed following 50–200 μM HLö-7 (Table 1).

4. Discussion

The two bispyridinium oximes HI-6 and HLö-7 had significant dose-dependent inhibitory effects on the potassium-evoked release of [^{3}H]-acetylcholine from the rat hippocampal slice preparation in vitro when the acetylcholinesterase activity was inhibited in the presence of soman. A lowering of the enhanced cholinergic activity could be achieved through the reduction of the release of acetylcholine from cholinergic nerves, thereby reducing the enhanced activation of both muscarinic and nicotinic cholinergic receptors in the brain. HI-6 effects such as this has previously been observed in cholinergic neurons in the lung (Aas et al., 1987), and in the rat diaphragm poisoned with crotylsarin used as an analogue of sarin and soman (Van Helden et al., 1996). Moreover, inhibition of the acetylcholinesterase activity in the central nervous system will result in increased cholinergic stimulation and a secondary activation of neurons releasing excitatory and inhibitory amino acid neurotransmittors such as glutamate and GABA, respectively (McDonough and Shih, 1993; 1995; Lallement et al., 1998). This will induce centrally mediated seizures and convulsions within a few minutes. Development of new drugs able to inhibit the release of acetylcholine, as well as glutamate, might contribute to a better medical treatment of organophosphorus poisoning (Van Helden and Beuters, 1999; Aas, 2003; Myhrer et al., 2003, 2005).

It is well established that 30–50% of the input from septum medialis and 50–75% of the input from the diagonal band of Broca are cholinergic (Amaral and Kurz, 1985; Wainer et al., 1985). These nerves constitute therefore a major cholinergic input to the hippocampus. Following soman intoxication in humans, a rapid inhibition of brain acetylcholinesterase activity will occur, with ageing of the acetylcholinesterase enzyme within approximately 2 min (Fleisher and Harris, 1965). Non-reactivating pharmacological effects of oximes may therefore have great importance in reducing the toxic effects of soman as previously suggested (Hamilton and Lundy, 1989; Van Helden et al., 1992).

In the present experiments, the acetylcholinesterase activity was significantly reduced to approximately 3% in the presence of both soman and 50 μM HI-6 or to 12% in the presence of both soman and 50 μM HLö-7. It is unlikely that an acetylcholinesterase activity of 3% is sufficient for hydrolysis of [^{3}H]-acetylcholine released from hippocampal cholinergic nerves, which means that the reduced amount of acetylcholine is probably due to the substantial reduction in the apparent evoked release of [^{3}H]-acetylcholine. One could argue that the relatively high reactivation of the acetylcholinesterase activity in the presence of 200 μM HLö-7 (an acetylcholinesterase activity of approximately 39%) was most certainly sufficient to reduce the concentration of [^{3}H]-acetylcholine in the perfusion buffer, and may thereby be the major mechanism for the apparent reduction of the [^{3}H]-acetylcholine release. On the other hand 50 μM HLö-7 resulted in an acetylcholinesterase activity of approximately 12%; however, a similar apparent reduction in the [^{3}H]-acetylcholine release was observed as following exposure to 200 μM HLö-7. It is therefore reasonable to believe that the reduced concentration of [^{3}H]-acetylcholine in the perfusion buffer was in fact an effect of HLö-7 on the cholinergic nerve terminals rather than an indirect effect through acetylcholinesterase reactivation and reduction of the acetylcholine concentration in the cholinergic synapses. Apparently, the latter effect by HLö-7 is not concentration dependent. Neverthless it is important to remember that a homogenate of hippocampus brain slices was used in the present study to determine acetylcholinesterase activity. A homogenate gives only an approximation and not an accurate measure of the specific acetylcholinesterase activity in the cholinergic synapses in the brain. Consequently, it cannot be excluded that some of

the apparent reduction in the [^{3}H]-acetylcholine release may be due partly to reactivation of the soman-inhibited synaptic acetylcholinesterase activity and hydrolysis of [^{3}H]-acetylcholine, and partly to a reduced release of [^{3}H]-acetylcholine in view of cholinergic activation of muscarinic receptors on the nerve terminals by acetylcholine.

Due to the acetylcholinesterase inhibition, the relative concentration of [^{3}H]-acetylcholine to [^{3}H]-choline in the perfusion buffer was significantly higher in the experiments where soman was present. The concentration of [^{3}H]-acetylcholine in control experiments in the presence of soman was approximately 50 fmol/ml perfusion buffer, while the spontaneous liberation of [^{3}H]-choline was only 10 fmol/ml perfusion buffer. This demonstrates that the observed HI-6 and HLö-7-induced reduction in the evoked [^{3}H]-acetylcholine release following soman exposure (Fig. 2) was relatively large compared to the increase in the evoked [^{3}H]-acetylcholine release observed in the presence of HI-6, HLö-7 and toxogonin in the experiments where soman was absent (Fig. 1). In these latter experiments, the [^{3}H]-choline concentration was much higher. The apparent spontaneous liberation of [^{3}H]-choline was approximately 55 fmol/ml perfusion buffer, while the evoked release of [^{3}H]-acetylcholine in the absence of soman was only about 4 fmol/ml perfusion buffer.

The observation that HI-6 and HLö-7 reduced the evoked release of acetylcholine suggests that these drugs have an agonistic function and not antagonistic properties on presynaptic cholinergic nerve terminals in the hippocampus or some yet unidentified function. Presynaptic cholinergic autoreceptors on cholinergic nerve terminals have previously been shown and characterised in many different tissues, including the central nervous system, and such receptors have been shown to be important in the regulation of the release of acetylcholine (Wessler, 1989; Bowman et al., 1990; Aas and Fonnum, 1986; Aas and Maclagan, 1990; Van Helden et al., 1996; Johnson et al., 2005). Neither toxogonin nor the two mono pyridinium oxime salts, P2S and 2-PAM, showed such agonistic pharmacological effects in the present study, indicating that these drugs do not have such intrinsic effects as HI-6 and HLö-7. In the absence of soman, however, toxogonin and HLö-7 enhanced the evoked release of [^{3}H]-acetylcholine several fold, while HI-6 had a much smaller effect on the release. Similarly, P2S and 2-PAM also enhanced the evoked release of [^{3}H]-acetylcholine, but only to a limited extent at low concentrations. Significant enhancements were observed at high concentrations of the oximes. The effect of the oximes was probably not due to a hemicholinium-3 like inhibition of the uptake of choline as previously suggested (Clement, 1979), because there was no alteration in the concentration of [^{3}H]-choline in the absence of soman. Another possible explanation is that the oximes might have antagonistic properties on pre-junctional muscarinic M_2 receptors in the absence of soman. Oximes might thereby reduce auto-inhibition of acetylcholine release. Whether this increase of potassium-evoked [^{3}H]-acetylcholine release induced by the oximes is of any significance is unknown.

It has been reported by others that oximes, such as 1,1-(4-hydroxy-iminopyridinium) trimethylene (TMB-4), HGG-42 and HGG-12, may have different effects on the evoked release of [^{3}H]-acetylcholine in the presence and absence of a cholinesterase inhibitor. In a paper by Kloog et al. (1986) in which the effects of oximes on the calcium dependent potassium evoked release of [^{3}H]-acetylcholine from slices isolated from rat brain stem were studied, an agonistic effect was described. This indicates that TMB-4, HGG-42 and HGG-12 may have agonistic properties on pre-junctional cholinergic muscarinic receptors. However, these experiments were performed in the absence of a cholinesterase inhibitor.

HI-6, HLö-7 and toxogonin are all bis-pyridinium aldoximes, while P2S and 2-PAM are mono-pyridinium aldoximes. Toxogonin has two aldoxime groups in the molecule; one aldoxime group in position four on each pyridinium ring. HI-6 and HLö-7 have several structural similarities, with one aldoxime and one carboxyamide group on each pyridinium ring respectively. HLö-7 bears an additional aldoxime group in position four on one of the pyridinium rings. It is possible that it is this similarity in the chemical structure of HI-6 and HLö-7 that plays an important role in reducing the release of acetylcholine in the presence of soman.

In summary, the results of the present work indicate that HI-6 and HLö-7 reduce the evoked release of [^{3}H]-acetylcholine following inhibition of the acetylcholinesterase activity by soman exposure. It is unlikely that the release-inhibition is due to the enhanced concentration of acetylcholine in the synapse causing a negative feedback via pre-junctional muscarinic receptors. This reduced release was not observed in the presence of toxogonin, P2S and 2-PAM. Such pre-junctional agonistic potency resulting in significant reduction of acetylcholine release, might reduce the toxic effects of soman and other organophosphates.

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