Norepinephrine stimulates arachidonic acid release from vascular smooth muscle via activation of cPLA₂

EDWARD F. LABELLE AND ERZSEBET POLYAK

Department of Physiology, Allegheny University of the Health Sciences, Allegheny University Hospital: Graduate, Philadelphia, Pennsylvania 19146

LaBelle, Edward F., and Erzsebet Polyak. Norepinephrine stimulates arachidonic acid release from vascular smooth muscle via activation of cPLA₂. Am. J. Physiol. 274 (Cell Physiol. 43):C1129—C1137, 1998.—The mechanism of agonist-activated arachidonate release was studied in segments of rat tail artery. Tail artery segments were prelabeled with [³H]arachidonate and then stimulated with norepinephrine (NE), and the radioactivity of the extracellular medium was determined. NE stimulated arachidonate release from the tissue without increasing arachidonic acid levels within cellular cytosol or crude membranes. About 90% of the extracellular radioactivity was shown to be unmetabolized arachidonate by TLC. Arachidonic acid release was not inhibited by the removal of the endothelium from the artery. NE exerted a half-maximal effect at a concentration of 0.2 µM. NE-stimulated arachidonate release was not inhibited by blockers of phospholipase C (U-73122), diacylglycerol lipase (RHC-80267), secretory phospholipase A₂ (mananolide), calcium-insensitive phospholipase A₂ (HELSS), or β-adrenergic receptors (propranolol). NE-stimulated arachidonic acid release was inhibited by blockers of cytosolic phospholipase A₂ (cPLA₂) (AACOCF₃), α₂-adrenergic receptors (prazosin), and specific G proteins (pertussis toxin). This indicated that NE stimulated arachidonate release from a specific smooth muscle cell to its nearby neighbor cells. Perhaps arachidonic acid extends the effect of NE on one specific smooth muscle cell and the hydrophilic choline derivatives released.

Many investigators have found that yet another phospholipase, known as phospholipase A₂ (PLA₂), exists in many mammalian tissues and that this phospholipase can be activated by agonists to release arachidonic acid from the tissues (1, 9). Arachidonic acid itself is the precursor of a series of eicosanoids that appear to play a role in many important processes (34). PLA₂ can also be activated by contractile agonists in smooth muscle (14, 29). Muthalif et al. (33) have demonstrated that NE could stimulate arachidonic acid release from cultured rabbit aortic smooth muscle cells via the activation of a specific PLA₂ known as cytosolic PLA₂ (cPLA₂). Many different isoforms of PLA₂ have been detected in mammalian tissues, such as cPLA₂, secretory PLA₂ (sPLA₂), and calcium-insensitive PLA₂ (iPLA₂) (8, 32). Some studies have shown that cPLA₂ appears most important in agonist-activated tissue (30, 39). These studies have suggested that cPLA₂ could be activated by pertussis toxin-sensitive G proteins (2, 36), by tyrosine phosphorylation (12), by calcium activation (12, 23), and by calcium-dependent translocation to the membrane (7). The low-molecular-weight sPLA₂ has been detected in many cell types, but since it is only active at millimolar levels of calcium, it is unlikely to mediate agonist effects within cells (6). iPLA₂ has been detected in vasopressin-activated cultured smooth muscle cells (29). There is also the possibility that arachidonic acid might be released via the activation of PLC and diacylglycerol lipase (17, 21).

The function of the released arachidonate and associated eicosanoids in smooth muscle is unclear. There is evidence that arachidonic acid can influence membrane ion channel activity (37, 48) and activate PKC (41), whereas certain eicosanoids, such as leukotrienes and thromboxanes, can activate surface receptors that release IP₃ and activate intracellular calcium release from subcellular stores (42, 43). Gong et al. (13) have shown that arachidonic acid can inhibit myosin light chain phosphatase, and they have suggested that this effect of arachidonic acid might be responsible for the GTP-dependent calcium sensitization of vascular smooth muscle (14).

The purpose of the current study was to determine whether the contractile agonist NE could stimulate the release of arachidonic acid from smooth muscle of rat tail artery and to determine the mechanism of this process. We find that NE can stimulate arachidonate release from this tissue and that the process appears to require activation of cPLA₂. This system also appears to be sensitive to pertussis toxin, which indicates the involvement of a G protein that is different from the G protein earlier shown to be required for the activation of PLC and PLD in this tissue (25, 28). There does not

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appear to be an agonist-dependent increase in free arachidonic acid levels within the smooth muscle cells.

MATERIALS AND METHODS

Male Sprague-Dawley rats (300–500 g) were purchased from Taconic Farms (Germantown, NY). The [3H]arachidonic acid, together with a kit used to measure levels of 6-ketoprostaglandin F1α (6-keto-PGF1α), was purchased from New England Nuclear (Boston, MA). BSA, NE, prazosin, isoproterenol, phenylephrine, proparanol, desipramine, and indomethacin were purchased from Sigma Chemical (St. Louis, MO). Manoalide, 1-(6-[17b-3-methoxyestra-1,3,5(10)-trien-17-yl]amino[4-hexyl]-1H-pyrrole-2,5-dione (U-73122), 1,6-bis(cyclohexyloximinocarbonyl-amino)-hexane (RHC-80267), arachidonyl trifluoromethyl ketone (AACOCF3), E-6-(bromo-4-hexyl)pyran-2-one (HELSS), tricyclodecan-9-yl xanthogenate·K (D609), and pertussis toxin were purchased from Biomol (Plymouth Meeting, PA). A monoclonal antibody directed against cPLA2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Arachidonic acid release measurements. Rats were killed by excess CO2 exposure, and the tail arteries were quickly removed. The arteries were rinsed with physiological saline solution (PSS) containing (in mM) 116.7 NaCl, 1.2 Na2HPO4, 4.5 KCl, 2.5 CaCl2, 1.2 MgSO4, 2.4 Na2SO4, 22.5 NaHCO3, 5 HEPES, and 5 d-glucose. The PSS solution was gassed with O2/CO2 (55:25:10; upper phase only). After the separation the thin-layer plates were sprayed with scintillation fluid and exposed to X-ray film, and radioactivity was measured with the assistance of a RIA kit. Segments of rat tail artery were incubated in the presence and absence of NE (10 µM) for 10 min at 37°C. Aliquots of the incubation medium were incubated for 16 h at 4°C with 0.01 µCi standard [3H]6-keto-PGF1α, together with antibody directed against 6-keto-PGF1α. The mixtures were treated with charcoal for 15 min and then were centrifuged at 1,000 g for 10 min. The radioactivity in the supernatant was measured and compared with radioactivity determined when [3H]prostaglandin was mixed with antibody and known amounts of nonlabeled prostaglandin.

Immunoblotting. The presence of cPLA2 in rat tail artery was determined by immunoblotting using the procedure of LaBelle et al. (25).

RESULTS

NE stimulated arachidonic acid release from segments of rat tail artery. Arachidonic acid release was also observed in the absence of NE.

The rate of efflux of arachidonic acid from the tissue was constant for ~12 min, in both the presence and absence of NE (Fig. 1A). NE significantly stimulated arachidonic acid release from rat tail artery after 30 s of treatment (Fig. 1B). When the extracellular medium obtained in the experiment shown in Fig. 1A was extracted with chloroform-methanol and the extract was separated by TLC, ~95% of the extracellular radioactivity was found to migrate with standard arachidonic acid (Fig. 2). When the production of a key cyclooxygenase metabolite of arachidonic acid (6-keto-PGF1α) was measured in segments of rat tail artery, we found that the radioactive material expelled from the tissue in the absence of agonist, whereas 0.34 ± 0.014 pmol/mg wet wt of 6-keto-PGF1α was released in the presence of NE (n = 4, P < 0.01). The cyclooxygenase inhibitor indomethacin (10 µM) failed to inhibit arachidonate release from rat tail artery, either in the presence or absence of NE (data not shown). This provided evidence that the radioactive material expelled from the tissue was only arachidonic acid and not a mixture of arachidonic acid plus a substantial amount of cyclooxygenase product. When rat tail artery was treated to remove endothelial cells, NE could still stimulate arachidonic acid release.
acid release from the tissue, which proved that arachidonic acid was being released from the smooth muscle cells of the artery and not the endothelium (Fig. 3). NE stimulated arachidonic acid release from rat tail artery half-maximally at a concentration of ~200 nM (Fig. 4). When the concentration of NE required to stimulate arachidonic acid release half-maximally was determined in the presence of desipramine (0.1 µM) to inhibit neuronal NE reuptake, no change in the sensitivity of the tissue to NE was observed (data not shown).

When rat tail artery segments were treated with buffer containing 80 mM KCl, the cell membranes were depolarized and calcium was induced to enter the cells via potential-sensitive channels (25). This resulted in the development of force by the tissue (25). The treatment of tail artery segments with 80 mM KCl-containing buffer did not influence arachidonic acid release from the tissue (Fig. 5).

Some investigators have suggested that agonists could stimulate arachidonic acid release from tissue via the stimulation of PLC followed by diacylglycerol lipase (11, 17). However, neither the phosphoinositide inhibitor U-73122 nor the diacylglycerol lipase inhibitor RHC-80267 could prevent NE from stimulating arachidonic acid release from rat tail artery (Fig. 6). This suggested that NE did not stimulate arachidonic acid release via either phosphoinositide or diacylglycerol lipase. When we measured the effects of U-73122 on NE-stimulated production of inositol phosphates in rat tail artery, this compound was shown to inhibit NE-activated increases in inositol monophosphate, inositol diphosphate, and IP₃ (data not shown). NE-stimulated arachidonic acid release from rat tail artery was also insensitive to inhibition by D609 (data not shown).

![Fig. 1. Effect of norepinephrine (NE) on arachidonic acid (AA) release from rat tail artery.](image)

![Fig. 2. TLC of extracellular radioactivity proves identity of AA.](image)
which has been shown to inhibit PC-specific PLC (40). This indicated that agonist-stimulated arachidonic acid release did not occur in response to stimulation of PC-specific PLC. We have shown that NE could stimulate phosphoinositidase in this tissue by the determination of IP3 production (15, 27), and we demonstrated PC-specific PLC activation by the determination of choline phosphate release (16).

The compound AACOCF3 has been shown to be a selective inhibitor of cPLA2 (30, 47). AACOCF3 blocks the effects of NE on arachidonic acid release in rat tail artery (Fig. 7). The concentration of AACOCF3 that half-maximally blocked the effects of NE on arachidonic acid release was 10 µM. The presence of cPLA2 in rat tail artery was proven by means of immunoblotting with an antibody to cPLA2 (Fig. 8). Neither the inhibitor of iPLA2 (HELSS) nor the inhibitor of sPLA2 (manoalide) could block NE-stimulated arachidonic acid release from rat tail artery (Fig. 9). This indicated that the stimulation of arachidonic acid release in this tissue by NE was most likely not mediated by either iPLA2 or sPLA2.

Pertussis toxin was shown to totally block NE-stimulated arachidonic acid release from rat tail artery (Fig. 10). This indicated that NE exerted its effects on arachidonic acid release via a pertussis toxin-sensitive G protein, most likely either G1 or G0. NE was shown to exert its effects on arachidonic acid release via the α-adrenergic receptor when the α1-adrenergic receptor...
inhibitor prazosin was shown to totally block NE-stimulated arachidonic acid release (Fig. 11). The β-adrenergic receptor propranolol failed to inhibit NE-stimulated arachidonic acid release, thereby proving that β-adrenergic receptors were not involved in this process (data not shown). Likewise phenylephrine, an α-adrenergic receptor agonist, stimulated arachidonic acid release from rat tail artery, whereas the β-receptor agonist isoproterenol failed to stimulate arachidonic acid release from this tissue (data not shown).

When rat tail artery segments prelabeled with [3H]arachidonic acid were stimulated with NE, homogenized, and separated into cytosol and crude membranes by centrifugation, NE was shown to have no stimulatory effects on the levels of arachidonic acid within the tissue (Table 1).

**DISCUSSION**

Many agonists have been shown to stimulate arachidonic acid release from different mammalian tissues (1, 9, 34). Arachidonic acid itself has been shown to serve as the precursor of a family of compounds known as eicosanoids (34). Both arachidonic acid and the eicosanoids have been shown to have effects on potassium channels (37), PKC (41), and receptor-mediated IP3 release (42, 43). It has also been suggested that arachidonic acid could inhibit myosin light chain phosphatase in smooth muscle and thereby enhance smooth muscle force (13, 14). Many investigators have found evidence for the direct activation of plasma membrane calcium channels by arachidonic acid in oligodendrocytes (44) as well as in smooth muscle cells (5, 48, 50).

There have been numerous reports that arachidonate release could be stimulated by the combined activities of the enzymes PLC and diacylglycerol lipase (11, 17). Other studies have suggested that arachidonate...
release was directly stimulated by PLA2 (4, 46). Several different PLA2 enzymes that might be important, such as sPLA2, cPLA2, and iPLA2, were found in mammalian tissues (1, 8, 9, 32, 34). The activation of cPLA2 in many tissues has been shown to be dependent on G protein activation (2, 36), tyrosine phosphorylation (12), calcium activation (12, 23), and membrane translocation (7). Mitogen-activated protein kinase has also been shown to activate cPLA2 in certain tissues (31) but not in others (3). Some very recent studies have indicated that cPLA2 activation might require PLD activation (21). It remains unclear just how arachidonic acid is produced in vascular smooth muscle during agonist activation.

Our data indicate that NE rapidly stimulates arachidonic acid release from the smooth muscle of rat tail artery. This process is activated at a fairly low concentration of NE (0.2 µM), which is much lower than the NE concentration shown to activate either PLC or PLD in our earlier studies (1–10 µM) (16, 27). Because 80 mM KCl failed to stimulate arachidonic acid release from rat tail artery, it could be concluded that arachidonic acid release might be involved in the mechanism of NE-induced force rather than a mere secondary consequence of force development in smooth muscle. Likewise, if KCl fails to stimulate arachidonic acid release, one can conclude that arachidonic acid release is not a simple consequence of increased levels of cytosolic calcium (25).

Our data also indicate that arachidonic acid release from vascular smooth muscle most likely does not require the activation of either phosphoinositidase C or PC-specific PLC combined with diacylglycerol lipase, since the inhibitors of these enzymes fail to block NE-activated arachidonic acid release (Fig. 6). The relative insensitivity of PLC and PLD to NE demonstrated in our earlier studies also suggests that arachidonic acid release was directly stimulated by PLA2. NE rapidly stimulates arachidonic acid release from rat tail artery. Presence of cPLA2 in rat tail artery was determined by immunoblotting using an antibody directed against cPLA2. These results were repeated twice with essentially identical results. Numbers on right are molecular weight.

Fig. 8. Identification of cPLA2 in rat tail artery. Presence of cPLA2 in rat tail artery was determined by immunoblotting using an antibody directed against cPLA2. These results were repeated twice with essentially identical results. Numbers on right are molecular weight.

Fig. 9. Effect of inhibitors of calcium-insensitive PLA2 and secretory PLA2, HELSS and manoalide, respectively, on AA release from rat tail artery. AA release from segments of rat tail artery was measured as described in legend to Fig. 1. Either HELSS (10 µM; A) or manoalide (10 µM; B) was included during the rinse step and the subsequent NE incubation (5–20 min) as described. Values are means + SE (n = 4). These results were repeated 6 times for HELSS and 3 times for manoalide, with essentially identical results.
Arachidonic acid release is not secondary to either PLC or PLD in our tissue. Finally, pertussis toxin has never inhibited PLC or PLD in rat tail artery (25, 28), whereas it does block arachidonic acid release (Fig. 10).

These experiments suggest that arachidonic acid release in rat tail artery does not rely on either PLC or PLD activation and might rely on PLA2 activation. Evidence in support of cPLA2 activation is shown in Fig. 7, wherein the selective inhibitor AACOCF3 (30, 47) blocks arachidonate release in rat tail artery. The insensitivity of this process to manoalide and HELSS suggests that neither sPLA2 nor iPLA2 are required during NE-activated arachidonic acid release in rat tail artery (19, 20). Manoalide has been shown to be selective for sPLA2 (20) and HELSS selective for iPLA2 (12). All of these inhibitors can easily penetrate cells since they are extremely hydrophobic (DMSO soluble). Lehman et al. (29) have shown that iPLA2 appeared to be activated by vasopressin in cultured smooth muscle cells (A10 cells). This may reflect a difference between agonists (NE vs. vasopressin) or a difference between cultured smooth muscle cells and intact smooth muscle. Muthalif et al. (33) introduced oligonucleotides complementary to mRNA specific for cPLA2 into cultured rabbit aortic smooth muscle cells, and these oligonucleotides were able to block the effects of NE on arachidonic acid release. This provided more evidence for a role of cPLA2 in agonist-activated smooth muscle cells. Wright and Malik (52) demonstrated that NE could stimulate prostacyclin release from intact rat aorta and that this process was sensitive to an inhibitor of PLA2 [7,7-dimethyl-(5Z,8Z)-eicosadienonic acid]. These results were consistent with our evidence that NE stimulates arachidonic acid release via PLA2 activation in intact rat tail artery. The direct demonstration of cPLA2 in rat tail artery by immunoblotting is consistent with its function in this tissue.

No increase in the concentration of arachidonic acid was observed within the cells of the rat tail artery after agonist activation. This would suggest that arachidonate is unlikely to ever reach levels high enough to

Table 1. Effect of norepinephrine on arachidonic acid levels within rat tail artery cells

<table>
<thead>
<tr>
<th>Arachidonic Acid, cpm</th>
<th>Cytosol</th>
<th>Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,248 ± 354</td>
<td>648 ± 147</td>
</tr>
<tr>
<td>NE (10 µM)</td>
<td>1,351 ± 251</td>
<td>625 ± 81</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3. Segments of rat tail artery were treated with [3H]arachidonic acid as described in legend to Fig. 1 and then were rinsed free of extracellular radioactivity and treated with or without norepinephrine (NE; 10 µM) for 20 min. The tissue was then homogenized and separated into cytosol and membranes, and the lipids were extracted and separated into classes as described in MATERIALS AND METHODS. Amount of arachidonic acid found in each fraction is reported. NE failed to exert a significant effect on arachidonate release in either cytosol or membranes (P > 0.7).
inhibit myosin light chain phosphatase in smooth muscle during agonist activation. Gong et al. (13) have shown that 300 \( \mu \text{M} \) arachidonic acid could inhibit myosin light chain phosphatase in permeable vascular smooth muscle. Khan et al. (24) have indicated that free arachidonate levels in tissues never get beyond low nanomolar levels, although a certain amount of this fatty acid might exist in the cytosol attached to fatty acid binding proteins (24). If NE can stimulate arachidonic acid release from rat tail artery, it must increase arachidonic acid levels transiently in certain membrane fractions, which was not detected in our study of crude membranes shown in Table 1. Further work is necessary to determine the arachidonic acid levels in more purified membranes from smooth muscle. PLA\(_2\) activation has been detected on the nuclear membrane (33), and Wolf et al. (51) have shown that iPLA\(_2\) activation on the membrane of subcellular stores has occurred in response to decreases in the calcium content of the stores. This indicates that PLA\(_2\) activation may not be limited to plasma membrane. Some 6-keto-PGF\(_{1\alpha}\) was released from tail artery in this study. However, the amount of arachidonate released was presumably much greater than the amount of 6-keto-PGF\(_{1\alpha}\), since no radioactivity was detected on the portion of the thin-layer plate where 6-keto-PGF\(_{1\alpha}\) would be expected to migrate (Fig. 2), and indomethacin failed to inhibit the release of radioactive material from the tissue. Other eicosanoids were no doubt released in very low amounts, but nearly all of the arachidonate released from the cells remained as free fatty acid, which suggests that arachidonate itself plays a role in this tissue. This does not rule out a role for the eicosanoids that might be released during our study.

Although this study does not elucidate the function of arachidonic acid in vascular smooth muscle, the possibility exists that it might be released from the cells to extend the function of NE beyond the synaptic cleft. Perhaps NE is itself metabolized very soon after being released from the neurons that impinge on smooth muscle, but arachidonic acid can diffuse quite a distance from the cell membrane directly under the synapse and exert effects on more distant smooth muscle cells.

In summary, we have demonstrated that NE stimulates arachidonic acid release from the rat tail artery. This process is sensitive to an inhibitor of cPLA\(_2\) and most likely results from cPLA\(_2\) activation, perhaps via pertussis toxin-sensitive G protein activation.

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Present address of E. Polyak: Univ. of Pennsylvania, Dept. of Cell and Developmental Biology, Philadelphia, PA 19104.

Address for reprint requests: E. F. LaBelle, Dept. of Physiology, Allegheny Univ. of the Health Sciences, 2900 Queen L., Philadelphia, PA 19129.

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