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Rapid attenuation of receptor-induced diacylglycerol and phosphatidic acid by phospholipase D-mediated transphosphatidylation: formation of bisphosphatidic acid

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Generation and attenuation of lipid second messengers are key processes in cellular signalling. Receptor-mediated increase in 1,2-diacylglycerol (DG) levels is attenuated by DG kinase and DG lipase. We here report a novel mechanism of DG attenuation by phospholipase D (PLD), which also precludes the production of another (putative) second messenger, phosphatidic acid (PA). In the presence of an alcohol, PLD converts phosphatidylcholine (PC) into a phosphatidylalcohol (by transphosphatidylation) rather than into PA. We found in bradykinin-stimulated human fibroblasts that PLD mediates transphosphatidylation from PC (donor) to the endogenous 'alcohol' DG (acceptor), yielding bis(1,2-diacylglycero)-3-*sn*-phosphate (bisphosphatidic acid; bisPA). This uncommon phospholipid is thus a condensation product of the phospholipase C (PLC) and PLD signalling pathways, where PLC produces DG and PLD couples this DG to a phosphatidyl moiety. Long-term phorbol ester treatment blocks bradykinin-induced activation of PLD and consequent bisPA formation, thereby unveiling rapid formation of DG. BisPA formation is rapid (15 s) and transient (peaks at 2-10 min) and is also induced by other stimuli capable of raising DG and activating PLD simultaneously, e.g. endothelin, lysophosphatidic acid, fetal calf serum, phorbol ester, dioctanoylglycerol or bacterial PLC. This novel metabolic route counteracts rapid accumulation of receptor-induced DG and PA, and assigns for the first time a physiological role to the transphosphatidylation activity of PLD, that is signal attenuation.

Key words: bisphosphatidic acid/diacylglycerol/phosphatidic acid/phospholipase D/transphosphatidylation

Introduction

1,2-Diacylglycerol (DG) is an important intracellular messenger whose primary function is activation of protein kinase C (PKC), an enzyme that modulates a wide array of cellular responses (Nishizuka, 1988). Phosphatidic acid (PA), generated in stimulated cells by the combined action of phospholipase C (PLC) and DG kinase and/or by phospholipase D (PLD), is thought to play a distinct, yet poorly defined second messenger role too (Tsai *et al.*, 1990; Bocckino *et al.*, 1991; Cook and Wakelam, 1991). In order to function properly, cells must exploit mechanisms to control levels of DG and PA at any time. Therefore, it is important to understand how these molecules are generated and attenuated.

We and others have found that stimulation of G protein-coupled receptors induces a biphasic formation of DG (reviewed by Billah and Anthes, 1990; van Blitterswijk *et al.*, 1991a; van der Bend *et al.*, 1992). An early, transient phase originates from breakdown of phosphoinositides by PLC, whereas a delayed, sustained phase is derived from phosphatidylcholine (PC) degradation. The early DG signal is rapidly attenuated by DG kinase, which yields PA, and, to a lesser extent, by DG lipase, which yields monoacylglycerol. The later-phase DG, however, is not converted by these enzymes (van Blitterswijk *et al.*, 1991a). Depending on cell type and agonist, DG can be generated from PC via two pathways: first, by the sequential activation of PLD and PA phosphohydrolase, and second, by a PC-specific PLC (PLC_c) (reviewed by Exton, 1990; Dennis *et al.*, 1991; Liscovitch, 1992). In human fibroblasts stimulated with the peptide bradykinin, we detected rapid (within 15 s) activation of PLD but no rapid formation of DG from PC by a PLC_c (van Blitterswijk *et al.*, 1991a,b). Downregulation of PKC in these cells by prolonged treatment with phorbol ester was found to block PLD activation completely, but unveiled a rapid bradykinin-induced DG formation from PC, apparently through a PLC_c (van Blitterswijk *et al.*, 1991a,b). We were intrigued by this finding and questioned whether some hidden DG-attenuating mechanism operates in normal cells, that is blocked in PKC-downregulated cells.

Here we describe a novel metabolic pathway by which second messenger DG levels are rapidly attenuated, and which is blocked in PKC-downregulated cells. The principle of this pathway is transphosphatidylation by PLD, hitherto regarded as an unphysiological reaction that depends on the presence of a primary alcohol. Here, endogenous DG appears to serve as the alcohol that couples to the phosphatidyl moiety through PLD. This is also the first demonstration that PLD-mediated transphosphatidylation has a physiological function.

Results

Bradykinin-induced formation of a novel phospholipid, hypothesized to be bisphosphatidic acid

Many investigators measure PLD activity by transphosphatidylation in the presence of ethanol, which yields phosphatidylethanol. This product is quantified by separation on TLC, often with ethyl acetate/isooctane/acetic acid/water (13:2:3:10, v/v) (Liscovitch and Amsterdam, 1989). Doing so, we found in human fibroblasts (HF cells) stimulated with bradykinin in the absence of ethanol an unknown phospholipid that runs on TLC at the same position as phosphatidylethanol. When adding a different alcohol, *n*-butanol, to cells during stimulation, we found two distinct, newly generated products that are clearly separated on TLC (Figure 1), i.e. phosphatidylbutanol (PBut) running ahead

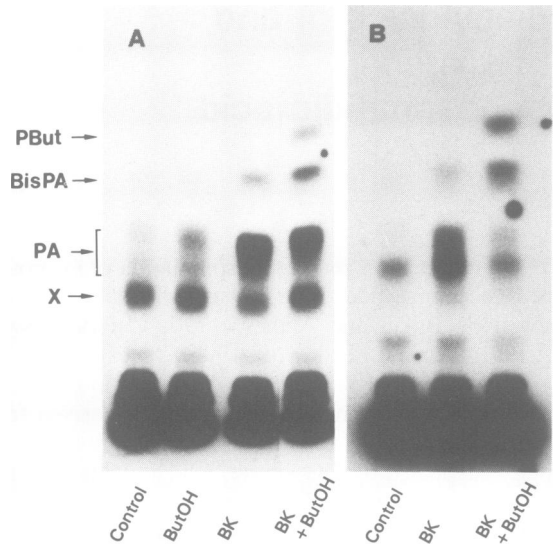

Fig. 1. Bradykinin-induced bisPA formation. HF cells were prelabelled with 5 μCi $^{32}P_i$ (**A**) or 0.5 μCi [^{32}P]lysoPC (**B**) and stimulated for 5 min with bradykinin (BK) in the presence or absence of 0.05% *n*-butanol (ButOH), as indicated. Autoradiographs (A and B) are from one and the same experiment on the same batch of cells. Exposure times were 18 h (A) and 40 h (B). Positions of PA, bisPA and phosphatidylbutanol (PBut) on the TLC plate are indicated (primuline-stained markers). X, unidentified spot, probably a mixture of acyl-phosphatidylglycerol and *N*-acyl-phosphatidylethanolamine (based on co-chromatography with reference lipids and radiolabelling with [^{14}C]ethanolamine). ^{32}P counts in bisPA spot (B, right lane) amounts to 0.3% of total phospholipids. The amount of bisPA generated varied considerably, from 0.02 to 1% of total phospholipids, between eight similar experiments. Note that PA is generated by DG kinase and PLD (A), or by PLD only (B) (see text).

of the novel product, above PA. This was visualized by autoradiography after prelabelling of cells with $^{32}P_i$ (Figure 1A) and with [^{32}P]lysophosphatidylcholine (lysoPC) (Figure 1B). Since the latter compound is readily converted in the cell to [^{32}P]PC, and since cellular ATP does not become labelled under these conditions (Pai *et al.*, 1988), the bradykinin-induced ^{32}P-labelled products cannot have been produced via DG kinase but must have been generated from labelled PC by PLD. Addition of *n*-butanol not only leads to PBut formation but also stimulates the formation of the novel product, both compounds being generated at the cost of PA (most clearly seen in Figure 1B).

Thus, we have two intriguing observations in bradykinin-stimulated cells: (i) the appearance of a novel, extremely hydrophobic phospholipid on TLC and (ii) the rapid PLC_c-mediated formation of DG in PKC-downregulated cells (see Introduction). Based on these observations we hypothesize that the novel phospholipid is bis(1,2-diacylglycero)-3-*sn*-phosphate (bisphosphatidic acid; bisPA), and that it is the product of a PLD-mediated transphosphatidylation reaction in which DG, generated by a PLC, serves as the nucleophilic alcohol (Figure 2). Indeed, this cellular product co-chromatographed with authentic tetrapalmitoyl-bisPA using TLC systems I and II (see Materials and methods) as well as a two-dimensional TLC system (Figure 3). The hypothesis of bisPA synthesis in stimulated cells, according to the scheme in Figure 2, was further tested by experiments described below.

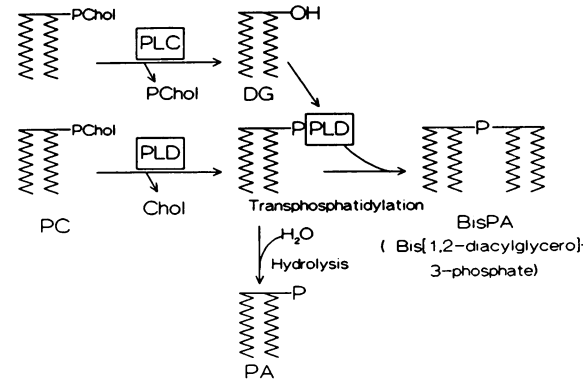

Fig. 2. BisPA formation from PC by PLD-mediated transphosphatidylation. Agonist-induced DG, generated by PLC, serves as the nucleophilic primary alcohol reacting with the phosphatidyl-PLD intermediate. BisPA is the condensation product of two signalling pathways, i.e. those mediated by the enzymes PLC and PLD (drawn here in boxes). Note that transphosphatidylation and hydrolysis are competitive reactions and that bisPA is generated at the cost of DG and PA. PChol, phosphocholine; Chol, choline.

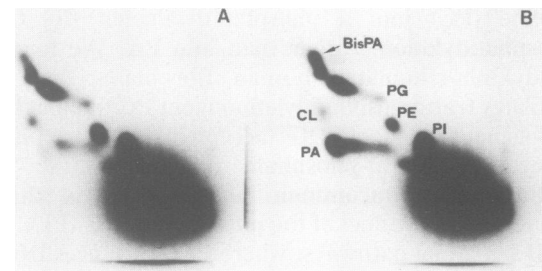

Fig. 3. Bradykinin-induced formation of [^{32}P]bisPA in cells prelabelled with [^{32}P]lysoPC (1 μCi per plate). Two-dimensional TLC separation of phospholipids was done as follows. First dimension (upwards): two runs with chloroform/methanol/0.88 M ammonia (60:60:5) up till 80% of the length of the plate; followed by ethyl acetate/isooctane/acetic acid/H_2O (13:2:3:10) full length. Second dimension (from right to left): chloroform/methanol/acetic acid/H_2O (50:30:8:4). **A**, control cells; **B**, bradykinin-stimulated (5 min) cells. Positions of PA, bisPA (both increased by bradykinin-induced PLD activity; see text), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and cardiolipin (CL) are indicated (comigrating with authentic markers). Note that the bulk radioactivity is in PC and lysoPC (near the origin). The spots just below bisPA were tentatively identified (by chemical/enzymatic degradation and co-chromatography with markers; not shown) as deacylated derivatives of bisPA.

BisPA formation depends on PKC and is induced by stimuli that simultaneously generate DG and activate PLD

Figure 4 shows PC breakdown induced by bradykinin and phorbol ester (PMA) in HF cells. Bradykinin activates PLD already within 15 s, as demonstrated by the release of [^{14}C]choline and by the formation of [^{3}H]myristoyl-phosphatidylbutanol (PBut; in the presence of *n*-butanol) from prelabelled cellular PC (Figure 4A and E). We have previously shown that choline release was not due to dephosphorylation of phosphocholine, and that optimal PLD activity is reached at 2 min bradykinin stimulation. Furthermore, a more pronounced PLD activity was obtained

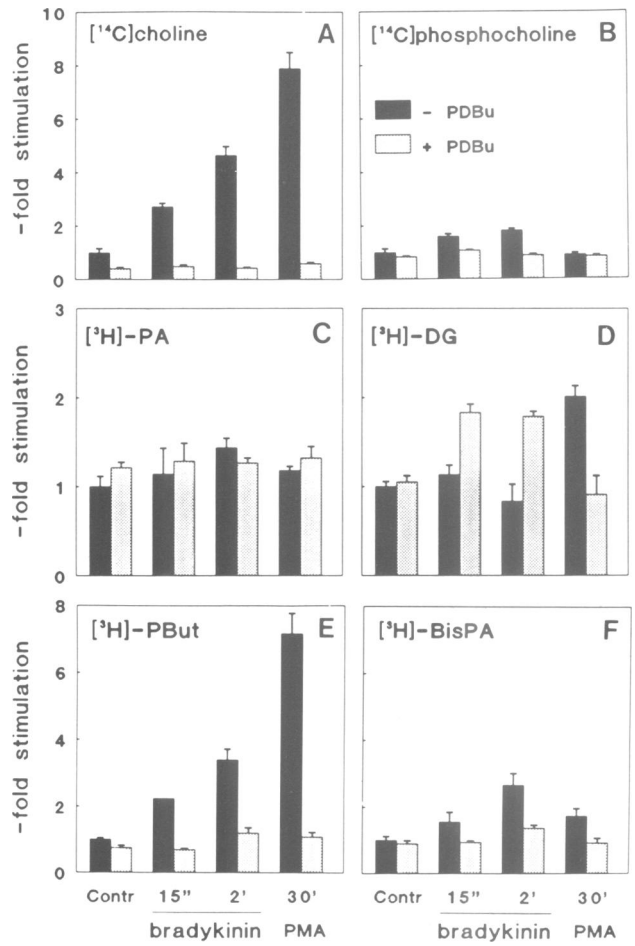

Fig. 4. Bradykinin- and PMA-induced products from PC. 'Downregulation' of PKC blocks PLD-mediated formation of free choline, phosphatidylbutanol (PBut) and bisPA and thereby unveils rapid DG formation by PLC. Quiescent HF cells were stimulated with 1 μM bradykinin or 100 nM phorbol ester (PMA) for the times indicated, in the presence of 0.05% *n*-butanol. Control cells were treated for 2 min with 0.05% *n*-butanol in HEPES medium. Cells had been prelabelled with 2 μCi [^{14}C]choline (48 h) and 2 μCi [^{3}H]myristic acid (4 h) per plate, and pretreated with phorbol dibutyrate (PDBu; 100 nM) for 20 h to downregulate PKC (shaded bars), or with the DMSO vehicle (solid bars). Lipids and choline catabolites were isolated as described in Materials and methods. Data are expressed as means ± S.D. (n = 3) relative to unstimulated controls. Radioactivity (d.p.m. ± S.D.; n = 3) in the (non-PKC-downregulated) controls were: (**A**) 1663 ± 249; (**B**) 37 553 ± 4871; (**C**) 4502 ± 524; (**D**) 3074 ± 166; (**E**) 628 ± 30; (**F**) 894 ± 108. For non-PKC-downregulated cells, radioactivity in bulk phospholipids was (320 ± 27) × 10^3 d.p.m. ^{3}H and (487 ± 34) × 10^3 d.p.m. ^{14}C. For PKC-downregulated cells, these phospholipid values were (361 ± 28) × 10^3 d.p.m. ^{3}H and (550 ± 48) × 10^3 d.p.m. ^{14}C.

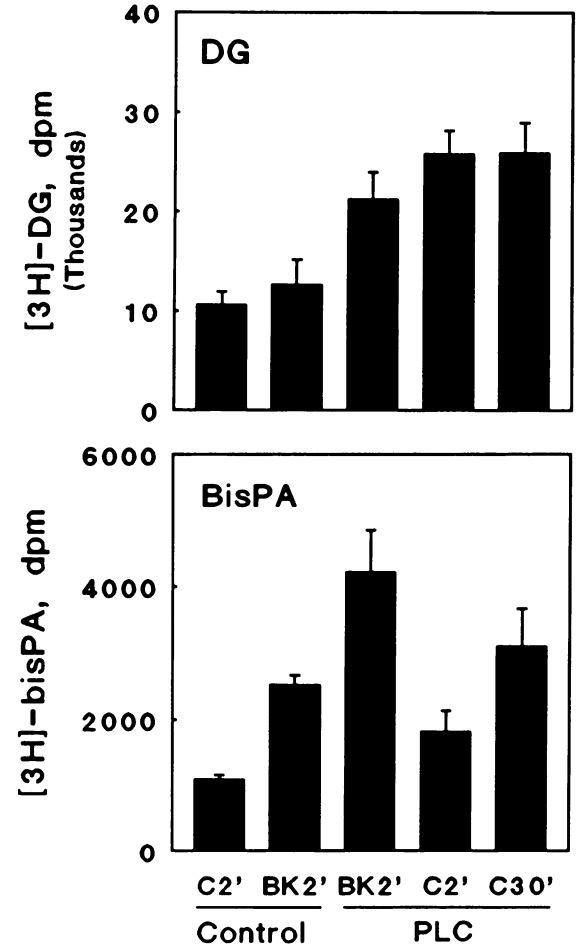

Fig. 5. Elevation of plasma membrane DG levels by treatment of cells with bacterial phospholipase C (PLC) (**above**) by itself generates bisPA and increases bradykinin-induced bisPA formation (**below**). Cells were labelled with 2 μCi [^{3}H]myristic acid for 4 h. Treatment with PLC (from *Bacillus cereus*, 1 U/ml) started 10 min prior to stimulation with bradykinin for 2 min (BK2') or control medium for 2 and 30 min (C2' and C30'). Lipids were isolated as described in Materials and methods.

by 30 min phorbol ester stimulation (van Blitterswijk *et al.*, 1991b). PLD activation by either stimulus is dependent on (downstream from) PKC, since long-term pretreatment with phorbol ester, known to downregulate at least several PKC isotypes, completely blocks choline release and PBut formation (Figure 4A and E). Figure 4 furthermore shows that the formation of [^{3}H]myristoyl-bisPA induced by bradykinin or PMA parallels that of [^{3}H]PBut and [^{14}C]choline, and is also blocked after downregulation of PKC, suggesting that the common enzyme activity underlying the formation of these products is PLD.

It is known that in the presence of a primary alcohol, PLD-mediated transphosphatidylation is favoured above PLD hydrolysis (Figure 2). This was also demonstrated for the present cell system (van Blitterswijk *et al.*, 1991b). It is, therefore, conceivable that agonist-induced formation of the transphosphatidylation products [^{3}H]PBut and [^{3}H]bisPA suppressed PLD-mediated [^{3}H]PA formation (Figure 4). Competition between these pathways (transphosphatidylation versus hydrolysis) is shown in Figure 1B for cells labelled with [^{32}P]lysoPC.

Downregulation of PKC unveiled a rapid bradykinin-induced formation of DG from PC that was not seen in control cells (Figure 4D). In contrast, phorbol ester-induced formation of DG, via the PKC-mediated PLD/PA phosphohydrolase pathway, was completely blocked (Figure 4D). Since under these conditions bradykinin-induced DG cannot be generated via PLD, and other phospholipids (including PA; Figure 4C), monoacyl- and triacylglycerols remained unaffected (results not shown), this DG is most likely produced by a PLC_c. The reaction scheme in Figure 2 then predicts that bradykinin-induced

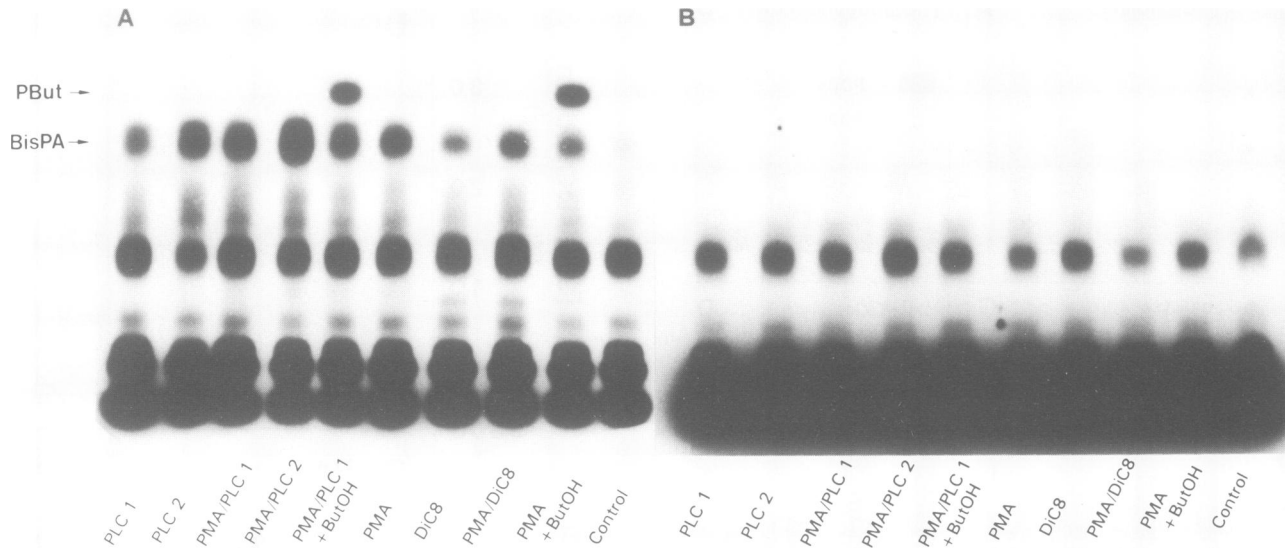

Fig. 6. BisPA induced by phorbol ester (PMA) and/or exogenous phospholipase C (PLC) and/or dioctanoylglycerol (DiC8) in native HF cells (**A**) and in PKC-downregulated cells (**B**). Cells were prelabelled with 5 μCi $^{32}P_i$ for 16 h and pretreated with phorbol dibutyrate (PDBu; 100 nM) (B), or with the DMSO vehicle (A) for 20 h. Cells were stimulated for 30 min with PMA (100 nM) and/or PLC (from *B.cereus*, 1 or 2 U/ml, as indicated) and/or DiC8 (75 μM), with or without 0.05% *n*-butanol (ButOH), as indicated. BisPA induction by PLC is dose-dependent: *B.cereus* PLC in concentrations of 0, 0.1, 0.5, 1.0 and 2.0 U/ml induced [^{32}P]bisPA in respective amounts of 295 (control value), 423, 687, 1012 and 1530 c.p.m. per 10^5 c.p.m. phospholipids (partly shown in this figure).

synthesis of bisPA by transphosphatidylation should occur at the cost of this PLC_c-generated DG. Indeed, the bradykinin data shown in Figure 4 panels D and F complement each other, at least in part: about half of the bradykinin-induced [^{3}H]DG in PKC-downregulated cells can be accounted for by a concomitant decrease in [^{3}H]bisPA. The other half is probably due to increased PLC_c activity, although this cannot be deduced directly from changes in radiolabelled phosphocholine, because background values were too high (Figure 4B).

The reaction scheme in Figure 2 also predicts that artificial elevation of DG levels in the plasma membrane should increase PLD-mediated bisPA formation. Indeed, short pretreatment of [^{3}H]myristate-labelled cells with bacterial PLC augments bradykinin-induced [^{3}H]bisPA (Figure 5). Bacterial PLC by itself is also capable of bisPA induction, most clearly seen at a longer incubation time (40 min in Figure 5). This is conceivable since DG initially formed by the bacterial PLC will activate PKC and, by consequence, PLD, which, in turn, uses the DG for transphosphatidylation. Similar observations were made in cells where DG levels were artificially raised with membrane-permeable dioctanoylglycerol (diC8) (results not shown for [^{3}H]myristate-labelled cells, but see Figures 6A and 8). Also in cells prelabelled with $^{32}P_i$ we observe bisPA formation by these treatments, as well as by the phorbol ester PMA (Figure 6A). BisPA formation induced by the exogenous PLC is dose-dependent, while stimulation with PMA plus PLC yields an amount of bisPA that is higher than obtained by the two stimuli alone. Figure 6A also shows that *n*-butanol added to cells during stimulation with PMA, together with or without bacterial PLC, generates PBut at the cost of bisPA. Conceivably, *n*-butanol competes with DG in the transphosphatidylation reaction. Figure 6 furthermore shows that 'downregulation' of PKC by prolonged treatment of cells with phorbol ester blocks bisPA formation induced by PMA, bacterial PLC or diC8, or a combination of these agents. This confirms our notion that stimulation of PLD activity and consequent bisPA formation in HF cells depends on PKC.

From the results described so far, we conclude that bisPA is produced by PLD in the presence of DG. In bradykinin-stimulated HF cells, bisPA synthesis attenuates the level of rapidly generated DG, and competes with PLD-mediated PA formation. BisPA can also be formed via artificial inducers/elevators of PLD and DG, in a dose-dependent fashion. In all cases, bisPA formation depends on PKC.

BisPA formation is agonist- and time-dependent

Figure 7 shows that bradykinin-stimulated bisPA formation in HF cells is transient. Using three different radioisotopes to label the phospholipids, bisPA levels were found to be maximal at 2–10 min stimulation and decayed thereafter. [^{3}H]arachidonate selectively (but not exclusively) incorporates into phosphoinositides (PI), and [^{14}C]palmitate into PC. We have previously estimated in such double-labelled cells to what extent DG originates from PI or PC by measuring $^3H/^{14}C$ ratios, differing by a factor 10 between these two phospholipids (van Blitterswijk *et al.*, 1991a). From the data in Figure 7, we conclude that DGs generated from both sources (DG_i and DG_c) are acceptors for transphosphatidylation (Figure 10). Thus, bisPA synthesis not only utilizes PC-derived DG, but also competes with PA formation by DG kinase (Figure 10). From the data in Figure 7, we calculate that the amount of [^{3}H]bisPA generated at 10 min bradykinin stimulation is 2752 d.p.m. (mean value). In this time period, the level of [^{3}H]PA rose from 5495 ± 766 to 11 080 ± 1485 d.p.m. (means of triplicates ± S.D.). The difference, i.e. 5585 d.p.m., can be taken as a measure of DG kinase activity (van Blitterswijk *et al.*, 1991a). We tentatively conclude that the degree of

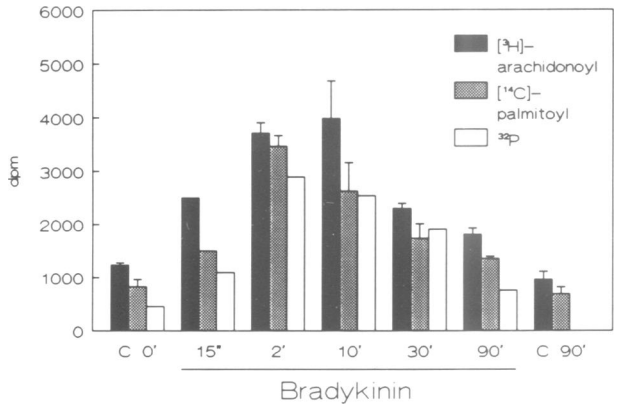

Fig. 7. Time-dependent bisPA formation induced by bradykinin. Cells were prelabelled with 2 μCi [^{3}H]arachidonic acid and 1 μCi [^{14}C]palmitic acid per dish for 2 days. In a parallel experiment cells were prelabelled overnight with 5 μCi $^{32}P_i$. Cells were stimulated with bradykinin or buffer (C) for the times (in seconds or minutes) indicated. Lipids were separated and the radioactivity in bisPA was determined. Data are means of triplicates with S.D. indicated. Radioactivity (dpm ± S.D.) incorporated in the total phospholipids represent (1122 ± 136) × 10^3 for ^{3}H and (798 ± 137) × 10^3 for ^{14}C.

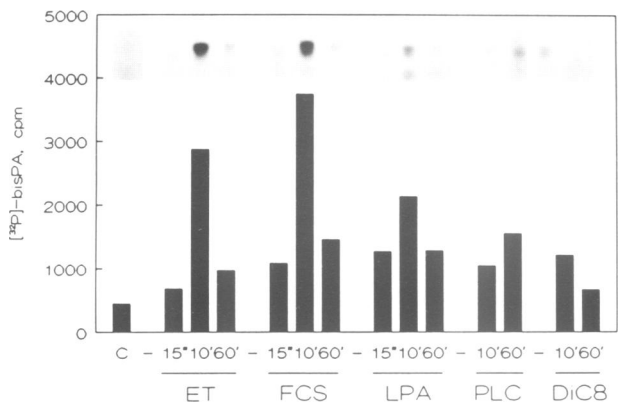

Fig. 8. Transient bisPA formation induced by various stimuli. ET, endothelin (0.1 μM); FCS, fetal calf serum (10%); LPA, lysophosphatidic acid (50 μg/ml); PLC, phospholipase C from *B.cereus* (1 U/ml); DiC8, dioctanoylglycerol (75 μM); C, unstimulated control. Cells were prelabelled overnight with 5 μCi $^{32}P_i$, and stimulated for the times (in seconds or minutes) indicated. Inserts: autoradiograph of corresponding bisPA spots.

attenuation of DG_i via the PLD-mediated bisPA pathway is about half of that effectuated via the DG kinase pathway.

BisPA was also transiently induced by other agonists (Figure 8), i.e. endothelin, fetal calf serum and lysophosphatidic acid, each being inducers of DG formation as well as PLD activity (Van Corven *et al.*, 1989, 1992; Van der Bend *et al.*, 1992). Figure 8 confirms that DG, artificially generated in the plasma membrane, induces bisPA formation (also shown in Figures 5 and 6A). BisPA induced by bacterial PLC is still elevated at 60 min stimulation, while that induced by diC8 has already decreased by that time, probably due to metabolic conversion of this type of DG in the plasma membrane (Bishop and Bell, 1986). Finally, bisPA formation is not restricted to HF cells, but occurred in other fibroblasts as well, i.e. in Rat-1 cells stimulated with endothelin (results not shown).

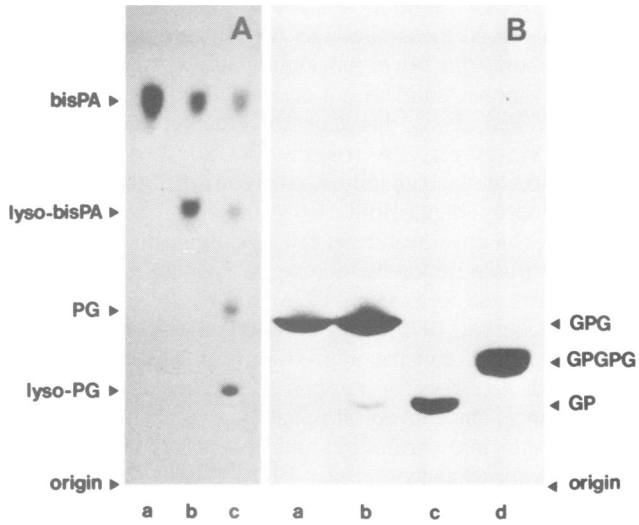

Fig. 9. Controlled breakdown of [^{32}P]bisPA by mild alkaline and phospholipase A_2 treatments. (**A**) Lane a, untreated control; lane b, digestion with phospholipase A_2 (porcine pancreas; 10 U/0.5 ml) for 1.5 h; lane c, with methylamine reagent for 8 min. Lipids were separated on a borate-impregnated TLC plate with chloroform/methanol/7 M ammonia (60:30:4). Unlabelled reference compounds, tetrapalmitoyl-bisPA (R_f = 0.75), phosphatidylglycerol (PG) and lyso-compounds were visualized by primuline staining (positions indicated). (**B**) Water-soluble deacylation products obtained by methylamine treatment (1 h) of ^{32}P-labelled bisPA (lane a), phosphatidylglycerol (lane b), PA (lane c), and bis(phosphatidyl)-glycerol (cardiolipin) (lane d), separated by cellulose-TLC (see Materials and methods). Positions of products from authentic reference compounds are indicated: GP, glycerophosphate; GPGPG, bis(glycerophospho)-glycerol; GPG, glycerophosphoglycerol (R_f = 0.34).

Identification of bisPA by chemical and enzymatic digestions

[^{32}P]BisPA, extracted from TLC plates, and unlabelled tetrapalmitoyl-bisPA were co-digested with phospholipase A_2 or by mild alkaline methylaminolysis. In each case, radioactive digestion products co-migrated on TLC with the unlabelled digestion products, i.e. lysobisPA, phosphatidylglycerol and lysophosphatidylglycerol (Figure 9A). The deacylated water-soluble 'backbone' molecule co-migrated on cellulose-TLC with glycerophosphoglycerol, generated from tetrapalmitoyl-bisPA or phosphatidylglycerol (Figure 9B). Furthermore, [^{3}H]myristate-bisPA was isolated and subjected to acetolysis (Renkonen, 1965; Brotherus and Renkonen, 1974). This yielded [^{3}H]myristoyl-acyl-acetylglycerol, co-migrating on TLC for >80% of the total counts with diacyl-monoacetylglycerol from reference bisPA and from PC, but not with monoacyl-diacetylglycerol, the acetolysis product from lysophospholipids (results not shown). We conclude that the radiolabelled cellular bisPA is indeed a polyglycero-phospholipid, distinct from phosphatidylglycerol and cardiolipin (Figures 3 and 9A and B), and that each of the four hydroxyl groups carries an acyl moiety.

Discussion

In this paper we have described a novel physiological function of PLD, that is the attenuation of rapidly formed second messenger DG levels by transphosphatidylation. For the cell, this is an important additional control mechanism,

next to DG kinase (and, to a lesser extent, DG lipase), to keep its second messenger DG level within certain limits. Furthermore, this novel metabolic pathway interferes with the two known pathways, i.e. via DG kinase and PLD-mediated hydrolysis, that generate a second (putative) lipid second messenger, PA (Figure 10). The product of this PLD-mediated transphosphatidylation, the unusual polyglycerophospholipid bis-(diacylglycero)phosphate (bisPA), is a novel feature in cell signalling. BisPA was first described as a minor membrane component in BHK cells (Brotherus and Renkonen, 1974) and bacteria (McAllister and De Siervo, 1975), but its function was unknown.

In vitro, PLD can also utilize more complex alcohols than butanol as acceptors for transphosphatidylation. Plant PLDs, for instance, can convert phosphatidylglycerol and phosphatidylinositol into cardiolipin and bis(phosphatidyl)inositol, respectively (Stanacev *et al.*, 1973; Clarke *et al.*, 1981). Another example is PLD from *Streptomyces* that can produce structural analogues of platelet activating factor *in vitro*, utilizing primary cyclic alcohols as phosphatidyl acceptors (Testet-Lamant *et al.*, 1992). In analogy, we found here *in vivo* that generation of bisPA in signal transduction depends on PLD activity and the availability of the endogenous 'alcohol' DG as acceptor for the phosphatidyl moiety.

The occurrence of bisPA formation in stimulated HF cells was supported by experiments where the two requisites for bisPA synthesis, i.e. endogenous DG and an active PLD, were modulated independently. An artificial increase of DG in the plasma membrane by treatment of cells with bacterial PLC or diC8 increases bisPA, while downregulation of PKC, the activator of PLD in this system, blocks bisPA formation. Furthermore, the chemical identity of this cellular PLD product as bisPA was proven by (i) co-chromatography with authentic reference bisPA on several TLC systems; and (ii) controlled chemical or enzymatic degradation, yielding apolar and water-soluble products that co-migrated on TLC with those obtained from authentic bisPA.

What is the impact of this PLD-mediated bisPA formation on cellular signalling? Particularly, how much does this metabolic pathway contribute to attenuation of DG and PA levels? The amount of bisPA generated in HF cells upon receptor stimulation is of similar magnitude to PBut, the other PLD transphosphatidylation product when *n*-butanol is present. They are both generated at the expense of the PLD hydrolysis product PA. Using [^{3}H]myristate-labelling of cellular PC, the amount of bisPA generated was of the same order of magnitude as DG rapidly induced by bradykinin in PKC-downregulated cells. Using [^{3}H]arachidonate, which selectively labels phosphoinositides, attenuation of bradykinin-induced [^{3}H]arachidonoyl-DG (DG_i) by PLD-transphosphatidylation was estimated to be about half of that effected by DG kinase. We should note, however, that the amount of bisPA formed was quite variable between different experiments, for reasons not yet understood. Therefore, we conclude from the present data that bisPA formation can substantially reduce or prevent receptor-generated DG and PA, but that the physiological conditions of cells for induction of consistent amounts of bisPA are difficult to control. We also conclude that PKC is essential for bisPA formation, and that it suppresses via this novel pathway the formation of its own activator, DG, by feedback regulation, like it controls agonist-induced DG levels via inhibition of phosphoinositide breakdown (Bishop *et al.*, 1990; van Blitterswijk *et al.*, 1991a) and activation of DG kinase (van Blitterswijk *et al.*, 1991a; Maroney and Macara, 1989) (Figure 10).

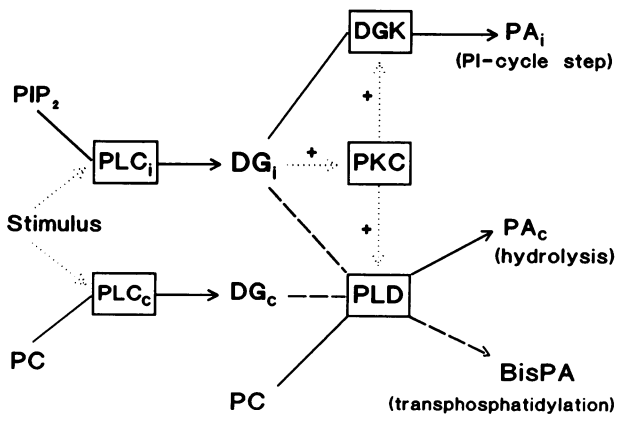

Fig. 10. Agonist-stimulated metabolic pathways that generate and attenuate diacylglycerols (DG_i and DG_c, from phosphoinositides and PC, respectively). PLD-mediated bisPA formation is the novel pathway (drawn with broken line) that, next to DG kinase (DGK), attenuates second messenger DG. It also competes with the known pathways (DGK and PLD) that generate the putative second messenger PA.

The effect of *n*-butanol, which was added to cells to estimate PLD activity, on bisPA formation, depended on the stimulus. In PMA-stimulated cells, formation of PBut generally occurs at the cost of bisPA, as expected when *n*-butanol and DG compete for transphosphatidylation. However, in bradykinin-stimulated cells, bisPA formation tends to be stimulated by *n*-butanol. Here, PLD-mediated formation of both bisPA and PBut (transphosphatidylation products) occurs at the cost of PA (PLD hydrolysis product). We have no straightforward explanation for these different effects. It is possible that bradykinin-stimulated and PMA-stimulated PLD activities pertain to distinct enzymes, which respond differently to *n*-butanol. The existence of two PLDs is also indicated by our finding that the former PLD activity is subject to rapid desensitization, while the latter is not (W.van Blitterswijk, unpublished results).

We have shown that bisPA can be induced by several agonists and that it is transient. How is it degraded in the cell? *In vitro*, we were unable to degrade bisPA with cabbage PLD or bacterial PLC (results not shown). However, bisPA could be partially digested with phospholipases A_2 (Figure 9) and A_1 (not shown). Therefore, the most likely route of degradation in the cell is deacylation by the phospholipase A type of enzyme(s). Intriguingly, on two-dimensional TLC (Figure 3) we find several ^{32}P-labelled spots closely together in the area just below bisPA, which we tentatively ascribe to tri- and diacyl-glycerophosphoglycerols, i.e. bisPA derivatives lacking one or two acyl chains, respectively. Most interestingly, these molecules, called semi-lysobisPA (or acyl-phosphatidylglycerol) and lysobisPA [or bis(monoacylglycero)-phosphate], respectively, have been found in secondary lysosomes of BHK cells (Brotherus and Renkonen, 1974; Somerharju *et al.*, 1977; Joutti, 1979), alveolar macrophages and liver (Mason *et al.*, 1972; Waite *et al.*, 1990; Hostetler *et al.*, 1992). In HF cells, bisPA is generated so rapidly (within seconds) upon surface-receptor stimulation that its synthesis is likely to occur in the plasma membrane. It remains to be

investigated whether the deacylated derivatives of bisPA in HF cells are indeed associated with endosomal/lysosomal compartments. If so, it would be tempting to speculate that bisPA is a 'sink' for redundant lipid second messengers (DG and PA), that is subsequently 'flushed' (degraded) via an endocytotic route.

Finally, we should consider the possibility that bisPA is a second messenger in its own right, although direct evidence for that notion is lacking at present. Future studies should reveal whether agonist-induced bisPA formation also occurs in other cell types, and should establish the role of bisPA in cell signalling more precisely.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was from Gibco. Bis(dipalmitoylglycero)-3-*sn*-phosphate (tetrapalmitoyl-bisPA) was purchased from Serdary (London, Ontario). Bis(monoacylglycero)-3-*sn*-phosphate (lysobisPA) was kindly provided independently by T.Y.Thuren/M.Waite and J.R.Wherrett. Bradykinin, oleoyl-3-*sn*-lysophosphatidic acid (LPA) and other lipids were from Sigma. Endothelin-1 was from Cambridge Research Biomedicals (Cambridge, UK). Phorbol-12-myristate 13-acetate (PMA) and phorbol-12,13-dibutyrate (PDBu) were from LC Services Corp. (Woburn, MA). [^{3}H]arachidonic acid, [^{14}C]palmitic acid, [^{3}H]myristic acid and [methyl-^{3}H]choline chloride were from New England Nuclear. Carrier-free [^{32}P]orthophosphate ($^{32}P_i$) was from Amersham. [^{32}P]lysoPC was prepared by phospholipase A_2 treatment (see below) of metabolically labelled [^{32}P]PC, which was extracted from cells and purified by two-dimensional TLC (see legend of Figure 3). Labelling of cells with 0.5 mCi $^{32}P_i$ eventually yielded ~3 μCi [^{32}P]lysoPC, which was purified by TLC and extracted from the plate according to Bligh and Dyer (1959). Phosphatidylethanol (PEt) and phosphatidylbutanol (PBut) were synthesized from egg PC by transphosphatidylation with ethanol and *n*-butanol, respectively, using cabbage PLD (Sigma) (Smith *et al.*, 1978). Silica gel 60 and PEI–cellulose F TLC plates were from Merck.

Cell culture, metabolic radiolabelling and stimulation

Early-passage diploid human foreskin fibroblasts (HF cells) were grown in monolayers in 55 mm glass tissue culture plates in DMEM supplemented with 7.5% (v/v) fetal bovine serum. The cells were subcultured at 3–4 day intervals. When cells had grown to 80–100% confluency, the medium was replaced with 2 ml DMEM, 0.25% fetal bovine serum containing 1 μCi [^{14}C]palmitic acid and 2 μCi [^{3}H]arachidonic acid and/or 2 μCi [^{3}H]- or [^{14}C]choline chloride. Prelabelling of the cells occurred to near isotopic equilibrium for 48 h. In other experiments, cells were prelabelled with 2 μCi [^{3}H]myristic acid for 4 h, which was found to be the optimal time to obtain the highest degree of selective labelling in the PC fraction of the phospholipids (85%). Labelling with sub-lytic concentrations of [^{32}P]lysoPC (0.3–1 μCi per dish) or with $^{32}P_i$ (5 μCi per dish) was performed in a phosphate-poor DMEM/EMEM (Flow Laboratories) 1:2 medium mixture for 17 h. In certain experiments PKC was downregulated by pretreating the cells for 20 h with 100 nM PDBu.

Prior to stimulation, the cells were rinsed twice with serum-free DMEM containing 0.1% bovine serum albumin (BSA), 20 mM HEPES, pH 7.4, and incubated in this medium for 1 h. Quiescent cells were stimulated in this medium (freshly added, without BSA) with 1 μM bradykinin, without or with 100 nM PMA in dimethyl sulfoxide (DMSO; 0.5% v/v final concentration) in the absence or presence of 0.05% *n*-butanol (for measuring PLD transphosphatidylation). ^{32}P-labelled cells did not undergo this change of medium, but were stimulated directly with agonist. Stimulations with other agonists/agents were as described in the figure legends. Cell stimulation was stopped by rapid aspiration of the medium, immediately followed by addition of 2 ml of chloroform/methanol (1:1) to the cells.

Lipid analysis

Detailed procedures have been described in previous papers (van Blitterswijk *et al.*, 1991a,b). Briefly, DG, PA and PEt or PBut or tetrapalmitoyl-bisPA carrier lipids (20–50 μg each) were added to the chloroform/methanol (1:1)/cell mixture to monitor chromatographic separations. After standing for 1 h, the above mixture was put into glass tubes, adjusted to a chloroform/methanol ratio of 2:1 and phase-separated by addition of 0.2 vol of H_2O according to Folch *et al.* (1957). The lipids present in the lower phase were dried under nitrogen, resuspended in 0.5 ml of hexane/methyl tert-butylether (MTBE)/acetic acid (100:5:0.1) and pre-separated on prepacked silica Sep-Pak columns (1 ml; Waters, Milford, MA), essentially according to Hamilton and Comai (1988). By this method triacylglycerols were first eluted with 6 ml hexane/MTBE/acetic acid (100:5:0.1), followed by elution of diacylglycerols with 6 ml hexane/MTBE/acetic acid (1:1:0.1) and subsequently phospholipids with 7 ml chloroform/methanol/0.1 M HCl (1:2:0.8).

1,2- and 1,3-diacyl-*sn*-glycerols were separated on borate-impregnated silica-TLC, using hexane/diethylether/methanol (4:1:1). Separation of PA, bisPA and PBut from the other phospholipids was done on silica-TLC using ethyl acetate/isooctane/acetic acid/water (13:2:3:10; two runs; system I) (van Blitterswijk *et al.*, 1991b), or on a two-dimensional TLC system (Figure 3, legend). Deacylation of bisPA (see below) was monitored on borate-impregnated silica plates using chloroform/methanol/7 M ammonia (60:30:4; system II). Lipid spots were visualized by spraying with primuline and/or autoradiography (Kodak XAR films; exposure time 1–4 days at −70°C). Lipids were quantified by scraping off the plates and liquid scintillation counting.

Phospholipid digestion methods

Controlled deacylation was performed by either of two methods: (i) alkaline O→N-transacylation with the monomethylamine reagent described by Clarke and Dawson (1981); or (ii) treatment with porcine pancreatic phospholipase A_2 (10 units; Sigma) in 0.5 ml 50 mM Tris–HCl (pH 7.4), 1 mM deoxycholate, 10 mM Mg^{2+}, for 1.5 h at 37°C under argon. Acetolysis was performed in acetic anhydride/acetic acid (2:3) at 145°C overnight, according to Renkonen (1965). The products were run on silica-TLC and developed in hexane/diethylether/methanol (80:40:4).

Analysis of water-soluble products

The aqueous extracts of choline metabolites were resolved by TLC, using 0.9% NaCl (in H_2O)/methanol/ammonia (50:50:5; two runs) (van Blitterswijk *et al.* (1991b). Commercial standards (from Sigma), choline (R_f = 0.22) and phosphocholine (R_f = 0.48) were cochromatographed and visualized in iodine vapour. Spots were scraped off and prepared for liquid scintillation counting. Water-soluble phosphorus-containing deacylation products from bisPA and from other glycerophospholipids were analysed by cellulose-TLC, developed in 1-propanol/ammonia/water (7:3:1; Poorthuis and Hostetler, 1975) and stained with a molybdate spray (Clarke and Dawson, 1981).

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