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Phospholipase-Mediated Inhibition of Spontaneous Oscillatory Uterine Contractions by Lindane *in Vitro*^{1,2}

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Abstract

Although regulation of uterine contractility is fundamental for parturition, mechanisms by which toxicants modify uterine muscle contractions remain poorly understood. In a previous cumulative concentration-response study, 10 μM lindane (γ -hexachlorocyclohexane) reduced contraction force and 30 μM lindane abolished contractions in Gestation Day 10 rat uterine strips when lindane was added to muscle baths at 10-min intervals. Other studies showed that brief (<10 min) exposures to 10–100 μM lindane inhibit gap junctions and activate phospholipase pathways in rat myometrial cells in culture. Consequently, lindane was used as a prototype toxicant with known uterine activity to investigate the hypothesis that activation of a specific phospholipase pathway provides a mechanistic link between inhibition of uterine contraction and inhibition of myometrial gap junctions. Uterine tissue and cells were pretreated with phospholipase pathway inhibitors to evaluate the role of phospholipase pathways in lindane's actions in the uterus. Concentrations of inhibitors were selected based on previous reports of effective concentrations for the enzyme activity and on pilot toxicity studies of the inhibitors on uterine contraction and gap junction communication. To monitor uterine contractions, longitudinal uterine strips were excised from Gestation Day 10 rats and suspended in isometric muscle baths, consistent with previous experiments. Exposure *in vitro* for 60 min to 10–50 μM lindane, an effective concentration range for the uterine responses of interest, revealed that 30 μM lindane rapidly abolished contractions. Subsequently, uterine strips were pretreated with phospholipase pathway inhibitors and then challenged with 30 μM lindane, the lindane concentration that elicited maximal inhibition of uterine contraction. Pretreatment with 20–50 μM of the phosphatidylinositol-specific phospholipase C inhibitor 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphorylcholine (ET-18-OCH₃) reversed lindane-induced inhibition of spontaneous uterine contractions. Gap junction intercellular communication was monitored by injecting the fluorescent dye Lucifer yellow into rat myometrial cells grown in culture and assessing dye transfer to adjacent cells using epifluorescence microscopy. Similar to uterine contraction, pretreatment of cell cultures with phospholipase C inhibitors (30 μM ET-18-OCH₃, 50 μM tricyclodecan-*p*-yl-xanthogenate · K [D609] or 50 μM tricyclodecan-*p*-yl-xanthogenate · K or 2-nitro-4-carboxyphenyl-*N,N*-dophenylcarbamate [NDCD]) partially reversed inhibition of dye transfer by 100 μM lindane, a

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lindane concentration previously shown to abolish myometrial Lucifer yellow dye transfer under similar culture conditions. In contrast, pretreatment with 20 μ M of bromoenol lactone (BEL) to inhibit the calcium-independent phospholipase A₂ or 100 mM ethanol to interrupt the phospholipase D pathway failed to prevent inhibition of spontaneous uterine contractions and inhibition of Lucifer yellow dye transfer by lindane (100 μ M). These data suggest that lindane inhibits myometrial gap junctions and spontaneous oscillatory contractions by a phospholipase C-mediated pathway.

Keywords

lindane; hexachlorocyclohexane; uterus; gap junctions; phospholipase; rat

Coordinated and forceful uterine contractions are necessary for parturition, yet few studies have examined mechanisms by which toxicants modify uterine muscle contractions (Loch-Caruso, 1999). The present study utilizes lindane, the γ -isomer of hexachlorocyclohexane, to investigate mechanisms of inhibition of spontaneous uterine contractions. Our laboratory initially investigated lindane's actions on uterine contraction because it was one of several organochlorine substances found in significantly elevated concentrations (15–136 ppb) in the blood lipids of women who had experienced spontaneous abortions or premature delivery compared with women who had undergone full-term pregnancy (7–39 ppb) (Saxena *et al.*, 1981; Wassermann *et al.*, 1982). However, *in vitro* exposure of rat uterine strips isolated from pregnant (Gestation Day 10) rats to cumulative concentrations of lindane at 10-min intervals was found to depress spontaneous contractions at 10 μ M lindane and abolish contractions at 30 μ M lindane (Criswell and Loch-Caruso, 1999). The inhibitory action of lindane was in contrast to stimulation of uterine contraction frequency that we observed with DDT (Juberg and Loch-Caruso, 1991; Juberg *et al.*, 1991, 1995) and polychlorinated biphenyl isomers (Tsai *et al.*, 1996; Bae *et al.*, 1999a,b, 2001), which were also elevated in the blood of women who experienced premature delivery or spontaneous abortion (Saxena *et al.*, 1981; Wassermann *et al.*, 1982). We subsequently used lindane as an inhibitor for probing physiologic and pathologic mechanisms of regulation of uterine contraction, and identified a previously unrecognized role for reactive oxygen species (Krieger and Loch-Caruso, 2001) and a previously unidentified myometrial phospholipase A₂ activity (Wang *et al.*, 2001).

Lindane is an insecticide used as a pharmacological agent in veterinary and human medicine to treat scabies and lice (Smith, 1991). Although lindane's use as an insecticide in agricultural, forestry, and household products has been restricted in the United States since 1977, lindane is a contaminant of 163 of the 1467 current or former U.S. EPA National Priorities List hazardous waste sites and its use as an insecticide continues in other countries (ATSDR, 1997). Lindane's insecticidal activity is ascribed to neuroexcitation subsequent to inhibition of GABA_A channels (Narahashi, 1976; Joy, 1982; Ogata *et al.*, 1988). Accordingly, exposure to excessive amounts of lindane through misuse or unintentional exposure causes seizures, convulsions, and other signs of intoxication in humans (Starr and Clifford, 1972; Munk and Nantel, 1977; Davies *et al.*, 1983), although actual amounts of lindane ingested were not determined in these reports. Rats administered a single oral dose of 30 mg lindane/kg exhibit convulsions within 10–30 min (Barron *et al.*, 1995), and less serious neuroexcitation is observed in rats administered a single oral dose of 10 or 20 mg lindane/kg (Llorens *et al.*, 1989; Llorens *et al.*, 1990).

In addition to neurotoxicity, lindane alters female reproductive processes in adult female rats. A single oral dose of 25 mg lindane/kg lengthens estrous cycles, and higher doses of 33 or 50 mg lindane/kg decrease sexual receptivity (Uphouse, 1987; Uphouse and Williams, 1989). Similarly, daily administration of 5–40 mg/kg lindane from 20 days of age until 110–125 days of age delayed the appearance of regular estrous cycles (Cooper *et al.*, 1989). Although lindane

exhibits weak estrogenic (Raizada *et al.*, 1980) and antiestrogenic effects (Chadwick *et al.*, 1988; Cooper *et al.*, 1989) *in vivo*, it has no significant affinity for the estrogen receptor (α subtype) (Laws *et al.*, 1994). In contrast, rats exposed to 10 mg lindane/kg/day for four generations showed no adverse reproductive effects (Palmer *et al.*, 1978). Although lindane induces neurological hyperexcitability through inhibition of GABA_A channels (Narahashi, 1976; Joy, 1982; Ogata *et al.*, 1988), *in vitro* exposure to the GABA_A channel inhibitor picrotoxin stimulates contraction of uterine strips from Gestation Day 10 rats (Criswell and Loch-Caruso, 1999). Because the picrotoxin response is opposite to the inhibitory response observed with lindane, this finding suggests that lindane-induced inhibition of uterine contraction is not mediated by GABA_A channel inhibition.

The uterine contractions necessary for parturition require the increased presence of gap junctions between the muscle cells of the myometrium (Garfield and Hayashi, 1981). The myometrial gap junctions facilitate coordination and synchronization of uterine contractions by providing low resistance intercellular pathways for the propagation of electrical and metabolic signals (Garfield and Yallampalli, 1994). Brief (4-min) exposures of myometrial cells in culture to 10–100 μ M lindane inhibit gap junction intercellular communication as measured by Lucifer yellow dye transfer (Criswell *et al.*, 1995). It has been suggested that lindane inhibits uterine contractions by inhibiting myometrial gap junction permeability (Criswell and Loch-Caruso, 1999), perhaps involving an oxidative stress-dependent mechanism (Krieger and Loch-Caruso, 2001), although the cellular pathway linking lindane-induced inhibition of myometrial gap junctions and contractility remains to be resolved.

Various studies have explored phospholipase-coupled pathways as mechanisms of regulation of gap junctions. Activation of the phosphatidylinositol (PI)-specific phospholipase C (PLC) pathway precedes uncoupling of gap junctions in striatal astrocytes (Giaume *et al.*, 1991). Protein kinase C (PKC) activation occurs downstream in the PLC and phospholipase D (PLD) pathways, and is associated with inhibition of gap junctions in several cell types and tissues (Murray and Fitzgerald, 1979; Yotti *et al.*, 1979; Menkes *et al.*, 1986; Sakata and Karaki, 1990; Sasaguri and Watson, 1990; Morrison and Vanhoutte, 1991; Watson and Karmazyn, 1991; Bastide *et al.*, 1994; Kenne *et al.*, 1994; Leibold *et al.*, 1994; Mikalsen and Sanner, 1994; Rivedal *et al.*, 1994; Kwak *et al.*, 1995; Kwak and Jongsma, 1996), including myometrium (Nnamani *et al.*, 1994; Criswell *et al.*, 1995). Arachidonic acid, which is generated as a consequence of phospholipase A₂ (PLA₂) activation, inhibits gap junctions, also (Cole *et al.*, 1985; Aylsworth *et al.*, 1986; Giaume *et al.*, 1989; de Haan *et al.*, 1994; Miyachi *et al.*, 1994; Criswell *et al.*, 1995; Hii *et al.*, 1995; Hayashi *et al.*, 1997; Lavado *et al.*, 1997). Lindane activates phospholipase signaling pathways in myometrium, as evidenced by lindane-stimulated generation of inositol phosphates (Criswell *et al.*, 1994), activation of PKC (Criswell *et al.*, 1995), and release of arachidonic acid (Criswell *et al.*, 1995; Wang *et al.*, 2001).

The current study investigates the hypothesis that lindane-induced activation of a specific phospholipase pathway inhibits spontaneous oscillatory uterine contractions through inhibition of myometrial gap junctions. Lucifer yellow dye transfer between myometrial cells in culture was used to monitor changes in lindane-induced inhibition of gap junction communication and uterine strips in muscle baths were used to monitor changes in lindane-induced inhibition of uterine contractions in response to treatment with inhibitors of phospholipase pathways.

MATERIALS AND METHODS

Chemicals

Lindane (γ -hexachlorocyclohexane, 99% purity) was obtained from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO), deoxyribonuclease I, type II collagenase, bovine

trypsin, propidium iodide, and NCDC (2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate) were also purchased from Sigma. The phospholipase inhibitors tricyclodecan-9-yl xanthogenate · K (D609), bromoenol lactone (BEL), and 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphorylcholine (ET-18-OCH₃) were obtained from Biomol (Plymouth Meeting, PA). Lucifer yellow was obtained from Molecular Probes (Eugene, OR). Myometrial cell culture medium (RPMI 1640) was from GibcoBRL (Gaithersburg, MD). Bovine calf serum (BCS) was purchased from HyClone (Logan, Utah).

Animals

Time-pregnant Sprague-Dawley rats were obtained from the breeding colony of the University of Michigan's Reproductive Sciences Program (Ann Arbor, MI) or from Harlan Laboratories (Indianapolis, IN). The rats were between 60 and 90 days of age and weighed between 180 and 220 g. The animals were housed at ambient temperature ($24 \pm 1^\circ\text{C}$) under a 12-h light schedule. The day of detection of spermatozoa in the vaginal smear was designated as Day 0 of pregnancy. Animals used in this study were at Gestation Day 10. This gestation day was selected for study because prior experiments, upon which this study was based, also used Gestation Day 10 rats (Criswell *et al.*, 1994, 1995; Criswell and Loch-Caruso, 1995; Criswell and Loch-Caruso, 1999; Wang *et al.*, 2001). The rats were cared for and handled in accordance with the *Guide for Care and Use of Laboratory Animals* (published by the National Academy of Science, 1996), the *Guiding Principles in the Use of Animals in Toxicology* (adopted by the Society of Toxicology in 1989), and protocols approved by the University Committee on Use and Care of Animals of the University of Michigan.

Measurement of uterine contractility

Gestation Day 10 rats were anesthetized with ether followed by exsanguination, a protocol required by collaborators with whom we shared tissue. The uteri were excised and trimmed free of embryos and fat. Spontaneous oscillatory uterine contractions were measured in standard isometric muscle baths by suspending longitudinal uterine strips (1 mm wide and 20 mm long) cut from the anti-mesometrial side of the midportion of the uterine horns. The baths contained physiologic salt solution composed of 116 mM NaCl, 4.6 mM KCl, 1.16 mM NaH₂PO₄ · H₂O, 1.16 mM MgSO₄ · 7H₂O, 21.9 mM NaHCO₃, 1.8 mM CaCl₂ · 2H₂O, 11.6 mM dextrose, and 0.03 mM CaNa₂EDTA at pH 7.4. The water-jacked baths were maintained at 37°C and aerated with a mixture of 95% O₂ and 5% CO₂. The uterine strips were tied to stationary posts at one end and to an isometric force transducer at the other end. Isometric contractions of uterine strips were recorded under constant passive force of 1.0 g. After a 40-min equilibration period, strips were challenged with 60 mM KCl to determine viability. Strips were then rinsed free of KCl and allowed to equilibrate for 3 to 7 h until regular spontaneous oscillatory contractions were established. Contractions were scored in 15-min intervals prior to and after the addition of test compounds. Force and frequency of contraction were defined as average maximum amplitude of contraction and average number of contractions per min, respectively, in each 15-min interval. Basal frequency and force were derived from the 15-min period before chemical exposure, and the data are presented as the percentage of basal force and frequency, respectively. Only amplitudes of contraction equal to or greater than 25% of the basal contraction were scored as contractions. Uterine responses to test compounds were determined after equilibration and establishment of regular, spontaneous oscillatory contractions.

Experimental conditions for evaluating lindane-induced inhibition of uterine contractions

Lindane was prepared as a stock solution in DMSO. To determine time- and concentration-dependent responses to lindane, uterine strips suspended in muscle baths were exposed to 10, 20, 30, 40, or 50 μM lindane and contractions were monitored over 15-min intervals of a 60-

min observation period. The concentration range of 10–50 μM lindane was selected because previous studies showed that this was an effective concentration range for inhibition of spontaneous uterine contractions, inhibition of myometrial gap junctions, and activation of myometrial phospholipase pathways (Criswell *et al.*, 1994, 1995; Criswell and Loch-Caruso, 1995, 1999). Limited clouding of the muscle bath solution was noted immediately after the addition of 40 or 50 μM lindane, but the solution quickly cleared within 2–3 min. Because the solubility limit of lindane is in the range of 40–70 μM at 35°C (Yalkowsky and He, 2002), lindane, within the concentration range used in this experiment, likely went into and remained in solution in the aqueous buffer at the 37°C temperature of the muscle bath. Solvent control strips were exposed to DMSO alone.

Experimental conditions for evaluating effects of phospholipase pathway inhibitors on uterine contractions

To investigate the role of phospholipase pathways, uterine strips were suspended in muscle baths and allowed to establish regular spontaneous oscillatory contractions before pretreatment with phospholipase pathway inhibitors. Concentrations of inhibitors used were observed in pilot toxicity studies to have no appreciable effects on uterine contraction when applied by themselves. After a 1-h pretreatment with phospholipase pathway inhibitors, the uterine strips were challenged with 30 μM lindane or with DMSO alone to serve as solvent controls. The lindane concentration of 30 μM was selected for this experiment because this concentration elicited maximal inhibition of uterine contraction in the previous experiment without changing the clarity of the buffer upon its addition. To investigate the role of phospholipase C, uterine strips in muscle baths were pretreated with the phosphatidylinositol-specific PLC inhibitor ET-18-OCH₃ at concentrations of 10, 20, 30, or 50 μM (prepared in DMSO) for 1 h. This inhibitor has an IC₅₀ of 1–10 μM and high specificity for PI-PLC compared with phosphatidylcholine-specific PLC and PLD (Powis *et al.*, 1992). The pretreatment with ET-18-OCH₃ was followed by a 60-min exposure to 30 μM lindane in the continued presence of ET-18-OCH₃. Subsequent experiments investigated the effects of inhibition of phospholipase A₂ and phospholipase D by pretreating uterine strips with 20 μM BEL (prepared in DMSO) or 100 mM ethanol (diluted in distilled deionized water), respectively, and then exposing the strips to 30 μM lindane or DMSO (solvent controls) in the continued presence of the inhibitors for 15 min. The PLA₂ inhibitor BEL has about a 1000-fold higher selectivity for calcium-independent PLA₂ compared with calcium-dependent PLA₂ (Hazen *et al.*, 1991). The concentration of BEL was selected based on previous experiments that showed that 1–10 μM BEL was an effective concentration range for inhibiting the calcium-independent PLA₂ activity in rat neutrophils (Tithof *et al.*, 1998; Wang *et al.*, 2001). Ethanol in high millimolar range (0.5–2%) effectively interrupts the PLD pathway by providing the primary alcohol for a transphosphatidyl reaction, thereby preventing further hydrolysis of phosphatidic acid to diacylglycerol (Rydzewska *et al.*, 1996; Vasta *et al.*, 1998). Uterine strips exposed to 30 μM ET-18-OCH₃ (prepared in DMSO) were included in this experiment, also.

Myometrial cell isolation and culture

Myometrial smooth muscle cells were isolated from midgestation (Day 10) Sprague-Dawley rats by methods previously described (Caruso *et al.*, 1990) with a modified digestion enzyme solution containing 100 $\mu\text{g}/\text{ml}$ deoxyribonuclease I, 150 $\mu\text{g}/\text{ml}$ type II collagenase, and 150 $\mu\text{g}/\text{ml}$ crude trypsin. Cells were seeded into flasks containing RPMI 1640 medium supplemented with 10% bovine calf serum (BCS) and maintained at 37°C in a 5% CO₂/95% air atmosphere. Growth medium was changed every other day, and cells were subcultured prior to confluence. All cells were used between passages three and five. To verify the smooth muscle character of the cultured cells, indirect immunofluorescence labeling with mouse anti- α -smooth muscle actin monoclonal antibody was performed as previously described (Caruso *et al.*, 1990), and 99–100% of cultured cells were labeled by the antibody.

Measurement of gap junction communication by Lucifer yellow dye transfer following microinjection

Myometrial cells were plated in 35-mm cell culture dishes at a density of 3×10^4 cells per dish in 2 ml of RPMI 1640 culture medium supplemented with 10% BCS and then incubated for 48 h at 37°C in a 5% CO₂/95% air atmosphere. Culture dishes were placed on a heated (37°C) stage of an inverted epifluorescence microscope (Nikon Diaphot, Garden City, NJ). Cell foci of three to nine cells were selected for dye transfer evaluation. A single cell of each selected cell cluster was injected with a mixed dye solution of 0.8% Lucifer yellow dye and 0.02% propidium iodide using an injection pressure of 6.5 psi for 200 ms. The dye solution was prepared in glucose-containing phosphate-buffered saline solution (0.9 mM CaCl₂, 2.68 mM KCl, 1.47 mM K₃PO₄, 0.5 mM MgCl₂, 8 mM Na₃PO₄, and 5 mM glucose, pH 7.4). Injections in any single dish were conducted over a 5-min period, and 10 to 20 cells were injected per dish. A minimum of 100 cells in 10 dishes was injected for each treatment group. After injection, cells were examined by epifluorescence microscopy for evidence of Lucifer yellow dye transfer. The G2A filter (510- to 560-nm excitation band and 590-nm barrier filter) was used to identify injected cells, indicated by propidium iodide red fluorescence in the nuclei. Using the B2A filter (450- to 490-nm excitation band and 520-nm barrier), the yellow fluorescence due to Lucifer yellow was visualized in the injected cell and, if dye transfer occurred, in adjacent cells. Only the primary neighboring cells directly in contact with the injected cell were scored. Functional gap junction intercellular communication was expressed as percentage of dye transfer, calculated as the number of cells in direct contact with the injected cell that exhibited Lucifer yellow fluorescence divided by the total number of primary neighboring cells in direct contact with the injected cell. Cells were scored for evidence of dye transfer during a 5-min evaluation period.

Experimental conditions for evaluating effects of phospholipase pathway inhibitors on myometrial gap junctions

Lucifer yellow dye transfer was monitored in myometrial cells in culture after treatment with phospholipase pathway inhibitors and lindane. The concentrations of the calcium-independent PLC inhibitor BEL (20 μ M), phosphatidylinositol-specific PLC inhibitor ET-18-OCH₃ (30 μ M), and the PLD pathway interrupter ethanol (100 mM) were selected to match those concentrations used in the contractility experiments. The inhibitor D609 was included in this experiment because it exhibits high specificity for phosphatidylcholine-specific PLC compared with phosphatidylinositol-specific PLC, PLA₂, and PLD (Muller-Decker, 1989; Schutze *et al.*, 1992). The concentration of D609 used in this experiment (50 μ M) is in the effective range for inhibition of phosphatidylcholine-specific PLC. Cells were also pretreated with NCDC, a PLC inhibitor that favors inhibition of PI-PLC (Walenga *et al.*, 1980) but with less specificity for this PLC isoform than ET-18-OCH₃. A concentration of 50 μ M NCDC was used in this experiment because a previous study showed that 20 μ M NCDC significantly attenuated PLC-dependent carbachol-induced contractions in Gestation Day 10 uteri *in vitro* (Bae *et al.*, 1999a). The inhibitors BEL, D609, ET-18-OCH₃, and NCDC were prepared in DMSO and ethanol was diluted in distilled deionized water.

After a 1-h pretreatment with phospholipase pathway inhibitors, the cells were exposed for 30 min to 100 μ M lindane or to DMSO in the continued presence of the inhibitors. The lindane exposure condition was selected based on prior studies showing that exposures to 100 μ M lindane for durations between 4 and 60 min abolish Lucifer yellow dye transfer in myometrial cell cultures (Criswell and Loch-Carusio, 1995; Criswell *et al.*, 1995). Two DMSO pretreatment solvent controls were included, one at the normal medium pH of 7.4 and one at the reduced pH of 7.0. The reduced pH solvent control was included for comparison with the D609-treated cultures because a medium pH of 7.0 was used to allow optimal activity of D609. No crystals were observed under phase microscopy in the medium after addition of lindane, likely related

to the solubility of lindane at 35°C (40–70 μM) (Yalkowsky and He, 2002), the medium temperature of 37°C, and the presence of 10% FBS in the medium. The phospholipase pathway inhibitor concentrations were observed in pilot toxicity studies to have no observable effects on myometrial cell morphology or ability to transfer Lucifer yellow dye. All treatments were conducted in RPMI 1640 culture medium supplemented with 10% BCS at 37°C in a 5% CO_2 /95% air atmosphere. Cells remained in the exposure media during dye injection and microscopic evaluation.

Statistical analysis

Data were summarized as means $\pm$ SEM and analyzed using SigmaStat software (SPSS Science, Chicago, IL). Analyses of the effects of various phospholipase inhibitors on dye transfer and uterine contraction during a single 15-min exposure (Table 1) were performed by one-way analysis of variance (ANOVA). Treatment-related effects on the time to cessation of contraction were analyzed by one-way ANOVA, also. The concentration-dependent effects of lindane and ET-18-OCH₃ were analyzed by two-way repeated measures ANOVA on ranks, with time as the repeated measure. Values expressed as percentages were rank-transformed prior to analysis because of the inherent nonparametric nature of percentage data. All pairwise *post hoc* multiple comparisons were performed by the Student-Newman-Keuls method. A *P* value of 0.05 was considered statistically significant.

RESULTS

Concentration-dependent effects of lindane on spontaneous oscillatory uterine contraction

To investigate the concentration-dependent effects of lindane on uterine contractions, the time required for complete inhibition of spontaneous oscillatory contractions was determined in longitudinal uterine strips suspended in muscle baths and exposed to lindane *in vitro*. The time required for complete inhibition of oscillatory uterine contractions decreased with increasing concentrations of lindane (Fig. 1). All control uterine strips exposed to the solvent DMSO (0 μM lindane) maintained contractions over the 60-min observation period and were assigned scores of 60 min. At 10 μM lindane, 66.7% of the strips contracted over the entire 60-min observation period, and these strips were assigned scores of 60 min. The 20, 30, 40, and 50 μM lindane exposures rapidly suppressed contractions within an average time of 15 min (significantly different from strips exposed to 0 μM or 10 μM lindane, $P < 0.05$; Fig. 1). Contractions ceased within the 60-min observation period in all uterine strips exposed to 30, 40, or 50 μM lindane.

Lindane's effects on contraction force (average maximum amplitude) and frequency (number of oscillations per min) were quantified over the recorded 60-min observation period for those uterine strips exposed to 0, 10, 20, or 30 μM lindane. Contraction force and frequency values are not shown for the higher lindane concentrations because contractions were abolished at concentrations at or exceeding 30 μM lindane. Control strips exposed to solvent (DMSO) did not exhibit significant changes in the force or frequency of contraction over the 60-min observation period. The lowest concentration tested, 10 μM lindane, produced a modest but significant reduction of peak contraction force after 15 min of exposure that intensified further after 45 min of exposure ($P < 0.05$; Fig. 2A). Decreases in contraction frequency did not occur until after 45 min of exposure to 10 μM lindane ($P < 0.05$; Fig. 2B), suggesting that the primary effect of lindane was on force generation rather than pacemaker activity. Exposure to 20 or 30 μM lindane rapidly and markedly reduced peak contraction force (Fig. 2A) and frequency (Fig. 2B) compared with solvent controls and in a time-dependent manner ($P < 0.05$), with contractions abolished in all strips after 30 min of exposure to 30 μM lindane.

Concentration-dependent effects of ET-18-OCH₃ on lindane-induced inhibition of uterine contraction

The concentration-dependent effects of the PI-PLC inhibitor ET-18-OCH₃ on lindane-induced inhibition of spontaneous oscillatory contractions were examined in longitudinal uterine strips exposed in muscle baths. Pretreatment with ET-18-OCH₃ (for 1 h) increased, in a concentration-dependent manner, the time required for cessation of spontaneous uterine contractions after initiation of exposure to 30 μ M lindane (Fig. 3). Strips exposed to solvent only (0 μ M) during the 1-h pretreatment period contracted an average of 11.8 min after initiation of exposure to lindane. Pretreatment with 20, 30, or 50 μ M ET-18-OCH₃, but not 10 μ M ET-18-OCH₃, significantly prolonged the mean time to cessation of contraction induced by lindane, compared with lindane-exposed strips pretreated with 0 μ M ET-18-OCH₃ (solvent controls) or 10 μ M ET-18-OCH₃ (Fig. 3; $P < 0.05$).

The reversal of lindane-induced inhibition of uterine contraction force and frequency is shown in Figs. 4A and 4B, respectively. After 15 min of exposure to 30 μ M lindane, uterine strips pretreated with 30 μ M or 50 μ M ET-18-OCH₃ contracted with greater force (Fig. 4A) and higher frequency (Fig. 4B) compared with time-matched solvent controls exposed to lindane without ET-18-OCH₃ pretreatment ($P \leq 0.05$). Pretreatment with 10 μ M ET-18-OCH₃ did not significantly alter the lindane-induced decreases in contraction force and frequency (Fig. 4A; $P < 0.05$).

Effects of inhibitors of different phospholipase pathways on lindane-induced inhibition of uterine contraction

Longitudinal uterine strips suspended in muscle baths were used to assess effects of inhibitors of various phospholipase pathways on lindane-induced inhibition of spontaneous oscillatory contractions *in vitro*. Exposure to lindane (30 μ M for 15 min) significantly decreased the average contraction frequency and force to 62.9 and 82.8% of basal values, respectively, compared with solvent controls (Table 1; $P < 0.05$). Pretreatment with the PI-PLC inhibitor ET-18-OCH₃ (30 μ M) prevented lindane-induced inhibition of frequency but not force of contraction under these experimental conditions. In contrast to ET-18-OCH₃, pretreatment with BEL (20 μ M) or ethanol (100 mM) did not reverse lindane-induced inhibition of contraction frequency or force (Table 1). However, ethanol pre-treatment further reduced the frequency of contraction during lindane exposure (Table 1; $P < 0.05$). Treatment with ET-18-OCH₃, BEL or ethanol alone produced no significant effects on contraction frequency or force (Table 1), indicating that the phospholipase pathway inhibitor treatments themselves did not significantly alter uterine contractions. These results show that neither the iPLA₂ inhibitor BEL nor the PLD pathway interrupter ethanol reversed lindane-induced inhibition of contraction frequency under experimental conditions in which the PI-PLC inhibitor ET-18-OCH₃ was effective.

Effects of phospholipase pathway inhibitors on lindane-induced inhibition of gap junctions

Intercellular transfer of Lucifer yellow dye between myometrial cells in culture was used to assess the effects of phospholipase pathway inhibitors on lindane-induced inhibition of gap junctions. As reported previously (Criswell *et al.*, 1995), lindane (100 μ M for 30 min) nearly abolished Lucifer yellow dye transfer, whereas treatment with DMSO (solvent control at pH 7.4) allowed transfer of dye to an average of 97.7% of adjacent cells (Fig. 5). Because the PC-PLC inhibitor D609 was included in this experiment and required a medium pH of 7.0 to allow optimal activity of D609, a control group at pH 7.0 was included for comparison with the D609-treated cultures. Dye transfer in this control group at pH 7.0 was not significantly different from that of the pH 7.4 controls (Fig. 5). Pretreatment (for 1 h) with the PC-PLC inhibitor D609 (50 μ M), the PI-PLC inhibitor ET-18-OCH₃ (30 μ M), or the nonspecific PLC inhibitor NCDC (50 μ M) significantly reversed lindane-induced inhibition of dye transfer from an

average of 1.1% in cultures treated with lindane alone to 43.3, 33.3, and 15.8%, respectively ($P < 0.05$; Fig. 5). However, neither the iPLA₂ inhibitor BEL (20 μ M) nor the PLD pathway interrupter ethanol (100 mM) prevented inhibition of dye transfer by lindane (Fig. 5). In addition, the inhibitors themselves had no significant effects on dye transfer (Fig. 5). Thus, inhibitors of the PLC pathway, but not the iPLA₂ or PLD pathway, reversed lindane-induced inhibition of Lucifer yellow dye transfer.

DISCUSSION

Gap junctions provide low resistance channels for direct cell-to-cell propagation of electrical and metabolic signals (Bennett *et al.*, 1991; Kumar and Gilula, 1996). In the uterus, as in cardiac muscle and vascular smooth muscle, functional gap junctions promote the development of coordinated oscillatory contractions (Garfield and Hayashi, 1981; Christ *et al.*, 1993; Oyamada *et al.*, 1994; Watts *et al.*, 1994; Tsai *et al.*, 1995; Delorme *et al.*, 1997). Lindane inhibits gap junction communication between myometrial cells in culture and inhibits spontaneous oscillatory contractions of uterine strips in muscle baths, as shown previously (Criswell and Loch-Caruso, 1995, 1999; Criswell *et al.*, 1995) and confirmed in the present report. Based on the model of uterine contraction developed by Garfield and his colleagues (Garfield *et al.*, 1978), it is suggested that lindane-induced inhibition of myometrial gap junction communication critically limits the intercellular transfer of signals necessary for coordination of oscillatory uterine contractions, and that, in the absence of transfer of these contraction-regulating signals, oscillatory contractions cease.

By demonstrating a concordance between effects of phospholipase pathway inhibitors on lindane-induced inhibition of myometrial gap junction communication and uterine contraction, the present study provides support for activation of phospholipase C as a mechanistic link between lindane's inhibitory actions on myometrial gap junctions and uterine contraction. The PI-PLC inhibitor ET-18-OCH₃, but not the iPLA₂ inhibitor BEL or the PLD pathway interrupter ethanol, reversed lindane-induced inhibition of uterine contraction and inhibition of myometrial gap junction intercellular communication, consistent with a role for PLC in lindane's inhibitory actions on myometrial gap junction communication and contractions. The finding that lindane-induced inhibition of myometrial gap junction communication was reversed by ET-18-OCH₃ is consistent with a previous report that the PI-PLC pathway mediates the uncoupling of gap junctions in striatal astrocytes (Giaume *et al.*, 1991).

In addition to ET-18-OCH₃, other PLC inhibitors reversed lindane-induced inhibition of gap junctions as measured by Lucifer yellow dye transfer. Specifically, the PC-PLC inhibitor D609 and the less specific PLC inhibitor NCDC partially reversed lindane-induced inhibition of gap junction communication, providing further support for PLC activation as a component of the mechanism by which lindane inhibits myometrial gap junctions. Moreover, because both ET-18-OCH₃ and D609 partially reversed lindane-induced inhibition of gap junction communication, both PI-PLC and PC-PLC may contribute to lindane-induced inhibition of myometrial gap junction communication. Efforts to investigate the effect of the PC-PLC inhibitor D609 on uterine contractility were unsuccessful because the experimental conditions required for generation of spontaneous oscillatory contractions were incompatible with the pH requirement for D609 activity.

Lindane increases hydrolysis of inositol phospholipids (Criswell and Loch-Caruso, 1995) and generation of 1,4,5-trisphosphate (Criswell *et al.*, 1995) in myometrial cells, indicating that myometrial PI-PLC activity is stimulated by lindane. Because PLC activation generates the PKC activator diacylglycerol, PLC activation may be linked to inhibition of myometrial gap junctions and contractility via diacylglycerol and subsequent PKC activation, as reported in rat striatal astrocytes (Giaume *et al.*, 1991). Further support for the speculated role for PKC is

derived from reports showing that PKC activation is associated with inhibition of gap junction communication in several cell types and tissues (Murray and Fitzgerald, 1979; Yotti *et al.*, 1979; Menkes *et al.*, 1986; Sakata and Karaki, 1990; Sasaguri and Watson, 1990; Morrison and Vanhoutte, 1991; Watson and Karmazyn, 1991; Bastide *et al.*, 1994; Kenne *et al.*, 1994; Leibold *et al.*, 1994; Mikalsen and Sanner, 1994; Rivedal *et al.*, 1994; Kwak *et al.*, 1995; Kwak and Jongsma, 1996), including myometrium (Nnamani *et al.*, 1994; Criswell *et al.*, 1995).

The iPLA₂ inhibitor BEL did not reverse lindane-induced inhibition of myometrial gap junction communication or uterine contractions, failing to support a mechanistic role for a BEL-sensitive iPLA₂ activity in lindane's inhibitory actions in the uterus. This result is consistent with the recent finding that lindane increases arachidonic acid release from myometrial cells by stimulating a BEL-insensitive calcium-independent PLA₂ activity (Wang *et al.*, 2001). Moreover, Wang *et al.* (2001) found no evidence for lindane activation of PLC or a calcium-dependent PLA₂ as a mechanism for lindane-induced myometrial release of arachidonic acid. Nonetheless, lindane stimulation of arachidonic acid release may be involved in inhibition of myometrial gap junctions (Criswell and Loch-Caruso, 1995) and uterine contractions (Criswell and Loch-Caruso, 1999) by an unidentified pathway, perhaps through actions on PKC, as observed in neural tissue (Sekiguchi *et al.*, 1987; Shearman *et al.*, 1989) and macrophages (Huang *et al.*, 1997). The mechanistic correlation between myometrial PLC activation and arachidonic acid release by lindane is under further investigation.

Because the PLD pathway interrupter ethanol did not reverse lindane-induced inhibition of myometrial gap junctions or uterine contractions, the present study did not support a mechanistic role for PLD in lindane's inhibitory actions in the uterus. The decreased contraction frequency that was observed in lindane-treated strips pretreated with ethanol is consistent with ethanol's ability to inhibit uterine contraction (Fuchs *et al.*, 1982) and our unpublished observation that ethanol enhances lindane-induced inhibition of myometrial gap junctions.

The observation of lindane-induced inhibition of myometrial gap junctions is consistent with reports that lindane inhibits gap junctions in myometrial cells (Criswell *et al.*, 1995), fibroblasts (Tsushimoto *et al.*, 1983), hepatocytes (Klaunig *et al.*, 1990), and liver epithelial cells (Guan *et al.*, 1995). In addition, lindane-induced inhibition of contractions of Gestation Day 10 uterus confirms our earlier report (Criswell and Loch-Caruso, 1999) and is consistent with reports that *in vitro* exposure to lindane inhibits contractions in vas deferens smooth muscle (Gandhi and Venkatakrishna-Bhatt, 1989; Gautam *et al.*, 1989) and, in a preliminary report, intestinal smooth muscle (Wooley *et al.*, 2000).

In previous studies, concentration-response curves of lindane-induced inhibition of uterine contractions were confounded by time because uterine strips in muscle baths were exposed to lindane in a cumulative manner (Criswell and Loch-Caruso, 1999). By analyzing contractions over a 60-min period in uterine strips exposed to single concentrations of lindane, the present study showed that the duration of exposure influences the concentration-dependence of the response. Although beyond the scope of the present investigation, further experiments could examine the possible explanations that increased duration of exposure may allow greater concentrations of lindane to accumulate in the uterine tissue or may allow greater time for the development of the inhibitory response.

The present study used midgestation (Day 10) uterine strips exposed to lindane *in vitro*, consistent with previous experiments that showed lindane-induced inhibition of uterine contraction (Criswell and Loch-Caruso, 1999). Because the uterus is not completely quiescent during pregnancy (Nathanielsz *et al.*, 1998) and uterine muscle activity during pregnancy can influence fetal development (Shinozuka *et al.*, 1999, 2000), inhibition of uterine contractility

during midgestation may have unrecognized adverse health consequences. Additionally, interference with uterine contractility during midgestation may result in complications for spontaneous and induced abortions. Furthermore, inhibition of uterine contractions has been observed in our laboratory in near-term Gestation Day 20 rat uterine strips exposed to 10 or 100 μ M lindane, with a prolonged inhibition continuing in the 100 μ M -exposed strips for at least 5 h after removal of exposure buffer (submitted for publication). These more recent findings indicate that lindane inhibits uterine contractions in the near-term uterus, also, when inhibition of uterine contractions might prevent, delay, or impede parturition.

Limited studies suggest the possibility that lindane may affect late gestation uterine muscle function *in vivo*. In preliminary experiments we observed that uterine strips excised from near-term (Gestation Day 20) pregnant rats exhibited depressed spontaneous, carbachol-induced, and oxytocin-induced contractions 24 h after a single subcutaneous injection of 4 or 6 mg lindane/kg body weight (unpublished). In women admitted to a hospital in India for obstetrical care who subsequently experienced preterm birth or spontaneous abortion, blood levels of lindane averaged 39.7 ppb (15–79 ppb range, $n = 15$) and 73.3 ppb (40–136 ppb range, $n = 10$), respectively (Saxena *et al.*, 1980, 1981). In another study of 17 Israeli women, significantly elevated concentrations of lindane (15 ppb) were detected in serum lipids of women who delivered prematurely compared with women who delivered at term (4.3 ppb) (Wassermann *et al.*, 1982). A limitation of both human studies is that other organochlorine substances were also significantly elevated in the blood of women who experienced preterm birth or spontaneous abortion, including DDT and polychlorinated biphenyl isomers that stimulate uterine contraction in rat uterine strips *in vitro* (Juberg and Loch-Caruso, 1991; Juberg *et al.*, 1991, 1995; Tsai *et al.*, 1996; Bae *et al.*, 1999a,b, 2001). Additionally, it is not clear that the stereoisomers of hexachlorocyclohexane were adequately separated in the residue analyses, and γ -hexachlorocyclohexane (20–200 μ M) stimulates, whereas lindane (γ -hexachlorocyclohexane) inhibits, oscillatory uterine contractions in rat pregnant uterus *in vitro* (Criswell and Loch-Caruso, 1999). These and other possible confounding factors were not adequately controlled in the analyses. Consequently, definitive studies in humans are lacking regarding the possible role of lindane and other hexachlorocyclohexane isomers in complications of childbirth.

Concentrations of lindane in blood lipids of pregnant women do not directly provide an adequate estimation of uterine exposure, in part because pregnancy significantly alters the distribution of several organochlorine substances, including hexachlorocyclohexanes. Specifically, lindane concentrations in extracted lipids of uteri of pregnant women at term were 2.0 ppm, threefold higher (0.67 ppm) than in adipose tissue and fivefold higher (0.40 ppm) than in maternal blood, showing a significant shift in the distribution of lindane from blood and adipose lipids to uterine lipids during pregnancy (Polishuk *et al.*, 1977). Although limited, these findings indicate that the uterine tissue concentrations of lindane increase during pregnancy, and that concentrations of lindane in blood lipids likely underestimate lipid concentrations in uterus during pregnancy.

Rat uterine strips exposed in muscle baths to cumulative concentrations of lindane up to 10 μ M lindane over a 50-min period accumulated 0.33 ppm lindane relative to wet weight and exhibited significantly decreased force of contraction (Criswell and Loch-Caruso, 1999). Although 2 ppm hexachlorocyclohexanes, including lindane, were measured in extracted lipids of uteri of pregnant women (Polishuk *et al.*, 1977), because the human uterine tissue concentrations are expressed on a lipid weight basis whereas the rat uterine strip concentrations are expressed on a wet weight basis, direct comparisons of these uterine concentrations are not possible. Furthermore, the small size and low relative abundance of tissue lipid of the rat uterine strips present technical challenges for measurement of extracted lipid weight following exposure in muscle baths (Criswell and Loch-Caruso, 1999). Even if feasible, comparisons of

the tissue concentrations would remain problematic due to the necessity for a different procedure for measurement of extracted lipid weight in the small uterine strips and variability introduced by lipid weight measurement procedures (Grimvall *et al.*, 1997). Moreover, uterine concentrations of lindane have not been reported in pregnant uterus of women with known exposures or in women who experienced prolonged or delayed onset of childbirth that might be related to lindane exposure. Consequently, measurement of uterine lindane concentrations in the rat uterine strips exposed in muscle baths provides limited information for human risk assessment purposes due to the lack of sufficient data on lindane residues in pregnant human uterus. Clearly, further investigations are required to determine whether lindane poses a reproductive risk to pregnant women by interfering with the development of uterine contractions during parturition.

The present study is the first demonstration that activation of a specific phospholipase cell signaling pathway couples lindane-induced inhibition of uterine oscillatory contractions to inhibition of myometrial gap junction communication. Because few studies have investigated mechanisms whereby toxicants modify uterine muscle function, these results advance our understanding of the potential for toxicants to disrupt physiological processes that require uterine contraction. Moreover, the present study reaffirmed the relationship between functional myometrial gap junctions and generation of oscillatory smooth muscle contractions by demonstrating a concordance between lindane-induced inhibition of myometrial gap junction communication and oscillatory uterine contractions. Although the applications of the present study to human risk assessment may not be direct, the knowledge that phospholipase C activation may interfere with the generation of uterine contractions by inhibiting myometrial gap junctions may find relevance indirectly as toxicants are evaluated for potential risks to pregnancy. Furthermore, because gap junctions are important in a wide variety of physiological functions and inhibition of gap junctions is associated with various dysfunctional and pathological conditions (Donaldson *et al.*, 1997; Rosenkranz *et al.*, 2000), the results of the present study of lindane actions in uterine muscle may have relevance to other tissues and other toxicants.

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FIG. 1.

The concentration-dependent effects of lindane on the time required for complete inhibition of spontaneous oscillatory contractions of uterine strips exposed *in vitro* to lindane. The time from the addition of lindane to complete cessation of contraction of each strip was recorded up to 60 min. Strips that contracted more than 60 min were assigned a score of 60 min. Strips exposed to 0 μM lindane were exposed to the solvent only (DMSO). The data are presented as the means $\pm$ SEM of 12 uterine strips. Data points sharing the same letter are not statistically different from each other ($P < 0.05$). Error bars not visible are contained within the symbols.

FIG. 2.

The time- and concentration-dependent effects of lindane on (A) force and (B) frequency of contraction of spontaneously oscillating uterine strips exposed to lindane *in vitro*. Bars not visible are data means equal to zero. Error bars not visible are too small to be displayed graphically. Groups sharing the same letter are not statistically different from each other ($P < 0.05$).

FIG. 3.

The concentration-dependent effects of ET-18-OCH₃ pretreatment on lindane-induced inhibition of spontaneous oscillatory uterine contractions. The time to complete cessation of spontaneous uterine oscillatory contraction was determined in uterine strips pretreated with ET-18-OCH₃ for 1 h prior to addition of 30 μM lindane (final concentration) to the ET-18-OCH₃-containing medium. The 0 μM concentration represents solvent (DMSO) controls. The data are presented as the means ± SEM of 12 uterine strips. Data points sharing the same letter are not statistically different from each other ($P < 0.05$).

FIG. 4.

The time- and concentration-dependent effects of ET-18-OCH₃ pretreatment on (A) force and (B) frequency of contraction of uterine strips exposed to lindane. Uterine muscle strips were pretreated with solvent (DMSO) alone (control) or ET-18-OCH₃ for 1 h prior to exposure to 30 μ M lindane. Pretreatment with ET-18-OCH₃ alone (10, 30, or 50 μ M) did not affect the force and frequency of contraction (data not shown). The data are expressed as the means $\pm$ SEM of 12 uterine strips. Bars not visible are data means equal to zero. Groups within each graph that share the same letter are not statistically different from each other ($P < 0.05$).

FIG. 5.

Effects of phospholipase pathway modulators on lindane-induced inhibition of myometrial gap junction communication as measured by Lucifer yellow dye transfer. Myometrial cells were exposed to 100 μ M lindane for 30 min with or without 1-h pretreatment with the phospholipase inhibitors BEL (20 μ M), D609 (50 μ M), ET-18-OCH₃ (30 μ M), NCDC (50 μ M), or ethanol (100 mM) (filled bars), or to inhibitors or DMSO (solvent controls) alone (open bars). The data are expressed as the means $\pm$ SEM for 10 dishes (100–120 injected cells). Different letters denote means statistically different from each other ($P < 0.05$).

TABLE 1

Effects of Phospholipase Pathway Modulators on Contractility of Uterine Muscle Strips^a

Treatment	Contraction frequency (% basal frequency) ^b	Contraction force (% basal force) ^b
DMSO (solvent control)	99.8 ± 2.5 ^c	108.2 ± 5.8 ^c
ET-18-OCH ₃	102.3 ± 6.7 ^c	96.2 ± 2.0 ^{c,d}
BEL	93.8 ± 4.1 ^c	98.5 ± 3.5 ^{c,d}
Ethanol	98.4 ± 7.6 ^c	97.6 ± 1.8 ^{c,d}
Lindane	62.9 ± 6.9 ^d	82.8 ± 3.1 ^e
ET-18-OCH ₃ + lindane	104.3 ± 6.7 ^c	79.4 ± 1.9 ^e
BEL + lindane	39.2 ± 14.2 ^{d,e}	86.1 ± 6.0 ^{d,e}
Ethanol + lindane	23.3 ± 3.3 ^e	78.1 ± 0.7 ^e

^a Uterine strips were exposed to 30 μ M lindane with or without 1-h pretreatments with BEL (20 μ M), ET-18-OCH₃ (30 μ M), or ethanol (100 mM), or were exposed to inhibitors or DMSO (solvent control) alone.

^b Data are expressed as means ± SEM of four uterine strips. Statistical analysis was performed by one-way ANOVA and group means were compared *post hoc* using the Student-Newman-Keuls test.

^c Different letters denote means statistically different from each other ($P < 0.05$) within each column.

^d Different letters denote means statistically different from each other ($P < 0.05$) within each column.

^e Different letters denote means statistically different from each other ($P < 0.05$) within each column.