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In vitro effects of toxogonin, HI-6 and HLö-7 on the release of [^{3}H]acetylcholine from peripheral cholinergic nerves in rat airway smooth muscle

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Abstract

The purpose of this work was to evaluate the possible non-reactivating effects of toxogonin (1,1'[oxybis(methylene)]bis[4-[(hydroxyimino)methyl]pyridinium]-dichloride), HI-6 (1-[[[(4-aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]pyridinium-dichloride) and HLö-7 (pyridinium, 1-[[[4-(aminocarbonyl)pyridino]methoxy]methyl]-2,4-bis-[(hydroxyimino)methyl]diiodide) on the release of acetylcholine from cholinergic nerves. The oximes have been tested in our rat bronchial smooth muscle model, with respect to the effects of oximes on the K^+ (51 mM)-evoked release of [^{3}H]acetylcholine in the presence and absence of soman (1.0 μM). Toxogonin (100 μM) had no effect on the K^+-evoked release of [^{3}H]acetylcholine in the presence or absence of soman (1.0 μM). Similar results were found for HI-6 (100 μM). In contrast, HLö-7 (100 μM) enhanced the K^+-evoked release of [^{3}H]acetylcholine in the absence of soman. In the presence of soman HLö-7 did not alter the release of [^{3}H]acetylcholine induced by K^+ stimulation. The potentiating effect of HLö-7 on the release of [^{3}H]acetylcholine could be blocked by the L-, N- and P-Ca^{2+} channel blockers verapamil (0.1 and 1.0 μM), ω-conotoxin GVIA (1.0 μM) and ω-agatoxin IV-A (0.2 μM), respectively. Muscarinic receptor antagonists (atropine (10 μM), pirenzepine (M_1) (1.0 μM) and methoctramine (M_2) (1.0 μM)) had no effects on the HLö-7 (100 μM)-enhanced release of [^{3}H]acetylcholine. Protein kinase inhibitors (H-7 (20 μM), calphostin C (1.0 μM) and KN-62 (10 μM)) inhibited the HLö-7 (100 μM)-enhanced K^+-evoked release of [^{3}H]acetylcholine. The results showed that only HLö-7 had a direct enhancing effect on the release of acetylcholine through activation or opening of Ca^{2+} channels and a subsequent protein phosphorylation in the nerve terminal.

Keywords: Acetylcholine; Ca^{2+} channel; HI-6; HLö-7; Toxogonin; Oxime; Phosphorylation; Protein kinase inhibitor; Soman

1. Introduction

Exposure to acetylcholinesterase inhibitors, such as organophosphorus compounds, impairs cholinergic neurotransmission in the central nervous system, in the autonomic nervous system as well as at the neuromuscular junction (Taylor, 1990). This intoxication is treated with a blocker of muscarinic receptors (atropine) supplemented with a reactivator of the acetylcholinesterase activity. Although a large number of such reactivators with a nucleophilic oxime group have been synthesised and tested against nerve agents (Taylor, 1990), there is still no single oxime effective in the therapy of nerve agent intoxication. Several oximes are known as reactivators of organophosphate-inhibited acetylcholinesterase. Toxogonin (obidoxime), pralidoxime (Taylor, 1990) and the asymmetric bis-pyridinium HI-6 ((1-[[[(4-aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]pyridinium-dichloride (DeJong and Wolring, 1980; DeJong et al., 1989; Wolthuis et al., 1981; Clement, 1982; Inns and Leadbeater, 1983; Boskovic et al., 1984; Clement et al., 1987; Lundy et al., 1992) are used as reactivators of nerve agent-inhibited acetylcholinesterase. HI-6 is among the most potent and least toxic reactivators of 'unaged' soman-inhibited acetylcholinesterase (Berends, 1987; DeJong and Wolring, 1980, 1984; Boskovic et al., 1984; Clement, 1983; Walday et al., 1993). HLö-7 (pyridinium, 1-[[[4-(aminocarbonyl)-pyridino]methoxy]methyl]-2,4-bis-[(hydroxyimino)methyl]-diiodide), is a potent reactivator of both soman- and tabun-inhibited acetylcholinesterase (DeJong et al., 1989; Eyer et al., 1992). Following exposure to soman, rapid dealkylation (aging) occurs, resulting in a non-reactivable enzyme (Loomis and Salafsky, 1963; Heilbronn and Tolagen, 1965; Wolthuis et al., 1981). This is the reason why

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oxime therapy has been considered to be of limited value against soman poisoning in humans. Recently, however, it has been reported that after lethal irreversible inhibition of acetylcholinesterase survival is possible on the basis of non-reactivating (pharmacological) effects of some of these oximes (Van Helden et al., 1991, 1994). A number of pharmacological effects of oximes have been described previously such as the opening of ion channels by HI-6 (Alkondon et al., 1988; Tattersall, 1993) and binding to nicotinic and muscarinic receptors (Clement, 1981; Bromfield et al., 1987; Su et al., 1983). It has previously been shown that HLö-7 improves respiration and circulation after high doses of tabun without any significant acetylcholinesterase reactivation indicating some other direct mechanisms by which HLö-7 functions as an antidote (Worek et al., 1994). It is, however, not yet clear which effects are essential for the functional recovery of neuronal transmission in spite of continued acetylcholinesterase inhibition. The aim of the present study was to evaluate the ability of toxogonin, HI-6 and HLö-7 to alter the release of acetylcholine from peripheral autonomic cholinergic nerves. Effects of oximes on the release of acetylcholine might be relevant in preventing or abolishing the expected accumulation of this transmitter upon acetylcholinesterase inhibition as suggested by Melchers et al. (1991).

2. Materials and methods

2.1. Animals

Male Wistar rats (200–300 g; from Møllegaard, Copenhagen, Denmark) were used. 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 two weeks before the start of the experiments under constant photoperiod conditions (12 h : 12 h, L : D) at a temperature of 20°C and 60% relative humidity. The sawdust bedding was replaced daily to ensure that the concentration of ammonia was kept at a very low level. The rats had no signs of symptoms of respiratory tract infections.

2.2. Chemicals

The chemicals were: verapamil hydrochloride, oxotremorine and ethopropazine hydrochloride (Sigma Chemical Co., MA, USA); pirenzepine hydrochloride (Boehringer Ingelheim, Germany); methoctramine hydrochloride, H-7 hydrochloride, HA-1004 hydrochloride, calphostin C, KN-62 (RBI, Natick, MA, USA); phorbol 12,13-dibutyrate (LC-laboratories, Woburn, MA, USA); atropine sulphate (Norsk Medisinal Depot, Oslo, Norway); ω-conotoxin GVIA (Research Biochemicals (MA, USA); ω-agatoxin IV-A (Latoxan, France); methyl-[^{3}H]choline chloride (New England Nuclear, Boston, MA, USA). Opti-fluor (Packard Instr., Groningen, Netherlands); toxogonin® (obidoxime) (1,1′[oxybis(methylene)]bis[4-[(hydroxyimino)methyl]pyridinium]-dichloride (Merck, Darmstadt, Germany); HI-6 (1-[[[(4-aminocarbonyl)pyridinio]-methoxy]-methyl]-2-[(hydroxyimino)methyl]pyridinium-dichloride) (Dr. Boulet, Defence Research Establishment Suffield, Ralston, Alberta, Canada); HLö-7 (pyridinium, 1-[[[4-(aminocarbonyl)pyridino]methoxy]-methyl]-2,4-bis[(hydroxyimino)methyl]diiodide) (Professor P. Eyer, Walter-Straub-Institut für Pharmakologie und Toxicologie der Ludwig-Maximilians-Universität München, Germany); soman (*O*-[1,2,2-trimethylpropyl]-methyl-phosphono-fluoridate, assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in this laboratory. All other chemicals were of analytical laboratory reagent grade.

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

Following decapitation of the animals, the bronchi were removed and transferred to the physiological buffer (see below). Pieces of bronchial smooth muscle tissue (five tissue pieces × 1 mg wet weight) were superfused in small chambers, as previously described by Aas and Fonnum (1986). Prior to the start of superfusion, the tissue was incubated for 60 min in 1.1 μM [^{3}H]choline chloride (370 GBq mmol^{-1}) in the superfusion medium in a shaking water-bath (25°C). The tissue was washed twice and superfused, by using a peristaltic pump with a flow rate of 200 μl min^{-1}, for 60 min prior to the collection of samples. The release of [^{3}H]acetylcholine was induced by raising the K^+ concentration for 5 min. 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) had the following composition (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 as buffer A, but contained 51 mM KCl, and the concentration of NaCl was reduced accordingly to keep the ionic strenght constant. The media were kept in a thermostatically controlled water-bath at 25°C during the experiments. KCl (51 mM) induced approximately 50% of the maximal release of [^{3}H]acetylcholine. A total of two (S1 and S3) or three (S1, S2 and S3) K^+ stimulations (51 mM) for 5 min were separated by 35 min superfusion with buffer A. The drugs were added prior to and during the second stimulation (S2) or instead of the second stimulation (S2) (time interval is given in the tables). Each experiment with drugs had its own control experiment. The collected fractions of the superfusion media were counted in an Opti-fluor scintillation cocktail for aqueous and non-aqueous samples. Release of ^{3}H during stimulation of tissues preincubated with [^{3}H]choline has been shown to be a good measure for [^{3}H]acetylcholine release (Richardson and Szerb, 1974).

Table 1
Effects of soman and Ca^{2+} channel blockers on the release of [^{3}H]acetylcholine

Experiment	S2	Stimulation S2	S1 (%)	S2 (%)	Significance of difference	S3 (%)	n
(A)	Control	51 mM KCl	100	79 ± 3		64 ± 3	12
(B)	1.0 μM soman	51 mM KCl	100	66 ± 3	(A) [c]	56 ± 1	6
(C)	Control, 0 M Ca^{2+}, 10 mM Mg^{2+}	51 mM KCl	100	24 ± 2	(A) [d]	87 ± 2	6
(D)	0.1 μM verapamil	51 mM KCl	100	102 ± 9	(A) [a]	93 ± 2	2
(E)	1.0 μM verapamil	51 mM KCl	100	83 ± 1	(A) [a]	81 ± 3	4
(F)	1.0 μM ω-conotoxin	51 mM KCl	100	68 ± 2	(A) [d]	65 ± 2	8
(G)	0.2 μM ω-agatoxin	51 mM KCl	100	94 ± 7	(A) [a]	77 ± 4	8

Bronchial smooth muscle was stimulated for 5 min three times consecutively with K^+ (51 mM) and voltage-dependent ion channel blockers present in the perfusion buffer (S2) (D-G). Verapamil was present 10 min before and during stimulation (D,E). ω-Conotoxin GVIA and ω-agatoxin IV-A were present 20 min before and during stimulation (S2) (F,G), respectively. The results are the means ± S.E.M. of n experiments. Significance of difference ([d] $P < 0.01$, [c] $P < 0.02$, [a] $P > 0.05$) from experiment in parentheses are shown.

2.4. Determination of acetylcholinesterase and pseudocholinesterase activities

The rat bronchi were homogenised (10% w/v) in 20 mM Na^+-K^+ phosphate buffer (pH 7.4) (glass/glass homogeniser), 20 strokes, 720 rpm (icecold) before determination of the acetylcholinesterase and pseudocholinesterase activities. Acetylcholinesterase activity was measured after inhibition of pseudocholinesterase activity with ethopropazine (Todrik, 1954). The cholinesterase activities were determined at 30°C with the radiochemical micromethod of Sterri and Fonnum (1978) in the presence and absence of soman (1.0 μM) and oxime (100 μM). Oxime was present 5 min before soman and in the presence of soman (10 min).

2.5. Statistics

Statistical analyses were done with Student's t-test (two-tailed). The fractional rate of evoked release of [^{3}H]acetylcholine, peak areas, as well as basal release before and after the depolarisation period and ratios between peak areas, were calculated. K^+-evoked [^{3}H]acetylcholine release was calculated by subtracting the basal release from the evoked release. The K^+-evoked release was always performed three times (S1, S2 and S3) consecutively and the K^+-evoked release of [^{3}H]acetylcholine was calculated as a percentage of that released in the first stimulation (S1) in each experiment. Mean and standard error of the mean (S.E.M.) were calculated for all data and n equals the number of experiments. Significance of difference between a control group and an experimental group were calculated by Student's t-test. Each experiment included a control group and an experimental group and they were always performed in parallel at the same time. [d]$P < 0.01$; [c]$P < 0.02$; [b]$P < 0.05$; [a]$P > 0.05$.

3. Results

3.1. General

K^+-evoked (51 mM) release of [^{3}H]acetylcholine from cholinergic nerves in the rat bronchial smooth muscle was Ca^{2+}-dependent and substantially reduced by a high concentration of Mg^{2+} (10 mM) (Table 1). The release of [^{3}H]acetylcholine was also reduced in the presence of the voltage-dependent Ca^{2+} channel blocker ω-conotoxin GVIA (1.0 μM) (blocker of N-type Ca^{2+} channels), but not in the presence of ω-agatoxin IV-A (blocker of P-type Ca^{2+} channels) or low concentrations of verapamil (0.1–1.0 μM) (blocker of L-type Ca^{2+} channels) (Table 1).

Table 2
Effects of toxogonin on the release of [^{3}H]acetylcholine

Experiment	S2	Stimulation S2	S1 (%)	S2 (%)	Significance of difference	S3 (%)	n
(A)	Control	51 mM KCl	100	79 ± 3		64 ± 3	12
(B)	Control		100	6 ± 2		93 ± 7	3
(C)	1.0 μM soman	51 mM KCl	100	66 ± 3	(A) [c]	56 ± 1	6
(D)	100 μM toxogonin		100	8 ± 1	(B) [a]	93 ± 1	6
(E)	100 μM toxogonin	51 mMKCl	100	88 ± 5	(A) [a]	66 ± 5	6
(F)	1.0 μM soman, 100 μM toxogonin	51 mM KCl	100	61 ± 2	(C) [a]	60 ± 2	4

Bronchial smooth muscle was stimulated for 5 min three times consecutively with K^+ (51 mM) (A,C,E,F) or twice (S1 and S3) without K^+ stimulation during the 5 min S2 period (B,D). Toxogonin was present 5 min before and during stimulation with K^+ (51 mM) (E), only during S2 (D) and in a 5 min period after exposure to soman (5 min) and during the following K^+ stimulation (51 mM) (F). Soman was present 5 min before K^+ (51 mM) stimulation (C). The results are the means ± S.E.M. of n experiments. Significance of difference ([c] $P < 0.02$, [a] $P > 0.05$) from experiment in parentheses are shown.

Table 3
Effects of HI-6 on the release of [^{3}H]acetylcholine

Experiment	S2	Stimulation S2	S1 (%)	S2 (%)	Significance of difference	S3 (%)	n
(A)	Control	51 mM KCl	100	79 ± 3		64 ± 3	12
(B)	Control		100	6 ± 2		93 ± 7	3
(C)	1.0 μM soman	51 mM KCl	100	66 ± 3	(A) [c]	56 ± 1	6
(D)	100 μM HI-6		100	5 ± 3	(B) [a]	86 ± 5	6
(E)	100 μM HI-6	51 mM KCl	100	85 ± 4	(A) [a]	68 ± 3	6
(F)	1.0 μM soman, 100 μM HI-6	51 mM KCl	100	72 ± 2	(C) [a]	60 ± 1	4

Bronchial smooth muscle was stimulated for 5 min three times consecutively with K^+ (51 mM) (A,C,E,F) or twice (S1 and S3) without K^+ stimulation during the 5 min S2 period (B,D). HI-6 was present 5 min before and during stimulation with K^+ (51 mM) (E), only during S2 (D) and in a 5 min period after exposure to soman (5 min) and during the following K^+ stimulation (51 mM) (F). Soman was present 5 min before K^+ (51 mM) stimulation (C). The results are the means ± S.E.M. of n experiments. Significance of difference ([c] $P < 0.02$, [a] $P > 0.05$) from experiment in parentheses are shown.

As previously shown (Aas et al., 1987) there was a slight reduction in the K^+-stimulated release of [^{3}H]acetylcholine in the presence of soman (1.0 μM) (Table 1).

3.2. The effects of toxogonin on the release of [^{3}H]-acetylcholine

Toxogonin (100 μM) had no effect on the K^+-evoked release of [^{3}H]acetylcholine and toxogonin (100 μM) did not induce release of [^{3}H]acetylcholine in the absence of K^+ ions (5.1 mM) (Table 2). Toxogonin (100 μM) had no effect on the soman (1.0 μM)-reduced K^+-evoked release of [^{3}H]acetylcholine (Table 2).

3.3. The effects of HI-6 on the release of [^{3}H]acetylcholine

HI-6 (100 μM) had no effect on the K^+-evoked release of [^{3}H]acetylcholine and HI-6 (100 μM) did not induce release of [^{3}H]acetylcholine in the absence of K^+ ions (5.1 mM) (Table 3). HI-6 (100 μM) had no effect on the soman (1.0 μM)-reduced K^+-evoked release of [^{3}H]acetylcholine (Table 3).

3.4. The effects of HLö-7 and Ca^{2+} channel blockers on the release of [^{3}H]acetylcholine

HLö-7 (1–100 μM) enhanced in a concentration-dependent manner the K^+-evoked release of [^{3}H]acetylcholine (Table 4). 100 μM HLö-7 enhanced the K^+-evoked release by approximately 24% compared to control. There was no alteration by HLö-7 of the soman (1.0 μM)-induced reduction of the release of [^{3}H]acetylcholine (Table 4). By exposing the tissue to a low Ca^{2+} concentration or by using voltage-dependent Ca^{2+} channel blockers (0.1–1.0 μM verapamil, 1.0 μM ω-conotoxin GVIA and 0.2 μM ω-agatoxin IV-A), it was shown that the enhanced

Table 4
Effects of HLö-7 and Ca^{2+} channel blockers on the release of [^{3}H]acetylcholine

Experiment	S2	Stimulation S2	S1 (%)	S2 (%)	Significance of difference	S3 (%)	n
(A)	Control	51 mM KCl	100	79 ± 3		64 ± 3	12
(B)	Control		100	6 ± 2		93 ± 7	3
(C)	1.0 μM soman	51 mM KCl	100	66 ± 3	(A) [c]	56 ± 1	6
(D)	100 μM HLö-7		100	7 ± 1	(B) [a]	72 ± 3	6
(E)	1 μM HLö-7	51 mM KCl	100	73 ± 2	(A) [a]	67 ± 2	6
(F)	5 μM HLö-7	51 mM KCl	100	83 ± 2	(A) [a]	76 ± 3	6
(G)	10 μM HLö-7	51 mM KCl	100	100 ± 7	(A) [b]	95 ± 10	6
(H)	100 μM HLö-7	51 mM KCl	100	103 ± 5	(A) [d]	81 ± 7	6
(I)	1.0 μM soman, 100 μM HLö-7	51 mM KCl	100	71 ± 3	(C) [a]	65 ± 2	10
(J)	0.1 mM Ca^{2+}, 10 mM HLö-7	51 mM KCl	100	18 ± 3	(A) [d]	90 ± 1	6
(K)	0.1 μM verapamil, 100 μM HLö-7	51 mM KCl	100	87 ± 2	(H) [b]	86 ± 3	4
(L)	1.0 μM verapamil, 100 μM HLö-7	51 mM KCl	100	79 ± 2	(H) [d]	73 ± 2	8
(M)	1.0 μM ω-conotoxin, 100 μM HLö-7	51 mM KCl	100	69 ± 5	(H) [d]	69 ± 3	8
(N)	0.2 μM ω-agatoxin, 100 μM HLö-7	51 mM KCl	100	73 ± 3	(H) [d]	57 ± 3	4

Bronchial smooth muscle was stimulated for 5 min three times consecutively with K^+ (51 mM) (A,C,E-N) or twice (S1 and S3) without K^+ stimulation during the 5 min S2 period (B,D). HLö-7 was present 5 min before and during stimulation with K^+ (51 mM) (E-N), only during S2 (D) and in a 5 min period after exposure to soman (5 min) and during the following K^+ stimulation (51 mM) (I). Soman was present 5 min before K^+ (51 mM) stimulation (C). Verapamil was present 10 min before and during K^+ (51 mM) (S2) stimulation (K,L). ω-Conotoxin GVIA and ω-agatoxin IV-A were present 20 min before and during K^+ (51 mM) stimulation (S2) (M,N), respectively. The results are the means ± S.E.M. of n experiments. Significance of difference ([d] $P < 0.01$, [c] $P < 0.02$, [b] $P < 0.05$, [a] $P > 0.05$) from experiment in parentheses are shown.

Table 5
Effects of HLö-7 and muscarinic receptor agonists and antagonists on the release of [^{3}H]acetylcholine

Experiment	S2	Stimulation S2	S1 (%)	S2 (%) of difference	Significance	S3 (%)	n
(A)	Control	51 mM KCl	100	79 ± 3		64 ± 3	12
(B)	10 μM oxotremorine	51 mM KCl	100	56 ± 2	(A) [d]	77 ± 2	6
(C)	10 μM atropine	51 mM KCl	100	97 ± 2	(A) [d]	103 ± 4	6
(D)	1.0 μM pirenzepine	51 mM KCl	100	86 ± 4	(A) [a]	63 ± 3	6
(E)	1.0 μM methoctramine	51 mM KCl	100	96 ± 5	(A) [c]	74 ± 5	6
(F)	100 μM HLö-7	51 mM KCl	100	103 ± 5		81 ± 7	6
(G)	10 μM oxotremorine, 100 μM HLö-7	51 mM KCl	100	58 ± 2	(F) [d] (A) [d]	83 ± 5	6
(H)	10 μM atropine, 100 μM HLö-7	51 mM KCl	100	100 ± 3	(F) [a] (A) [d]	96 ± 3	6
(I)	1.0 μM pirenzepine, 100 μM HLö-7	51 mM KCl	100	90 ± 3	(F) [a] (A) [c]	68 ± 2	6
(J)	1.0 μM methoctramine, 100 μM HLö-7	51 mM KCl	100	101 ± 5	(F) [a] (A) [d]	79 ± 7	6

Bronchial smooth muscle was stimulated for 5 min three times consecutively with K^+ (51 mM) (A-J). Oxotremorine, atropine, pirenzepine and methoctramine were present 10 min before and during K^+ (51 mM) stimulation (S2). HLö-7 was present 5 min before and during stimulation with K^+ (51 mM) (F-J). The results are the means ± S.E.M. of n experiments. Significance of difference ([d] $P < 0.01$, [c] $P < 0.02$, [a] $P > 0.05$) from experiment in parentheses are shown.

release of [^{3}H]acetylcholine induced by HLö-7 could be reduced significantly compared to experiments with only HLö-7 (Table 4).

3.5. The effects of HLö-7 and muscarinic receptor agonists and antagonists on the release of [^{3}H]acetylcholine

The unspecific muscarinic receptor blocker atropine (10 μM) and the specific muscarinic M_2 receptor antagonist methoctramine (1.0 μM) enhanced significantly the K^+-evoked release of [^{3}H]acetylcholine (Table 5). The M_1 specific receptor antagonist pirenzepine (1.0 μM) had no effect, while the unspecific muscarinic receptor agonist oxotremorine (10 μM) reduced the release of [^{3}H]acetylcholine (Table 5), as previously described (Aas and Fonnum, 1986). Combination of HLö-7 (100 μM) with any of the three muscarinic receptor antagonists had no significant effects on the potentiating effects of HLö-7 on the K^+-evoked release of [^{3}H]acetylcholine (Table 5). Oxotremorine (10 μM), on the other hand, reduced the K^+-evoked release of [^{3}H]acetylcholine significantly in the presence of HLö-7 (100 μM) (Table 5).

3.6. The effects of protein kinase inhibitors and activators, and HLö-7 on the release of [^{3}H]acetylcholine

Phorbol 12,13-dibutyrate (100 nM and 1.0 μM), an activator of the protein kinase C activity, enhanced the K^+-evoked (51 mM) release of [^{3}H]acetylcholine by approximately 42% and 78%, respectively.

Table 6
Effects of HLö-7 and protein kinase inhibitors and activators on the release of [^{3}H]acetylcholine

Experiment	S2	Stimulation S2	S1 (%)	S2 (%)	Significance of difference	S3 (%)	n
(A)	Control	51 mM KCl	100	79 ± 3		64 ± 3	12
(B)	20 μM H-7	51 mM KCl	100	80 ± 2	(A) [a]	68 ± 2	6
(C)	20 μM HA 1004	51 mM KCl	100	87 ± 4	(A) [a]	75 ± 3	8
(D)	1.0 μM calphostin C	51 mM KCl	100	84 ± 3	(A) [a]	77 ± 6	6
(E)	10 μM calphostin C	51 mM KCl	100	69 ± 2	(A) [c]	50 ± 7	5
(F)	1.0 μM KN-62	51 mM KCl	100	91 ± 8	(A) [a]	79 ± 4	6
(G)	10 μM KN-62	51 mM KCl	100	66 ± 2	(A) [d]	50 ± 4	6
(H)	100 μM HLö-7	51 mM KCl	100	103 ± 5		81 ± 7	6
(I)	20 μM H-7, 100 μM HLö-7	51 mM KCl	100	85 ± 3	(H) [c]	80 ± 2	6
(J)	20 μM HA 1004, 100 μM HLö-7	51 mM KCl	100	92 ± 3	(H) [a]	80 ± 3	9
(K)	1.0 μM calphostin C, 100 μM HLö-7	51 mM KCl	100	78 ± 2	(H) [d]	68 ± 7	6
(L)	10 μM calphostin C, 100 μM HLö-7	51 mM KCl	100	76 ± 4	(H) [d]	55 ± 4	7
(M)	10 μM KN-62, 100 μM HLö-7	51 mM KCl	100	65 ± 2	(H) [d]	52 ± 4	6
(N)	100 nM phorbol 12,13-dibutyrate	51 mM KCl	100	121 ± 3	(A) [d]	78 ± 2	6
(O)	1.0 μM phorbol 12,13-dibutyrate	51 mM KCl	100	157 ± 2	(A) [d]	81 ± 6	6

Bronchial smooth muscle was stimulated for 5 min three times consecutively with K^+ (51 mM) (A-O). H-7, HA 1004, calphostin C and KN-62 were present 15 min before and during K^+ (51 mM) stimulation (S2). HLö-7 was present 5 min before and during stimulation with K^+ (51 mM) (H-M). Phorbol 12,13-dibutyrate was present 5 min before and during stimulation with K^+ (S2). The results are the means ± S.E.M. of n experiments. Significance of difference ([d] $P < 0.01$, [c] $P < 0.02$, [a] $P > 0.05$) from experiment in parentheses are shown.

The unspecific protein kinase C inhibitor H-7 (20 μM), which inhibits protein kinase C directly and not via interaction with Ca^{2+} or phospholipids, had no effects on a control stimulation with K^+, but reduced the HLö-7 (100 μM)-enhanced K^+-evoked (51 mM) release of [^{3}H]acetylcholine by approximately 18% (Table 6). The apparently more specific protein kinase C inhibitor calphostin C had no effect (1.0 μM) or a small inhibiting effect (10 μM) on the control stimulation with K^+ (51 mM), but reduced significantly the HLö-7-enhanced K^+-evoked (51 mM) release of [^{3}H]acetylcholine by approximately 25% (1.0 μM) and 27% (10 μM), respectively. Inhibition of the Ca^{2+}/calmodulin-dependent protein kinase II activity by KN-62 (10 μM) reduced the control evoked release of [^{3}H]acetylcholine with K^+ (51 mM) by about 13% (Table 6). The HLö-7-enhanced K^+-evoked (51 mM) release of [^{3}H]acetylcholine was, moreover, reduced by 38% compared to control without HLö-7.

The inhibitor of cAMP and cGMP dependent protein kinases, HA 1004 (20 μM), which has a low affinity for protein kinase C, had no effects on the control evoked release of [^{3}H]acetylcholine with K^+ (51 mM). The HLö-7 (100 μM)-enhanced K^+-evoked (51 mM) release of [^{3}H]acetylcholine was neither reduced in the presence of HA-1004 (20 μM) (Table 6).

3.7. Determination of acetylcholinesterase and pseudocholinesterase activities

There was an almost complete inhibition of the acetylcholinesterase and pseudocholinesterase activities in the presence of soman (1.0 μM). The acetylcholinesterase and pseudocholinesterase activities were inhibited by 93.5% and 100%, respectively. In the presence of toxogonin (100 μM) and soman (1.0 μM) the acetylcholinesterase and pseudocholinesterase activities were inhibited by 96% and 100%, respectively. In the presence of HI-6 (100 μM) and soman (1.0 μM) the acetylcholinesterase and pseudocholinesterase activities were inhibited by 89.5% and 100%, respectively. In the presence of HLö-7 (100 μM) and soman (1.0 μM) the acetylcholinesterase and pseudocholinesterase activities were inhibited by 97% and 98.2%, respectively.

4. Discussion

The aim of this work was to study the molecular effects of toxogonin, HI-6 and HLö-7 on cholinergic nerves, which are unrelated to reactivation of the acetylcholinesterase activity. Previously, toxogonin, HI-6 and HLö-7 have been shown to prolong survival time in rats following a lethal injection with the organophosphate crotylsarin (Van Helden et al., 1994) and this prolongation was shown not to be due to a reactivation of the acetylcholinesterase enzyme activity. This means that the oximes probably have other effects in vivo unrelated to enzyme reactivation which are relevant for survival. This is of importance since such 'non-reactivating' effects may be of therapeutic relevance in the treatment of nerve agent intoxication (Oldiges and Schoene, 1970; Schoene et al., 1976; Smith and Muir, 1977; Hamilton and Lundy, 1989; Van Helden et al., 1992).

The present experiments have shown that neither toxogonin nor HI-6 had any effects on the release of [^{3}H]acetylcholine both in a control and a K^+-depolarised muscarinic cholinergic synapse. An effect of HI-6 on the release of acetylcholine has previously been suggested as one of the possible mechanisms in the neruromuscular junction in the rat diaphragm (Melchers et al., 1991), where HI-6 restored the failure of the neuromuscular transmission following tetanic contractions after irreversible acetylcholinesterase inhibition. Although there may be differences in this respect between the cholinergic synapses in smooth muscle and striated muscles (Wessler, 1989), and differences depending on whether or not the junctional acetylcholinesterases in both types of synapses are inhibited (Bowman et al., 1990; Wessler, 1989) the present results suggest that the effect of these oximes in the rat diaphragm is probably due to a post-junctional effect in the neuromuscular junction rather than an alteration of a prejunctional mechanism. The oximes might have an effect directly on the nicotinic receptors in the diaphragm as previously suggested (Alkondon et al., 1988, Tattersall, 1993).

As earlier shown (Aas et al., 1987) soman reduces the K^+-evoked release of [^{3}H]acetylcholine from cholinergic nerves. This effect of soman was not altered by the presence of toxogonin, HI-6 or HLö-7, providing further evidence to the fact that the oximes do not alter the prejunctional release of acetylcholine during acetylcholinesterase inhibition. The effect of soman as well as the effect of the muscarinic receptor agonist oxotremorine on the K^+-evoked release of [^{3}H]acetylcholine is most likely only due to activation of prejunctional muscarinic receptors (Aas and Maclagan, 1990). The enhanced synaptic concentration of acetylcholine, due to inhibition of the acetylcholinesterase activity, activates therefore the prejunctional muscarinic M_2 receptors, since methoctramine (an M_2 receptor antagonist) and not pirenzepine (an M_1 receptor antagonist) increased the release of [^{3}H]acetylcholine (Aas and Maclagan, 1990).

HLö-7, in contrast to what was observed in the presence of toxogonin and HI-6, enhanced the K^+-evoked release of [^{3}H]acetylcholine in a concentration-dependent manner. In order to study any effects of HLö-7 on Ca^{2+} ion channels, blockers of these channels were employed. In vivo, it is probably the N-type Ca^{2+} channel which is the most important ion channel in the regulation of the release of neurotransmitters (Dunlap et al., 1995). This suggestion is supported by the present data, which clearly showed that both the L-type and P-type Ca^{2+} channels obviously play

a less important physiological role in the regulation of acetylcholine release from cholinergic nerves in the airways. The present experiments with HLö-7 showed, in contrast to control experiments without oxime, that the HLö-7-enhanced release of [^{3}H]acetylcholine was reduced by verapamil (0.1–1.0 μM) (L-type Ca^{2+} channel blocker), ω-conotoxin GVIA (1.0 μM) (N-type Ca^{2+} channel blocker) and ω-agatoxin IV-A (0.2 μM) (P-type Ca^{2+} channel blocker). This means that HLö-7 might generate a new or open existing silent or active L-, N- or P-type endogenous Ca^{2+} channels in the nerve terminal membrane and thereby increase the influx of Ca^{2+} and subsequently increase the release of acetylcholine. HLö-7, but not toxogonin and HI-6, might therefore increase the quantal content and thereby the concentration of acetylcholine in the synaptic cleft and enhance the post-junctional stimulation during soman intoxication.

In order to study the molecular mechanism of the HLö-7-enhanced K^+-evoked release of [^{3}H]acetylcholine, several inhibitors of protein kinases were employed. Protein kinase C may play an important role in vivo in the release and/or in the modulation of the release of acetylcholine, as has previously been shown for protein kinase C in the 4-aminopyridine-evoked release of neurotransmitter glutamate (Barrie et al., 1991). Such a possible role for protein kinase C in the release of acetylcholine has been shown in the present study. This conclusion can be drawn from the results in which phorbol 12,13-dibutyrate (an activator of protein kinase C) significantly enhanced the K^+-evoked release of [^{3}H]acetylcholine, and from the results where calpostin C (a potent and selective inhibitor of protein kinase C) inhibited the HLö-7-enhanced K^+-evoked [^{3}H]acetylcholine release. The cAMP- and cGMP-dependent protein kinase inhibitor HA 1004 has a low affinity for protein kinase C and serves, therefore, as an excellent negative control of H-7, which blocks both protein kinase C and cAMP-dependent protein kinases. HA 1004 had no effect on the HLö-7-enhanced K^+-evoked release of [^{3}H]acetylcholine, while H-7 reduced the release to some extent. This reduction is probably due to the effect of H-7 on the protein kinase C activity. It is therefore likely that protein kinase C plays a more important role than protein kinase A in the effect of HLö-7 on the release of [^{3}H]acetylcholine. In addition to the effect on protein kinase C, HLö-7 might enhance the K^+-evoked release of neurotransmitter through activation of Ca^{2+}/calmodulin-dependent protein kinase II, an enzyme known to phosphorylate synapsin I (Greengard et al., 1987). Thus, increasing the phosphorylation of synapsin I should increase the neurotransmitter release. This effect of HLö-7 was illustrated by using KN-62, which is a selective inhibitor of the rat brain Ca^{2+}/calmodulin-dependent protein kinase II where the inhibition is by binding directly to the calmodulin site of the enzyme (Tokumitsu et al., 1990).

In conclusion, HLö-7 enhances the release of [^{3}H]acetylcholine by opening of L-, N- or P-type Ca^{2+} channels in the nerve terminal membrane. Following depolarisation the enhanced influx of Ca^{2+} activates protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase. This protein kinase activation is by some yet unknown mechanism coupled to the release of acetylcholine and probably also to the presynaptic muscarinic M_2 receptor.

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