Stimulation of Pregnant Rat Uterine Contraction by the Polychlorinated Biphenyl (PCB) Mixture Aroclor 1242 May Be Mediated by Arachidonic Acid Release through Activation of Phospholipase A2 Enzymes

JEEHYEON BAE, MARC PETERS-GOLDEN, and RITA LOCH-CARUSO
Toxicology Program, Department of Environmental and Industrial Health (J.B., R.L-C.), and Division of Pulmonary and Critical Care Medicine, Department of Medicine, (M.P.-G.), The University of Michigan, Ann Arbor, Michigan

Accepted for publication December 30, 1998 This paper is available online at http://www.jpet.org

ABSTRACT
The polychlorinated biphenyl (PCB) mixture Aroclor 1242 (A1242) increases frequency of contractions of pregnant rat uteri, suggesting a possible mechanism for decreased gestational age and increased spontaneous abortion in women and animals exposed to PCBs. In the present study, we hypothesized that A1242-induced stimulation of uterine contraction is mediated by arachidonic acid released by phospholipase A2 (PLA2) enzymes. Isometric uterine contraction was measured in longitudinal uterine strips isolated from gestation day 10 rat. Pretreatment of uterine strips with the PLA2 inhibitor (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS) or manoalide, or an inhibitor of the G protein of (bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS) or manoalide, or an inhibitor of the G protein of PLA2, isotetrandrine, completely prevented the increase of contractile frequency induced by 50 μM A1242. However, the phospholipase C inhibitors 2-nitro-4-carboxyphenyl-N,N,N-triphenylcarbamate (NDCD) and neomycin were unable to block stimulation of uterine contraction by A1242. In accordance, A1242 (100 μM) did not release inositol phosphates from myo-[3H]inositol-labeled myometrial cells, whereas myometrial cells pretreated with [3H]arachidonic acid released arachidonic acid in a concentration- and time-dependent manner after exposure to A1242 (10–100 μM). A1242 significantly stimulated arachidonic acid release in the absence of extracellular calcium, although the release was attenuated. Analysis of the eicosanoids released by A1242 indicated that only 0.83% of released [3H]arachidonic acid was metabolized to eicosanoids and 99.07% remained as free arachidonate. Uterine contraction increased in strips exposed to exogenous arachidonic acid (1–100 μM). This study suggests that A1242 stimulates contraction in pregnant rat uterus by a mechanism involving PLA2-mediated arachidonic acid release, and that arachidonic acid, rather than eicosanoids, may mediate A1242 uterotonic action in the uterus.

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants previously used widely in industry (Safe, 1992). Exposure to PCBs is associated with spontaneous abortion and decreased gestational length in women (Leoni et al., 1989; Taylor et al., 1989) and nonhuman primates (Arnold et al., 1990). Timely and effective uterine contraction is a critical component of parturition. Increased frequency of uterine contractions before term is associated with preterm birth in women (Bell, 1983).

In previous studies, we showed that noncoplanar ortho-substituted PCB congeners (Tsai et al., 1996) and PCB mixtures (Bae and Loch-Caruso, 1996) stimulate spontaneous oscillatory contractions of pregnant rat uterine muscle, suggesting a possible mechanism for disturbing the maintenance of pregnancy to term. Uterine muscle activity may be stimulated by many factors, including prostanoids (PGs) metabolized from liberated arachidonic acid (Huszar and Roberts, 1982) and calcium released from intracellular stores by inositol 1,4,5-trisphosphate (Carsten and Miller, 1985). Released arachidonic acid can be metabolized by cyclooxygenases, lipooxygenases, or cytochrome P-450 enzymes (for review, see Piomelli, 1996). Most studies of uterine contraction

ABBREVIATIONS: A1242, Aroclor 1242; BCS, bovine calf serum; carbachol, carbamylcholine chloride; CMF-PBS, calcium/magnesium-free PBS; DMSO, dimethyl sulfoxide; HELSS, (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; HETE, hydroxyeicosatetraenoic acid; 12-HHT, 12-hydroxyheptadecatrienoic acid; PG, prostaglandin; 6-k-PGF1α, 6-keto-PGF1α; LT, leukotriene; NCDC, 2-nitro-4-carboxyphenyl-N,N,N-triphenylcarbamate; PCB, polychlorinated biphenyl; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; TXB2, thromboxane B2.
have focused on eicosanoids rather than arachidonic acid per se. However, in many cell types arachidonic acid itself serves as a second messenger (Promelli, 1996). Arachidonic acid can be released by phospholipase A2 (PLA₂) directly, by sequential action of phospholipase C (PLC) and diacylglycerol lipase, or by phospholipase D (PLD) and subsequent actions of phosphatidic acid phosphohydrolase and diacylglycerol lipase (see review, Dennis, 1983). In uterine tissues, both PLA₂ and PLC are involved in releasing arachidonic acid (Flint et al., 1986; Schrey et al., 1987). The activity of PLD has been detected in myometrium (Dokhac et al., 1995), but its ability and significance in releasing arachidonic acid in uterus is unknown. Others have shown that PCBs increase arachidonic acid release in neutrophils (Tithof et al., 1996) and stimulate inositol phosphate production from neutrophils and cerebellar granule cells (Tithof et al., 1995; Shafer et al., 1996). Therefore, in this study we investigated whether A1242-induced stimulation of uterine contraction is through the PLA₂- or PLC-mediated arachidonic acid release. Furthermore, we explored whether arachidonic acid or its metabolites are responsible for the uterotropic response elicited by A1242.

Materials and Methods

Chemicals. Aroclor 1242 (A1242) was purchased from Ultra Scientific (North Kingstown, RI). Manoalide, isotetrandrine, arachidonic acid, A23187, and (E)-6-(bromomethylene)tetrahydro-3(1-naphthalenyl)-2H-pyran-2-one (HELSS) were obtained from Biomol (Plymouth Meeting, PA). BSA, 2-nitro-4-carboxyphenyl-V,N-diphenylurea, neomycin, carbamylcholine chloride (carbachol), EDTA, EGTA, deoxyribonucleases I, and II type collagenase were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]Arachidonic acid (210 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Mγo-[2-3H]inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). HPLC standards of 6-keto-PGF₁α, PGF₂α, thromboxane B₂, PGE₂, PGD₂, leukotriene (LT) B₄, LTB₄, LTD₄, 12-hydroxyheptadecatrienoic acid (12-HHT), 15-hydroxyeicosatetraenoic acid (15-HETE), 12-HETE, 5-HETE, and free arachidonic acid were purchased from Cayman Chemical (Ann Arbor, MI). C₁₈ Sep-Pak cartridges were purchased from Waters Associates (Milford, MA). Dowex AG1-X8 (100–200 mesh, formate form) was obtained from BioRad Labs (Rockville Center, NY). RPMI medium and crude trypsin were obtained from Gibco BRL (Grand Island, NY) and defined, iron-supplemented bovine calf serum (BCS) was from HyClone (Logan, UT). Arachidonic acid stock was divided into aliquots and stored at −20°C, and arachidonic acid solution was made fresh for each experiment while aerated with N₂. All chemicals were dissolved in water (di H₂O), except carbachol and neomycin, which were dissolved in dimethyl sulfoxide (DMSO) except carbachol and neomycin, which were dissolved in deionized water (di H₂O). Final exposure concentrations of DMSO did not exceed 0.1%.

Preparation of Uterine Strips. Female Sprague-Dawley rats aged 60 to 90 days and weighing 180 to 220 g were mated with males. Pregnant (gestation day 10) rats were obtained from Harlan (Indianapolis, IN) or the colony of the Reproductive Science Program at the University of Michigan. The animals were housed at 24 ± 1°C under a 12-h light/12-h dark schedule. Pregnant rats were anesthetized with ether followed by exsanguination, a protocol required by collaborators with whom we shared tissue. After isolating uteri, embryos were removed. Longitudinal smooth muscle strips 1 mm wide by 20 mm long were cut from the mid-portions of horns that contained four implantation sites.

Measurement of Spontaneous Oscillatory Contractions. The uterine strips were suspended in standard muscle baths that contained physiological 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-jacketed bath was maintained at 36°C and aerated with a mixture of 95% O₂ and 5% CO₂. The uterine strip was tied with surgical silk to a stationary post at one end and to an isometric force transducer at the other end. Isometric contractions of strips were monitored under constant passive force of 1.0 g. After a 40-min equilibration period, strips were challenged with 60 mM KCl to determine viability and maximum KCl-induced contraction force. After rinsing out the KCl, strips were allowed to equilibrate for 2 to 5 h to establish regular spontaneous oscillatory contractions. Contractions were measured by frequency (number of contraction/relaxation cycles in a 10-min period), because this was the most prominent parameter affected by A1242. After equilibration, the 10-min interval before any treatment was termed the basal frequency.

Measurement of Chemical-Induced Oscillatory Contractions. Because the frequency of contraction was monitored during an 80-min exposure (Bae and Loch-Caruso, 1996), uterine strips were exposed to A1242 for 60 min. The last 10-min interval was taken for analysis. Because development of maximal effect is more rapid for carbachol, for these experiments muscle strips were treated for 30 min and the last 10-min segment was taken for analysis. Also, uterine strips were exposed to DMSO (solvent controls) or HELSS, manoalide, isotetrandrine, NCDC, or neomycin for either 50 min for the carbachol experiments or 80 min for the A1242 experiments. For muscle strips treated with both an inhibitor and A1242 or carbachol, strips were treated with each inhibitor for 15 to 20 min before exposure to either A1242 or carbachol for 60 or 30 min, respectively. In the arachidonic acid concentration-effect experiment, uterine strips were exposed to a single concentration of arachidonic acid for at least 1 h. Contraction profiles between 2 and 12 min were taken for analysis because some strips showed tetanic contractions during the first 2 min, whereas contractions were oscillatory for up to 1 h after that period. All data were normalized with respect to basal frequency of contraction and expressed as percent of basal frequency.

Myometrial Cell Isolation and Culture. Myometrial smooth muscle cells were isolated and cultured by methods established previously (Loch-Caruso et al., 1992) with a modified digestion enzyme solution. Uteri were removed from rats on gestational day 10 as described in the preparation of uterine strips section, except that excised uteri were placed in calcium/magnesium-free Hanks’ balanced salt solution containing 136.9 mM NaCl, 5.4 mM KCl, 0.4 mM KH₂PO₄, 0.3 mM NaH₂PO₄, 4.2 mM NaHCO₃, 20 mM HEPES, 0.6 mM EDTA, and 5.6 mM glucose at pH 7.2. Uterine tissue was minced and digested in an enzyme digestion solution containing 100 µg/ml deoxyribonucleases I, 150 µg/ml type II collagenase, and 150 µg/ml crude trypsin at 37°C for 45 min. Cells were further dissociated by repeated pipetting and the resulting cell suspension was filtered and centrifuged (150g) for 5 min at 4°C. The resulting pellet was resuspended and centrifuged (200g for 5 min at 4°C) twice in calcium/magnesium-free PBS (CMF-PBS) containing 2.68 mM KCl, 1.50 mM K₂PO₄, 136.9 mM NaCl, and 8.1 mM Na₂PO₄ at pH 7.2. Greater than 95% of the myometrial cells were viable, according to trypan blue exclusion by the cells. Cells were seeded into flasks containing RPMI medium and 10% bovine calf serum, and cultured at 37°C in a 95% air and 5% CO₂ atmosphere. Cultures were examined for immunofluorescence labeling of α-smooth muscle-specific actin, an actin isoform selectively expressed in smooth muscle (Sklalli et al. 1986). The purity of the smooth muscle cell cultures was verified by the labeling of all cells with α-smooth muscle-specific actin (raised in mouse) and anti-mouse IgG (whole molecule) fluorescent isothiocyanate conjugate (raised in goat), using a previously described protocol (Loch-Caruso et al., 1992). Both antibodies were obtained from Sigma Chemical Co. Media were changed every 1 to 2 days. Cells were subcultured every 7 days before confluence using 0.25% crude tryp-
sin (supplemented with 0.1% EDTA) dissolved in CMF-PBS. Passages two to five of cultured cells were used for experiments.

**Labeling Cells with [3H]Arachidonic Acid.** The cultured myometrial cells were dissociated with 0.25% crude trypsin with 0.1% EDTA in CMF-PBS and seeded at a density of 150 × 10^5 cells/35-mm plate containing RPMI medium with 10% BCS. After a 24-h incubation, the medium was removed and the cell layer was rinsed with medium. Myometrial cells were then incubated in medium supplemented with 10% BCS in the presence of 1 μCi/ml [3H]arachidonic acid for an additional 24 h. To remove unlabeled [3H]arachidonic acid, the cell layer was carefully rinsed three times with RPMI medium, and the cells were incubated for 1 h in RPMI with 10% BCS.

**Determination of [3H]Arachidonic Acid Release.** After removing the medium, cells were exposed to A1242 or carbachol in RPMI medium containing 0.1% BSA to trap released arachidonic acid. To measure [3H]arachidonic acid release in the absence of extracellular calcium, cells were exposed to A1242 in calcium-free HEPES-buffered physiological salt solution (135 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 1 mM HEPES, and 5 mM dextrose at pH 7.4) supplemented with 1 mM EGTA and 0.1% BSA. Following treatment, medium was collected and mixed with scintillation cocktail and radioactivity was determined by scintillation counting (Beckman, Fullerton, CA). Radiolabeled cells were either scraped from the plates or dislodged with 0.25% crude trypsin dissolved in CMF-PBS. The radioactivity in the cells was used to determine total incorporation of [3H]arachidonic acid. Data were expressed as percent of [3H]arachidonic acid release relative to the total incorporated dpm.

**Measurement of Inositol Phosphate Accumulation.** Accumulation of inositol phosphates was measured using methods described previously (Thompson and Fisher, 1990). Inositol phospholipids were labeled by incubation of cells with myo-[3H]inositol (10 μCi/ml) for 60 to 72 h. After this incubation, cells were harvested in Puck’s D1 solution (Honegger and Richelson, 1976) and collected by centrifugation (300g for 1 min at 4°C). Myometrial cells were resuspended in oxygenated buffer A solution (142 mM NaCl, 30 mM HEPES, 5.6 mM KCl, 3.6 mM NaHCO3, 2.2 mM CaCl2-2H2O, 1.0 mM MgCl2-6H2O, and 5.6 mM glucose, pH 7.4) and centrifuged again as before. Cells were resuspended in buffer A with 30 mM LiCl. Aliquots of cells were transferred to reaction tubes and were unexposed or exposed to DMSO (solvent controls), A1242, or carbachol and incubated for 60 min in a 37°C water bath. After 60 min of incubation, the reaction was stopped by adding 0.5 ml of 20% trichloroacetic acid while on ice. After centrifugation (1600g for 5 min at 4°C), the pellets were saved for lipid analysis and the supernatants were aspirated for total inositol phosphates measurement. Supernatants were vortexed with 2 ml of water-saturated diethyl ether, and the upper phase was aspirated. This separation process was repeated five times. For binding of labeled inositol phosphates, the lower phase was neutralized with 1 M NaHCO3 to pH 7, mixed with 1.7 ml of H2O and 0.5 ml of formate resin slurry, and centrifuged (1600g for 3 min at 4°C). The separated resin was vortexed with unlabeled myo-inositol. After the resin settled, the supernatant was aspirated and this process was repeated four more times. By adding 1 ml of elution buffer (0.1 M formic acid and 1.2 M ammonium formate), all inositol phosphates were stripped from the resin. After centrifugation (1600g for 5 min at 4°C), aliquots of supernatant were removed and mixed with scintillation cocktail, and radioactivity was measured by a scintillation counter (Packard, Meriden, CT). For lipid analysis, 0.5 ml of water and 1.5 ml of chloroform were added to the trichloroacetic acid-precipitated pellet extracts and centrifuged. Aliquots of the organic phase were then taken and mixed with scintillation cocktail to determine radioactivity by a scintillation counter. The data were presented as the percent of inositol phosphates released relative to the total radioactivity into lipid.

**Statistical Analysis.** Results are expressed as means ± S.E.M. Data were analyzed by one-way ANOVA and group means were compared post hoc using Student-Newman-Keuls’ test, except for the HPLC data, which were analyzed by t test (Sigma Stat, Jandel Scientific, CA). For all studies, a p value less than .05 was considered statistically significant.

**Results**

**A1242-Stimulated Release of Arachidonic Acid in Pregnant Rat Myometrial Cells.** [3H]Arachidonic acid-labeled myometrial cells were treated with different concentrations of A1242 for 30 min and released [3H]arachidonic acid was measured. Cells that were not exposed to A1242 but were treated with DMSO alone served as solvent controls. Under normal calcium conditions, solvent controls released 4.56 ± 0.91% of total incorporated arachidonic acid, whereas myometrial cells exposed to 10, 30, 50, 80, or 100 μM A1242 for 30 min released increasing amounts of arachidonic acid in a concentration-dependent manner to 4.75 ± 0.38, 5.99 ± 1.07, 8.84 ± 0.64, 10.81 ± 1.86, and 14.50 ± 0.98%, respectively (Fig. 1). The release of arachidonic acid was significantly greater at 50, 80, and 100 μM A1242 compared with controls (p < .05). When myometrial cells were incubated under calcium-free conditions (calcium-free physiological salt solution with 1 mM EGTA), A1242 was still able to liberate arachidonic acid in a concentration-dependent manner, with significant increases observed at 80 and 100 μM (p < .05; Fig. 1). In the absence of extracellular calcium, arachidonic acid release was significantly attenuated by treatment with 100 μM A1242 compared with the release of arachidonic acid in the presence of extracellular calcium (p < .05; Fig. 1).
addition, A1242 (100 μM) stimulated arachidonic acid release in a time-dependent manner, with arachidonic acid release significantly increased at 30, 60, and 90 min compared with controls (p < .05; Fig. 2A). In contrast, the muscarinic agonist carbachol was less effective than A1242 in releasing arachidonic acid. Compared with controls, carbachol released significant amounts of arachidonic acid at 100, 500, and 1000 μM to 4.75 ± 0.26, 4.72 ± 0.42, and 4.93 ± 0.14%, respectively (p < .05; Fig. 2B). These values, however, were not statistically different from each other.

**HPLC Profiles of A1242-Liberated Arachidonic Acid and Eicosanoids.** Figure 3 shows representative radioactivity elution profiles from HPLC analysis of [3H]arachidonic acid-preloaded myometrial cells treated with DMSO (solvent controls), A1242, A23187, or both A1242 and A23187. Control myometrial cells released small amounts of labeled arachidonic acid and eicosanoids, in which 92.40 ± 1.43% of total release was arachidonic acid, 6.71 ± 1.69% was 6-k-PGF1α, the stable metabolite of prostacyclin, and the remainder consisted of unidentified metabolites (0.07884 ± 0.00731% of incorporated dpm) (Table 1). Total radioactivity released by cells exposed to 100 μM A1242 for 30 min was 16-fold higher than that seen in control cells (1.28567 ± 0.10123% of incorporated dpm; Table 1). Nearly all (99.07 ± 0.19%) of the released arachidonic acid in response to A1242 remained as free arachidonic acid; small amounts of 6-k-PGF1α and 12-HETE, a product of 12-lipoxygenase metabolism, were also detected (Table 1). On the other hand, release of total arachidonic acid and eicosanoids from myometrial cells treated with the calcium ionophore A23187 (5 μM) for 20 min was 6-fold higher than controls (0.49773 ± 0.05690% of incorporated dpm). However, in contrast to A1242, considerable amounts of metabolites of arachidonic acid remained as produced and only 76.86 ± 0.79% of total release was arachidonic acid itself. The cyclooxygenase products detected were thromboxane B2 (TXB2), PGF2α, PGE2, 6-k-PGF1α, and 12-HHET. Also, A23187 produced 15-HETE, 12-HETE, and 5-HETE, metabolites of 15-, 12-, and 5-lipoxygenase, respectively. To determine whether A1242 inhibited formation of arachidonic acid metabolites, cells were pretreated with A1242 for 10 min and, in its continuous presence, exposed to 5 μM A23187 for an additional 20 min. A23187 stimulation of eicosanoid formation was significantly reduced in the presence of A1242 (p < .05), with 96.97 ± 0.50% of incorporated dpm detected as arachidonic acid. Even though cotreatment with A1242 and A23187 further increased total radioactivity release 29.3-fold over control, 1.8-fold over A1242 alone, and
TABLE 1
Release of arachidonic acid and its metabolites by myometrial cells exposed to Aroclor 1242 and calcium ionophore A23187

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Arachidonic acid or % of incorporation</th>
<th>% of total release</th>
<th>% of incorporation</th>
<th>% of total release</th>
<th>% of incorporation</th>
<th>% of total release</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1242 + A23187</td>
<td>6-k-PGF₁α</td>
<td>0.02352</td>
<td>0.00587</td>
<td>0.02352</td>
<td>0.00587</td>
<td>0.02352</td>
</tr>
<tr>
<td>A23187</td>
<td>TXB2</td>
<td>0.00612</td>
<td>0.00150</td>
<td>0.00612</td>
<td>0.00150</td>
<td>0.00612</td>
</tr>
<tr>
<td>A23187</td>
<td>PGF2α</td>
<td>0.00087</td>
<td>0.00221</td>
<td>0.00087</td>
<td>0.00221</td>
<td>0.00087</td>
</tr>
<tr>
<td>A23187</td>
<td>PGE2</td>
<td>0.00051</td>
<td>0.00131</td>
<td>0.00051</td>
<td>0.00131</td>
<td>0.00051</td>
</tr>
<tr>
<td>A23187</td>
<td>12-HHT</td>
<td>0.00099</td>
<td>0.00264</td>
<td>0.00099</td>
<td>0.00264</td>
<td>0.00099</td>
</tr>
<tr>
<td>A23187</td>
<td>15-HETE</td>
<td>0.00103</td>
<td>0.00314</td>
<td>0.00103</td>
<td>0.00314</td>
<td>0.00103</td>
</tr>
<tr>
<td>A23187</td>
<td>12-HETE</td>
<td>0.00457</td>
<td>0.01384</td>
<td>0.00457</td>
<td>0.01384</td>
<td>0.00457</td>
</tr>
<tr>
<td>A23187</td>
<td>5-HETE</td>
<td>0.00048</td>
<td>0.00145</td>
<td>0.00048</td>
<td>0.00145</td>
<td>0.00048</td>
</tr>
<tr>
<td>A23187</td>
<td>Total metabolites</td>
<td>0.06179</td>
<td>0.00318</td>
<td>0.06179</td>
<td>0.00318</td>
<td>0.06179</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. from three experiments. Asterisks indicate results are significantly different from controls (p < 0.05).
PLA\textsubscript{2} inhibitors may have nonspecific inhibitory effects on uterine contraction, each inhibitor was tested for changes in spontaneous oscillatory contraction of uterine muscle strips. The stimulatory activity of A1242 was blocked and contractions were inhibited to 54.3 \pm 20.3\% by a 20-min pretreatment with 5 \textmu M HELSS, a calcium-independent PLA\textsubscript{2} inhibitor (Hazen et al., 1991) (Fig. 6A). Uterine contraction was not significantly changed by HELSS alone (108.0 \pm 8.7\%) compared with solvent (DMSO) control (Fig. 6A). Pretreatment with the secretory PLA\textsubscript{2} inhibitor (Lombardo and Dennis, 1985) manoalide (3 \textmu M) for 20 min also prevented the A1242-induced stimulatory effect on uterine contraction (115.8 \pm 10.6\%), and manoalide itself (125.1 \pm 4.1\%) showed no statistically significant effect on contraction compared with solvent (DMSO) control strips (Fig. 6A). In addition, isotetrandrine (10 \textmu M), an inhibitor of PL\textsubscript{A2}-coupled G protein (Hashizume et al., 1991), blocked increased contractile frequency of muscle strips by A1242 to 65.6 \pm 19.2\%, but did not significantly alter contractile activity by itself (88.4 \pm 6.4\%) compared with solvent (DMSO) controls (Fig. 6A).

Additional experiments examined the effects of individual inhibitors on 5 \textmu M carbachol-stimulated contraction. Carbachol was included as a positive control, because it released inositol phosphates significantly (see Fig. 4) but had a weak arachidonic acid-releasing effect in myometrial cells (see Fig. 2B). Uterine strips treated with 5 \textmu M carbachol for 30 min showed a similar extent of contraction stimulation as 50 \textmu M A1242-exposed uterine strips, 198.2 \pm 8.1\% of basal frequency (Fig. 6B). Pretreatment with HELSS (5 \textmu M) or isotetrandrine (10 \textmu M) did not significantly alter stimulation of contraction by carbachol to 178.0 \pm 7.3\% and 167.8 \pm 6.7\%, respectively. However, uterine strips exhibited attenuated carbachol-induced increases in the presence of 3 \textmu M manoalide (142.7 \pm 6.7\%; p < .05). Nonetheless, the frequency of uterine contraction was significantly increased by carbachol in the presence of manoalide in comparison with (DMSO) solvent controls (Fig. 6B). Because earlier experiments demonstrated that carbachol induced a small, yet significant, arachidonic acid release in pregnant rat myometrial cells, this decreased carbachol response in strips pretreated with manoalide is possibly related to blockage of carbachol-stimulated arachidonic acid release by the PLA\textsubscript{2} inhibitor. Alternatively, the attenuation of the carbachol response by manoalide may result from inhibition of PLC, because manoalide has also been shown to inhibit PLC (Bennett et al., 1987).

**Effect of PLC Inhibitors on A1242-Stimulated Uterine Contraction.** In contrast to PLA\textsubscript{2} inhibitors, the presence of the phosphoinositide-PLC inhibitor (Walenga et al., 1989) NCDC (20 \textmu M) did not attenuate the A1242-induced stimulation of uterine contraction (189.9 \pm 21.5\%; Fig. 6A). Additionally, NCDC treatment alone did not significantly alter contraction frequency compared with solvent (DMSO) controls (77.1 \pm 11.6\%; Fig. 6A). Similar results were obtained by pretreating uterine strips with another phosphoinositide-PLC inhibitor (Lodhi et al., 1979), neomycin (300
Inhibition of carbachol-induced stimulation was observed in significantly altered compared with DMSO solvent controls, carbachol-stimulated contraction frequency (88.1 ± 5.0%; Fig. 6B). Compared with the response of NCDC alone on which may also contribute the uterotonic effect of carbachol.

Pathways, including the release of arachidonic acid (Shuttleworth, 1996) and calcium influx through activation of voltage-operated calcium channels (Kamishima et al., 1992), which may also contribute the uterotonic effect of carbachol.

**Discussion**

The present study investigated possible mechanisms by which A1242 stimulates uterine contraction. This study showed that A1242 increased arachidonic acid release from cultured pregnant rat myometrial cells, and that most of the arachidonic acid released by A1242 was not metabolized to eicosanoids but remained as free arachidonate. Additionally, PLA2 inhibitors, but not PLC inhibitors, prevented A1242-induced stimulation of oscillatory uterine contraction.

We demonstrated previously that the PCB mixture A1242 increases the frequency of oscillatory contractions of pregnant rat uteri with in vitro exposures of 50 or 100 μM A1242 (Bae and Loch-Caruso, 1996). Preliminary data show that two uterine strips exposed to 100 μM A1242 for 1 h in the muscle baths had a residue concentration of 59.5 ± 7.5% (mean ± S.E.M.). The average PCB concentration in extracted lipids from uterine muscle of women in labor have the highest level of PCBs in extractable fat (Mitchell and Lundin-Schiller, 1990). However, little attention has been paid to arachidonic acid per se as a modulator of uterine function.

An interesting finding of this study is that most of the arachidonic acid released by exposure to A1242 was not subsequently metabolized to other eicosanoids, similar to previous observations in neutrophils (Tithof et al., 1998). In contrast, the calcium ionophore A23187 produced various eicosanoids, indicating that the absence of arachidonic acid metabolites by A1242 exposure is not due to the lack of metabolizing enzymes in myometrial cells. One possible explanation for these results is that inhibition of cyclooxygenase and lipoxygenase activities by A1242 may prevent metabolism of released arachidonic acid. Alternatively, A1242 and A23187 may activate calcium-independent and -dependent PLA2, respectively, that act on different pools of phospholipids and subsequently promote different fates of the released arachidonate, as proposed by Tithof et al. (1998) for neutrophils. However, myometrial cells cotreated with A1242 and the calcium ionophore A23187 released less 6-k-PGF1α and PGE2 than A23187 alone, even though the cotreatment released arachidonic acid to a greater extent than did treatment with A23187 alone. These results suggest that A1242 inhibits arachidonate metabolism. Moreover, because the cotreatment with A1242 and A23187 also increased the percentage of incorporated [3H]arachidonate released as 5-HETE, A1242 may inhibit different lipoxygenases and cyclooxygenases to different extents.

The role of arachidonic acid in cellular signaling has received increased consideration (for review, Piomelli, 1996). In the present study, the arachidonic acid and eicosanoid HPLC profile demonstrated that exposure of myometrial cells to A1242 produced almost exclusively arachidonic acid. These results suggest that in the uterus free arachidonic acid itself acts as a second messenger. Addition of exogenous arachidonic acid to uterine strips in muscle baths immediately increased uterine contraction. The possible role of arachidonic acid metabolites in the stimulated uterine contraction induced by arachidonic acid was not able to be determined, because inhibitors of cyclooxygenase and lipoxygenase abolished spontaneous oscillatory contraction by themselves (data not shown). However, release of eicosanoids by A1242 from uterine muscle strips could be directly examined by the
analysis of medium collected from the muscle bath under conditions where uterine strips are exposed to A1242.

Studies have revealed that the uterus, as well as other organs, expresses cytosolic PLA<sub>2</sub> and type-I and type-II secretory PLA<sub>2</sub><sub>s</sub> (Bennett et al., 1993; Prigent-Tessier et al., 1996). Although the presence of a calcium-independent PLA<sub>2</sub> has not been confirmed in myometrium, myometrial PLA<sub>2</sub> activity has been demonstrated in the absence of calcium (Khouja and Jones, 1992), suggesting that uterine PLA<sub>2</sub> activity is not calcium-dependent. Calcium-independent PLA<sub>2</sub> activity has been shown to exist in every mammalian tissue examined, suggesting their potential importance in cellular functions (Ackermann and Dennis, 1995). HELSLS is a potent inhibitor of the cytosolic calcium-independent PLA<sub>2</sub> (Hazen et al., 1991). Significant release of arachidonic acid in the absence of extracellular calcium by A1242 and blockade of A1242-induced stimulation of contraction by HELSLS strongly suggest involvement of calcium-independent PLA<sub>2</sub>s, at least the cytosolic form, in the myometrial A1242 response.

In contrast to other PLA<sub>2</sub><sub>s</sub>, calcium-independent PLA<sub>2</sub>s do not require calcium for their activity. Calcium-independent PLA<sub>2</sub>s have been shown to exist in every mammalian tissue examined, suggesting their potential importance in cellular functions (Ackermann and Dennis, 1995). HELSLS is a potent inhibitor of the cytosolic calcium-independent PLA<sub>2</sub> (Hazen et al., 1991). Significant release of arachidonic acid in the absence of extracellular calcium by A1242 and blockade of A1242-induced stimulation of contraction by HELSLS strongly suggest involvement of calcium-independent PLA<sub>2</sub>s, at least the cytosolic form, in the myometrial A1242 response.

Low molecular weight PLA<sub>2</sub>s, generally described as secretory PLA<sub>2</sub>s, are present both in soluble and membrane forms. The soluble form of secretory PLA<sub>2</sub> is released into the extracellular space upon stimulation and binds to its transmembrane receptor in the plasma membrane where it releases arachidonic acid (Ishizaki et al., 1994). A cell-associated form of secretory PLA<sub>2</sub> also has been shown to be involved in agonist-induced arachidonic acid liberation (Per- nas et al., 1991). Manoalide is an inhibitor of type I and II secretory PLA<sub>2</sub> activities (Lombardo and Dennis, 1985). In the present study, the inability of A1242 to increase uterine contraction in the presence of manoalide suggests that secretory PLA<sub>2</sub>-mediated arachidonic acid release likely is involved in A1242-induced stimulation of uterine contraction. However, the exact secretory PLA<sub>2</sub> isozyme involved and its localization remain for further study.

The action of the PLA<sub>2</sub> inhibitors seemed relatively specific for A1242-mediated stimulation of uterine contraction, because the inhibitors by themselves showed little effect on uterine contraction frequency. Furthermore, the PLA<sub>2</sub> modulators were not able to prevent stimulation by carbachol, which activates PLC as its major pathway in uterine contractile stimulation (Marc et al., 1986). A1242 and the PCB congener 2,2′-dichlorobiphenyl stimulate inositol phosphate production in neutrophils and cerebellar granule cells, respectively (Tithof et al., 1995; Shaper et al., 1996). However, A1242 did not increase inositol phosphate release from cultured pregnant myometrial cells, although carbachol did produce significant increases. In accordance, pretreatment of uterine strips with the PLC inhibitors NCDC or neomycin did not prevent A1242-induced stimulation of contractions but did significantly attenuate contraction by carbachol. Therefore, increased contraction frequency by carbachol is mediated mainly by a PLC pathway that is not a mechanism for the stimulated uterine contraction elicited by A1242.

The uterus develops spontaneous oscillatory contractions under appropriate conditions. Intracellular calcium increases that arise spontaneously or by agonist stimulation cause uterine smooth muscle to contract. Arachidonic acid modulates various ion channels that could have an impact on muscle contraction. In particular, its ability to activate voltage-operated calcium channels (Vacher et al., 1989) suggests a possible means by which arachidonic acid could increase uterine contractile frequency. Studies from our laboratory indicate that A1242 induces intracellular calcium and depolarizes myometrial cells, lending support to this idea (Bae and Loch-Caruso, 1998). However, the exact mechanism by which A1242-released arachidonic acid acts as an uterotonergic agonist remains for further study.

In summary, the results of the present study suggest that the increase of pregnant uterine contractile frequency by A1242 may be mediated by arachidonic acid per se, liberated by activation of PLA<sub>2</sub> enzymes, including calcium-independent and secretory PLA<sub>2</sub>s.

Acknowledgments

We thank Dr. Craig Harris for providing uterine tissues, Rob McNish for his technical assistance with HPLC, Edward McEwen for his help in isositol phosphates measurements, and Chwen-Ting Wang for helpful discussions.

References


Carroll J, Crooke ST and Miller DJ (1985) Inhibition of Ca<sup>2+</sup>-transporting microsomes derived from uterine sarcoplasmic reticulum. Biochem Biophys Res Commun 130:1027–1031.


Send reprint requests to: Dr. Rita Loch-Caruso, Toxicology Program, Department of Environmental and Industrial Health, The University of Michigan, 1420 Washington Heights, Room M6112, Ann Arbor, MI 48109-2029. E-mail: rlc@umich.edu