Regulation of Diacylglycerol Metabolism by Vasoconstrictor Hormones in Intact Small Arteries

Jacqueline Ohanian, Ashley Izzard, Mark Littlewood, and Anthony Heagerty

The initiation of receptor-mediated small artery contraction is dependent on inositol 1,4,5-trisphosphate-stimulated release of stored calcium. The role of the other product of inositol lipid hydrolysis, 1,2-diacylglycerol, in maintaining contraction remains controversial. Therefore, we have determined the contractile response of rat subcutaneous small arteries (<300 μm i.d.), when mounted as ring preparations in a myograph, to noradrenaline, angiotensin II, KCl-induced membrane depolarization, and a cell-permeable diglyceride, dioctanoylglycerol. In parallel experiments, the conversion of this diglyceride to dioctanoylphosphatidate was studied in 32P-labeled vessels. Dioctanoylglycerol produced a slow-onset sustained contraction that was dependent on extracellular calcium. This was accompanied by the generation of the lipid dioctanoylphosphatidate. Noradrenaline and KCl induced rapid-onset sustained contractions and increased the production of dioctanoylphosphatidate (75% and 91%, respectively). In addition, dioctanoylglycerol levels were reduced (41%) after noradrenaline stimulation, suggesting activation of diacylglycerol kinase. In contrast, the contractile response to angiotensin II was transient, and this agonist did not significantly affect the conversion of dioctanoylglycerol to phosphatidate. Noradrenaline markedly increased (fourfold) the formation of endogenous phosphatidate, whereas endogenous 1,2-diacylglycerol was increased (47%) with angiotensin II. These results demonstrate that phosphatidate formation is regulated by vasoconstrictor hormones during receptor-mediated contraction, independent of phosphodiesterase mass. Modulation of the levels of lipid second messengers downstream from phospholipid hydrolysis may represent a mechanism by which agonists that act through the same signaling network produce divergent contractile responses. (Circulation Research 1993;72:1163-1171)

KEY WORDS • diacylglycerol • contractile response • noradrenaline • angiotensin II • diacylglycerol kinase

The binding to their receptors of vasoconstrictor hormones such as noradrenaline (NA) and angiotensin II (Ang II) links these agonists to a common signaling system and activates phospholipase C (PLC), which hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate. This results in the production of two second messengers, inositol 1,4,5-trisphosphate (Ins 1,4,5-P3) and 1,2-diacylglycerol (1,2-DAG).1 There is good evidence to suggest that Ins 1,4,5-P3 can initiate contraction by stimulating the release of calcium from intracellular stores.2 However, the sequence of events that sustains contraction in vascular smooth muscle is still unclear. 1,2-DAG is known to serve as a second messenger in protein kinase C (PKC)-mediated signal transduction,3 but whether PKC plays a role in vascular smooth muscle contraction is not established. It has been proposed that PKC enhances contraction by increasing calcium influx and the phosphorylation of myosin light chain kinase4,5 or, conversely, that it reduces contraction by negative feedback on phospholipid breakdown.6-8 There is now a growing body of evidence to support an alternative mechanism for sustained smooth muscle contraction via calcium sensitization. It has been shown that phosphatase activity is decreased during tonic contraction by a guanine nucleotide–binding protein (G protein)–mediated effect, leading to prolonged phosphorylation of the 20-kd light chains of myosin and increased calcium sensitivity.9,10 As yet, the second messengers involved in this sensitization process are unknown, although recent studies have implicated arachidonic acid.11 It should be noted that 1,2-DAG is an important source of 1) arachidonic acid, which is an essential precursor of prostaglandins and eicosanoids, and 2) phosphatidic acid (PA). The functional role of PA in signal transduction is uncertain. However, this lipid has been shown to increase calcium influx and efflux,12-14 to stimulate PLC hydrolysis of phospholipids,15,16 to cause contraction of vascular smooth muscle cells,17 to be involved in carbachol-induced contraction of guinea pig taenia coli,18 and to induce cell proliferation,19 possibly by its ability to interact with GTPase-activating protein.19 Recently, some of these observations have been ascribed to contamination of PA with lyso-PA,20 although this is not resolved fully.21 Therefore, the mechanisms that regu-
late the levels of PA in contractile tissues are of considerable interest.

Previously, we have reported that the vasoconstrictor hormones NA and Ang II have different effects on the accumulation of 1,2-DAG and PA during the onset of contraction in rat small arteries. This suggested that differential metabolism of 1,2-DAG might be important in discriminating the effects of these pressor agents that act on distinct receptors but are coupled to the same transmembrane signaling system. In the present study, we have extended these findings by examining the role of diacylglycerol (DAG) kinases in the production of PA in intact vessels, using the cell-permeable short-chain diglyceride diacylglycerol (diC3), and by measuring the production of the novel lipid dioctanoylphosphatic acid (PA8). We have studied both basal diC3 metabolism and the effects of the vasoconstrictor hormones NA and Ang II. Concomitant functional studies show that, in this arterial preparation, NA produced a sustained contraction but that the Ang II response was transient. From our results, we propose that vasoconstrictor hormones may influence the production of PA through a receptor-mediated effect on DAG kinase(s). The differences in the metabolism of 1,2-DAG that result may underlie the distinct contractile profiles observed with these agonists.

**Materials and Methods**

**Animals**

Adult female Wistar and Sprague-Dawley rats (approximately 200 g body weight) were used for all the experiments described.

**Tissue Preparation**

The animals were killed by stunning followed by cervical dislocation. Two subcutaneous small arteries (<300 μm i.d.) supplied by the axillary artery were dissected from each rat and cleaned of adherent fat and connective tissue. In experiments determining the levels of 1,2-DAG, the tissues were transferred to an Eppendorf tube containing 100 μl culture medium (M199) composed of (mM) NaCl 128, KCl 5.4, MgSO4 • 7H2O 0.8, Na2HPO4 • 2H2O 0.34, CaCl2 • 2H2O 1.3, NaHCO3 4.2, KH2PO4 0.44, Fe(NO3)3 • 9H2O 0.002, glucose 5.6, and HEPES 25. The tissues were left to equilibrate at 37°C in a shaking water bath for 1 hour. In experiments determining the levels of PA, vessels were prelabeled with [32P]PA, as previously described. Briefly, vessels were first phosphate-depleted by incubation in phosphate-free HEPES buffer containing (mM) NaCl 129, KCl 5.8, MgSO4 • 7H2O 0.8, CaCl2 • 2H2O 1.26, NaHCO3 4.2, Fe(NO3)3 • 9H2O 0.002, glucose 5.6, and HEPES 25 for 1 hour at 37°C. They were then transferred to an Eppendorf tube containing 100 μl of this buffer plus 250 kBq [32P]PO4 and incubated at 37°C for a further hour. We have previously shown that this period of prelabeling is optimum for the incorporation of [32P]orthophosphate into intracellular PA in these tissues.

**Incubation and Stimulation Conditions**

After the 60-minute incubation in M199 or [32P]PO4 buffer, 768 μl of either prewarmed M199 or phosphate-free HEPES buffer containing diC3 at the required concentration (diluted from a 50-mM stock in dimethyl sulfoxide) or 0.5% dimethyl sulfoxide (the maximum concentration used in any experiment in the study) as control was added. The addition of this volume of buffer returned the phosphate concentration to normal (0.78 mM). The tissues were then incubated with diC3 for 10 minutes at 37°C. After this period, they were transferred to either 100 μl prewarmed M199 or normal HEPES buffer (0.78 mM PO4, 288 kBq·ml⁻¹) and, after 1 minute, stimulated by the addition of 100 μl prewarmed vehicle (M199 or normal phosphate HEPES buffer), 100 μl NA (final concentration, 15 μM), 100 μl Ang II (final concentration, 100 nM), or 1 ml KCl (final concentration, 125 mM; molar substitution with NaCl) for 20 seconds. Agonists were used at these concentrations to produce a maximal contraction in this preparation and to ensure maximal activation of the phosphoinositide signaling system.

**Lipid Extraction**

After the 20-second stimulation, the tissue was removed and homogenized in 0.5 ml chloroform/methanol (1:2 [vol/vol]) for 1,2-DAG or chloroform/methanol/ HCl (20:40:1 [vol/vol/vol]) for [32P]PA. The homogenate was left on ice for 10 minutes, and then 0.5 ml chloroform and 0.5 ml distilled water were added. The mixture was agitated and spun at 12,000g for 3 minutes. The upper phase was removed and discarded, and the lower lipid-containing phase was transferred to a glass tube and dried under a stream of N2 gas. The neutral lipid samples were reconstituted in 250 μl chloroform layered with N2 gas and stored at −70°C. The acidic lipid samples were immediately separated by thin-layer chromatography (TLC) (see below). The residual tissue pellet was dissolved in 500 μl of 2 M NaOH, and protein content was estimated by the method of Lowry et al. Previsably, we have shown that the contractile state of our tissue does not affect either the solubility of the protein or the amount of lipid extracted; therefore, we used sample protein content to normalize our results.

**Quantitation of 1,2-sn-DAG**

1,2-DAG was quantified by enzymatic conversion of 1,2-DAG to [32P]PA using *Escherichia coli* DAG kinase and [γ-32P]ATP, according to the method of Preiss et al. Known amounts of [14C],1,2-dioleoyl-sn-DAG were run with each assay, as previously described, to quantify the conversion. A standard curve for the conversion of diC3 to [32P]PA8 by *E. coli* DAG kinase was constructed over the range of 50–500 pmol. The formation of [32P]PA8 was linear and quantitative over this range of substrate (data not shown).

**Separation of [32P]PA and [32P]PA8**

TLC was used to separate the dioctanoyl-PA from the endogenous species of PA. Samples of PA, from either in vitro conversion or endogenous labeling, were spotted onto oxalate-coated silica gel 60 TLC plates, as previously described. The plates were developed in a solvent mixture of ethyl acetate/acetone/acidic acid/2,2,4-trimethyl pentane (9:2.4:5 [vol/vol/vol]). Authentic [32P]PA8 (prepared by enzymatic conversion of diC3 to [32P]PA8) was run on each plate as a standard. [32P]PA was visualized by autoradiography, scraped from the plate, and determined by liquid scintillation counting. This system gave a clear separation of PA and PA8 with
refractory index (RF) values of 0.36 and 0.2, respectively. For determination of extracted 1,2-DAG, the amount of 1,2-DAG converted to PA was calculated from the specific activity of the $[^{32}P]ATP$ and the sample volumes. Results are expressed as picomoles of DAG per milligram protein. Endogenously labeled PA is expressed as disintegrations per minute per milligram protein.

**Specific Activity of Intracellular $[^{32}P]ATP$**

After incubation with $[^{32}P]PO_4$, as described above, vessels were homogenized in 500 μl ice-cold 50% tri-chloroacetic acid and left on ice for 5 minutes. The homogenate was spun at 12,000g for 3 minutes. The supernatant was washed five times with 5 ml diethyl ether and diluted to 2 ml with added water. The pellet was dissolved in 2 M NaOH, and protein content was determined by the method of Lowry et al. The sample was injected onto a Partisil 10 SAX high-performance liquid chromatography (HPLC) column (250×4.6 mm, Technicol, Stockport, UK) through a sample injector fitted with a 2-ml sample loop. The column was eluted at 1.2 ml.min$^{-1}$ with a nonlinear preprogrammed gradient of water/1.2 M ammonium formate/orthophosphoric acid (pH 3.7). Ultraviolet absorbance was monitored at 254 nm to detect ATP. The fractions corresponding to the ATP peak were collected, and radioactivity was determined by liquid scintillation counting. The total counts in the peak were divided by the concentration of ATP (determined from known standards) to give the specific activity of $[^{32}P]ATP$ in disintegrations per minute per nanomole.

**Determination of Contractile Response**

After dissection, segments of small arteries were mounted as ring preparations in a myograph. After mounting, the segments were maintained in physiological salt solution (PSS) containing (mM) NaCl 119, KCl 4.7, MgSO$_4$, 7H$_2$O 1.17, KH$_2$PO$_4$ 1.18, NaHCO$_3$ 2.5, CaCl$_2$ 2.5, glucose 5.5, and EDTA 0.026, pre-gassed with 5% CO$_2$ in O$_2$ at 37°C for 1 hour, and then set to an internal circumference at which they were held just under tension. The resting tension–internal circumference relation was then determined, and the vessels were set to a normalized internal circumference, which was 90% of that which the vessel would have when relaxed and under a transmural pressure of 100 mm Hg. The vessels were then activated with NA (10 μM) in K$^+$-PSS (equimolar substitution of KCl for NaCl) twice, followed by NA in PSS, K$^+$-PSS, and finally NA in K$^+$-PSS. Each solution was applied for 2 minutes before washing out with PSS. Vessels were allowed to relax between these activations. The vessels were then equilibrated in HEPES buffer (composition as for M199) before stimulation with the following agonists: 15 μM NA, 100 nM Ang II, 100 μM diC$_8$, and 125 mM KCl±1 μM prazosin. The agonists were applied in HEPES buffer for 5 minutes, and the responses were recorded with a chart recorder (Grass Instrument Co., Quincy, Mass.). A minimum of 15 minutes was allowed for a recovery period between each agent. In the experiments using prazosin, the vessels were preincubated with prazosin for 5 minutes before stimulation with KCl. The response to 100 μM diC$_8$ was recorded over a period of 10 minutes in the presence and absence of calcium in the external medium.

**Materials**

M199 was purchased from GIBCO, Paisley, Scotland. NA hydrochloride, Ang II, and prazosin hydrochloride were supplied by Sigma Chemical Co., Poole, UK. Dioctanoyl-sn-glycerol and cardiolipin were supplied by Avanti Polar Lipids, Birmingham, Ala. DAG kinase (E. coli) was supplied by Lipidex, Inc., Westfield, N.J. Radioisotope $[^{32}P]$dioleoylphosphatidylcholine (specific activity, 4 GBq·mmol$^{-1}$) was supplied by Du Pont, New England Nuclear, Boston; $[^{32}P]$ATP (specific activity, 370 MBq·μl$^{-1}$) was from Amersham, Buckinghamshire, UK; and $[^{32}P]PO_4$ (specific activity, 18.5 TBq·mmol$^{-1}$) was from ICN Flow, High Wycombe, UK. TLC plates (Merck 5721) were purchased from British Drug House, Poole, UK.

**Statistical Analyses**

Results are expressed as mean±SEM, and comparisons between basal and agonist-induced changes were made by Student’s $t$ test.

**Results**

**Conversion of diC$_8$ to PA$_8$**

We chose diC$_8$ to study the formation of phosphatidate in intact small arteries because it is a cell-permeable diglyceride that is rapidly and predominantly converted to PA in other cell systems. Also, by use of TLC it is possible to separate the PA$_8$ from endogenous PA in the cell extracts and therefore use it as a marker of DAG kinase activity in situ. Indeed, to verify that the PA$_8$...
produced by in situ phosphorylation is the same as that produced by in vitro phosphorylation of diC₈, we used HPLC to demonstrate that both forms coeluted exactly (Figure 1). Incubation of small arteries with 100 μM diC₈ resulted in the rapid appearance of PA₈, which was maximal at 5 minutes and remained stable up to 15 minutes, after which there was a slight decline to 30 minutes (Figure 2). In a further set of experiments, the vessels were incubated with 100 μM diC₈ for 10 minutes and then transferred to medium without diC₈ to follow the metabolism of this lipid. The tissue levels of diC₈ fell rapidly and were virtually undetectable after 15 minutes. However, the diC₈ did not accumulate as PA₈, because levels of this lipid fell in parallel with substrate (Figure 3). Incubation of the arteries with increasing concentrations of diC₈ (from 10 to 100 μM) resulted in dose-related increases in extracted diC₈ and PA₈ (Table 1). The increase in the diglyceride levels (sevenfold from 10 to 100 μM) was greater than the increase in the corresponding phosphatidate (twofold from 10 to 100 μM).

To study the effects of agonists on the conversion of 1,2-DAG to PA, we preincubated the vessels with varying concentrations of diC₈ and then measured the levels of [32P]PA₈ when the vessels were stimulated to contract. This protocol was chosen, rather than determining the rate of appearance of [32P]PA₈ with a single-dose level of diC₈, because of the very rapid metabolism of PA₈ in our tissue. All stimulations were performed in the absence of diC₈ in the external medium to facilitate the measurement of tissue diC₈. However, because this would result in the measurement of [32P]PA₈ under non–steady-state conditions, we also determined the levels of [32P]PA₈ in the continued presence of 100 μM diC₈. There was a lower basal incorporation of 32P into [32P]PA and [32P]PA₈ in the continued presence of diC₈ in the external medium (Table 2). This most probably reflects the difference in the rat strain used for these experiments (Wistar versus Sprague-Dawley). However, the [32P]PA/[32P]PA₈ ratio was 3 for both rat strains, indicating a similar uptake of diC₈ and conversion to [32P]PA₈.

Removal of diC₈ from the external medium did not alter the levels of [32P]PA and [32P]PA₈ during the NA (15 μM) stimulation (Table 2). However, the lower control values of the group in the presence of diC₈ (100 μM) resulted in an apparently greater increase in the accumulation of 32P in these phospholipids during stimulation with this agonist (–sixfold in the presence of diC₈ compared with –twofold when diC₈ was removed from the medium). Ang II stimulation did not significantly alter the levels of [32P]PA or [32P]PA₈ when diC₈ was present or absent from the external medium (Table 2). These results demonstrate that removal of diC₈ from the incubation medium before stimulation did not significantly alter the effects of the agonists on [32P]PA and [32P]PA₈ levels.

In addition, since the accumulation of 32P in PA would be dependent on the specific activity of [32P]ATP, we measured this parameter in our preparation. After 20 seconds of agonist stimulation, there was no change in the specific activity of [32P]ATP (control, 5.7±0.6 dpm×10⁶·nmol ATP⁻¹; 15 μM NA, 7.0±0.4 dpm×10⁶·nmol ATP⁻¹ [n=4]; 100 nM Ang II, 5.0±0.3 dpm×10⁶·nmol ATP⁻¹ [n=2]). Therefore, we are confident that changes in [32P]PA reflect changes in PA mass.
**Effects of NA and Ang II**

The doses of agonist used are those that elicit a maximal contractile response in these vessels. A time point of 20 seconds of stimulation was chosen because this is during the initial phase of contraction and is also the time when the greatest differences in lipid profiles have been observed. Figure 4 illustrates the mechanical response of these small arteries to the agonists. Both agents induced a contraction. However, the response was of smaller magnitude and not maintained with Ang II. These contractile profiles are in agreement with other functional studies in small arteries.29–31

NA (15 μM) increased the levels of [32P]PA8 after incubation with 50 μM (NA, +61%; p<0.05) or 100 μM (NA, +75%; p<0.02) diC8 (Figure 5). The levels of diC8 were reduced after stimulation with NA (−46%, p<0.02 with 100 μM diC8 only) (Figure 6). Stimulation for 20 seconds with Ang II (100 nM) did not markedly affect either the accumulation of [32P]PA8 or the levels of diC8 (Figures 5 and 6).

**Effects of Membrane Depolarization With Potassium**

It has been shown that raised levels of intracellular calcium will inhibit PA CTP transferase and thus slow the metabolism of PA through the phosphoinositol cycle.32 Therefore, we determined the effects of potassium depolarization (125 mM KCl), a maneuver that raises intracellular calcium levels.33 This level of KCl (125 mM) induced a rapid and sustained contraction in small arteries, similar to the response seen with NA (Figure 4), and an increase in the production of [32P]PA8 (+91%, p<0.01) (Figure 7). However, in the presence of the α1-adrenoceptor antagonist prazosin (1 μM), the effect on [32P]PA8 was abolished (Figure 7). This indicated that the effects on PA levels with potassium alone were due to the action of catecholamines released by depolarization of nerve terminals in the vessel wall. In the presence of prazosin (1 μM), the initial phase of the contractile response was also slightly reduced, approximately 50% (Figure 4), showing a contribution of catecholamines to the contraction.

**Effects of diC8**

Incubation of small arteries with 100 μM diC8 for 10 minutes resulted in the slow development of tension (Figure 4). Replacement of the external medium with "nominally free calcium" solution abolished the response, implying that calcium influx was involved in the contraction. At none of the dose levels of diC8 was there an effect on basal levels of endogenous 1,2-DAG or [32P]PA (Table 1).

**Effects of diC8 on 1,2-DAG and [32P]PA During Agonist Stimulation**

The levels of endogenous [32P]PA were markedly elevated after a 20-second stimulation with NA (15 μM) but were unaffected by Ang II (100 nM) in the absence of diC8 (Table 3), in agreement with our previous results.22 Preincubation with increasing concentrations of diC8 did not alter this response (Table 3), indicating that elevation of cellular 1,2-DAG levels with a synthetic diglyceride did not change the agonist-induced

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**Table 1. Effects of Dioctanoylglycerol on the Basal Levels of Endogenous 1,2-Diacylglycerol and [32P]Phosphatidic Acid and the Accumulation of [32P]Dioctanoylphosphatidic Acid**

<table>
<thead>
<tr>
<th>Vessel extract</th>
<th>diC8 concentration (μM)</th>
<th>0</th>
<th>10</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>diC8 (nmol·mg protein⁻¹)</td>
<td></td>
<td>...</td>
<td>0.036±0.01</td>
<td>0.170±0.04</td>
<td>0.272±0.04</td>
</tr>
<tr>
<td>1,2-DAG (nmol·mg protein⁻¹)</td>
<td></td>
<td>1.45±0.25</td>
<td>1.28±0.20</td>
<td>1.49±0.32</td>
<td>1.38±0.06</td>
</tr>
<tr>
<td>[32P]PA8 (dpm·mg protein⁻¹)</td>
<td></td>
<td>...</td>
<td>5.013±704</td>
<td>6.438±1.349</td>
<td>9.641±1.623</td>
</tr>
<tr>
<td>[32P]PA (dpm·mg protein⁻¹)</td>
<td></td>
<td>22.83±1.973</td>
<td>24.60±3.394</td>
<td>23.49±2.899</td>
<td>28.29±4.351</td>
</tr>
</tbody>
</table>

Values are mean±SEM from a minimum of five separate experiments.

Small arteries were incubated with varying concentrations of diC8, and then the lipids were extracted. Mass levels of 1,2-DAG and diC8 and the incorporation of [32P]PO4 into PA and PA8 were determined as described in "Materials and Methods."

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**Table 2. Effects of Agonist Stimulation on the Accumulation of [32P]Phosphatidic Acid and [32P]Dioctanoylphosphatidic Acid in the Presence and Absence of Dioctanoylglycerol in the External Medium**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[32P]PA (dpm·mg protein⁻¹)</th>
<th>+diC8</th>
<th>−diC8</th>
<th>[32P]PA8 (dpm·mg protein⁻¹)</th>
<th>+diC8</th>
<th>−diC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 μM NA</td>
<td>75.408±9.314*</td>
<td>94.58±6.963*</td>
<td>16.86±2.017*</td>
<td>25.37±2.278*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 nM Ang II</td>
<td>27.74±1.780</td>
<td>12.49±1.762</td>
<td>12.70±7.01</td>
<td>6.35±6.90</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PA, phosphatic acid; PA8, dioctanoylphosphatidic acid; diC8, dioctanoylglycerol; NA, noradrenaline; Ang II, angiotensin II. Values are mean±SEM from a minimum of five experiments.

Small arteries were prelabeled with [32P]PO4 and then incubated for 10 minutes with 100 μM diC8. The tissues were then stimulated with NA or Ang II either in the presence (+diC8) or absence (−diC8) of 100 μM diC8 in the external medium. The incorporation of [32P]PO4 into PA and PA8 was determined as described in "Materials and Methods."

* p<0.001 and tp<0.05 vs. corresponding control value by Student’s t test.
Effects of KCl on 1,2-DAG and [32P]PA

In the absence of diC₈, membrane depolarization with KCl resulted in an increase in [32P]PA. However, this effect was attenuated by blockade of α₁-adrenoceptors, indicating that it was due to neuronal catecholamine release (Table 4). After incubation for 10 minutes with 100 μM diC₈, there was a similar increase in [32P]PA that again was attenuated by prazosin. There was no change in endogenous 1,2-DAG levels in the presence of KCl either alone or in the presence of prazosin or diC₈ (100 μM) (data not shown).

Discussion

diC₈ is a cell-permeable diglyceride that has been used to study the metabolism of 1,2-DAG in intact cells.²⁸,³⁴,³⁵ The objective of the present investigation was to examine the conversion of diC₈ to the novel lipid PA₈ in response to vasoconstrictor hormones. Previously, we have shown that NA and Ang II have different effects on PA formation in small arteries. In this study, we extend this finding by demonstrating that these agonists differentially affect the metabolism of diC₈.

In agreement with studies in other cell types,²⁸,³⁴,³⁵ diC₈ was rapidly phosphorylated to produce PA₈, the levels of which remained stable for up to 15 minutes. The PA₈ was efficiently metabolized, as shown by its complete disappearance 15 minutes after the removal of substrate from the incubation medium. The uptake of diC₈ by the arteries was dose-related; there was a sevenfold increase in vessel diC₈ for a 10-fold increase in external concentration. In contrast, PA₈ levels were increased only twofold, further suggesting active metabolism of this novel lipid. We were unable to determine the pathway of PA₈ metabolism in our tissue because of the relatively low degree of [3P]PA radiolabeling of this lipid. However, in two previous studies, [H]diC₈ was shown to accumulate in other phospholipids²⁸,³⁵ with a slower time course than the appearance of [32P]PA.²⁸ Bishop and Bell further separated this phospholipid fraction and demonstrated a significant incorporation of radiolabel into phosphatidylinositol with initially far less radioactivity in phosphatidylcholine. Similarly, Severson and See-Cheong observed only slight incorporation of [H]diC₈ into phosphatidylcholine in rabbit aortic smooth muscle cells. Therefore, it is most likely that the PA₈ in our preparation was incorporated into phosphatidylinositol, presumably by the enzyme CTP-PA cytidyl transferase. Obviously, this is an active metabolic pathway in small arteries, implying that the levels of PA are tightly controlled in nonstimulated tissue. These data also show that diC₈ is a substrate for DAG kinase in such small vessels.

KCl did not alter either diC₈ or endogenous 1,2-DAG metabolism, implying that elevation of intracellular calcium and membrane depolarization did not affect...
DAG kinase or CTP–PA cytidyl transferase activity. These results are different from those of Lapetina et al., who demonstrated that elevated calcium decreased CTP–PA cytidyl transferase activity, leading to PA accumulation. Recently, a study in leukocytes in vitro has shown that elevated calcium inhibits PA phosphohydrolase, resulting in increased PA levels. The activities of these two enzymes would have to be measured directly in small arteries to resolve these discrepancies.

Next, we studied the effects of the vasoconstrictor hormones NA and Ang II on the conversion of diC₈ to PA₆. Increased phosphorylation of diC₈ in response to agonist would be consistent with activation of DAG kinase. In addition, because the concentration of the synthetic DAG would be unaltered by hormone stimulation, changes in its phosphorylation would reflect agonist-dependent changes in DAG kinase activity independent of 1,2-DAG mass. However, MacDonald et al. have previously shown that during platelet-derived growth factor stimulation of Swiss 3T3 cells the endogenous 1,2-DAG produced competes with didecanoyl glycerol (diC₁₀), actually inhibiting the phosphorylation of this synthetic diglyceride. Therefore, we measured both diC₈ and [³²P]PA₆ levels and endogenous 1,2-DAG and [³²P]PA during agonist stimulation to ensure similar phosphorylation of these lipids. There was a marked increase in [³²P]PA₆ levels after NA treatment. This appeared to be due to increased phosphorylation as diC₈ levels were decreased; a similar effect on endogenous 1,2-DAG and [³²P]PA was seen. These results indicate that the increased PA levels were due to activation of DAG kinase by NA stimulation. This is in agreement with a study in rat liver in which phenylephrine (an α₁-adrenoreceptor agonist) increased DAG kinase activity. In contrast, Ang II did not alter the production of either [³²P]PA₆ or endogenous [³²P]PA, implying that this agonist had little effect on diglyceride or phosphatidate metabolism. Therefore, the results with these two agonists suggest that NA activated DAG kinase and Ang II did not. In addition, diC₈ was phosphorylated in a similar manner to endogenous diglycerides, indicating that there was no competition between substrates. This may suggest activation of both membrane-bound arachidonate-specific and nonspecific soluble kinases by NA. The DAG kinases present in small arteries and the effect of vasoconstrictor hormones on their activities are currently under investigation.

Exposure of the vessels to high K⁺ to raise intracellular calcium caused an increase in [³²P]PA, which was abol-

![Graph showing the effect of agonists on diocanoylglycerol (diC₈) levels in resistance vessels. Vessels were incubated with increasing concentrations of diC₈. The vessels were then stimulated for 20 seconds in the absence of diC₈ with 15 μM noradrenaline (○), 100 nM angiotensin II (△), and vehicle (□). The lipids were extracted, and the levels of diC₈ were determined as described in “Materials and Methods.” The results are expressed as mean±SEM from a minimum of five separate experiments. *p<0.05 vs. vehicle as determined by Student’s t test.](http://circres.ahajournals.org/)

![Bar graphs showing the effects of elevation of intracellular calcium with KCl on the levels of dioctanoylglycerol (diC₈) and [³²P]dioctanoylphosphatidic acid ([³²P]PA₆) in small arteries. DAG, diacylglycerol; Pz, prazosin. Vessels were prelabeled with [³²P]PO₄ (phosphatidic acid only) and then incubated with diC₈ (100 μM) for 10 minutes. They were then exposed to 125 mM KCl±1 μM Pz for 20 seconds in the absence of diC₈. The lipids were extracted, and the levels of diC₈ (panel A) and [³²P]PA₆ (panel B) were determined as described in “Materials and Methods.” The results are expressed as mean±SEM from a minimum of five separate experiments. Open bars indicate control (basal) values; hatched bars indicate 125 mM KCl; and crosshatched bars indicate 125 mM KCl+1 μM Pz. *p<0.05 vs. basal level as determined by Student’s t test.](http://circres.ahajournals.org/)
lished by α₁-adrenoreceptor blockade with prazosin. These data further confirm that catecholamines acting on α₁-receptors activate DAG kinase, an effect that is not solely a result of elevated intracellular calcium or membrane depolarization. In neuronal tissues, K⁺-induced depolarization increased inositol phosphates, implicating PLC activation. This effect was not entirely due to secondary release of neurotransmitters. There was no evidence that K⁺ depolarization activated PLC in small arteries, since neither 1,2-DAG nor [32P]PA levels were altered. This is in agreement with a study in cultured vascular smooth muscle cells, in which K⁺ depolarization did not increase the levels of [32P]PA. Therefore, there would appear to be differences between tissues in the effects of depolarization on PLC activity.

**Arterial Contractile Responses**

There were major differences in the contractile responses to Ang II compared with NA and KCl. Ang II induced a variable and nonsustained contraction. Similar responses to Ang II have been reported by other workers in different vascular beds. The lack of a sustained contraction does not appear to be explained by a termination of the Ang II response, e.g., receptor uncoupling, internalization of receptors, or Ang II degradation, because we have shown the sustained production of 1,2-DAG for time periods up to 5 minutes. Therefore, it would appear that the generation of inositol phosphates and arachidonate containing 1,2-DAG is not sufficient to ensure a maintained contraction in small arteries. The stimulated production of other vasoactive substances such as PA in the case of NA may be necessary to sustain contraction. Whether PA modulates tonic contraction through direct mechanisms or indirectly through an effect on calcium levels remains to be investigated. Also, it should be noted that incubation of resistance arteries with 100 μM diC₈ resulted in the slow development of tension over a 10-minute period. This response was dependent on extracellular calcium and similar to that seen with phorbol esters in small arteries, suggesting that activation of PKC may have occurred. However, it is also possible that the PA₈ formed was involved in the contraction; certainly the results with Ang II suggest that elevation of 1,2-DAG levels alone do not necessarily result in sustained contraction. There was no alteration in basal levels of endogenous 1,2-DAG and [32P]PA during incubation with diC₈. Therefore, increased cellular DAG and, presumably, activation of PKC did not affect basal levels of these lipids, indicating

**Table 3. Effects of Agonist Stimulation on the Levels of Endogenous [32P]Phosphatidic Acid in the Presence of Dioctanoylglycerol**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 μM diC₈</th>
<th>10 μM diC₈</th>
<th>50 μM diC₈</th>
<th>100 μM diC₈</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22,834±1,973</td>
<td>24,601±3,394</td>
<td>23,495±2,899</td>
<td>28,294±4,350</td>
</tr>
<tr>
<td>15 μM NA</td>
<td>86,754±8,819</td>
<td>77,156±6,853</td>
<td>66,413±6,299</td>
<td>75,408±9,314</td>
</tr>
<tr>
<td>100 nM Ang II</td>
<td>24,513±1,878</td>
<td>23,348±1,875</td>
<td>24,474±1,659</td>
<td>27,744±1,780</td>
</tr>
</tbody>
</table>

PA, phosphatidic acid; diC₈, dioctanoylglycerol; NA, noradrenaline; Ang II, angiotensin II. Values are mean±SEM from a minimum of five separate experiments.

Small arteries were prelabeled with [32P]PO₄ and then incubated with diC₈ at increasing concentrations for 10 minutes. The vessels were stimulated for 20 seconds with vehicle (control), NA, or Ang II. The lipids were then extracted, and the levels of [32P]PA were determined as described in “Materials and Methods.”

*p<0.01 vs. corresponding control value by Student’s t test.

**Figure 8. Bar graph showing the effects of dioctanoylglycerol (diC₈) on the levels of endogenous 1,2-diacylglycerol (1,2-DAG) during agonist stimulation of small arteries.** Vessels were incubated for 10 minutes with diC₈ (100 μM) and then stimulated for 20 seconds in the absence of diC₈ with vehicle (basal, open bar), 15 μM noradrenaline (NA, hatched bar), and 100 nM angiotensin II (AI, crosshatched bar). The lipids were extracted, and the mass levels of 1,2-DAG were determined as described in “Materials and Methods.” The results are expressed as mean±SEM from a minimum of five separate experiments. *p<0.05 and **p<0.001 vs. basal value as determined by Student’s t test.

**Table 4. Effects of Elevation of Intracellular Calcium With KCl, in the Presence and Absence of α₁-Adrenoreceptor Blockade, on Endogenous [32P]Phosphatidic Acid**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 μM diC₈</th>
<th>100 μM diC₈</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22,292±1,774</td>
<td>28,294±4,350</td>
</tr>
<tr>
<td>125 mM KCl</td>
<td>36,124±2,935*</td>
<td>36,043±1,675</td>
</tr>
<tr>
<td>125 mM KCl + 1 μM Pz</td>
<td>27,696±2,706</td>
<td>33,188±4,314</td>
</tr>
</tbody>
</table>

PA, phosphatidic acid; diC₈, dioctanoylglycerol; Pz, prazosin. Values are mean±SEM from a minimum of five separate experiments.

Small arteries were prelabeled with [32P]PO₄ and then incubated with vehicle or diC₈ (100 μM) for 10 minutes. They were then exposed to KCl+Pz for 20 seconds. The lipids were extracted, and the levels of [32P]PA were determined as described in “Materials and Methods.”

*p<0.003 vs. corresponding control value by Student’s t test.
that DAG kinase activity was not modulated by PKC per se. This confirms observations reported in other tissues.38,42,43

In summary, we have demonstrated differential regulation of the metabolism of diglycerides during contraction induced by two vasoactive agonists. It is possible that this is one way by which there is cell recognition of a particular ligand when the inositol phospholipid signaling system used is common to many stimuli. In addition, the route of production of metabolites and their identity may be pertinent to the rate of contraction observed in vascular smooth muscle and the degree to which it can be sustained.

Acknowledgment

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