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Selective effects of carbamate pesticides on rat neuronal nicotinic acetylcholine receptors and rat brain acetylcholinesterase

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Abstract

Effects of commonly used carbamate pesticides on rat neuronal nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes have been investigated using the two-electrode voltage clamp technique. The potencies of these effects have been compared to the potencies of the carbamates to inhibit rat brain acetylcholinesterase. The potency order of six carbamates to inhibit $\alpha4\beta4$ nicotinic receptors is fenoxycarb > EPTC > carbaryl, bendiocarb > propoxur > aldicarb with IC50 values ranging from 3 μM for fenoxycarb to 165 μM for propoxur and >1 mM for aldicarb. Conversely, the potency order of these carbamates to inhibit rat brain acetylcholinesterase is bendiocarb > propoxur, aldicarb > carbaryl ≫ EPTC, fenoxycarb with IC50 values ranging from 1 μM for bendiocarb to 17 μM for carbaryl and ≫1 mM for EPTC and fenoxycarb. The $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha3\beta2$ nicotinic acetylcholine receptors are inhibited by fenoxycarb, EPTC, and carbaryl with potency orders similar to that for $\alpha4\beta4$ receptors. Comparing the potencies of inhibition of the distinct subtypes of nicotinic acetylcholine receptors shows that the $\alpha3\beta2$ receptor is less sensitive to inhibition by fenoxycarb and EPTC. The potency of inhibition depends on the carbamate as well as on a combination of α and β subunit properties. It is concluded that carbamate pesticides affect different subtypes of neuronal nicotinic receptors independently of acetylcholinesterase inhibition. This implicates that neuronal nicotinic receptors are additional targets for some carbamate pesticides and that these receptors may contribute to carbamate pesticide toxicology, especially after long-term exposure.

Keywords: Carbamate pesticides; Neuronal nicotinic acetylcholine receptor; Rat brain acetylcholinesterase; *Xenopus* oocytes; Two-microelectrode voltage clamp

Introduction

Carbamates, all derived from the basic structure of carbamic acid, represent a broad variety of compounds, which have a number of applications. They are widely applied as insecticides, herbicides, and fungicides. Many of these chemicals are potential neurotoxicants, particularly following occupational, accidental, or intentional exposure. Acute toxic symptoms of carbamate poisoning, e.g., miosis, urination, diarrhea, diaphoresis, lacrimation, salivation, and excitation of the central nervous system (O'Malley, 1997), are generally caused by inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7), which leads to accumulation of acetylcholine (ACh) (for review see Ecobichon, 2001). Studies on chronic exposure to carbamate insecticides and case reports of long-term exposure give equivocal results. Some show no effects after chronic exposure (Risher et al., 1987), whereas others describe memory impairment (Dési et al., 1974; Ruppert et al., 1983; Ecobichon, 2001), degenerative polyneuropathy (Umehara et al., 1991), neurobehavioral effects (Moser, 1999), and other neurological disorders (Dési et al., 1974; Dickoff et al., 1987). An extensive survey of the toxicology of the common insecticide carbaryl reports a variety of reversible neurobehavioral and neurotoxic effects in vertebrates, all associated with acute poisoning symptoms (Cranmer, 1986). Overall, it ap-

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pears that at least some carbamate esters may initiate neurological and behavioral changes at dose levels that produce few overt signs of acute toxicity or significant reduction in nervous tissue AChE activity (Ecobichon, 2001). Mechanisms involved in these changes, however, remain to be elucidated.

Previous research showed evidence of carbamates interacting with receptors of the cholinergic system. The carbamate physostigmine and related cholinesterase inhibitors displace the muscarinic ACh receptor agonist [^{3}H]oxotremorine-M from its receptors in rat cortex and brain stem homogenates (Van den Beukel et al., 1997; Lockhart et al., 2001). Additional evidence has been presented to suggest that some of these cholinesterase inhibitors are muscarinic ACh receptor agonists (Volpe et al., 1985; Lockhart et al., 2001). Agonistic, antagonistic, potentiating, and inhibitory effects of physostigmine and related cholinesterase inhibitors on nicotinic ACh receptors (nAChRs) have also been described. Low concentrations of physostigmine and neostigmine agonize or potentiate, whereas high concentrations of these drugs block neuronal nAChR channels (Storch et al., 1995; Nagata et al., 1997; Van den Beukel et al., 1998). High concentrations of physostigmine also block the ion channel of muscle-type nAChRs (Wachtel, 1993). Various cholinesterase drugs with similar potentiating and inhibitory actions on neuronal nAChRs appear to be competitive ligands at the agonist recognition sites of muscle and neuronal types of nAChR (Sherby et al., 1985; Svensson and Nordberg, 1997; Zwart et al., 2000). For physostigmine the mechanism involves a competitive interaction with the agonist recognition site of the nAChR (Zwart et al., 2000). The potentiation occurs when low concentrations of ACh and physostigmine are combined, i.e., when a fraction of the nAChRs is occupied by a single ACh and a single physostigmine molecule simultaneously. At high concentrations physostigmine blocks the response by occupying both agonist recognition sites as well as by noncompetitive ion channel block. The degrees of potentiation and inhibition were shown to differ between drugs and to depend on receptor subunit composition (Zwart et al., 1999, 2000).

Some carbamate insecticides, e.g., the cholinesterase inhibitors aminocarb, aldicarb, and carbaryl tested at a concentration of 100 μM, displace [^{3}H]ACh from muscle-type nAChRs in *Torpedo* electric organ membranes (Eldefrawi and Eldefrawi, 1983). Like neostigmine, carbaryl causes a concentration-dependent potentiation and inhibition of neuronal nAChR channels in rat pheochromocytoma PC12 cells (Nagata et al., 1997). An observed enhancement of cholinergic transmission in guinea pig myenteric plexus preparations has been proposed to be associated with an effect of the dithiocarbamate fungicide propineb on ganglionic and not on muscle-type nAChRs (Marinovich et al., 2002).

The resemblance in the effects of carbamate pesticides and carbamate drugs on nicotinic receptor model systems suggests that the pesticides may also affect cholinergic neurotransmission. In order to test this hypothesis we have investigated effects of a range of carbamate pesticides on neuronal nAChRs heterologously expressed in *Xenopus laevis* oocytes. The potencies of carbamate effects on the nAChRs are compared to the potencies of rat brain AChE inhibition and the differential sensitivities of specific subtypes of ganglionic and brain neuronal nAChRs have been investigated. The combined results demonstrate that several of the carbamates investigated more potently inhibit neuronal nAChRs than brain AChE and that ganglionic and brain nAChRs are inhibited by carbamates in a compound- and receptor-specific way.

Methods

Chemicals. ACh (acetylcholine chloride), collagenase type I, DMSO (ACS reagent), MS-222 (3-amino benzoic acid ethyl ester, methane sulfonate salt), NaCl, and neomycin solution (10 mg neomycin/ml in 0.9% NaCl) were obtained from Sigma (St. Louis, MO). Aldicarb (2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime), aminocarb (4-(dimethylamino)-3-methylphenolmethylcarbamate ester), bendiocarb (2,2-dimethyl-1,3-benzodioxol-4-ol methylcarbamate), carbaryl (1-naphthalenol methylcarbamate), carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate), dioxacarb (2-(1,3-dioxolan-2-yl)phenyl methylcarbamate), EPTC (*S*-ethyl *N*,*N*-dipropylthiocarbamate), fenoxycarb (ethyl[2-(4-phenoxyphenoxy)ethyl]carbamate), methomyl (*N*-[[(methylamino)carbonyl]oxy]ethanimidothioic acid methyl ester), oxamyl (2-(dimethylamino)-*N*-[[(methylamino)-carbonyl]oxy]-2-oxoethanimidothioic acid methyl ester), pirimicarb (dimethylcarbamic acid 2-(dimethylamino)-5,6-dimethyl-4-pyrimidinyl ester), and propoxur (2-(1-methylethoxy)phenol methylcarbamate) were purchased from Riedel de Haën (Seelze, Germany). $CaCl_2$ (1 M solution), MgCl (1 M solution), $MgSO_4$, $NaHCO_3$, and NaOH were purchased from BDH Laboratory Supplies (Poole, England). $Ca(NO_3)_2$, Hepes, and KCl were from Merck (Darmstadt, Germany). For AChE assays, acetylthiocholine iodide, 5,5′-dithio-bis-(2-nitro)benzoic acid, ethopropazine, Triton X-100, physostigmine, and EDTA were from Sigma. NaCl, K_2HPO_4, and KH_2PO_4 were bought from Merck and Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) was obtained from Boehringer (Mannheim, Germany).

Saline solutions for electrophysiology were prepared with distilled water and solutions for AChE assays were prepared with Milli-Q filtered water (Millipore SA, Molsheim, France). Stock solutions (0.1 M) of aldicarb, aminocarb, bendiocarb, carbaryl, carbofuran, dioxacarb, EPTC, fenoxycarb, methomyl, oxamyl, pirimicarb, and propoxur were prepared in DMSO. cDNAs of nicotinic receptor subunits ligated into the pSM plasmid vector containing the SV40 viral promotor were a kind gift from Dr. J.W. Patrick (Baylor College of Medicine, Houston, TX).

Brain AChE preparation. Male Wistar rats (~350 g, $n = 5$; Harlan, Horst, The Netherlands) were decapitated and the brain was removed. The tissue was homogenized (900 rpm, 10% w/v homogenate) in ice-cold buffer containing 50 mM Tris, 1 M NaCl, 5 mM EDTA, and 1% (v/v) Triton X-100, pH 7.4, and subsequently centrifuged for 10 min at 36,000*g* in a Ti50 rotor at 4°C (L8-70; Beckman Coulter, Fullerton, CA). The supernatant was kept on ice and used within 3 h. An aliquot was drawn to determine the protein content according to Bradford (1976). The protein content ranged from 30 to 37 mg/ml.

Brain AChE inhibition. The stock solutions of the AChE inhibitors were diluted in a phosphate buffer (8 mM KH_2PO_4 and 48 mM K_2HPO_4, pH 7.4) to obtain work standards containing 0.1 μM to 10 mM of the inhibitor. Aliquots (25 μl) of the work standard were added to 225 μl of brain homogenate. After incubation for 1 min at 37°C, 25-μl samples were drawn, added to 250 μl ice-cold phosphate buffer, mixed, and rapidly frozen in liquid nitrogen. These samples were stored at −70°C until analysis. Thawed samples were appropriately diluted and were assayed in quadruplicate for AChE activity using a 96-well microplate modification of the Ellman method (Bueters et al., 2003). Ethopropazine (10 μM) was used as a specific inhibitor of butyrylcholinesterase.

Receptor expression in oocytes. X. laevis frogs were obtained from AmRep (Breda, The Netherlands) and kept in standard aquaria (0.5 × 0.4 × 1 m; 1–15 per aquarium) with a normal 12-h light/dark cycle (lights on at 7:00 a.m. and lights off at 7:00 p.m.). The water in the aquaria (copper-free, pH 7, 1.25 mmol CaO/L, 24°C) was continuously refreshed at a rate of ~1000 L/day. The animals were fed three times a week on earthworms (Hagens, Nijkerkerveen, The Netherlands). Mature female frogs were anesthetized by submersion in 0.2% MS-222 and ovarian lobes were surgically removed. All experimental procedures involving animals were approved by a local ethical committee and were in accordance with Dutch law. Oocytes were defolliculated manually after treatment with collagenase type I (1.5 mg/ml calcium-free Barth's solution) for 1.5 h at room temperature. Plasmids coding for α3, α4, β2, and β4 subunits of rat neuronal nAChRs (Boulter et al., 1987; Duvoisin et al., 1989) were dissolved in distilled water. Stock solutions containing α4 or α3 and β2 or β4 subunit cDNAs at a 1:1 molar ratio were injected into the nuclei of stages V and VI oocytes within 8 h after harvesting, using a Drummond microinjector. Approximately 1 ng of each plasmid containing α and β cDNA was injected in a total injection volume of 18.4 nl/oocyte. After injection the oocytes were incubated at 19°C in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM Hepes, and 50 μg/ml neomycin. Experiments were performed on oocytes after 3–6 days of incubation (Zwart and Vijverberg, 1997).

Electrophysiology. Oocytes were clamped using two microelectrodes (0.5–2.5 MΩ) filled with 3 M KCl and a custom-built voltage clamp amplifier with high-voltage output stage (Stühmer, 1992; Zwart and Vijverberg, 1997). The external saline was clamped at ground potential by means of a virtual ground circuit using an Ag/AgCl reference electrode and a Pt/Pt-black current passing electrode. Membrane current was measured with a current-to-voltage converter incorporated in the virtual ground circuit. The membrane potential was held at −40 mV and all experiments were performed at room temperature (21–23°C). Oocytes were placed in a silicon tube (3 mm i.d.), which was continuously perfused with saline solution (in mM: 115 NaCl, 2.5 KCl, 1 $CaCl_2$, and 10 Hepes, pH 7.2 with NaOH) at a rate of approximately 20 ml/min. Aliquots of concentrated stock solutions of ACh in distilled water were added to the saline immediately before the experiments. Compounds were applied by switching between control and compound-containing saline using a servomotor-operated valve. The carbamates were applied for 20 s during the 40-s ACh application. Agonist applications were alternated by 5 min of superfusion with agonist-free saline to allow the receptors to recover completely from desensitization. In order to minimize adsorption of carbamates in the superfusion system, glass reservoirs and Teflon (PTFE) tubing (4 × 6 mm, Rubber, Hilversum, The Netherlands) were used. Membrane currents were low-pass filtered (eight-pole Bessel; −3 dB at 0.3 kHz), digitized (12 bits, 1024 samples per record), and stored on disk for off-line computer analysis (Zwart and Vijverberg, 1997).

Data analysis. Amplitudes of ion currents were measured and normalized to the amplitude of ACh-induced control responses (100%) to adjust for differences in receptor expression levels between oocytes and for small variations in response amplitude over time. The percentage of inhibition of the ACh-induced ion current by the carbamates was calculated from the quotient of the amplitude of the response after 20 s coapplication of the carbamate and that of the control response at the same time point. Standard concentration–effect curves were fitted to the experimental data according the Hill equation: $E = 100/[1 + (C/\mathrm{IC50})^{n_H}]$, where E represents the percentage response, C the concentration carbamate, IC50 the concentration that reduces the response by 50%, and n_H is the Hill slope. Each inhibition curve was fitted to data obtained from a single experiment. The mean concentration–effect curves are drawn using the calculated mean estimated values of the IC50 and Hill slope from n experiments. All data are represented as mean ± SD of n oocytes or brains. GraphPad Prism software (version 3.00) was used to fit the data and to assess statistical significance.

Results

Effects of carbamates on rat α4β4 nAChRs

The effects of six carbamates, i.e., aldicarb, bendiocarb, carbaryl, EPTC, fenoxycarb, and propoxur, on rat $\alpha4\beta4$ neuronal nAChRs have been investigated in detail. In oocytes expressing the rat $\alpha4\beta4$ nAChR, superfusion with external solution containing a near maximum effective concentration of 1 mM ACh evoked typical ligand-gated ion currents. The carbamate pesticides were coapplied with ACh during the ACh-evoked response in order to assess possible potentiating or inhibitory actions. In the concentration range of 0.1 μM to 1 mM, all six carbamates inhibited the $\alpha4\beta4$ nAChR-mediated ion current to some extent. Coapplication of the solvent DMSO at concentrations up to 0.1% (v/v) with ACh did not result in detectable effects. Marginal inhibitory effects were observed for 0.3 and 1% DMSO, i.e., solvent concentrations associated with the carbamate concentrations of 0.3 and 1 mM, respectively. The concentration-dependent inhibition of rat $\alpha4\beta4$ nAChR-mediated ion current by EPTC and propoxur is illustrated in Fig. 1. The kinetics of the responses in Fig. 1 illustrate that the rate of onset of inhibition increased with the concentration of the carbamates. At wash-out of the carbamates the reverse was observed. The rate of recovery of the ACh-induced ion current decreased with the concentration of the carbamates. On the wash-out of high concentrations of carbamates, the ACh-induced ion current did not fully recover before termination of the superfusion with ACh. The results in Fig. 1 fail to show potentiation of the ACh-induced ion current, which might be expected from previously published results on physostigmine, neostigmine, and carbaryl (Nagata et al., 1997; Zwart et al., 1999, 2000). Further screening of the effects of carbamates on the $\alpha4\beta4$ nAChRs revealed that 1 μM ACh-induced ion currents were unaffected by all six carbamates and by six additional carbamates, i.e., aminocarb, carbofuran, dioxacarb, methomyl, oxamyl, and pirimicarb, tested at a concentration of 1 μM. Conversely, some potentiation of 1 μM ACh-induced ion currents was occasionally observed with 100 μM aldicarb, pirimicarb, and propoxur, but the potentiating effects were too variable to be statistically significant.

The concentration-dependent inhibitory effects of aldicarb, bendiocarb, carbaryl, EPTC, fenoxycarb, and propoxur on 1 mM ACh-induced ion currents mediated by rat $\alpha4\beta4$ nAChRs are summarized in Fig. 2. Concentration–effect curves fitted to the data yielded IC50 values ranging from 3 μM for fenoxycarb to >1 mM for aldicarb and Hill slopes close to 1.5. The results show that the different carbamates have distinct potencies to inhibit the rat $\alpha4\beta4$ nAChR-mediated ion current with a potency order fenoxycarb > EPTC > carbaryl, bendiocarb > propoxur > aldicarb.

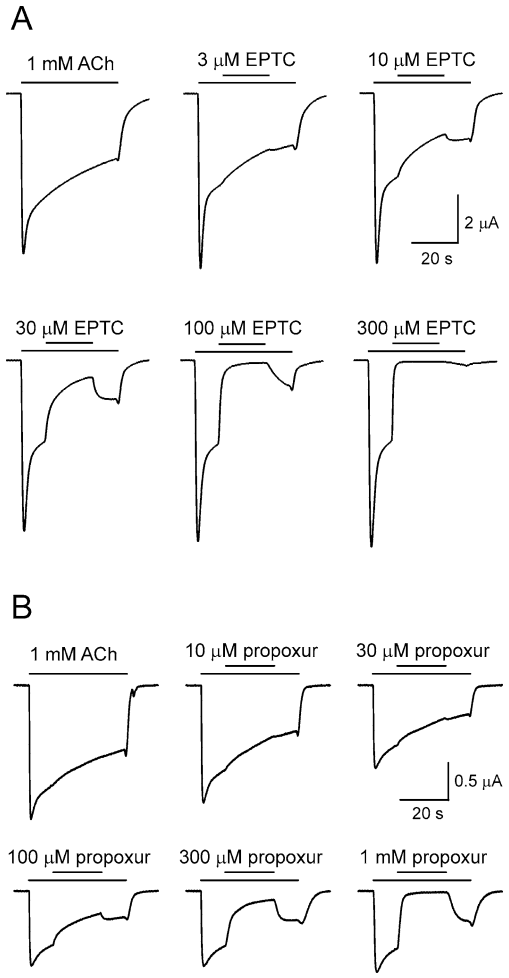


Fig. 1. Concentration-dependent inhibitory effects of carbamate pesticides on rat neuronal $\alpha4\beta4$ nAChRs expressed in *X. laevis* oocytes. The traces illustrate that coapplications of the indicated concentrations of (A) EPTC and (B) propoxur with 1 mM ACh result in a reduction of the agonist-induced inward current. Note that EPTC is the more potent inhibitor causing nearly complete inhibition when applied at a concentration of 100 μM, whereas the same concentration of propoxur causes less than half maximum inhibition of $\alpha4\beta4$ nAChR-mediated ion current. The horizontal bars on top of the traces indicate the periods of superfusion with external solution containing 1 mM ACh and/or carbamate at the concentrations indicated. Calibration bars apply to all traces in each panel.

Effects of carbamates on rat brain AChE

The inhibitory effects of the different carbamate pesticides on rat brain AChE activity were also determined for comparison with the demonstrated effects on the nAChR. Rat brain homogenate was incubated with increasing concentrations of the carbamates aldicarb, bendiocarb, carbaryl, EPTC, fenoxycarb, propoxur and physostigmine. The concentrations of the compounds ranged from 10 nM to 1 mM. The solvent DMSO, at the maximum final concentration of 1% (v/v) in these experiments, did not noticeably affect the rat brain AChE activity. The concentration-dependent reduction of rat brain AChE activity by the carbamates is shown in Fig. 3. Concentration–effect curves were fitted to

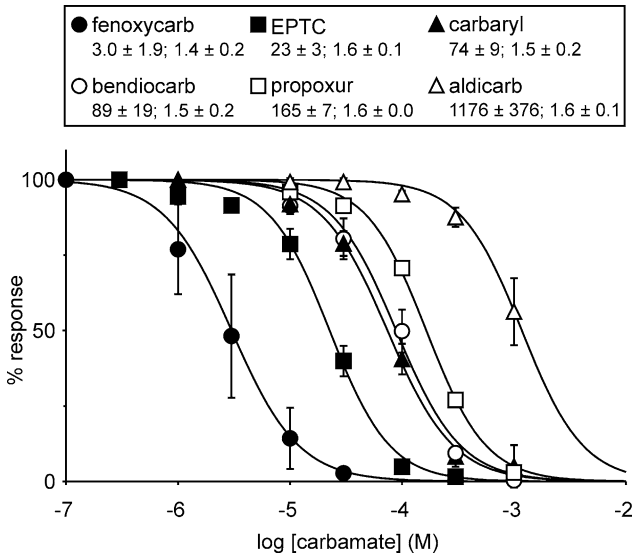


Fig. 2. The concentration dependence of inhibition of ion current mediated by rat $\alpha4\beta4$ nAChRs expressed in *Xenopus* oocytes by fenoxycarb, EPTC, carbaryl, bendiocarb, propoxur, and aldicarb. The inhibitory effects were measured in experiments in which carbamates were coapplied with 1 mM ACh as illustrated in Fig. 1. The data are depicted as mean ± SD. Error bars smaller than the size of the symbols are not shown. The lines are drawn according to the mean parameters of three to seven inhibition curves fitted to data from different oocytes. Estimated values of IC50 (μM) and n_H (mean ± SD) are given below the name of each compound.

the data according to the Hill equation. Most carbamate pesticides inhibited the rat brain AChE activity virtually completely, with IC50 values ranging from 1 μM for bendiocarb to 17 μM for carbaryl. For the AChE inhibitor physostigmine, which was tested as a reference compound, an IC50 value of 0.4 μM was obtained. In contrast, fenoxycarb and EPTC up to concentrations of 1 mM did not cause a marked reduction of the AChE activity. The low potencies of fenoxycarb and EPTC precluded the fitting of inhibition curves and the estimation of IC50 values, which were well beyond 1 mM. The potency order for rat brain AChE inhibition by the carbamates is physostigmine, bendiocarb > propoxur, aldicarb > carbaryl ≫ EPTC, fenoxycarb (Fig. 3).

Selectivity of carbamate effects for distinct subtypes of neuronal nAChRs

Effects of the three most potent inhibitors of the $\alpha4\beta4$ nAChR-mediated ion current, i.e., fenoxycarb, EPTC, and carbaryl (see Fig. 2), on other subtypes of neuronal nAChR were also investigated. To this purpose the $\alpha4$ nAChR subunit, which is abundant in the central nervous system (Whiting et al., 1991), and the $\alpha3$ nAChR subunit, which is strongly expressed in autonomic ganglia (see De Biasi, 2002), were coexpressed with the $\beta2$ or the $\beta4$ nAChR subunit in *Xenopus* oocytes. The effects were assessed in voltage clamp experiments in which various concentrations of the three carbamates were coapplied with a near maximum-effective concentration (0.3–1 mM) of ACh to oocytes expressing the distinct nAChR subtypes. In these experiments all observed effects were inhibitory. The effects of 3 μM fenoxycarb and 100 μM carbaryl on $\alpha4\beta4$, $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha3\beta2$ nAChRs are illustrated in Fig. 4. The results of three to seven experiments for each of the carbamates on each of the nAChR subtypes are shown in Fig. 5. Inhibition curves, according to the Hill equation, were fitted to the data and the mean and SD of the estimated IC50 values and Hill slopes were calculated. Two-way ANOVA of the IC50 values showed that the carbamates have different potencies to inhibit neuronal nAChRs ($F(2,32) = 196$; $p < 0.0001$) and that the distinct nAChR receptor subtypes have different sensitivities to inhibition by the carbamates ($F(3,32) = 6.69$; $p < 0.01$). In addition, interactions between specific carbamates and specific nAChR subtypes also appear to be involved ($F(6,32) = 7.82$; $p < 0.0001$). The results in Fig. 5 show that the carbamates inhibit $\alpha4\beta4$, $\alpha4\beta2$, and $\alpha3\beta4$ nAChRs with the potency order fenoxycarb > EPTC > carbaryl. All receptor subtypes show a similar sensitivity to carbaryl. Conversely, the $\alpha3\beta2$ nAChR is less sensitive to inhibition by fenoxycarb and EPTC than the other receptor subtypes. As a consequence, the potency order of the carbamates to inhibit $\alpha3\beta2$ nAChRs is fenoxycarb > EPTC, carbaryl.

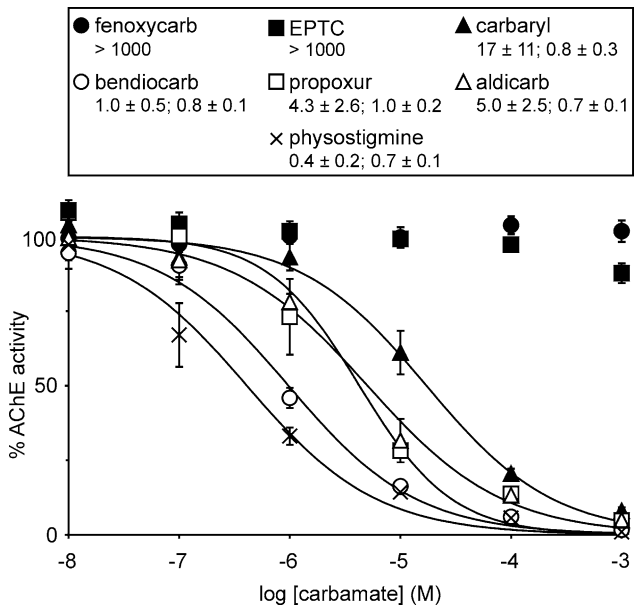


Fig. 3. The concentration-dependent inhibition of rat brain AChE by the carbamate pesticides bendiocarb, propoxur, aldicarb, carbaryl, EPTC, and fenoxycarb. For comparative purpose the effects of physostigmine are also depicted. The data are depicted as mean ± SD. Error bars smaller than the size of the symbols are not shown. The drawn lines are the mean inhibition curves obtained from four to five independent experiments. Estimated values of IC50 (μM) and n_H (mean ± SD) are given below the name of each compound.

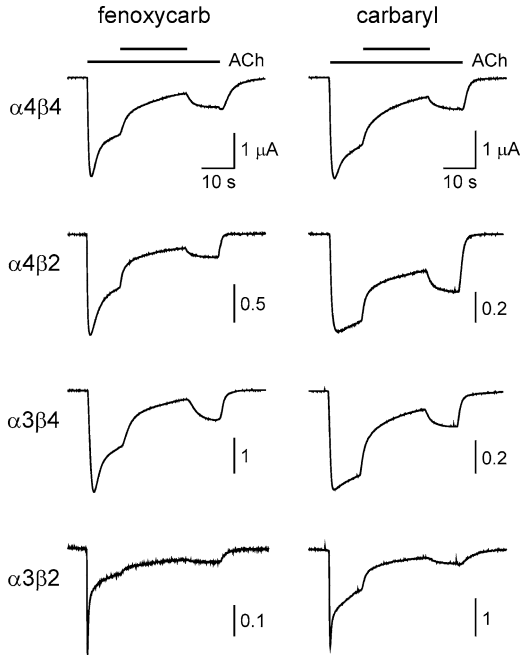
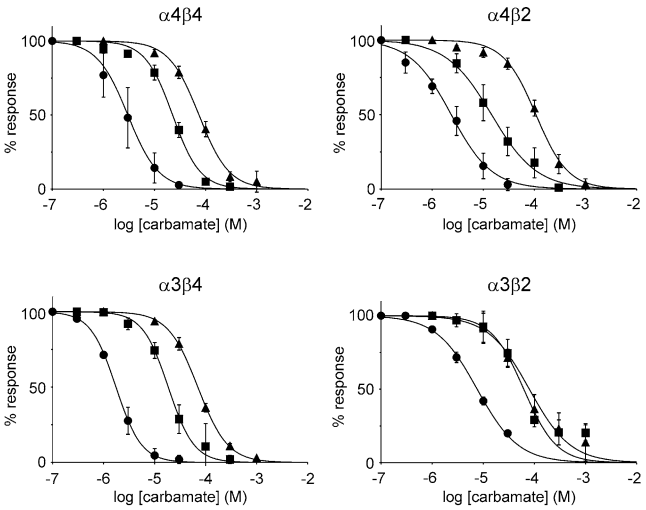


Fig. 4. Comparative effects of fenoxycarb and carbaryl on rat $\alpha4\beta4$, $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha3\beta2$ neuronal nAChR subtypes expressed in *X. laevis* oocytes. Ion currents were evoked by superfusion with external solution containing a near maximum-effective concentration of ACh. The carbamates fenoxycarb (3 μM; left column of traces) or carbaryl (100 μM; right column of traces) were coapplied during the ACh-evoked response. The periods of application of ACh and carbamates are indicated on top of the traces. Note that the effect of 3 μM fenoxycarb on $\alpha3\beta2$ is markedly less than its effect on the other nAChR subtypes, whereas 100 μM carbaryl inhibits all nAChR subtypes to a similar extent. Each trace was obtained from a different oocyte and all vertical calibrations are expressed in μA. The horizontal calibrations apply to all traces.

Discussion

The results demonstrate that carbamate pesticides interact directly with rat neuronal nAChRs. The predominant effect observed is an inhibition of ACh-induced ion currents in transfected *X. laevis* oocytes. Investigation of the effects on $\alpha4\beta4$ nAChR-mediated ion currents showed that the various carbamates tested have a wide range of inhibitory potencies. The more potent pesticides were fenoxycarb, EPTC, and carbaryl, with IC50 values ranging from 3 to 74 μM. For six carbamates, which were shown to be effective on $\alpha4\beta4$ nAChRs, the potencies for inhibition of rat brain AChE were also determined. As expected, most carbamates were relatively potent inhibitors of AChE, with IC50 values ranging from 1 μM for bendiocarb to 17 μM for carbaryl. The insecticide fenoxycarb, which was designed as a juvenile hormone analogue (Grenier and Grenier, 1993), and the herbicide EPTC were ineffective as AChE inhibitors. When comparing the potency order of the effects on $\alpha4\beta4$ nAChRs, i.e., fenoxycarb > EPTC > carbaryl, bendiocarb > propoxur > aldicarb, with that of the effects on rat brain AChE, i.e., bendiocarb > propoxur, aldicarb > carbaryl ≫ EPTC, fenoxycarb, it is striking that the more potent inhibitors of the nAChR are the less potent inhibitors of AChE.

In view of the excitatory symptoms of carbamate poisoning and since the acute toxicity of the carbamate pesticides correlates well with their potency to inhibit AChE (Ecobichon, 2001), it is unlikely that the inhibitory effects on nAChRs contribute to the acute poisoning syndrome. However, as outlined in the introduction, some carbamates may also induce a variety of neurological changes at dose levels producing no overt signs of acute toxicity (Ecobichon, 2001). This, and the supersensitivity of AChE knockout mice to the organophosphate AChE inhibitors DFP and VX (Xie et al., 2000; Duysen et al., 2001), suggest the involvement of other targets than AChE in carbamate neurotoxicity. In addition, chronic use of dithiocarbamate drugs in the treatment of alcoholism in humans may provoke several neurological and behavioral changes (Vaccari et al., 1998). Effects on glutamate and dopamine release (Vaccari



	α4β4	α4β2	α3β4	α3β2
● **fenoxycarb**				
IC_{50} (μM)	$3.0 \pm 1.9^{\S}$	$2.4 \pm 0.7^{\S\S}$	$1.8 \pm 0.3^{\S\S}$	7.6 ± 0.6
n_H	1.4 ± 0.2	1.1 ± 0.2	1.7 ± 0.3	1.1 ± 0.1
■ **EPTC**				
IC_{50} (μM)	$23 \pm 3^{*,\S\S\S}$	$14 \pm 3^{\S\S\S}$	$18 \pm 3^{\S\S\S}$	62 ± 3
n_H	1.6 ± 0.1	1.0 ± 0.1	1.7 ± 0.4	1.4 ± 0.8
▲ **carbaryl**				
IC_{50} (μM)	74 ± 9	107 ± 23	70 ± 8	80 ± 27
n_H	1.5 ± 0.2	1.4 ± 0.2	1.5 ± 0.0	1.1 ± 0.5

Fig. 5. The concentration dependence of inhibition of ion currents mediated by rat $\alpha4\beta4$, $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha3\beta2$ neuronal nAChR subtypes expressed in *X. laevis* oocytes by fenoxycarb, EPTC, and carbaryl. The inhibitory effects were measured in experiments in which various concentrations of the carbamates were coapplied with a near maximum-effective concentration of ACh as illustrated in Fig. 4. The drawn lines are the mean inhibition curves obtained from three to seven oocytes. The data are depicted as mean ± SD. Error bars smaller than the size of the symbols are not shown. Estimated values of IC50 and n_H (mean ± SD) are given at the bottom. Statistical significance of the data was assessed by ANOVA and Bonferroni's multiple comparison test. *Difference with $\alpha4\beta2$ nAChR ($p < 0.05$).§, §§, §§§Difference with $\alpha3\beta2$ ($p < 0.05$, 0.01, and 0.001, respectively).

et al., 1996, 1998), muscarinic ACh receptors (Van den Beukel et al., 1997), as well as effects on neuronal nAChRs, might all contribute to chronic carbamate toxicity. Although relations between cholinesterase inhibition or receptor effects and neurotoxicity (other than the acute syndrome) of carbamates cannot yet be established, all of these mechanisms are relevant for future toxicological research. The latter is important in view of the recent interest in potential chronic adverse effects of cholinesterase inhibitors, whether pesticides, warfare agents, or drugs (Soreq and Seidman, 2001).

Although the mechanisms relating to AChE inhibition have been described in detail (for review see Soreq and Seidman, 2001), the nature of the inhibitory effect of the carbamate pesticides on nicotinic ACh receptors is less evident. It has been shown that high concentrations of some carbamates, e.g., aminocarb, aldicarb, and carbaryl, displace [^{3}H]ACh from *Torpedo* electric organ nAChRs (Eldefrawi and Eldefrawi, 1983). These data and the potentiation of 30–100 μM carbachol-induced ion currents in rat PC12 cells by carbaryl (Nagata et al., 1997) indicate that carbamates may act competitively or allosterically. The nAChRs in *Torpedo* electric organ are muscle-type nAChRs (for review see Karlin, 2002). PC12 cells express heteromeric neuronal nAChRs composed of α3, α5, β2, β3, and β4 subunits (Rogers et al., 1992) as well as homomeric α7 nAChRs (Drisdel and Green, 2000). The present results show that the carbamates cause inhibitory effects on four distinct subtypes of neuronal nAChRs investigated, i.e., $\alpha4\beta2$, $\alpha3\beta2$, and $\alpha3\beta4$. These neuronal nAChRs differ from the muscle-type nAChR and are not representative for the heterogeneous pool of nAChRs expressed in PC12 cells. It has been shown previously that the degrees of potentiation and inhibition of neuronal nAChRs by the carbamate physostigmine depend on agonist and physostigmine concentrations as well as on the specific subtype of neuronal nAChR (Zwart et al., 2000). Similar principles might account for the divergence between the present results on the effects of a number of carbamates on rat nAChRs expressed in oocytes and previous results obtained with nAChRs natively expressed in rat PC12 cells (Nagata et al., 1997). The exact mechanisms by which the carbamate drugs and pesticides modulate nAChR function remain a topic of our current investigations.

The results demonstrate that carbamate pesticides inhibit the $\alpha4\beta4$, the $\alpha4\beta2$, and the $\alpha3\beta4$ nAChR subtypes to a similar extent. However, the $\alpha3\beta2$ nAChR appears to be less sensitive for inhibition by fenoxycarb and EPTC than the other subtypes of nAChR (see Fig. 5). In a previous study comparing the effects of the ganglionic antagonists hexamethonium, mecamylamine, pentolinium, and trimetaphan on $\alpha3\beta2$ and $\alpha3\beta4$ nAChRs, the $\alpha3\beta2$ receptor also appeared the less sensitive receptor subtype (Cachelin and Rust, 1995). The present and the previous results show that α as well as β nAChR subunits contribute to the inhibitory effect of distinct classes of cholinergic compounds.

Although the $\alpha3\beta2$ ganglionic subtype of nAChR appears to be less sensitive for inhibition by some carbamates, the physiological consequences and toxicological implications of the differential sensitivity of nAChR subtypes remain unclear. Some information on the roles of specific nAChR subunits in behavior and development comes from studies on knockout mice. Age-related effects on learning, observed in the behavioral assessment of β2 subunit knockout mice, indicate a role of the β2 nAChR subunit to prevent dysfunction of cholinergic neurons in aged mice (for review see Cordero-Erausquin et al., 2000). The same authors review the involvement of α4 and β2 subunits in the reinforcing effects of nicotine and the pivotal role of the α3 subunit in peripheral, autonomic control. The findings indicate that some subtypes of neuronal nAChR are involved in the long-term maintenance or adaptation of neuronal activity. Therefore, potential chronic effects of acutely nontoxic levels of carbamate pesticides remain a topic of toxicological significance.

In conclusion, the results demonstrate that carbamate pesticides, like carbamate drugs, affect nAChRs independently of AChE inhibition. Thus, it appears that nAChRs are an additional, non-AChE target for the carbamate pesticides. The findings suggest that the effects on neuronal nAChRs contribute to the toxicology of some carbamate pesticides and these effects may contribute to long-term changes in the nervous system.

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