A Noncompetitive, Sequential Mechanism for Inhibition of Rat \( \alpha4\beta2 \) Neuronal Nicotinic Acetylcholine Receptors by Carbamate Pesticides

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The mechanism by which carbamate pesticides inhibit rat \( \alpha4\beta2 \) nicotinic acetylcholine (ACh) receptors (nAChRs) expressed in Xenopus laevis oocytes has been investigated using the two-electrode voltage clamp technique. Carbaryl, S-ethyl N,N-dipropylthiocarbamate (EPTC), and fenoxycarb inhibit ACh-induced ion currents in a concentration-dependent way. EPTC and fenoxycarb inhibit ion currents induced by 1 mM ACh with 3-fold to 5-fold higher potency than ion currents induced by 1 \( \mu \)M ACh. The potency of carbaryl appears to be independent of ACh concentration. Fenoxycarb displaces \(^3\)H-epibatidine bound to \( \alpha4\beta2 \) (nAChRs) with a \( K_i \) of 750 \( \mu \)M, which is much higher than the functional IC\(_{50}\) of 2.3–11 \( \mu \)M. This shows that the inhibition of ion current by the carbamate is a noncompetitive effect. Inhibition by fenoxycarb is independent of the state of the ion channel. The rate of onset of inhibition is enhanced, and the rate of reversal of inhibition is reduced, when the concentration of fenoxycarb is increased. The rate of reversal of inhibition is also reduced when the period of exposure to fenoxycarb is increased. The time- and concentration-dependent inhibition of nAChR-mediated ion current by fenoxycarb is accounted for by a two-step mechanism involving a rapid blocked state and a sequential nAChR-mediated ion current by fenoxycarb is accounted for by a two-step mechanism involving a rapid blocked state and a sequential

Key Words: neuronal nicotinic acetylcholine receptor; carbamate pesticide; noncompetitive inhibition; voltage clamp; Xenopus oocyte; epibatidine binding.

Carbamate pesticides, widely applied as insecticides, herbicides, and fungicides, may cause a variety of symptoms in mammals and humans. Acute carbamate poisoning generally causes inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7), which impairs the hydrolysis of released acetylcholine (ACh) and leads to excessive cholinergic symptoms. Studies on chronic exposure to carbamate pesticides report several neurological sequelae of unknown origin (for review, see Ecobichon, 2001; Moser, 1999) and suggest that additional mechanisms, distinct from inhibition of AChE, are involved in carbamate neurotoxic effects. In addition to inhibition of AChE, several carbamates have been shown to interact with cholinergic receptors. The carbamate physostigmine and related cholinesterase inhibitors interact with muscarinic ACh receptors (Lockhart et al., 2001; Van den Beukel et al., 1997), as well as nicotinic ACh receptors (nAChRs). The mechanism of interaction of the carbamate physostigmine and related drugs with nicotinic receptors has been thoroughly investigated. Low concentrations of physostigmine and analogues agonize or potentiate neuronal nAChRs, whereas high concentrations of these drugs block neuronal nAChRs. These effects appear to be caused by competitive and noncompetitive interactions with the nAChRs (Storch et al., 1995; Nagata et al., 1997; Van den Beukel et al., 1998; Zwart et al., 2000). However, the mechanism of action of carbamate pesticides on nAChRs is less well known.

The fungicide propineb enhances cholinergic transmission in guinea pig myenteric plexus preparations, presumably by an effect on ganglionic nAChRs and not on muscle type nAChRs (Marinovich et al., 2002). Several carbamate insecticides, e.g., the cholinesterase inhibitors aminocarb, aldicarb, and carbaryl at 100 \( \mu \)M, displace \(^3\)H-ACh from muscle type nAChRs in Torpedo electric organ membranes (Eldefrawi and Eldefrawi, 1983). Additionally, carbaryl concentration-dependently potentiates and inhibits neuronal nAChR channels in rat pheochromocytoma PC12 cells (Nagata et al., 1997). Systematic investigation of the effects of a number of carbamate pesticides on defined subtypes of neuronal nAChRs expressed in Xenopus laevis oocytes has demonstrated relatively high potencies of fenoxycarb, S-ethyl N,N-dipropylthiocarbamate (EPTC), and carbaryl to inhibit the ACh-induced ion current (Smulders et al., 2003). The resemblance in effects of carbamate pesticides and carbamate drugs on nAChRs might suggest that they have similar mechanism of action. However, the specific mechanism of action of carbamate pesticides remains to be elucidated. Because the nature of the effects of carbamate pesticides on neuronal nAChRs is mainly inhibitory (Smulders et al., 2003), we have focused on the mechanism of inhibition by several of the more potent carbamates, i.e., carbaryl, EPTC, and fenoxycarb and have investigated their concentration-dependent effects on rat \( \alpha4\beta2 \) nAChRs heterologously expressed in Xenopus laevis oocytes.

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MATERIALS AND METHODS

Animals and materials. Xenopus laevis (AmRep, Breda, The Netherlands) were kept in standard aquaria and fed three times a week on earthworms (Hagens, Nijkerkerveen, The Netherlands). All experimental procedures involving animals were approved by a local ethics committee and were in accordance with Dutch law. Acetylcholine chloride (ACh), collagenase type I, dimethylsulfoxide (DMSO; ACS reagent), 3-amino benzoic acid ethyl ester, methane sulfonate salt (MS-222), NaCl, and neomycin solution (10 mg neomycin/ml in 0.9% NaCl) were obtained from Sigma (St. Louis, MO). Carbaryl (1-naphthalenyl methyl-carbamate), fenoxycarb (ethyl[2-4-phenoxypyrenyl]ethylcarbamate), and EPTC (S-ethyl N,N-diarylpropylthiocarbamate) were purchased from Riedel-de Haën (Seelze, Germany). CaCl₂ (1 M solution), MgCl₂ (1 M solution), MgSO₄, NaHCO₃, and NaOH were purchased from BDH Laboratory Supplies (Poole, England). Ca(NO₃)₂, HEPES and KCl were from Merck (Darmstadt, Germany). 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).

Receptor expression in oocytes. Mature female frogs were anesthetized by submersion in 0.2% MS-222 and ovarian lobes were surgically removed. 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 α4 and β2 subunits of rat neuronal nAChRs (Boulter et al., 1987; Duvoisin et al., 1989), dissolved in distilled water at a 1:1 molar ratio, were injected with a Drummond microinjector into the nuclei of stage V and stage VI oocytes within 8 h after harvesting. The volume injected was 18.4 nl/oocyte (~0.1 ng of each plasmid). After injection, the oocytes were incubated at 19°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM MgCl₂, 0.82 mM MgSO₄, 15 mM HEPES, and 50 μg/ml neomycin] for 3–6 days before experiments were performed (Zwart and Vijverberg, 1997).

3H-Epibatidine binding to oocyte homogenates. Batches of 50–80 frozen oocytes expressing rat α4β2 nAChRs were thawed and homogenized in a Potter homogenizer in an excess of buffer A (50 mM Tris·HCl pH 7, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, and 2 mM phenylmethylsulfonyl fluoride), centrifuged for 60 min at 30,000 × g, and rinsed twice. The homogenates were resuspended in the same buffer containing 20 μg/ml of the protease inhibitors leupeptin, bestatin, pepstatin A, and aprotinin. Receptor expression ranged from 172 to 286 fmol/mg protein, which corresponds to an average nAChR density of 41 to 50 fmol/oocyte (44.3 ± 1.6; mean ± S.E.M., n = 5).

Preliminary time course experiments were performed before saturation and competition analyses to determine the time required for 3H-epibatidine to reach equilibrium with the rat α4β2 nAChRs. In saturation binding experiments, aliquots of oocyte homogenates were incubated overnight at 4°C with 0.005–2.5 nM 3H-epibatidine. Nonspecific binding was determined in parallel in the presence of 100 nM unlabeled epibatidine. At the end of incubation, samples were filtered on GFC filters pre-soaked in polyethylenimine through a Brandell apparatus and counted in a β counter. To test the ability of fenoxycarb to inhibit 3H-epibatidine binding, fenoxycarb dissolved in DMSO was diluted in buffer A just before use. Serial dilutions were pre-incubated for 30 min at room temperature with homogenates containing α4β2 nAChRs. Subsequently, 0.05 nM 3H-epibatidine was added for overnight incubation at 4°C.

Electrophysiology. Oocytes were placed in a silicone tube (inner diameter 3 mm), which was continuously perfused with saline solution (115 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 10 mM HEPES in distilled water, pH 7.2 with NaOH) at a rate of ~20 ml/min, resulting in a fluid velocity around the oocyte of ~0.6 mm/s. Ion currents evoked by high concentrations of ACh activated with a time constant of 0.3–0.4 s. Oocytes were voltage clamped using two microelectrodes (0.5–2.5 MΩ) filled with 3 M KCl (Stühmer, 1992; Zwart and Vijverberg, 1997). The membrane potential was held at −40 mV unless noted otherwise. All experiments were performed at room temperature (21±23°C).

Aliquots of frozen, concentrated stock solutions of 1 M ACh in distilled water and of 0.1 M of carbaryl, EPTC, and fenoxycarb in DMSO were thawed and added to the saline immediately before the experiments. Compounds were applied by switching the perfusate from control to compound-containing saline using a servomotor-operated valve. Agonist applications were alternated by 5 min of superfusion with agonist-free saline to allow the receptors to recover completely from desensitization. To minimize adsorption of carbamates in the perfusion system, glass reservoirs and Teflon tubing (PTFE; 4 × 6 mm, Rubber, Hilversum, The Netherlands) were used. Membrane currents were low-pass filtered (8-pole Bessel; −3 dB at 0.3 kHz), digitized (12 bits, 1024 samples/record), and stored on disk for off-line computer analysis (Zwart and Vijverberg, 1997).

Data analysis. Amplitudes of ion currents were normalized to those of ACh-induced control responses to adjust for differences in receptor expression levels between oocytes and for small variations in response amplitude over time. Standard concentration-effect curves were fitted to the experimental data according to the Hill equation:

\[ E = \frac{100}{1 + \left(\frac{[C]}{IC_{50}}\right)^{n_H}} \]

where \( E \) is the percentage response, \([C]\) is the carbamate concentration, \( IC_{50} \) is the carbamate 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 mean values of the \( IC_{50} \) and Hill slope calculated from 3–4 experiments. All data are represented as mean ± S.D. of n oocytes. GraphPad Prism 3.0 software was used to fit the data and to assess statistical significance.

The binding parameters were estimated from saturation binding data and fit to data obtained from a single experiment using non-linear least-squares procedure using the LIGAND program.

RESULTS

Effects of Carbamates on Neuronal Type nAChRs.

The effects of carbaryl, EPTC and fenoxycarb have been investigated in detail in oocytes expressing the rat α4β2 neuronal type nAChR. Large inward currents were evoked by superfusion with external solution containing the near maximum-effective concentration of 1 mM ACh. During the ACh-evoked responses carbamate pesticides were coapplied with ACh for a short period to assess their effects on rat α4β2 nAChRs. The carbamates generally inhibit the α4β2 nAChR-mediated ion current evoked by high concentrations of ACh (Fig. 1). Effects of carbaryl, EPTC and fenoxycarb on rat α4β2 nAChRs were also assessed at the low concentration of 1 μM ACh. The low concentration of ACh elicits ion currents with amplitudes that are less than 10% of the maximum ACh response in the same oocyte (Zwart et al., 2000). At this low concentration of ACh, carbaryl caused a similar inhibition, but EPTC and fenoxycarb caused smaller inhibitory effects than at high agonist concentration (Fig. 1). With low concentrations of EPTC, some potentiation of the 1 μM ACh-induced ion current...
was also observed (not shown). In general, the reversal of the EPTC-induced inhibition was too slow for repeated applications to the same oocyte, because a complete reversal of effects was not obtained, even after prolonged washing. The results suggest that the effects of the carbamate pesticides depend on the concentration of the agonist ACh used to evoke the response.

**Dependence of the Carbamate Effects on Agonist Concentration**

Concentration-effect curves of the inhibition of 1 mM (Smulders et al., 2003) and 1 μM ACh-induced ion currents mediated by rat α4β2 nAChRs by carbaryl, EPTC, and fenoxy carb are shown in Figure 2. The solvent DMSO, coapplied at concentrations up to 0.1% (v/v) with ACh, did not cause detectable effects. DMSO at the highest concentrations of 0.3% and 1%, i.e., solvent concentrations associated with the carbamate concentrations of 0.3 mM and 1 mM, respectively, caused 8–13% inhibition when applied alone. For carbaryl and fenoxy carb, inhibition was the only effect observed in the

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**FIG. 1.** Effects of carbamate pesticides on rat neuronal α4β2 nAChR expressed in Xenopus laevis oocytes. Coapplication of carbaryl, EPTC, and fenoxy carb with 1 μM ACh (left column) results in a reduction of the agonist-induced inward current. At the much higher concentration of 1 mM ACh (right column), the inhibitory effects of fenoxy carb and EPTC are enhanced and the effect of carbaryl is similar to that obtained at 1 mM ACh. The horizontal bars on top of the traces indicate the period of superfusion with external solution containing ACh (dashed bars) and carbamate (solid bars) at the concentrations indicated. Each trace is from a different oocyte. Note that the maximum amplitude of the ACh-induced ion current depends on the receptor expression level, which varies between oocytes.

**FIG. 2.** Concentration-effect curves for the inhibition of rat α4β2 nAChR mediated ion current by (A) fenoxy carb, (B) EPTC, and (C) carbaryl. Ion currents were evoked by low (1 μM; open circles) and high (1 mM; filled circles) concentrations of ACh. The solid lines are the mean concentration-effect curves fitted to the data of 3–4 oocytes according to the Hill equation (eq. 1). Data points and vertical bars are the mean ± S.D. of the experimental data. Estimated values for IC50 and Hill slope are summarized in Table 1. The concentration-effect curves at 1 mM ACh are after Smulders et al. (2003).
complete concentration range of 0.1 μM–1 mM, whereas EPTC caused some potentiation in the lower concentration range and inhibition at higher concentrations. Inhibition curves, according to the Hill equation (eq. 1), were fitted to the data. Calculated mean and S.D. of the estimated IC50 values and Hill slopes are summarized in Table 1. The different carbamates have distinct potencies to inhibit the nAChR-mediated ion current with an overall potency order: fenoxycarb > EPTC > carbaryl. For the more potent inhibitors fenoxycarb and EPTC a clear left shift of the inhibition curves is observed when the agonist concentration is raised from 1 μM to 1 mM (Fig. 2; Table 1).

Competition Binding of Fenoxycarb with 3H-Epibatidine

The dependence of the inhibitory effects of the carbamate pesticides on agonist concentration might indicate that both interact with the same sites on the nAChR. Therefore, radioligand binding experiments have been performed on membranes of oocytes transfected with rat α4β2 nAChRs to investigate possible competition between fenoxycarb, the more potent carbamate, and 3H-epibatidine, a radiolabeled agonist of the nAChR. Saturation binding (Fig. 3A) yielded a Kd of 46 pM 3H-epibatidine (coefficient of variation 11%) and a Bmax of 237 ± 30 fmol/mg protein (mean ± SEM, n = 5). The binding data were analyzed for a single vs. two-site interaction, but there was no statistical better fit for the two-site model. Nonspecific binding, determined in parallel by means of incubation in the presence of unlabeled epibatidine, amounted to 5–15% of total binding. Analysis of the specific binding data yielded linear Scatchard plots, demonstrating the presence of a single class of high-affinity epibatidine binding sites in membranes of oocytes transfected with rat α4β2 nAChRs. No specific 3H-epibatidine binding was found in membranes prepared from untransfected oocytes.

Specific 3H-epibatidine binding to oocyte homogenates was displaced by increasing concentrations of fenoxycarb in competition binding experiments (Fig. 3B). The displacement experiments yielded a Ki value for fenoxycarb of 750 μM (coefficient of variation 16%). Similar experiments with ACh yielded a Ki value for the displacement of 3H-epibatidine by ACh of 39 nM (coefficient of variation 13%). These results show that the agonist epibatidine bound to the agonist recognition sites of rat α4β2 nAChRs is displaced by fenoxycarb, but only at concentrations that are much higher than those required to inhibit the ACh-induced ion current.

### Table 1

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>[ACh]</th>
<th>IC50 (μM)</th>
<th>nH</th>
</tr>
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<tbody>
<tr>
<td>Carbaryl</td>
<td>1 μM</td>
<td>90 ± 7</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>107 ± 23</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>EPTC</td>
<td>1 μM</td>
<td>35 ± 0</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>13 ± 4*</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Fenoxycarb</td>
<td>1 μM</td>
<td>11 ± 3</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>2.3 ± 0.7**</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

All values are mean ± S.D. of parameter estimates obtained from three or four independent experiments. *p < 0.001 and **p < 0.01 as compared to the effect at lower agonist concentration on the same receptor subtype (one-way ANOVA). Data on 1 mM ACh were refitted after Smulders et al. (2003).
Noncompetitive Mechanisms

The binding experiments show that fenoxycarb has a very low affinity for the agonist recognition site. This suggests that inhibition of the ACh-induced ion current by fenoxycarb, which occurs at a several orders of magnitude lower concentration, is due mainly to noncompetitive effects. Therefore, various possibilities for noncompetitive inhibition of the neuronal type nAChR by the carbamate fenoxycarb have been investigated. To assess whether the carbamate itself is the agent causing the inhibitory effect, experiments were performed in which ACh and fenoxycarb were coapplied and subsequently washed out, either separately or together. The result shows a rebound tail current, indicating reversal of block only when fenoxycarb is washed out (Fig. 4A). On washout of ACh, the remaining inward current rapidly declined to zero irrespective of whether fenoxycarb remained present or not and rebound tail currents were not observed. These results show that fenoxycarb blocks the ion current and rules out the possibility that the inhibitory effect is caused by an enhancement of the channel blocking potency of ACh in the presence of fenoxycarb.

The possible alternatives of open and closed channel block were also evaluated. The results in Figure 4B show that fenoxycarb, at a concentration that causes nearly complete block when applied during the ACh-induced response, also causes a strong inhibitory effect when applied before application of ACh. These results demonstrate that the presence of the agonist is not required for the inhibitory effect to occur, and that open channel block cannot account for the noncompetitive, inhibitory effect of fenoxycarb. In addition, the degree of inhibition appeared to be independent of the membrane potential over the range from −100 mV to −40 mV (Fig. 5). In separate experiments, performed on oocytes expressing α4β4 nAChRs, it was demonstrated that inward current evoked at a membrane potential of −30 mV is blocked in exactly the same way as outward current evoked at +30 mV (not shown).

Onset and Reversal of Inhibition

The kinetics of the effects of fenoxycarb show that the rate of onset of inhibition increased with carbamate concentration (Fig. 6A). Values of the apparent fast and slow rate constants of the onset of inhibition were obtained by fitting eq. 2 to the data as shown in the inset of Figure 6B and plotted against fenoxycarb concentration. An inverse relation between carbamate concentration and the rate of reversal of inhibition was obtained on washout (Fig. 6B). The rate of reversal of inhibition of the ACh-induced ion current decreased with increasing concentration and with an increasing period of application of the carbamate immediately before washout (Fig. 6C). After high concentrations of carbamates and after long periods of exposure, the inhibitory effect did not fully reverse on washout of the carbamate before termination of superfusion with ACh (Fig. 6, insets). These
... results demonstrate that the inhibition by the carbamate pesticides cannot be accounted for on the basis of a first order process. The simplest model to describe the dual rate of onset and the concentration-dependent rate of reversal of inhibition is a two-step sequential chemical equilibrium between carbamate and nAChR:

\[ \begin{align*}
    C + R & \leftrightarrow CR \\
    & \leftrightarrow CR^* \\
    k_{on} & \quad k_{+1} \\
    k_{off} & \quad k_{-1}
\end{align*} \]

In the model R is the activated nAChR, C the carbamate molecule, CR is a rapidly reversible (low-affinity) blocked state of the receptor, and CR* is a slowly reversible (high-affinity) blocked state. Note that the transition from CR to CR* and vice versa occurs without binding or dissociation of the carbamate. The first step is considered to be fast as compared to the second. Forward rate constants \( k_{on} \) (M\(^{-1}\) s\(^{-1}\)) and \( k_{+1} \) (s\(^{-1}\)) and backward rate constant \( k_{off} \) (s\(^{-1}\)) and \( k_{-1} \) (s\(^{-1}\)) were estimated by fitting the following relations between the concentration of the carbamate \([C]\) and the observed rapid (\( k_{fast} \)) and slow (\( k_{slow} \)) components of onset of the inhibitory effect (e.g., Zhao et al., 1999):

\[ k_{fast} = k_{off} + k_{on}[C] \] (3a)

\[ k_{slow} = k_{-1} + k_{+1}/(1 + K_{d,1}/[C]). \] (3b)

Note that with \( K_{d,1} = k_{off}/k_{on} \) and \( K_{d,2} = k_{-1}/k_{+1} \) this accounts for a concentration-dependent inhibitory effect with an apparent affinity of:

\[ K_{d,apparent} = K_{d,1}/(1 + 1/K_{d,2}) \] (3c)

Regression lines according to eq. 3a and eq. 3b in Figure 6A show an approximate compliance of the effects observed with the two-step sequential mechanism of scheme 3. The rate constants for scheme 3 obtained from the kinetic fits are \( k_{on} = 0.044 \) M\(^{-1}\) s\(^{-1}\), \( k_{off} = 0.81\) s\(^{-1}\), \( k_{+1} = 0.23\) s\(^{-1}\), and \( k_{-1} = 0.06\) s\(^{-1}\). Thus \( K_{d,1} = k_{off}/k_{on} = 18.4\) M\(^{-1}\) and \( K_{d,2} = k_{-1}/k_{+1} = 0.26\) and the apparent affinity of the overall process of inhibition by fenoxycarb, according to eq. 3c, \( K_{d,apparent} = 3.8\) M\(^{-1}\). This is almost the same as the IC\(_{50}\) value of 2.3 M\(^{-1}\) for the inhibition curve of fenoxycarb (Table 1).

Because the fitting of the fast and slow rate constants is confounded by desensitization, a more accurate parameter estimate was obtained from fitting the two-step model presented in scheme 3 including exponential desensitization of the receptors using ACSL software (Aegis Software Group, Huntsville, AL; Fig. 7A). This yielded values for \( K_{d,1} = 5.8\) M\(^{-1}\), \( K_{d,2} = 0.21\) and \( K_{d,apparent} = 1.0\) M\(^{-1}\). The calculated value for \( K_{d,apparent} \) is very similar to the apparent affinity of fenoxycarb obtained from the inhibition curves (Table 1). In a second stage, the onset and the reversal of inhibition by a range of concentrations of fenoxycarb was simulated using Berkeley Madonna software (Version 8.0.1; Berkeley, CA). The simulation was performed using...
concentrations of ACh. Competition binding experiments demonstrate that the apparent affinity of fenoxycarb for the epibatidine binding sites on the nAChR is very much lower ($K_i = 750\ \mu M$) than that of inhibition of the ion current ($IC_{50} = 2.3–11\ \mu M$). The very low affinity of fenoxycarb for the agonist recognition site assessed by ligand binding (Fig. 3) suggests that a noncompetitive mechanism is responsible for the inhibitory effect of the carbamate on the nAChR-mediated ion current. Because binding experiments are performed on supposedly desensitized nAChRs, it cannot be excluded apriori that the affinity of fenoxycarb for the agonist recognition site is much higher in nondesensitized nAChRs than in desensitized nAChRs. However, in that case it would be expected that the potency of fenoxycarb would be higher at low, i.e., nondesensitizing, as compared to high, desensitizing concentrations of ACh. The observation of the reverse relation between fenoxycarb potency and agonist concentration (Fig. 2) provides an additional argument for the noncompetitive nature of the inhibitory effect.

From the inhibition of rat $\alpha_4\beta_2$ nAChRs by fenoxycarb in the presence and in the absence of the agonist (Fig. 4) and from the absence of voltage dependence of the inhibitory effect (Fig. 5), it is concluded that channel opening is not required for block and that the carbamate inhibits the ACh-induced ion current independent of the state of the ion channel. Furthermore, the biphasic kinetics of onset of inhibition and the concentration-dependence and time-dependence of the kinetics of reversal of inhibition (Fig. 6) demonstrate that the inhibitory effect is more complex than a simple first order mechanism and suggests a two-step mechanism. A model originally developed in enzyme kinetics, i.e., a sequential two-step equilibrium with a rapidly reversible association and dissociation of the carbamate followed by a slowly reversible transition (Zhao et al., 1999), fits the kinetic data very well and also predicts the features of concentration-dependent inhibition (Fig. 7). A particularly attractive feature of the model is that it accounts for the slow reversal of the inhibitory effect after exposure to high concentration of carbamate and after prolonged exposure. A similar slow, concentration-dependent reversal has been reported before for the inhibition of human muscle type nAChRs expressed in TE-671 cells by the philanthotoxin PhTX-(12). Based on the effects of philanthotoxins, it was concluded that these toxins act on two functionally distinct sites, one outside the ion channel associated with a slowly reversible inhibitory effect and another inside the ion channel associated with rapidly reversible open channel block (Brier et al., 2003). Despite the apparent homology between the effects of philanthotoxins and those presently observed for fenoxycarb, the present data are not indicative of open channel block as observed with PhTX-343. The effect of fenoxycarb is not voltage dependent, and is independent of the direction of the current through the ion channel. The same has been observed for PhTX-(12), which is a weak open channel blocker and is supposed to enhance

![Data](image1.png)  
**FIG. 7.** Fit of the experimental data of fenoxycarb inhibiting $\alpha_4\beta_2$ nAChRs with a two-step mechanism. (A) The left panel shows the kinetic data of onset and reversal of inhibition of 1 mM ACh-induced ion current by 30 $\mu M$ fenoxycarb applied for periods of 3, 6, 12, and 20 s. The superimposed traces represent the slowly decaying phase of the ACh-induced response and are from the same oocyte. The control trace, without fenoxycarb, is marked “c.” The right panel shows the results of fitting the two-step inhibition scheme to the entire data set using ACSL software. All currents were assumed to desensitize with the same rate constant, which was also estimated from the data and amounted to 0.019 s$^{-1}$. Other rate constants estimated from the fit are: $k_{on} = 0.052\ \mu M^{-1}\ s^{-1}$, $k_{off} = 0.30\ s^{-1}$, $k_{1} = 0.13\ s^{-1}$, and $k_{2} = 0.027\ s^{-1}$. (B) The left panel shows the kinetics of onset and reversal of inhibition of 1 mM ACh-induced ion current by 0.3, 1, 3, 10, and 30 $\mu M$ fenoxycarb applied for a period of 20 s. The superimposed traces are from the same oocyte. The right panel shows the results of simulating the two-step inhibition scheme to the exact kinetic parameters as in A and with the same range of concentrations of fenoxycarb as in the left panel. Dashed bars denote superfusion of 1 mM ACh for each superfused set of current traces. Solid bars denote superfusion with fenoxycarb at a concentration of 30 $\mu M$ for different periods in A and at the different concentrations mentioned for a period of 20 s in B.

### DISCUSSION

The results demonstrate that the carbamate pesticides fenoxycarb and EPTC inhibit rat $\alpha_4\beta_2$ type neuronal nAChRs in a way that depends on the concentration of the agonist ACh, which is used to activate the ligand-gated ion channels. It is remarkable that these inhibitory effects, which are the more potent effects on nAChRs of a range of carbamate pesticides (Smulders et al., 2003), are enhanced in the presence of high as compared to low
receptor desensitization (Brier et al., 2003). Another recent study of the kinetics of block of human muscle type nAChR-gated ion channels expressed in HEK 293 cells by tacrine also concludes that single-site channel block cannot account for the effects observed, whereas multiple-site sequential block models do. Like fenoxycarb in the present study, tacrine was shown to interact with the agonist recognition site of the nAChR, but only at much higher concentrations than those required to inhibit the ion channels (Prince et al., 2002). The present model (scheme 3) is a simple sequential two-step mechanism, comparable to the mechanisms evaluated for tacrine (Prince et al., 2002). The model for fenoxycarb includes a single blocking site only, and an additional slow transition, which leads to a second blocked state or to a desensitized state of the receptor. Based on the present results, a desensitized state is the more likely explanation, because this would account for the enhanced potency of fenoxycarb and EPTC at elevated ACh concentrations (Fig. 2). This is corroborated by the observation that the reversal of the effect of fenoxycarb depends on whether fenoxycarb is applied in the absence or in the presence of ACh (Fig. 4C). After application in the absence of ACh, the reversal was close to 50%, whereas only ~25% reversal was observed when the same concentration of fenoxycarb was applied for the same period together with the same concentration of ACh. The slow transition, which leads to a second blocked state or to a desensitized state of the nAChRs is a general property of nicotinic agonists. Exposure to ACh promotes the second blocked/desensitized state (CR*) of the nAChR (Fig. 4C, left panel). PhTX-sal indicates that ACh promotes the second blocked/desensitized state together with the same concentration of ACh. The slow transition, which leads to a second blocked state or to a desensitized state of the receptor. Based on the present results, a desensitized state is the more likely explanation, because this would account for the enhanced potency of fenoxycarb and EPTC at elevated ACh concentrations (Fig. 2). This is corroborated by the observation that the reversal of the effect of fenoxycarb depends on whether fenoxycarb is applied in the absence or in the presence of ACh (Fig. 4C). After application in the absence of ACh, the reversal was close to 50%, whereas only ~25% reversal was observed when the same concentration of fenoxycarb was applied for the same period together with the same concentration of ACh. The slow transition, which leads to a second blocked state or to a desensitized state of the nAChR (Fig. 4C, left panel). PhTX-sal indicates that ACh promotes the second blocked/desensitized state (CR*) of the nAChR (Fig. 4C, left panel). PhTX-sal indicates that ACh promotes the second blocked/desensitized state (CR*) of the nAChR (Fig. 4C, left panel).

The similarities of the effects of fenoxycarb with those of the natural toxin PhTX and of the Alzheimer drug tacrine indicate that the model presented may find future application to account for the inhibitory mechanisms of a wider class of compounds than the carbamate pesticides. Like tacrine, various cholinesterase-inhibiting carbamate drugs noncompetitively inhibit ACh-induced ion currents at high concentrations (e.g., Zwart et al., 2000). The mechanism of this noncompetitive inhibitory effect has not been resolved thus far. Characterization of the nature of the binding site(s) involved in the similar effects of the various compounds and the exact location on the nAChR could provide important support for a general, unifying mechanism of inhibition. Enhancement of the probability of the desensitized state of the nAChRs is a general property of nicotinic agonists. Exposure to chronic low levels of nicotine, which induce nAChR desensitization, has previously been shown to lead to alterations in the expression level of α4β2 nAChR in human brain (Breese et al., 1997). It seems not unlikely that carbamates that promote nAChR desensitization may also alter nAChR expression levels. The extent to which this effect occurs and its relation to symptoms of chronic exposure to carbamate pesticides remains to be investigated.

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