Norepinephrine and endothelin activate diacylglycerol kinases in caveolae/rafts of rat mesenteric arteries: agonist-specific role of PI3-kinase

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Submitted 25 October 2006; accepted in final form 29 December 2006

Clarke CJ, Ohanian V, Ohanian J. Norepinephrine and endothelin activate diacylglycerol kinases in caveolae/rafts of rat mesenteric arteries: agonist-specific role of PI3-kinase. Am J Physiol Heart Circ Physiol 292: H2248–H2256, 2007. First published January 5, 2007; doi:10.1152/ajpheart.01170.2006.—The phosphatidylinositol (PI) signaling pathway mediates norepinephrine (NE)- and endothelin-1 (ET-1)-stimulated vascular smooth muscle contraction through an inositol-trisphosphate-induced rise in intracellular calcium and diacylglycerol (DG) activation of protein kinase C (PKC). Subsequent activation of DG kinases (DGKs) metabolizes DG to phosphatic acid (PA), potentially regulating PKC activity. Because precise regulation and spatial restriction of the PI pathway is necessary for specificity, we have investigated whether this occurs within caveolae/rafts, specialized plasma membrane microdomains implicated in vascular smooth muscle contraction. We show that components of the PI signaling cascade-phosphatidylinositol 4,5-bisphosphate (PIP2), PA, and DGK-θ are present in caveolae/rafts prepared from rat mesenteric small arteries. Stimulation with NE or ET-1 induced [33P]PIP2 hydrolysis solely within caveolae/rafts. NE stimulated an increase in DGK activity in caveolae/rafts alone, whereas ET-1 activated DGK in caveolae/rafts and noncaveolae/rafts; however, [33P]PA increased in all fractions with both agonists. Previously, we reported that NE activated DGK-θ in a phosphatidylinositol 3-kinase (PI3-kinase)-dependent manner; here, we describe PI3-kinase-dependent DGK activation and [33P]PA production in caveolae/rafts in response to NE but not ET-1. Additionally, PKB, a potential activator of DGK-θ, translocated to caveolae/rafts in response to NE but not ET-1, and PI3-kinase inhibition prevented this. Furthermore, PI3-kinase inhibition reduced the sensitivity of contraction to NE but not ET-1. Our study shows that caveolae/rafts are major sites of vasoconstrictor hormone activation of the PI pathway in intact small arteries and suggest a link between lipid signaling events within caveolae/rafts and contraction.

Signal transduction; vascular smooth muscle; lipid second messengers; phosphatidylinositol 3-kinase

The phosphatidylinositol (PI) signaling pathway is important in vascular smooth muscle (VSM) function. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC) produces the second messengers 1,4,5-inositol 1,4,5-trisphosphate [Ins (1,4,5)P3] and diacylglycerol (DG). Ins (1,4,5)P3 stimulates release of store calcium, initiating contraction through the myosin light chain kinase pathway. DG is the physiological activator of protein kinase C (PKC), implicated in calcium sensitization of force (17, 44).

Diacylglycerol kinases (DGKs) phosphorylate DG to produce phosphatic acid (PA). In nonstimulated cells, their activity is low, allowing DG to be used for glycerolipid biosynthesis, but on activation of the PI pathway, DGK activity increases. This drives the conversion of DG generated by PI-PLC to PA, presumably inactivating PKC, although it could also sustain signaling by increasing PIP2 formation (reviewed in Ref. 48). PA is also a bioactive lipid with several putative targets, such as sphingosine kinase 1 (11), Raf-1 (18, 41), phosphatases (24), and phosphoinositide 4-phosphate 5-kinase (23). Aside from interaction with protein effectors, PA may also play a role in signal transduction as a precursor of phosphoinositide lipids, necessary for replenishment of PI pools after agonist-stimulation of PLC (48).

It is well known that subcellular localization and targeting of signaling molecules are important for regulating signal transduction in biological systems, and a major mechanism occurs through interaction with lipids at specific membrane sites (reviewed in Ref. 46). However, there is relatively sparse information on the generation of lipids at specific membrane sites. Lipid rafts are plasma membrane microdomains enriched in cholesterol and sphingolipids. Although biochemically similar, caveolae are a subset of lipid rafts identified by their characteristic flask-shaped morphology and the presence of caveolins. Caveolae/lipid rafts are implicated in signal transduction, owing to their high content of signaling proteins such as G protein-coupled receptors (GPCRs), heterotrimeric and small G proteins, and PKC (40). However, differences in morphology and associated proteins suggest that these domains may serve distinct or indeed complementary functions. Caveolae/lipid rafts are abundant in smooth muscle and endothelial cells and have been implicated in VSM contraction (2, 5, 25). In nonmuscle cells, PI lipids have been identified in lipid rafts (20, 38) and in A431 cells, and PIP2 hydrolysis in response to GPCR agonists and growth factors occurs solely at these sites (38). Furthermore, a recent study in platelets reported the presence of DGK activity in lipid rafts and showed a thrombin-stimulated increase in PA levels in these domains (8), demonstrating agonist-dependent PA generation within lipid rafts. These studies suggest that caveolae/rafts may be important sites for signaling through the PI pathway.

The aim of this study was to investigate whether vasoconstrictor agonist activation of PI signaling was spatially restricted within plasma membrane microdomains in vascular tissue. We show that norepinephrine (NE) and endothelin-1 (ET-1) induce PIP2 hydrolysis solely within caveolae/rafts of rat mesenteric small arteries (RMSAs), leading to activation of DGK and generation of PA in these domains. Furthermore,
inhibition of DGK activation and reduction of PA production within these domains correlated with reduced sensitivity of contraction, suggesting that localization of PI signaling to caveolae/rafts is linked to the regulation of VSM contractility.

MATERIALS AND METHODS

The investigation was carried out in accordance with The University of Manchester Animal Experimentation Guidelines and the UK Animals (Scientific Procedures) Act 1986. Experiments were performed with the approval of the Review Board of the University of Manchester and the Home Office.

Animals and incubation conditions. Adult female Sprague-Dawley rats (8–10 wk of age, 180–220 g body wt) were used for all experiments. The mesentery artery was excised and placed in ice-cold physiological salt solution. Mesenteric small arteries (internal diameter <400 μm) were cleaned of adherent fat and connective tissue and dissected from the mesenteric bed. Unless stated otherwise, arteries were equilibrated in 1 ml of tissue culture medium M199 (GIBCO) for 1 h at 37°C before stimulation with NE (15 μM) or ET-1 (100 nM) for various time points.

Isolation of caveolae/raft microdomains. Caveolae/raft-enriched microdomains were purified from RMSAs according to the method of Song et al. (45) with the following modifications. Arteries were homogenized in 500 μl of 0.5 M Na2CO3 (pH 11) in ground glass homogenizers and further homogenized sequentially with an Omni homogenizer (3 × 10 s, 20,000 rpm at 10-s intervals) and sonication (3 × 20 s at 20-s intervals); all steps were carried out on ice. Aliquots of homogenate were removed for protein estimation by the Bradford Assay (9). A 450-μl aliquot of homogenate, protein concentration ~1 mg/ml, was mixed with 450 μl 80% sucrose in MES-buffered saline [MBS; 25 mM MES (pH 6.5) and 0.15 M NaCl] and placed at the bottom of a polyallomer centrifuge tube. A discontinuous sucrose gradient was formed by layering 700 μl of 35% sucrose in MBS + 0.25 M Na2CO3 (pH 11) on top, followed by 625 μl of 5% sucrose in MBS + 0.25 M Na2CO3 (pH 11), and centrifuging at 55,000 rpm (160,000 g) for 1 h at 4°C (TLC-55 rotor, Beckman TL100 centrifuge). From the top of the gradient, 13 fractions of 175 μl were removed. The noncaveolae/raft pellet was removed and resuspended in fraction 13.

Immunoblotting. Proteins in the individual fractions were precipitated with 5% trichloroacetic acid and processed for SDS-PAGE and Western blot analysis with the appropriate primary antibody. Signals were developed by horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Pierce). Signal intensity was quantitated with 5% trichloroacetic acid and processed for SDS-PAGE and conventional TLC on 0.25-mm-thick Silica Gel 60 plates. For cholesterol, plates were developed in chloroform-methanol-acetic acid-formic acid-dH2O (70:30:12:4:2 vol/vol/vol/vol/vol) followed by hexane-diisopropylether-acetic acid (130:70:4 vol/vol) (28). To visualize cholesterol, plates were stained with 8% cupric acetate in 3% phosphoric acid and heated to 100°C for 30 min. [33P]PO4 was separated from other [33P]-labeled phospholipids on oxalate-coated TLC plates (37) and developed in chloroform-methanol-ammonium hydroxide-dH2O (17:13:2:2.8:1 vol/vol/vol/vol) (19). PA was separated as described previously (37). Radiolabeled lipids were visualized by electronic autoradiography (InstantImager, Perkin Elmer) and identified by use of cochromatographed standards stained with I2 vapour.

Assay of DGK activity. DGK activity at the membrane was extracted and assayed using the n-octyl-β-D-glucopyranoside (OBG)-mixed micelle assay as previously described for rat subcutaneous small arteries (35). Bradford assays (17) were performed on extracted samples to ensure that equal protein amounts were used in each assay. Following sucrose density centrifugation, fractions were pooled as caveolae/rafts (fractions 2–5), noncaveolae/rafts 1 (fractions 6–9), and noncaveolae/rafts 2 (fractions 10–13). Membranes were recovered by diluting pooled fractions threefold in 0.2 M MBS [pH 6.5; 25 mM MES (pH 6.5) and 0.15 M NaCl] and centrifuged at 100,000 g for 2 h. Concentrated membrane pellets were washed in 0.2 M MES (pH 6.0) and resuspended in DGK homogenization buffer, and DGK activity was extracted and assayed using the OBG-mixed micelle assay as previously described (35). Since protein levels were too low to measure in individual caveolae/raft fractions, samples were normalized by loading equal protein amounts onto sucrose gradients before centrifugation.

Measurement of contractile responses. Contractile responses of small arteries to NE and ET-1 were measured by using pressure myography. Briefly, segments of small artery (<350 μm ID) were cannulated and mounted in a Living Systems pressure myograph as described previously (34). Following equilibration for 45 min in physiological salt solution (pH 7.4, gassed with 5% CO2-95% O2) at 37°C and 20 mmHg intraluminal pressure, the intraluminal pressure was raised to 70 mmHg and the vessel was left to stabilize for a minimum of 15 min before construction of cumulative concentration response curves to ET-1 (0.03–300 nM) and NE (0.1–15 μM). Lumen diameter was measured 2 min following the addition of agonist, immediately before the addition of the next concentration. To study the effect of phosphatidylinositol 3-kinase (PI3-kinase) inhibition, arteries were incubated in 10 μM LY-294002 or 0.1% DMSO (vehicle) for 1 h before cumulative addition of NE or ET-1. NE concentration response curves in the presence of vehicle and inhibitor were obtained from a single vessel segment, for ET-1 individual segments were used for each treatment.

Materials. LY-294002 and ET-1 were purchased from Calbiochem, and TLC plates (Merck 5721) and “HiPerSolv” grade solvents were obtained from VWR International (Leics, UK). [33P]PO4 (specific activity, 370 MBq/ml) was from Amersham International (Amersham, Bucks, UK). γ-[33P]ATP was from MP Biomedicals. Monoclonal anti-caveolin-1 clone 2297 was from W. van Blitterswijk (Netherlands Cancer Institute, Amsterdam, The Netherlands). NE, PA standard, β-Cholera toxin-horseradish peroxidase conjugate, and all other chemicals were supplied by Sigma Chemicals.

Statistical analysis. Comparisons between two groups were analyzed by Student’s t-test. Repeated-measures ANOVA was used for comparison between multiple groups. P < 0.05 was considered statistically significant with n indicating the number of experiments.

RESULTS

Caveolae/raft isolation from RMSAs. Caveolae/raft fractions are characterized by their buoyancy due to high-lipid content and enrichment with cholesterol (rafts/caveolae), caveolin-1 (caveolae), and the ganglioside GM1 (rafts) (15, 26, 40). Accordingly, we used these markers to identify caveolae/raft fractions.
Caveolin-1, cholesterol, and GM₁ were predominantly localized to fractions 2–5 at the 5–35% sucrose interface (66 ± 5%, 44 ± 2%, and 54 ± 4% of total, respectively), identifying these fractions as enriched in caveolae/rafts (Fig. 1) in agreement with published data (45).

**Distribution of [³³P]PIP₂.** Previous studies in cultured cells have reported an enrichment of PIP₂ in caveolae/rafts (20, 38). Therefore caveolae/rafts were isolated from [³³P]-labeled RMSAs, and fractions were analyzed for [³³P]PIP₂. Since PIP₂ is a minor membrane phospholipid (6), fractions were pooled into caveolae/raft (fractions 2–5), noncaveolae/raft-1 (fractions 6–9), and noncaveolae/raft-2 (fractions 10–13). In nonstimulated arteries, 52 ± 4% of [³³P]PIP₂ was found within caveolae/raft fractions, and the remaining 50% was distributed between noncaveolae/raft fractions 1 and 2 (Fig. 2A).

NE- and ET-1-stimulated [³³P]PIP₂ hydrolysis and PA production localizes to caveolae/rafts. The presence of [³³P]PIP₂ in caveolae/rafts suggested that they might be sites of agonist-induced [³³P]PIP₂ hydrolysis. To investigate this, [³³P]-labeled RMSAs were stimulated with NE (15 μM, 20 s or 5 min) or ET-1 (100 nM, 20 s or 10 min), caveolae/rafts were isolated, and [³³P]PIP₂ content was analyzed. Time points chosen corresponded to the initial and sustained phases of RMSA contraction to these agonists (36, 37). Agonist concentrations were those which induce maximum contraction in RMSAs (Refs. 35 and 36, and see Fig. 8).

**Fig. 2.** Caveolae/rafts are enriched in [³³P]phosphatidylinositol 4,5-bisphosphate (PIP₂) and are sites of norepinephrine (NE) and endothelin 1 (ET or ET-1)-stimulated [³³P]PIP₂ hydrolysis. RMSAs were labeled with [³³P], stimulated with NE (15 μM), ET-1 (100 nM), or distilled water (dH₂O), and caveolae/rafts were isolated. Lipids were extracted from fractions, pooled as fractions 2–5 caveolae/raft, fractions 6–9 noncaveolae/raft-1, and fractions 10–13 noncaveolae/raft-2. [³³P]PIP₂ content was analyzed as described in MATERIALS AND METHODS. A: basal distribution of [³³P]PIP₂. Data are expressed as means ± SE %total [³³P]PIP₂ (n = 5 experiments). B: effect of NE on [³³P]PIP₂ levels. Data are expressed as means ± SE %basal PIP₂ level in each of the individual fractions, where basal PIP₂ = 100% (*P < 0.05 compared with basal, from a minimum of 4 separate experiments). C: effect of ET-1 on [³³P]PIP₂ levels. Data are expressed as means ± SE %basal PIP₂ level in each of the individual fractions, where basal PIP₂ = 100% (*P < 0.05 compared with basal, from a minimum of 5 separate experiments).
hydrolysis (27); therefore, the greater increase in PA may reflect this additional source of DG. Incubation with 2% butan-1-ol to block PLD-derived PA (30) had no effect on either basal or agonist-stimulated [33P]PA levels (not shown), confirming that the [33P]PA was derived from the DGK pathway. We also found that 40.1 ± 2.4% of [33P]PA was localized to caveolae/rafts (fractions 2–5) with 26.2 ± 1.0% in noncaveolae/rafts-1 (fractions 6–9) and 25.1 ± 2.8% in noncaveolae/rafts-2 (fractions 10–13). NE increased [33P]PA levels in caveolae/rafts rapidly, within 20 s and in a biphasic manner peaking at 1 min, returning to basal at 2 min before increasing again at 5 min. A similar pattern was seen in noncaveolae/raft fractions, although levels of [33P]PA were already decreasing at 1 min in these fractions (Fig. 3B). ET-1 stimulation also increased [33P]PA levels in caveolae/raft and in noncaveolae/raft fractions within 20 s, and levels remained elevated up to 10 min (Fig. 3C). Finally, NE-induced [33P]PA production was mimicked by 10 μM phenylephrine and inhibited by preincubation with prazosin (10 μM), demonstrating coupling through α1-adrenoceptors (Fig. 4) in agreement with our previous study (37). ET-1-induced [33P]PA production was inhibited by BQ-123, indicating coupling through ETα receptors (Fig. 4).

**Mechanisms involved in NE-and ET-1-stimulated [33P]PA formation in caveolae/rafts.** We have previously shown that NE stimulation increases DGK activity in rat small arteries, resulting in the formation of PA (35, 37), and here we show that ET-1 also stimulates membrane-associated DGK activity (Fig. 5). Accordingly, we investigated whether DGK was
involved in the production of $[^{33}P]PA$ in caveolae/rafts following NE or ET-1 stimulation. In nonstimulated RMSAs, 24 ± 2% of DGK activity was localized to caveolae/rafts with 30 ± 2% and 47 ± 4% in noncaveolae/rafts-1 and -2, respectively. NE increased DGK activity 1.59 ± 0.28-fold in caveolae/rafts with no significant effect observed in noncaveolae/rafts, whereas ET-1 increased DGK activity 1.40 ± 0.18-fold in caveolae/rafts and 1.37 ± 0.12-fold in noncaveolae/rafts-1 with no significant effect observed in noncaveolae/rafts-2 (Fig. 6). Membrane-associated DGK activity measured after high-pH Na$_2$CO$_3$ extraction confirmed that the caveolea/raft extraction protocol had no effect on the magnitude of DGK activation (not shown).

DGKs exist as multiple isoforms, and in RMSAs, NE activates DGK- in a PI3-kinase-dependent manner (49). In this study we confirmed the presence of DGK in a PI3-kinase-dependent manner (49). NE-stimulated DGK activity (Table 1) that was localized to caveolae/rafts (Fig. 6A) and pretreatment of RMSAs with 10 M LY-294002, a concentration that blocks PI3-kinase activity in NE-stimulated RMSAs (49), inhibited NE-stimulated membrane-associated DGK activity (Table 1) that was localized to caveolae/rafts (Fig. 6B). Furthermore, PI3-kinase inhibition had no effect on basal $[^{33}P]PA$ levels in any fraction but reduced NE-stimulated $[^{33}P]PA$ levels in caveolae/rafts and noncaveolae/rafts-2 (Fig. 6C). However, LY-294002 had no effect on ET-1-stimulated DGK activity (Table 1) or $[^{33}P]PA$ levels in any of the fractions (Fig. 6C).

To further explore the PI3-kinase-dependent regulation of DGK activity in caveolae/rafts, we studied the localization of PKB since we previously demonstrated a NE-stimulated DGK activity associated with PKB (49). In nonstimulated arteries, 5.3 ± 0.5% of PKB localized to caveolae/raft fractions. NE increased PKB levels approximately threefold in caveolae/rafts and twofold in noncaveolae/raft-1 fractions with a corresponding decrease in noncaveolae/raft-2 fractions. However, with ET-1, although there was a tendency for PKB to increase in caveolae/raft and noncaveolae/raft-1 fractions and to decrease in noncaveolae/raft-2, this effect was not statistically significant (Fig. 7B) and possibly reflected a general redistribution toward the membrane. Stimulation times were chosen to reflect peak DGK activity in response to these agonists (NE, 1 min; and ET-1, 5 min) (Fig. 5) (35). Furthermore, pretreatment with LY-294002 (10 M) had no effect on basal PKB distribution but significantly reduced the NE-stimulated PKB association with caveolae/rafts (Fig. 7, C and D).

**Effect of PI3-kinase inhibition on contractile responses.** To investigate the functional significance of PI3-kinase inhibition, the effects of LY-294002 on RMSA tension development in response to NE and ET-1 were studied. Cumulative concentration-response curves to ET-1 and NE were obtained in the presence and absence of LY-294002 (10 M; Fig. 8). PI3-kinase inhibition decreased the contractile response to NE at submaximal concentrations (Fig. 8A) and increased the EC$_{50}$ from 2.12 ± 0.28 to 4.57 ± 0.47 M ($P < 0.05$), demonstrating altered sensitivity. The effects of LY-294002 on maximal contraction (~27% inhibition) were comparable with the effects of this inhibitor on $[^{33}P]PA$ in caveolae/rafts (~23% inhibition; Figs. 8A and 6B), showing a good agreement between PA levels and the contractile response. In contrast, PI3-kinase inhibition had no effect on ET-1-stimulated contraction (Fig. 8B), again agreeing with the lack of effect of LY-294002 on $[^{33}P]PA$ levels with this agonist (Fig. 6B).

![Image of DGK activity](http://ajpheart.physiology.org/)

**Fig. 6. The effect of phosphatidylinositol 3-kinase (PI3K) inhibition on NE and ET-1-stimulated DGK activity and $[^{33}P]PA$ production in caveolae/rafts and noncaveolae/rafts.** A: immunoblot of DGK-9 distribution in sucrose density fractions 1–13, representative of 3 independent experiments. B: RMSAs were stimulated with vehicle (dH$_2$O, 5 min, $n = 5$ experiments), ET-1 (100 nM, 5 min, $n = 5$ experiments), or NE (15 M, 1 min, $n = 5$ experiments) or incubated for 1 h with LY-294002 (LY, 10 M) before stimulation with NE (15 M, 1 min, $n = 4$ experiments). Caveolae/rafts and noncaveolae/rafts were prepared as fractions 2–5 caveolae/rafts, fractions 6–9 noncaveolae/raft-1, and fractions 10–13 noncaveolae/raft-2, and DGK activity was extracted and assayed as described in MATERIALS AND METHODS. Data are means ± SE (in pmol PA·min$^{-1}$·mg$^{-1}$ extract$^{-1}$). *$P < 0.05$ compared with vehicle. C: $[^{33}P]PA$-labeled RMSAs were incubated in the presence or absence of 10 M LY-294002 for 60 min before stimulation with vehicle (dH$_2$O, 5 min), NE (15 M, 1 min), or ET-1 (100 nM, 5 min); membrane fractions were isolated, and $[^{33}P]PA$ content was analyzed as described in MATERIALS AND METHODS. Data are expressed as means ± SE (in cpm), normalized for total protein, from a minimum of 3 experiments. *$P < 0.05$, NE compared with LY + NE.

<table>
<thead>
<tr>
<th>DGK Activity, pmol PA·min$^{-1}$·mg$^{-1}$</th>
<th>Protein</th>
<th>Vehicle</th>
<th>LY-294002</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal</strong></td>
<td>15</td>
<td>54 ± 2</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>NE-1 min</td>
<td>6</td>
<td>102 ± 16</td>
<td>76 ± 12*</td>
</tr>
<tr>
<td>ET-1, 1 min</td>
<td>5</td>
<td>63 ± 5</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>ET-1, 5 min</td>
<td>4</td>
<td>118 ± 14</td>
<td>109 ± 9</td>
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Values are means ± SE, $n$, number of experiments. Rat mesenteric small arteries were stimulated with norepinephrine (NE, 15 M) or endothelin-1 (ET-1, 100 nM) in the presence of vehicle (0.1% DMSO) or LY-294002 (10 M) for the times indicated, and membrane-associated diacylglycerol kinase (DGK) activity was measured as described in MATERIALS AND METHODS. PI3-kinase, phosphatidylinositol 3-kinase. *$P < 0.05$, LY-294002 compared with vehicle.

Table 1. The effect of PI3-kinase inhibition on membrane-associated DGK activity
DISCUSSION

Caveolae/rafts are plasma membrane microdomains, abundant in smooth muscle and endothelial cells and implicated in signal transduction, owing to their enrichment with signaling proteins (26, 40). In this study, we have identified PIP2 and PA in caveolae/rafts isolated from RMSAs and evaluated alterations in the levels of these lipids after NE and ET-1 stimulation. Additionally, we have demonstrated agonist-specific activation of DGK-θ and redistribution of PKB to these sites by NE but not ET-1. Our data suggest that, in vascular tissue, caveolae/rafts are sites of vasoconstrictor-induced activation of the PI signaling cascade and that the generation of lipid second messengers at these sites is necessary for modulating the contractile response.

Using a detergent-free method (45), we prepared buoyant membranes from RMSAs enriched in three caveolae/raft fractions markers: cholesterol, caveolin-1, and GM1. These fractions contained 52% of the total [33P]PIP2, in agreement with another study where [3H]inositol was used to label PIP2 in A431 cells (20). However, we cannot say whether PIP2 is preferentially localized in rafts or caveolae, because our preparation is partially localized in rafts or caveolae, because our preparation is not ET-1. Our data suggest that, in vascular tissue, caveolae/rafts are sites of vasoconstrictor-induced activation of the PI signaling cascade and that the generation of lipid second messengers at these sites is necessary for modulating the contractile response.

We also found [33P]PA (40 ± 2.4% of total) and DGK activity (24 ± 2% of total) present in caveolae/rafts. Given that the protein content of caveolae/rafts is markedly lower than noncaveolae/raft fractions (26, 45) and, indeed, was below detection levels in our preparation, this suggests that caveolae/rafts contain relatively high DGK activity in nonstimulated RMSAs. This is in good agreement with a study in platelets where DGK activity and PA were found in lipid rafts (8) and in contrast to a study in MDCK cells where DGK activity was absent from these domains (20). These differences could reflect our use of intact tissue versus cultured cells, DGK assay conditions, or conditions used for preparation of lipid rafts, because the composition of these fractions is critically dependent on the detergents and their concentrations used for extraction (1). However, given that PA is metabolized by lipid phosphate phosphohydrolases, the activity of which has been detected in caveolae from rat lung (31), this is indicative of caveolae/rafts being sites of PA production and metabolism in tissues, further strengthening the view that caveolae/rafts are sites of phospholipid signaling.

Agonist stimulation of PIP2 hydrolysis and PA production in caveolae/rafts. There is considerable evidence that cells contain agonist-responsive and -unresponsive pools of PIs that are compartmentalized at the plasma membrane (reviewed in Ref. 29). This is considered to be an important regulatory mechanism, because although the most documented function of PIP2 is that of a precursor for Ins(1,4,5)P3 and DG, PIP2 can also directly interact with specific domains in proteins to regulate their activity, and it can also act as a substrate for PI3-kinase to generate additional second messengers. A consequence of this is that PIP2 has a pivotal role in regulating many cellular functions such as actin cytoskeleton reorganization, cell migration, vesicle trafficking, and ion channel activity in addition to the classical regulation of intracellular calcium and PKC activity (reviewed in Refs. 3, 12, 21). Therefore, compartmentalisation of PIP2 into specific cellular pools is essential to lend selectivity to its diverse functions. Our observation that vasoconstrictors stimulate PIP2 hydrolysis solely within caveolae/rafts shows that agonist responsive and unresponsive pools of PIP2 do exist in vascular tissue. This agrees with a study in A431 cells where bradykinin-induced PIP2 hydrolysis was confined to lipid rafts (38) and is also consistent with the reported caveolae localization of the α1-adrenoceptor and ET1 receptor (10, 16). NE and ET-1 stimulation also increased [33P]PA levels in caveolae/rafts within the same time frame as PIP2 hydrolysis. However, [33P]PA production was not re-
The initial transient increase in \(^{33}\text{P}\)PA in caveolae/rafts in different sites in the plasma membrane is being undertaken. This issue, a detailed analysis of PA species produced at or by hydrolysis of phospholipids other than PIP2. To resolve noncaveolae/raft fractions by the action of other DGK isoforms, vessels were treated with vehicle (0.01% DMSO) or 10 \(\mu\)M LY for 60 min before construction of a cumulative concentration response to NE (\(n = 8\) experiments; A) or ET-1 (\(n = 4\) experiments; B). Data are means ± SE. *\(P < 0.05\) by ANOVA and Bonferroni posttest.

stricted to caveolae/rafts, suggesting that either DG or PA is capable of diffusing away from the initial site of production. Alternatively, there may be a generation of DG and \(^{33}\text{P}\)PA in noncaveolae/raft fractions by the action of other DGK isoforms or by hydrolysis of phospholipids other than PIP2. To resolve this issue, a detailed analysis of PA species produced at different sites in the plasma membrane is being undertaken.

PI3-kinase-dependent activation of DGK in caveolae/rafts. The initial transient increase in \(^{33}\text{P}\)PA in caveolae/rafts induced by NE was consistent with the transient activation of DGK-\(\theta\) by this agonist (49). Recently, we showed that NE activates PI3-kinase/PKB signaling in RMSAs and that this pathway is essential for activation of DGK-\(\theta\) (49). Here we show that caveolae/rafts are sites where PI3-kinase/PKB regulates DGK activity and provides evidence that DGK-\(\theta\) is involved in the response. Moreover, we also show that PI3-kinase is involved in the contractile response to NE, suggesting a link between PI3-kinase/PKB activation of DGK in caveolae/rafts and contraction. Another study in VSM cells has shown that mechanosensitive activation of PI3-kinase/PKB-dependent signaling requires intact caveolae (42), suggesting these membrane domains are important for this signaling pathway in smooth muscle. However, although PI3-kinase inhibition completely inhibited NE-stimulated DGK activity in caveolae/rafts, there was only a partial reduction of \(^{33}\text{P}\)PA levels. This probably reflects the presence of other constitutively active DGK isoforms such as DGK-\(\epsilon\) (48). In contrast, ET-1 induced a sustained increase in DGK activity and \(^{33}\text{P}\)PA production in caveolae/rafts that was independent of PI3-kinase activity. This suggests that ET-1 activates different DGK isoforms to NE, demonstrating differential regulation of DGK and DG metabolism by vasoconstrictor hormones in vascular tissues. However, this is unlikely to be a type 1 DGK isoform (\(\alpha, \beta, \text{and } \gamma\)) since calcium removal or the DGK inhibitor R-59949 had no effect on ET-1-stimulated DGK activity (data not shown).

Caveolae/rafts and the contractile response. There is growing evidence that caveolae and caveolins are important for VSM contractility. For instance, caveolin knockout models are reported to have aberrations in endothelial-dependent relaxation, contractility, and maintenance of myogenic tone (reviewed in Ref. 25), whereas disruption of caveolae by cholesterol sequestration inhibits smooth muscle contraction by many agonists (reviewed in Ref. 5). Cholesterol depletion of rat-tail artery was reported to disrupt ET-1-stimulated contraction (14), and this was subsequently reported to be through a disruption of calcium influx (4). However, there is little consensus in the data concerning the role of caveolae in \(\alpha_1\)-adrenergic contractile responses. In caveolin-1 null mice, normal (13) or blunted (39) contraction to \(\alpha_1\)-adrenergic stimulation was reported, although the latter was due to enhanced endothelial relaxation rather than an alteration of smooth muscle response. Cholesterol depletion also suggests that \(\alpha_1\)-adrenergic contraction is not dependent on intact caveolae (5), but two recent reports challenge this view, demonstrating blunted contractile responses to \(\alpha_1\)-adrenergic stimulation in ferret aorta (22) and RMSAs (43) after cholesterol depletion. This suggests tissue-specific differences.

Here we observed NE- and ET-1-stimulated activation of the PI signaling cascade, important for contraction, in caveolae/rafts (schematically represented in Fig. 9). Furthermore, with both agonists, there was a direct correlation with the effects of LY-294002 on contraction and \(^{33}\text{P}\)PA levels in caveolae/rafts. With ET-1, both contraction and \(^{33}\text{P}\)PA were unaffected by PI3-kinase inhibition, whereas LY-294002 attenuated NE-stimulated maximal contraction (27% inhibition) and comparably reduced \(^{33}\text{P}\)PA levels in caveolae/rafts (23% inhibition; Figs. 6C and 8A). These data suggest a link...
between signaling events within caveolae/rafts and contraction. This also suggests that generation of PA within caveolae/rafts by GPCR agonists may be a common component of the signaling pathway leading to VSM contraction. Finally, although our tissue contains endothelial cells in addition to smooth muscle, the latter are the predominant cell type in our tissue, and because both α1-adrenoceptors and ETA receptors are mainly expressed on smooth muscle cells, it is most probable that the changes we observed are of smooth muscle origin.

In conclusion, our data indicate that caveolae/rafts appear to be major sites for NE- and ET-1-induced PI signaling in RMSAs and suggest that DGK activation and PA production within these domains may be linked to a regulation of the contractile response. We also show that differences in PI3-kinase dependence of DGK activation and PA production between the two agonists were further reflected in the sensitivity of NE- but not ET-1-stimulated contraction to PI3-kinase inhibition. Interestingly, since PI3-kinase has been implicated in increased vascular tone in hypertension (32, 33), this suggests a pathway by which NE signaling may be deregulated in VSM in pathological states.

ACKNOWLEDGMENTS

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GRANTS

This study was funded by the British Heart Foundation.

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