Central Cholinesterase Inhibition Enhances Glutamatergic Synaptic Transmission

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Kozhemyakin M, Rajasekaran K, Kapur J. Central cholinesterase inhibition enhances glutamatergic synaptic transmission. J Neurophysiol 103: 1748–1757, 2010. First published January 27, 2010; doi:10.1152/jn.00949.2009. Central cholinergic overstimulation results in prolonged seizures of status epilepticus in humans and experimental animals. Cellular mechanisms of underlying seizures caused by cholinergic stimulation remain uncertain, but enhanced glutamatergic transmission is a potential mechanism. Paraoxon, an organophosphate cholinesterase inhibitor, enhanced glutamatergic transmission on hippocampal granule cells synapses by increasing the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) in a concentration-dependent fashion. The amplitude of mEPSCs was not increased, which suggested the possibility of enhanced action potential-dependent release. Analysis of EPSCs evoked by minimal stimulation revealed reduced failures and increased amplitude of evoked responses. The ratio of amplitudes of EPSCs evoked by paired stimuli was also altered. The effect of paraoxon on glutamatergic transmission was blocked by the muscarinic antagonist atropine and partially mimicked by carbachol. The nicotinic receptor antagonist α7-bungarotoxin did not block the effects of paraoxon; however, nicotine enhanced glutamatergic transmission. These studies suggested that cholinergic overstimulation enhances glutamatergic transmission by enhancing neurotransmitter release from presynaptic terminals.

INTRODUCTION

Cholinergic over-stimulation of the CNS causes prolonged seizures in humans and in experimental animals. Inhibition of cholinesterase, the acetylcholine degrading enzyme, by organophosphate insecticides such as parathion and malathion, and more potent nerve agents such as sarin and soman, causes elevation of central acetylcholine levels (McDonough and Shih 1997). Seizures were observed in humans exposed to nerve-agent poisoning during the Iran-Iraq war and in the Tokyo subway attacks where sarin and VX were used (Nozaki et al. 1996; Okumura et al. 1996). Peripheral injection of high doses of pilocarpine led to prolonged seizures in experimental animals. Stimulation of central muscarinic receptors is a well-established method for inducing prolonged seizures in experimental animals. Peripheral injection of high doses of pilocarpine lead to prolonged seizures commonly referred to as status epilepticus (Turski et al. 1989; Turski 1983). Pretreatment with lithium followed by pilocarpine injection also leads to prolonged seizures and neuropathology (Honchar et al. 1983). The cellular mechanisms underlying seizures induced by cholinergic stimulation remain unclear. Functional imaging studies in experimental animals suggest that during these seizures, limbic structures such as the amygdala, piriform cortex, entorhinal cortex, and hippocampus are activated (Clifford et al. 1987). Extensive cell damage and loss is activated in these limbic regions during seizures induced by cholinergic stimulation (Fujikawa 1996; Honchar et al. 1983).

It has been suggested that cholinergic overstimulation leads to increased glutamatergic transmission. The pattern of neuronal activation associated with seizures caused by cholinergic stimulation is similar to that observed with kainic-acid-induced seizures (Lothman and Collins 1981; Lothman et al. 1985). NMDA receptor antagonists protect against neuronal loss caused by cholinergic seizures (Fujikawa et al. 1994). Neurochemical measurements of tissue glutamate levels and extracellular glutamate levels during seizures induced by cholinergic stimulation have yielded mixed results. Tissue glutamate levels appear to be diminished, whereas extracellular glutamate levels are increased during these seizures (McDonough and Shih 1997). Some electrophysiological studies have suggested that muscarinic agonists suppress glutamate release from presynaptic terminals (De Sevilla et al. 2002). Stimulation of nicotinic receptors in the hippocampus enhances glutamate release from presynaptic terminals and enhances synaptic transmission (Alkondon and Albuquerque 2004; Gray et al. 1996; Radcliffe and Dani 1998; Sharma and Vijayaraghavan 2003). However, the effect of muscarinic stimulation or that of organophosphates and nerve agents on glutamatergic transmission in the hippocampus has not been investigated in detail.

Anticholinesterase nerve agents are restricted-use chemicals and surrogates are needed for studies in civilian laboratories. Paraoxon and its derivative parathion are commonly used as organophosphate pesticides; accidental and intentional human poisoning with these agents lead to symptoms identical to nerve agent exposure: sweating, dizziness, vomiting, diarrhea, seizures, cardiac arrest, respiratory arrest, and, in extreme cases, death (Garcia et al. 2003). In guinea pig hippocampal slices, paraoxon causes seizures similar to those caused by the nerve gas soman (Harrison et al. 2004). In this study, we studied the effects and mechanism of action of paraoxon on glutamatergic transmission in the hippocampus.

METHODS

Slice preparation

All studies were performed according to protocols approved by the University of Virginia Animal Use and Care committee. Animals were anesthetized with isoflurane before decapitation and followed by quick removal of the brain, which was sectioned to 350 μm using a Vibratome at 4°C in an oxygenated slicing solution. The solution contained the following (in mM): 120 sucrose, 65.5 NaCl, 2 KCl, 1.1 KH2PO4, 25 NaHCO3, 10 d-glucose, 1 CaCl2, and 5 MgSO4. The slices were stored in oxygenated artificial cerebrospinal fluid (ACSF)
containing the following (in mM): 119 NaCl, 2.5 KCl, 1 KH2PO4, 25 NaHCO3, 10 d-glucose, 2.5 CaCl2, and 1.3 MgSO4; osmolarity was 290–300 in the chamber. Slices were held in this chamber at room temperature (24°C) for 60 min before transfer to the recording chamber on the stage of a microscope (Olympus, Tokyo, Japan). All chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise.

**Whole cell recording**

Whole cell patch-clamp recordings were performed using infrared differential interference contrast (IR-DIC) microscopy and a ×40 water-immersion objective to visually identify dentate granule cells. Slices were continuously superfused with ACSF solution saturated with 95% O2–5% CO2 at room temperature.

Patch electrodes (tipped resistances, 3–6 MΩ) were pulled from borosilicate glass (Sutter Instruments, Novato, CA) on a horizontal Flaming-Brown microelectrode puller (Model P-97, Sutter Instruments), using a three-stage pull protocol. Electrode tips were filled with a filtered internal recording solution consisting of (in mM) 117.5 CsMeSO4, 10 2-hydroxyethyl piperazine-N2-[ethanesulfonic acid] (HEPES), 0.3 N-[glycyl-bis (a-aminomethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 15.5 CsCl, 1.0 MgCl2, pH 7.3 (with CsOH); osmolarity was 290–300 mosM. The electrode shank contained (in mM) 4 ATP Mg2+ salt, 0.3 GTP Na+ salt, and 5 QX-314.

Neurons were voltage clamped to −60 mV for the duration of the recording, which was between 20 and 30 min. Whole cell capacitance and series resistance were compensated by 80% at 10-ms lag. Recording was performed when series resistance after compensation was ≤20 MΩ. Access resistance was monitored with a 10-ms, 10-mV test pulse once every 2 min, and if the series resistance increased by 25% at any time during the experiment, then the recording was terminated. Currents were filtered at 5 kHz, digitized using a Digidata 1322 digitizer (Molecular Devices, Sunnyvale, CA), and acquired using Clampex 8.2 software (Molecular Devices).

Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from granule cells after blocking the GABA_A receptor with the antagonist picrotoxin (50 μM). In preliminary experiments, a combination of 6-cyano-7-nitroquinolinoxide-2,3-dione (CNQX) and 2-amino-5-phosphonovaleric acid (APV) blocked all EPSCs. Miniature EPSCs (mEPSCs) were recorded by blocking action potentials with 1/9262 antagonist picrotoxin (50 mM) 4 ATP Mg2+ osmolarity was 290–300 mosM. The electrode shank contained (in mM) 4 ATP Mg2+ salt, 0.3 GTP Na+ salt, and 5 QX-314.

Data analysis

The digitized current traces were analyzed with MiniAnalysis (Synaptosoft, Decatur, GA). To detect sEPSCs and mEPSCs, a detection threshold three times root mean square (RMS) of baseline noise was used. After detection, frequency and peak amplitude of EPSCs were analyzed for individual neurons. Each detected event in the 10- to 30-min recording was visually inspected to remove false detections.

The dose response curve for paraoxon was generated using a biphasic sigmoidal function in Graphpad Prism software.
of paraoxon ($n = 44$) ranging from 0.01 nM to 30 μM. Paraoxon was found to increase sEPSC frequency in a concentration-dependent manner. This effect can be described by means of a biphasic sigmoidal curve (Fig. 1E). The EC₅₀ and Hill slope values were derived from the Eq. 1 that best fit the observed data by the least-square fit method. The EC₅₀ of the first phase of best fit was 0.3 nM and the Hill coefficient ($I$) was 1.76. The EC₅₀ of the second phase was near 3 μM with a Hill coefficient ($I$) of 1.21.

The effects of paraoxon could not be reversed by washing out the drug. Therefore we tested whether ambenonium, a reversible high-affinity cholinesterase inhibitor, could mimic the effects of paraoxon on increasing the frequency of sEPSCs. Application of ambenonium (500 nM) significantly increased (133% increase, $P < 0.001, n = 5$) the sEPSC frequency (from 0.69 ± 0.1 to 1.61 ± 0.7 Hz), which returned to baseline levels (0.60 ± 0.1 Hz) on washout of the drug from the bath (Table 1). A lower concentration of ambenonium (250 nM) also reversed the increased frequency.

### Table 1. Impact of Cholinergic agonists on sEPSC parameters

<table>
<thead>
<tr>
<th>Condition</th>
<th>Frequency, Hz</th>
<th>Peak Amplitude, pA</th>
<th>Rise Time (90–90%), ms</th>
<th>Decay Time (90–10%), ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (8)</td>
<td>1.15 ± 0.22</td>
<td>15.22 ± 1.09</td>
<td>4.92 ± 0.48</td>
<td>25.89 ± 4.1</td>
</tr>
<tr>
<td>Paraoxon (3 μM)</td>
<td>1.70 ± 0.40*</td>
<td>23.35 ± 0.21*</td>
<td>4.74 ± 0.72</td>
<td>29.44 ± 5.86</td>
</tr>
<tr>
<td>Baseline (7)</td>
<td>0.75 ± 0.19</td>
<td>16.16 ± 0.19</td>
<td>2.90 ± 0.40</td>
<td>10.6 ± 1.30</td>
</tr>
<tr>
<td>Carbachol (50 μM)</td>
<td>1.14 ± 0.26**</td>
<td>15.30 ± 0.14**</td>
<td>3.03 ± 0.34</td>
<td>10.72 ± 1.28</td>
</tr>
<tr>
<td>Baseline (5)</td>
<td>1.49 ± 0.37</td>
<td>14.5 ± 2.74</td>
<td>3.38 ± 0.6</td>
<td>17.96 ± 5.90</td>
</tr>
<tr>
<td>Nicotine (1 μM)</td>
<td>1.84 ± 0.36*</td>
<td>15.55 ± 2.76*</td>
<td>3.43 ± 0.46</td>
<td>20.56 ± 7.71</td>
</tr>
<tr>
<td>Baseline (4)</td>
<td>0.58 ± 0.11</td>
<td>15.43 ± 1.89</td>
<td>2.31 ± 0.27</td>
<td>10.97 ± 1.01</td>
</tr>
<tr>
<td>Ambenonium (250 μM)</td>
<td>1.08 ± 0.31*</td>
<td>15.93 ± 2.02</td>
<td>2.26 ± 0.39</td>
<td>11.14 ± 1.63</td>
</tr>
<tr>
<td>Baseline (5)</td>
<td>0.69 ± 0.10</td>
<td>14.23 ± 1.73</td>
<td>3.54 ± 0.75</td>
<td>11.70 ± 0.43</td>
</tr>
<tr>
<td>Ambenonium (500 μM)</td>
<td>1.61 ± 0.70***</td>
<td>17.50 ± 1.80</td>
<td>2.49 ± 0.44</td>
<td>11.82 ± 2.10</td>
</tr>
</tbody>
</table>

Values are means ± SD. $n$ are in parentheses. sEPSC, spontaneous excitatory postsynaptic potential. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$. 

The ordinate depicts frequency of sEPSCs in the presence of paraoxon expressed as the percent fraction of that before paraoxon application. The solid line, the best fit of the concentration response relationship to a biphasic sigmoidal function.
ibly enhanced the frequency of sEPSCs by 86% (from 0.58 ± 0.11 to 1.08 ± 0.31 Hz, \( P < 0.05, n = 4; \) Table 1).

**Paraoxon effect on mEPSCs**

Because sEPSCs consist of action potential-dependent and action potential-independent events (mEPSCs), we tested whether paraoxon enhanced the amplitude of mEPSCs. To test whether paraoxon increased the action potential-independent release of glutamate, 1 \( \mu \)M tetrodotoxin was added to the external solution to record mEPSCs. After recording mEPSCs from granule cells for 5 min, 3 \( \mu \)M paraoxon was applied (Fig. 2, A and B). The frequency and amplitude of mEPSCs both before and after application of paraoxon were then compared. Paraoxon increased the frequency of mEPSCs in five cells from 1.08 ± 0.08 to 1.32 ± 0.34 Hz (\( P < 0.05; n = 5; \) Fig. 2, B and C). In contrast, application of paraoxon did not significantly change the mean amplitudes of mEPSCs (11.56 ± 1.19 vs. 12.00 ± 1.16 pA; \( P > 0.05; n = 5; \) Fig. 2, B and D). Paraoxon did not alter the rise time of mEPSCs (2.80 ± 0.11 vs. 2.78 ± 0.11 ms) and decay time (13.70 ± 0.56 vs. 12.91 ± 0.39 ms).

**Paraoxon altered glutamate release**

One interpretation of the finding that paraoxon increased sEPSC amplitude (Fig. 3A) but not mEPSC amplitude is that the total amount of neurotransmitter released in response to a single action potential was increased or the number of release sites activated per action potential was increased. Amplitude distribution histograms have been used to analyze changes in presynaptic release mechanisms (Edwards et al. 1990). We therefore investigated whether the distributions of sEPSC amplitudes were different before and after application of paraoxon by resolving the distribution of sEPSCs into multiple peaks. The amplitudes of sEPSCs recorded from control neurons (\( n = 8 \)) were pooled and fitted to an equation for multiple Gaussians using Origin software. The amplitude distribution histogram could be best fit to a sum of 2 Gaussians. The peaks of the Gaussians were centered at 11.48 and 17.6 pA (Fig. 3B). These Gaussians comprised 43.59 and 56.41% of the overall population of sEPSCs. The amplitude distribution histogram of sEPSCs recorded after application of paraoxon was best fit to 3 Gaussians (Fig. 3C), centered at 11.68, 18.96, and at 34.02 pA, and corresponding overall populations were 32.63, 44.58, and 22.79%, respectively.

This analysis suggested that paraoxon caused the appearance of a new, larger peak in the amplitude distribution histogram. Paraoxon did not cause a shift in the location of the first peak of the amplitude distribution histogram, and it did not cause separation between subsequent peaks to change. Combination of these findings was consistent with increased neurotransmitter release and argued against changes in postsynaptic receptors resulting in increased sEPSC amplitude. These studies on sEPSCs were carried out in combined entorhinal cortex-hippocampal slices; therefore cell bodies of both input neurons were intact. Increased frequency of action potentials in presynaptic neurons could explain some observed changes in sEPSCs.

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**FIG. 2.** Paraoxon enhances the frequency of action-potential-independent miniature EPSCs (mEPSCs). A and B: representative mEPSC recordings obtained from dentate granule cells before (control) and after application of paraoxon. C and D: cumulative probability plots of mEPSC frequency (C) and amplitude (D) obtained by pooling data from neurons before (---) and after (- - -) application of paraoxon.

**FIG. 3.** Presynaptic changes underlie paraoxon-induced increase in sEPSC amplitude. A: representative trace of averaged sEPSCs before (---) and after (- - -) application of paraoxon. B and C: amplitude distribution histograms obtained from sEPSCs recorded before (B) and after (C) application of paraoxon. Note the appearance of an additional peak of larger sEPSC after paraoxon application.
To control the contribution of presynaptic neurons, we stimulated the perforant path, the afferent fibers from entorhinal cortex, to test whether eEPSCs were affected by paraoxon.

A minimally effective stimulation pulse (0.2 V; 10 μs) was applied to the perforant pathway by means of a glass electrode filled with ASCF, and evoked responses were recorded. During baseline recording from a cell, 44 stimuli were delivered and 10 failed to evoke a response (22.7% failure rate; see Fig. 4A). After application of paraoxon, 60 stimuli were delivered, and there were 11 failures (18.7%). A decrease in failure rate (total number of failures/stimuli of trials; \(N_0/N\)) was evident in all those cells that showed an increase in the eEPSC amplitude following paraoxon application. The amplitudes of minimal stimulus eEPSCs were measured in cells before and after application of paraoxon. Application of 3 μM paraoxon increased the mean amplitude of eEPSCs from 23.20 ± 1.43 to 35.93 ± 1.58 pA (\(P < 0.05; n = 7\); Fig. 4B). Paraoxon also increased decay time from 8.07 ± 1.96 to 14.52 ± 2.68 ms (\(P < 0.05; n = 7\)) but did not alter rise time (1.37 ± 0.31 vs. 1.60 ± 0.30 ms; \(P > 0.05; n = 7\)). These observations support the hypothesis that paraoxon acts at presynaptic sites and increases action potential-dependent release.

To further confirm that paraoxon enhanced EPSCs by presynaptic mechanisms, paired stimuli were delivered to evoke EPSCs. Figure 4C shows representative averaged pairs of eEPSCs generated at 50-ms intervals using a half-maximal stimulus intensity (the mean of maximal eEPSC was 550 ± 96 pA). For the eight cells, the mean amplitudes of the first peak eEPSC at baseline was 166.8 ± 24.06 pA, and of the second peak it was 203.1 ± 29.17 pA. The paired-pulse facilitation at baseline was 123.1 ± 4.59%, and after application of PXN, it was 90.84 ± 4.63%. Thus paraoxon diminished the degree of paired pulse facilitation by 26.5 ± 5.52% (\(n = 8; P < 0.001\); Fig. 4D).

Nicotinic enhancement of EPSCs

Previous studies demonstrate that paraoxon enhances synaptic transmission and that this enhancement could be mediated by muscarinic receptors, nicotinic receptors, or other mechanisms independent of these receptor subtypes. To determine the type of cholinergic receptor mediating the effect of paraoxon, we examined nicotinic and muscarinic agonists and antagonists. We first tested whether the nicotinic receptor agonist nicotine mimicked the action of paraoxon. Nicotine (1 μM) increased the frequency of sEPSCs (Fig. 5, A, B, and D) and their amplitudes (Table 1). Nicotine did not significantly change the rise and decay time constant of sEPSCs (each comparison \(P > 0.05; n = 5\)).

Increased sEPSC frequency caused by nicotine was completely reversed by 50 nM α-bungarotoxin (αBGT), which binds to the nicotinic acetylcholine receptor with high affinity (Fig. 5, A–D). Preapplication of αBGT prevented nicotine-induced increase in frequency and amplitudes of sEPSCs (Table 2). However, preapplication of αBGT did not prevent increase in sEPSC frequency caused by the application of paraoxon (Table 2; Fig. 5, E–H). Additionally, when αBGT was applied after application of paraoxon, changes in amplitudes and frequency of sEPSCs caused by paraoxon were not reversed (Table 2).

Muscarinic enhancement of EPSCs

We tested whether blocking muscarinic receptors selectively would prevent the action of the paraoxon. Atropine, a muscarinic antagonist, was applied after a 5-min recording of
carbachol only partially mimicked the effects of paraoxon.

duced increase in sEPSC frequency and amplitude. Carbachol application could not overcome the effect of atropine (Fig. 7, A–C). Similarly, application of paraoxon in the presence of atropine did not change eEPSC amplitudes (Fig. 7, D–F) and did not increase the failure rate (15.9%). Moreover, when atropine was applied after paraoxon, it reversed paraoxon action on eEPSC amplitudes from 40.28 ± 2.07 to 32.40 ± 2.54 pA (ANOVA test P < 0.01; n = 4; Fig. 7F).

We then tested whether, like paraoxon, muscarinic agonists acted on EPSCs evoked with minimal stimulation. Carbachol increased mean amplitudes of eEPSCs from 18.43 ± 2.60 pA (P < 0.001; n = 7; Fig. 8, A and B) and reduced the failure rate of evoked responses from 13.3 to 8% (B).

### DISCUSSION

The present study demonstrated that the cholinesterase inhibitor paraoxon enhanced glutamatergic transmission on hippocampal granule cells synapses by increasing the frequency and amplitudes of sEPSCs, principally through presynaptic mechanisms. Furthermore, the effect of muscarinic receptor blockade on EPSCs evoked by minimal stimulation was then studied. The application of the muscarinic antagonist atropine reduced eEPSC amplitudes from 27.44 ± 3.37 to 24.80 ± 2.98 pA (Fig. 7, A–C) and increased the failure rate from 7.2 to 12.2%. Carbachol application did not change eEPSC amplitudes (Fig. 7, A–C).

### TABLE 2. Impact of paraoxon, carbachol, and nicotine

<table>
<thead>
<tr>
<th></th>
<th>Frequency, Hz</th>
<th>Peak Amplitude, pA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Muscarinic Receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (4)</td>
<td>1.27 ± 0.17</td>
<td>14.61 ± 2.62</td>
</tr>
<tr>
<td>Atropine</td>
<td>1.072 ± 0.23</td>
<td>14.74 ± 2.47</td>
</tr>
<tr>
<td>Atropine + carbachol</td>
<td>1.19 ± 0.33</td>
<td>14.51 ± 0.85</td>
</tr>
<tr>
<td>Baseline (5)</td>
<td>0.54 ± 0.14</td>
<td>13.85 ± 1.22</td>
</tr>
<tr>
<td>Atropine</td>
<td>0.46 ± 0.17</td>
<td>13.62 ± 1.62</td>
</tr>
<tr>
<td>Atropine + paraoxon</td>
<td>0.55 ± 0.20</td>
<td>14.79 ± 1.44</td>
</tr>
<tr>
<td>Carbachol</td>
<td>1.49 ± 0.43*</td>
<td>17.76 ± 1.69</td>
</tr>
<tr>
<td>Carbachol + atropine</td>
<td>1.15 ± 0.48</td>
<td>15.45 ± 1.08</td>
</tr>
<tr>
<td>Baseline (4)</td>
<td>1.18 ± 0.32</td>
<td>13.77 ± 1.60</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>1.84 ± 0.60*</td>
<td>15.01 ± 2.03*</td>
</tr>
<tr>
<td>Paraoxon + atropine</td>
<td>1.21 ± 0.36</td>
<td>14.69 ± 1.57</td>
</tr>
</tbody>
</table>

| **B. Nicotinic receptors** |               |                    |
| Baseline (5)              | 1.01 ± 0.34   | 18.63 ± 1.93       |
| α-bungarotoxin            | 1.17 ± 0.22   | 15.65 ± 3.08       |
| α-bungarotoxin + nicotine | 1.28 ± 0.32   | 15.68 ± 2.84       |
| Baseline (4)              | 1.21 ± 0.16   | 12.04 ± 0.09       |
| α-bungarotoxin            | 1.31 ± 0.18   | 12.04 ± 0.10       |
| α-bungarotoxin + paraoxon | 1.69 ± 0.15*  | 11.88 ± 0.10       |
| Baseline (n = 5)          | 1.49 ± 0.38   | 14.59 ± 2.74       |
| Nicotine                  | 1.73 ± 0.42*  | 15.55 ± 2.76*      |
| Nicotine + α-bungarotoxin | 1.51 ± 0.45   | 14.75 ± 1.74       |
| Baseline (4)              | 1.59 ± 0.19   | 11.31 ± 0.09       |
| Paraoxon                  | 2.36 ± 0.33** | 12.08 ± 0.27*      |
| Paraoxon + α-bungarotoxin | 2.28 ± 0.30*  | 12.24 ± 0.42*      |

The impact of the cholinergic agonists paraoxon, carbachol, and nicotine coapplied with antagonists before and after application of antagonists of muscarinic or nicotinic receptors on sEPSC frequency and amplitude. *P < 0.05; **P < 0.005 using repeated measures ANOVA test.
atropine and paraoxon (by paraoxon increased both action-potential-dependent and presynaptic mechanisms). Paraoxon increased EPSC amplitude by altered part by muscarinic receptors. Similarly, muscarinic receptor agonists were found to enhance excitatory neurotransmission.

Paraoxon increased EPSC amplitude by altered presynaptic mechanisms

The results of this study suggest that cholinergic stimulation by paraoxon increased both action-potential-dependent and-independent neurotransmitter release from presynaptic terminals. However, neither paraoxon nor carbachol altered the amplitude of mEPSCs. Further evidence for a presynaptic locus of action is from our observation that neither paraoxon nor carbachol altered the location of the first peak or separation between peaks of the amplitude distribution histograms (Edwards et al. 1990). The amplitude and frequency of sEPSCs was increased by paraoxon. An obvious explanation for increased frequency of sEPSCs recorded from dentate granule cells would be increased action potentials in the presynaptic neurons in the entorhinal cortex and dentate hilus, especially because we had studied combined entorhinal cortex–hippocampus slices. Major excitatory input to dentate granule cells is provided by layer II and III of the entorhinal cortex (Steward and Scoville 1976). Muscarinic stimulation causes depolarization and oscillatory activity in layer II and V neurons in the entorhinal cortex (Klink and Alonso 1997a,b). It is therefore likely that the increased sEPSC frequency observed was in part due to an increased number of action potentials from the entorhinal cortex neurons. The muscarinic antagonist atropine is also known to reduce action potential bursting evoked by organophosphates in the CA1 region of hippocampal slices (Harrison et al. 2004). Indeed, examination of the effect on paraoxon on the firing rate of neurons indicated that the drug enhanced neuronal firing rate. Alternately, increased amplitude of sEPSC could also be a result of the unmasking of previously undetected synapses. However, additional mechanism might be operational. Presynaptic mechanisms such as an increased probability of release (per action potential) maybe an alternate explanation for the increase in sEPSC amplitude. Three lines of evidence further suggested that paraoxon acted on these presynaptic mechanisms: it enhanced the amplitude of evoked EPSCs, reduced their failure rate, and changed the response to paired-pulse stimulation. Increased amplitude of sEPSCs could be due to postsynaptic mechanisms such as an increased number of postsynaptic receptors as reported recently. However, mEPSC amplitude and other measures of action-potential-independent release were not affected in our studies.

Modulation of transmitter release by muscarinic activation

Application of selective agonists and antagonists of nicotinic or muscarinic receptors suggest that muscarinic receptors are involved in the process of modulation of glutamatergic transmission by paraoxon. The action of paraoxon on sEPSCs was in part mediated by muscarinic receptor activation because carbachol had a similar action and atropine blocked this effect. Interestingly, carbachol partially mimicked the effects of paraoxon on sEPSC properties because carbachol diminished sEPSC amplitude in contrast to that of paraoxon, perhaps due to the rapid desensitization of nicotinic receptors following carbachol application. Nevertheless, the occlusion of paraoxon actions by atropine confirms muscarinic involvement in the actions of paraoxon. We also observed that carbachol application did not alter sEPSC amplitude but increased the amplitude of eEPSCs. We believe that rapid desensitization of nicotinic receptors by carbachol could have masked the drugs actions on muscarinic receptor-mediated EPSC components; more so because sEPSC comprises both action-potential-dependent and-independent events. In contrast, evoked responses generated by electrical stimulation of presynaptic afferents are comprised...
solely of summated action-potential-dependent EPSCs, thereby more accurately demonstrating the effects of carbachol. Activation of muscarinic receptors can modulate neurotransmitter release by inhibition of calcium or potassium channels. Activation of muscarinic receptors modulates both calcium and M-type potassium currents in sympathetic ganglia (Brown and Adams 1980; Selyanko et al. 2000). Increased neurotransmitter release in response to paraoxon and muscarinic stimulation is unlikely to be due to inhibition of N- or L-type Ca\(^{2+}\) channels because this action would diminish neurotransmitter release. In contrast, inhibition of M-current is likely to enhance neurotransmitter release by broadening action potentials. The molecular pathways involved in the inhibition of L- and N-type Ca\(^{2+}\) channels and M-type potassium channels are distinct, and independent modulation of these channels is possible (Liu et al. 2006).

Molecular cloning of various genes for K\(^{+}\) channels, identification of channel gene mutations that lead to human benign familial neonatal convulsions, and reconstitution studies suggest that M current is mediated by KCNQ2/KCNQ3 channels (Cooper and Jan 2003; Shapiro et al. 2000). These channels activate and deactivate at sub threshold membrane potentials and modulate burst generation and after-hyperpolarization in pyramidal neurons (Yue and Yaari 2004). These channels appear to be...
present on the initial segment of the axon and on the presynaptic terminals of hippocampal neurons. It was recently proposed that these channels could modulate neuronal excitability and transmitter release (Vervaeke et al. 2006). Future studies with M current channel openers and antagonists could test whether muscarinic actions on glutamate release are mediated in part by these channels.

Some previous studies have suggested that muscarinic receptor stimulation inhibits glutamate release from presynaptic terminals. In one study of the Schaffer collateral-CA1 synapse, the cholinergic agonist carbamylcholine increased the failure rate without changes in the EPSC amplitude (de Sevilla et al. 2002). The suppression of evoked responses was insensitive to manipulations increasing the probability of release, such as paired-pulse facilitation, increases in temperature, and increases in the extracellular Ca\(^{2+}\):Mg\(^{2+}\) ratio. The authors suggested that muscarinic receptors diminished the release probability at the Schaffer collateral-CA1 synapses. The differences in conclusions of this study and ours may be due to the differences in synapses studied (Schaffer collateral-CA1 vs. perforant path-granule cell) or varied methods used to stimulate afferent inputs and to record EPSCs.

In addition to actions via muscarinic receptors, paraoxon appears to have a direct action on glutamate release from the presynaptic terminal. Paraoxon increased the frequency of action potential-independent mEPSCs recorded from dentate granule cells. However, carbachol did not increase the frequency of mEPSCs. Furthermore, though paraoxon increased the amplitude of sEPSCs, whereas neither carbachol nor arioniomium increased sEPSC amplitudes. Further, the muscarinic antagonist atropine only partially blocked the effect of paraoxon on evoked and spontaneous EPSCs. This action of paraoxon could represent direct, cholinesterase-independent action. Indeed, cholinesterase inhibition-independent stimulation of neurotransmitter release has been described previously (Rocha et al. 1996).

**Paraoxon-induced seizures**

Cholinesterase inhibitors and muscarinic agonists such as pilocarpine have been long known to cause prolonged, persistent seizures, but there are few studies exploring the mechanisms of seizure induction by these agents. There is basal release of acetylcholine in the hippocampus. Cholinesterase inhibitors increase ambient acetylcholine levels in the brain rapidly by inhibiting its breakdown (Shi and McDonoug 1997). These studies report a threefold increase in ambient acetylcholine levels in the cortex after administration of cholinesterase inhibitors. Microdialysis probes placed in the dorsal hippocampus near the dentate gyrus collect ~0.2–0.4 pmol of acetylcholine in 20 min. These levels are increased during exploration and learning and subject to modulation under physiological and pathological conditions (Jope and Morriseit 1986; Jope et al. 1987; Mitsushima et al. 2009). Increased levels of acetylcholine acting via muscarinic receptors could in turn strengthen postsynaptic excitatory transmission by increased the surface expression of AMPA receptors on dendritic spines (de Sevilla et al. 2008).

The current study suggests increased glutamate release from presynaptic terminals as a mechanism for inducing prolonged seizures and status epilepticus. Seizures consist of recurrent synchronized bursting of neurons. Conditions for generating these bursts have been studied extensively in vitro with hippocampal slice preparations and in cultures. Results of these experiments have been simulated in computer models of neuronal networks, which suggested that seizure-like events arise from two basic mechanisms: sustained dendritic depolarization and increased axonal and presynaptic terminal excitability (Traub et al. 1984, 1996). The present study provides evidence that cholinergic and muscarinic stimulation cause increased presynaptic activity.

The increased glutamate release demonstrated in the current study is similar to that reported in the in vitro low magnesium-induced recurrent bursting model (Mangan and Kapur 2004; Mody et al. 1987). Other chemicals that cause focal seizures in experimental animals and humans appear to act via other mechanisms: GABA\(_A\) receptor antagonism by drugs such as penicillin, pentylenetetrazol, and bicuculline, and glutamate receptor activators by drugs such as kainic acid, homocysteine, and NMDA (Velisek 2006).

In summary, these studies demonstrate that the organophosphate paraoxon causes increased excitatory neurotransmitter release by a combination of increased presynaptic firing and increased vesicular release at presynaptic terminals via the activation of muscarinic receptors, and a direct action that may contribute to generating seizures.

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**REFERENCES**


CHOLINGERGIC OVERSTIMULATION ENHANCES GLUTAMATERIC TRANSMISSION


