Diffential Muscarinic Receptor Binding of Acetylcholinesterase Inhibitors in Rat Brain, Human Brain and Chinese Hamster Ovary Cells Expressing Human Receptors

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ABSTRACT

Displacement of muscarinic radioligands by the cholinesterase inhibitors parathion, paraoxon, physostigmine and phenyl saligenin cyclic phosphate was examined in rat cortex and brain stem, human cortex and brain stem, and in Chinese hamster ovary (CHO) cells expressing human M2 or M4 muscarinic acetylcholine receptors. None of the cholinesterase inhibitors tested significantly affected binding of the antagonist [3H]oxoquinuclidinyl benzilate. However, the agonist [3H]oxotremorine-methiodide ([3H]oxo-M) was displaced by all compounds tested in a differential manner. Parathion only marginally displaced [3H]oxo-M binding with pKᵢ values <5 in all tissue or cell types. In rat brain paraoxon, physostigmine and phenyl saligenin cyclic phosphate displaced [3H]oxo-M with pKᵢ values of 7.5, 7.0 and 6.1, respectively. The cholinesterase inhibitors displaced [3H]oxo-M in human brain at 15- to 250-fold higher concentrations, that is with pKᵢ values of 6.3, 4.6 and 4.2, respectively.

Maximal displacement of [3H]oxo-M varied between 25% and 95%, depending on the species and the compound. Human receptors in brain and in CHO cells were equally sensitive to displacement of [3H]oxo-M by parathion, physostigmine and phenyl saligenin cyclic phosphate. However, paraoxon displaced [3H]oxo-M at ≥35-fold lower concentrations from human receptors in brain than in CHO cells. In conclusion, the data show that cholinesterase inhibitors interfere with agonist binding to muscarinic acetylcholine receptors. The species-selectivity of the displacement appears to result from differences between rat and human muscarinic acetylcholine receptors. In addition, for paraoxon marked differences exist between the sensitivity of human muscarinic acetylcholine receptors in brain tissue and of those expressed in clonal CHO cells.

OPs, known as insecticides and nerve gases, and carbamates, which may be used as insecticides as well as in the treatment of glaucoma (Soman and Dube, 1989) and Alzheimer’s disease (Levy et al., 1994; Whitehouse, 1993), are known for their stimulatory effects on cholinergic transmission. OPs and carbamates inhibit AChE and thereby cause accumulation of ACh in the synaptic cleft. Subsequently, ACh receptors are overstimulated leading to the neurotoxic symptoms, or possibly to the therapeutic effect. OP toxicity is characterized by pronounced interspecies variability, resulting from differences in toxicokinetic as well as toxicodynamic factors (Wallace, 1992). Carbamates may also have species-selective actions (Tune et al., 1988; Ioannou et al., 1988).

The acute toxicity of OPs cannot be explained by their AChE-inhibiting action. The AChE-inhibiting potency does not always correlate with the acute in vivo toxicity (Chambers, 1992). Furthermore, oximes with the highest antidote potency against OPs are the poorest reactivators of inhibited AChE (Bedford et al., 1989). Soman-induced depression of the EPSPs in rat superior cervical ganglia is not influenced by pretreatment with the irreversible AChE inhibitor diisopropyl fluorophosphate, which causes ≥98% inhibition of AChE (Yarowsky et al., 1984). OPs and other AChE-inhibitors may also bind to mAChR and act as agonists. At nanomolar concentrations paraoxon and chlorpyrifos oxon displace [3H]CD bound to mAChR and inhibit forskolin-stimulated formation of cAMP in rat striatum (Jett et al., 1989).
Paraoxon also displaces [³H]NMS from mACHR and stimulates phosphatidyl inositol turnover to a level twice basal in human neuroblastoma SK-N-SH cells (Katz and Marquis, 1992). Therefore, direct effects on mACHR appear to be also involved in the mechanism of action of these compounds.

Multiple subtypes of mACHR (M1–M5) are distinguished, based on the molecular structure and pharmacological characteristics (Brann et al., 1993). mACHR subtypes exhibit distinct distribution patterns in the central and peripheral nervous system. Rat and human cerebral cortex, for example, contain a mixture of M1, M2, M3 and M4 receptors, although receptor subtype distribution patterns may vary between the rat and human nervous system. In addition, the molecular structure of identified mACHR subtypes differs between species (Bonner, 1989; Tietje and Nathanson, 1991), possibly leading to altered functional properties or drug sensitivities. Differential actions of OPs and carbamates at mACHR of different species or at distinct receptor subtypes within a species, might account for species-selective effects of CHI.

In this study, mACHR binding of the OPs parathion, para-oxon and PSP and of the carbamate Phy was examined, by analyzing displacement of the muscarinic antagonist [³H]QNB and of low concentrations of the agonist [³H]oxo-M. These CHI were selected because of their known properties. The insecticide parathion causes limited AChE inhibition compared to its neurotoxic metabolite paraoxon. PSP is the active metabolite of tri(O-cresyl)phosphate, and inhibits AChE as well as NTE which is associated with delayed neuropathy. Phy inhibits AChE in a reversible manner. Using cerebral cortex and brain stem of rat and human origin, as well as CHO cells stably transfected to express human M2 or M4 mACHR, species- and subtype-selective binding of the selected AChE-inhibiting compounds to mACHR was analyzed.

Materials and Methods

Membrane preparations. Adult male Wistar rats (Harlan, Zeist, The Netherlands) were killed by decapitation, the brains were rapidly removed and the cerebral cortex and brain stem were dissected. The tissues were homogenized in 10% m/v ice-cold 0.32 M sucrose, using a homogenizer with Teflon pestle (Braun-Melsungen, Melsungen, Germany), and centrifuged at 2700 rpm for 12 min at 4°C (Sorvall RC28S, SM-24 rotor, Sorvall, Wilmington, DE). The supernatant was centrifuged at 19,000 rpm for 10 min at 4°C. The resulting pellet was homogenized in ice-cold Hepes buffer (in mM, Hepes 20, MgCl₂ 10, pH 7.4) for [³H]oxo-M experiments or in ice-cold Tris buffer (in mM, Tris 50, MgCl₂ 10, EDTA 0.5, pH 7.4) for [³H]QNB experiments.

Human postmortem brain tissue was from four patients, three women at the age of 35, 60 and 87 and a man at the age of 63, without apparent psychiatric or neurological disease. The brains were stored at 4°C before dissection of the frontal cortex andpons (brain stem) within 10 hr postmortem. The dissected cortex and brain stem were stored at −70°C up to 2 months until homogenization after the same procedure as with rat brain.

CHO cells were stably transfected to express the human M2 and M4 mACHR, using the expression vector pKCRE, and subsequently cloned by N. J. Stam (NV Organon, Oss, The Netherlands). Frozen pellets of CHO cells were homogenized using a Polytron homogenizer (Kinematica, Kriens-Lucerne, Switzerland) in ice-cold Hepes or Tris buffer for [³H]oxo-M and [³H]QNB experiments, respectively.

Membrane preparations treated to depleted AChE. When indicated, membrane preparations were treated to depleted endogenous AChE. The different tissues or cells were homogenized as described above, except for the last homogenization step in which milli-Q water was used instead of buffer. The membrane preparations were frozen and thawed 10 times, to release endogenous ACh from synaptosomes and vesicles (Whittaker et al., 1964). The membranes were pelleted by centrifugation at 19,000 rpm for 10 min at 4°C, washed with ice-cold Hepes buffer, pelleted again and finally homogenized in ice-cold Hepes buffer.

These membrane preparations were used in binding experiments that were carried out as described below. The next day the relative activity of the cytoplasmic enzyme LDH of the membrane preparations and supernatants, before and after treatment with 1% Triton X-100, was assayed, according to the method of Bergmeyer et al. (1965).

Binding. Approximately 450, 150 and 250 mg protein of rat brain, human brain and CHO cells, respectively, was used per incubation with 0.5 nM [³H]oxo-M or [³H]QNB and with AChE inhibitor, atropine or buffer in a total volume of 0.5 ml. This mixture was incubated shaking for 1 hr at room temperature, which was long enough to reach equilibrium. Incubation was terminated by vacuum filtration and 10 s washing with ice-cold Tris buffer (4.95 mM Tris, pH 7.4) over 0.05% polyethyleneimine-presoaked glass fiber filters (double thickness printed filtermat B, Wallac Oy, Turku, Finland), using a Skatron Micro Cell Harvester (Skatron Instruments AS, Norway).

Using a T-tray heatsealer (Wallac Oy) scintillation sheets (thick Meltilex B/SH, Wallac Oy) were sealed to the filters. Filter bound radioactivity was counted for 2 to 3 min using a 1205 beta plate counter (Wallac Oy).

Specific binding was calculated by subtracting nonspecific binding in the presence of 1 μM atropine from total binding. Nonspecific binding ranged from 15 to 40% at the highest concentrations of radioligands used. Saturation binding of the radioligands to the membrane preparations was determined in triplicate in the presence of increasing concentrations of the radioligands. Displacement of radioligand binding to mACHR by the CHI was determined by analyzing specific binding of the radioligands in triplicate in the presence of paraoxon, PSP or Phy (100 pM–10 μM final concentration) or of parathion (10 pM–10 μM final concentration), compared to specific control binding.

The exact concentrations of the radioligands were determined by 3 min counting of triplicate samples of the radioligand with scintillation liquid (Ultima Gold MV, Packard Instruments, Meriden, CT) using a Packard-tricarb liquid scintillation counter.

Protein concentration of the different tissues was determined according to the Bradford method (Bradford, 1976), using a kit (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard.

Data analysis. Data were analyzed using GraphPad software (GraphPad Software Inc., San Diego, CA). Saturation binding data were converted to Scatchard plots, from which affinity constants Kᵢ and Bₘᵢ values were determined as 1/slope and the x-axis intercept, respectively. Similar results were obtained by nonlinear regression, using the function Bₘᵢ* x/Kᵢ + x. The pKi values were calculated from the IC₅₀ values using the equation pKi = −log (IC₅₀/(1 + [F]/Kᵢ)), where [F] is the concentration of free ligand (Cheng and Prusoff, 1973). For statistical analysis the Student’s t test was used. Significance of interspecies differences of pKi values was determined for the same as well as for different brain structures.

AChE. The AChE activity in rat brain membranes was determined radiometrically according to the method of Johnson and Russell (1975). AChE activity was measured as the hydrolysis of [³H]ACh to [³H]-labeled acetate during 15 min incubation at room temperature. The activity was related to known esterase activity of Electric Eel AChE (Sigma Chemical Co., St Louis, MO). Rat brain
AChE activity was measured in the absence and presence of oxo-M and QNB at a concentration range of 10 pM to 1 μM.

**Compounds.** The CHI used were: O,O-diethyl-O-4-nitrophenylphosphorothioate (parathion, Riedel-de Haen, Seelze, Germany, purity ≥98%), O,O-diethyl-O-4-nitrophenylphosphate (paraoxon, Riedel-de Haen, purity ≥97%), PSP (kindly provided by Dr. M. Ehrlich, Blacksburg, VA, purity 100%) and physostigmine (eserine, hemisulphate salt, Sigma, purity ≥99%). [3H]oxo-M and [3H]QNB were obtained from Du Pont NEN (Boston, MA).

**Results**

**Saturation binding.** For each tissue or cell type saturation binding of [3H]oxo-M and [3H]QNB was determined. [3H]QNB is a nonspecific muscarinic antagonist that binds to all mAChR with high affinities, whereas [3H]oxo-M exhibits high affinity binding to predominantly M2 and M4 receptors (Richards and Van Giersbergen, 1995; McKinney et al., 1989; Van Giersbergen and Leppik, 1995). Saturation curves and Scatchard plots of [3H]oxo-M binding to membrane preparations of the different tissues or cells were obtained. The Kᵅ and Bₘₐₓ values of [3H]oxo-M binding to the different membrane preparations were determined using Scatchard analysis and are listed in table 1. Similar results were obtained by nonlinear regression (not shown). The Kᵅ values of [3H]QNB binding were in the same range as those of [3H]oxo-M binding for all preparations, with maximally one order lower Kᵅ values. Maximal [3H]oxo-M binding was 12 to 40% of [3H]QNB binding.

**Displacement by CHI.** Displacement of the radioligands [3H]QNB and [3H]oxo-M by parathion, paraoxon, Phy and PSP was determined at 0.5 nM [3H]QNB and [3H]oxo-M. At these concentrations radioligand binding was not saturated. In CHO cells specific binding of the muscarinic antagonist [3H]QNB was not affected by any of the CHI tested at concentrations ranging from 10 pM to 100 μM. In rat brain specific [3H]QNB binding was not affected by parathion and paraoxon, and 10 to 40% was displaced by Phy and PSP at the highest concentration of 100 μM. In human brain, at 100 μM, only paraoxon and Phy displaced [3H]QNB by 25 to 40 and 45 to 83%, respectively. Specific binding of the agonist [3H]oxo-M, however, was displaced with lower IC₅₀ values in a differential manner, depending on the compound and the cell type. Figure 1 shows that in rat cortex membranes paraoxon displaced [3H]oxo-M with an IC₅₀ of 60 nM, although [3H]QNB binding was not affected by 0.1 nM to 100 μM paraoxon. Representative displacement curves of specific [3H]oxo-M binding in rat brain stem, human brain stem and CHO cells expressing human M2 mAChR by all CHI tested are shown in figure 2. All fitted data are summarized in table 2.

Table 1: Specific binding of [3H]oxo-M to mAChR in different cell or tissue types

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Kᵅ (nM)</th>
<th>Bₘₐₓ (pmol/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain stem</td>
<td>1.04</td>
<td>0.67</td>
</tr>
<tr>
<td>Rat cortex</td>
<td>0.70</td>
<td>0.56</td>
</tr>
<tr>
<td>Human brain stem</td>
<td>3.28</td>
<td>0.19</td>
</tr>
<tr>
<td>Human cortex</td>
<td>3.27</td>
<td>0.19</td>
</tr>
<tr>
<td>CHO M2</td>
<td>1.41</td>
<td>1.23</td>
</tr>
<tr>
<td>CHO M4</td>
<td>1.89</td>
<td>82.0</td>
</tr>
</tbody>
</table>

Paraoxon displaced [3H]oxo-M with a significant different pKᵅ value (P < .05) in rat than in human brain (table 2). In CHO cells expressing M2 and M4 receptors displacement by paraoxon occurred at ≤560- and ≤35-fold higher concentrations than in rat and human brain, respectively, resulting in significant different pKᵅ values (P < .005). However, parathion only marginally affected specific [3H]oxo-M binding in all membrane preparations. At the highest concentration of 10 μM only 19, 11 and 48% of [3H]oxo-M was displaced in rat brain, human brain and M2-expressing CHO cells, respectively. In M4-expressing CHO cells parathion had a pKᵅ value of 5.3.

Phy and PSP displaced [3H]oxo-M at, respectively, 250- and 80-fold lower concentrations in rat than in human brain, with significant different (P < .001) pKᵅ values (table 2). In CHO cells expressing human M2 or M4 receptors pKᵅ values of Phy and PSP were equal (P > .14) to those in human brain. For all compounds pKᵅ values in cortex and brain stem within the same species were equal (P > .18; table 2). Phy also had equal pKᵅ values in CHO cells expressing M2 and M4 receptors (P = .29).

Specific [3H]oxo-M binding was not always completely displaced by the CHI (fig. 2). The percentage of maximal displacement by paraoxon and PSP was significant higher (P < .01 and <.02, respectively) in rat brain than in human brain (table 3). Conversely, Phy displaced a significant lower percentage (P < .02) in rat than in human brain. A reliable estimate of maximal displacement by parathion in rat and human brain, and by OPs in CHO cells could not be obtained, because there was only limited displacement at the highest concentrations used.

In summary, these data showed that CHI displaced bind-
ing of the agonist [3H]oxo-M to mAChR in a differential manner, depending on the cell-type and the compound.

The origin of the differential displacement by CHI of [3H]oxo-M from rat and human mAChR, and by paraoxon from human mAChR in brain and expressed in CHO cells was further explored. Human brain was stored at −70°C until homogenization, while rat brain was used immediately after dissection. To test whether differences in storage cause differential displacement of [3H]oxo-M, binding was also performed with rat cortex that was stored frozen like human brain. Compared to fresh cortex specific control binding (in the absence of CHI) of previously frozen cortex was decreased by about 40%. Radioligand displacement by parathion, paraoxon and PSP had equal pKi values (≤5, 7.4 and 6.2, respectively) as in fresh rat cortex (≤5, 7.5 and 6.1). Phy displaced [3H]oxo-M in stored cortex at a somewhat higher concentration (pKi value 6.6) than in fresh cortex concentration (pKi value 7.1). The percentages of maximal displacement were about 20% lower with all CHI tested. However, these differences between fresh and stored rat cortex are not in accordance with those observed between rat and human brain and therefore do not explain the species differences.

AChE. In rat brain the concentrations of the CHI tested that displace [3H]oxo-M were in the same range as those that inhibit AChE (Ward et al., 1993; Moriearty and Becker, 1992; Tang et al., 1994). Several possible pathways of interactions of the compounds with AChE which could interfere with [3H]oxo-M binding were examined.

To test whether nonhydrolyzed, endogenous ACh affects [3H]oxo-M, experiments were performed with membrane preparations treated to deplete endogenous ACh, as described in “Materials and Methods.” LDH activity was deter-

**Table 2**

Displacement of specific [3H]oxo-M binding to muscarinic receptors by AChE inhibitors

<table>
<thead>
<tr>
<th>pKi Values (±SD)</th>
<th>Rat brain stem</th>
<th>Rat cortex</th>
<th>Human brain stem</th>
<th>Human cortex</th>
<th>Human M2 CHO</th>
<th>Human M4 CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parathion</td>
<td>&lt;5 (&lt;0.05)</td>
<td>&lt;5 (&lt;0.05)</td>
<td>&lt;5 (&lt;0.05)</td>
<td>&lt;5 (&lt;0.05)</td>
<td>&lt;5 (&lt;0.05)</td>
<td>5.31 (±0.38)</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>7.57a (±0.05)</td>
<td>7.47a (±0.15)</td>
<td>6.45b (±0.56)</td>
<td>6.17b (±0.80)</td>
<td>&lt;4 (±0.05)</td>
<td>4.75a (±0.35)</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>6.91d (±0.13)</td>
<td>7.12d (±0.25)</td>
<td>4.53e (±0.20)</td>
<td>4.75e (±0.39)</td>
<td>4.47e (±0.20)</td>
<td>4.79e (±0.39)</td>
</tr>
<tr>
<td>PSP</td>
<td>6.11f (±0.27)</td>
<td>6.12f (±0.31)</td>
<td>4.52g (±0.23)</td>
<td>&lt;4 (±0.20)</td>
<td>&lt;4 (±0.28)</td>
<td>4.26g (±0.19)</td>
</tr>
</tbody>
</table>

Data are means of three to six triplicate experiments ± S.D. Different superscript letters at the same row indicate significant differences. a vs. b: P < .05; a,b vs. c: P < .005; d vs. e: P < .001; f vs. g: P < .005.

**Fig. 2.** CHI differentially displace [3H]oxo-M depending on the species and cell or tissue type used. Representative displacement curves of [3H]oxo-M binding to mAChR in membranes from rat brain stem, human brain stem and human-M2-expressing CHO cells of several CHI. Points are means of three determinations, bars represent S.E.M.
TABLE 3
Percentages of maximal displacement of [3H]oxo-M by CHI

<table>
<thead>
<tr>
<th></th>
<th>% Maximal Displacement (± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Rat brain stem</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>*</td>
</tr>
<tr>
<td>Physopestigmine</td>
<td>63% (±12)</td>
</tr>
<tr>
<td>PSP</td>
<td>69% (±7)</td>
</tr>
</tbody>
</table>

* A percentage of maximal displacement could not be determined, because of limited displacement at the highest concentrations used, i.e., 10 μM parathion and 100 μM PSP.

Data are means of three to six triplicate experiments ± S.D. Different superscript letters at the same row indicate significant differences. a vs. b: P = .01; c vs. e and d vs. f: P < .02; g vs. h: P < .02.

The CHI paraoxon, Phy and PSP differentially displaced the muscarinic agonist [3H]oxo-M from rat and human mAChR. The sensitivity to parathion was low in all cell or tissue types. For the rat [3H]oxo-M was displaced by CHI at concentrations in the submicromolar range, similar to concentrations inhibiting AChE (Ward et al., 1993; Moriearty and Becker, 1992; Tang et al., 1994). Agonist binding to human mAChR both in human brain and in transfected CHO cells was affected by considerably higher CHI concentrations.

TABLE 4
[3H]oxo-M displacement by CHI in membrane preparations treated to deplete AChE

<table>
<thead>
<tr>
<th></th>
<th>pK₅ Values (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat cortex</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Physopestigmine</td>
<td>7.63 (±0.04)</td>
</tr>
<tr>
<td>PSP</td>
<td>6.69 (±0.08)</td>
</tr>
<tr>
<td></td>
<td>5.68 (±0.13)</td>
</tr>
</tbody>
</table>

Data are means of two triplicate experiments ± S.D.

Discussion

The CHI paraoxon, Phy and PSP differentially displaced the muscarinic agonist [3H]oxo-M from rat and human mAChR. The sensitivity to parathion was low in all cell or tissue types. For the rat [3H]oxo-M was displaced by CHI at concentrations in the submicromolar range, similar to concentrations inhibiting AChE (Ward et al., 1993; Moriearty and Becker, 1992; Tang et al., 1994). Agonist binding to human mAChR both in human brain and in transfected CHO cells was affected by considerably higher CHI concentrations.

Esterase Inhibitors Bind to mAChR

Percentage of maximal displacement of [3H]oxo-M by CHI

- Paraoxon: 71% (±7)
- Physopestigmine: 63% (±12)
- PSP: 69% (±7)

Data are means of three to six triplicate experiments ± S.D. Different superscript letters at the same row indicate significant differences. a vs. b: P = .01; c vs. e and d vs. f: P < .02; g vs. h: P < .02.

Muscarnic agonists displace radioligand agonists at 100- to 1000-fold lower concentrations than radioligand antagonists (Sharif et al., 1995; Van Giersbergen and Leppik, 1995; Dong et al., 1995). Therefore, the differential displacement of [3H]oxo-M and [3H]QNB suggests that the CHI studied act as agonists on mAChR. In rat striatum paraoxon (Jett et al., 1991) and chlorpyrifos oxon (Huff et al., 1994) have been reported to inhibit forskolin-induced cAMP formation with an IC₅₀ of 300 and 155 nM, respectively, which is a partial atropine-sensitive effect. In addition, paraoxon at 0.1 nM stimulates phosphatidyl inositol turnover in SK-N-SH neuroblastoma cells, which is partially sensitive to muscarinic agonists (Katz and Marquis, 1992).

AChE. In rat brain the concentrations of the compounds that inhibit AChE correlated with and were in the same range as those that displace [3H]oxo-M (Ward et al., 1993; Moriearty and Becker, 1992; Tang et al., 1994). A similar correlation was not found between the inhibition of human AChE and displacement of [3H]oxo-M binding from human mAChR (Moriearty and Becker, 1992). Brain membrane preparations as well as CHO cells contain considerable amounts of membrane-bound AChE (I. van den Beukel and J. J. Zijlstra, unpublished observations).

Several possible pathways of interaction with AChE interfering with [3H]oxo-M binding were ruled out. 1. Specific radioligand binding was not likely to result from binding to AChE, as oxo-M and QNB had no effect on AChE activity. 2. Because [3H]QNB binding was not affected, AChE were not down-regulated by nonhydrolyzed ACh. 3. Elevated levels of ACh are unlikely to displace [3H]oxo-M. From endogenous ACh in rat cortex (53 pmol/mg wet weight, Molenaar et al., 1973), it was calculated that total release without hydrolysis would result in an ACh concentration of 245 nM during the incubation. However, because in rat brain AChE is maximally inhibited at the IC₅₀ value of [3H]oxo-M displacement (resulting in maximal ACh levels) radioligand displacement at higher concentrations cannot correspond with further increased levels of ACh. In human brain the concentration of Phy that maximally inhibits AChE is even 100-fold lower than the IC₅₀ value of displacement (Moriearty and Becker, 1992). The small shift of some of the binding curves in membranes treated to deplete ACh might have been an effect of the freeze/thaw procedure itself.

Comparison of esterase inhibition for a range of OPs resulted in no clear differences between rodent and human AChE (Veronesi et al., 1997, in press). This further corroborates the notion that the presently observed effects of CHI are governed by a mechanism distinct from esterase inhibition. However, differential steric hindrance of agonist binding due to CHI binding to AChE in close proximity to mAChR.
in the membrane can presently not be excluded and could explain the observed effects.

**Differential displacement of [³H]oxo-M in rat and human brain.** In rat brain the CHI paraaxon, Phy and PSP displaced the agonist [³H]oxo-M at 15- to 250-fold lower concentrations than in human brain. Further, paraaxon and PSP maximally displaced a higher percentage, while Phy displaced a lower percentage of [³H]oxo-M in rat than in human brain. Displacement of the M2 muscarinic agonist [³H]CD in rat brain is also reported to be incomplete, as paraaxon maximally displaced [³H]CD by 50% (Ward et al., 1993; Jett et al., 1991).

Although freezing had some minor effects on displacement by the CHI, the difference in storage of rat and human brain does not account for the differences in displacement observed. Species-selective displacement could result from different molecular structures of mAChR between species or from different mAChR subtypes and different subtype distribution patterns between species. High-affinity binding of [³H]oxo-M occurs primarily to M2 and M4 receptors. Oxo-M binds with 10- to 100-fold higher affinities to M2 and 3- to 10-fold higher affinities to M4 receptors, compared to M1 and M3 receptors (Richards and Van Giersbergen, 1995; McKinney et al., 1989; Van Giersbergen and Leppik, 1995). Human and rat cortex contain a mixture of M1, M2, M3 and M4 receptors, although in rat brain stem M2 receptors are predominantly expressed (Flyn et al., 1995; Wall et al., 1991a; Li et al., 1991; Wall et al., 1991b; Yasuda et al., 1993; Levey et al., 1991). However, pKᵢ values and maximal displacement were equal in cortex and brain stem within a species. It is thus not likely that the CHI distinguish M2 and M4 receptors, and muscarinic subtype-selectivity does not explain the species-differences observed. Therefore, it appears that the different displacement in rat and human brain is caused by different molecular structures and properties of mAChR between species.

**Differential displacement of [³H]oxo-M by paraaxon from human mAChR in brain and CHO cells.** Surprisingly, paraaxon displaced specific [³H]oxo-M binding at ≥35-fold lower concentrations from human mAChR in brain than in CHO cells. The sensitivities to the other CHI studied were equally low in both membrane preparations. Further, CHI were far more potent in rat brain than in CHO cells transfectected with human mAChR. Chlorpyrifos oxon also displaces [³H]oxo-M at 15- to 250-fold lower concentrations than from human mAChR (predominantly M1 and M3) in the human SK-N-SH neuroblastoma cell line (Richards and Van Giersbergen, 1995; Baumgold and White, 1989; Wall et al., 1991b), although the expression level is similar (Lambert et al., 1989). Further research will be needed to understand the influence of the microenvironment on the pharmacological and functional properties of receptors.

Species and subtype differences in mAChR structure are mainly found in the third cytoplasmic (i3) domain of the mAChR, implicated in G-protein coupling (Bonner, 1989). CHI might affect the interaction of mAChR and G-proteins. This would be in agreement with the observation that antagonist binding is not inhibited by CHI (Huff et al., 1994; Ward et al., 1993; Bakry et al., 1988; present results), and that functional effects of CHI on mAChR are only partly blocked by muscarinic antagonists (Jett et al., 1991; Huff et al., 1994; Katz and Marquis, 1992). Furthermore, OP effects on mAChR can be prevented by sodium fluoride affecting G-proteins (Ehrlich et al., 1994). Differences in agonist displacement between rat and human brain and between human receptors in brain and CHO cells therefore might be due to different molecular structures of the i3 domains and to other G-proteins present in the different cell types.

Concluding, the CHI paraaxon, Phy and PSP species-selectively displace [³H]oxo-M at lower concentrations from rat than from human mAChR. The high affinity found suggests that interaction with mAChR might play a role in the neurotoxicity of these compounds, known for their AChE inhibition. The results with paraaxon show that properties of human mAChR in postmortem brain and in CHO cells are not necessarily the same.

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**References**


Esterase Inhibitors Bind to mAChR


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