Membrane-associated diacylglycerol kinase activity is increased by noradrenaline, but not by angiotensin II, in arterial smooth muscle

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In rat small arteries, noradrenaline stimulates the sustained production of arachidonoyl-phosphatidic acid, whereas there is only a slight and transient increase with angiotensin II [Ohanian, Ollerenshaw, Collins and Heagerty (1990) *J. Biol. Chem.* **265**, 8921–8928]. Diacylglycerol kinase (DGK) is the enzyme responsible for generating phosphatidic acid from 1,2-diacylglycerol (DAG). To investigate whether agonists influence DGK activity, we have studied this enzyme in both particulate and soluble fractions prepared from rat small arteries. Soluble DGK activity was inhibited by octyl glucoside. Therefore a deoxycholate assay was used for this fraction, whereas an octyl glucoside mixed-micelle assay was used to examine particulate fractions. Particulate DGK selectively phosphorylated long-chain DAG at a rate 2.5–3-fold higher than that for the synthetic

substrate dioctanoylglycerol. In contrast, the substrate preference of the soluble isoenzyme(s) was: dioctanoylglycerol > arachidonoyl-DAG = dioleoylglycerol. Stimulation of intact arteries with noradrenaline (15 μ M) increased membrane-associated DGK activity 3-fold, transiently. Angiotensin II (100 nM) stimulation did not alter the DGK activity of this fraction. The activity of the soluble DGK was increased by both agonists, but only transiently. These results demonstrate that rat small arteries contain a membrane-associated DGK which metabolizes arachidonoyl-containing substrate. Also, the activity of this enzyme is regulated differentially by vasoconstrictor hormones. It is concluded that modulation of DGK activity may represent one point at which agonists using the same signal-transduction pathway may tailor the cellular response.

INTRODUCTION

Vasoconstrictor hormones appear to initiate their actions through the hydrolysis of phosphatidylinositol lipids in vascular smooth muscle [1–4]. Receptor-mediated activation of phospholipase C (PLC) with subsequent hydrolysis of PtdIns(4,5) P_2 releases two second messengers, Ins(1,4,5) P_3 and 1,2-*sn*-diacylglycerol (1,2-DAG). The former causes the release of Ca^{2+} from intracellular stores and is recognized to be the trigger for smooth-muscle contraction [5]. A key element in signal transduction, 1,2-DAG, is the physiological activator of protein kinase C (PKC) [6]. In addition there is evidence that phosphatidic acid (PA), a metabolite of 1,2-DAG, may be involved in cell signalling. PA is a potent mitogen in cells [7], and stimulates PtdIns(4,5) P_2 formation [8] as well as PLC activity [9,10]. Also it has been implicated in PA-dependent protein phosphorylation in rat tissues [11]. After receptor stimulation, two routes for PA production have been proposed: (1) phosphorylation of 1,2-DAG catalysed by diacylglycerol kinases (DGK), and (2) phospholipase D hydrolysis of phospholipids, in particular phosphatidylcholine. However, recent studies indicate that the latter occurs early, is transient and therefore may not contribute significantly to PA formation [12,13]. Phosphorylation by DGK may be the preferred route.

Multiple isoenzymes of DGK have been identified in both cytosolic and membrane fractions of mammalian cells [14–19]. The best characterized is an 86 kDa cytosolic DGK, which has been isolated and cloned from both human white blood cells [20] and pig thymus [21]. In addition, a membrane-bound DGK with preference for arachidonate-containing DAG has been reported in Swiss 3T3 cells [22], and an enzyme with similar properties has been isolated from baboon tissues [17]. The significance of these

multiple DGKs is unclear, and it is not known whether they play distinct roles during cell signalling. However, several reports indicate that DGKs may be regulated during receptor activation. For instance, the soluble enzyme has been shown to translocate from the cytosol to the membrane in a Ca^{2+} -dependent manner [23], and an increase in membrane 1,2-DAG content also induces translocation [24]. The purified pig 80 kDa enzyme is phosphorylated by PKC and cyclic-AMP-dependent kinase *in vitro* [25]. The phosphorylated enzyme associates with phosphatidylserine (PS) membranes, which appears to stabilize its activity [25]. Indeed, in Swiss 3T3 cells phorbol ester treatment also induced translocation to the membrane [26]. More recently the 86 kDa human cytosolic DGK has been shown to be phosphorylated not only by cyclic-AMP-dependent kinase, PCK α and PKC ϵ , but also by the epidermal-growth-factor receptor [27]. Ca^{2+} -mobilizing hormones stimulate DGK activity in rat liver membranes [28] and in guinea-pig parotid cells [29]. Van Blitterswijk and co-workers [30] have shown that down-regulation of PKC decreased the conversion of arachidonate-DAG into PA during bradykinin stimulation of human fibroblasts. These results indicate that PKC may regulate the levels of its activator through an effect on DGK. However, there have been no studies of DGK in vascular tissue.

Previously we have shown that the profile of lipid second messengers produced during contraction in small arteries is agonist-specific: arachidonate-enriched DAG, which is largely derived from inositol lipid breakdown, was increased by angiotensin II but unchanged by noradrenaline [4]. In contrast, arachidonate-enriched PA accumulated with time in noradrenaline-stimulated arteries, whereas this lipid was only slightly and transiently increased with angiotensin II. Subsequent studies using the cell-permeant diacylglycerol dioctanoylglycerol (diC $_8$),

Abbreviations used: 1,2-DAG, 1,2-*sn*-diacylglycerol; DGK, diacylglycerol kinase; PA, phosphatidate; PKC, protein kinase C; PLC, phospholipase C; OBG, octyl β -glucopyranoside; diC $_8$, dioctanoyl-*sn*-glycerol; PS, phosphatidylserine; (in DAGs): 18:0, stearate; 18:1, oleate; 20:4, arachidonate.

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which is rapidly phosphorylated to give dioctanoyl-PA, have shown that noradrenaline increases the production of this lipid, whereas angiotensin II does not [31]. These results suggested that agonists are able to regulate differentially the levels of PA through an effect on DGK. The present work shows, by using particulate and soluble fractions, that small arteries contain membrane-associated DGK with preference for long-chain DAG as substrate and that the activity of this enzyme is increased after stimulation by noradrenaline, but not by angiotensin II.

EXPERIMENTAL

Preparation of small-artery homogenates

Adult female Sprague–Dawley rats (200 g body wt.) were used for all the experiments described. The animals were killed by stunning, following by cervical dislocation. Two subcutaneous small arteries of internal diameter < 300 μ m, supplied by the axillar artery, were dissected from each rat, and cleaned of adherent fat and connective tissue. Three vessels (6–8 mg wet wt. of tissue) were used for each sample. The tissue was placed in 200 μ l of tissue-culture medium (M199) containing (mM) NaCl (128), KCl (5.4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.34), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.3), NaHCO_3 (4.2), KH_2PO_4 (0.44), $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (0.002), glucose (5.6), Hepes (25), and left to equilibrate in a water bath at 37 °C for 30 min. Subsequently, either 200 μ l of prewarmed vehicle (M199) or 200 μ l of noradrenaline (final concn. 15 μ M) or 200 μ l of angiotensin II (final concn. 100 nM) was added. The tissues were removed at various time points, as specified in the text, and homogenized in a hand-held glass homogenizer in 100 μ l of ice-cold homogenization buffer (20 mM Mops, pH 7.2, 0.25 M sucrose, 1 mM dithiothreitol, 1 mM EGTA, 1 mg/ml leupeptin). The homogenates were separated immediately into particulate and supernatant fractions as detailed below.

Preparation of particulate and supernatant fractions from small arteries

The tissue homogenate was centrifuged at 300 000 *g* for 10 min in a Beckman TL100 centrifuge. The supernatant was removed and used as the soluble fraction. The pellet was washed with 200 μ l of homogenization buffer, then resuspended in 40 μ l of homogenization buffer.

Detergent extraction of DGK activity

DGK activity was extracted from the particulate fraction by the method of Lemaitre et al. [17]. Briefly, 40 μ l of extraction buffer [20 mM Mops, pH 7.2, 4% (w/v) octyl β -glucopyranoside (OBG), 0.6 M KCl, 20% (v/v) glycerol, 1 mM dithiothreitol] were added to the particulate suspension. The mixture was left on ice for 30 min, then centrifuged at 300 000 *g* for 10 min. This treatment resulted in 90–100% of the activity being recovered in the supernatant.

Assay of DGK activity

To introduce 1,2-DAG, hydrophobic substrate, into a reaction mixture, it is necessary to use a detergent. However, the activities of DGK isoenzymes included in a crude enzyme preparation are affected differentially by the detergents employed. For instance, the 80 kDa soluble DGK from pig thymus gives the highest

activity with deoxycholate, but is suppressed by OBG [15], whereas the arachidonoyl-specific enzyme is most active in an OBG mixed-micelle system [17]. In light of these findings, we have employed two separate assay systems as described below.

(a) Soluble fraction

The assay used was a modification of the deoxycholate method of Kanoh et al. [32]. The reaction mixture (final volume 50 μ l) contained 50 mM Tris/HCl (pH 7.4), 100 mM NaCl, 20 mM NaF, 1 mM dithiothreitol, 1 mM EGTA, 1 mM deoxycholate, 10 mM MgCl_2 , 0.5 mM DAG, 0.56 mM PS, 1 mM [32 P]ATP (20–30 d.p.m./pmol) and soluble fraction. A DAG suspension was prepared first; the calculated amount of either stearate (18:0)/arachidonate (20:4)-DAG or diC_8 and PS taken from a 10 mg/ml stock solution in chloroform was dried in a glass tube under a stream of O_2 -free N_2 gas. Deoxycholate from a 10 mM stock solution, and assay buffer from a 5 $\times$ stock solution (250 mM Tris/HCl, pH 7.4, 500 mM NaCl, 100 μ M NaF, 5 mM dithiothreitol, 5 mM EDTA, 50 mM MgCl_2), were added. The solution was sonicated in a bath-type sonicator for 5 min to suspend the DAG substrate. The suspension was pipetted into a microfuge tube (30 μ l per sample); 10 μ l of [32 P]ATP was added and the mixture warmed to 30 °C. The reaction was started by adding 10 μ l of soluble fraction (approx. 10 μ g of protein). The reaction time was 3 min.

(b) Particulate fraction

The assay used was the OBG mixed-micelle assay of Lemaitre and Glomsett [33]. The reaction mixture (final volume 35 μ l) contained 55–60 mM Mops (pH 7.2), 18 mM MgCl_2 , 1.1 mM dithiothreitol, 73 mM OBG, 0.52 mM [32 P]ATP (100–200 d.p.m./pmol), 2.9 mM DAG, 3.3 mM PS, 5.7% glycerol and 86 mM KCl. A mixed-micelle solution containing 10.15 mM DAG, 11.55 mM PS and 222 mM OBG was prepared first. The calculated amounts of either 18:0/20:4-DAG or diC_8 and PS taken from a 10 mg/ml stock solution in chloroform were dried in a glass tube under a stream of O_2 -free N_2 gas, and immediately resuspended in a freshly prepared solution of 222 mM OBG. The solution was sonicated for 30 s in a bath-type sonicator and vortex-mixed at frequent intervals until a clear solution was formed. The reaction mixture consisted of 5 μ l of assay buffer (0.375 mM Mops, pH 7.2, 0.125 mM MgCl_2 and 6 mM dithiothreitol) mixed with 10 μ l of particulate extract (approx. 10 μ g of protein) and 10 μ l of mixed micelle solution. The reaction was started by addition of 10 μ l of [32 P]ATP (1.82 mM ATP, 100–200 d.p.m./pmol), and left for 3 min at room temperature.

Determination of [32 P]PA formed

The reaction in either assay was terminated by the addition of 300 μ l of chloroform/methanol/10 M HCl (66:33:1, by vol.), and washed with 2 $\times$ 250 μ l of pure upper phase (chloroform/methanol/water, 3:48:47, by vol.). The resultant organic phase was dried under a stream of O_2 -free N_2 gas and reconstituted in 12 μ l of chloroform. A 10 μ l portion of the sample was spotted on to a heat-activated silica-gel 60 t.l.c. plate and developed in chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by vol.). Authentic PA was run as a standard and [32 P]PA was located by autoradiography. The spot corresponding to PA was scraped from the t.l.c. plate into scintillation vials, 2 ml of scintillant (Ultima Gold MV) was added and radioactivity was determined by liquid-scintillation counting.

Expression of results

The results are expressed as pmol of PA formed/min per mg of protein. Protein was determined by the method of Lowry et al. [34].

In the mixed-micelle assay, DAG concentration is expressed as mole fraction, defined as the ratio of the DAG concentration to the concentration of OBG in micelles, using a critical micelle concentration (CMC) of 25 mM. The concentration of deoxycholate was less than the CMC; in this assay DAG concentration is expressed as mM.

Data analysis

The results are expressed as means $\pm$ S.E.M., and comparisons between groups were made by Student's *t* test.

Materials

Tissue-culture medium M199 was from Gibco BRL (Paisley, Scotland, U.K.). DiC₈ and PS were from Avanti Polar Lipids (Birmingham, AL, U.S.A.) [γ -³²P]ATP (sp. radioactivity 370 MBq/ml) was from Amersham International (Amersham, Bucks., U.K.). 18:0/20:4-DAG, noradrenaline hydrochloride, angiotensin II and all other reagents were from Sigma Chemical Co. (Poole, Dorset, U.K.). Silica-gel 60 t.l.c. plates (Merck 5721) and h.p.l.c.-grade solvents were purchased from British Drug Houses (Poole, Dorset, U.K.). Ultima Gold MV scintillant and scintillation vials were from Canberra-Packard (Pangbourne, Berks., U.K.).

RESULTS

An assay method ideal for all DGK isoenzymes has not yet been developed: therefore two separate assays were used to measure DGK activity in rat small arteries. Enzyme activity was detected in the soluble fraction in the presence of deoxycholate and in the particulate fraction in the presence of OBG. No DGK activity was detected in the soluble fraction by the OBG mixed-micelle assay. This is in agreement with a previous study demonstrating inhibition of pig thymus cytosolic 80 kDa DGK activity by OBG [15]. Under the conditions of the assays described in the Experimental section, the incorporation of [³²P]ATP into both dioctanoyl-PA and 18:0/20:4-PA were linear with respect to time and added protein (results not shown). The original deoxycholate assay did not include PS in the reaction mixture, because the pig 80 kDa DGK is unaffected by this lipid [32]. However, addition of PS stimulated the activity of DGK in the soluble fraction of rat small arteries up to 0.56 mM. Above this concentration the enzyme was inhibited (Figure 1). DGK activity was determined over a range of DAG concentrations. Maximal activity was observed at 6 mol% in the OBG assay with particulate DGK and at 0.5 mM in the deoxycholate assay with soluble DGK (Figures 2a and 2b).

Substrate specificity

The substrate preference of rat small-artery DGK was determined by using three different 1,2-*sn*-DAGs, 18:0/20:4-DAG, 18:1/18:1-DAG and diC₈, at 6 mol% for the particulate fraction and 0.5 mM for the soluble fraction. The soluble DGK(s) showed the highest activity when diC₈ was the substrate (Figure 3). The two endogenous unsaturated 1,2-DAG species were

phosphorylated similarly (Figure 3). In contrast, the DGK in particulate fractions demonstrated a 2.5–3-fold preference for long-chain DAG compared with diC₈ (Figure 3). At the higher substrate concentrations (6–12 mol%) there was a modest decrease in the phosphorylation of 18:1/18:1-DAG (Figure 2a).

Figure 1 Effect of increasing PS concentration on soluble DGK activity

Soluble fractions were prepared from small arteries, and DGK activity was measured with the deoxycholate assay as described in the Experimental section. Each point was determined in duplicate, and the data are representative of two separate experiments. Substrate used: ★, diC₈; ○, 18:0/20:4-DAG.

Figure 2 Effect of increasing concentrations of DAG on DGK activity

Particulate and soluble extracts of DGK were prepared from small arteries and enzyme activity was determined as described in the Experimental section, with one of three substrates as indicated. (a) Particulate activity. DAG concentrations are expressed as mol% of OBG in micellar form; the data are means $\pm$ S.E.M. of three separate experiments performed in duplicate. (b) Soluble activity. DAG concentrations are expressed in mM; the data are means of duplicates from one assay; similar results were obtained in two further assays.

Figure 3 Comparison of the rates of phosphorylation of different DAGs by the particulate and soluble DGKs of small arteries

Particulate and soluble extracts of DGK were prepared from small arteries and enzyme activity was determined as described in the Experimental section. Three substrates were used: 18:0/20:4-DAG, 18:1/18:1-DAG, and diC_8 . The data are means $\pm$ S.E.M. from a minimum of six separate experiments performed in duplicate: * $P < 0.05$, ** $P < 0.05$ compared with 18:0/20:4-DAG, by Student's t test.

Figure 4 Effects of noradrenaline stimulation on DGK activity in small arteries

Small arteries were stimulated with noradrenaline (15 μM) for various times up to 5 min. The reaction was terminated, particulate and soluble fractions were prepared and DGKs were assayed as explained in the Experimental section, with two separate substrates: $\circ$, 18:0/20:4-DAG; $\square$, diC_8 . (a) Particulate enzyme activity; (b) soluble enzyme activity. The data are means $\pm$ S.E.M. from a minimum of five separate experiments assayed in duplicate: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ by Student's t test.

Effects of agonist stimulation of DGK activity

The effects of two vasoconstrictor hormones, noradrenaline and angiotensin II, on DGK activity were investigated. These agonists

Figure 5 Effects of angiotensin II stimulation on DGK activity in small arteries

Small arteries were stimulated with angiotensin II (100 nM) for various times up to 5 min. The reaction was terminated, particulate and soluble fractions were prepared and DGKs were assayed as explained in the Experimental section, with two separate substrates: $\circ$, 18:0/20:4-DAG; $\square$, diC_8 . (a) Particulate enzyme activity; (b) soluble enzyme activity. The data are means $\pm$ S.E.M. from a minimum of five separate experiments assayed in duplicate: * $P < 0.05$ by Student's t test.

were chosen because after receptor activation they both induce an immediate increase in $\text{Ins}(1,4,5)\text{P}_3$ [1,3]. However, their effects on 1,2-DAG and PA differ, such that arachidonate-containing DAG accumulates with angiotensin II, whereas arachidonate-containing PA accumulates with noradrenaline [4]. Whether these effects are due to an effect on DGK has not been studied.

(a) Noradrenaline

When intact small arteries were stimulated with 15 μM noradrenaline, there was a transient increase in DGK activity in both the soluble and particulate fractions (Figure 4). The increase in soluble enzyme activity was observed at a single time point (45 s), and the magnitude of the increase was similar for both substrates tested (Figure 4b). However, the increase in membrane-associated DGK activity showed a different time course, such that enhanced activity was observed as early as 20 s after stimulation and the effect was maintained up to 90 s (Figure 4a). In addition, the increased rate of phosphorylation was greater when 18:0/20:4-DAG (+161%) was used as substrate compared with diC_8 (+116%).

(b) Angiotensin II

Stimulation of intact small arteries with angiotensin II (100 nM) did not alter the DGK activity of the particulate fraction at any time point up to 5 min with either substrate (Figure 5a). In contrast, and similar to the effect of noradrenaline, there was an

Table 1 Effect of diC_8 and AlF_4^- on small-artery DGK activity

Small arteries were incubated for 10 min with either diC_8 (100 μM) or AlF_4^- (10 μM). Enzyme activity was then determined in the particulate fraction with 18:0/20:4 DAG as substrate and in the soluble fraction with diC_8 , as described in the Experimental section. The results are means $\pm$ S.E.M. (NS, not significant, determined by Student's *t* test).

	[^{32}P]PA formed (pmol/min per mg of protein)	
	Particulate	Soluble
Basal ($n = 8$)	62 $\pm$ 12	88 $\pm$ 42
DiC_8 ($n = 4$)	43 $\pm$ 10 (NS)	77 $\pm$ 21 (NS)
AlF_4^- ($n = 4$)	119 $\pm$ 36 (NS)	60 $\pm$ 24 (NS)

increase (approx. 2.5-fold) in the activity of the soluble enzyme after 45 s of stimulation only (Figure 5b). Unlike noradrenaline, this effect was only seen when diC_8 was used as substrate (Figure 5b).

In order to investigate these effects, two further treatments were studied. (1) The membrane DAG content was increased by incubating the intact arteries with diC_8 (100 μM for 10 min) [31]. There was no change in either the particulate or soluble DGK activity after incubation with diC_8 (Table 1). (2) The intact arteries were incubated for 10 min with 10 μM AlF_4^- , a treatment which increases intracellular Ca^{2+} in small arteries [35]. Again, there was no significant change in DGK activity in either cellular fraction (Table 1).

DISCUSSION

The observation that arachidonate-enriched PA was formed in noradrenaline-stimulated small arteries led us to suggest that DGK may be involved in the metabolism of inositol-derived 1,2-DAG [4]. Further experiments using the cell-permeant diacylglycerol diC_8 confirmed that there was an active DGK pathway in small arteries [31]. However, the complexity of lipid metabolism during agonist stimulation, as well as various routes of 1,2-DAG and PA removal and interconversion, make it difficult to attribute unequivocally the rises in PA seen with noradrenaline to increased DGK activity. In the present study we have established that small arteries contain DGKs which are differentially distributed and which have different activity depending on the detergent used in the assay. The particulate fraction of this tissue contains a DGK with preference for long-chain diacylglycerols and had highest activity in an OBG mixed-micelle assay. MacDonald and colleagues have reported that membrane-bound arachidonoyl-specific DGK activity is present in Swiss 3T3 cells [22], and recently such an enzyme was isolated from baboon brain [17]. In addition, a detailed study of the metabolism of membrane 1,2-DAGs in NIH 3T3 cells has shown that arachidonoyl-DAG was preferentially incorporated into PA and phosphatidylinositol, a pathway which was blocked by the DGK inhibitor R59022 [36]. However, using permeabilized smooth-muscle cells, derived from rat thoracic aorta, Chuang and co-workers [37] did not observe preferential phosphorylation of arachidonate-containing DAG. Furthermore, Goto and Kondo [38] recently isolated a 90 kDa DGK from the rat, which is membrane associated and expressed in intestinal smooth muscle; again this isoenzyme did not show preferential phosphorylation of arachidonate-containing sub-

strate. Therefore, whether rat smooth-muscle tissues contain an arachidonoyl-specific DGK is not clear. There was a tendency for greater phosphorylation of 18:0/20:4-DAG compared with 18:1/18:1-DAG at the higher concentrations of substrate in the particulate fraction of rat small arteries. Assuming that arachidonoyl-specific DGK is involved in the metabolism of DAG produced during receptor activation, this enzyme would be expected to be located in the plasma membrane. However, owing to the small amount of tissue in our preparation we were unable to prepare purified plasma membranes. Therefore, the presence of this enzyme may have been masked by isotypes located in other membranes in the particulate fraction.

DGK activity was also present in the soluble fraction of rat small arteries. This enzyme(s) differed from the membrane-bound species in both substrate specificity and detergent preference. In fact, the activity of this enzyme was completely suppressed by OBG, but was stimulated by deoxycholate. Similar findings have been reported with the soluble enzyme from other tissues [15]. Although greatest activity was recorded when diC_8 was the substrate, there was no difference in the phosphorylation of the two endogenous long-chain species, 18:0/20:4 and 18:1/18:1-DAG, suggesting non-specificity. It is possible that the DGKs had greater access to diC_8 in the deoxycholate assay system, due to greater solubility of this lipid compared with endogenous hydrophobic DAGs, resulting in an apparent preference for diC_8 as substrate. OBG mixed micelles provide a single homogeneous phase incorporating enzyme, DAG and lipid cofactor, and under these conditions DGK activity is not limited by delivery of the water-insoluble substrate. Therefore this system is superior for determining both enzyme activity and substrate preference. However, the inhibition of soluble DGK by OBG precluded the use of a micelle delivery system. Due to the small amounts of tissue available, it was not possible to purify and characterize these enzymes further. However, our results indicate the presence of multiple isoenzymes of DGK in rat small arteries, in agreement with data from other tissues [16,17,19].

The presence of a membrane-bound arachidonoyl-specific DGK in many tissues [17,22] suggests its involvement in the PtdIns cycle. Enhanced phosphorylation of endogenous arachidonoyl-DAG has been demonstrated in Swiss 3T3 cells stimulated with platelet-derived growth factor [22]; however, those authors concluded that this effect was due to increased 1,2-DAG production and not increased DGK activity. In contrast, an increase in DGK activity has been reported in guinea-pig parotid cells stimulated with carbachol [29] and in liver membranes after phenylephrine treatment [28]. Those studies did not determine whether this was an effect on arachidonoyl-DGK or non-specific DGKs. Our data demonstrate that noradrenaline stimulation of small arteries results in an increase in particulate DGK activity when measured *in vitro*. This effect was rapid, being observed within 20 s of stimulation. It was also transient, as enzyme activity had returned to basal at 3 min. The response was agonist-specific, as angiotensin II had no effect. These data are in agreement with our previous studies in intact small arteries showing that noradrenaline markedly increased arachidonoyl-PA levels, whereas angiotensin II caused only a small and transient rise [4]. However, we and others have shown that the increase in PA after α_1 -adrenergic stimulation is sustained, implying that metabolism of this lipid is slow [4,39]. This is supported by the fact that arachidonate 1,2-DAG levels were increased after 5 min of noradrenaline stimulation but not at 20 s, in agreement with an early but transient increase in arachidonoyl-DGK activity. The mechanism(s) by which noradrenaline stimulation increased membrane DGK activity is unclear. It is unlikely to be due to increased production of 1,2-

DAG, as angiotensin II increases levels of this lipid [4]. Furthermore, both agonists increase intracellular Ca^{2+} in vascular smooth muscle [40], suggesting that this messenger was not involved. Future studies will focus on the mechanisms regulating this enzyme during agonist stimulation.

In contrast with their effects on particulate DGK, both agonists stimulated soluble enzyme activity. This effect was only seen at one time point (45 s) and was of similar magnitude for both hormones, suggesting a non-specific effect on the soluble enzyme. One possibility is that elevated intracellular Ca^{2+} stimulated this DGK activity. Certainly it is known that the 80 kDa soluble enzyme contains Ca^{2+} -binding sites and that its activity is increased *in vitro* by Ca^{2+} [23]. It has also been suggested that phosphorylation of DGK by PKC during agonist stimulation increases enzyme activity [29,30]. However, whether soluble DGKs are involved in the phosphorylation of 1,2-DAG produced during agonist stimulation is not known. The soluble enzyme has been shown to translocate to the membrane in response to increased Ca^{2+} [23], increased membrane 1,2-DAG content [24] and phorbol ester treatment [26]. However, there was no evidence to suggest that translocation of soluble DGK occurred in small arteries during stimulation with either noradrenaline or angiotensin II. MacDonald and co-workers also found no evidence that cytosolic DGK translocated to the membrane in Swiss 3T3 cells stimulated by platelet-derived growth factor [22]. In an attempt to investigate whether increased membrane DAG content or elevated intracellular Ca^{2+} would alter DGK activity or distribution in small arteries, we treated our tissue with diC_8 or AIF_4^- . Neither stimulus affected DGK activity. Further studies are required to determine the mechanisms of non-specific DGK activation in small arteries.

Our data suggest that DGK is a key component in signal-transduction pathways in small arteries. In addition, the demonstration that membrane-associated DGK is differentially activated by vasoconstrictor hormones implies that this may be one point at which the levels of lipid second messengers, and by inference PKC activation, are regulated. Future studies must focus on the mechanisms involved in DGK activation.

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