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This grant proposes to determine whether organophosphate insecticides act upon the cholinergic system in the lungs increasing cholinergic neurotransmission and causing airway hyperresponsiveness, which is characteristic of asthma. Guinea pigs were either treated acutely with a high dose or chronically (7 days) with a low does of the organophosphate chlorpyrifos sc. Electrical stimulation of the vagus nerves caused frequency-dependent bronchocontriction that was significantly potentiated in animals treated with chlorpyrifos. M2 muscarinic autoreceptors, which normally inhibit release of acetylcholine from cholinergic nerves were dysfunctional in the chlorpyrifos-treated animals. The function of M3 muscarinic receptors on airway smooth muscle was not altered by cholorpyrifos treatment. In addition, the high but not the lower dose of chlorpyrifos significantly inhibited acetylcholinesterase activity, further contributing to airway hyperreactivity. These data demonstrate that organophosphate pesticides are capable of causing airway hyperreactivity by inhibiting neuronal M2 receptor function. A second series of experiments has demonstrated that 2 additional organophosphates, diazinon and parathion also inhibit M2 receptor function and cause airway hyperreactivity, experiments to determine the lowest level of exposure are underway. Thus, we have confirmed that organophosphates cause airway hyperactivity by inhibiting M2 receptor function on the cholinergic nerves and by inhibiting acetylcholinesterase activity.

14. SUBJECT TERMS
Organophosphates insecticides, asthma, bronchial hyperresponsiveness, cholinergic function

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INTRODUCTION

Over the past 20 years there has been a significant increase in the incidence of asthma in industrialized nations. Over the same timeframe, insecticide usage has increased significantly, not only in agricultural settings, but also in the inner cities. One of the most commonly used classes of insecticides is the organophosphates and a number of clinical reports and epidemiological studies have linked exposure to organophosphates to airway hyperreactivity and asthma. However, the mechanisms by which these insecticides cause changes in airway function remain unknown. In the lung, cholinergic nerves in the vagi mediate airway tone and reactivity. These nerves release acetylcholine onto M3 muscarinic receptors causing contraction of airway smooth muscle resulting in bronchoconstriction. Vagally induced bronchoconstriction is limited by autoinhibitory M2 muscarinic receptors on parasympathetic nerves. We have shown that previously that neuronal M2 receptors are dysfunctional in animal models of asthma. 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. Since organophosphates are known to alter cholinergic function in the brain, we tested whether organophosphate insecticides can alter neuronal M2 muscarinic receptor function in the lungs and induce hyperreactivity.

BODY:

There are 3 specific aims:

1. To determine whether organophosphates cause airway hyperreactivity.
2. To determine the mechanism of action by which the organophosphates induced hyperreactivity
3. To determine whether exposure to organophosphates exacerbated airway hyperreactivity following antigen challenge.

Results: We have tested the ability of three different organophosphate insecticides to induce airway hyperreactivity by preventing neuronal M2 receptors from inhibiting release of acetylcholine in the lungs. We have used chlorpyrifos (70 and 390 mg/kg), parathion (10, 1 and

0.1 mg/kg) and diazanon (75 and 0.75mg/kg). None of these organophosphates at any of the doses used resulted in any apparent signs of cholinergic intoxication within 24 hours following the injections. Neither did any of them, or the peanut oil used to dilute them, alter baseline pulmonary inflation pressure (all ranged within 90-105 mmH2O), resting heart rate (all ranged within 290-310 beats/min) or resting blood pressure (all ranged within diastolic: 41-48 mmHg) in vagotomized, anesthetized guinea pigs.

Chlorpyrifos: We have demonstrated that chlorpyrifos administered either as 390mg/kg for 24 hours or 70mg/kg for 7 days, significantly potentiated vagally induced bronchoconstriction in anesthetized guinea pgs. Vagally induced bronchoconstriction was not altered in animals receiving vehicle alone (peanut oil) relative to controls. Chlorpyrifos treatment did not alter bronchoconstriction induced by methacholine, demonstrating that it had no effect at the level of the airway smooth muscle.

In contrast, acetylcholine induced bronchoconstriction was significantly increased in the 390mg/kg chlorpyrifos for 24 hour animals but not in the animals treated with 70 mg/kg for 7 days. We measured the effect of chlorpyrifos on acetylcholinesterase. Chlorpyrifos treatment at 70mg/kg for 7 days had no effect on lung acetylcholinesterase. However acute treatment with 390mg/kg chlorpyrifos for 24 hour inhibited lung acetylcholinesterase by 50% and blood acetylcholinesterase by nearly 90%. Thus it is likely that potentiation of acetylcholine induced bronchoconstriction by the higher dose of chlorpyrifos was mediated by decreased acetylcholinesterase.

Since we demonstrated that neuronal M2 receptor function was inhibited by both chlorpyrifos dosing regimes, we concluded that chlorpyrifos induced hyperreactivity following 390mg/kg for 24 hours or 70mg/kg for 7 days was mediated by loss of neuronal M2 receptor function. At the higher dose (390mg/kg for 24 hours) decreased acetylcholinesterase also contributed to hyperreactivity. These results have been published in a manuscript (Mechanisms of organophosphate insecticide-induced airway hyperreactivity. Fryer AD, Lein PJ, Howard AS, Yost BL, Beckles RA, Jett DA. Am J Physiol Lung Cell Mol Physiol. 2004 May;286(5):L963-9. Epub 2004 Jan 02: copy attached in appendix).

Parathion, Diazanon: To test whether the ability of chlorpyrifos to inhibit neuronal M2 receptor function and cause airway hyperreactivity was limited to only chlorpyrifos or was a property of all organophosphates we tested two additional organophosphates and compared them to permethrin, a non-organophosphate insecticide; all insecticides were suspended in peanut oil and administered subcutaneously for 24 hours prior to physiological measurements.

Neither of parathion nor permethrin potentiated either acetylcholine or methacholine induced bronchoconstriction (figure 1; center and right panels). However, both parathion, 10mg/kg and diazanon, 75 mg/kg caused hyperreactivity to electrical stimulation of the vagus nerves (left panel, figure 1). In contrast, permethrin, a non-organophosphate insecticide did not potentiate vagally induced bronchoconstriction-suggesting that insecticide induced hyperreactivity is limited to the organophosphates.

We tested whether parathion, 10mg/kg and diazanon 75 mg/kg could inhibit the function of the neuronal M2 muscarinic receptors. Both significantly shifted the dose response curve to pilocarpine to the right demonstrating that both could inhibit neuronal M2 receptor function (figure 2). Thus both organophosphates inhibit M2 receptor function. This might be one mechanism by which organophosphates induce airway hyperreactivity.

However, both does of organophosphates in these doses significantly inhibited acetylcholinesterase in the lungs and blood (Table 1).

Non-acetylcholinesterase inhibiting doses of Parathion and Diazanon: To assess whether non-acetylcholinesterase inhibiting does could also induced airway hyperreactivity. Doses that were 100 fold lower were tested. Diazanon at 0.75 mg/kg sc still induced airway hyperreactivity (figure 3) and inhibited function of the neuronal M2 receptors (figure 5). However, this does did not inhibit acetylcholinesterase in the blood or lungs (table 1).

The 100 fold lower dose of parathion (0.1mg/kg sc) did not cause airway hyperreactivity (figure 4), so an intermediate does was tested. 1.0 mg/kg parathion did not inhibit acetylcholinesterase (table 1), but did cause hyperreactivity (figure 4) and loss of neuronal M2 receptor function (figure 6) Thus, both parathion and diazanon even at doses that do not inhibit acetylcholinesterase cause hyperreactivity and loss of M2 function. These experiments clearly

delineate between hyperreactivity due to inhibition of acetylcholinesterase and hyperreactivity due to inhibition of M2 receptor function. Organophosphates appear to induced hyperreactivity via loss of M2 receptor function.

Key Research Accomplishments:

1: Chlorpyrifos, parathion, and diazanon each cause airway hyperreactivity to electrical stimulation of the vigil at doses that do not inhibit acetylcholinesterase.

2: Chlorpyrifos, parathion, and diazanon do not cause airway hyperreactivity to intravenous methacholine, a muscarinic agonist not metabolized by acetylcholinesterase.

3. Even at doses that do not inhibit acetylcholinesterase, the function of inhibitory M2 receptors on the parasympathetic nerves is inhibited by chlorpyrifos, parathion, and diazanon. This would increase release of acetylcholine and may be one of the mechanisms of chlorpyrifos induced hyperreactivity.

4. At high doses chlorpyrifos, parathion, and diazanon will inhibit acetylcholinesterase. This is reflected in increased bronchoconstriction to iv acetylcholine in the lungs and is another mechanism for airway hyperreactivity.

Reportable outcomes:

We have published one manuscript on the effects of chlorpyrifos. Mechanisms of organophosphate insecticide-induced airway hyperreactivity. Fryer AD, Lein PJ, Howard AS, Yost BL, Beckles RA, Jett DA. Am J Physiol Lung Cell Mol Physiol. 2004 May;286(5):L963-9. Epub 2004 Jan 02: copy attached in appendix

At this point we are writing a manuscript containing the data on parathion, diazanon and permethrin that is presented in table 1 and figures 1-6

Conclusions:

Airway hyperreactivity was measured in guinea pigs 24 hr after injection with chlorpyrifos parathion or diazanon sc. We tested a range of doses, including doses that do not inhibit acetylcholinesterase. Even at these low doses, electrical stimulation of the vagus nerves caused a frequency-dependent bronchoconstriction that was significantly potentiated in animals treated with any of the organophosphates.

Neuronal M2 receptor function was tested with pilocarpine, which inhibits vagally induced bronchoconstriction in control animals. In animals treated with the organophosphates, the pilocarpine dose-response curve was sifted significantly to the right, demonstrating decreased responsiveness of the neuronal M2 receptors. The function of M3 muscarinic receptors on airway smooth muscle was not altered by either chlorpyrifos parathion or diazanon. These data demonstrate that organophosphate insecticides are capable of causing airway hyperreactivity by inhibiting neuronal M2 receptor function at doses that do not inhibit acetylcholinesterase.

Thus we have completed aim 1 and 2 for three of the organophosphates. We are currently undertaking aim 3 and examining the interaction of organophosphates on antigen sensitized guinea pigs.

Table 1:

Achesterace activity (Specific Activity umole thiocholine/min/mig protein)

TREATMENT	LUNGS	BLOOD
Peanut oil	**1.8±0.2**	**2.3±0.1**
Diazanon 75mg/kg	0.8±0.2*	1.0±0.055*
Diazanon 7.5mg/kg	1.8±0.3	2.5±0.03
Parathion 10mg/kg	1.0±0.3	1.2±0.03*
Parathion 1.0mg/kg	1.6±0.4	2.0±0.2

Figure 1
Organophosphate pesticides potentiate vagally induced bronchoconstriction

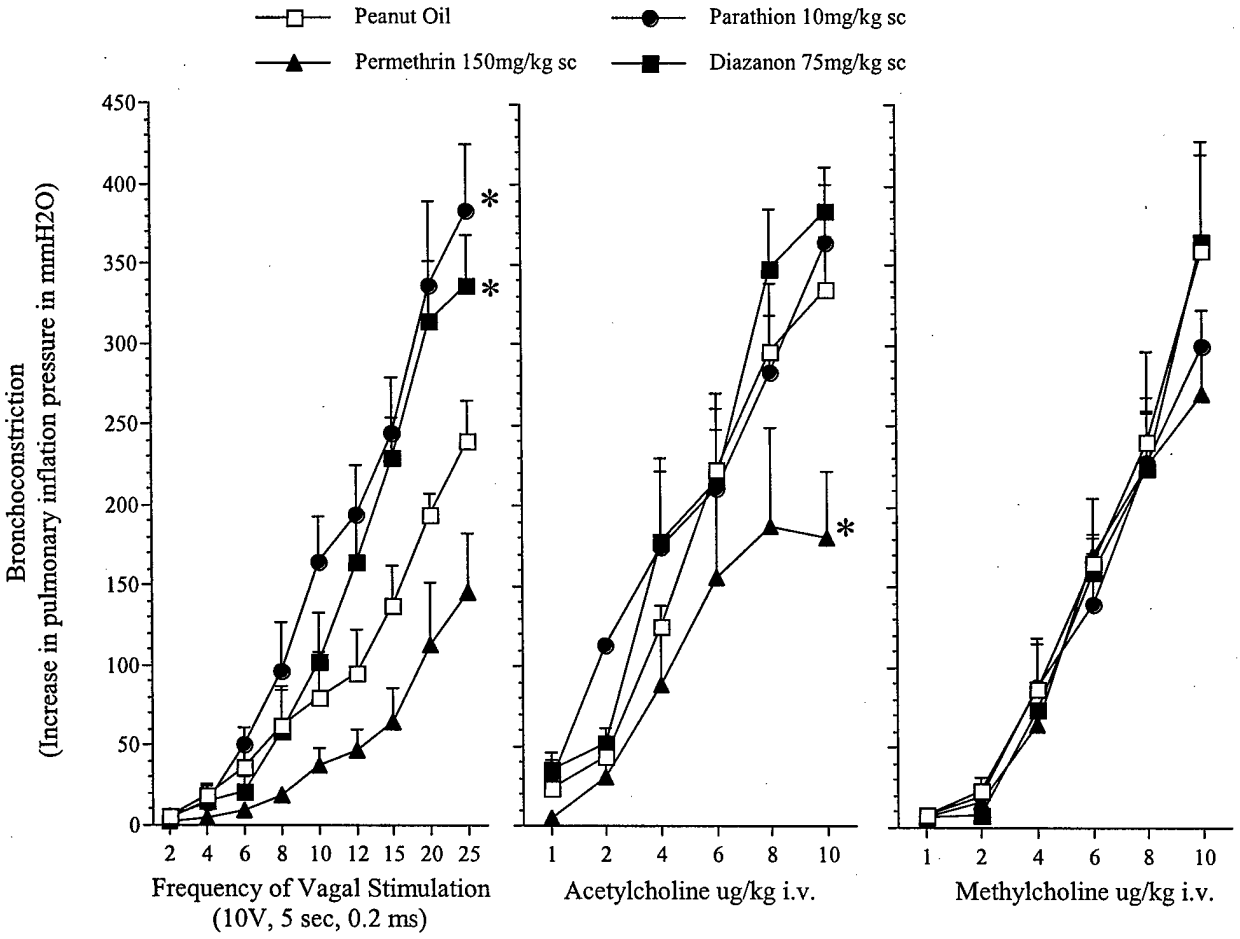

Fig. 1. Electrical stimulation of the vagus nerves (1-25 Hz, 10V, 0.2ms, 5 second train), acetylcholine and methacholine produced frequency and dose dependent bronchoconstriction in the lungs, measured as an increase in pulmonary inflation pressure (open squares). Vagally induced bronchoconstriction (left panel) was significantly increased in animals treated with either parathion (filled circles) or diazanon (filled squares) for 24 hours. Permethrin (filled triangles) did not potentiate vagally induced bronchoconstriction. Neither acetylcholine (middle panel) nor methacholine (right panel) induced bronchoconstriction were penetrated by any of the organophosphates. Each point is the mean +/-SEM of 5-8 animals; *significantly different from control.

Figure 2
M2 receptor function is inhibited by organophosphate pesticides
*significantly different from peanut oil, ANOVA repeated measures.

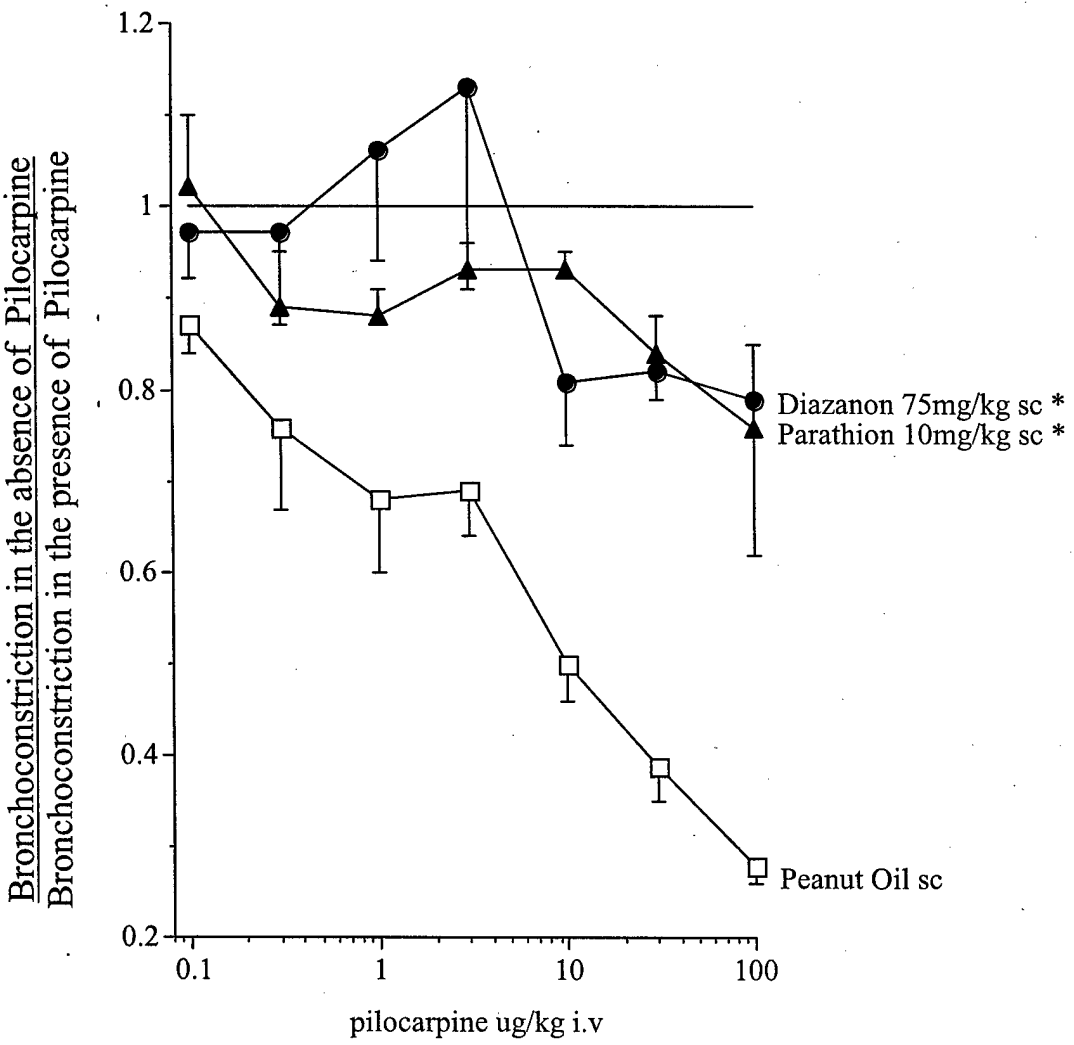

Fig. 2. Neuronal M_2 receptor function was tested using pilocarpine. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals treated with peanut oil for 24 hours (open circles) demonstrating functional M_2 receptors. In the animals treated with either 75mg/kg diazanon (filled circles) or 10mg/kg parathion (filled triangles) for 24 hours pilocarpine did not inhibit vagally induced bronchoconstriction indicating neuronal M_2 muscarinic receptor dysfunction. Each point is the mean +/- SEM of 5 animals; *significantly different from control.

Figure 3

Diazanon 0.75-75 mg/kg sc Causes Airway Hyperreactivity

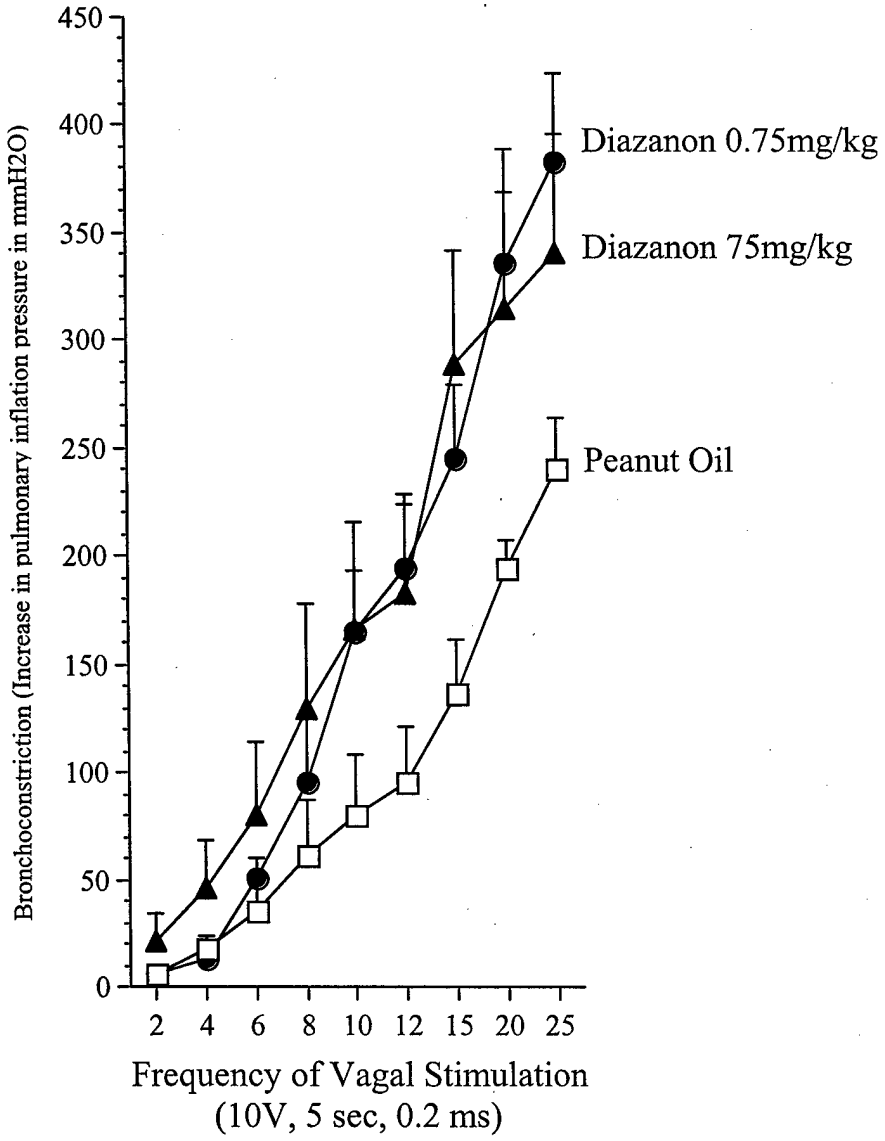

Fig. 3. Electrical stimulation of the vagus nerves (1-25 Hz, 10V, 0.2ms, 5 second train) produced frequency and dose dependent bronchoconstriction in the lungs, measured as an increase in pulmonary inflation pressure (open squares). Vagally induced bronchoconstriction was significantly increased in animals treated with diazanon for 24 hours. Each point is the mean +/-SEM of 5-8 animals; *significantly different from control.

Figure 4
Parathion at doses equal to and greater than 1.0 mg/kg cause airway hyperreactivity.

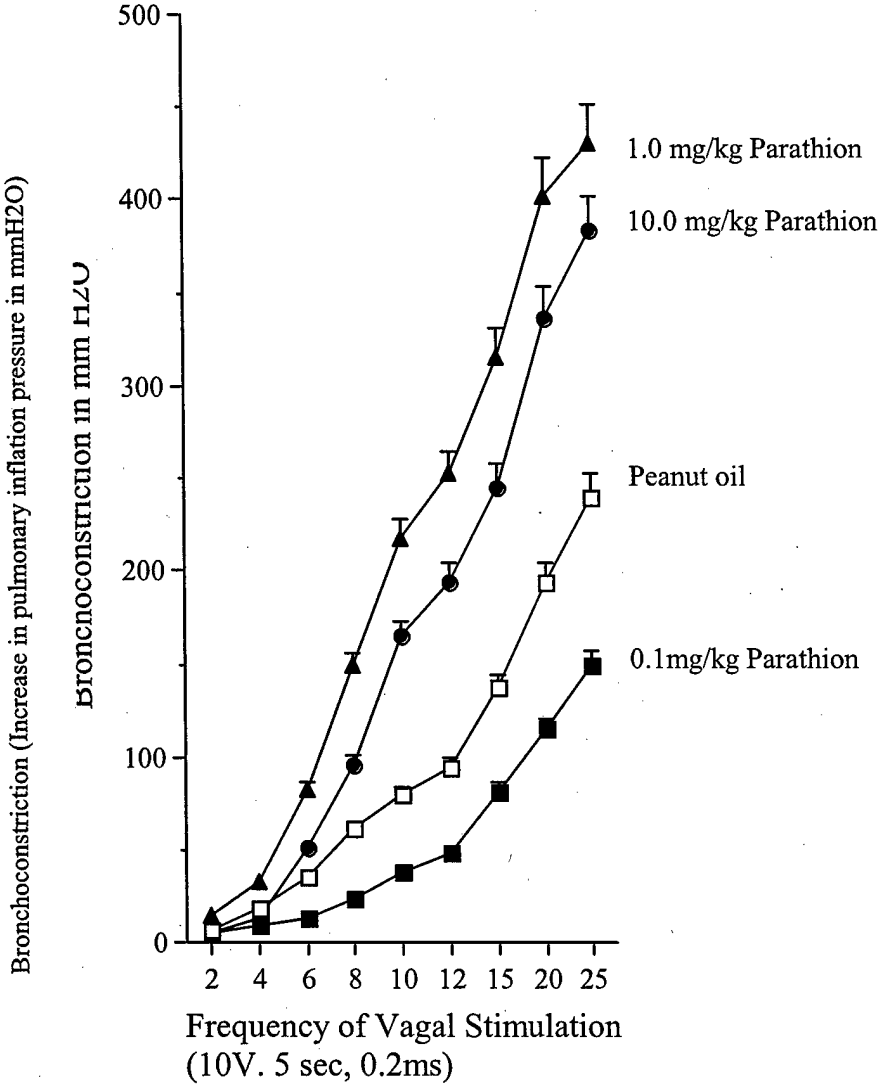

Fig. 4. Electrical stimulation of the vagus nerves (1-25 Hz, 10V, 0.2ms, 5 second train) produced frequency and dose dependent bronchoconstriction in the lungs, measured as an increase in pulmonary inflation pressure (open squares). Vagally induced bronchoconstriction was significantly increased in animals treated with 1-10 mg/kg parathion for 24 hours. 0.1 mg/kg parathion did not potentiate vagallyinduced bronchoconstriction. Each point is the mean +/-SEM of 5-8 animals; *significantly different from control.

Figure 5
Diazanon at doses ranging from 0.75 to 75 mg/kg sc inhibit the function of neuronal M2 muscarinic receptors.

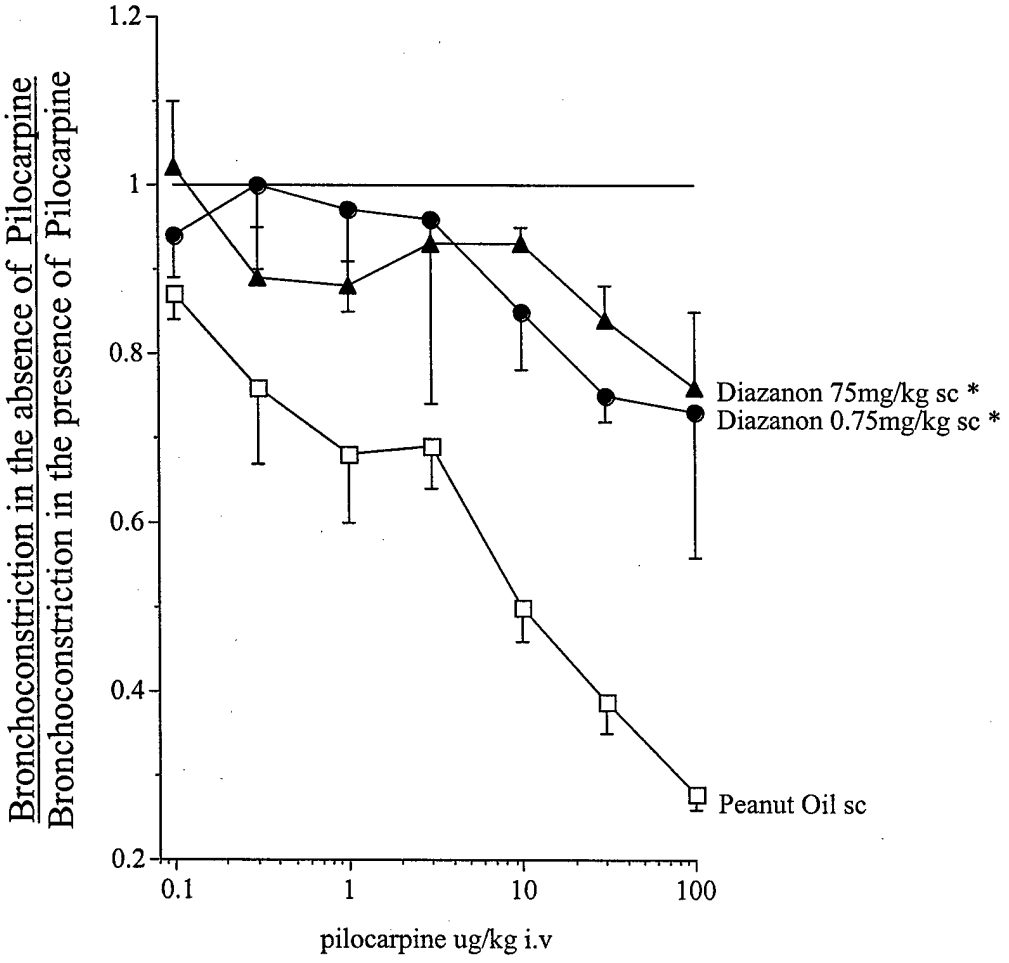

Fig. 5 Neuronal M_2 receptor function was tested using pilocarpine. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals treated with peanut oil for 24 hours (open squares) demonstrating functional M_2 receptors. In the animals treated with either 75mg/kg diazanon (filled circles) or 0.75mg/kg diazanon (filled triangles) for 24 hours pilocarpine did not inhibit vagally induced bronchoconstriction indicating neuronal M_2 muscarinic receptor dysfunction. Each point is the mean +/- SEM of 5 animals; *significantly different from control.

Figure 6
Parathion at 1.0 and 10.0 mg/kg inhibits the function of neuronal M2 muscarinic receptors.

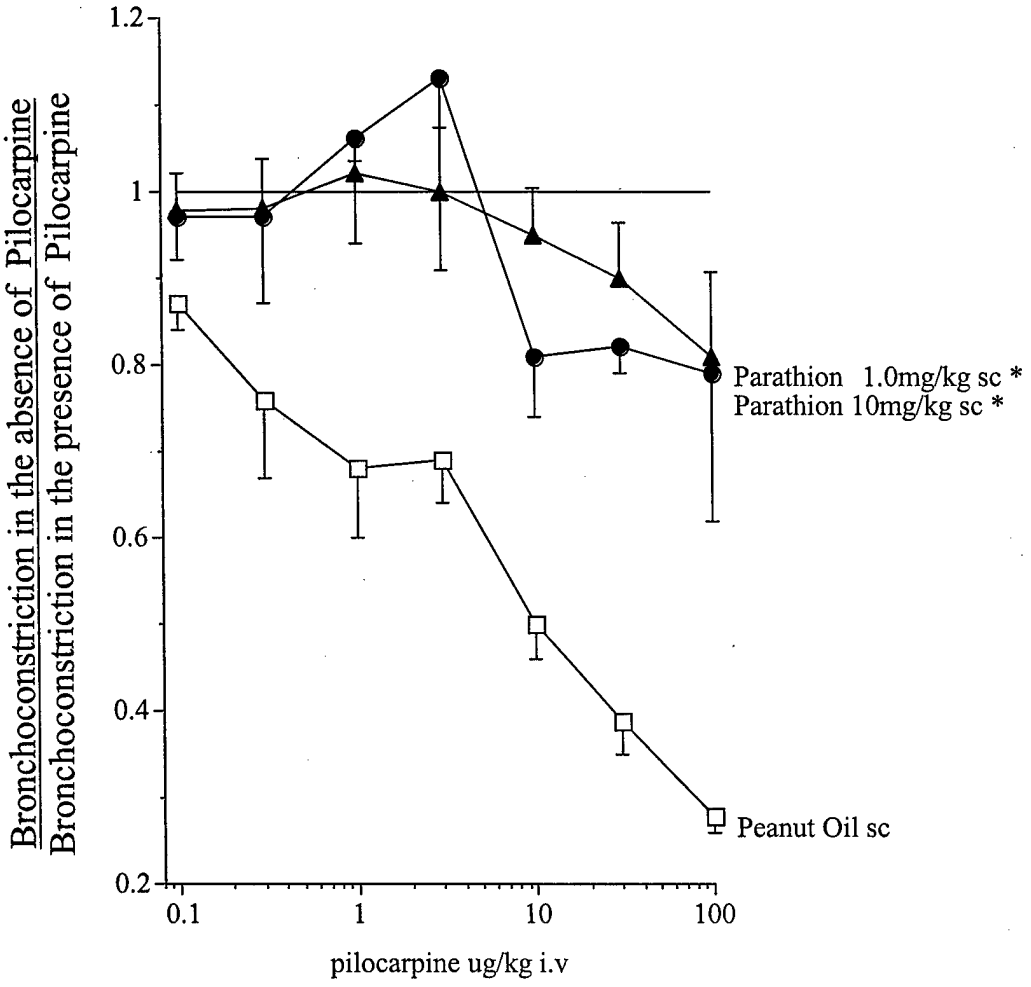

Fig. 6 Neuronal M_2 receptor function was tested using pilocarpine. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals treated with peanut oil for 24 hours (open squares) demonstrating functional M_2 receptors. In the animals treated with either 1.0mg/kg parathion (filled circles) or 10mg/kg parathion (filled triangles) for 24 hours pilocarpine did not inhibit vagally induced bronchoconstriction indicating neuronal M_2 muscarinic receptor dysfunction. Each point is the mean +/- SEM of 5 animals; *significantly different from control.

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Mechanisms of organophosphate insecticide-induced airway hyperreactivity

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Fryer, Allison D., Pamela J. Lein, Angela S. Howard, Bethany L. Yost, Rondell A. Beckles, and David A. Jett. Mechanisms of organophosphate insecticide-induced airway hyperreactivity. *Am J Physiol Lung Cell Mol Physiol* 286: L963–L969, 2004. First published January 2, 2004; 10.1152/ajplung.00343.2003.—It has been suggested that pesticide exposure may be a contributing factor underlying the increased incidence of asthma in the United States and other industrialized nations. To test this hypothesis, airway hyperreactivity was measured in guinea pigs exposed to chlorpyrifos, a widely used organophosphate pesticide. Electrical stimulation of the vagus nerves caused frequency-dependent bronchoconstriction that was significantly potentiated in animals 24 h or 7 days after a single subcutaneous injection of either 390 mg/kg or 70 mg/kg of chlorpyrifos, respectively. Mechanisms by which chlorpyrifos may cause airway hyperreactivity include inhibition of acetylcholinesterase (AChE) or dysfunction of M3 muscarinic receptors on airway smooth muscle or of autoinhibitory M2 muscarinic receptors on parasympathetic nerves in the lung. AChE activity in the lung was significantly inhibited 24 h after treatment with 390 mg/kg of chlorpyrifos, but not 7 days after injection of 70 mg/kg of chlorpyrifos. Acute exposure to eserine (250 μg/ml) also significantly inhibited lung AChE but did not potentiate vagally induced bronchoconstriction. Neuronal M2 receptor function was tested using the M2 agonist pilocarpine, which inhibits vagally induced bronchoconstriction in control animals. In chlorpyrifos-treated animals, pilocarpine dose-response curves were shifted significantly to the right, demonstrating decreased responsiveness of neuronal M2 receptors. In contrast, chlorpyrifos treatment did not alter methacholine-induced bronchoconstriction, suggesting that chlorpyrifos does not alter M3 muscarinic receptor function on airway smooth muscle. These data demonstrate that organophosphate insecticides can cause airway hyperreactivity in the absence of AChE inhibition by decreasing neuronal M2 receptor function.

asthma; pesticide; muscarinic receptor; cholinesterase

OVER THE PAST 20 YEARS there has been a significant increase in the incidence of asthma in industrialized nations, particularly in children in urban settings (33). Over this same period, the use of insecticides, particularly organophosphate insecticides, has increased significantly not only in agricultural (26, 45, 71, 75) but also residential and urban settings (6, 15, 50, 73, 74). A number of clinical and epidemiological studies have linked exposure to organophosphates to airway hyperreactivity and other symptoms of asthma (12, 19, 35, 59). However, the association between organophosphates and asthma has not been tested in an experimental model system and the mechanism(s) by which this class of insecticides causes changes in airway function remains speculative.

In the lung, cholinergic nerves in the vagi mediate airway tone and reactivity. These nerves release acetylcholine onto M3 muscarinic receptors causing contraction of airway smooth muscle, resulting in bronchoconstriction. Vagally induced bronchoconstriction is limited by autoinhibitory M2 muscarinic receptors on parasympathetic nerves (28, 54). Previous studies have shown that neuronal M2 receptors are dysfunctional in animal models of asthma (29, 31, 37) and in patients with asthma (55). 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.

Organophosphates are known to alter cholinergic function in the brain (53, 60). The generally accepted mechanism of organophosphate neurotoxicity following acute exposure to high doses is inhibition of acetylcholinesterase (AChE). It has been proposed that this same mechanism underlies the effects of organophosphate insecticides on bronchoconstriction (14, 67). However, there is evidence that in the brain, low-level doses of organophosphate pesticides that do not inhibit AChE may alter cholinergic neurotransmission via direct effects on muscarinic and nicotinic receptor function (1, 7, 36, 39, 40, 42–44). These observations led us to test whether chlorpyrifos (Dursban, Lorsban), a commonly used organophosphate with documented widespread human exposure (15, 17, 25, 34, 48), potentiates vagally induced bronchoconstriction by increasing cholinergic drive in the lungs via inhibition of AChE and/or alteration of neuronal M2 or airway smooth muscle M3 muscarinic receptor function.

METHODS

Animals. Specific pathogen-free male guinea pigs (300–350 g) were shipped from Hilltop Lab Animals (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 The Johns Hopkins University Animal Care and Use Committee.

Chlorpyrifos and eserine exposure. Chlorpyrifos (*o,o*-diethyl *o*-[3,5,6-trichloro-2-pyridinol] phosphorothionate, 99.5% pure) was purchased from Chem Service (West Chester, PA) and used within 1 mo of purchase with interim storage as recommended by the manufacturer. Chlorpyrifos dissolved in peanut oil at 70 mg/kg or 390 mg/kg or an equal volume (300 μl) of peanut oil alone was administered to guinea pigs by subcutaneous injection in the subscapular region. Although the most significant routes of chlorpyrifos exposure in humans are oral ingestion and inhalation (30), subcutaneous dosing is commonly used in mechanistic studies of chlorpyrifos and other organophosphates (13, 16, 62, 70). Subcutaneous administration of

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chlorpyrifos allows gradual release into the systemic circulation (63), which closely resembles human exposures (30). The guinea pig throat anatomy occludes a stomach tube more so than with rats or mice. Thus the subcutaneous method is also much less stressful to a guinea pig relative to gavage. Animals dosed with chlorpyrifos were monitored for signs of cholinergic intoxication (tremors, altered gait, and excessive excretions) at 1 and 24 h following injections. Guinea pigs given 70 mg/kg sc were tested for airway hyperreactivity 7 days postinjection, whereas animals receiving 390 mg/kg sc were tested 24 h later. These dosing paradigms were found to inhibit lung AChE activity by 0 and 50%, respectively (see Fig. 1), approximating the organophosphate exposures typically observed in humans, which include chronic exposure to low doses that have negligible effect on AChE or acute/subacute exposure to doses that significantly inhibit AChE (30). A 50% inhibition of AChE was chosen as a target value since this level of AChE inhibition is not uncommon in acutely exposed humans (30), and overt toxicity to chlorpyrifos in rodents becomes manifest when brain AChE is inhibited by >60% (57). Vehicle controls treated with peanut oil subcutaneously were tested 7 days later, and since the physiological values obtained with these animals did not differ from those measured in animals that were tested 24 h after saline injections (see Fig. 2), peanut oil controls were not performed for the cohort of animals tested 24 h after chlorpyrifos injection. Eserine was dissolved in sterile saline and administered at a dosage of 0.25 mg/kg iv to anesthetized animals 15 min before physiological measurements, as described below.

Anesthesia and measurement of pulmonary inflation pressure. Guinea pigs were anesthetized (1.5 g/kg urethane ip), and blood pressure and heart rate were measured from the carotid artery. Both jugular veins were cannulated for administration of drugs. Both vagus nerves were cut and placed on electrodes under oil. Succinylcholine (10 μg/kg iv) was infused to paralyze the animals, and they were ventilated via a tracheal cannula with a positive pressure constant volume (1 ml/100 g body wt and 100 breaths/min). Pulmonary inflation pressure (P_{pi}) was measured via a side arm at the trachea; bronchoconstriction was measured as the increase in P_{pi} over the pressure produced by the ventilator as previously described (28, 29, 37).

Measurement of vagally induced bronchoconstriction. Noradrenaline was depleted 25 min before the start of the experiment with guanethidine (10 mg/kg iv). Both vagi were stimulated (0.2 ms, 10 V, 1–25 Hz, 5-s duration at 2-min intervals) producing frequency-dependent bronchoconstriction and bradycardia due to release of acetylcholine onto postjunctional M2 muscarinic receptors in the heart and postjunctional M3 muscarinic receptors in the lungs. Both vagally induced bronchoconstriction and bradycardia could be abolished by atropine (1 mg/kg iv).

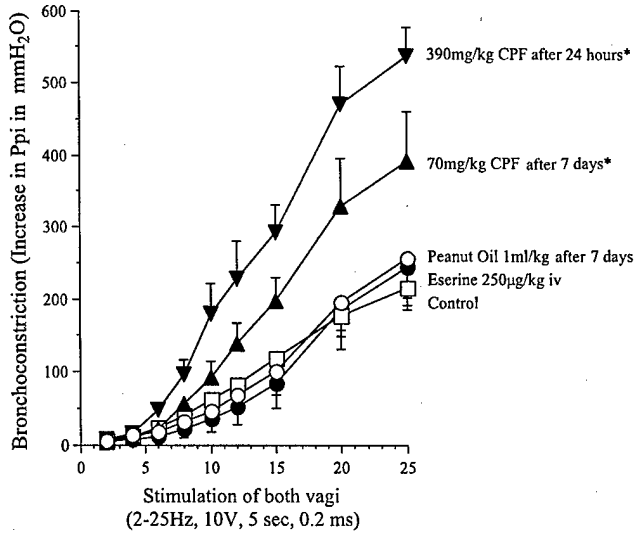

Fig. 2. Electrical stimulation of the vagus nerves (1–25 Hz, 10 V, 0.2 ms, 5-s train) produced frequency-dependent bronchoconstriction in the lungs, measured as an increase in pulmonary inflation pressure (P_{pi}; □). Vagally induced bronchoconstriction was significantly increased in animals 7 days after a single dose of 70 mg/kg of chlorpyrifos (▲) and 24 h after a dose of 390 mg/kg of chlorpyrifos (▼). Frequency response curves in animals 7 days after treatment once with peanut oil vehicle (○) or acutely with 250 μg/kg of eserine (iv; ●), a nonorganophosphate AChE inhibitor, were not different from control animals. Each point is the mean ± SE of 5–8 animals. *Significantly different from control.

Measurement of neuronal M2 and smooth muscle M3 muscarinic receptor function. The function of neuronal M2 receptors was determined using a standard assay (28, 29, 37): the ability of the muscarinic agonist pilocarpine to inhibit the bronchoconstrictor response to vagal stimulation at 2 Hz. 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 (28). The effect of pilocarpine on vagally induced bronchoconstriction is reported as the ratio of bronchoconstriction in the presence of pilocarpine to bronchoconstriction in the absence of pilocarpine. Decreased inhibition by pilocarpine of vagally induced bronchoconstriction, manifested as a shift to the right of the dose-response curve, indicates M2 receptor dysfunction (28, 29, 37).

In vagotomized guinea pigs, parasympathetic nerves that release acetylcholine and contain M2 receptor are absent. To assess the effects of organophosphates on M3 muscarinic receptor function in airway smooth muscle and to bypass endogenous acetylcholine release from parasympathetic nerves, we measured bronchoconstriction in vagotomized guinea pigs in response to methacholine (1–10 μg/kg iv), which is not readily metabolized by AChE (11, 56), and acetylcholine (1–8 μg/kg iv). The dose-response curve of bronchoconstriction in response to acetylcholine was compared with that of methacholine as well as with vagally induced bronchoconstriction to determine whether changes in vagally induced bronchoconstriction occur via changes in the nerves, in AChE activity, or postjunctional M3 receptor function.

AChE assay. Immediately after the completion of physiological measurements, lungs, brain, and heparinized blood samples were

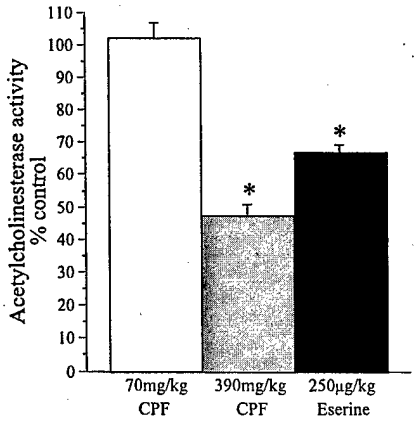

Fig. 1. Mean acetylcholinesterase (AChE) levels in the lungs of guinea pigs treated with vehicle were 3.8 ± 0.15 μmol substrate·min^{-1}·mg^{-1} protein. Treatment of animals with a single dose of 70 mg/kg of chlorpyrifos (CPF) did not inhibit AChE activity in the lungs 7 days postinjection (open bar). In contrast, 390 mg/kg of chlorpyrifos after 24 h (gray bar) and 250 μg/kg of eserine (solid bar) significantly inhibited AChE activity in the lungs. Each point is the mean ± SE of 5–8 animals presented as a % of control. *Significantly different from control.

obtained for determination of AChE activity via the standard Ellman assay (23) using DTNB and acetylthiocholine iodide (ASChI) as the substrate. Assays were run against blanks containing DTNB. The reaction was started with the addition of ASChI after equilibration for 2–3 min. Hydrolysis of ASChI was determined by monitoring the change in absorbance at 405 nm. To inhibit pseudocholinesterase activity, 100 μM tetraisopropyl pyrophosphoramide was included in the assay. Data from lung and brain samples were normalized using protein concentration determined using the bicinchoninic 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 as determined using a hemacytometer.

Statistics. Data are expressed as means ± SE. Frequency, pilocarpine, methacholine, and acetylcholine dose-response curves were analyzed using a two-way analysis of variance for repeated measures. Baseline heart rates (beats/minute), blood pressures (mmHg), P_{pi} (mmH_2O), and changes in P_{pi} (mmH_2O before pilocarpine administration) as well as AChE activity levels (as % of control) were analyzed using ANOVA (Statview 4.5; Abacus Concepts, Berkeley, CA). A *P* value of 0.05 was considered significant.

RESULTS

Treatment with chlorpyrifos did not result in any apparent signs of cholinergic intoxication in guinea pigs 1 or 24 h after subcutaneous injections of 70 or 390 mg/kg. AChE activity in the lungs was not altered 7 days after a single injection of 70 mg/kg of chlorpyrifos (Fig. 1). However, 24 h after a single injection of 390 mg/kg of chlorpyrifos, lung AChE was significantly inhibited by 50%. Acute exposure to 250 μg/kg of eserine, a nonorganophosphate anticholinesterase, caused a level of AChE inhibition that was not significantly different from that observed with the higher dose of chlorpyrifos (Fig. 1).

Neither chlorpyrifos nor peanut oil altered baseline P_{pi} (control 91 ± 7 mmH_2O, peanut oil 96 ± 5 mmH_2O, 70 mg/kg of chlorpyrifos 87 ± 7 mmH_2O, 390 mg/kg of chlorpyrifos 96 ± 5 mmH_2O), resting heart rate (control 281 ± 11 beats/min, peanut oil 271 ± 8 beats/min, 70 mg/kg of chlorpyrifos 268 ± 9 beats/min, 390 mg/kg of chlorpyrifos 300 ± 7 beats/min), or resting diastolic blood pressure (control 45 ± 2.3 mmHg, peanut oil 41 ± 2 mmHg, 70 mg/kg of chlorpyrifos 46 ± 2 mmHg, 390 mg/kg of chlorpyrifos 56 ± 2.4 mmHg) in vagotomized, anesthetized guinea pigs.

Electrical stimulation of both vagi (1–25 Hz) caused a frequency-dependent increase in bronchoconstriction that was significantly potentiated in animals 24 h after a single injection of 390 mg/kg of chlorpyrifos or 7 days after a single injection of 70 mg/kg of chlorpyrifos (Fig. 2). However, a greater increase in bronchoconstriction was observed in animals that received the acute high-dose chlorpyrifos treatment. In contrast, eserine, which significantly inhibited AChE (Fig. 1), did not potentiate vagally induced bronchoconstriction. Vagally induced bronchoconstriction was not altered in animals receiving vehicle alone (peanut oil) relative to control animals.

Neuronal M2 receptor function was tested in chlorpyrifos-treated animals using the muscarinic agonist pilocarpine. Before pilocarpine was administered, simultaneous electrical stimulation of both vagus nerves (2 Hz, 0.2 ms, 5–20 V, 22 s at 1-min intervals) produced transient bronchoconstriction (measured as an increase in P_{pi}) that did not differ among groups (control 27.6 ± 0.2 mmH_2O, peanut oil 19.8 ± 3 mmH_2O, 70 mg/kg of chlorpyrifos 18.6 ± 5 mmH_2O, 390 mg/kg of chlorpyrifos 24.6 ± 6 mmH_2O). In guinea pigs treated with peanut oil, pilocarpine (1–100 μg/kg iv) dose dependently inhibited vagally induced bronchoconstriction, demonstrating that the neuronal M2 receptors are functional (Fig. 3, ○). The effect was identical to saline-treated controls (not shown), demonstrating that injection of peanut oil subcutaneously for 7 days did not alter the function of neuronal M2 receptors. The dose-response curve to pilocarpine was shifted significantly to the right in animals 24 h after treatment with chlorpyrifos at 390 mg/kg. A lesser, but still significant, rightward shift was observed 7 days after treatment with 70 mg/kg of chlorpyrifos. Shifting of the pilocarpine dose-response curve to the right is consistent with decreased function of the M2 receptors.

To test the direct response of M3 receptors on airway smooth muscle to muscarinic agonists, bronchoconstriction induced by intravenous methacholine, which is not rapidly metabolized by AChE, and by intravenous acetylcholine was measured in vagotomized guinea pigs. Chlorpyrifos treatment did not alter methacholine-induced bronchoconstriction (Fig. 4*A*). Because M2 receptors on vagus nerves were not present in these vagotomized guinea pigs, the absence of an effect on methacholine-induced bronchoconstriction indicates that chlorpyrifos does not affect the ability of agonists to interact with postjunctional M3 muscarinic receptors. Acetylcholine-induced bronchoconstriction was significantly increased in animals treated with 390 mg/kg of chlorpyrifos (Fig. 4*B*). Eserine at a dosage of 250 μg/kg also potentiated acetylcholine-induced bronchoconstriction, and although the eserine dose response did not differ significantly from the control dose-response curve, it also did not differ significantly from the dose-response curve obtained from animals treated with 390 mg/kg of chlorpyrifos. Thus eserine potentiated bronchoconstriction to an intermediate level between that of control

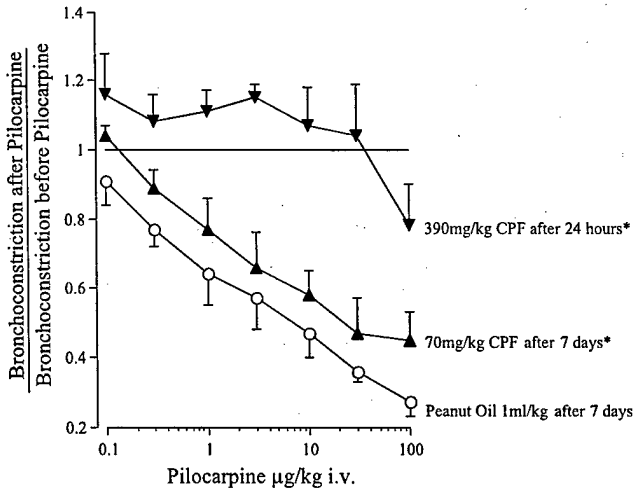

Fig. 3. Neuronal M2 receptor function was tested using pilocarpine. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals 7 days after treatment with peanut oil vehicle (○), demonstrating the presence of functional M2 receptors. The effect of pilocarpine was shifted significantly to the right in animals 7 days after treatment with 70 mg/kg of chlorpyrifos (▲). In the animals treated with 390 mg/kg of chlorpyrifos (▼), pilocarpine did not inhibit vagally induced bronchoconstriction, indicating neuronal M2 muscarinic receptor dysfunction after 24 h. Each point is the mean ± SE of 5 animals. *Significantly different from control.

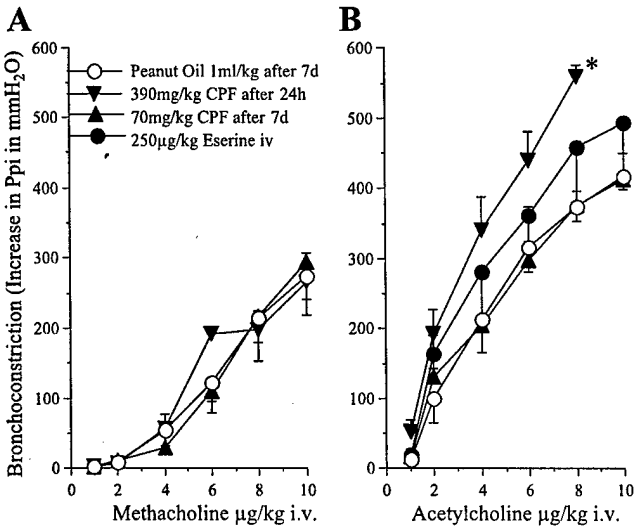

Fig. 4. *A*: methacholine (1–10 µg/kg iv)-induced bronchoconstriction in vagotomized guinea pigs was not different between peanut oil (○)- or chlorpyrifos-treated guinea pigs (7 days after 70 mg/kg of chlorpyrifos, ▲; and 24 h after 390 mg/kg of chlorpyrifos, ▼). *B*: acetylcholine (1–10 µg/kg iv)-induced bronchoconstriction in vagotomized guinea pigs was significantly potentiated by 390 mg/kg of chlorpyrifos, whereas 70 mg/kg of chlorpyrifos had no effect. Acute treatment with 250 µg/kg of eserine (iv, ●) caused a small but not statistically significant potentiation of acetylcholine-induced bronchoconstriction. Each point is the mean ± SE of 5 animals. *Significantly different from control; d, days.

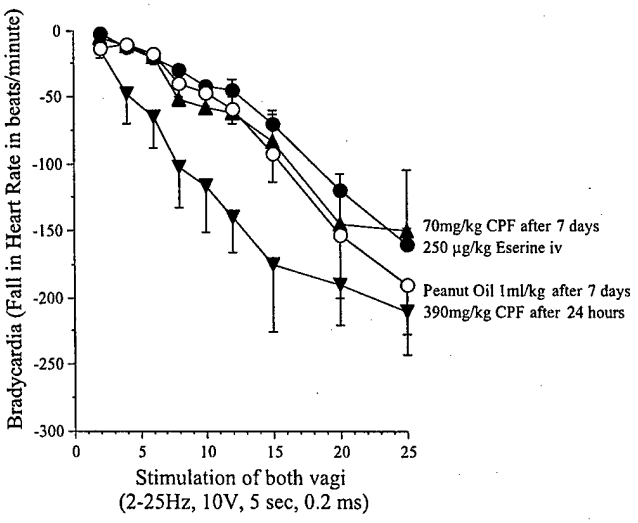

Fig. 5. Electrical stimulation of the vagus nerves (1–25 Hz, 10 V, 0.2 ms, 5-s train) produced frequency-dependent bradycardia in the heart measured as a fall in heart rate (○). Vagally induced bradycardia in animals 7 days after treatment with 70 mg/kg of chlorpyrifos (▲) or acutely with 250 µg/kg of eserine (●) was not significantly different from peanut oil-treated animals. However, vagally induced bradycardia in animals treated with 390 mg/kg of chlorpyrifos after 24 h (▼) was shifted to the left. Up until 20 Hz, the shift was significantly different from oil control; however, at 20 and 25 Hz, the fall in heart rate was approaching maximum, and the differences were no longer significant. Each point is the mean ± SE of 5–8 animals.

animals and animals treated with 390 mg/kg of chlorpyrifos. Treatment of animals with 70 mg/kg of chlorpyrifos for 7 days had no effect on acetylcholine-induced bronchoconstriction. The potentiation of acetylcholine-induced bronchoconstriction by the higher dose of chlorpyrifos and by eserine is consistent with the inhibition of AChE by these treatments (see Fig. 1).

M2 muscarinic receptors are also present in the heart. Thus we measured vagally induced bradycardia to determine whether organophosphate insecticides alter M2 muscarinic receptors in tissues other than the lung. In the heart, stimulation of the vagus nerves (1–25 Hz) produces bradycardia that is frequency dependent (Fig. 5). Treatment with 70 mg/kg of chlorpyrifos did not alter the vagally induced fall in heart rate relative to control. However, in the animals treated with 390 mg/kg of chlorpyrifos for 24 h, the frequency response curve was potentiated at frequencies <20 Hz (Fig. 5). At 20 and 25 Hz, the fall in heart rate approaches maximum, and the differences are no longer significant. However, the potentiation of vagally induced bradycardia by the higher dose of chlorpyrifos does not appear to be mediated by inhibition of AChE since eserine at a concentration that significantly inhibits AChE does not potentiate vagally induced bradycardia (Fig. 5). Methacholine- and acetylcholine-induced bradycardia was not altered by either high- or low-dose chlorpyrifos treatment (Fig. 6) despite inhibition of AChE by the higher dose of chlorpyrifos.

DISCUSSION

In humans, exposure to organophosphate insecticides and other pesticides has been associated with a variety of respiratory symptoms, including decreased forced expiratory volume in 1 min, wheeze, cough, and shortness of breath (3, 19, 35, 46, 47, 65, 67). Similarly, it has been reported that organophosphate insecticides induce bronchospasm in a variety of animals (20, 32, 66). Our data provide more direct evidence of a causal link between organophosphate exposure and airway hyperreactivity. Specifically, we observed that vagally induced bronchoconstriction in guinea pig lungs is significantly potentiated

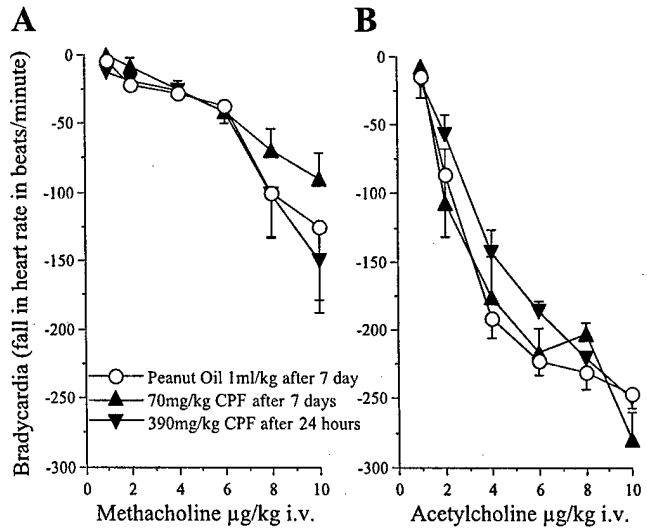

Fig. 6. *A*: methacholine (1–10 µg/kg iv)-induced bradycardia in vagotomized guinea pigs was not different between peanut oil (○)- or chlorpyrifos-treated guinea pigs (7 days after 70 mg/kg of chlorpyrifos, ▲; and 24 h after 390 mg/kg of chlorpyrifos, ▼). *B*: acetylcholine (1–10 µg/kg iv)-induced bradycardia in vagotomized guinea pigs was not different between peanut oil (○)- or chlorpyrifos-treated guinea pigs (7 days after 70 mg/kg of chlorpyrifos, ▲; and 24 h after 390 mg/kg of chlorpyrifos, ▼). Each point is the mean ± SE of 5 animals.

following either acute (24 h after 390 mg/kg of chlorpyrifos) or chronic (7 days after 70 mg/kg) exposure to chlorpyrifos.

It has been proposed that if organophosphates contribute to asthma, it is likely due to inhibition of AChE (14, 24, 67), which is the principal mechanism underlying acute organophosphate neurotoxicity. Support for this hypothesis includes observations that not only organophosphate insecticides, but also other structurally unrelated AChE-inhibiting insecticides, such as carbaryl, enhance airway hyperreactivity in rats (20) and humans (67). However, two observations from our studies suggest mechanisms other than AChE inhibition mediate chlorpyrifos effects on vagally induced bronchoconstriction. First, our data indicate that animals tested 7 days after receiving 70 mg/kg of chlorpyrifos (subcutaneously) exhibit airway hyperreactivity in the absence of AChE inhibition. Second, acute administration of the nonorganophosphate eserine at a dose (250 μg/ml) that significantly inhibits AChE does not potentiate vagally induced bronchoconstriction. Similarly, a poor correlation has been noted between cholinesterase inhibition and toxic effects in the brain by some organophosphate insecticides (22), prompting investigations of alternative mechanisms of neurotoxicity. These observations are important because they suggest that toxicity from some organophosphates may occur below thresholds of exposure normally defined by AChE inhibition.

Neuronal M2 muscarinic receptors limit release of acetylcholine from the vagus nerves in the lungs (28). Pharmacological blockade of neuronal M2 receptors increases release of acetylcholine from the nerves (5, 27), which potentiates vagally induced bronchoconstriction (28, 29, 37). Our data show that neuronal M2 receptor function is inhibited by both high and low doses of chlorpyrifos, consistent with other findings that organophosphate insecticides act on muscarinic receptors in the brain (1, 36, 38, 43). This effect of chlorpyrifos on vagally induced bronchoconstriction is dependent on the dosing regimen. Vagally induced bronchoconstriction was significantly greater in animals treated with the high dose of chlorpyrifos relative to animals treated with the low dose (Fig. 2). A similar dependency was observed for the effects of chlorpyrifos on M2 receptor function as determined by pilocarpine dose-response curves (Fig. 3). In contrast, neither dose of chlorpyrifos changed the response to intravenous methacholine, demonstrating that the function of M3 muscarinic receptors on airway smooth muscle was not altered in animals in which M2 receptors mediating ACh release were not present. Selective loss of neuronal M2 receptor function in the lungs is also associated with other models of airway hyperreactivity, including antigen challenge (29), viral infection (37), and exposure to ozone (31), suggesting that decreased M2 receptor function on airway nerves is a generalized mechanism underlying airway hyperreactivity.

The ability of organophosphate insecticides to inhibit neuronal M2 receptors may not be restricted to the lungs. Organophosphate insecticides have been shown to inhibit muscarinic receptor binding in the brain (1, 7, 36, 39, 40, 43, 44) and bind to a subpopulation of M2 receptors in the heart (68). In the heart, M2 receptors are present on parasympathetic nerves that supply the heart where they function to inhibit release of acetylcholine (52, 58) as well as on cardiac muscle where they mediate bradycardia (10, 51). The single high dose of chlorpyrifos potentiated vagally induced bradycardia but not bradycardia induced by intravenous administration of acetylcholine. This is consistent with loss of neuronal M2 receptor function on parasympathetic nerves in the heart resulting in increased release of acetylcholine, which potentiates vagally induced bradycardia. Neither dose of chlorpyrifos altered bradycardia induced by acetylcholine or methacholine administered intravenously, indicating that the postjunctional M2 receptors are not sensitive to organophosphate insecticides, and this is consistent with the sensitivity of neuronal M2 receptors we observed in the lung. These data are also consistent with previous observations that the function of the neuronal M2 receptors in the heart are inhibited by systemic administration of double-stranded RNA, which does not alter the function of postjunctional M2 receptors (9). Thus the neuronal receptors appear to be more vulnerable to inhibition than the postjunctional receptors.

The mechanism(s) by which organophosphate insecticides alter M2 receptor function in the lungs have yet to be elucidated. Mechanisms by which these compounds alter muscarinic receptor function in neurons include downregulation of muscarinic receptor expression (39, 40), modulation of ligand binding to muscarinic receptors (38, 43, 44), and alteration of signal transduction pathways downstream of muscarinic receptor activation (8, 36, 72). In vitro studies of cardiac M2 muscarinic receptors have demonstrated that acute exposure to the oxon metabolite of chlorpyrifos alters ligand binding via diethylphosphorylation of the receptor itself (7). Whether these mechanisms underlie the effects of organophosphates on neuronal M2 receptor function in the lung has yet to be determined.

Data presented here indicate that organophosphate insecticides potentiate vagally induced bronchoconstriction via disruption of the cholinergic control of airway responsiveness. A significant finding from our studies is that chlorpyrifos altered neuronal M2 receptor function in the lung at concentrations that did not inhibit AChE. Although the threshold concentration for this effect was not determined in our studies, it has been shown that ligand binding to muscarinic receptors in the brain (43) as well as signaling pathways downstream of muscarinic receptor binding (64) can be disrupted by very low (nanomolar to picomolar) concentrations of organophosphate insecticides. These data suggest that exposure to not only occupational, but also environmental, levels of these compounds may have biological consequences. The significance of these findings to public health is heightened by evidence of widespread human exposure to chlorpyrifos and other organophosphate insecticides (34, 45).

Children represent a potentially sensitive subpopulation with respect to asthma. Thus it is of great concern that 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 with a 4% detection rate for the herbicide atrazine (2). Many of the organophosphate insecticides have been restricted or banned due to their developmental neurotoxicity in animals. However, many of these compounds, including chlorpyrifos, are still used commercially in both agricultural settings and urban environments. These pesticide usage patterns correlate positively with reports of high incidence of asthma morbidity in agricultural workers (3, 35, 41, 46, 67, 69, 76) and in residents of the inner cities of the United States (18, 33, 49, 61). The coincident rise in the incidence of

asthma (33) and in the use of organophosphate insecticides (26, 45, 71, 75) suggests a potential causal relationship between these two observations, which is corroborated by our data obtained using an animal model of airway hyperreactivity. Our data raise significant questions regarding the current use of organophosphate insecticides in the inner cities to control cockroach antigen (6, 15, 50, 73, 74), which itself has been associated with asthma (4, 21), and suggest that exposure to insecticides may be contributing to rather than ameliorating asthma.

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