Action of an irreversible acetylcholine esterase inhibitor, soman, on muscarinic hyperpolarization in cat bladder parasympathetic ganglia

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1 Intracellular recording techniques were used to examine the action of an irreversible acetylcholine esterase (AChE) inhibitor, soman, on the hyperpolarizations mediated through muscarinic cholinergic receptors in cat bladder parasympathetic neurones.

2 Soman (0.1–10 μM) depressed the amplitude and prolonged the duration of the muscarinic slow inhibitory postsynaptic potential (s-i.p.s.p.) elicited by a preganglionic tetanus (40 Hz for 1 s) in the presence of mecamylamine (20 μM), phentolamine (1 μM) and caffeine (1 mM), in a dose-dependent manner. The effect of soman on the amplitude of the s-i.p.s.p. was partially reversible, while the effect on the duration was irreversible.

3 Soman hyperpolarized the membrane and decreased input resistance, but this effect could not account for soman-induced inhibition of the s-i.p.s.p.

4 Soman depressed the amplitude and prolonged the duration of a muscarinic hyperpolarization induced by pressure application of acetylcholine (ACh) in the presence of mecamylamine, phentolamine and caffeine. The time course of this effect paralleled that on the synaptically-evoked muscarinic s-i.p.s.p.

5 A reversible AChE inhibitor, pyridostigmine (10–100 μM), also depressed the amplitude and prolonged the duration of a muscarinic hyperpolarization induced by either preganglionic stimulation or ACh pressure application. These actions were reversible, and not accompanied by a significant change in membrane potential or input resistance.

6 The inhibitory action of soman (1 μM) on the muscarinic hyperpolarization was prevented by pyridostigmine (10 μM), but not by atropine (1 μM).

7 These results demonstrate that soman prolongs not only the muscarinic hyperpolarization, but also inhibits its amplitude through a postsynaptic action, probably through AChE inhibition, in cat bladder parasympathetic neurones.

Introduction

It is known that chronic treatment with irreversible organophosphorus acetylcholine esterase (AChE) inhibitors, DFP (diisopropylfluorophosphate; Yamada *et al.*, 1983; Sivam *et al.*, 1983) and soman (pinacolylmethylphosphonofluoridate; Churchill *et al.*, 1984; Aas *et al.*, 1987), reduces the number of muscarinic acetylcholine (ACh) receptors (see Russell & Overstreet, 1987, for review). This is attributed to persistent cholinomimetic activity resulting from AChE inhibition (Klein *et al.*, 1979). Such down-regulation of muscarinic receptors was not observed after acute administration (Sivam *et al.*, 1983; Aas *et al.*, 1987). These results were obtained from binding studies of muscarinic ACh receptors. To our knowledge, there are few electrophysiological studies which examine an effect of AChE inhibitors on a muscarinic response. Recently, we found with intracellular recording techniques that the application of soman immediately inhibited the membrane hyperpolarization mediated by the muscarinic receptors in cat bladder parasympathetic neurones (Kumamoto & Shinnick-Gallagher, 1988). It is well known that AChE inhibitors induce not only the persistence of ACh in the synaptic cleft due to AChE inhibition, but also other actions (see Karczmar, 1984, for review), including a blockade of the nicotinic ACh receptor-ion channel complex (Kuba *et al.*, 1974; Albuquerque *et al.*, 1987). Therefore, the acute soman-induced depression of muscarinic hyperpolarization may be due to an action independent of AChE inhibition. This study was undertaken to examine elec-

trophysiologically the mechanism underlying the decrease in muscarinic response immediately following application of soman to cat bladder parasympathetic neurones. In order to determine the involvement of AChE inhibition in the action of soman, the effect of a reversible AChE inhibitor, pyridostigmine, on the muscarinic hyperpolarization was also examined.

Methods

Adult cats of either sex were used in all experiments. The urinary bladder was dissected from the cat under anaesthesia induced by an intraperitoneal injection of α -chloralose (66 mg kg⁻¹) and pentobarbitone (5.5 mg kg⁻¹). A single ganglion and accompanying preganglionic nerve were visualized under a microscope by inflating the urinary bladder with warm saline injected through a polyethylene catheter inserted into the urethra. The ganglion and preganglionic nerve were dissected free of surrounding tissue. The isolated preparation was placed in a physiological solution gassed with 95% O₂ – 5% CO₂ at room temperature and remained viable for at least two days.

Drug application

The ganglion with preganglionic nerve was transferred to an organ bath of approximately 0.5 ml in volume and continuously superfused at a rate of about 3 ml min⁻¹ with preheated oxygenated Krebs solution maintained at 35–36°C at the

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Table 1 Recovery of the amplitude and the duration of the muscarinic hyperpolarization from soman (0.01–10 μM) treatment

	Control (pre-soman)	15–35 (0.01 μM)	Recovery* (post-soman) Treatment time (min)		
			20–30 (0.1 μM)	10–15 (1 μM)	5–10 (10 μM)
Amplitude (mV)	11.9 $\pm$ 0.5 (68)	10.4 $\pm$ 1.0 (5)	6.3 $\pm$ 1.4 (6)	5.7 $\pm$ 0.6 (16)	5.1 $\pm$ 1.3 (5)
Duration (s)	4.8 $\pm$ 0.2 (68)	7.7 $\pm$ 0.6 (5)	26.9 $\pm$ 5.2 (6)	28.5 $\pm$ 3.7 (16)	41.1 $\pm$ 15.2 (5)

The muscarinic hyperpolarization (s-i.p.s.p.) was elicited by a preganglionic tetanus (40 Hz for 1 s) in the presence of mecamylamine (20 μM), phentolamine (1 μM) and caffeine (1 mM). The stimulus strength used was one at which a maximal s-i.p.s.p. was induced. Each value is expressed as mean $\pm$ s.e.mean. The number in parentheses is the number of cells examined. * Soman, 0.1, 1 and 10 μM , superfusion with different treatment times had a comparable inhibitory effect on the s-i.p.s.p. (see Figure 1c, and Figures 2 and 3 in Kumamoto & Shinnick-Gallagher, 1988), whereas a 15–35 min treatment with 0.01 μM soman had almost no effect on the amplitude of s-i.p.s.p. Measurements during recovery were obtained 0.2–6 h after soman treatment.

recording site. Drugs were dissolved in the superfusate. The exchange of the superfusing solution was complete within 2.5 min. Acetylcholine (ACh) was applied by pressure pulse (10–20 psi pressure; 3–50 ms duration; Picospritzer, General

Valve) from low-resistance micropipettes (filled with 0.1 mM ACh Cl) placed close to the neurone impaled by the micro-electrode.

Electrical measurements

Neurons on the surface of the ganglion were impaled with glass microelectrodes (50–100 M Ω) filled with 0.5 M K₂SO₄. Electrical activity was recorded by an Axoclamp-2A pre-amplifier. Synaptic responses were elicited by applying rectangular pulses, of 0.5 ms duration and various strengths, to the preganglionic nerve held within a small polyethylene tube filled with the physiological solution and containing a Ag/AgCl wire. Data were stored on a video cassette, by use of a Video Cassette Format Instrumentation Recorder (Model 420B, A. R. Vetter Co.), for subsequent analysis. Figures were reproduced from pen recorder tracings. All data are expressed as means $\pm$ s.e.mean. Statistical significance was determined by Student's paired *t* test and *P* values less than 0.05 were considered significant. In all cases *n* refers to number of cells examined.

Solutions

The normal Krebs solution had the following composition (in mM): NaCl 117, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.5. Drugs used were mecamylamine HCl, atropine sulphate, caffeine, acetylcholine Cl (Sigma, St. Louis, MO, U.S.A.), phentolamine HCl (Summit, NJ, U.S.A.) and pyridostigmine bromide (Hoffman-La Roche, Nutley, NJ, U.S.A.). Soman was obtained from the U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, Maryland, U.S.A.

Results

In Krebs solution containing the nicotinic receptor antagonist, mecamylamine (20 μM), as well as phentolamine (1 μM) and caffeine (1 mM) which blocked α -adrenergic (Akasu *et al.*, 1986b) and purinergic (Akasu *et al.*, 1984) slow hyperpolarizations, stimulation of the preganglionic nerve trunk repetitively (40 Hz for 1 s) elicited a slow muscarinic inhibitory postsynaptic potential (s-i.p.s.p.; Gallagher *et al.*, 1982; Shinnick-Gallagher *et al.*, 1987; Kumamoto, 1989). The amplitude of the s-i.p.s.p. was 11.9 $\pm$ 0.5 mV (*n* = 68; resting membrane potential (RMP) = -56.8 $\pm$ 0.8 mV), and its duration was 4.8 $\pm$ 0.2 s at maximal stimulus strengths (see Table 1).

Figure 1 Soman depressed the muscarinic hyperpolarizations (s-i.p.s.p.s) elicited by a preganglionic tetanus (40 Hz for 1 s) in the presence of mecamylamine (20 μM), phentolamine (1 μM) and caffeine (1 mM), in a dose-dependent manner. (a) Dose-response curve for the action of soman. Each point shows the mean of the % changes in the amplitude of the s-i.p.s.p. 5 min after initiating soman superfusion. Vertical lines show s.e.mean (at 0.1 μM soman the s.e.mean was within the size of the symbol). Corresponding durations of s-i.p.s.p. at 0.01, 0.1 and 1 μM soman were 103 $\pm$ 3, 117 $\pm$ 3 and 280 $\pm$ 31% of control, respectively. Since soman (10 μM) almost completely abolished the s-i.p.s.p., it was difficult to assess accurately the prolongation of the s-i.p.s.p. Values in parentheses are numbers of cells tested. The stimulus strength used was one at which a maximal s-i.p.s.p. was elicited. (b) Effect of soman (0.01 μM) on the s-i.p.s.p. 28 min after initiation of treatment. The resting membrane potential (RMP: -48 mV) remained constant during soman superfusion. (c) Action of soman (10 μM) on the s-i.p.s.p. This effect was accompanied by a membrane hyperpolarization (7 mV); RMP = -68 mV. Stimulus strength used was 50 V. Input resistance (R_{in}) was monitored by anelectronic potentials (downward deflection) resulting from injection of constant current hyperpolarizing pulses (100 ms duration). The presynaptic tetanus was applied at (○) shown under each trace.

Action of soman on the s-i.p.s.p. elicited by the preganglionic tetanus

The addition of the irreversible acetylcholine esterase (AChE) inhibitor, soman, to the superfusate depressed and prolonged the muscarinic s-i.p.s.p. (Figures 1, 2, 3 and 5; Kumamoto & Shinnick-Gallagher, 1988). In general, the higher the concentration of soman, the faster its onset of action. The inhibitory effect of soman on the amplitude of s-i.p.s.p. was observed more quickly with increased concentrations. A high concentration ($10\ \mu\text{M}$) of soman rapidly depressed the s-i.p.s.p. within 3 min after initiation of treatment (Figure 1c; see Figures 2 and 3 in Kumamoto & Shinnick-Gallagher, 1988, for actions of soman 1 and $0.1\ \mu\text{M}$, respectively) and this was accompanied by a prolongation of the s-i.p.s.p. In contrast, superfusion of a low concentration ($0.01\ \mu\text{M}$) of soman for 28 min had almost no effect on amplitude, but slowed the rising phase of the s-i.p.s.p. when compared to the control response (Figure 1b). A comparison of the % change in the amplitude of the s-i.p.s.p. produced by soman (0.01 – $10\ \mu\text{M}$) 5 min after the commencement of soman superfusion showed that the action of soman was clearly dose-dependent (Figure 1a). After removal of soman from the superfusion solution, the amplitude of the s-i.p.s.p. partially recovered with a slow time course, but the duration remained prolonged (Table 1).

The magnitude of the muscarinic s-i.p.s.p. was dependent on the stimulus strength applied to the preganglionic nerve trunk (cf. Kumamoto, 1989). The amplitude increased with increasing stimulus strength and attained a maximum value (Figure 2; $n = 14$). Soman ($1\ \mu\text{M}$) depressed this maximum response (Figure 2; $n = 4$), suggesting that soman treatment elicited a non-competitive decrease in the muscarinic synaptic response.

Figure 2 Effect of soman ($1\ \mu\text{M}$) on the muscarinic hyperpolarization (s-i.p.s.p.) elicited by preganglionic tetani (40 Hz for 1 s) of various stimulus strengths in the presence of mecamylamine ($20\ \mu\text{M}$), phentolamine ($1\ \mu\text{M}$) and caffeine ($1\ \text{mM}$). (A) Muscarinic s-i.p.s.p.s are plotted against stimulus strengths of the preganglionic tetani in control ($\circ$; RMP = $-53\ \text{mV}$), and 10 min ($\bullet$; RMP = $-54\ \text{mV}$) after the preparation was treated with soman for 10 min (depressed the s-i.p.s.p.s almost completely). Curves were drawn by eye. (B and C) Records, (a) to (c), in (B) and (d) to (f), in (C) are sample traces of s-i.p.s.p.s in control and after washout of soman, respectively, at corresponding stimulus strengths shown in (a) to (f) of (A). The value next to each record indicates the stimulus strength used to elicit the s-i.p.s.p.s. Downward deflections are anelectronic potentials resulting from constant current hyperpolarizing pulses (100 ms duration) as a measure of R_{in} . The preganglionic tetanus was applied at ($\circ$) shown under each trace.

Figure 3 Effect of soman ($1\ \mu\text{M}$) on muscarinic hyperpolarizations elicited by a preganglionic tetanus (40 Hz for 1 s; 50 V stimulus strength) and by puff application (50 ms duration; 10 psi pressure) of acetylcholine (ACh) in the presence of mecamylamine ($20\ \mu\text{M}$), phentolamine ($1\ \mu\text{M}$) and caffeine ($1\ \text{mM}$). (A) A muscarinic s-i.p.s.p. ($\circ$) induced by a preganglionic tetanus was depressed by soman with a time course parallel to that for the muscarinic hyperpolarization ($\bullet$) elicited by puff application of ACh. (B) Records (a) to (f) of (A), respectively. RMP: $-56\ \text{mV}$ in control; $-65\ \text{mV}$ during soman superfusion for 7 min; $-61\ \text{mV}$, 27 min after wash of soman. R_{in} was monitored by anelectronic potentials (downward deflection) resulting from injection of constant current hyperpolarizing pulses (120 ms duration). The preganglionic tetanus and ACh puff were applied at ($\circ$) and ($\bullet$), respectively, shown under each trace.

Soman ($1\ \mu\text{M}$) hyperpolarized the membrane ($6.5 \pm 1.1\ \text{mV}$, $n = 13$; $P < 0.001$; RMP = $-58.2 \pm 2.2\ \text{mV}$) and decreased input resistance ($89.0 \pm 4.0\%$, $n = 13$, of control; $P < 0.02$) in the presence of mecamylamine, phentolamine and caffeine. Injection of current to return the membrane potential to the control value did not affect the soman-induced inhibition of the s-i.p.s.p. Measurements of membrane potential and input resistance obtained 0.45–6 h after washout of soman were not significantly different from those of the control, although the soman-induced inhibition and prolongation of the s-i.p.s.p. persisted.

Action of soman on the muscarinic hyperpolarization elicited by puff application of acetylcholine

Soman also depressed and prolonged muscarinic hyperpolarization elicited by puff application of acetylcholine (ACh; Figures 3 and 4). The maximal amplitude ($10.0 \pm 1.1\ \text{mV}$, $n = 6$; RMP = $-54.0 \pm 2.4\ \text{mV}$) and duration ($6.3 \pm 1.2\ \text{s}$) of the muscarinic hyperpolarization elicited by ACh puff application were, respectively, $46 \pm 4\%$ and $240 \pm 34\%$, of control 5 min after the initiation of soman ($1\ \mu\text{M}$) superfusion. The effect of soman on the amplitude of the ACh hyperpolarization was not significantly different from its effect ($52 \pm 3\%$; Figure 1a) on the synaptically-evoked maximal muscarinic s-i.p.s.p. ($P > 0.10$). Furthermore, muscarinic hyperpolarizations elicited by ACh released from either the nerve terminal or a micropipette located near the neurone were depressed by soman with a similar time course (Figure 3; $n = 4$).

The effect of soman on the relationship between the duration of the ACh pressure application and the amplitude of the muscarinic hyperpolarization is plotted in Figure 4. The amplitude of the muscarinic hyperpolarization increased with

Figure 4 Effect of soman ($1 \mu\text{M}$) on the muscarinic hyperpolarizations elicited by acetylcholine (ACh) puff application (15 psi pressure) of various durations in the presence of mecamylamine ($20 \mu\text{M}$), phentolamine ($1 \mu\text{M}$) and caffeine (1 mM). (A) The amplitude of the muscarinic hyperpolarization is plotted against the duration of the ACh pressure application in control ($\circ$; RMP = -56 mV) and 15 min after initiating soman superfusion (RMP = -60 mV ; $\bullet$: responses measured when the RMP was returned to -56 mV by injecting a cathodal current). Curves are drawn by eye. (B) Records (a) to (f) are sample traces of muscarinic hyperpolarizations elicited by ACh puff applied with durations corresponding to those shown in the graph. ACh puff was applied at ($\bullet$) shown under each trace. Downward deflections are anelectronic potentials elicited by constant current hyperpolarizing pulses (100 ms duration) as a measure of R_{in} .

longer ACh puff durations and saturated to a steady value ($n = 19$); soman ($1 \mu\text{M}$) depressed this maximum value (Figure 4; $n = 3$). This suggests that soman treatment results in a non-competitive decrease of the muscarinic response.

Soman does not affect the extrapolated reversal potential of the s-i.p.s.p.

Muscarinic s-i.p.s.ps (amplitude: $12.1 \pm 1.6 \text{ mV}$, $n = 11$; duration: $5.4 \pm 0.5 \text{ s}$; RMP = $-58.8 \pm 1.8 \text{ mV}$) decreased with membrane hyperpolarization and the extrapolated reversal potential was $-99.9 \pm 2.9 \text{ mV}$ ($n = 11$; cf. Gallagher *et al.*, 1982); this value was not significantly different from that, $-103.8 \pm 3.2 \text{ mV}$ ($n = 13$), in neurones in which muscarinic s-i.p.s.ps ($6.6 \pm 0.7 \text{ mV}$; $39.1 \pm 6.4 \text{ s}$; RMP = $-59.2 \pm 1.6 \text{ mV}$) had been depressed and prolonged by treatment with soman ($1 \mu\text{M}$; $P > 0.10$; Figure 5). The relationship between the amplitude of the s-i.p.s.p. and membrane potential was linear in control and after soman treatment, suggesting that the effect of soman on the muscarinic hyperpolarization was not voltage-dependent.

Effect of a second application of soman

After washout of soman, the amplitude of the depressed muscarinic s-i.p.s.p. partially reversed over a long period of

Figure 5 Extrapolated reversal potential of the muscarinic hyperpolarization (s-i.p.s.p.) elicited by a preganglionic tetanus (40 Hz for 1 s; 50 V stimulus strength) in the presence of mecamylamine ($20 \mu\text{M}$), phentolamine ($1 \mu\text{M}$) and caffeine (1 mM) in control (RMP = -50 mV), and 34 min (RMP = -60 mV) after an 8 min treatment with soman ($1 \mu\text{M}$) during which the s-i.p.s.p. was almost completely depressed. (A) The s-i.p.s.ps are plotted against membrane potential in control ($\circ$) and after wash of soman ($\bullet$). This cell had a more hyperpolarized reversal potential of s-i.p.s.p. compared to the mean value (-99.9 mV) obtained from many cells, or the equilibrium potential for K^+ (-95.3 mV ; Akasu *et al.*, 1986a) estimated from the reversal potential of the after-hyperpolarization of the action potential. This may be due to the fact that sites of synapse involved in the production of the s-i.p.s.p. exist in regions remote from the soma into which the microelectrode was impaled. (B and C), Records, (a) to (c), in (B) and (d) to (f), in (C) are sample traces of s-i.p.s.ps in control and after washout of soman, respectively, recorded at membrane potentials corresponding to those shown in (a) to (f) of (A). The value next to each record indicates the membrane potential at which s-i.p.s.p. was measured. The preganglionic tetanus was applied at ($\circ$) shown under each trace.

time (Table 1). However, the partially-recovered s-i.p.s.p. (amplitude: $4.5 \pm 0.8 \text{ mV}$, $n = 5$; duration: $30.9 \pm 11.1 \text{ s}$; RMP = $-61.5 \pm 2.6 \text{ mV}$) was not further inhibited by a second application of soman (amplitude: $4.1 \pm 0.6 \text{ mV}$, $n = 5$; $P > 0.10$; not shown). An enhanced nicotinic depolarization produced by applying soman in normal Krebs solution (Kumamoto & Shinnick-Gallagher, 1988) was also unaffected by further application of soman (not shown).

Action of pyridostigmine on the muscarinic hyperpolarization

The reversible AChE inhibitor, pyridostigmine, (Taylor, 1984) also depressed and prolonged the muscarinic s-i.p.s.p. elicited by a preganglionic tetanus, in a dose-dependent manner (Figure 6a). This action was not accompanied by a significant change in membrane potential (from -54.3 ± 1.3 to $-56.1 \pm 1.9 \text{ mV}$, $n = 12$) or input resistance ($97 \pm 4\%$ of control, $n = 11$) even when pyridostigmine was applied at a high concentration ($100 \mu\text{M}$). The affinity of pyridostigmine at inhibiting the s-i.p.s.p. was less than that of soman by a factor of over 1000.

Pyridostigmine ($100 \mu\text{M}$) also depressed the amplitude ($8.5 \pm 1.6 \text{ mV}$, $n = 4$; RMP = $-52.5 \pm 1.5 \text{ mV}$) of the maximal muscarinic hyperpolarization elicited by pressure

Figure 6 Dose-response curves for the effect of pyridostigmine on the muscarinic hyperpolarizations observed in the presence of mecamylamine (20 μM), phentolamine (1 μM) and caffeine (1 mM). (a) Muscarinic hyperpolarizations (s-i.p.s.p.s) were elicited with a preganglionic tetanus (40 Hz for 1 s). Values in parentheses are numbers of cells tested. Each point represents the mean of the % changes in the amplitude (○) and duration (●) of the s-i.p.s.p. 10 min during treatment with pyridostigmine. The vertical lines show s.e.mean unless it was within the size of symbol. The dotted lines indicate 100%. The stimulus strength used was one at which a maximal s-i.p.s.p. was elicited. (b) Effect of pyridostigmine (100 μM) on muscarinic hyperpolarizations elicited by acetylcholine (ACh) applied in a pressure pulse (40 ms duration; 18 psi pressure). Muscarinic hyperpolarizations were depressed and prolonged 10 min after adding pyridostigmine to the superfusion solution (middle) compared to control (left). The muscarinic hyperpolarization returned to control value 20 min after washout of pyridostigmine (right). RMP (= -49 mV) remained constant throughout pyridostigmine superfusion. Downward deflections are anelectronic potentials resulting from constant current hyperpolarizing pulses (100 ms duration) as a measure of R_{in} . The puff of ACh was applied at (●) shown under each trace.

application of ACh by $52 \pm 9\%$ ($P < 0.02$) and prolonged the duration (5.0 ± 0.5 s) by $105 \pm 18\%$ ($P < 0.01$; Figure 6b). The inhibitory effect of pyridostigmine on the muscarinic hyperpolarization induced by a pressure pulse was not significantly different from that, $58 \pm 7\%$ (Figure 6a), for a synaptically-evoked maximal muscarinic s-i.p.s.p. ($P > 0.10$). These data indicate that pyridostigmine acts on the post-synaptic membrane.

Figure 7 shows the time course of the action of pyridostigmine (100 μM) on the amplitude and duration of the muscarinic s-i.p.s.p. elicited by a preganglionic tetanus. The inhibitory effect on the amplitude and the prolonged duration of the s-i.p.s.p. observed with pyridostigmine treatment were almost completely reversed 40 min after washout of pyridostigmine; this is consistent with the well-known reversible inhibitory action of pyridostigmine on AChE (Taylor, 1984). The amplitude of the s-i.p.s.p. recovered faster from the effects of pyridostigmine than the duration. This may be due to the fact that the duration of s-i.p.s.p. is more sensitive to pyridostigmine than the amplitude (see Figure 6a).

Protection from soman effects by pyridostigmine

Since both soman and pyridostigmine depressed and prolonged muscarinic hyperpolarizations, it was possible that these actions of soman could be altered by pretreatment with pyridostigmine. When soman (1 μM) was applied in the presence of pyridostigmine (10 μM), the amplitude of the s-i.p.s.p. was depressed to $33 \pm 7\%$ ($n = 5$) of control (in post-pyridostigmine) 10 min after the initiation of soman super-

Figure 7 Reversible effects of pyridostigmine (100 μM) treatment on the muscarinic hyperpolarization (s-i.p.s.p.) elicited by a preganglionic tetanus (40 Hz for 1 s) in the presence of mecamylamine (20 μM), phentolamine (1 μM) and caffeine (1 mM). (A) Mean amplitude (hatched columns) and duration (open columns) of the s-i.p.s.p.s obtained from five cells in control (RMP = -54.2 ± 2.5 mV), during and after pyridostigmine treatment. Vertical bars denote s.e.mean. Dotted lines show the averaged amplitude and duration of s-i.p.s.p. in control. (B) Sample traces of s-i.p.s.p.s elicited by a preganglionic tetanus (50 V stimulus strength) in control, 10 min during, and 10, 20, 30 and 40 min after pyridostigmine treatment. RMP (= -50 mV) remained constant throughout pyridostigmine superfusion. Downward deflections are anelectronic potentials resulting from constant current hyperpolarizing pulses (100 ms duration) as a measure of R_{in} . The preganglionic tetanus was applied at (○) shown under each trace.

fusion (not shown); this depression was significantly greater than that ($6 \pm 3\%$, $n = 9$; 10 min during soman superfusion) observed when soman (1 μM) was applied alone ($P < 0.01$). Furthermore, the amplitude of the s-i.p.s.p. recovered to $70 \pm 8\%$ ($n = 5$) of control (in post-pyridostigmine) 20 min after washout of soman when pyridostigmine was still present (not shown). This amount of reversal was also greater than the corresponding recovery ($35 \pm 9\%$, $n = 8$) observed following soman application which was not preceded by pyridostigmine superfusion ($P < 0.05$). After a wash of 0.5–4 h after all the treatments, the remaining s-i.p.s.p. had an amplitude and a duration of 11.6 ± 1.6 mV ($n = 8$; RMP = -52.8 ± 2.1 mV) and 14.4 ± 2.6 s, respectively. This s-i.p.s.p. was prolonged compared to control (in pre-pyridostigmine; 4.8 ± 0.2 s, $n = 68$; Table 1; $P < 0.01$), but had an amplitude comparable to that of control (11.9 ± 0.5 mV; Table 1; $P > 0.1$). These results indicate that pyridostigmine protects against the irreversible actions of soman on the amplitude of the muscarinic hyperpolarization.

Does atropine inhibit the action of soman?

The muscarinic receptor antagonist, atropine, blocks the muscarinic s-i.p.s.p. in cat bladder neurones (Gallagher *et al.*, 1982; Kumamoto, 1989). It was possible that the action of soman was direct, on the same site as atropine and thus, soman might depress the hyperpolarization by blocking the muscarinic receptor. Superfusion of atropine (1 μM) completely blocked the s-i.p.s.p. within a few minutes. When soman (1 μM) was applied for 10 min to the atropine-treated preparation and then soman and atropine were washed out over 1 h, the

muscarinic hyperpolarization was depressed (6.4 ± 1.1 mV, $n = 12$; RMP = -57.3 ± 1.6 mV) and prolonged (34.4 ± 5.8 s). These values were not significantly different from the corresponding values (Table 1; $P > 0.10$) observed after treatment with only soman ($1 \mu\text{M}$). However, even in the presence of atropine, soman ($1 \mu\text{M}$) hyperpolarized the membrane by 4.7 ± 1.5 mV ($n = 3$; RMP = -57.3 ± 2.3 mV).

Discussion

The present results demonstrate that in cat bladder parasympathetic neurones, the irreversible acetylcholine esterase (AChE) inhibitor, soman, depresses the amplitude and prolongs the duration of the slow muscarinic inhibitory postsynaptic potential (s-i.p.s.p.), elicited by a preganglionic tetanus in a dose-dependent manner. The threshold concentration for prolonging the duration of the s-i.p.s.p. was lower than the concentration required to depress its amplitude. The prolongation of s-i.p.s.p. by soman is expected from its ability to inhibit AChE and thus increase the lifetime of acetylcholine (ACh) in the synaptic cleft. Soman also induced a membrane hyperpolarization associated with an increased input conductance. The observed changes in these membrane properties could not explain the soman-induced inhibition of s-i.p.s.p.

Soman-induced membrane hyperpolarization

Membrane potential changes induced by soman seem to depend on the preparations used. In rat superior cervical (Yarowsky *et al.*, 1984) or bullfrog sympathetic (Heppner & Fiekers, 1988) ganglia, soman produced membrane depolarization, accompanied by a decrease in input resistance; the former action was different from that in cat bladder parasympathetic neurone. The soman-induced hyperpolarization in bladder neurones was not due to an excessive concentration of ACh in the synaptic cleft resulting from an inhibition of AChE activity, because the hyperpolarization was not blocked by atropine. Furthermore, the reversible AChE inhibitor, pyridostigmine, did not hyperpolarize the membrane. It is also unlikely that this action of soman was due to release of endogenous (noradrenergic or purinergic) neurotransmitters known to hyperpolarize these neurones (cf. Akasu *et al.*, 1984; 1986b; Kumamoto, 1989) because this action of soman was observed in the presence of phentolamine and caffeine. Hyperpolarization following soman superfusion was also observed in drug-free Krebs solutions (Kumamoto & Shinnick-Gallagher, 1988). Although these data do not rule out the possibility that some unknown substance may be released by soman, they do suggest that the membrane hyperpolarization may be due to a direct action of soman.

Soman-induced depression of muscarinic response

In the superior cervical ganglion (Yarowsky *et al.*, 1984) or phrenic nerve-hemidiaphragm (Andersen *et al.*, 1987) of rat, it has been suggested that soman has a presynaptic action. It is unlikely that the inhibition of the muscarinic s-i.p.s.p. observed with soman is due to a decrease in ACh release from nerve terminals, since soman also depressed the muscarinic hyperpolarization elicited by exogenous application of ACh. Furthermore, the depolarization mediated by nicotinic receptors in cat bladder neurones was not depressed, but enhanced by soman (Kumamoto & Shinnick-Gallagher, 1988). These data indicate that soman acts on the postsynaptic membrane to inhibit the muscarinic hyperpolarization.

In cat bladder parasympathetic neurones, the muscarinic s-i.p.s.p. is due to activation of a K channel (Gallagher *et al.*, 1982; Shinnick-Gallagher *et al.*, 1987). Soman may affect the distribution of ions across the membrane and thus depress the

muscarinic response. However, the extrapolated reversal potential for the muscarinic s-i.p.s.p. was unchanged although the s-i.p.s.p. was depressed.

Soman may reduce the number of muscarinic receptors acutely, as has been suggested with chronic soman treatment (Churchill *et al.*, 1984; Aas *et al.*, 1987). Alternatively, if soman increased the dissociation constant for ACh binding to the muscarinic receptor, the muscarinic response would be depressed. The soman-induced inhibition of the maximal amplitude of the muscarinic s-i.p.s.p. elicited by preganglionic stimulation or ACh application, i.e., non-competitive inhibition of muscarinic response, suggested that the number of muscarinic receptors may be affected by soman. Alternatively, this observation may be due to a decrease in the number of functional muscarinic receptors even if the number of receptors themselves is not changed. Aas *et al.* (1987) demonstrated that acute exposure to soman reduced the maximal value of the contraction induced by a muscarinic action of ACh without changing the number of muscarinic receptors in bronchial smooth muscle.

It has been suggested that soman may interact directly with sites on the nicotinic ACh receptor macromolecule (Albuquerque *et al.*, 1987). Therefore, soman may reduce the muscarinic response by acting at a site other than at the AChE active centre. This target site is probably not the site where atropine acts on the muscarinic receptor, because atropine failed to inhibit the effect of soman in bladder neurones. If soman blocks the open state of a K channel involved in the muscarinic response, treatment with soman would result in a decrease in the number of functional muscarinic receptors. Classically, agents associated with non-competitive antagonism of responses induce an open channel block of the receptor-channel complex which is voltage-dependent (cf. Ascher *et al.*, 1979). This is probably not the mechanism of action of soman, because the effect of soman on the muscarinic s-i.p.s.p. was not voltage-dependent.

Is the effect of soman due to AChE inhibition? The effect of soman may be due to persistent cholinomimetic activity resulting from AChE inhibition. Consistent with this hypothesis is our finding that the reversible AChE inhibitor, pyridostigmine, also depressed the amplitude of the muscarinic hyperpolarization; this effect reversed on washing, a result clearly different from the partially reversible action of soman on the amplitude of the muscarinic hyperpolarization. Thus, inhibition of muscarinic response can be observed not only with soman, but also with other AChE inhibitors.

It is known that the action of an irreversible inhibitor on AChE can be prevented by a reversible inhibitor (Harris *et al.*, 1978; Van Meter *et al.*, 1978; Yamada *et al.*, 1983; McGee & Brezenoff, 1987). The irreversible action of soman on bladder neurones was also prevented by pretreatment with pyridostigmine. These results suggest that the effects of soman may be due to inhibition of AChE activity, resulting in an accumulation of ACh in the synaptic cleft and an excessive excitation of muscarinic receptors. It has been suggested that this extreme stimulation of muscarinic receptors results in a down-regulation or loss of muscarinic receptors (Klein *et al.*, 1979). Feigenbaum & El-Fakahany (1984) have observed a decrease in the number of binding sites for muscarinic receptors following a 30 min preincubation of neuroblastoma cells with a muscarinic agonist. Furthermore, a down-regulation of muscarinic receptors has been shown for chronic treatment of organophosphate AChE inhibitors such as DFP (Yamada *et al.*, 1983) and soman (Churchill *et al.*, 1984). The loss of muscarinic receptors produced by chronic DFP treatment was antagonized by the muscarinic receptor antagonist, atropine (Yamada *et al.*, 1983), suggesting that excessive stimulation of receptors was the causative factor. In addition, a soman-induced depression of synaptic transmission in superior cervical ganglia was shown to be inhibited by atropine pretreatment (Yarowsky *et al.*, 1984). In contrast, the soman-induced inhibition of the muscarinic hyperpolarization in

bladder neurones was not suppressed by pretreatment with atropine. Thus, the inhibition of AChE may not necessarily account for this action of soman. However, because AChE activity was still inhibited during washout of atropine, it is possible that the persistent cholinergic activity could still lead to an acute down-regulation of muscarinic receptors.

Recovery from soman action

After washout of soman, the amplitude of the muscarinic hyperpolarization partially recovered, although the duration remained prolonged. This finding is consistent with observations of spontaneous recovery of ganglionic (Lukomskaya *et al.*, 1980) or neuromuscular (Van Dongen *et al.*, 1988) transmission following treatment with irreversible AChE inhibitors. Recovery from soman treatment may occur through an unknown mechanism independent of *de novo* synthesis of

AChE. When soman was applied to bladder neurones a second time, the partially-recovered muscarinic response was not depressed further. The lack of an effect of a second application of organophosphorus AChE inhibitors has also been demonstrated for EEG seizure elicited by DFP in rabbits (Van Meter *et al.*, 1978) and for inhibition of neuromuscular transmission by soman in rats (Van Dongen *et al.*, 1988). These data also suggest that the soman-induced depression of the muscarinic hyperpolarization in bladder neurones is due to inhibition of AChE.

In conclusion, soman not only prolongs the muscarinic hyperpolarization, but also depresses its amplitude in a non-competitive manner; both effects are probably due to inhibition of AChE. A direct action of soman to hyperpolarize the neuronal membrane may not result from AChE inhibition.

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