Potentiated bradykinin-induced increase of 1,2-diacylglycerol generation and phospholipase D activity in human senescent fibroblasts

Elisabetta MEACCI,* Valeria VASTA, Paola FARAONI, Marta FARNARARO and Paola BRUNI

Department of Biochemical Sciences, University of Firenze, Viale Morgagni 50, 50134 Firenze, Italy

1. The comparative study of the effect of bradykinin (BK) in young and old IMR-90 human fibroblasts shows that old cells are characterized by a reduced increase in 1,2-diacylglycerol (1,2-DAG) generation upon stimulation after short-term treatment and a significant higher increase after long-term agonist treatment. BK-induced activation of phospholipase D (PLD), the major enzyme involved in sustained 1,2-DAG generation, was 2.5-fold higher in old cells, strongly suggesting that it is involved in the potentiated increase of 1,2-DAG formation. The increased activation of PLD by BK in old cells was specific, since in parallel experiments the effect of thrombin was not significantly different in young and old cells. PLD activity in old cells was only reduced by down-regulation of protein kinase C (PKC) activity, in contrast to what was observed in young cells where it was completely abolished. This indicates that the enzyme activity in

old cells was partially PKC-independent. BK was also able to increase the release of [¹⁴C]ethanolamine, a water-soluble product of hydrolysis of phosphatidylethanolamine (PtdEtn), through PLD activation in young and old cells. The BK effect was significantly higher in old cells and, very likely, PKC-independent, since phorbol 12-myristate 13-acetate failed to induce PtdEtn hydrolysis. 2. The present results indicate that the PLD/1,2-DAG pathway is specifically potentiated by BK in old fibroblasts, demonstrating that the formation of positive effectors of PKC activation is not necessarily decreased in cellular senescence. It remains to be established whether the increased generation of DAG upon BK stimulation plays any role in the altered PKC signalling pathway which characterizes old fibroblasts.

INTRODUCTION

For quite a long time the study of the complex process of aging has benefited from the use of cultured human fibroblasts that undergo cellular senescence *in vitro* after a limited number of population doublings [1,2]. Old cells show a reduced rate of proliferation and reduced responsiveness to mitogens, but despite the intensive investigation, the molecular mechanisms involved are far from being understood. A number of studies indicate that receptor numbers for the major growth factors are unaltered in old cells and conceivably the molecular defect responsible for the impaired transduction of the mitogenic signal is distal from the receptor [3–5].

In this context, during the last few years attention has been paid to the possible alterations in the signal transduction pathways which might be responsible for the onset of senescent phenotype. Indeed, it has been shown that the reduced proliferative response to growth factors present in serum is accompanied by a reduction in the activity of phospholipase C γ (PLC γ) [6], which, by activation through tyrosine kinase receptors, generates the key messengers 1,2-diacylglycerol (1,2-DAG) and inositol phosphates.

It is well known that 1,2-DAG can also be generated by the rapid conversion of phosphatidic acid produced by phospholipase D (PLD) acting on phosphatidylcholine (PtdCho) [7,8]. There is also evidence of a PtdCho-specific PLC which may produce 1,2-DAG directly [9]. Moreover, 1,2-DAG cellular content appears to be crucial for the control of the activity of protein kinase C (PKC), which is widely recognized as an enzyme

family with a pivotal role in the intracellular signalling network and which is also critical in the regulation of cell growth and differentiation [10,11]. In view of the central role of the PKC signalling pathway, several studies have tried to identify a possible involvement of this enzyme in the appearance of cellular senescence. In this regard, it has been shown that in old fibroblasts serum-induced translocation of PKC from cytosol to plasma membrane, as well as the enzyme-induced expression of *c-fos* proto-oncogene, are less efficient [12,13]. On the other hand, it has also been demonstrated that phorbol 12-myristate 13-acetate (PMA), the direct activator of PKC, is able to translocate in the same way the enzyme in young and old cells, suggesting that the molecular defect responsible for the onset of old phenotype might reside in the abnormal generation of enzyme effectors [12]. It has also been observed that old fibroblasts contain higher amounts of 1,2-DAG [13,14] and that the serum-induced increase in 1,2-DAG formation is strongly reduced [15], or abolished [13], in these cells. These results indicate that in old fibroblasts a defect in the generation of 1,2-DAG following activation of tyrosine kinase receptors by serum growth factors occurs. This change is potentially responsible for alterations in the signalling pathway leading to mitogenesis through PKC activation.

PKC activation may also be triggered by the increase in 1,2-DAG levels induced by G-protein-coupled receptors. In this respect, bradykinin (BK), a peptide released during the inflammatory response, is known to induce a large increase in 1,2-DAG content in cultured fibroblasts by interacting with specific G-protein-coupled receptors [16,17] and the subsequent activation of PKC mediates the action of the peptide on metabolism [18].

Abbreviations used: PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; 1,2-DAG, 1,2-diacylglycerol; PMA, phorbol 12-myristate 13-acetate; BK, bradykinin; DMEM, Dulbecco's modified minimal essential medium; FBS, fetal bovine serum; PtdCho, phosphatidylcholine; PtdEtOH, phosphatidylethanol; PtdEtn, phosphatidylethanolamine; PDL, population doubling level.

* To whom correspondence should be addressed.

With the aim of evaluating the effectiveness of the G-protein-mediated signal-generating pathway in old cells, in the present study the effect of BK on 1,2-DAG generation in young and old IMR-90 human fibroblasts and the involvement of PLD were also examined.

MATERIALS AND METHODS

Materials

BK, PMA, indomethacin, propranolol, Dulbecco's modified minimum essential medium (DMEM), PBS, fetal bovine serum (FBS), Dowex-50 W and other biochemicals were obtained from Sigma (Italia). [9-10-³H(N)]Myristic acid (0.4–2.2 TBq/mmol), [2-³H]glycerol (185–740 GBq/mmol), [1,2-¹⁴C]ethanolamine hydrochloride (37–185 MBq) were obtained from New England Nuclear (Dreiech, Germany). The Silica gel 60 plates for TLC were obtained from Merck (Darmstadt, Germany).

Cell culture

Human IMR-90 fibroblasts [19] at different population doubling levels (PDLs) were obtained from Medical Research (Camden, NJ, U.S.A.): fibroblasts at low PDL (< 26) and high PDL (> 42) were defined as young and old cells respectively. The cells were maintained in DMEM containing 5.5 mM glucose, antibiotics and 10% (v/v) FBS, at 37 °C in an atmosphere of 5% CO₂. Cell cultures were routinely tested for contaminating mycoplasma using a kit from Boehringer (Mannheim, Germany).

BK-Induced 1,2-[³H]DAG formation in IMR-90 fibroblasts

Young and old IMR-90 fibroblasts were grown to confluence in 6-well dishes; confluent cells were made quiescent by incubation in DMEM containing 0.5% FBS for 48 h, and labelled for the last 24 h with [³H]glycerol (5 µCi/ml) or [³H]myristic acid (5 µCi/ml). The medium was then aspirated, the monolayers were washed twice with PBS and equilibrated for 1 h in DMEM with 0.5% FBS. Cell cultures were then stimulated with agonists and the incubations were terminated by aspirating the medium, washing twice with ice-cold PBS and immediately adding 1 ml of ice-cold methanol. Cells were collected by scraping and the dishes washed with an additional 1 ml of ice-cold methanol. Lipids were then extracted essentially as described by Bligh and Dyer [20] except that water was replaced with 1 M NaCl. The lower phase was dried under a stream of nitrogen and dissolved in a small volume of chloroform/methanol (2:1, v/v). The 1,2-[³H]DAG was separated by TLC on silica gel 60 plates with benzene/diethyl ether/ethanol/acetic acid (25:20:1:1, by vol.) followed by hexane/diethyl ether (47:3, v/v) as described previously [21]. Unlabelled standards were run together with samples, and lipid spots corresponding to 1,2-DAG were detected by exposure to iodine vapour, marked and scraped into scintillation vials after allowing evaporation of the iodine. The samples received 0.5 ml of methanol and liquid scintillation cocktail to measure radioactivity.

Measurement of the distribution of radioactivity in cellular phospholipids after labelling with [³H]glycerol or [³H]myristic acid

After lipid extraction approx. 600 µl of lower phase was dried under a stream of nitrogen, dissolved in a small volume of chloroform/methanol (2:1, v/v) and analysed by TLC using the following solvent: chloroform/methanol/acetic acid/water (100:30:35:3, by vol.). Unlabelled compounds were used as standards to localize the individual phospholipids.

Measurement of PLD activity in IMR-90 fibroblasts

PLD activity was determined as previously described by measuring [³H]phosphatidylethanol ([³H]PtdEtOH) produced via PLD-catalysed transphosphatidylation in [³H]myristic acid-labelled cells [22]. IMR-90 fibroblasts were grown in 6-well dishes, confluent cells were made quiescent by incubating in DMEM containing 0.5% FBS for 48 h and then labelled with [³H]myristic acid (5 µCi/ml) for 16–18 h; in the experiments on down-regulation of PKC, cells were labelled for 36 h. The cells were washed, incubated in a fresh medium for 2 h and then stimulated in the presence of 0.5% ethanol added 5 min before the addition of the agonists. Incubations were performed at 37 °C and terminated by scraping the cells into 1 ml of methanol. Lipid extraction was performed as described above. [³H]PtdEtOH was separated from other phospholipids by TLC on silica gel 60 plates with the organic phase of the solvent system: ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, by vol.).

Measurement of release of water-soluble products of phosphatidylethanolamine (PtdEtn) hydrolysis

Quiescent fibroblasts were incubated in the presence of [¹⁴C]ethanolamine (0.3 µCi/ml) for 48 h. Fibroblasts were then washed and incubated in a fresh medium containing 5 mM unlabelled ethanolamine for 3 h (to decrease the level of water-soluble ¹⁴C-labelled phospholipid precursors). Cells were harvested from 100-mm-diam. dishes, centrifuged for 5 min at 1000 rev./min and kept in suspension [(4–6) × 10⁶ cells/ml] at 37 °C for 20 min in DMEM containing unlabelled ethanolamine (1 mM) and phosphoethanolamine (1 mM). Cells were then stimulated for 30 min with agonists and incubations were terminated by the addition of HClO₄ (0.45 M final concentration); the tubes were vortexed and centrifuged for 2 min in a microfuge. The supernatant was neutralized with K₂CO₃ and utilized for the chromatographic separation of ethanolamine metabolites. The pellets were dissolved in 100 mM Tris/HCl, pH 7.5, sonicated and used to determine radioactivity in the lipid fraction. Fractionation of labelled ethanolamine metabolites was performed on Dowex-50 W (H⁺ form)-packed columns (1 ml bed volume) with minor modifications of the procedure described by Cook and Wakelam [23]; phosphoethanolamine was eluted with 10 ml of water and ethanolamine was eluted with 12 ml of 1 M HCl.

RESULTS

BK-Induced 1,2-[³H]DAG generation in young and old fibroblasts

Young and old IMR-90 human fibroblasts were incubated with [³H]glycerol for 24 h to metabolically label phospholipids, and the time-course of 1,2-[³H]DAG generation upon stimulation with BK was evaluated. As reported in Figure 1, in young and old fibroblasts the profile of 1,2-[³H]DAG generation was biphasic and the early rise in the compound was less marked than the later one. Old cells were characterized by a lower increase in 1,2-[³H]DAG at 30 s of stimulation and by a remarkably higher increase at later times of incubation. The effectiveness of the peptide to promote 1,2-[³H]DAG generation by phospholipid hydrolysis was evaluated at different concentrations of the agonist; as is shown in Figure 2 the concentration dependence of the effect was similar in both types of cells and approx. 1 nM BK was required to induce a half-maximal effect.

The altered proliferative response of old fibroblasts has been related to the increased arachidonic acid metabolism which characterizes these cells [24] and it has been demonstrated that

Figure 1 Time-course of BK-induced 1,2-[³H]DAG formation in young and old IMR-90 fibroblasts

Quiescent young (□) and old (○) fibroblasts were labelled with [³H]glycerol for 24 h and stimulated with 1 μM BK for the indicated times. 1,2-[³H]DAG was measured as described in the Materials and methods section. The radioactivity incorporated in the controls was: for total phospholipids 1101580 ± 43900 d.p.m. and 1128300 ± 63700 d.p.m. per dish and for 1,2-DAG 15058 ± 761 d.p.m. and 16300 ± 1678 d.p.m. per dish of young and old cells respectively and did not change significantly during the experiment. The percentage distribution of radioactivity in phospholipids was: 61.4 ± 1.2% PtdCho, 11 ± 0.9% phosphatidylinositol, 4.5 ± 0.5% phosphatidylserine, 19.5 ± 0.3% PtdEtn, 3.6 ± 0.8% phosphatidic acid in young cells and 58 ± 2.4% PtdCho, 14.6 ± 2.3% phosphatidylinositol, 4.2 ± 2.4% phosphatidylserine, 20.4 ± 0.6% PtdEtn, 3.1 ± 0.4% phosphatidic acid in old cells. Data are means ± S.E.M. of at least three independent experiments performed in duplicate. The effect of BK in young and old fibroblasts was always significant with Student's *t* test, except at 1 min in old cells. Statistical significance reported in the Figure refers to the difference in 1,2-DAG increase between young and old IMR-90 fibroblasts (* *P* < 0.05, ** *P* < 0.01).

Figure 2 Dose dependence of BK effect on 1,2-[³H]DAG formation in young and old IMR-90 fibroblasts

Quiescent young (□) and old (○) fibroblasts, labelled with [³H]glycerol for 24 h, were stimulated for 30 min in the presence of the indicated concentrations of BK. 1,2-[³H]DAG was measured as described in the Materials and methods section. Data are means ± S.E.M. of three independent experiments performed in duplicate.

the cyclo-oxygenase inhibitor, indomethacin, is able to reduce the phenomenon significantly. It is known that BK induces a significant stimulation of prostanoid production [25], which is

Table 1 Effect of BK on PLD activity in young and old IMR-90 fibroblasts

Young and old IMR-90 fibroblasts were labelled with [³H]myristic acid for 16–18 h in DMEM containing 0.5% FBS. The cells were preincubated with 0.5% ethanol for 5 min and then stimulated with 1 nM or 1 μM BK for 15 min. [³H]PtdEtOH was resolved by TLC as described in the Materials and methods section. The radioactivity of total phospholipids was 345480 ± 17300 d.p.m. and 307930 ± 32600 d.p.m. per dish in young and old cells respectively; the radioactivity of PtdEtOH in controls was 1385 ± 151 d.p.m. and 1449 ± 101 d.p.m. per dish in young and old cells respectively. Values represent means S.E.M. of four and five independent experiments performed in duplicate in young and old fibroblasts respectively. The effect of BK was significant with Student's *t* test (*P* < 0.001) in both cell types. Statistical significance refers to the difference in increase between young and old cells (** *P* < 0.01).

	[³ H]PtdEtOH formation (% of total labelled phospholipid)	
	Young cells	Old cells
Control	0.52 ± 0.07	0.40 ± 0.04
1 nM BK	1.29 ± 0.23	3.35 ± 0.27**
1 μM BK	1.43 ± 0.13	3.85 ± 0.34**

potentiated in old fibroblasts [26]. Since it has been shown that prostaglandin F_{2α} is able to induce sustained 1,2-[³H]DAG formation in fibroblasts [27] it was conceivable that the increased effect of BK on 1,2-DAG generation in old cells could result from the concomitant activation of the arachidonate signalling pathway. Experiments analogous to those reported in Figure 1 performed in the presence of 5 μM indomethacin showed that responsiveness to BK was not affected (results not shown), thus suggesting that prostanoids do not exert a regulatory role on 1,2-[³H]DAG production induced by BK.

PLD activity in young and old fibroblasts stimulated with BK

The involvement of PLD in the sustained increased generation of 1,2-[³H]DAG in old fibroblasts at later times of BK treatment was investigated using propranolol, which is reported to reduce the generation of 1,2-[³H]DAG through the PLD/phosphatidate phosphohydrolase pathway by the selective inhibition of phosphatidate phosphohydrolase [7,28]. Unexpectedly, pre-treatment of young and old fibroblasts with propranolol (200 μM) increased basal levels of 1,2-[³H]DAG and did not reduce the BK effect (results not shown). Therefore the compound appears to be unsuitable as an inhibitor of the PLD/phosphatidate phosphohydrolase pathway since its action is not confined to phosphatidate phosphohydrolase inhibition but probably affects other steps in 1,2-[³H]DAG metabolism, as previously observed [29].

PLD activity was determined in young and old fibroblasts by measuring the accumulation of [³H]PtdEtOH produced by the transphosphatidyl reaction catalysed by PLD in the presence of ethanol (Table 1). PLD activity was significantly stimulated by 1 nM or 1 μM BK at 15 min in both young and old fibroblasts. It is worth noting that [³H]PtdEtOH accumulation was highly augmented in old fibroblasts, reaching values approx. 2.5-fold higher than those detected in their younger counterparts. Treatment of fibroblasts with 200 μM propranolol increased [³H]PtdEtOH formation 2-fold, confirming the previous observation [29] on the stimulatory role of the compound on PLD activity (results not shown).

In different studies [30] it has been shown that PKC activation is a prerequisite necessary for agonist stimulation of PLD activity, even though PKC-independent activities have also been described [31]. To verify whether a similar control exists in BK-induced

Table 2 Effect of down-regulation of PKC on the stimulation of PLD activity by BK and thrombin in young and old IMR-90 fibroblasts

Confluent young and old IMR-90 fibroblasts were labelled with [^3H]myristic acid for 36 h. Cells were incubated with 500 nM PMA or vehicle (0.5% DMSO) for the last 16 h and then treated with 1 μM BK or 0.1 unit/ml thrombin for 15 min in the presence of 0.5% ethanol. Values represent means $\pm$ S.E.M. of three experiments performed in duplicate. Statistical significance, determined with Student's *t* test, refers to the difference in [^3H]PtdEtOH formation between young and old fibroblasts (**P* < 0.05).

		^3H PtdEtOH formation (% of basal)	
		PMA pretreatment	
		Young cells	Old cells
BK (1 μM)	—	166 $\pm$ 59	392 $\pm$ 32*
Thrombin (0.1 unit/ml)	—	171 $\pm$ 50	119 $\pm$ 39
BK (1 μM)	+	-11 $\pm$ 0.3	110 $\pm$ 25*
Thrombin (0.1 unit/ml)	+	-23 $\pm$ 0.8	27 $\pm$ 0.5

PLD activation, [^3H]PtdEtOH accumulation was measured in fibroblasts pretreated with 500 nM PMA for 16 h to down-regulate PKC [18] and then stimulated with BK (Table 2). Down-regulation of PKC completely abolished the effect of BK on PLD activity in young fibroblasts and only reduced the effect by 60% in old cells. Thrombin, another agonist known to act through a G-protein-coupled receptor [32], was also able to increase PLD activity, but the response of young and old fibroblasts was not significantly different. In contrast to what was observed for BK, down-regulation of PKC completely abolished the effect of thrombin on PLD activity in both young and old fibroblasts.

Phospholipid origin of BK-induced sustained 1,2- ^3H DAG formation

BK-induced 1,2- ^3H DAG formation at 30 min was measured in cells labelled with [^3H]myristic acid or with [^3H]glycerol in parallel. As can be seen from Table 3, BK potentiation of 1,2- ^3H DAG formation in old cells was less marked in cells labelled with [^3H]myristic acid, which is preferentially incorporated into PtdCho [33], than in [^3H]glycerol-labelled cells. This finding suggests that the potentiated effect of BK on sustained 1,2- ^3H DAG formation in old cells could be the result of hydrolysis of other phospholipid precursors besides PtdCho. Since PtdEtn may represent another potential source for agonist-induced 1,2-DAG formation [30] this point was investigated measuring the release of water-soluble products of PtdEtn hydrolysis. Young and old fibroblasts were incubated for 48 h with

Table 4 Effect of BK and PMA on release of water-soluble products of PtdEtn hydrolysis in young and old IMR-90 fibroblasts

Young and old IMR-90 fibroblasts were labelled with [^{14}C]ethanolamine for 48 h in DMEM containing 0.5% FBS as described in the Materials and methods section. Cells were stimulated with 1 μM BK or 50 nM PMA for 30 min. The radioactivity incorporated in the controls was: for [^{14}C]PtdEtn 290 000 $\pm$ 71 000 d.p.m. and 200 000 $\pm$ 29 000 d.p.m. per dish and for [^{14}C]ethanolamine 7987 $\pm$ 840 d.p.m. and 6091 $\pm$ 477 d.p.m. per dish of young and old cells respectively. Data are means $\pm$ S.E.M. of four experiments. The effect of PMA was not significant; the effect of BK was significant with Student's *t* test (*P* < 0.05 and *P* < 0.001 in young and old cells respectively). Statistical significance reported refers to the difference in increase between young and old cells (***P* < 0.01).

		^{14}C Ethanolamine (% of [^{14}C]PtdEtn)	
		Young cells	Old cells
Control		3.20 $\pm$ 0.35	2.90 $\pm$ 0.27
1 μM BK		4.40 $\pm$ 0.39	4.80 $\pm$ 0.46**
50 nM PMA		4.00 $\pm$ 0.30	3.70 $\pm$ 0.68

[^{14}C]ethanolamine to specifically label PtdEtn and the ability of BK and PMA to promote at 30 min the release of water-soluble products of PtdEtn hydrolysis, was determined. BK effectively activated the release of [^{14}C]ethanolamine in young and old fibroblasts, whereas PMA was ineffective (Table 4). It is worth noting that the efficacy of BK was doubled in old fibroblasts compared with young cells, thus indicating that the phospholipase activity addressed to PtdEtn was stimulated to a greater extent. Chromatographic fractionation of water-soluble products of PtdEtn hydrolysis demonstrated that BK did not induce [^{14}C]phosphoethanolamine release thus suggesting that PLD was activated rather than PLC.

DISCUSSION

A number of recent studies have indicated that an impairment of the PKC signalling pathway triggered by growth factors acting on tyrosine kinase receptors occurs in old fibroblasts that may be involved in the onset of the senescent phenotype [12,13,15]. In the present study we examined whether in old fibroblasts the generation of 1,2- ^3H DAG, the physiological activator of PKC, is altered following G-protein-coupled receptors occupancy. The time-course of the effect of BK treatment on 1,2- ^3H DAG accumulation in young and old IMR-90 human fibroblasts was characterized by a biphasic increase similar to what was pre-

Table 3 Effect of BK on the formation of 1,2- ^3H DAG in young and old IMR-90 fibroblasts labelled with [^3H]glycerol or [^3H]myristic acid

Confluent cells, labelled with [^3H]glycerol or [^3H]myristic acid as described in the Materials and methods section, were treated with 1 μM BK for 15 min. 1,2- ^3H DAG content was measured as described in the Materials and methods section. The radioactivity incorporated in 1,2-DAG in the controls was 26297 $\pm$ 2470 d.p.m. and 20357 $\pm$ 1740 d.p.m. per dish of young cells and 9250 $\pm$ 735 d.p.m. and 9506 $\pm$ 715 d.p.m. per dish of old cells labelled with [^3H]myristic acid or [^3H]glycerol respectively. Data are means $\pm$ S.E.M. of three independent experiments performed in duplicate. When the cells were prelabelled with [^3H]myristic acid the percentage distribution of radioactivity in phospholipids was: PtdCho 77 $\pm$ 2.4%, phosphatidylinositol 2.6 $\pm$ 2.3%, phosphatidylserine 2 $\pm$ 2.4%, PtdEtn 5.1 $\pm$ 0.6%, phosphatidic acid 3.8 $\pm$ 0.4% in young cells and PtdCho 84.2 $\pm$ 1.2%, phosphatidylinositol 1.8 $\pm$ 0.9%, phosphatidylserine 5 $\pm$ 0.5%, PtdEtn 6.3 $\pm$ 0.3%, phosphatidic acid 5 $\pm$ 0.8% in old cells. In the cells prelabelled with [^3H]glycerol, the percentage of distribution in phospholipids was as reported in the legend of Figure 1.

		1,2- ^3H DAG (% of labelled lipids)			
		^3H Myristic acid-labelled fibroblasts		^3H Glycerol-labelled fibroblasts	
		Young cells	Old cells	Young cells	Old cells
Control		3.34 $\pm$ 0.23	3.03 $\pm$ 0.06	1.43 $\pm$ 0.09	1.30 $\pm$ 0.07
BK (1 μM)		5.44 $\pm$ 0.23 (63%)	5.86 $\pm$ 0.58 (93%)	4.10 $\pm$ 0.16 (187%)	5.71 $\pm$ 0.26 (339%)

viously observed in other studies [16,17]. The 1,2- ^3H DAG generation in the early phase of BK stimulation, known to be due to PLC hydrolysis of phosphatidylinositol, was reduced in old cells. This suggests the presence of a defect in this signalling system similar to the one detected following serum stimulation [6]. Sustained 1,2- ^3H DAG accumulation in the late phase of BK stimulation was, unexpectedly, significantly higher in old cells, reaching values 2.5-fold higher than those measured in young cells. On the other hand, upon stimulation with serum [13,15] an impairment in the generation of the second phase of 1,2- ^3H DAG in old fibroblasts was observed. PtdCho has been recognized as the primary source of sustained 1,2- ^3H DAG generation induced by BK. Several reports indicate that PLD is the major enzymic activity involved in the hydrolysis of PtdCho [34] and that the phosphatidic acid produced is rapidly converted into 1,2-DAG [7,8]. In addition, BK has been shown to activate PLD [17,35] in cultured fibroblasts. The observed potentiated 1,2- ^3H DAG increase induced by BK in old fibroblasts was suggestive of an increased PLD activity. Indeed, PLD was found to be stimulated to a higher extent by BK in these cells and this may well account for the concomitant increase in 1,2- ^3H DAG production. The present results do not rule out that PtdCho-PLC may contribute to 1,2- ^3H DAG formation upon BK treatment as previously reported [17]; however, it seems that this phospholipase plays a secondary role compared with PLD [34]. No difference in PLD activity was detected between young and old cells stimulated with thrombin, which is also able to induce a 1,2-DAG increase in cultured fibroblasts [36]. Therefore old cells appear to be characterized by a specific potentiation of the BK-induced PLD/1,2-DAG pathway.

The maximal activation of PLD was elicited in young and old cells by 1 nM BK, whereas the same concentration exerted only half-maximal effect on 1,2- ^3H DAG accumulation, thus suggesting that other steps, such as the one catalysed by phosphatidate phosphohydrolase, may be under the control of the peptide and limit 1,2- ^3H DAG production. The evaluation of the role of PKC in the BK-induced PLD activation indicates that, in contrast to what was observed in young cells, in old fibroblasts PLD activity is not completely PKC-dependent. This suggests that a different PLD isoform is present or, alternatively, that an alteration in the regulatory mechanisms of the enzyme occurs.

The experimental evidence indicates that the potentiated effect of BK on 1,2- ^3H DAG formation in old cells may also result from the hydrolysis of other phospholipid precursors besides PtdCho. Recent studies suggest that different PLD isoenzymes exist. These possibly differ in substrate specificity and in the regulation by membrane-generated signals such as PKC activation [30]. In particular, PLD activities have been studied and shown to be either PtdCho-specific [8,37], or able to hydrolyse both PtdCho and PtdEtn [38]. The present results show that BK activates the release of [^{14}C]ethanolamine, a water-soluble product of PtdEtn hydrolysis, and that the effect is increased in old cells. The observation that PMA does not activate PtdEtn hydrolysis strongly suggests that a PKC-independent PLD, addressed to PtdEtn, is activated by BK. This PLD activity seems, therefore, different from PtdEtn-PLD activities described in NIH-3T3 fibroblasts [39] and in human breast carcinoma cells [40] which are activated by PMA treatment.

The present results demonstrate that the signalling pathway triggered by the G-protein-coupled BK receptor in old human fibroblasts is significantly potentiated, since a higher increase in sustained 1,2- ^3H DAG formation was observed in these cells.

This increase may result from the concomitant higher stimulation of PLD activity. The potentiation of PLD activity in old cells appears to be specific for BK and partially independent of PKC activation. These findings are in contrast with the hypothesis that, in aging cells, PLD activity is inhibited thus resulting in a general defect in PLD/DAG pathway activation [13]. Moreover, they demonstrate that the agonist-induced formation of positive effectors leading to PKC activation is not necessarily decreased in old cells. It remains to be determined whether the potentiated increase in DAG induced by BK in old fibroblasts provokes an alteration in the PKC signalling pathway or whether it exerts some other regulatory role.

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