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Epidermal Growth Factor Receptor Stimulation of Diacylglycerol Kinase

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The epidermal growth factor receptor (EGFR) activates formation of the phospholipid signal messenger phosphatidic acid (PA) on ligand binding. We explored the effects of chronic EGF stimulation on cellular PA in NIH3T3 cells expressing intact EGFR a mutant EGFR (EGFRvIII). The presence of EGFRvIII increased PA levels to twice those induced by chronic EGFR activation. Fatty acid methyl ester analysis revealed a marked increase in oleic acid containing PA. No apparent increase in phospholipase D (PLD) activity was detected, and diacylglycerol (DAG) kinase assays demonstrated a marked preference for dioleoyl DAG in the presence of activated EGFR or EGFRvIII. Levels of PA which were lower than would be predicted by DAG kinase activation are explained by increased phosphatidate phosphohydrolase activity. Specific inhibitors of EGFR kinase and DAG kinase suppressed DAG kinase activation and PA production by EGFRvIII. EGFR kinase activation by chronic exposure to ligand or by deletional mutation stimulates formation of a specific form of signalling PA.

Phosphatidic acid (PA) is an intracellular phospholipid second messenger stimulated by an array of ligands. PA mediates multiple biological effects in the absence of conversion to other second messengers such as diacylglycerol (DAG) and exogenous PA induces DNA synthesis, myc/fos expression and hormone secretion (1, 2). Cellular targets for PA include PI-4-kinase, PKCζ, MAP kinase, protein tyrosine phosphatases and raf-1. (3-9). The potency of PA in cell systems in which species containing different fatty acyl chains have been tested generally demonstrates a greater effect for species containing longer fatty acids with greater degrees of unsaturation (10-12).

Production of PA from membrane phospholipids may be mediated by the sequential activities of phospholipase C (PLC) and DAG kinase or by PLD. Ligand stimulation of EGFR stimulates PA production through either or both of these pathways (13-17). Similarly, the oncogenic *v-erbB* gene product, a mutated homologue of EGFR, stimulates PA production through the PLC/DAG kinase cascade (18). EGFR activation also stimulates phosphorylation of a transfected DAG kinase construct, although no increase in activity was demonstrated (19).

A form of EGFR (EGFRvIII), which is structurally similar to the *v-erbB* gene product, has been cloned from human glioblastoma and is expressed in a wide variety of human tumors (20-24). This 145 kDa receptor undergoes spontaneous homodimerization, autophosphorylation and mediates transformation in vitro and in vivo (25). Initial studies revealed elevated levels of PA in NIH3T3 cells transfected with EGFRvIII. We then demonstrated that chronic EGFR stimulation or the presence of EGFRvIII preferentially stimulates formation of oleoyl containing PA. A DAG kinase activity specific for dioleoyl DAG increases concordant with the increase in oleoyl containing PA and without any change in PLD activity. Chronic EGF exposure stimulates DAG kinase activity but also increases endogenous phosphatidate phosphohydrolase (PPH) activity, partially suppressing PA levels. Specific inhibitors of the EGFR kinase and DAG kinase block activation of the observed DAG kinase activity, confirming dependence on receptor kinase and sensitivity to R59022. This study demonstrates new findings that an oncogenic form of EGFR stimulates a specific DAG kinase activity and in parallel suppresses catabolism of the resulting form of PA, resulting in

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Abbreviations: DAG, diacylglycerol; EGFR, epidermal growth factor receptor; FAME, fatty acid methyl ester; PA, phosphatidic acid; PLC, phospholipase C; PLD, phospholipase D; PPH, phosphatidate phosphohydrolase.

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constitutive expression of a mitogenic lipid second messenger.

MATERIALS AND METHODS

Materials. [γ-^{32}P]-ATP and [^{32}P]-orthophosphate were from Dupont-NEN (Boston, MA) [^{3}H]-oleic acid was from Moravek (Brea, CA), and EGF was from Peprotech (Rocky Hill, NJ). Diacylglycerol, phosphatidic acid, lysophosphatidic acid and phosphatidylbutanol were from Avanti Polar lipids (Alabaster, AL), FAME were from Matreya (Pleasant Gap, PA). R59022 and AG1478 were from Calbiochem (La Jolla, CA). Silica gel G 60 and silica gel G plates were from Alltech (Deerfield, IL). All other reagents were from Sigma (St. Louis, MO).

Cell culture. HC2 20d2/c (HC2) and CO12 20c2/b (CO12) were generated and maintained as described (25). Cells were plated at 10^6 cells per 25 cm^2 plate in DMEM/10% FCS, 200 units/ml penicillin, 200 μg/ml streptomycin, glutamate and grown for 2 days. Monolayers were then washed once with PBS and serum starved in DMEM/0.1% fatty acid free BSA for 24 hr. Cells were labelled by removing complete medium, washing once with serum free medium and then labelled to isotopic equilibrium in DMEM containing [^{32}P]-orthophosphate (100 μCi/ml in phosphate free medium) or [^{3}H]-oleic acid (1 μCi/ml in 0.1% fatty acid free BSA).

DAG kinase assay. The deoxycholate based assay was done essentially as described (26) with modifications by Walsh et al. (27). Briefly, cells were lysed in 1 mM deoxycholate, 100 mM Tris-HCl, 20 mM NaF, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA (pH 7.0). 100 μg of lysate was added to a reaction mix with final concentration of 1mM deoxycholate, 1mM DAG, 1.5 mM [γ-^{32}P]-ATP, 100 mM Tris-HCl (7.4), 20 mM NaF, 10 mM $MgCl_2$, 1mM DTT, 1mM EGTA in 16 $\times$ 100 mm glass tubes. Samples were briefly vortexed and the reaction was allowed to proceed for 15 min. at 25° C. Reactions were quenched with 2 ml of ice cold methanol/chloroform (1:1, v/v) containing 60 μl/ml of 12N HCl. 1 ml of 1% perchloric acid/0.1% phosphoric acid in H_2O/methanol (7:1, v/v) was added and mixtures briefly vortexed followed by removal of the upper aqueous phase. The organic phase was then washed twice more with 1 ml of the same solution and 650 μl of the final organic phase was assayed by Cerenkov counting.

Phospholipid extraction. Whole cell phospholipids were extracted by modification of Bligh and Dyer using 0.4M HCl as the aqueous wash when PA was assayed (28-30). Extracts were then separated on preactivated silica gel 60A plates impregnated with 0.25 M oxalic acid in the solvent system chloroform/methanol/12 N HCl (87:13:0.5, v/v/v). DAG analysis was carried out on silica gel G in the solvent system hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The position of lipids was determined by iodine exposure and detection of standards and/or autoradiography followed by scraping of silica gel spots and quantitated by scintillation counting (Cerenkov counting for [^{32}P]-orthophosphate).

Phospholipase D analysis. PLD activity was assayed essentially as described (31) by quantitation of the formation of labelled phosphatidylbutanol.

Capillary GC. The identity of acyl chain content in PA was determined by capillary GC after esterification with BF3/methanol (32). FAME were then analyzed on a Hewlett-Packard 5790A gas chromatograph using a Supelco Omegawax 250 column, 30 m $\times$ 0.25 mm ID, using nitrogen as carrier gas. Sample runs were performed with the injector and detector at 200 and 250° C, respectively, and the programmed run was 140° C for 2 min., 4° C/min. to 250° C for 4 min., FAME were detected by FID. Peaks were identified using standard FAME.

Unless otherwise indicated all experiments were performed at least three times and representative data are presented.

TABLE 1

EGFR and EGFRvIII Stimulated PA Production

Cells	Agonist	Time	% control
CO12	None		100 ± 14
	EGF	15 min	114 ± 9
		30 min	116 ± 6
		60 min	108 ± 3
		6 hrs	112 ± 3
		12 hrs	130 ± 10
		24 hrs	142 ± 2
HC2	None		340 ± 4

HC2 and CO12 cells were labelled with 100 mCi/ml of [^{32}P]-orthophosphate in serum and phosphate free medium for 24 hours in the presence or absence of EGF (25 ng/ml). Cellular lipids were extracted and labelled phospholipids quantitated as described in "Materials and Methods." PA was normalized to total phospholipid radioactivity (% d.p.m.) and data expressed as % of unstimulated CO12 control which was 0.73 ± 0.14% of total [^{32}P]-orthophosphate label. Data points represent duplicate cultures.

RESULTS

The ligand dependent activation of EGFR has been demonstrated to stimulate PA production in a variety of cell contexts (13-16, 33). We followed the production of PA by labelling of lipid pools with [^{32}P]-orthophosphate followed by exposure to agonist as described in "Materials and Methods". As shown in Table 1, [^{32}P]-orthophosphate labelled PA formation in HC2 cells containing the EGFRvIII mutant was 340% of basal PA in unstimulated control CO12 cells. EGF (25 ng/ml) stimulation for 24 hr. of CO12 cells which contained intact EGFR resulted in levels approximately 50% higher than those of controls but this was still significantly lower than that found in HC2 cells. Because HC2 cells express extremely low levels of intact EGFR, they do not respond to EGF (34). CO12 cells exposed to EGF for shorter periods did not have significantly elevated levels of PA.

Because the fatty acid constituents of PA have been found to be critical to the induction of mitogenesis and to its ability to modulate other enzyme activities we characterized the fatty acid components of PA in unstimulated CO12 and HC2 cells (10, 12). As shown in Table 2, the presence of EGFRvIII resulted in a 2-fold increase in the content of the monoenoic fatty acid oleate in isolated PA fractions. This increase was relatively specific with only minor changes in other saturated and unsaturated fatty acids that were assayed. We subsequently labelled cells with [^{3}H]-oleic acid to confirm preferential labelling. As demonstrated in Table 3 [^{3}H]-oleic incorporation is 132 ± 9% that of unstimulated controls in cells exposed to EGF for 24 hrs and 210 ± 2% in

TABLE 2

Fatty Acid Composition of PA from CO12 and HC2 Cells

Fatty acid	CO12 (Average % ± STD)	HC2 (Average % ± STD)
14:0	13.7 ± 2.7	7.8 ± 3.2
16:0	33.7 ± 1.8	35.0 ± 0.3
16:1	13.5 ± 4.9	12.6 ± 0.3
18:0	17.9 ± 4.6	14.6 ± 2.5
18:1	10.8 ± 4.1	23.1 ± 1.8
18:2	2.6 ± 1.6	1.4 ± 1.9
20:4	7.9 ± 1.5	4.8 ± 0.3

Cellular lipids were extracted and separated on TLC, the silica gel corresponding to PA was scraped and PA reextracted. PA was then methylated as described in "Materials and Methods" and FAME were separated by capillary GC. Data expressed as perfect of total detected FAME. C18:3, C20:2, C20:3, C22:4 were less than 2% in both cell types.

HC2 cells. DAG levels were also assayed in parallel and no significant changes due to the presence of EGFRvIII or EGF exposure were present (data not shown).

Agonists or oncogenes typically stimulate PA production from membrane phospholipids through the serial activation of phospholipase C and DAG kinase or activation of PLD. We initially evaluated the contribution of PLD by measuring the production of radiolabelled phosphatidylbutanol catalyzed by PLD (31, 35). HC2 or CO12 cells were prelabelled with [^{3}H]-oleic acid followed by incubation with 30 mM 1-butanol, and formation of phosphatidylbutanol was quantitated. Prelabelled cells did not demonstrate any increase in phosphatidylbutanol production over extended incubations in the presence of butanol in the presence or absence of EGF (Table 4). The presence of butanol in medium during labelling of HC2 cells also did not suppress elevated levels of [^{3}H]-oleic PA (data not shown).

DAG kinase has been postulated to be an important mediator of PA production in response to EGF (13, 15-17, 33). EGFR stimulation of cells transfected with an isoform of DAG kinase resulted in a rapid tyrosine phosphorylation of DAG kinase, suggesting modulation of the enzyme by EGFR (19). Because PLD activation did not appear to be responsible for upregulation of PA we characterized DAG kinase activities for different DAG isoforms in unfractionated cell extracts from CO12 and HC2 cells in an attempt to identify the source of oleoyl PA.

Basal levels of DAG kinase activity for the different DAG isoforms varied from 0.035 nmol/mg/min. to 0.15 nmol/mg/min. with the highest activity towards dioleoyl DAG (Figure 1). Stimulation of intact EGFR for 24 hours or the presence of EGFRvIII increased DAG kinase activity for dioleoyl DAG nearly equivalently, with a maximal activity of 0.42 nmol/mg/min. DAG kinase activity did not increase significantly in CO12 cells in response to EGF for more than 12 hours (data not shown).

Chronic stimulation of intact EGFR and EGFRvIII appeared to be equally capable of activating DAG kinase activity for dioleoyl DAG, but PA levels were lower in chronically stimulated cells. We postulated that the presence of mutant receptor may have similar effects to those mediated by other transforming oncogenes, that is to suppress phosphatidate phosphohydrolase activity, thereby allowing increased levels of PA to accumulate (36). We indirectly tested this hypothesis by including the phosphatidate phosphohydrolase inhibitor propranolol during labelling of cells (37). As shown in Figure 2, the presence of propranolol increased levels of PA in both cell lines, however it had the greatest effect in CO12 cells exposed to EGF, increasing PA levels by 240%, whereas in HC2 cells the increase was less than 100%. These data suggest that PPH actively suppresses PA levels during chronic EGF stimulation and the presence of EGFRvIII inhibits PPH activity, increasing cellular levels of PA.

Inhibitors of EGFR and DAG kinase were used to confirm that these enzymes were responsible for the observed activities. The tyrphostin AG1478 is a potent and specific inhibitor of EGFR kinase activity in vitro, with an IC_{50} of 0.003 nM (38). AG1478 will inhibit kinase activity and autophosphorylation mediated by EGFRvIII and completely reverses the transformed phenotype mediated by EGFRvIII in HC2 cells at 0.5-1 μM within 24 hrs. (RBM, DKM,

TABLE 3

[^{3}H]-Oleic Acid Labelling of PA from CO12 and HC2 Cells

Cells	Agonist	Time	% control
CO12	None		100 ± 5
	EGF	15 min	86 ± 16
		30 min	84 ± 11
		60 min	102 ± 10
		6 hrs	93 ± 12
		12 hrs	120 ± 6
		24 hrs	132 ± 9
HC2	None		210 ± 2

Cellular lipids were labelled with 1 mCi/ml of [^{3}H]-oleic acid in serum free medium for 24 hours and exposed to vehicle or EGF 25 ng/ml for indicated periods of time. Lipids were then extracted and PA separated and analyzed as described in "Materials and Methods." PA was normalized to total phospholipid radioactivity (% d.p.m.) and data expressed as % of unstimulated CO12 control which was 1.41 ± 0.07% of total [^{3}H]-oleic acid label. Data points represent duplicate samples.

TABLE 4

Phospholipase D Response to EGF Exposure or EGFRvIII

	CO12			HC2		
EGF exposure (min)	0	5	60	0	5	60
PBut (% control)	100 ± 8	112 ± 11	90 ± 3	101 ± 12	89 ± 2	88 ± 12

Cellular lipids were labelled with 1 mCi/ml of [^{3}H]-oleic acid in serum free medium for 24 hours, washed and preincubated with 1-butanol (30 mM) for 15 min. followed by exposure to EGF 25 ng/ml or vehicle. Lipids were then analyzed as described in "Materials and Methods." Phosphatidylbutanol formation is expressed as % of unstimulated CO12 control.

unpublished observations). As has been demonstrated by other investigators, kinase inhibition in intact cells generally requires higher concentrations in order to overcome competition with intracellular ATP (39). The DAG kinase inhibitor R59022 suppresses DAG kinase and although it has been used to inhibit DAG kinase in intact cells, in our hands effective concentrations were nonspecifically toxic (data not shown). We tested the ability of AG1478 to inhibit the effect of EGFRvIII and chronic EGF exposure on PA formation and DAG kinase activation and the effect of R59022 on DAG kinase activity in cell lysates. As shown in Figure 3, the addition of AG1478 at 1 μM, where essentially 100% of cells remain viable, inhibited levels of [^{32}P]-orthophosphate labelled PA in CO12 and HC2 cells to almost the level of unstimulated controls. AG1478 exposure during serum starvation also suppressed DAG kinase activity for dioleoyl DAG to approximately that of unstimulated cells although the addition of the tyrphostin to lysates had no effect on activity (Figure 4 and data not shown). The addition of the DAG kinase inhibitor R59022 (1 μM) to unfractionated cell lysates for 15 min. prior to DAG kinase assay completely suppressed activity for dioleoyl DAG (Figure 4). These data confirm that EGFR kinase activity is necessary for formation of labelled PA and activation of DAG kinase and that an appropriate DAG kinase inhibitor can suppress the observed activity.

DISCUSSION

Generation of PA in response to ligand or activated oncogenes occurs over different time courses depending on the agonist and cell context. The mechanisms underlying elevations in this phospholipid second messenger are also varied and may involve both activation of synthesis and suppression of the catabolism of PA. In this study we have shown that prolonged EGFR stimulation or the presence of EGFRvIII is capable of increasing the mitogenic second messenger PA through a specific increase in DAG kinase activity for the substrate dioleoyl DAG. The presence of mutant receptor also inhibits phosphatidate phosphohydrolase activity, suppressing one metabolic pathway for PA. Inhibitors of EGFR tyrosine kinase activity and DAG kinase block the induction of DAG kinase activity, demonstrating that the

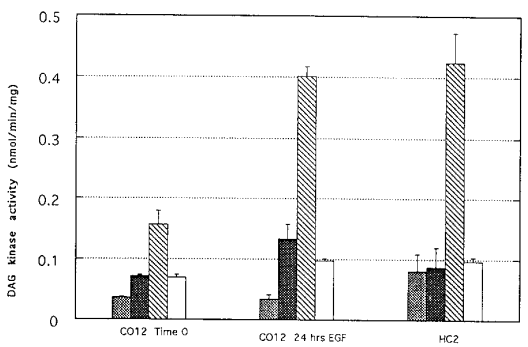

FIG. 1. EGFR stimulation of DAG kinase activity. Lysates from cells treated with vehicle or EGF (25 ng/ml × 24 hrs) were incubated with the DAG species indicated in the presence of [γ-^{32}P]-ATP and incorporation into PA quantitated as described in "Materials and Methods." DAG species: 16:0-16:0 (▨), 16:0-18:1 (▩), 18:1-18:1 (▩), 18:0-20:4 (□).

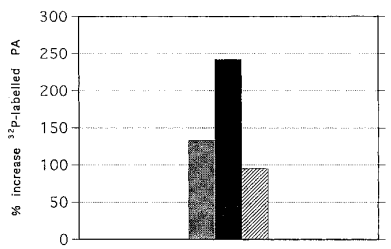

FIG. 2. Propranolol effect on PA in CO12 and HC2 cells. CO12 cells incubated with vehicle (▨) or EGF (25 ng/ml × 24 hrs) (■) and HC2 cells (▨) were labelled as per Table 1 in the presence and absence of propranolol (100 μM). Lipids were then extracted and PA assayed as per "Materials and Methods" and the % increase in PA production in response to propanolol was determined.

mutant EGFR is the proximal cause for increased PA. Stimulation of intact EGFR increases PA and DAG kinase activity, however PPH appears to partially suppress total intracellular PA. EGFRvIII resembles the transforming oncogenic proteins fps and ras which elevate intracellular levels of PA through activation of PA synthesis and concurrent suppression of PPH, although production of PA appears to be stimulated through PLD (36).

The contribution of PA to transformation by EGFRvIII is more difficult to assess. The addition of exogenous dioleoyl PA to untransformed NIH3T3 cells increases cell refractility, rounding and proliferation (2). However the inability to inhibit metabolism of PA to other second messengers which also have biologic effects (i.e. DAG or lysophosphatidic acid) makes the significance of this finding inferential. Acyl chain length and unsaturation of phospholipids influence the physical state of lipid species within membranes, affecting membrane fluidity, permeability and stability. Acyl chain constituents may also modify the interaction of lipids with potential protein targets. Stacey and coworkers demonstrated that PA containing unsaturated fatty acids as well as fatty acids alone could inhibit GTPase activating protein activity of different proteins, presumably through the critical micellar concentration of these lipids (40-42). PA influences the activation of components of several protein and lipid signal transduction cascades and it seems likely that modulation of these cascades contributes to the biologic phenotype mediated by EGFRvIII. Characterization of PA effects on these potential substrates, i.e. raf-1, PI-4-kinase, MAP kinase and the atypical PKC's may reveal how PA contributes to the transformation and mitogenesis mediated by EGFR kinase activity.

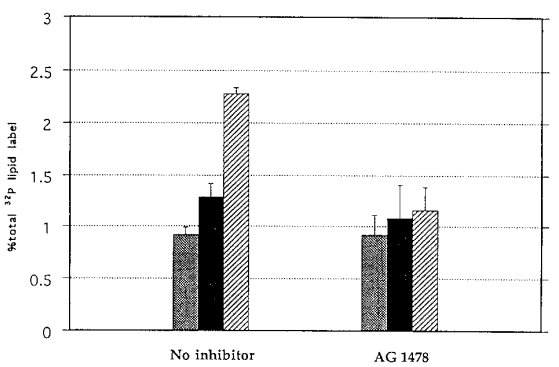

FIG. 3. PA formation in the presence of tyrphostin AG1478. CO12 cells incubated with vehicle (▨) or EGF (25 ng/ml × 24 hrs) (■) and HC2 cells (▨) were labelled as per Table 1 in the presence and absence of AG1478 (1 μM). Lipids were then extracted and PA assayed as per "Materials and Methods."

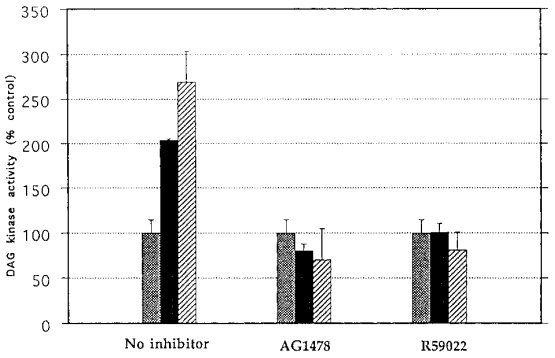

FIG. 4. DAG kinase activity in the presence of tyrphostin AG1478 and DAG kinase inhibitor R59022. Lysates from CO12 cells incubated with vehicle (▨) or EGF (25 ng/ml × 24 hrs) (■) and HC2 cells (▨) were incubated with dioleoyl DAG in the presence of [γ-^{32}P]-ATP and incorporation into PA was quantitated as described in "Materials and Methods." Intact cells were incubated with AG1478 (1 μM) during starvation and lysates with DAG kinase inhibitor R59022 (1 μM) as indicated.

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