Fatty acids inhibit growth-factor-induced diacylglycerol kinase α activation in vascular smooth-muscle cells

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We have previously shown that unsaturated fatty acids amplify platelet-derived-growth-factor (PDGF)-induced protein kinase C (PKC) activation in vascular smooth-muscle cells (VSMCs). Diacylglycerol-induced PKC activation is normally terminated by diacylglycerol kinases (DGKs). We thus hypothesized that fatty acids act by inhibiting a DGK. Fractionation of VSMC extracts demonstrated that the DGK α isoform was the major DGK activity present. PDGF markedly increased the DGK activity of cultured cells. An inhibitor selective for the DGK α isoform, R59949 [3-{2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone], abolished the growth-factor-induced increase in DGK activity, but had little effect on basal activity. PDGF thus selectively activates DGK α . Epidermal growth factor and α -thrombin

stimulated total DGK activity similarly to PDGF. Activation by epidermal growth factor was sensitive to R59949, again suggesting involvement of DGK α . However, the α -thrombin-induced activity was unaffected by this agent. Unsaturated fatty acids inhibited growth-factor-induced DGK α activation, but had no effect on basal activity. Fatty acids also amplified the PDGF-induced increase in cell diacylglycerol content. These results indicate that inhibition of DGK α contributes to fatty-acid-induced amplification of PKC activation. Increased levels of fatty acids in diabetes may thus contribute to chronic PKC activation associated with this disorder.

Key words: diabetes mellitus, non-esterified fatty acids, platelet-derived growth factor, protein kinase C, vascular smooth muscle.

INTRODUCTION

Diacylglycerol kinases (DGKs) phosphorylate *sn*-1,2-diacylglycerol (DAG) released during stimulus-induced phosphoinositide turnover [1–3]. As such, DGKs attenuate DAG-mediated protein kinase C (PKC) activation. Cells express multiple DGK isoforms. Sequence comparisons reveal a conserved DGK catalytic domain, but varied regulatory motifs, suggesting divergent mechanisms of regulation [1–3]. Several groups have reported stimulation of DGK activities by a variety of signals [4–11]. Most of these studies did not identify the specific DGKs involved. Further complicating DGK regulation are observations that only some DAG pools are targeted by DGKs [11–13]. During cell stimulation, DGKs appear to selectively phosphorylate an arachidonate-enriched DAG pool deriving from phosphoinositide (PI) turnover [11–13]. DGKs thus initiate PI resynthesis in addition to attenuating PKC activation.

In diabetes, chronic PKC activation has been linked to vascular changes leading to multiple complications [14,15]. Pharmacological PKC activation with phorbol esters mimics diabetes-induced effects on the vasculature, including increased trans-endothelial permeability [16], enhanced synthesis of extracellular-matrix proteins [17] and inhibition of cytosolic Ca²⁺ signalling [18,19]. Conversely, PKC inhibition can attenuate some of these same events [20]. Such findings strongly implicate PKC in the complications of diabetes. The origin of diabetes-

induced DAG and its role in PKC activation is less clear. Diabetes increases the flux of label from glucose into DAG, indicating that *de novo* synthesis contributes to increased DAG concentrations [14,21]. Circulating non-esterified ('free') fatty acids (NEFA) are increased in people with diabetes [22]. We initially hypothesized that NEFA activate PKC in the diabetic vasculature by increasing *de novo* DAG synthesis. However, induction of *de novo* DAG synthesis by exposing vascular smooth muscle cells (VSMCs) to fatty acids did not correlate with PKC activation [23]. This suggested that *de novo* DAG synthesis and PKC activation are independent events. Growth factors activate DAG-dependent PKCs by increasing PI turnover to DAG [24]. Diabetes impairs flux through the PI cycle [25]. Increased PI hydrolysis is thus unlikely to contribute to DAG accumulation or PKC activation in diabetes. Inhibition of a DGK could activate PKC. Given the role of DGKs in PI resynthesis, DGK inhibition could also contribute to decreased PI turnover. In fact, Nobe et al. have reported markedly reduced DGK activities in aortas and kidneys from diabetic rats [26]. Several other tissues did not exhibit this change. Inhibition of a DGK may thus contribute to diabetes-related PKC activation.

We now demonstrate that DGK α is the major DGK isoform in carotid smooth muscle and cultured VSMCs. We also demonstrate that DGK α activity is selectively stimulated by platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). α -Thrombin also stimulated DGK activity in these cells,

Abbreviations used: DAG, *sn*-1,2-diacylglycerol; DGK, diacylglycerol kinase; diC₁₀, *sn*-1,2-didecanoylglycerol; EGF, epidermal growth factor; NEFA, non-esterified ('free') fatty acid; FGFb, fibroblast growth factor-2; MEM, (Earle's) minimal essential medium; PA, phosphatidic acid; PDGF, platelet-derived growth factor; PI, phosphoinositide; PKC, protein kinase C; R59949, 3-{2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone; VSMC, vascular smooth-muscle cell.

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but this appeared to reflect activation of another DGK isoform. Unsaturated NEFA inhibited growth factor-induced increases in DGK α activity. This effect correlated with their ability to amplify PDGF-induced PKC activation, which we have previously reported [23]. These results indicate that inhibition of DGK α activation contributes to the amplification of PKC activation by unsaturated NEFA and that inhibition of DGK α activation may contribute to PKC activation in diabetes.

EXPERIMENTAL

Materials

[γ - 32 P]ATP and [32 P]P_i were from Du Pont–New England Nuclear, Wilmington, DE, U.S.A. *sn*-1-Palmitoyl-2-oleoyl phosphatidylcholine, *sn*-1,2-didecanoyl phosphatidylcholine, *sn*-1-palmitoyl-2-oleoyl phosphatidylserine and *sn*-1,2-dioleoyl phosphatidylglycerol were from Avanti Polar Lipids, Birmingham, AL, U.S.A. R59949 [3-{2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone; DGK inhibitor II] was from Calbiochem, La Jolla, CA, U.S.A. Recombinant human PDGF (BB dimer), human epidermal growth factor (EGF) and human fibroblast growth factor-2, 146-amino-acid variant (FGFb), were from R&D Systems, Minneapolis, MN, U.S.A. Human α -thrombin, prepared by the method of Fenton et al. [27] was a gift of Dr. Carolyn Patterson, Indiana University School of Medicine, Indianapolis, IN, U.S.A. Carbachol, phenylephrine, and angiotensin II were from Sigma, St Louis, MO, U.S.A. *sn*-1-Palmitoyl-2-oleoylglycerol and *sn*-1,2-didecanoylglycerol (diC₁₀) were prepared by digestion of the corresponding phosphatidylcholines with *Bacillus cereus* phospholipase C [28]. Fatty acids and PMA were from Sigma. *Escherichia coli* strain WH1061, harbouring the inducible plasmid pSD005 that encodes a synthetic *E. coli* DGK with an N-terminal histidine tag, was a gift of Dr James Bowie, University of California at Los Angeles, Los Angeles, U.S.A. [29].

Cell culture

Primary cultures of VSMCs were prepared from porcine carotid arteries as described previously [23]. For experiments, 5×10^4 cells/ml from passages 3–7 were seeded into 35-mm- or 100-mm-diameter dishes in 1.5 or 12 ml of (Earle's) minimal essential medium (MEM) supplemented with 10% (v/v) fetal-calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. The culture medium was replaced every 48 h. Cells reached near confluence after 7 days. They were rendered quiescent by washing once with antibiotic-free, low-phosphate MEM, which was prepared by supplementing phosphate-free MEM (Gibco BRL, Grand Island, NY, U.S.A.) with 50 μ M NaH₂PO₄, 0.1% fetal-calf serum and 20 mM Hepes, pH 7.4. The cells were then incubated in the same medium for 24 h. Stock solutions of fatty acids and albumin were prepared as described previously [23] and added directly to culture media to give the desired final concentrations. The final albumin concentration in all experiments was 50 μ M (0.33%).

In situ assay of DGK activity

DGK activity was assayed in intact cells by monitoring the phosphorylation of exogenously added diC₁₀, a short-chain DAG analogue [30]. Quiescent cells in 35-mm-diameter dishes were labelled by adding 50 μ Ci/dish of [32 P]P_i to the culture medium. After 10 min, diC₁₀ was added to a final concentration of 50 μ M (10 μ l of 7.5 mM diC₁₀ in ethanol). After an additional 10 min,

the cells were stimulated with 10 ng/ml PDGF (10 μ l of 1.5 μ g/ml in MEM). Stimulation with other growth factors was performed in an identical fashion. Final concentrations used were: EGF, 40 ng/ml; α -thrombin, 20 NIH (National Institutes of Health) units/ml; FGFb, 25 ng/ml; angiotensin II, 100 nM; carbachol, 100 μ M; and phenylephrine, 100 μ M. After 20 min incubation with PDGF, the medium was aspirated and cells were rinsed once with ice-cold 10 mM Tris/HCl/0.85% NaCl. The cells were harvested by scraping them into 0.75 ml of 0.1 M HCl and transferring them to a glass test tube. Lipids were then extracted by adding 2.8 ml of methanol/chloroform (2:1, v/v). After 30 min on ice, 0.95 ml of CHCl₃ and 0.95 ml of 0.1 M HCl were added and the phases separated by centrifugation. To perform thin layer chromatography (TLC), a fixed volume of the lower phase was carefully removed and evaporated under a stream of N₂. The residue was dissolved in 20 μ l of CHCl₃ and 10 μ l of that solution spotted onto silica gel 60 plates (E. Merck, Darmstadt, Germany). The plates were developed in ethyl acetate/iso-octane/acetic acid/water (45:20:12:6, by vol.), which separates didecanoyl phosphatidic acid (didecanoyl PA) from endogenous, long-chain PA [30]. Spots corresponding to didecanoyl [32 P]PA were detected by autoradiography, scraped off and counted for radioactivity in a liquid-scintillation analyser. To normalize activities, parallel experiments were performed using unstimulated control cells, the activity of which was taken to be 100% (basal DGK activity). The extent of DGK α stimulation by PDGF, expressed as a percentage of the basal DGK activity, did not vary by more than 10% when multiple determinations were performed with a single set of VSMC plates. It ranged from 80 to 150% with different VSMC preparations, with higher stimulation generally observed in earlier passages. As described in the Results section below, the increase in DGK activity with PDGF stimulation reflects activation of DGK α , while the basal diC₁₀ phosphorylation is due to the activity of other DGK isoforms.

DAG and diC₁₀ mass assays

Recombinant, histidine-tagged *E. coli* DGK for use in these assays was prepared from *E. coli* strain WH1061/pSD005 by Ni(II)-chelate chromatography [31]. Endogenous long-chain DAG in VSMC extracts was assayed as described previously [23]. To assay diC₁₀, cells in 60-mm-diameter plates were chilled on ice and the medium aspirated. Cell monolayers were washed twice with 5 ml of ice cold Dulbecco's PBS without Ca²⁺ or Mg²⁺. They were then harvested by scraping and the lipids extracted into neutral methanol/chloroform as described by Preiss et al. [32]. DiC₁₀ in these extracts was determined using a radioenzymic assay with *E. coli* DGK [32]. Short-chain DAG analogues are phosphorylated by *E. coli* DAG kinase similarly to naturally occurring DAG [33]. To separate didecanoyl PA from the PA derived from endogenous long-chain DAG present in these cells, the radiochemical products were separated by TLC on silica-gel-60 plates, which were developed with ethyl acetate/iso-octane (2,2,4-trimethylpentane)/acetic acid/water (45:20:12:6, by vol.) as described above. Spots corresponding to didecanoyl [32 P]PA were detected by autoradiography, scraped off and counted in a liquid-scintillation analyser. A solution of diC₁₀ calibrated using a standard ester assay [34] was used to prepare a standard curve.

In vitro DGK activity assays

In vitro DGK activity assays utilized Triton X-100/hexadecylsulphobetaine mixed micelles to disperse and deliver the DAG substrate [35]. This system also permitted dispersal of R59949 for

studies of *in vitro* inhibition [35]. To examine for arachidonoyl DAG-selective DGK (DGK ϵ), activities were determined separately with *sn*-1-palmitoyl-2-oleoylglycerol and *sn*-1-stearoyl-2-arachidonoylglycerol [36,37]. Ca^{2+} activation of VSMC DGKs was determined in an octyl glucoside/phosphatidylserine assay as described previously [35].

Antibody preparation and immunoblotting

To prepare polyclonal anti-DGK α antibodies, residues 2–336 of porcine DGK α , which encompass the entire regulatory region, were expressed as a glutathione S-transferase fusion in *E. coli* [38]. Immunization of rabbits with the recombinant protein and collection of antisera were performed by Alpha Diagnostic International, San Antonio, TX, U.S.A. Two rabbits each received five 150 μg injections of antigen at 2-week intervals. The first injection was in 0.5 ml of complete Freund's adjuvant and the others in 0.5 ml of incomplete adjuvant. Antisera were collected at weeks 7, 9, 11 and 13. Specific reactivity toward DGK α , purified from porcine thymus [35], was confirmed by immunoblotting. To prepare immunoblots, samples were electrophoresed on SDS/7.5% PAGE gels and transferred to nitrocellulose. Bound antibodies were detected using an enhanced chemiluminescence kit (ECL[®]; Amersham, Arlington Heights, IL, U.S.A.) and horseradish-peroxidase-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Immunoprecipitation of DGK α was performed by adding 0.2% Triton X-100 to 100 μl of the pooled MonoQ peak fractions (see below). The samples were pre-cleared with 20 μl of Protein A/G-agarose (Santa Cruz) for 30 min and then incubated with 3 μl of anti-DGK α antibody. After 30 min, 30 μl of Protein A/G-agarose was added and the incubation continued another 2 h. The immunocomplexes were pelleted and the supernatant saved for DGK assay. The Protein A/G-agarose immunocomplexes were washed twice with buffer containing 0.2% Triton X-100, eluted, and examined by immunoblotting. Control immunoprecipitations were performed with 3 μl of pre-bleed antisera instead of anti-DGK α .

Chromatographic resolution of DGK activities from carotid smooth muscle

All purification steps were carried out at 4 °C. Porcine carotid arteries were harvested and the adventitia removed. They were cut open lengthwise and the endothelial layer scraped off. The media were minced into small pieces with razor blades. These were homogenized in a blender and a Polytron homogenizer with 3 vol. of 20 mM Tris/HCl (pH 7.4)/0.25 M sucrose/2.5 mM EGTA/1 mM MgCl_2 /1 mM PMSF/1 mM dithiothreitol/0.05 mM ATP/10 $\mu\text{g}/\text{ml}$ aprotinin/10 $\mu\text{g}/\text{ml}$ leupeptin. This homogenate was centrifuged at 1000 g for 10 min to pellet nuclei and unbroken cells. The postnuclear supernatant was further fractionated into cytosol and membranes by centrifugation at 100 000 g for 60 min. The cytosolic supernatant was diluted to a protein concentration of 5 mg/ml in the same buffer with the NaCl concentration adjusted to 100 mM and addition of Triton X-100 to 0.02%. The membranes were suspended at a protein concentration of 5 mg/ml in the same buffer with 100 mM NaCl and 1% Triton X-100. The membranes and cytosol (2 mg of protein) were fractionated over a Mono Q HR 5/5 column (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The column was eluted with a 30 ml gradient of 100–500 mM NaCl in the same buffer. The Triton concentration in the eluting buffer was 0.02% in all cases. The flow rate was 0.2 ml/min and 0.5 ml fractions were collected. The conductivity of the column effluent was monitored. Peak fractions were pooled and stored at -70°C .

Sulphopropyl (SP)-Sephacrose chromatography of DGK activities recovered from Mono Q was performed as previously described [35].

RESULTS

DGK α is the major DGK activity in vascular smooth muscle

Individual cells may express multiple DGK isoforms [1–3]. To characterize the DGK activities in vascular smooth muscle, cytosol and membrane fractions were prepared from medial layers of porcine carotid arteries and fractionated over Mono Q. Cytosol contained 85% of the total DGK activity and the membranes 15%. The major cytosolic activity was eluted at 100 mM NaCl, and the peak fractions were immunoreactive with anti-DGK α antibodies (Figure 1A). A second cytosolic DGK activity, which did not react with the DGK α antibodies, was eluted at 250 mM NaCl. The membrane fractions also contained two peaks of DGK activity (Figure 1B). The first, which was eluted at 100 mM NaCl, again corresponded to DGK α . Partitioning of DGK α between membranes and cytosol has been previously noted [39,40]. The membrane activity that was eluted at 190 mM NaCl was selective for *sn*-2-arachidonoyl DAG, indicating that it is DGK ϵ [36,37]. These observations are consistent with a previous report that multiple animal tissues express three chromatographically resolvable DGKs, one of which is a membrane-bound, arachidonate-selective isoform [41]. Both the cytosolic and membrane-bound forms of DGK α were

Figure 1 Chromatographic separation of VSMC DGK activities

Cytosol and membrane fractions of porcine carotid media were prepared and the DGK activities resolved over Mono Q as described in the Experimental section. The first peak of activity eluted from Mono Q was also rechromatographed over SP-Sephacrose. Active fractions were examined for DGK α immunoreactivity. Western blots of individual column fractions with anti-DGK α antibodies are shown above the activity profiles. The M_r of the immunoreactive band in membrane fractions 23 and 24 does not correspond to DGK α , indicating that it is non-specific. (A) Shows the Mono Q elution profile of DGK activities in VSMC cytosol, (B) shows the Mono Q elution profile of DGK activities in VSMC membranes, and (C) shows the SP-Sephacrose elution profile of the first DGK activity eluted from Mono Q. ●, Assayed with palmitoyl-oleoyl DAG; △, assayed with stearoyl-arachidonoyl DAG.

Table 1 Properties of VSMC DGK

Peak fractions from the columns shown in Figure 1 were pooled and examined for arachidonate selectivity, stimulation by 20 μM Ca^{2+} and inhibition by 2 mol% R59949 as described previously [35]. Numbers of the column fractions pooled for assays are also indicated. Results are means $\pm$ S.E.M. for two independent measurements

	Fractions assayed	Arachidonate selectivity (fold)	Stimulation by Ca^{2+} (fold)	Inhibition by R59949 (%)
Cytosol				
Peak 1	14–17	0.93 ± 0.13	2.34 ± 0.14	70.6 ± 2.6
Peak 2	36–37	0.86 ± 0.03	0.84 ± 0.05	16.7 ± 2.1
Membrane				
Peak 1	14–17	1.00 ± 0.11	1.93 ± 0.18	71.1 ± 2.3
Peak 2	25–27	2.88 ± 0.52	0.93 ± 0.06	16.5 ± 0.4

stimulated by Ca^{2+} and inhibited by R59949, but these agents did not affect the other DGK activities (Table 1). We have previously shown R59949 to be selective for Ca^{2+} -dependent DGK isoforms, including DGK α [35]. The *in vitro* inhibition observed with the VSMC DGKs is in excellent agreement with our previous demonstration of 68% inhibition of porcine DGK α , but only minimal inhibition of several Ca^{2+} -independent DGKs from a variety of animal tissues [35]. Membranes and cytosol from cultured VSMCs were also immunoreactive with anti-DGK α antibodies (results not shown).

To confirm that the activity eluted at 100 mM NaCl consisted largely of DGK α , the pooled peak fractions were immunoprecipitated with anti-DGK α antibody. About 85% of the activity was precipitated. Moreover, the activity remaining in the supernatant was inhibited by R59949 and activated by Ca^{2+} , similarly to the total peak activity. The activity eluted from Mono Q at 100 mM NaCl was also fractionated over SP-Sepharose. Only a single peak of DGK activity, which was co-eluted with DGK α immunoreactivity at 190 mM NaCl, was observed (Figure 1C). This is consistent with our previous observation that DGK α from porcine thymus is eluted from SP-Sepharose at 200 mM NaCl [35]. These results indicate that the peak of DGK activity eluted at 100 mM NaCl consists largely or entirely of DGK α , and that this isoform represents the major DGK activity in vascular smooth muscle.

DGK α is selectively activated by PDGF in cultured VSMCs

A variety of stimuli increase DGK activities in cultured cells [4–10]. PDGF stimulates a DGK activity in VSMCs [42]. We examined the effect of PDGF on VSMC DGK activity using an *in situ* assay based on phosphorylation of exogenously supplied diC_{10} , a short-chain DAG analogue. This method has been employed by several groups to measure DGK activities in living cells [12,30,43–45]. diC_{10} behaves similarly to naturally occurring DAG in *in vitro* assays, and its *in vivo* phosphorylation provides a measure of DGK activity that does not include PA arising from endogenous DAG [30]. As shown in Figure 2, PDGF induced a rapid increase in diC_{10} phosphorylation, indicating that is stimulating DGK activity in these cells. Note that this assay measures only phosphorylation of diC_{10} , the long-chain PA arising from phosphorylation of endogenous DAG being removed by the TLC step. As short-chain fatty acids are not normal constituents of cell phospholipids, PA arising from pathways other than DAG phosphorylation is also excluded. These results thus indicate that PDGF is directly stimulating DGK activity. If the diC_{10} concentration added to the medium was reduced to 10 or 25 μM , the diC_{10} [^{32}P]PA produced in

Figure 2 Stimulation of VSMC *in situ* DGK activity by PDGF

The increase in activity in response to PDGF was abolished by the DGK α -selective inhibitor R59949. This agent had no effect on basal DGK activity. *P* values are as follows: control versus PDGF only, $n = 14$, $P < 10^{-9}$; PDGF + R59949 versus R59949 only, $n = 6$, not significant; control versus R59949 only, not significant.

response to PDGF was $68 \pm 10\%$ and $103 \pm 6\%$ of that observed with 50 μM diC_{10} . Total diC_{10} associated with the cells increased linearly with added diC_{10} (results not shown). These results indicate that the concentration of diC_{10} used for these experiments is saturating the PDGF-stimulated DGK activity, and that changes in endogenous DAG are unlikely to significantly alter diC_{10} phosphorylation.

As shown above, among the DGK activities in VSMCs, DGK α is selectively inhibited by R59949. Although R59949 and the related compound, R59022, only partially inhibit DGK activities *in vitro*, *in vivo* studies have noted nearly complete inhibition of sensitive DGK activities by these agents [4,7,42,45]. R59949 abolished the PDGF-induced increase in diC_{10} phosphorylation, but had only a small effect on basal DGK activity (Figure 2). These results indicate that PDGF selectively activates DGK α , and that other DGKs account for the basal activity in unstimulated VSMCs.

We also examined whether other stimuli can activate DGK in these cells. Stimulation of diC_{10} phosphorylation by EGF was

Figure 3 Stimulation of VSMC *in situ* DGK activity by α -thrombin

The increase in activity in response to 20 NIH units/ml of α -thrombin was unaffected by R59949. *P* values are as follows: control versus thrombin only, $P < 0.001$; control versus thrombin + R59949, $P < 0.001$; R59949 only versus thrombin only, $P < 0.0005$; R59949 only versus thrombin + R59949, $P < 0.0005$; control versus R59949 only, not significant; thrombin versus thrombin + R59949, not significant; $n = 6$ for all experiments.

Figure 4 Effect of PKC depletion on PDGF stimulation of DGK activity

The left panel shows the effect of PKC depletion (phorbol ester, 24 h) on PDGF stimulation of *in situ* DGK activity in VSMCs. The right panel shows Western blots confirming depletion of the DAG-dependent PKC α and ϵ isoforms. *P* values are as follows ($n = 4$): control versus PDGF only, $P < 0.002$; phorbol ester versus PDGF + phorbol ester, $P < 0.001$; control versus phorbol ester, not significant; PDGF versus PDGF + phorbol ester, $P < 0.05$.

similar to that with PDGF. FGFb also stimulated DGK activity, but only about 25% over basal. For both of these growth factors, the increase in diC₁₀ phosphorylation was completely inhibited by R59949, suggesting that they are also activating DGK α . α -Thrombin stimulated diC₁₀ phosphorylation similarly to PDGF (Figure 3). This stimulation was unaffected by R59949, suggesting that α -thrombin is activating a different DGK. These

results also confirm the strong *in vivo* selectivity of R59949 among DGK isoforms [35]. No effect on diC₁₀ phosphorylation was observed with angiotensin II, carbachol or phenylephrine (results not shown).

PKC can phosphorylate DGKs [46–48]. Moreover, phorbol esters have been reported to stimulate cytosol-to-membrane translocation of DGK activities [8,9]. It is thus possible that one or more PKC isoforms mediate PDGF-induced DGK α activation. It is also possible that diC₁₀ used for the *in situ* DGK assay activates a PKC, which in turn activates one of the DGKs that contribute to the basal activity we observed. To test these possibilities, we examined PDGF stimulation of DGK activity in VSMCs depleted of DAG-dependent PKCs by PMA pretreatment. Cultured porcine VSMCs express the DAG-dependent α and ϵ PKC isoforms, as well as the DAG-independent PKC ζ [23]. Phorbol-ester treatment depleted PKC α and PKC ϵ (Figure 3). PKC depletion had only small effects on basal and PDGF-stimulated DGK activities (Figure 4). This indicates that PDGF-induced activation of DGK α does not require a DAG-dependent PKC and that the basal, unstimulated activity observed in these cells does not reflect activation of the other DGKs via diC₁₀-induced activation of PKC α or PKC ϵ . Direct PKC stimulation by short-term PMA treatment (0–20 min) was also without effect on diC₁₀ phosphorylation (results not shown). Other groups have also reported that DGKs can be activated independently of PKC [11,12,49]. These findings indicate that PDGF-dependent DGK α activation is not mediated by classical (α) or novel (ϵ) PKCs.

Figure 5 Effects of fatty acids on growth-factor-induced DGK α activation in VSMCs

Cells were pretreated for 4 h with 150 μ M NEFA bound to 50 μ M albumin and then stimulated with 10 ng/ml PDGF or 40 ng/ml EGF for 20 min. DGK activity was determined using the *in situ* assay as described in the Experimental section. Results in each panel are means $\pm$ S.E.M. for multiple independent determinations with and without growth factor or fatty acid treatment as indicated. *P* values for growth-factor-treated cells versus fatty-acid-plus-growth-factor-treated cells are as follows: (A), PDGF, oleate, $n = 11$, $P < 0.0001$; (B), PDGF, palmitate, $n = 9$, not significant; (C), PDGF, elaidate, $n = 6$, $P < 0.002$; (D) EGF, oleate, $n = 4$, $P < 2 \times 10^{-7}$.

Figure 6 Effects of NEFA on membrane/cytosol distribution of DGK α

Quiescent VSMCs were exposed to fatty acids for 4 h. Membrane and cytosol fractions were then prepared as described previously [23] and examined for DGK α by immunoblotting.

Table 2 Effects of NEFA on VSMC DiC₁₀ levels

VSMCs in 60-mm-diameter plates were treated with diC₁₀ for 20 min according to the *in situ* DGK activity protocol, except that the [³²P]P_i was omitted. The cells were then harvested and diC₁₀ assayed as described in the Experimental section. The diC₁₀ recovered from the cells represents 1.1% of the total added. Results are means $\pm$ S.E.M. for four independent determinations. *P* values were not significant for any comparison.

Cell treatment	DiC ₁₀ content (pmol)
No-NEFA control	829 $\pm$ 27
Oleate	819 $\pm$ 13
Palmitate	801 $\pm$ 26

Fatty acids inhibit growth-factor-induced DGK α activation

We have previously shown that oleate amplifies PDGF-induced activation of PKC α and PKC ζ [23]. Palmitate did not have this effect. Addition of NEFA to *in vitro* assays can stimulate DGK activities [50,51]. Treatment with oleate or palmitate for 4 h had little effect on basal diC₁₀ phosphorylation (Figures 5A and 5B). However, oleate inhibited the PDGF-induced increase in DGK activity by 60% (Figure 5A). Palmitate, by contrast, had only a small effect (Figure 5B). Inhibition of DGK α activation by oleate, but not palmitate, is consistent with our previous finding that that oleate, but not palmitate, amplifies PDGF-induced PKC activation in VSMCs [23]. Elaidate, the *trans*-unsaturated analogue of oleate, also inhibited DGK activation (Figure 5C). Elaidate amplified PDGF-induced PKC α and PKC ζ activation similarly to oleate (results not shown). Oleate also inhibited the stimulation of DGK activity by EGF (Figure 5D). The extent of inhibition was similar to, or slightly greater than, that seen with PDGF-stimulated DGK activity. Oleate caused only modest (30%) inhibition of the increase in DGK activity stimulated by α -thrombin (results not shown). Fatty acids had no effect on the cell content of DGK α , as estimated by immunoblots (Figure 6). Varying the time of oleate exposure from 10 min to 24 h also had no effect on DGK levels (results not shown). Fatty acids did not alter the concentration of diC₁₀ associated with the cells (Table 2), indicating that the effects on *in situ* DGK activity do not reflect diversion of diC₁₀ into other pathways. These results are consistent with the hypothesis that unsaturated NEFA amplify PDGF-induced PKC activation by impairing the activation of DGK α .

Figure 7 Effect of oleic acid on the PDGF-induced increase in DAG

Quiescent VSMCs were treated with oleic acid for 4 h and then stimulated with PDGF. DAG masses were determined at various times after PDGF addition. Data are means $\pm$ S.E.M. for four independent experiments. Symbols used: ○, cells treated with 150 μ M oleic acid and 50 μ M albumin; ●, control cells treated with 50 μ M albumin only. *P* values were significant at 5 min (*P* < 0.05) and 10 min (*P* < 0.02).

Fatty acids amplify PDGF-induced DAG accumulation

PDGF activates a PI-dependent phospholipase C that cleaves PIs to produce DAG [24]. If inhibition of DGK α contributes to the NEFA-induced amplification of PKC activation, then there might be an increase in the DAG peak occurring after PDGF stimulation. As shown in Figure 7, oleate pretreatment, which we have previously shown to have no effect on basal DAG, doubled the increase in DAG after PDGF stimulation. This is consistent with our previous observation that oleate has no effect on basal PKC activity, but amplifies PKC activation in response to PDGF stimulation [23].

DISCUSSION

We have demonstrated that PDGF and EGF selectively activate DGK α in cultured VSMCs. α -Thrombin activated a different DGK isoform. The present study thus contributes to the growing body of evidence that DGK α [7,49,52] and other DGKs [48,49,53] are individually regulated by extracellular signals. DGKs phosphorylate only DAG arising from stimulated PI turnover [11–13]. As conversion of DAG into other lipids, such as phosphatidylcholine or triacylglycerol, does not proceed through a PA intermediate, DGKs channel PI-derived DAG back into PI resynthesis. DGK inhibition may thus contribute to alterations in inositol-lipid metabolism associated with diabetes [25]. However, further studies will be required to demonstrate this.

Diabetes is associated with development of micro- and macro-angiopathies. Chronic activation of PKC appears to play a critical role in these complications [14]. Work in our laboratory has shown that NEFAs, which are elevated in diabetes, can modulate PKC activation in vascular smooth muscle [23]. We have now shown that fatty acids impair the normal activation of DGK α by PDGF. Reduced DGK α activity may thus contribute to chronic PKC activation in the diabetic vasculature. Consistent with this inhibition of a retinal DGK with R59949 elicits microvascular changes similar to those in early diabetes [54]. Moreover, activation of a vascular DGK by high concentrations of α -tocopherol may contribute to prevention of diabetes-induced PKC activation by this agent [55–57]. Such results indicate that DGK inhibition mimics diabetes-induced vascular changes, but

do not prove that this mechanism is operating in diabetes. In this regard, Nobe et al. have shown that the total DGK activities of aortas and kidneys from streptozotocin-diabetic rats are markedly reduced [26]. A variety of other tissues exhibited no change or modest increases in DGK activity. It should be noted that the changes in DGK activity caused by fatty acids, while modest, are similar in magnitude to the changes in PKC activity that have been reported in diabetes. Our findings thus suggest that impaired DGK α activation in response to local growth factors in the diabetic vasculature may have contributed to the decrease in DGK activity observed by Nobe and co-workers [26].

How NEFAs inhibit DGK α activation is unknown. Fatty acids and their metabolites exert numerous effects on signalling pathways that may directly or indirectly modulate DGKs [58]. DGK α is the only Ca^{2+} -activated DGK in VSMCs (Table 1). Unsaturated fatty acids, including oleate, inhibit Ca^{2+} influx through capacitative and voltage-operated channels [59,60]. Saturated fatty acids do not have these effects. Modulation of Ca^{2+} influx by different NEFAs thus correlates with their inhibition of DGK α activation. Diabetes has been shown to similarly impair Ca^{2+} signalling [18,19]. Fatty-acid-induced dysregulation of Ca^{2+} signalling and inhibition of DGK α activation may thus contribute to chronic PKC activation in diabetes.

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