Plasmenylethanolamine is the major storage depot for arachidonic acid in rabbit vascular smooth muscle and is rapidly hydrolyzed after angiotensin II stimulation
(plasalogens/phosphatidylethanolamine/phosphatidylcholine)

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Communicated by William D. Phillips, January 3, 1989

ABSTRACT The present study demonstrates that rabbit aortic intimal smooth muscle cells contain the majority of their endogenous arachidonic acid mass in plasmenylethanolamine molecular species. To demonstrate the potential significance of these plasmenylethanolamines as substrates for the smooth muscle cell phospholipases that are activated during agonist stimulation, aortic rings were prelabeled with [3H]arachidonic acid and stimulated with angiotensin II. Although the specific activities of the choline and inositol glycerophospholipid pools were similar, when the labeling interval, ethanolamine glycerophospholipids had a specific activity of only 20% of the specific activity of choline and inositol glycerophospholipids. Despite the marked disparity in the specific activities of these three phospholipid classes, angiotensin II stimulation resulted in similar fractional losses (35-41%) of [3H]arachidonic acid from vascular smooth muscle choline, ethanolamine, and inositol glycerophospholipid classes. Reverse-phase HPLC demonstrated that >60% of the [3H]arachidonic acid released from ethanolamine glycerophospholipids after angiotensin II stimulation originated from plasmenylethanolamine molecular species. Taken together, the results demonstrate that the major phospholipid storage depot for arachidonic acid in vascular smooth muscle cells are plasmenylethanolamine molecular species which are important substrates for the phospholipase(s) that are activated during agonist stimulation.

Icosanoids are important regulators of vascular smooth muscle cell contractile state, hypertrophy, and proliferation (1-6). Accordingly, significant attention has focused on the biochemical events responsible for the liberation of arachidonic acid from endogenous smooth muscle cell lipids during signal transduction. Arachidonic acid release from cellular phospholipids may occur directly in a single catalytic step by the action of phospholipase A2 or by a multistep sequential pathway initiated by phospholipase C and culminated by the sequential actions of diglyceride and monoglyceride lipases (7-9). Although recent studies demonstrated that radiolabeled arachidonic acid was released from choline, ethanolamine, and inositol glycerophospholipid in cultured smooth muscle cells after agonist stimulation, quantification of the amount of released radiolabeled arachidonic acid from each pool suggested that different phospholipid classes were primarly responsible for radiolabeled arachidonic acid release (10-13). Since the biochemical and physiologic properties of cultured smooth muscle cells differ substantially from properties of vascular smooth muscle cells in intact tissue, the present study was performed to assess the distribution of arachidonic acid mass in phospholipids and the fractional turnover of individual phospholipid molecular species in intact vascular smooth muscle. The results demonstrate that the majority of arachidonic acid mass in vascular smooth muscle is present in plasmenylethanolamine molecular species and that substantial amounts of arachidonic acid in smooth muscle plasmenylethanolamines are rapidly mobilized after angiotensin II stimulation.

MATERIALS AND METHODS

Extraction of Aortic Intimal Smooth Muscle Glycerophospholipids. The intimal layer of aortic smooth muscle was prepared according to the method of Ross (14) from New Zealand rabbit and mongrel dog aorta denuded of endothelium. The intimal layer was frozen at liquid nitrogen temperature and pulverized. An aliquot of the powdered tissue was taken for subsequent protein analysis and the remaining tissue was extracted by the method of Bligh and Dyer (15).

Preparation of Aortic Rings, [3H]Arachidonic Acid Labeling, and Angiotensin II Stimulation. Thoracic aortas were removed from New Zealand rabbits, denuded of endothelial cells, and extravascular fat was dissected. The aortas were cut into rings (≈0.5 cm wide) and incubated for 12 hr at 37°C in 20 ml of modified Krebs-Henseleit buffer at pH 7.3 continuously equilibrated with O2/CO2 (95:5, vol/vol) containing 118 mM NaCl, 4.7 mM KCl, 3.0 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 0.5 mM Na EDTA, 15 mM glucose, and 20 μCi of [3H]arachidonic acid (1 Ci = 37 GBq). The aortic rings next were rinsed twice with modified Krebs-Henseleit buffer without [3H]arachidonic acid and then were incubated for 15 min with modified Krebs-Henseleit buffer containing 100 nM angiotensin II or saline. Incubations were terminated by freeze clamping blotted tissue. The tissue was pulverized at liquid nitrogen temperature, the wet weight was determined, a sample was taken to determine the wet/dry weight ratio, and tissue was extracted by the Bligh and Dyer method (15). During the extraction procedure, 1 × 10^6 dpm of [3H]lysophosphatidylcholine and 500 nmol of 1,2-diiodoacetic acid were added as internal standards.

Phospholipid Separation and Analyses. Phospholipids were resolved by using an Ultrasphere-Si column (4.5 × 250 mm; 5 μm; Beckman) as the stationary phase with an initial linear gradient over 10 min from a mobile phase of hexane/isopropanol/water (48.5:48.5:3, vol/vol) to hexane/isopropanol/water (47.75:47.75:4.5, vol/vol). The composition of the latter mobile phase was held constant for 8 min followed by a step change to hexane/isopropanol/water (46.5:46.5:7, vol/vol). The mass of each phospholipid class was quanti-

Abbreviations: GPE, sn-glycero(3)phosphoethanolamine; GPC, sn-glycero(3)phosphocholine.

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RESULTS

Phospholipid Class and Subclass Analysis of Aortic Smooth Muscle Intimal Lipids. The predominant phospholipid classes in rabbit aortic smooth muscle cell intima were choline glycerophospholipids (50%) and ethanolamine glycerophospholipids (34%) (Table 1). Aortic smooth muscle cell intima also contained substantial amounts of sphingomyelin (9%) as well as modest amounts of inositol (4%) and serine (3%) glycerophospholipids. Capillary gas chromatographic analysis of individual phospholipid classes demonstrated that ethanolamine glycerophospholipids were more enriched in plasmalogen molecular species (70%) (Table 2, line 6). In contrast, choline glycerophospholipids contained only small amounts (7%) of plasmalogen molecular species (line 3). No significant amounts (<1%) of the dimethyl acetals of palmitic, stearic, or oleic aldehydes were isolated after acid methanolation of inositol or serine glycerophospholipids. Reverse-phase HPLC analysis of the Vitride-reduced and benzoylated derivatives of rabbit vascular smooth muscle cell choline and ethanolamine glycerophospholipids revealed that these classes contained <3% alkyl ethers (Table 1). The plasmalogen content of ethanolamine and choline glycerophospholipids in canine intimal aortic smooth muscle was similar to that found in rabbit intima (Table 2, lines 7 and 8). In addition, the majority of arachidonic acid mass in smooth muscle cells was present in ethanolamine glycerophospholipids (58%), while smaller amounts were present in choline (36%) and inositol (6%) glycerophospholipids (Table 1).

Regiospecificity of the Aliphatic Constituents of Ethanolamine and Choline Glycerophospholipids from Aortic Intimal Smooth Muscle. The regiospecific decylation of phospholipids catalyzed by *Naja naja* phospholipase A2 was exploited to identify the distribution of aliphatic constituents located at the sn-1 and sn-2 positions. Phospholipase A2 treatment of vascular smooth muscle cell ethanolamine glycerophospholipids resulted predominantly in the generation of lysophospholynlethanolamine and free arachidonic acid (Table 2, lines 6 and 5, respectively). Comparisons of the ratios of lysophospholynlethanolamine to lysophosphatidylethanolamine demonstrated that 73% of vascular smooth muscle cell ethanolamine glycerophospholipids was composed of plasmalogen molecular species (line 6). In contrast, phospholipase A2 treatment of vascular smooth muscle cell choline glycerophospholipids resulted predominantly in the production of lysophosphatidylycholine (containing palmitic acid) (line 3) as well as the production of oleic, linoleic, and arachidonic free fatty acids (line 2).

Reverse-Phase HPLC Separation of Rabbit Aortic Smooth Muscle Cell Ethanolamine and Choline Glycerophospholipids. To further document the predominance of plasmalogens in ethanolamine glycerophospholipids derived from vascular smooth muscle cells by an independent method and to compare the amounts of arachidonic acid in plasmalogen and diacyl molecular species, reverse-phase HPLC was used (Fig. 1A). The major UV-absorbing peaks eluted at 58, 63, and 95 min, which correspond to plasmalogen molecular species with arachidonic acid at the sn-2 position and palmitic, oleic, and stearic vinyl ethers at the sn-1 position (identified by comparisons with authentic standards). Another UV-absorbing peak was present at 80 min, corresponding to a diacyl phospholipid molecular species with arachidonic acid at the sn-2 position and stearic acid at the sn-1 position. Although the integrated UV absorbances do not quantitatively represent the mass of each molecular species, comparisons between different subclasses containing arachidonic acid (i.e., peaks 1, 2, and 5 for plasmalogen molecular species versus peak 4 for diacyl molecular species) are approximate reflections of mass since the integrated UV absorbance is approximately proportional to the number of double bonds present in a given species (18).

Reverse-phase HPLC analysis of choline glycerophospholipids (Fig. 1B) demonstrated the presence of diacyl molecular species containing arachidonic acid peaks 1 and 4) and linoleic (peak 2) and oleic (peak 3) acids at the sn-2 carbon. Since these fatty acids contain different numbers of olefinic bonds, comparisons of the integrated UV absorbance are not reliable indicators of relative mass.

Fast Atom Bombardment Mass Spectrometry of Intimal Aortic Smooth Muscle Glycerophospholipids. Fast atom bombardment mass spectrometry of aortic intimal smooth muscle ethanolamine glycerophospholipids demonstrated major protonated parent ions (MH)+ of m/z 750 and 752 corresponding to molecular species containing the vinyl ether of oleic and stearic aldehydes at the sn-1 carbon and arachidonic acid esterified to the sn-2 carbon (Fig. 2A). The parent ion at m/z 724 corresponds to a plasmalynlethanolamine, which contains the vinyl ether of palmitic aldehyde at the sn-1 carbon and arachidonic acid at the sn-2 carbon. The only major phosphatidylethanolamine molecular species detected had a parent ion (MH)+ present at m/z 768, which corresponds to 1-stearoyl-2-arachidonyl-GPE. Peaks below m/z 700 in this spectra as well as spectra of choline glycerophospholipids result from desorption of glycerol oligomers or represent glycerophospholipid fragmentation products. Fast atom bombardment mass spec-
Table 2. Fatty acid and aldehyde profiles of native and phospholipase A<sub>2</sub>-treated phospholipids of intimal smooth muscle from rabbit aorta and canine aorta

<table>
<thead>
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<th></th>
<th>16:0 (D)</th>
<th>16:0 (F)</th>
<th>18:0 (D)</th>
<th>18:0 (F)</th>
<th>18:1 (D)</th>
<th>18:1 (F)</th>
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<td>1</td>
<td>13</td>
<td>1</td>
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<td>Rabbit PLA&lt;sub&gt;2&lt;/sub&gt;-CGP-FA</td>
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<td>20</td>
<td>ND</td>
<td>4</td>
<td>ND</td>
<td>31</td>
<td>27</td>
<td>ND</td>
<td>19</td>
<td>ND</td>
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<tr>
<td>Rabbit PLA&lt;sub&gt;2&lt;/sub&gt;-CGP-LPC</td>
<td>5</td>
<td>56</td>
<td>2</td>
<td>32</td>
<td>ND</td>
<td>5</td>
<td>ND</td>
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</tr>
<tr>
<td>Rabbit EGP</td>
<td>11</td>
<td>4</td>
<td>24</td>
<td>11</td>
<td>4</td>
<td>10</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Rabbit PLA&lt;sub&gt;2&lt;/sub&gt;-EGP-FA</td>
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<td>4</td>
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</tr>
<tr>
<td>Rabbit PLA&lt;sub&gt;2&lt;/sub&gt;-EGP-LPE</td>
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<td>9</td>
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<td>14</td>
<td>7</td>
<td>4</td>
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<td>26</td>
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<td>5</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>29</td>
<td>11</td>
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Choline and ethanolamine glycerophospholipids (CGP and EGP, respectively) from rabbit and canine aortic intima were purified by straight-phase HPLC, subjected to acid-catalyzed methanolation, and analyzed by capillary gas chromatography. Products of phospholipase A<sub>2</sub>-treated CGP and EGP were separated by TLC and subjected to acid-catalyzed methanolation. PLA<sub>2</sub>-CGP(EGP)-FA and PLA<sub>2</sub>-CGP(EGP)-LPC are the fatty acid and lysophospholipid products of phospholipase A<sub>2</sub> treatment, respectively. D, dimethyl acetel; F, fatty acid methyl ester; ND, not detectable. Values are expressed in % weight.

chromatography of choline glycerophospholipids demonstrated predominant parent ions at m/z 758 and 760 corresponding to 1-palmitoyl-2-linoleoyl-GPC [-sn-glycero(3)phosphocholine] and 1-palmitoyl-2-oleyl-GPC, respectively (Fig. 2B). Aortic intimal smooth muscle inositol glycerophospholipids contained major negative ions (M−1)<sup>−</sup> at m/z 883 and 885 corresponding to 1-oleoyl-2-arachidonyl-GPI [-sn-glycero(3)phospho(1)-<sup>−</sup>1-myoinositol] (20%) and 1-stearoyl-2-arachidonyl-GPI (80%) (data not shown).

**Radiolabeling of Aortic Smooth Muscle Phospholipids and Angiotensin II-Stimulated Release of Incorporated [3H]Arachidonic Acid.** Rabbit aortic rings incorporated substantial amounts of radiolabeled arachidonic acid into choline, ethanolamine, and inositol glycerophospholipids during the 12-hr labeling interval (Table 3). Despite the fact that ethanolamine glycerophospholipids contained the majority of arachidonic acid mass in rabbit vascular smooth muscle, the choline glycerophospholipid pool contained >3 times as much radiolabeled arachidonic acid after the labeling interval. Accordingly, the specific activity of arachidonic acid in each major glycerophospholipid pool was substantially different. Choline and inositol glycerophospholipids had a specific activity =5-fold that of the specific activity of ethanolamine glycerophospholipids (Table 3). It should be noted that initial studies demonstrated that the phospholipid composition of aortic rings was virtually identical to that of the intimal layer of aorta. Despite the marked disparities in the specific activities of these three phospholipid classes, subsequent incubation of prelabeled rabbit aortic rings with 100 nM angiotensin II resulted in the release of similar fractional percentages of radiolabeled arachidonic acid from choline, ethanolamine, and inositol glycerophospholipid classes (35–41% loss from each pool) (Table 3). Direct quantification of released radiolabeled arachidonic acid demonstrated that choline and ethanolamine glycerophospholipids together were responsible for >90% of the released radiolabeled arachidonic acid. To identify the individual molecular species that incorporated radiolabeled arachidonic

![Fig. 1](image1.png)

**Fig. 1.** Reverse-phase HPLC of aortic intimal ethanolamine and choline glycerophospholipids. Intimal ethanolamine and choline glycerophospholipids (~300 nmol) were purified by straight-phase HPLC, injected onto an octadecyl silica column, and eluted with a mobile phase composed of methanol/acetonitrile/water (90:5:2.5:7, vol/vol) containing 20 mM choline chloride. UV absorbance was monitored at 203 nm. The molecular identities of the ethanolamine glycerophospholipids (A) are 1-O-hexadec-1'-enyl-2-icosodec-5',8',11',14'-enoyl-GPE (peak 1), 1-O-octadec-1'-,9'-enyl-2-icosodec-5',8',11',14'-enoyl-GPE (peak 2), 1-O-hexadec-1'-enyl-2-octadec-9',12'-enoyl-GPE (peak 3), 1-octadecanoyl-2-icosodec-5',8',11',14'-enoyl-GPE (peak 4), and 1-O-octadec-1'-enyl-2-icosodec-5',8',11',14'-enoyl-GPE (peak 5). The molecular identities of choline glycerophospholipids (B) are 1-hexadecanoyl-2-icosodec-5',8',11',14'-enoyl-GPE (peak 1), 1-hexadecanoyl-2-octadec-9',12'-enoyl-GPE (peak 2), 1-hexadecanoyl-2-octadec-9',14'-enoyl-GPE (peak 3), and 1-octadecanoyl-2-icosodec-5',8',11',14'-enoyl-GPE (peak 4).

![Fig. 2](image2.png)

**Fig. 2.** Fast atom bombardment mass spectrometry of aortic intimal ethanolamine and choline glycerophospholipids. HPLC-purified intimal ethanolamine (A) and choline (B) glycerophospholipids (~300 nmol) were dissolved in 20 µl of chloroform/methanol (1:1, vol/vol) and 2 µl was mixed with 3 µl of glycerol on a copper probe. Fast atom bombardment mass spectrometry was performed as described in Materials and Methods.
Defatted and endothelium-denuded aortic rings were labeled for 12 hr with [3H]arachidonic acid (1 μCi/ml) in 20 ml of modified oxygenated Kreb–Henseleit (K–H) buffer at 37°C. Extracellular [3H]arachidonic acid was subsequently removed by two sequential 15-min incubations at 37°C in 25 ml of modified oxygenated K–H buffer. The aortic rings were incubated for 15 min in modified K–H buffer containing either 100 nM angiotensin II (ALL) or vehicle (saline). Aortic lipids were extracted by the Bligh and Dyer (15) method, at which time 1 × 10⁶ dpm of [14C]sphingosydicholine was added (internal standard). Individual phospholipid classes were resolved by straight-phase HPLC and incorporated radiolabel was quantitated by liquid scintillation spectrometry. The values for radiolabel incorporated into each phospholipid pool are the means ± SEM for four separate experiments and are expressed as cpm/g (dry weight). RSA, relative specific activity (ratio of [3H]arachidonic acid cpm to nmol of arachidonoylated phospholipid; the RSA of choline GPL was normalized to 1); GPL, glycerophospholipids.

\( P < 0.05 \) for control vs. ALL-treated aortic rings.

\( P < 0.01 \) for control vs. ALL-treated aortic rings.

Table 3. Distribution of [3H]arachidonic acid in aortic phospholipids before and after incubation with angiotensin II

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ALL</th>
<th>% change</th>
<th>RSA</th>
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<tbody>
<tr>
<td>Choline GPL</td>
<td>413,010 ± 48,400</td>
<td>255,220 ± 55,890*</td>
<td>-38</td>
<td>1</td>
</tr>
<tr>
<td>Ethanolamine GPL</td>
<td>130,820 ± 8,210</td>
<td>77,510 ± 4,420</td>
<td>-41</td>
<td>0.20</td>
</tr>
<tr>
<td>Inositol GPL</td>
<td>63,630 ± 11,350</td>
<td>41,310 ± 9,120*</td>
<td>-35</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Acid during the labeling interval and those that released [3H]arachidonic acid after agonist stimulation, reverse-phase HPLC was used. As expected, radiolabeled arachidonic acid was predominantly incorporated into 1-palmitoyl-2-arachidonyl-GPC (75%) with smaller amounts present in 1-stearoyl-2-arachidonyl-GPC as well as other choline glycerophospholipid subclasses (data not shown). Angiotensin II stimulation resulted in comparable fractional decreases in each radiolabeled choline glycerophospholipid molecular species. Reverse-phase HPLC of radiolabeled ethanolamine glycerophospholipids demonstrated that plasmenyl species contained the majority of radiolabeled arachidonic acid (70%), while smaller amounts (30%) were present in phosphatidyethanolamine molecular species (Fig. 3). Surprisingly, the distribution of [3H]arachidonic acid in plasmenyl molecular species was similar in plasmenylethanolamines containing the vinyl ether of palmitaldehyde and stearaldehyde at the sn-1 position, although the relative mass distribution of these two molecular species was substantially different. Analysis of individual radiolabeled molecular species of ethanolamine glycerophospholipids that contributed to the release of radiolabeled arachidonic acid after angiotensin II stimulation was made by comparing the amounts of [3H]arachidonic acid in individual molecular species after normalization to the recovery of internal standard as described in Materials and Methods (Fig. 3). Statistically significant losses (\( P < 0.02; n = 3 \)) of arachidonoylated plasmenylethanolamine molecular species (peaks 3, 4, and 6) as well as arachidonoylated phosphatidyethanolamine molecular species (peak 5) were observed. The fractional percentage of hydrolysis of each ethanolamine glycerophospholipid subclass was similar, demonstrating that the phospholipase(s) responsible for the release of arachidonic acid from vascular smooth muscle phospholipids during angiotensin II stimulation effectively hydrolyzes both phosphatidyethanolamine and plasmenylethanolamine substrates.

![Fig. 3. Reverse-phase HPLC of [3H]arachidonic acid-labeled ethanolamine glycerophospholipids from rabbit aortic rings incubated with and without 100 nM angiotensin II. Aortic rings were prelabeled with [3H]arachidonic acid and subsequently incubated with or without 100 nM angiotensin II. Cellular phospholipids were extracted as described in Materials and Methods in the presence of 500 nmol of 1,2-dioctadec-8’,11’,14’-enoyl-GPE (internal standard). Ethanolamine glycerophospholipids were purified by straight-phase HPLC, injected onto an octadeyl silica column, and eluted with methanol/acetonitrile/water (90:5:2.5, vol/vol) containing 20 mM choline chloride. Column eluents were collected in 1-min fractions and their radioactivity was determined by liquid scintillation spectrometry.](image-url)
DISCUSSION

The results of this study demonstrate that ethanolamine glycerophospholipids in aortic smooth muscle cells from both dogs and rabbits are predominantly composed of plasmalogen molecular species. Over 63% of plasmenylethanolamine molecular species in rabbit aorta contain arachidonic acid esterified to the sn-2 position as ascertained by capillary gas chromatography, fast atom bombardment mass spectrometry, and reverse-phase HPLC. Although plasmenylethanolamines are an important storage depot for arachidonic acid in other tissues such as platelets, Madin–Darby kidney cells, and endothelial cells (19–21), the fact that plasmenylethanolamines represent the major phospholipid storage depot for arachidonic acid in vascular smooth muscle cells has previously gone unrecognized. Furthermore, the loss of arachidonic acid from plasmenylethanolamine during angiotensin II stimulation suggests an important biologic role for plasmenylethanolamine molecular species as substrates for the phospholipases that are activated during signal transduction in smooth muscle cells. Considering the potential role of iocanoids in the development of atherosclerotic plaques, smooth muscle cell hypertrophy, and smooth muscle cell injury, it seems likely that hydrolysis of arachidonoylated plasmenylethanolamines may contribute to the pathophysiological sequelae of these processes.

Since radiolabeled arachidonic acid is rapidly lost from plasmenylethanolamine molecular species within minutes of angiotensin II stimulation of rabbit aortic rings, vascular smooth muscle cells must contain phospholipases that are activated after agonist stimulation that can effectively utilize plasmenylethanolamine as substrate. However, quantitation of the relative contribution of the plasmenylethanolamine pool to arachidonic acid release in these studies, as well as those of others, requires assumptions about the relationship between radiolabeled ethanolamine glycerophospholipids and nonradiolabeled ethanolamine glycerophospholipids. If the simplifying assumption is made that the fractional turnover of radiolabeled ethanolamine glycerophospholipids is representative of the turnover of ethanolamine glycerophospholipids in vascular smooth muscle cells in general, then it seems likely that the ethanolamine glycerophospholipid pool is a substantial contributor to the release of arachidonic acid mass during signal transduction. Since significant subcellular compartmentation of radiolabeled ethanolamine glycerophospholipid molecular species into metabolically distinct compartments may occur, these extrapolations, as with extrapolations from any radiolabeling experiment with intact cells, should be interpreted cautiously.

Recently, the importance of alterations in membrane dynamics as a modulator of transmembrane ion flux has become increasingly appreciated (22). The molecular dynamics of plasmalogen molecular species differ substantially from that of their diacyl counterparts (23). Furthermore, the recent demonstration of plasmalogen selective phospholipases (24, 25) has suggested an important role of these moieties as substrates of the phospholipases that are activated during signal transduction. The results of the present study demonstrate that smooth muscle cells contain substantial amounts of arachidonoylated plasmenylethanolamine molecular species, which likely contribute to smooth muscle cell function both through their effects on membrane dynamics and as important substrates for the phospholipases activated during angiotensin II stimulation.

This research was supported in part by National Institutes of Health Grant HL 41250 and an American Heart Association Grant-in-Aid. D.A.F. is an American Heart Association Missouri Affiliate Fellow. R.W.G. is the recipient of an Established InvestigatorAward from the American Heart Association. We are grateful for the support of the Washington University Mass Spectrometry Resource, which is funded by National Institutes of Health Grant RR 00954.