Organophosphorus Insecticides Induce Airway Hyperreactivity by Decreasing Neuronal M2 Muscarinic Receptor Function Independent of Acetylcholinesterase Inhibition

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We previously demonstrated that the organophosphorus (OP) insecticide chlorpyrifos potentiates vagally induced bronchoconstriction independent of acetylcholinesterase (AChE) inhibition by decreasing the function of neuronal M2 muscarinic receptors that normally inhibit acetylcholine release from parasympathetic nerves supplying airway smooth muscle. However, it has been reported that different OPs may not affect muscarinic receptors equally. To determine if the effects of chlorpyrifos on airway hyperreactivity can be generalized to other OPs, we tested whether parathion and diazinon also inhibit neuronal M2 receptor function resulting in airway hyperreactivity. In control animals, the M2 agonist pilocarpine inhibits vagally induced bronchoconstriction in a dose-related manner. Treatment of guinea pigs with either parathion (1–10 mg/kg, sc) or diazinon (0.75–75 mg/kg, sc) shifted pilocarpine dose-response curves significantly to the right, indicating loss of neuronal M2 receptor function. These OP treatments also significantly potentiated vagally induced bronchoconstriction. Treatments that did not decrease M2 receptor function (parathion at 0.1 mg/kg, sc, or the non-OP insecticide permethrin at 150 mg/kg, sc) also did not cause airway hyperreactivity. None of the OP treatments altered bronchoconstriction induced by iv acetylcholine or methacholine in vagotomized guinea pigs, suggesting that OP-induced airway hyperreactivity is not due to altered function of muscarinic receptors on airway smooth muscle or to AChE inhibition. AChE assays of lung, blood, and brain confirmed that parathion and diazinon decreased M2 function at concentrations that did not inhibit AChE. These data suggest that multiple diethyl phosphorothionate OPs cause airway hyperreactivity via a common mechanism of M2 receptor dysfunction independent of AChE inhibition.

Key Words: organophosphorus pesticides; asthma; M2 muscarinic receptor; airway hyperreactivity.

Asthma prevalence and severity has increased over the past two decades, with the greatest increase occurring in children and adolescents living in urban environments (Hartert and Peebles, 2000; Weitzman et al., 1992). Over this same period, the use of insecticides, particularly organophosphorus (OP) insecticides, has increased, not only in agricultural environments (Fenske et al., 2002; Koch et al., 2002; USDA, 2003; Wilhoit et al., 1999) but also significantly in residential and urban settings (Berkowitz et al., 2003; CDC, 2003; Lu et al., 2001; Weisenburger, 1993; Whaytt et al., 2002). A number of clinical and epidemiological studies have linked OP exposure to symptoms associated with asthma including airway hyperreactivity and wheezing (Bryant, 1985; Deschamps et al., 1994; Hoppin et al., 2002; O’Malley, 1997; Salam et al., 2004). The biological mechanism proposed to explain OP effects on asthma was inhibition of acetylcholinesterase (AChE, E.C. 3.1.1.7) resulting in decreased hydrolysis of acetylcholine (Casarett and Doull, 1975; Sethilselvan et al., 1992), which could then increase bronchoconstriction via activation of M3 muscarinic receptors on airway smooth muscle (Coulson and Fryer, 2003; Roffel et al., 1990, 1994).

In support of epidemiological evidence linking OP exposure to asthma, we have recently established that chlorpyrifos, a widely used OP, induces airway hyperreactivity in a guinea pig model (Fryer et al., 2004). However, our data show that chlorpyrifos potentiates vagally induced bronchoconstriction in the absence of AChE inhibition. Rather, the mechanism involves decreased function of inhibitory M2 muscarinic receptors on the parasympathetic nerves supplying airway smooth muscle. Vagally induced bronchoconstriction normally is limited by these autoinhibitory M2 muscarinic receptors (Coulson and Fryer, 2003; Fryer and Maclagan, 1984; Minette and Barnes, 1988). Loss of M2 receptor function leads to increased release of acetylcholine from the parasympathetic nerves, resulting in potentiation of vagally mediated bronchoconstriction, which contributes to airway hyperreactivity. OP-induced inhibition of M2 receptor function and the consequent airway hyperreactivity are consistent with previous studies demonstrating that neuronal M2 receptors are dysfunctional in animal models of asthma (Fryer and Wills-Karp, 1991; Gambone et al., 1994;...
There are reports of OPs interacting with muscarinic receptors in brain tissue at doses that do not inhibit AChE (Katz and Marquis, 1989); however, these effects appear not to be consistent across brain regions or between different OPs (Pope, 1999). Recent in vitro studies using slice cultures from rat striatum indicated that the active metabolite of chlorpyrifos increased acetylcholine release via inhibition of autoinhibitory muscarinic receptors (Liu et al., 2002), which is consistent with our observations of chlorpyrifos effects on cholinergic neurotransmission in the airway. However, these striatal studies also found that, in the presence of AChE inhibitors, the active metabolites of the OPs parathion and methyl parathion act like muscarinic agonists to inhibit acetylcholine release. These data raise a question of whether observations of chlorpyrifos-induced airway hyperactivity via decreased M2 receptor function can be generalized across the OP class of insecticides. To address this question, we tested two different OPs, diazinon and parathion, which have different profiles of toxicity (Ecobichon, 2001; Moser, 1995). Although EPA-mandated restrictions for residential use of diazinon (Spectracide®) have recently been phased in, it remains a commonly used insecticide in the United States (USDA, 2003; Whitmore et al., 2003). Moreover, there is evidence of widespread exposure to diazinon in the general population (Barr et al., 2004; Whyatt et al., 2002). Parathion was included in this study despite the fact that EPA cancelled all uses of this pesticide in 1992, because its toxicological properties in both humans and animals are well known, and there are previous reports that parathion induces symptoms of asthma in experimental animals (Segura et al., 1999). Pyrethroids are a widely used class of non-OP pesticides that do not act via cholinesterase inhibition and have been shown to either not affect or increase muscarinic receptor function (Abou-Donia et al., 2004; Ahlborn et al., 1994; Eriksson and Nordberg, 1990; Husain et al., 1994). For these reasons, and because there is concern that pyrethroids may exacerbate asthma (Landrigan et al., 1999), we included permethrin in our tests of pesticide effects on airway hyperreactivity.

MATERIALS AND METHODS

Animals. Specific pathogen-free male guinea pigs (300–350 g) were shipped from Hilltop Lab Animals Inc. (Scottsdale, PA) in filtered crates, housed in high-efficiency particulate-filtered air, and fed a normal diet (Prolab; Agway, Syracuse, NY). All protocols were approved by Animal Care and Use Committees at the Johns Hopkins and Oregon Health and Science Universities.

Pesticide exposures. Parathion (o,o-diethyl-o-p-nitrophenyl phosphorothioate, 99.5% pure), diazinon (o,o-diethyl-o-[2-isopropyl-4-methyl-6-pyridinyl] phosphorothioate, 99.5% pure), and permethrin (3-phenoxbenzyl-[(RS)-cis/trans-3,2,2-dichlorovinyl]-2,2-dimeth) were purchased from Chem Service (West Chester, PA) and used prior to the expiration date, with interim storage as recommended by the manufacturer. Pesticides dissolved in peanut oil or an equal volume (300 μl) of peanut oil alone were administered to guinea pigs by subcutaneous (sc) injection in the subscapular region. Subcutaneous dosing is commonly used in mechanistic studies of OPs (Bushnell et al., 1991; Chiappa et al., 1995; Pope et al., 1992; Stanton et al., 1994) and is proposed to result in gradual release of the pesticide into the systemic circulation (Pope et al., 1991), which approximates most human exposures (Gallo and Lawryk, 1991). The highest doses of diazinon and parathion tested in these studies were determined to be those that caused a 50% inhibition of AChE in guinea pig lungs. The dose of permethrin used in these studies is within one order of magnitude of the amount of permethrin absorbed by guinea pig dermis (40 mg/kg) following a single application of 5% permethrin cream, which is a standard formulation for treating scabies (Franz et al., 1996), and approximately one-tenth the dermal ID₅₀ reported for rats (approximately 4000 mg/kg) and rabbits (approximately 2000 mg/kg). Animals dosed with parathion or diazinon were monitored for signs of cholinergic intoxication (tremors, altered gait, and excessive excretions) at 1 and 24 h following injections. In addition, effects on physiological parameters (heart rate, blood pressure) under basal conditions were monitored in animals treated with pesticides prior to initiating experiments. Physiological measurements of lung function were carried out 24 h post injection, and since previous studies demonstrated that lung function in guinea pigs treated with peanut oil does not differ from that seen in saline-treated controls (Fryer et al., 2004), only peanut oil controls are reported herein.

Anesthesia and measurement of pulmonary inflation pressure. Guinea pigs were anesthetized with 1.5 g/kg urethane (ip). Heart rate and blood pressure were measured from the carotid artery. The trachea was cannulated, and the animals were ventilated via a tracheal cannula with a positive pressure constant volume (1 ml per 100 g body weight and 100 breaths/minute). The jugular veins were cannulated, and the nicotinic receptor antagonist succinylcholine (10 μg/kg/min, iv) infused to paralyze the animals. Pulmonary inflation pressure (Ppi) was measured from a side arm at the trachea; bronchoconstriction was measured as the increase in Ppi over the pressure produced by the ventilator as previously described (Fryer and Maclagan, 1984; Fryer and Wills-Karp, 1991; Jacoby and Fryer, 1991).

Measurement of vagally induced bronchoconstriction. All animals received guanethidine (10 mg/kg, iv) prior to the start of the experiment to deplete noradrenaline. Both vagus nerves were cut. The distal ends were placed on electrodes under oil and were stimulated at 2-min intervals (0.2 ms, 10 V, 1–25 Hz, 5-sec duration), producing frequency-dependent bronchoconstriction due to release of acetylcholine onto postjunctional M3 muscarinic receptors in the lungs and postjunctional M2 muscarinic receptors in the heart. Both vagally induced bronchoconstriction and bradycardia could be abolished by atropine (1 mg/kg, iv).

Measurement of neuronal M2 muscarinic receptor function. The function of neuronal M2 receptors was determined by measuring the ability of the muscarinic agonist, pilocarpine, to inhibit bronchoconstriction in response to vagal stimulation at 2 Hz. Pilocarpine is a muscarinic agonist with selectivity for prejunctional M2 versus postjunctional M3 receptors in vivo (Fryer and Maclagan, 1984; Langley, 1878), thus, pilocarpine inhibits vagally induced bronchoconstriction via stimulation of the neuronal M2 receptors at doses that are 100-fold less than the doses required to cause bronchoconstriction by stimulating postjunctional M3 receptors (Fryer and Maclagan, 1984). The effect of pilocarpine on vagally induced bronchoconstriction is reported as the ratio of bronchoconstriction in the absence of pilocarpine. A shift to the right of the pilocarpine dose-response curve indicates decreased M2 receptor function (Fryer and Maclagan, 1984; Fryer and Wills-Karp, 1991; Jacoby and Fryer, 1991).

Measurement of postjunctional muscarinic receptor function. Intravenous injection of acetylcholine (1–10 μg/kg) was used to assess the function of the postjunctional M3 receptors in the lungs and postjunctional M2 receptors in the heart. To determine if AChE inhibition influenced the response to acetylcholine, these experiments were repeated using methacholine (1–10 μg/kg, iv), an agonist that is less susceptible to hydrolysis by cholinesterases than acetylcholine (Bruning et al., 1996; Norell et al., 1993). Since muscarinic agonists also initiate a reflex bronchoconstriction (Delpierre et al., 1983; Wagner and Jacoby, 1999), these experiments were performed in vagotomized animals.
**ACHE assay.** Since OP inhibition of AChE differs between lung and brain within any given species (Lessire et al., 1996), we measured AChE activity in the lung in addition to AChE activity in the brain and blood, both of which are commonly used biomarkers of OP toxicity. Immediately following the completion of physiological measurements, lungs, brain, and heparinized blood samples were collected for determination of AChE activity via the standard Ellman assay (Ellman et al., 1961) using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (AChI) as the substrate. Lung and brain samples were homogenized in lysis buffer (0.1 M phosphate, pH 8.0) containing 0.1% Triton using a Dounce homogenizer, centrifuged at 13,400 × g, and the supernatant collected for analysis. Assays were run against blanks containing DTNB.

The reaction was started with the addition of AChI after equilibration for 5 min. Hydrolysis of AChI was determined by monitoring the change in absorbance at 405 nm. To inhibit pseudocholinesterase activity, 100 µM tetraisopropyl pyrophosphoramide (iso-OMPA) was included in the assay. Data from lung and brain samples were normalized using protein concentration as determined using the BCA assay according to the manufacturer’s directions (Pierce, Rockford, IL). AChE activity in blood samples was normalized according to the number of red blood cells (RBC) as determined using a hemacytometer.

**Statistics.** Data are expressed as mean ± standard error of the mean (SEM). Frequency, pilocarpine, methacholine, and acetylcholine dose-response curves were analyzed using a two-way analysis of variance for repeated measures. Baseline heart rates (beats/min), blood pressures (mmHg), pulmonary inflation pressure (Ppi), and changes in Ppi were analyzed using a two-way analysis of variance (ANOVA; Statview 4.5, Abacus Concepts, Inc., Berkley, CA); a p value ≤ 0.05 was considered significant.

**RESULTS**

Guinea pigs were injected sc with one of two OPs, parathion (0.1–10 mg/kg) or diazinon (0.75–75 mg/kg), or with the non-OP insecticide, permethrin (150 mg/kg), 24 h prior to physiological measurements. None of these treatments caused any apparent signs of cholinergic intoxication. In anesthetized, vagotomized guinea pigs, baseline pulmonary inflation pressure (Ppi) did not differ among animals treated with the highest doses of parathion, diazinon, permethrin, or the vehicle (peanut oil) control (peanut oil, 87 ± 8 mmH2O; parathion, 109 ± 3.8 mmH2O; diazinon, 98 ± 4 mmH2O; permethrin, 97.5 ± 5 mmH2O). Neither were there differences among treatment groups in resting heart rate (peanut oil, 320.9 ± 9 beats/min; parathion, 342.5 ± 9 beats/min; diazinon, 321 ± 15 beats/min; permethrin, 308 ± 11 beats/min) or in resting systolic/diastolic blood pressure (peanut oil, 38.5 ± 3 mmHg/21 ± 3.8 mmHg; parathion, 50 ± 7.5 mmHg/28 ± 5.9 mmHg; diazinon, 42.5 ± 8.8 mmHg/23.5 ± 6.4 mmHg; permethrin, 47.5 ± 2.2 mmHg/25.8 ± 4 mmHg) in vagotomized guinea pigs.

Neuronal M2 receptor function was measured using the muscarinic agonist pilocarpine. Prior to administering pilocarpine, simultaneous electrical stimulation of both vagus nerves (2 Hz, 0.2 ms, 5–15 Volts, 22 sec at 1-min intervals) produced transient bronchoconstriction (measured as an increase in Ppi) that was not different among groups (peanut oil, 33.0 ± 2.6 mmH2O; parathion 10–0.1 mg/kg, 24.2 ± 2.7 mmH2O; 19.8 ± 3 mm H2O, 22.1 ± 4 mmH2O; diazinon 75 and 0.75 mg/kg, 32.1 ± 3.8 mmH2O, 20.9 ± 5.4 mmH2O). In guinea pigs treated with peanut oil, pilocarpine (1–100 µg/kg, iv) decreased vagally induced bronchoconstriction in a dose-dependent manner, demonstrating that the neuronal M2 muscarinic receptors are functional (Fig. 1, open squares). The ability of pilocarpine to decrease vagally induced bronchoconstriction was significantly inhibited in animals treated with 10 or 1.0 mg/kg, but not 0.1 mg/kg, parathion (Fig. 1, left side, filled symbols). Similarly, diazinon, at both 75 mg/kg and at a 100-fold lower dose of 0.75 mg/kg blocked the ability of pilocarpine to decrease vagally induced bronchoconstriction (Fig. 1, right side, filled symbols). Thus, both OPs inhibited the function of autoinhibitory neuronal M2 receptors.

To determine if blockade of neuronal M2 receptor function is related to airway hyperreactivity, vagally induced bronchoconstriction was measured in guinea pigs treated with these same doses of parathion and diazinon. Electrical stimulation of both vagi (1–25 Hz) caused a frequency-dependent increase in bronchoconstriction in peanut oil-treated animals (Fig. 2, open squares). Vagally induced bronchoconstriction was significantly potentiated in animals treated with parathion at 10 or 1 mg/kg, but not in animals treated with parathion at 0.1 mg/kg (Fig. 2, open squares).

**FIG. 1.** Parathion and diazinon inhibit neuronal M2 receptor function. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals treated with peanut oil (open squares; both panels) demonstrating functional M2 receptors. In animals treated with either 1.0 or 10.0 mg/kg sc parathion (left panel), or with 0.75 or 75 mg/kg sc diazinon (right panel), pilocarpine did not inhibit vagally induced bronchoconstriction, indicating neuronal M2 muscarinic receptor dysfunction. A lower dose of parathion (0.1 mg/kg, sc, closed squares, left panel) did not inhibit M2 receptor function. Each point is the mean ± SEM of four to six animals; *significantly different from peanut oil control.
left panel, filled symbols). Both doses of diazinon potentiated vagally induced bronchoconstriction (Fig. 2, center panel, filled symbols). Thus, both OPs significantly potentiated vagally induced bronchoconstriction, but only at doses that inhibited M2 receptor function. In contrast, the non-OP insecticide, permethrin, did not potentiate vagally induced bronchoconstriction, but significantly attenuated it instead (Fig. 2, right panel, filled triangles).

To test the effect of the OPs and of permethrin on the responsiveness of airway smooth muscle and the function of the post-junctional M3 muscarinic receptors on airway smooth muscle, bronchoconstriction was induced in vagotomized guinea pigs by iv acetylcholine (Fig. 3). To test whether the response to acetylcholine was affected by OP-induced inhibition of AChE, these experiments were repeated using iv methacholine (Fig. 4), which is not rapidly metabolized by AChE. Neither the OPs, parathion and diazinon, nor the non-OP, permethrin, potentiated either acetylcholine- or methacholine-induced bronchoconstriction (Figs. 3 and 4). Thus, the OPs did not enhance the ability of the M3 receptors to respond to agonists or enhance the ability of airway smooth muscle to contract. That there was no difference between methacholine- and acetylcholine-induced bronchoconstriction would suggest that inhibition of AChE is not a mechanism for OP-induced hyperreactivity at these doses.

M2 muscarinic receptors are also present in the heart. Thus, we measured bradycardia to determine whether OP insecticides alter M2 muscarinic receptors in tissues other than the lung. In the heart, stimulation of the vagus nerves (1–25 Hz) produced bradycardia that is frequency dependent (open squares, left panel, Fig. 5). Both parathion (10 mg/kg; closed circles) and diazinon (75 mg/kg; closed squares) potentiated vagally induced bradycardia (left panel, Fig. 5). In contrast, permethrin did not alter vagally induced bradycardia (closed triangles, left panel, Fig. 5). Similar to the lung, OP-induced potentiation of vagally induced bradycardia does not appear to be mediated by inhibition of AChE, since acetylcholine-induced bradycardia was not altered by either OP or by permethrin (center panel, Fig. 5). Neither was this due to any alteration in the ability of post-junctional M2 receptors to decrease heart rate, since methacholine-induced bradycardia was not different among treatment groups (right panel, Fig. 5).

Only the highest dose of each OP significantly inhibited AChE activity in the lung and in the blood (Fig. 6). Neither 1.0 mg/kg or 0.1 mg/kg parathion or 0.75 mg/kg diazinon, or 150 mg/kg permethrin inhibited AChE activity in the periphery (Fig. 6). Brain AChE was inhibited by 10 and 1.0 mg/kg, but not by 0.1 mg/kg parathion. Neither diazinon nor permethrin inhibited brain AChE activity at the doses tested (Fig. 6).

**DISCUSSION**

Similar to our previous observations of chlorpyrifos (Fryer et al., 2004), we found that both parathion and diazinon caused...
FIG. 3. Parathion and diazinon do not alter airway response to iv acetylcholine. Acetylcholine (1–10 µg/kg, iv) induced a dose-dependent bronchoconstriction in vagotomized guinea pigs (peanut oil controls: open circles; all panels) that was not potentiated by parathion (0.1–10.0 mg/kg, sc; filled shapes, left panel) or by diazinon (0.75–75 mg/kg, sc; filled shapes, center panel). In contrast, the non-OP insecticide, permethrin (150 mg/kg, sc; filled triangles, right panel) inhibited acetylcholine-induced bronchoconstriction. Each point is the mean ± SEM of four to five animals; *significantly different from peanut oil control.

FIG. 4. Methacholine (1–10 µg/kg, iv) induced a dose-dependent bronchoconstriction in vagotomized guinea pigs (peanut oil controls: open circles; all panels) that was not altered by parathion (filled shapes; left panel), diazinon (filled shapes; center panel), or permethrin (right panel). Each point is the mean ± SEM of four to five animals; *significantly different from peanut oil control.
airway hyperreactivity in guinea pigs, as evidenced by potentiation of vagally induced bronchoconstriction. These effects are not due to changes in either postjunctional M3 receptors or airway smooth muscle contractility, since methacholine-induced bronchoconstriction was not potentiated by either parathion or diazinon. Both doses of diazinon tested in these studies (0.75 and 75 mg/kg, sc) elicited comparable potentiation of vagally induced bronchoconstriction, and a no-effect level was not determined for this OP in these studies. However, the effects of parathion were dose-related in that the higher (1.0 and 10 mg/kg, sc) but not the lowest (0.1 mg/kg, sc) doses tested caused airway hyperreactivity. A similar dose range of parathion (3.2–17 mg/kg, ip) has been reported to increase lung resistance and to augment respiratory secretions in the rabbit lung (Segura et al., 1999). In contrast to effects observed with the OPs, treatment with the non-OP insecticide permethrin, attenuated vagally induced bronchoconstriction. Together, these data suggest that induction of airway hyperreactivity is common to diethyl phosphorothionate organophosphorus compounds, but is not a generalized property of all pesticides.

Studies of OP neurotoxicity have indicated that acute effects of these compounds are mediated primarily by AChE inhibition (Pope, 1999). Several observations from our studies rule out AChE inhibition as the mechanism underlying the potentiation of vagally induced bronchoconstriction by parathion and diazinon. First, direct measurements of AChE activity in lungs, blood, and brain indicated that parathion and diazinon inhibited AChE in a dose-related manner, but this did not correlate with airway hyperreactivity. Second, although inhibition of AChE by pharmacological cholinesterase inhibitors has been shown to potentiate bronchoconstriction in response to acetylcholine (Colbatch and Halmagyi, 1963; Daly and Schweitzer, 1951), neither diazinon nor parathion potentiated bronchoconstriction induced by iv acetylcholine in vagotomized guinea pigs (Fig. 3), even at concentrations that caused 50% or more inhibition of AChE in the lung and blood (Fig. 6). The observation that these OPs induce airway hyperreactivity independent of AChE inhibition is important because it indicates that OP-induced airway hyperreactivity occurs below thresholds of toxic exposure that are currently defined by AChE inhibition.

In contrast, the non-OP insecticide permethrin attenuated vagally induced bronchoconstriction. Although we did not test the ability of permethrin to interact with neuronal M2 receptors, it has been reported that permethrin can increase M2 receptor function (Abou-Donia et al., 2004; Ahlbom et al., 1994; Eriksson and Nordberg, 1990; Husain et al., 1994), which would be consistent with our observations of its effects on airway hyperreactivity. An unexpected finding was that permethrin attenuated acetylcholine-induced bronchoconstriction. The mechanism underlying this effect is not known.
Neuronal M₂ muscarinic receptors limit release of acetylcholine from parasympathetic nerves in the lungs (Fryer and Maclagan, 1984). Pharmacological blockade of neuronal M₂ receptors increases release of acetylcholine from these nerves (Baker et al., 1992; Fryer et al., 1996), which potentiates vagally induced bronchoconstriction (Fryer and Maclagan, 1984; Fryer and Wills-Karp, 1991; Jacoby and Fryer, 1991). Our data show that neuronal M₂ receptor function is inhibited by both parathion and diazinon at doses that cause airway hyperreactivity. In contrast, a dose of parathion that does not affect M₂ receptor function also does not alter vagally induced bronchoconstriction. Thus, OP-induced inhibition of neuronal M₂ receptor function mediates airway hyperreactivity. Loss of neuronal M₂ receptor function in the lungs is also associated with other models of airway hyperreactivity including antigen challenge (Fryer and Wills-Karp, 1991), viral infection (Jacoby and Fryer, 1991), and exposure to ozone (Gambone et al., 1994), suggesting that decreased M₂ receptor function on airway nerves is a generalized mechanism underlying airway hyperreactivity.

The ability of OPs to inhibit neuronal M₂ receptors may not be restricted to the lungs. OPs compete for binding to muscarinic receptors in the brain (Abdallah et al., 1992; Bomser and Casida, 2001; Huff et al., 1994; Jett et al., 1993, 1994; Katz and Marquis, 1989, 1992) and in the heart (Silveira et al., 1990). In the heart, M₂ receptors are present on both cardiac muscle, where they mediate bradycardia (Brode et al., 2001; Maeda et al., 1988), and parasympathetic nerves that supply the heart, where they function to inhibit release of acetylcholine (Manabe et al., 1991; Oberhauser et al., 2001). Both parathion (10 mg/kg, sc) and diazinon (75 mg/kg, sc) potentiated bradycardia induced by vagal stimulation but not bradycardia induced by iv administration of acetylcholine or methacholine, suggesting inhibition of prejunctional neuronal M₂ receptors, but not postjunctional cardiac M₂ receptors. Similarly, the function of the neuronal M₂, but not postjunctional M₂, receptors in the heart is inhibited by chlorpyrifos (Fryer et al., 2004) and by systemic administration of double-stranded RNA (Bowerfind et al., 2002). Thus neuronal M₂ receptors appear to be more vulnerable to inhibition than postjunctional M₂ receptors.

The interaction of OPs with pre- and postjunctional muscarinic receptors in the brain is complex. OPs have been demonstrated to antagonize muscarinic receptors either via direct effects on the receptors themselves (Fitzgerald and Costa, 1992; Huff et al., 2001; Katz and Marquis, 1989; Liu et al., 2002; Zhu et al., 1991) or indirectly by decreasing receptor number in response to increased acetylcholine resulting from AChE inhibition (Cioffi and el-Fakahany, 1986; Zhu et al., 1991). Conversely, it has also been reported that OPs stimulate neuronal muscarinic receptors either directly (Liu et al., 2002; Ward and Mundy, 1996) or indirectly as a result of increased synaptic levels of acetylcholine consequent to AChE inhibition (Kibbinger and Wessler, 1980; Liu et al., 2002). Reports of OPs directly stimulating muscarinic receptors were derived from functional studies of striatum (Liu et al., 2002) and frontal cortex (Ward and Mundy, 1996). Although there are M₂ receptors on presynaptic nerves in both the striatum (Hersch et al., 1994) and cortex (Levey et al., 1991), studies in knockout mice suggest that it is the M₄, and not the M₂ receptors, that are functionally significant in inhibiting acetylcholine release in these brain regions (Zhang et al., 2002). These observations raise the possibility that earlier reports of direct stimulation of muscarinic receptors by OPs reflect effects on M₄ rather than M₂ receptors. When considered together with our findings that OPs inhibit neuronal M₂ receptor function in the lungs and heart, these data strongly suggest that M₂ and M₄ receptors are differentially affected by OPs.

The mechanism(s) by which OPs inhibit M₂ receptor function are not yet known. Because parathion and diazinon decreased...
M2 receptor function at doses that did not inhibit AChE, it seems unlikely that either indirect stimulation or decreased expression of M2 receptors secondary to AChE inhibition underlie M2 receptor inhibition. Although it is possible that AChE was acutely inhibited at the time of administration, causing persistent downregulation of M2 receptors 24 h later, this seems unlikely because the function of the postjunctional M2 receptors on the heart (see Fig. 5) was not inhibited 24 h after administration of OPs. Furthermore, the OPs were administered not as a single bolus dose, but rather subcutaneously in oil, a method that allows for gradual release of the OPs in the systemic circulation (Pope et al., 1991). It is also not the case that M4 receptors mediate OP-induced airway hyperreactivity, since M4 receptors are not expressed on parasympathetic nerves in the lungs (Fryer et al., 1996). Therefore, it seems likely that OPs directly inhibit neuronal M2 receptor function. Whether they do so by downregulation of muscarinic receptor expression (Jett et al., 1993, 1994), modulation of ligand binding to M2 receptors (Jett et al., 1991; Katz and Marquis, 1989, 1992), or alteration of signal transduction pathways downstream of muscarinic receptor (Bomser et al., 2002; Huff et al., 1994; Schuh et al., 2002; Ward and Mundy, 1996) has yet to be determined. A significant difference between these earlier published studies and our findings is that the former reported the potency of OP binding to muscarinic receptors as comparable to that of OP binding to acetylcholinesterase (AChE), whereas our data suggest that OP interactions with neuronal M2 receptors in airways occur at lower doses than those required to inhibit AChE activity in the lung or blood.

Data presented here confirm that the diethyl phosphorothionate OP insecticides cause airway hyperreactivity via a common mechanism of disrupting negative feedback control of cholinergic regulation in the lungs. Thus, we have shown that not only chlorpyrifos (Fryer et al., 2004), but also diazinon and parathion, inhibit neuronal M2 receptor function in the lung at concentrations that do not inhibit AChE. Since the resting vagal tone in guinea pig lungs is approximately 10–15 Hz (Myers and Undem, 1996), our data suggest that loss of M2 receptor function results in increased basal release of acetylcholine. Furthermore, irritation of the lung results in reflex bronchoconstriction that is mediated by the parasympathetic nerves (Carr and Undem, 2003; Undem and Carr, 2002), and loss of M2 receptor function also increases reflex bronchoconstriction (Costello et al., 1999; Evans et al., 2000). M2 receptors on parasympathetic nerves supplying glands in the airways also regulate mucin secretion in the airways (Ramnarine et al., 1996; Rogers, 2001). Thus OPs could potentiate basal tone, reflex bronchoconstriction, and mucus secretion, all of which are characteristics of asthma.

Use of OP insecticides has increased significantly in urban and agricultural settings over the past 30 years (Fenske et al., 2002; Koch et al., 2002; USDA, 2003; Wilhoit et al., 1999), coincident with an increase in asthma (Hartert and Peebles, 2000; Weitzman et al., 1992). Children represent a potentially sensitive subpopulation with respect to asthma, and there is evidence of wide-spread exposure of children to OPs. Screens of fetal exposure to OP pesticides have detected chlorpyrifos (8.26 μg/ml), diazinon (13 μg/ml), and parathion (2.3 μg/ml) in meconium (Ostrea et al., 2002). Recent studies in Seattle found that of 110 preschool children from 96 households of varying cultures, family income, and housing type, all excreted OP metabolites in their urine (Curl et al., 2003; Lu et al., 2001). Similarly, in a sample of 84,000 children across the United States, the urinary levels of chlorpyrifos metabolites were above the detection limit 98% of the time, compared to a 4% detection rate for the herbicide atrazine (Adgate et al., 2001). Not only is there widespread exposure of children to these insecticides, but data collected as part of the most recent National Health and Nutrition Examination Survey (NHANES) indicated that across all racial and ethnic groups, urinary concentrations of OP metabolites in children 6–11 years of age were consistently significantly higher than in adults (Barr et al., 2004). Consistent with these conclusions, chlorpyrifos residues have been shown to persist in the home for up to 2 weeks after a single application, with potential exposure to infants and children reaching levels 60–120 times greater than the U.S. EPA recommended reference levels (Fenske et al., 1990; Gurunathan et al., 1998). The U.S. EPA has determined the dermal and acute dietary NOAEL (no observed adverse effect level) for diazinon to be 1 mg/kg/day and 0.25 mg/kg/day, respectively, based on plasma cholinesterase inhibition (U.S. EPA, 2000). We observed airway hyperreactivity in response to diazinon (sc) at a dose that did not inhibit plasma cholinesterase, suggesting that diazinon may exert adverse effects on human lung function at doses considered safe under current EPA guidelines. These data suggest that exposure to these compounds may contribute to the observed increase in asthma prevalence over the past 30 years (Hartert and Peebles, 2000; Weitzman et al., 1992).

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