Differential pathways (phospholipase C and phospholipase D) of bradykinin-induced biphasic 1,2-diacylglycerol formation in non-transformed and K-*ras*-transformed NIH-3T3 fibroblasts

Involvement of intracellular Ca^{2+} oscillations in phosphatidylcholine breakdown

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Bradykinin (BK) induced a biphasic increase in 1,2-diacylglycerol (DAG) in both K-*ras*-transformed fibroblasts (DT) and the parent NIH-3T3 cells. The first phase was coincident with the increase in $\text{Ins}(1,4,5)\text{P}_3$ resulting from $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis, and the second, sustained, phase was derived from phosphatidylcholine (PtdCho) hydrolysis. In NIH-3T3 cells, stimulation by BK induced greater production of choline than phosphocholine in [^3H]choline-labelled cells and appreciable phosphatidylethanol (PtdEtOH) formation in [^3H]myristic acid-labelled cells, suggesting that PtdCho was hydrolysed mainly by a phospholipase D (PLD) activity. Pretreatment with propranolol, an inhibitor of phosphatidate phosphohydrolase, markedly diminished the second DAG accumulation, supporting the above notion. In DT cells, BK induced predominantly phosphocholine generation and little PtdEtOH formation, indicating that the PtdCho hydrolysis was due to a phospholipase C (PLC) activity. The BK-induced oscillations in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) observed in single DT cells [Fu, Sugimoto, Oki, Murakami, Okano & Nozawa (1991) FEBS Lett. 281, 263–266] were detected as a sustained $[\text{Ca}^{2+}]_i$ elevation when assayed in a cell suspension. A receptor-operated Ca^{2+} channel blocker, SK&F 96365, suppressed both the BK-induced phosphocholine generation and the sustained $[\text{Ca}^{2+}]_i$ elevation in a similar dose-dependent manner. These results thus suggested that oscillations in $[\text{Ca}^{2+}]_i$ are involved in the activation of PtdCho-specific PLC in DT cells.

INTRODUCTION

The *ras* proto-oncogenes encode highly conserved, membrane-associated, low-molecular-mass GTP-binding proteins which are thought to be important in pathways regulating cell proliferation and differentiation [1]. Genes with point mutations encode modified proteins (*v-ras*) which are resistant to inactivation by GTPase-activating protein [2] and cause sustained activation of unknown *ras* targets, leading to oncogenic transformation. Some earlier studies have demonstrated enhanced phosphoinositide turnover in *ras*-transformed cells. By overexpressing *ras* proteins, coupling of bombesin or bradykinin (BK) receptors to phospholipase C (PLC) was amplified [3,4], and the agonist-independent stimulation of PLC was increased in cells transformed by an activated N- or H-*ras* gene [5]. Huang *et al.* [6] showed that the increased phosphoinositide turnover was due to altered protein kinase activity rather than to the PLC activity itself. When steady-state levels of $\text{PtdIns}(4,5)\text{P}_2$, inositol phosphates and 1,2-diacylglycerol (1,2-DAG) were examined in some fibroblast cell lines before and after transformation with three different *ras* genes, decreased levels of phosphoinositide and enhanced levels of inositol phosphates and 1,2-DAG were observed after transformation [7]. On the other hand, sustained elevation of 1,2-DAG without increased formation of inositol phosphates in *ras*-transformed fibroblasts suggested other sources of DAG than phosphoinositides [8,9].

There is increasing evidence that DAG plays an important role in the signal transduction of mitogenic stimuli such as thrombin,

fibroblast growth factor, platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and lectins [10–14]. The quantitative measurement of 1,2-DAG mass content [15] has enabled us to recognize biphasic 1,2-DAG formation in various cell types stimulated with agonists [13,16]. The first, rapid, phase coincides with $\text{Ins}(1,4,5)\text{P}_3$ formation, and the second, delayed, accumulative phase occurs in the absence of $\text{Ins}(1,4,5)\text{P}_3$. It is thus thought that, whereas the first transient production of DAG is due to $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis, the second sustained production results from phosphatidylcholine (PtdCho) breakdown. In fact, agonist-induced breakdown of PtdCho has been reported in various cells such as hepatocytes [17], MDCK cells [18], HL-60 granulocytes [19] and fibroblasts [20]. Two possible pathways have been suggested for the generation of 1,2-DAG from PtdCho [21]. The activation of PtdCho-specific PLC generates 1,2-DAG and phosphocholine [22], and the activation of phospholipase D (PLD) produces choline and phosphatidic acid (PtdOH), which is then converted to 1,2-DAG via PtdOH phosphohydrolase [23]. The elevation of the steady-state 1,2-DAG level in *ras*-transformed cells can be accounted for by one or both of these two pathways. The stimulation of PtdCho hydrolysis has been demonstrated in rat fibroblasts transfected with a temperature-permissive K-*ras* oncogene [24], in Swiss 3T3 cells loaded with oncogenic *ras* p21 [25], and in *Xenopus laevis* oocytes microinjected with oncogenic *ras* p21 [26]. This may indicate that transformation by *ras* activates PtdCho hydrolysis.

In addition to 1,2-DAG, Ca^{2+} signals are known to be important in cell proliferation, but the relationship between Ca^{2+}

Abbreviations used: BK, bradykinin; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; 1,2-DAG, 1,2-diacylglycerol; DT cells, K-*ras*-transformed NIH-3T3 cells; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; fura-2/AM, fura-2 acetoxymethyl ester; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; HBSS, Hepes-buffered saline; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PtdOH, phosphatidic acid; PtdEtOH, phosphatidylethanol; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; SK&F 96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl-1H-imidazole hydrochloride.

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and oncogenic transformation remains poorly understood. Recently we have found that BK, bombesin and fetal calf serum induced periodic changes in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) ($[\text{Ca}^{2+}]_i$ oscillations) in K-*ras*-transformed fibroblasts (DT cells) [27]. Electrophysiological investigations revealed BK-induced oscillations in membrane potential which were associated with $[\text{Ca}^{2+}]_i$ changes in cells expressing the transforming H-*ras* oncogene [28]. Collin *et al.* [29] have shown that microinjection of H-*ras* into snail neurons caused a sustained voltage-sensitive inward Ca^{2+} current, suggesting that a Ca^{2+} channel might be a target for *ras* oncogenes.

In the present study we have examined the pathways of 1,2-DAG generation, especially the delayed accumulative phase of its biphasic production induced by BK stimulation in NIH-3T3 fibroblasts and the corresponding *ras*-transformed (DT) cells. The results obtained here suggest that the sustained 1,2-DAG formation in DT cells is mediated via the PtdCho-specific PLC pathway, whereas in parent NIH-3T3 cells it is mainly mediated via PtdCho-specific PLD followed by the activation of PtdOH phosphohydrolase. Furthermore, it is also postulated that $[\text{Ca}^{2+}]_i$ oscillations are involved in the activation of PtdCho-specific PLC in DT cells.

EXPERIMENTAL

Materials

BK and H-7 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); fura-2 and fura-2 acetoxymethyl ester (fura-2/AM) were from Dojin Laboratories (Kumamoto, Japan); SK&F 96365 was kindly supplied by Dr. J. E. Merritt (SmithKline-Beecham Pharmaceuticals); [9,10- ^3H]myristic acid (39.3 Ci/mmol) was from Du Pont-New England Nuclear; [methyl- ^3H]choline chloride (85 Ci/mmol) was from American Radiolabeled Chemical (St. Louis, MO, U.S.A.); silica gel 60 plates were from Merck (Darmstadt, Germany), and silica gel LK6D was from Whatman Chemical Separation Inc. (Clifton, NJ, U.S.A.). All other chemicals were obtained from commercial sources and were of the highest quality available.

Cell cultures

Parent NIH-3T3 cells and DT cells were used in these experiments. Ras protein is overexpressed in DT cells, as described by Noda *et al.* [30]. These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum [27]. Special care was taken to maintain the cell lines at subconfluent densities, and cultures were replaced at 1 month intervals from frozen stocks. For stimulation by BK, cells were cultured in DMEM containing 10% fetal calf serum for 48 h and then in DMEM containing 1% fetal calf serum for another 48 h prior to adding the agonist.

Assay of PtdIns(4,5) P_2 -specific PLC activity

Cells were grown in 100 mm dishes, and subconfluent cultures were washed three times with cold phosphate-buffered saline (PBS; 2.68 mM-KCl/1.38 mM- KH_2PO_4 /147 mM-NaCl/4.29 mM- Na_2HPO_4 , pH 7.4) and scraped with a rubber policeman. Cell homogenates were prepared by sonication in extraction buffer consisting of 20 mM-Tris/HCl (pH 7.4), 2 mM-phenylmethanesulphonyl fluoride, 2 mM-EDTA and 1 μg of leupeptin/ml. PLC activity was measured as described previously [31]. Briefly, the reaction mixture (68 μl) contained 50 mM-Tris/maleate buffer (pH 6.5), 80 mM-KCl, 1 mg of sodium deoxycholate/ml, 0.2 mM-[^3H]PtdIns(4,5) P_2 (25000 d.p.m.) and 50 μg of cell protein. A final Ca^{2+} concentration of 1 μM was used for the assay of

PtdIns(4,5) P_2 -specific PLC activity. After incubation for 10 min at 37 °C, the reaction was terminated by addition of 0.34 ml of chloroform/methanol/conc. HCl (100:100:0.6, by vol.) and 0.1 ml of 5 mM-EGTA/1 M-HCl solution. The mixture was vortex-mixed and then centrifuged for 10 min at 2000 *g*. A 0.2 ml portion of the upper aqueous phase was carefully transferred to a vial and mixed with 6 ml of scintillation fluid, and radioactivity was determined in a liquid scintillation counter.

Assay of protein kinase C (PKC) activity

Cells were grown in 100 mm dishes and subconfluent cultures were washed twice with cold PBS and scraped with a rubber policeman into PBS. The cytosol and the membrane fractions were prepared as described previously [32]. Phosphorylation activity was measured by the incorporation of [^{32}P]ATP into histone H1 in 3 min at 30 °C. PKC activity was defined as the difference between the phosphorylation activity in the presence of 1 mM- CaCl_2 , 64 μM -phosphatidylserine (PtdSer) and 1.3 μM -diolein and that in the presence of 1 mM-EGTA instead of Ca/PtdSer/DAG.

The protein contents were measured using the Bio-Rad protein assay, with γ -globulin as standard.

Determination of mass content of Ins(1,4,5) P_3

Cells were grown in 24-well plates and the subconfluent cultures were washed three times with DMEM, followed by the addition of 400 μl of DMEM and 120 μl of 10% (w/v) HClO_4 , and the mixture was kept on ice for 20 min and then neutralized with ice-cold 1.53 M-KOH/75 mM-Hepes for 20 min. The samples were centrifuged at 2000 *g* for 10 min to remove the KClO_4 precipitate. The supernatant (100 μl) was taken for measuring mass contents of Ins(1,4,5) P_3 with the IP_3 assay kit from Amersham. The standard curve was linear from 0.19 to 25 pmol of Ins(1,4,5) P_3 .

Determination of mass content of DAG

Cells were seeded in 12-well plates and subconfluent cultures were rapidly washed twice with Hepes-buffered salt solution (HBSS) (145 mM-NaCl, 25 mM-Hepes, pH 7.35, 5 mM-KCl, 1 mM- CaCl_2 , 1 mM- MgCl_2 , 10 mM-glucose and 0.1% BSA) and incubated with or without BK (100 nM) for the indicated times. The reaction was terminated with ice-cold methanol. Lipids were then extracted essentially as described by Bligh & Dyer [33], except that 0.2 M-KCl/5 mM-EDTA was used instead of 2 M-KCl. The mass content of DG in the cellular extract was measured using *Escherichia coli* DAG kinase (Amersham) essentially as described previously [34]. Briefly, the DG in the lipid extract prepared from cells was quantitatively converted to [^{32}P]PtdOH by incubation at 30 °C for 60 min with the kinase in the presence of [^{32}P]ATP. Following extraction steps to remove unreacted [^{32}P]ATP, [^{32}P]PtdOH was separated by t.l.c. (silica gel 60) using a solvent system of chloroform/methanol/acetic acid/water (30:15:4:2, by vol.). The area corresponding to [^{32}P]PtdOH was located by autoradiography and then scraped off the plates, and radioactivities were determined in a liquid scintillation counter.

Extraction and analysis of lipids from cells prelabelled with [^3H]myristic acid

Cells were prelabelled with 0.5 μCi of [^3H]myristic acid/ml of medium for 48 h. The reaction was terminated, and cellular lipids were extracted and analysed as described previously [34].

Extraction and analysis of choline metabolites from cells prelabelled with [^3H]choline

Cells were exposed to 0.5 μCi of [^3H]choline/ml of medium for 48 h. The incubation was terminated by adding ice-cold meth-

anol, and the aqueous and organic phases were separated as described previously [34]. The aqueous phase was dried by centrifuging (2000 g, 90 min) under vacuum (Taitec VC-960 centrifugal concentrator and VD-600 freeze dryer) and resuspended in 30 μ l of 50 % (v/v) ethanol, and then spotted on to silica gel LK6D and run in methanol/0.5 % NaCl/28 % NH₃ (50:50:1, by vol.) by the method of Yavin [35]. Standards of choline, phosphocholine and glycerophosphocholine were co-chromatographed and identified using I₂ vapour. Phospholipids were separated and analysed as described above [34].

Assay of [Ca²⁺]_i in cell populations and in single cells

[Ca²⁺]_i was measured in fura-2-loaded cells as described previously [27,32].

Data analysis

Statistical differences between control and test values were analysed by Student's *t* test.

RESULTS

DG mass content and PKC activity in K-*ras*-transformed fibroblasts and parent NIH-3T3 cells

During exponential growth in the presence of 10 % fetal calf serum, the mass content of DG in cells was significantly higher than that in NIH-3T3 cells (Table 1). Since the DG assay system measure 1-alkyl-2-acylglycerol as well as 1,2-DAG, we differentially quantified these two types of DG in samples treated with *Rhizopus arrhizus* phospholipase A₁, which hydrolyses the C-1 acyl chain [36]. In both DT and NIH-3T3 cells, most of the total DG was of the diacyl type (1,2-DAG), with the ether type being at most 10–15 %. As reported previously [37,38], the sustained high level of 1,2-DAG causes down-regulation of PKC in *ras*-transformed cells. Since no significant differences were seen in protein content between NIH-3T3 cells and DT cells (240–260 μ g of protein/10⁶ cells), the PKC activity was considerably lower in DT cells than in the parent NIH-3T3 cells (Table 1).

In order to determine the source(s) of the higher 1,2-DAG level in DT cells, we first examined phosphoinositide metabolism. The mass content of Ins(1,4,5)P₃, another product of

Table 1. Mass contents of DG and Ins(1,4,5)P₃ and activities of PtdIns(4,5)P₂-specific PLC and PKC in K-*ras*-transformed (DT) and non-transformed NIH-3T3 cells

DT and NIH-3T3 cells were cultured in 10 % fetal calf serum containing DMEM for 48 h before harvesting. Detection of mass contents of total DG and Ins(1,4,5)P₃, and measurement of activities of PKC and PtdIns(4,5)P₂-specific PLC, were performed as described in the Experimental section. Values represent means $\pm$ S.E.M. (*n* = 6). Significant differences compared with non-transformed NIH-3T3 cells: * *P* < 0.05, ** *P* < 0.01.

	NIH-3T3 cells	DT cells
DG content (pmol/10 ⁶ cells)	191.0 $\pm$ 21.6	333.1 $\pm$ 32.0*
Ins(1,4,5)P ₃ content (pmol/10 ⁶ cells)	22.1 $\pm$ 3.7	13.8 $\pm$ 3.3
PtdIns(4,5)P ₂ -specific PLC activity (nmol/min per mg of protein)	16.9 $\pm$ 3.4	8.2 $\pm$ 1.3**
PKC activity (pmol/min per mg of protein)		
Membrane	102.4 $\pm$ 5.2	44.9 $\pm$ 11.2*
Cytosol	781.0 $\pm$ 91.6	343.1 $\pm$ 24.8*

Table 2. Choline metabolites in K-*ras*-transformed (DT) and non-transformed NIH-3T3 cells

DT and NIH-3T3 cells were prelabelled with [³H]choline (0.5 μ Ci/ml) in 10 % fetal calf serum containing DMEM for 48 h and then incubated in isotope-free medium for 6 h before harvesting. The incubation was terminated by adding ice-cold methanol. Choline metabolites were extracted and analysed as described in the Experimental section. The total radioactivities incorporated were 162 150 $\pm$ 9535 d.p.m./10⁵ NIH-3T3 cells and 148 370 $\pm$ 7340 d.p.m./10⁵ DT cells. Values are means $\pm$ S.E.M. (*n* = 4); significant differences from non-transformed cells: ** *P* < 0.01.

	³ H radioactivity (d.p.m./10 ⁵ cells)	
	NIH-3T3 cells	DT cells
Aqueous fractions		
Choline	371 $\pm$ 56	126 $\pm$ 3**
Phosphocholine	12 060 $\pm$ 178	46 805 $\pm$ 274**
Glycerophosphocholine	1 469 $\pm$ 89	5 484 $\pm$ 400**
Lipid fractions		
PtdCho	130 650 $\pm$ 417	76 070 $\pm$ 1176**
Lyso-PtdCho	3 277 $\pm$ 289	1 057 $\pm$ 98**
Sphingomyelin	9 572 $\pm$ 392	7 195 $\pm$ 1193

PtdIns(4,5)P₂ hydrolysis catalysed by PLC, was quantitatively measured using an Ins(1,4,5)P₃-specific binding protein (Table 1). Compared with NIH-3T3 cells, DT cells contained much less Ins(1,4,5)P₃, when measured in either serum-starved cells or exponentially growing cells in the presence of 10 % fetal calf serum. These results were reflected in the activity of PLC in hydrolysing PtdIns(4,5)P₂, which was much lower in DT cells than that in non-transformed NIH-3T3 cells (Table 1), indicating that phospholipid(s) other than PtdIns(4,5)P₂ were a major source of 1,2-DAG accumulation in DT cells.

Next, both types of cells were incubated with [³H]choline in DMEM containing 10 % fetal calf serum for 48 h to accomplish steady-state labelling, by which time more than 75 % of total radioactivity was incorporated into PtdCho: 150 101 $\pm$ 9381 d.p.m./10⁵ cells (mean $\pm$ S.D., *n* = 3) in NIH-3T3 cells and 152 092 $\pm$ 5590 d.p.m./10⁵ cells (*n* = 3) in DT cells. These cells were further incubated in the radioactivity-free medium for 6 h, after which the radioactivity in PtdCho in DT cells was greatly decreased, appearing instead in water-soluble fractions, mainly phosphocholine (Table 2). Furthermore, the distribution of radioactivity in phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine was the same in the two types of [³H]glycerol-labelled fibroblasts. These findings suggested that PtdCho breakdown was greater in DT cells, thereby resulting in the persistently high level of 1,2-DAG.

Biphasic formation of 1,2-DAG by BK stimulation

Addition to BK (100 nM) to serum-starved DT and NIH-3T3 cells resulted in a biphasic increase in the level of 1,2-DAG (Fig. 1a), which was more noticeable in the latter cells than in the former cells. It was also observed that only the diacyl type of DG responded to BK in both DT and NIH-3T3 cells; no increase in ether-linked DG was seen after stimulation by BK (results not shown). The first 1,2-DAG peak in NIH-3T3 cells was observed at 10 s and in DT cells at 30 s after stimulation. These initial rapid 1,2-DAG peaks were well correlated with the transient Ins(1,4,5)P₃ formation (Fig. 2). The peak levels of initial 1,2-DAG formation (about 500 pmol/10⁶ cells) were similar in both DT and NIH-3T3 cells.

In the second phase of 1,2-DAG formation in NIH-3T3 cells, a large peak (about 810 pmol/10⁶ cells) was obtained at 3 min;

Fig. 1. Time course of BK-induced 1,2-DAG formation in K-ras-transformed (DT) and non-transformed NIH-3T3 cells

DT cells (○) and NIH-3T3 cells (●) were stimulated with 100 nM-BK for the indicated times, after incubation in medium containing 1 % fetal calf serum for 48 h. The cellular 1,2-DAG mass content was measured as described in the Experimental section. Values are the means of duplicate determinations from two separate experiments (a) or means $\pm$ S.E.M. ($n = 6$) (b).

Fig. 2. Time course of BK-induced Ins(1,4,5)P₃ formation in K-ras-transformed (DT) and non-transformed NIH-3T3 cells

DT cells (○) and NIH-3T3 cells (●) were stimulated with 100 nM-BK for the indicated times, after incubation in medium containing 1 % fetal calf serum for 48 h. The mass content of Ins(1,4,5)P₃ was determined as described in the Experimental section. Values are the means of duplicate determinations from two separate experiments.

this rapidly decreased in 10 min, followed by a sustained level of 1,2-DAG for several hours (Fig. 1b). DT cells also exhibited the second peak (about 540 pmol/10⁶ cells) at 3 min, followed by a slower decrease leading to the sustained level.

In order to identify the source of the second phase of 1,2-DAG formation, we examined choline metabolites in [³H]choline-labelled cells stimulated with BK. As shown in Fig. 3, a rapid formation of choline and a small gradual increase in phosphocholine were observed in NIH-3T3 cells. However, DT cells displayed an opposite profile: phosphocholine formation

Fig. 3. Time course of BK-induced generation of choline metabolites in K-ras-transformed (DT) and non-transformed NIH-3T3 cells labelled with [³H]choline

DT cells (○) and NIH-3T3 cells (●) were labelled with [³H]choline (0.5 μ Ci/ml) in medium containing 1 % fetal calf serum for 48 h and then incubated with 100 nM-BK in HBSS for the indicated times. Choline metabolites were extracted and analysed for [³H]choline (a) and [³H]phosphocholine (b) as described in the Experimental section. Results are means $\pm$ S.E.M. ($n = 6$). ³H radioactivities of unstimulated DT and NIH-3T3 cells (10⁶ cells/assay) were 318 $\pm$ 24 and 531 $\pm$ 36 d.p.m. (means $\pm$ S.E.M., $n = 6$) for choline, and 7639 $\pm$ 369 and 5503 $\pm$ 305 d.p.m. for phosphocholine respectively.

Table 3. BK-induced PtdEtOH formation in K-*ras*-transformed (DT) and non-transformed NIH-3T3 cells

DT cells and NIH-3T3 cells were labelled with [³H]myristic acid (0.5 μ Ci/ml) for 48 h in DMEM containing 1% fetal calf serum. After washing in HBSS, the cells (10⁵ cells/assay) were preincubated in the absence or presence of H-7 (50 μ M) for 30 min and with 0.5% ethanol for 5 min, and were then incubated with or without 100 nM-BK for 3 min. [³H]PtdEtOH was resolved by t.l.c. as described in the Experimental section. Values represent means $\pm$ S.E.M. ($n = 6$); significant differences from non-transformed cells: * $P < 0.05$, ** $P < 0.01$.

	PtdEtOH formation (d.p.m./assay)	
	NIH-3T3 cells	DT cells
Control	328 $\pm$ 75	170 $\pm$ 20
BK (100 nM)	1030 $\pm$ 130**	280 $\pm$ 35*
H-7 (50 μ M) for 30 min + BK (100 nM)	492 $\pm$ 45	265 $\pm$ 30*

was more rapid and greater. Phosphocholine can be generated in cells either by hydrolysis of PtdCho or during its biosynthesis through choline kinase. In our study, such BK-induced phosphocholine formation in DT cells is not accounted for by the action of choline kinase on basal choline. If this were the case, a concomitant decrease in choline should parallel the increase in phosphocholine after stimulation by BK; however, the choline level was in fact increased (Fig. 3a). Another possibility, that choline kinase may act on choline produced by PLD, could also be ruled out by the fact that PtdCho-specific PLD activity was lower in DT cells than that in NIH-3T3 cells, as demonstrated in Table 3. Furthermore, we measured phosphocholine formation in DT cells that were either untreated or treated with 100 nM-BK by brief labelling with [³H]choline for 10 min. However, no changes were detected in the [³H]phosphocholine level in BK-stimulated cells or unstimulated cells (results not shown).

Thus it appears that BK activates different pathways of PtdCho

breakdown in the parent and *ras*-transformed NIH-3T3 cells. To assess this further, NIH-3T3 cells and DT cells were labelled with [³H]myristic acid (0.5 μ Ci/ml) for 48 h for its preferential incorporation into PtdCho. In fact, approx. 70% of the total radioactivity was incorporated into the PtdCho fraction. These radiolabelled cells were stimulated with BK (100 nM) in the presence of ethanol (0.5%) for measurement of phosphatidylethanol (PtdEtOH) formation, which is a specific marker for activation of PLD. BK induced marginal PtdEtOH production in DT cells, whereas it caused a more than 3-fold increase in NIH-3T3 cells (Table 3). Furthermore, pretreatment for 10 min with an inhibitor of PtdOH phosphohydrolase, i.e. propranolol (200 μ M), decreased the second peak of BK-induced 1,2-DAG formation by 70% in NIH-3T3 cells, but it had little effect on 1,2-DAG production in DT cells (Table 4). These findings suggested that BK-induced PtdCho breakdown responsible for the sustained DAG formation was mainly through the activation of PtdCho-specific PLD in NIH-3T3 cells, and through the activation of PtdCho-specific PLC in DT cells.

Modulation by PKC of BK-induced PtdCho breakdown in DT and NIH-3T3 cells

It has been reported that PtdCho breakdown is regulated by PKC in several cell systems [21]. Different PKC activities in DT and NIH-3T3 cells were thought to be attributable, to some extent, to the differential pathways of PtdCho breakdown induced by BK. Pretreatment with 50 μ M-H-7, a PKC inhibitor [39], suppressed the activation of PKC by phorbol 12-myristate 13-acetate (PMA) in NIH-3T3 cells, as inferred by a marked inhibition of 80 kDa protein phosphorylation as previously reported [27]. Under these conditions, as shown in Table 3, BK-induced PtdEtOH formation in [³H]myristic acid-labelled NIH-3T3 cells was largely prevented, but PtdEtOH formation in DT cells was not affected. Moreover, in NIH-3T3 cells, H-7 also decreased BK-induced [³H]choline generation [540 $\pm$ 36 d.p.m./10⁵ cells (mean $\pm$ S.D.; $n = 3$) with H-7 pretreatment, versus 1209 $\pm$ 290 d.p.m./10⁵ cells ($n = 3$) without H-7 pretreatment]. These findings suggested that activity of PtdCho-specific PLD in NIH-3T3 cells is largely dependent on the activation of PKC.

Involvement of oscillations in [Ca²⁺]_i in the sustained 1,2-DAG accumulation in DT cells

Our recent study demonstrated that BK elicited oscillations in [Ca²⁺]_i in DT cells (approx. 60% of total cells examined), but not in NIH-3T3 cells, when examined by digital imaging analysis [27]. The Ca²⁺ oscillations continued for at least 2 h in the presence of both BK and extracellular Ca²⁺, with changes in frequency and amplitude (Figs. 4a–4c). It was interesting to note that measurement of [Ca²⁺]_i in cell suspension revealed a persistent elevation of [Ca²⁺]_i following the Ins(1,4,5)P₃-mediated [Ca²⁺]_i spike in BK-stimulated DT cells (Fig. 4d), which lasted for several hours (results not shown). This prolonged elevation at the intermediate [Ca²⁺]_i level reflected the [Ca²⁺]_i oscillations observed in single cells. A recently developed inhibitor of receptor-operated Ca²⁺ channels, SK&F 96365 [40], was found to inhibit the BK-induced [Ca²⁺]_i oscillations, whereas the blocker affected neither the basal level of [Ca²⁺]_i nor the Ins(1,4,5)P₃-mediated rise in [Ca²⁺]_i in both types of cells (results not shown). Consequently, SK&F 96365 was applied to DT cells to examine whether [Ca²⁺]_i oscillations are involved in PLC-mediated PtdCho breakdown. DT cells which had been loaded with fura-2 for assay of [Ca²⁺]_i, or labelled with [³H]choline to examine the choline metabolites, were treated with various concentrations of SK&F 96365 for 10 min and then stimulated with 100 nM-BK. The results in Fig. 5 show that increasing concentrations of SK&F 96365 progressively suppressed the two BK-induced

Table 4. Effect of propranolol on BK-induced 1,2-DAG formation in K-*ras*-transformed (DT) and non-transformed NIH-3T3 cells

After incubation in medium containing 1% fetal calf serum for 48 h, DT and NIH-3T3 cells were washed twice with HBSS and pretreated with or without 200 μ M-propranolol for 10 min, and then stimulated with 100 nM-BK for the indicated times. The mass content of 1,2-DAG was analysed as described in the Experimental section. Values represent means $\pm$ S.E.M., $n = 5$. The mass contents of 1,2-DAG in unstimulated DT and NIH-3T3 cells were 317 $\pm$ 25 and 173 $\pm$ 21 pmol/10⁶ cells respectively. Significant difference from non-transformed cells: ** $P < 0.01$.

	1,2-DAG formation (pmol/10 ⁶ cells)	
	– Propranolol	+ Propranolol
NIH-3T3 cells		
BK (100 nM) for 10 s	484.3 $\pm$ 34.0	401.5 $\pm$ 21.1
BK (100 nM) for 3 min	702.1 $\pm$ 30.5	345.2 $\pm$ 9.3**
DT cells		
BK (100 nM) for 30 s	506.3 $\pm$ 14.2	496.2 $\pm$ 30.1
BK (100 nM) for 3 min	577.1 $\pm$ 16.2	570.8 $\pm$ 21.5

Fig. 4. Effect of BK on $[Ca^{2+}]_i$ in single cells and in a cell population of K-ras-transformed (DT) cells

(a) Typical trace of $[Ca^{2+}]_i$ in single cells stimulated with 100 nM-BK; (b) $[Ca^{2+}]_i$ trace 1 h after BK stimulation; (c) $[Ca^{2+}]_i$ trace 2 h after BK stimulation; (d) DT cells in suspension were stimulated with BK (100 nM). $[Ca^{2+}]_i$ was measured in suspension (d) or in single cells (a-c) as described in the Experimental section. Traces shown are representative examples of typical responses of single cells or cell populations from at least four similar separate experiments.

Fig. 5. Effects of SK&F 96365 on BK-induced phosphocholine generation and $[Ca^{2+}]_i$ increase in a population of K-ras-transformed (DT) cells

DT cells labelled with $[^3H]$ choline or loaded with fura-2/AM were pretreated with SK&F 96365 for 10 min prior to stimulation with BK (100 nM) for 3 min. $[^3H]$ Phosphocholine (○) was analysed as described in the legend to Fig. 2; BK-induced phosphocholine generation without SK&F 96365 pretreatment was 5727 ± 259 d.p.m. (mean $\pm$ S.E.M., $n = 6$) above baseline (control). The $[Ca^{2+}]_i$ (■) of cells in suspension was measured as described in the legend to Fig. 4; in the absence of SK&F 96365, the BK-induced elevation of $[Ca^{2+}]_i$ in the second sustained phase was 350 ± 25 nM (mean $\pm$ S.E.M., $n = 4$) above baseline (control).

events: the sustained $[Ca^{2+}]_i$ elevation due to oscillations, and the generation of $[^3H]$ phosphocholine. The SK&F 96365 concentration required to inhibit the sustained $[Ca^{2+}]_i$ elevation by 50% was $3.5 \mu M$, and $[^3H]$ phosphocholine production was inhibited by 50% in the presence of $4.3 \mu M$ -SK&F 96365. However, this drug did not affect production of $[^3H]$ choline metabolites in NIH-3T3 cells (results not shown). These findings indicated the possibility that oscillations in $[Ca^{2+}]_i$ in BK-stimulated DT cells play an important role in the activation of PtdCho-specific PLC.

DISCUSSION

A number of recent studies suggest that PtdCho hydrolysis, catalysed by either PLC or PLD, is an important signalling pathway in the control of cell growth and tumour transformation [21,22,24,41,42]. In the present study, we have obtained evidence indicating that PtdCho-specific PLC causes the prolonged phase of 1,2-DAG accumulation and that its activation is associated with $[Ca^{2+}]_i$ oscillations in a K-ras-transformed NIH-3T3 cell line stimulated with BK.

When serum-starved NIH-3T3 and DT cells were stimulated with BK (100 nM), biphasic 1,2-DAG production was observed in these two cell types, being more predominant in NIH-3T3 cells (Fig. 1). The first peak of 1,2-DAG was coincident with $Ins(1,4,5)P_3$ formation (Fig. 2), indicating the contribution of phosphoinositide hydrolysis to the transient 1,2-DAG production. Analysis of PtdCho metabolism indicated that the primary source for the second prolonged elevation of 1,2-DAG was PtdCho in DT and NIH-3T3 cells stimulated with BK.

In NIH-3T3 cells, BK-induced $[^3H]$ choline generation peaked at 3 min after stimulation, which was coincident with the second peak of 1,2-DAG formation. Also, BK elicited a 3-fold increase in PtdEtOH formation in $[^3H]$ myristic acid-labelled cells, which is a good parameter of PLD activity [43]. Furthermore, pretreatment with propranolol, an inhibitor of PtdOH phosphohydrolase, considerably lowered the second 1,2-DAG accumulation. These results strongly suggested that the sequential actions of PtdCho-specific PLD and PtdOH phosphohydrolase are mainly responsible for the second, sustained, elevation of 1,2-DAG in BK-stimulated NIH-3T3 cells. Several studies have demonstrated that PtdCho-specific PLD was activated by several mitogens, such as EGF, PDGF, bombesin, vasopressin and BK in fibroblasts [41,44-48], and also that PMA activated the enzyme via PKC [21,41,44,45]. In our immunoblotting study using specific antibodies for PKC isoenzymes, it was shown that BK induced translocation of PKC α from the cytosol to the membrane in NIH-3T3 cells after 3 min stimulation (result not shown). Pretreatment for 30 min with the PKC inhibitor H-7 significantly inhibited both PtdEtOH formation (Table 3) and choline formation in response to BK, supporting the notion that activation of PtdCho-specific PLD is dependent on PKC, and that a subsequent positive feedback loop consists of PKC and PtdCho-specific PLD/PtdOH phosphohydrolase in fibroblasts [44,45,49].

On the other hand, in K-ras-transformed (DT) cells, BK induced a sustained 1,2-DAG accumulation whose peak was less sharp and lower than that in NIH-3T3 cells. It was also shown that the generation of phosphocholine rather than choline was predominant, and PtdEtOH formation was very low. Unlike NIH-3T3 cells, pretreatment of DT cells with propranolol failed to inhibit 1,2-DAG formation induced by BK (Table 4), which suggested that PtdCho hydrolysis was principally mediated via PLC and not PLD. Deficient activation of PtdCho-specific PLD in DT cells could be, at least in part, explained by the partial down-regulation of PKC, which was caused by the consistent high 1,2-DAG content (Table 1). Furthermore, in DT cells BK did not induce detectable translocation of PKC α (results not

shown), and PMA induced the phosphorylation of a 80 kDa protein [27]. It has been reported that down-regulation of PKC attenuated PtdCho hydrolysis by PLD in various cell systems, including fibroblasts [21,44,45,49].

Previous investigations have suggested that PtdCho breakdown by the PLC pathway is associated with *ras*- and *src*-oncogene products [9,24,42]. However, the mechanism by which these oncogene products induce activation of PtdCho-specific PLC is still unknown. Quilliam *et al.* [50] have postulated that the effects of *ras* protein on phospholipid metabolism may not be due to its action as a GTP-binding protein that couples directly to phospholipase(s).

Several lines of evidence have been presented to indicate that Ca²⁺ is implicated in PtdCho breakdown via PLC. For example, Ca²⁺ is required for ATP-induced phosphocholine formation in hepatocyte membranes [51], and hydrolysis of PtdCho by partially purified preparations of PLC from heart [52] and promonocytes [53] is enhanced by adding Ca²⁺.

We have previously reported that BK, a locally produced peptide hormone, caused different [Ca²⁺]_i responses in DT and NIH-3T3 cells [27]. In DT cells, but not NIH-3T3 cells, BK induced a prolonged elevation of [Ca²⁺]_i in the cell population or persistent [Ca²⁺]_i oscillations in single cells after the initial [Ca²⁺]_i spike (Fig. 4). For maintenance of the oscillations, the continuous presence of both the agonist BK and the extracellular Ca²⁺ was necessary (result not shown). From the findings that the voltage-dependent Ca²⁺ channel may not be involved in BK-induced [Ca²⁺]_i oscillations [54] and that an inhibitor of a receptor-operated Ca²⁺ channel, SK&F 96365, prevented the [Ca²⁺]_i oscillations, it is conceivable that the Ca²⁺ influx through a receptor-operated Ca²⁺ channel contributes to the repetitive [Ca²⁺]_i increases (oscillations).

In the present study we have produced evidence which suggests that the prolonged activation of PtdCho-specific PLC is caused by the repetitive [Ca²⁺]_i increases in DT cells stimulated with BK. Pretreatment with EGTA (1 mM for 3 min) completely abolished the BK-induced [Ca²⁺]_i oscillations [27], as well as the second sustained 1,2-DAG formation (346.5 ± 22.4 pmol/10⁶ cells, mean ± S.E.M., *n* = 4) in DT cells. In addition, SK&F 96365 dose-dependently suppressed both the generation of phosphocholine and the sustained [Ca²⁺]_i elevation in DT cells in an identical manner (Fig. 5). Thus it is tempting to speculate that the agonist-induced [Ca²⁺]_i oscillations in *ras*-transformed cells are closely associated with the regulation of PtdCho-specific PLC activity leading to sustained 1,2-DAG accumulation. It is also reasonable to consider that both the sustained 1,2-DAG elevation and the long-lasting [Ca²⁺]_i oscillations cause down-regulation of PKC, and that down-regulated PKC activity is unable to activate the PtdCho-specific PLD pathway for DAG production. However, PKC is essential for BK-induced [Ca²⁺]_i oscillations [27], which would contribute to the prolonged 1,2-DAG formation via PtdCho-specific PLC in DT cells.

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