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Stimulation of phosphatidylcholine breakdown and diacylglycerol production by growth factors in Swiss-3T3 cells

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The effect of a number of growth factors on phosphatidylcholine (PtdCho) turnover in Swiss-3T3 cells was studied. Phorbol 12-myristate 13-acetate (PMA), bombesin, platelet-derived growth factor (PDGF) and vasopressin rapidly stimulated PtdCho hydrolysis, diacylglycerol (DAG) production, and PtdCho synthesis. Insulin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulated PtdCho synthesis, but not its breakdown, whereas epidermal growth factor (EGF) and bradykinin were without effect. Stimulation of PtdCho hydrolysis by the above ligands resulted in increased production of phosphocholine and DAG (due to phospholipase C activity) and significant amounts of choline, suggesting activation of a phospholipase D as well. CDP-choline and glycerophosphocholine levels were unchanged. Down-regulation of protein kinase C with PMA (400 nM, 40 h) abolished the stimulation of PtdCho hydrolysis and PtdCho synthesis by PMA, bombesin, PDGF and vasopressin, but not the stimulation of PtdCho synthesis by insulin and $PGF_{2\alpha}$. PtdCho hydrolysis therefore occurs predominantly by activation of protein kinase C (either by PMA or PtdIns hydrolysis) leading to elevation of DAG levels derived from non-PtdIns(4,5)P_2 sources. PtdCho synthesis occurs by both a protein kinase C-dependent pathway (stimulated by PMA, PDGF, bombesin and vasopressin) and a protein kinase C-independent pathway (stimulated by insulin and $PGF_{2\alpha}$). DAG production from PtdCho hydrolysis is not the primary signal to activate protein kinase C, but may contribute to long-term activation of this kinase.

INTRODUCTION

One of the earliest events to occur in a cell during mitogenic stimulation by growth factors such as platelet-derived growth factor (PDGF) and bombesin is activation of protein kinase C by diacylgycerol (DAG). Until recently, agonist-stimulated PtdIns(4,5)P_2 hydrolysis was thought to be the main source of DAG for protein kinase C activation (Berridge, 1984). It has recently become clear that other phospholipids, especially PtdCho, may provide an alternative source for DAG production (e.g. Besterman *et al.*, 1986). For example, both interleukin-1 (in Jurkat cells; Rosoff *et al.*, 1988) and the *ras* oncogene (Lacal *et al.*, 1987; Price *et al.*, 1989*b*) can increase DAG by elevating PtdCho hydrolysis in the absence of PtdIns(4,5)P_2 hydrolysis. Similarly, interleukin-3 (in FDCP-Mix1 cells; Whetton *et al.*, 1988) can activate protein kinase C by a mechanism which does not involve inositol phosphate production. However, most ligands appear to stimulate both PtdInsP_2 and PtdCho hydrolysis. These include formylmethionyl-leucylphenylalanine in HL-60 granulocytes (Pai *et al.*, 1988), adrenaline (epinephrine) in MDCK cells (Slivka *et al.*, 1988), PDGF in 3T3-LI cells (Besterman *et al.*, 1986), vasopressin in REF52 cells (Cabot *et al.*, 1988*a*) and bombesin in Swiss-3T3 cells (Muir & Murray, 1987). PMA also stimulates PtdCho hydrolysis in a number of different cell types, e.g. MDCK cells (Daniel *et al.*, 1986), REF52 cells (Cabot *et al.*, 1988*a*), HeLa cells (Paddon & Vance, 1980), NG108-15 cells (Liscovitch *et al.*, 1987) and Swiss-3T3 cells (Takuwa *et al.*, 1987). Agonist stimulation of PtdCho breakdown may therefore occur by a mechanism involving protein kinase C (Pelech *et al.*, 1984; Kolesnick & Paley, 1987). This increased PtdCho turnover is presumably caused by activation of a PtdCho-specific phospholipase C (Besterman *et al.*, 1986), although a phospholipase D may also be involved (Cabot *et al.*, 1988*b*).

As well as increasing the hydrolysis of PtdCho, PMA and other mitogens can stimulate the synthesis of PtdCho (Warden & Friedkin, 1984; Kolesnick & Paley, 1987) by increasing the activity of the key enzyme choline-phosphate cytidyltransferase (EC 2.7.7.15) (Paddon & Vance, 1980; Pelech *et al.*, 1984; Kolesnick, 1987).

The contribution of PtdCho hydrolysis to the generation of DAG (and subsequent activation of protein kinase C) has not been systematically investigated in any detail. We have used Swiss-3T3 cells to study the effect of a variety of growth factors on both the synthesis and breakdown of PtdCho. The present study shows that mitogens which activate protein kinase C (e.g. PDGF, PMA, bombesin and vasopressin) stimulate PtdCho hydrolysis, DAG production and PtdCho synthesis. Ligands such as insulin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulate only the synthetic pathway. PtdCho turnover may, therefore, be an important source of DAG during agonist stimulation of early and late signalling events.

EXPERIMENTAL

Materials

Radioisotopes and PDGF were from Amersham

Abbreviations used: MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; PMA, phorbol 12-myristate 13-acetate ('TPA'); PDGF, platelet-derived growth factor; EGF, epidermal growth factor; Me_2SO, dimethyl sulphoxide; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline (10 mM-potassium phosphate/0.14 M-NaCl, pH7.2); BSA, bovine serum albumin; PtdCho, phosphatidylcholine.

International. Dulbecco's modified Eagle's medium (DMEM), antibodies, trypsin and fetal-calf serum were from Gibco. PMA, vasopressin, PGF_{2a}, bradykinin, insulin, EGF, bombesin and other chemicals were from Sigma. Silica-gel 60 t.l.c. plates were from Merck.

Methods

Cells and labelling protocols. Swiss-3T3 cells were grown and maintained as previously described (Morris *et al.*, 1989). For choline labelling, 30 mm-diameter dishes of confluent cells were labelled overnight in serum-free DMEM containing [*methyl*-^{3}H]choline (5 μCi/ml). For glycerol labelling, cells were seeded at 5×10^4 cells per 30 mm dish in 1 ml of DMEM containing 10% (v/v) fetal-calf serum and [1(3)-^{3}H]glycerol (20 μCi/ml) and allowed to reach confluence.

Assays for choline metabolites. The medium was removed, the cells washed once with phosphate-buffered saline (PBS) and incubated in 1 ml of modified Hanks medium containing Tris (100 mM; pH 7.0), glucose (10 mM), BSA (fatty-acid-free; 1 mg/ml) and unlabelled choline (1 mM) for 20 min. Cells were washed again in PBS and incubated for a further 20 min in fresh modified Hanks medium. The medium was removed and the cells washed in 1 ml of PBS, then incubated in 400 μl of Hanks medium (without choline) with or without growth factors for the appropriate time. To terminate the incubation, the medium was removed, the reaction arrested with chloroform/methanol (1:2, v/v) and the aqueous and lipid phases separated as described by Morris *et al.* (1989). Aliquots of the lipid phase and the incubation medium were counted for radioactivity by liquid-scintillation spectrometry. The aqueous phase of the chloroform/methanol extract was dried, resuspended in 50% ethanol, spotted on to silica-gel 60 plates and run in 0.6% NaCl/methanol/35% NH_3 (100:100:3, by vol.) to separate choline metabolites (Yavin, 1976). Standards were also run and identified with iodine vapour. Spots corresponding to choline, phosphocholine, glycerophosphocholine and CDP-choline were cut out and counted for radioactivity as described previously (Price *et al.*, 1989*b*).

Assay for DAG. After labelling with [1(3)-^{3}H]glycerol, cells were washed three times in PBA and then incubated for 2×20 min in modified Hanks medium as in the choline experiments. Incubations with growth factors and termination of the reactions were also as for the choline experiments. Usually three dishes were combined per experiment. The chloroform (lipid) phase was evaporated under N_2 and resuspended in chloroform/methanol (1:2, v/v). Half was counted for radioactivity and the other half spotted on to silica-gel 60 plates and run in hexane/diethyl ether/methanol/acetic acid (90:30:3:2) against mono-(MAG), di- and tri-(TAG) acylglycerol standards (Habenicht *et al.*, 1981). Spots were identified with iodine vapour, cut out and counted for radioactivity.

Calculation of results. Results are expressed as radioactivity in a particular choline (or glycerol) metabolite as a percentage of the total radioactivity in the lipid phase (termed '% c.p.m.'). Choline-labelled cells typically contained 100000 c.p.m./dish, and glycerol-labelled cells 10000 c.p.m./dish.

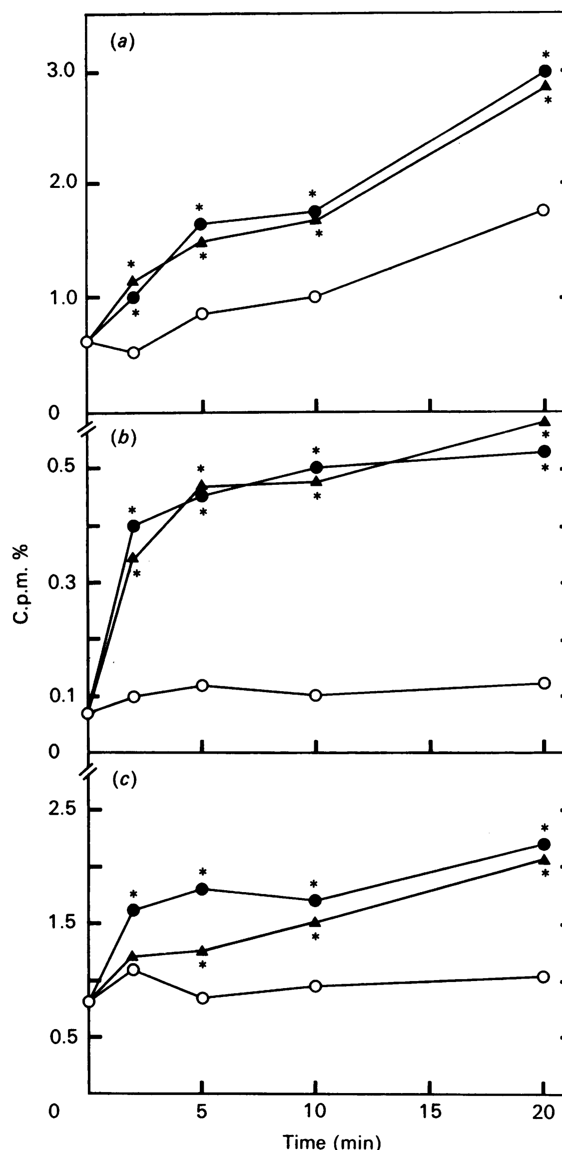

Fig. 1. Effect of bombesin and PMA on PtdCho breakdown

Cells were labelled and incubated as described under 'Methods'. Bombesin (40 nM) or PMA (100 nM) was added at zero time. (*a*) [^{3}H]Choline released to the medium; (*b*) [^{3}H]choline present in cells; (*c*) [^{3}H]phosphocholine present in cells. ○, Control; ●, bombesin; ▲, PMA. 'C.p.m. %' means radioactivity in choline or phosphocholine as a percentage of that in phosphatidylcholine. *indicates $P < 0.05$ in two-sided t test ($n = 6$) compared with controls (○).

RESULTS

Quiescent monolayers of Swiss-3T3 cells were labelled overnight with [*methyl*-^{3}H]choline, and the water-soluble choline metabolites were separated. The relative distribution of label between the metabolites in resting cells was: choline (51%); phosphocholine (30%); glycerophosphocholine (10%); CDP-choline (9%). Phosphocholine and choline make up the largest pools of choline metabolites and were found to be the most sensitive to ligand stimulation. Fig. 1(*c*) shows that both bombesin and PMA rapidly increase intracellular

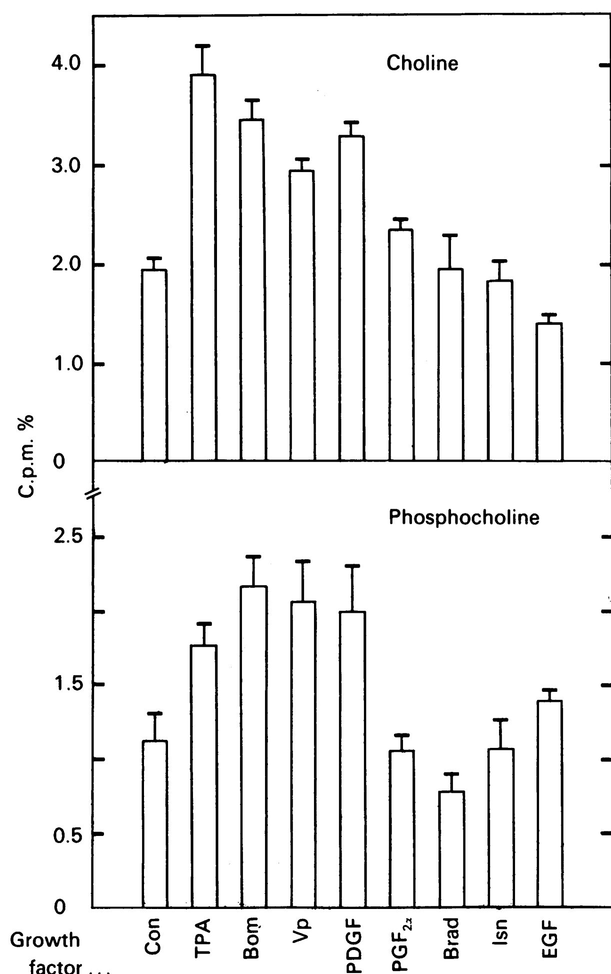

Fig. 2. Changes in PtdCho turnover caused by various growth factors

Cells were labelled as described under 'Methods' and incubated for 20 min with the above ligands. Choline produced is the sum of intracellular and extracellular. PMA (100 nM), bombesin (Bom, 50 nM), vasopressin (Vp, 20 ng/ml), PDGF (40 ng/ml), $PGF_{2\alpha}$ (300 ng/ml), bradykinin (Brad, 1 μM), EGF (10 ng/ml), and insulin (Isn, 1 μg/ml) were used at the concentrations cited (Con = control). Results are means ± S.E.M. (n = 6–8). 'C.p.m. %' is defined in Fig. 1.

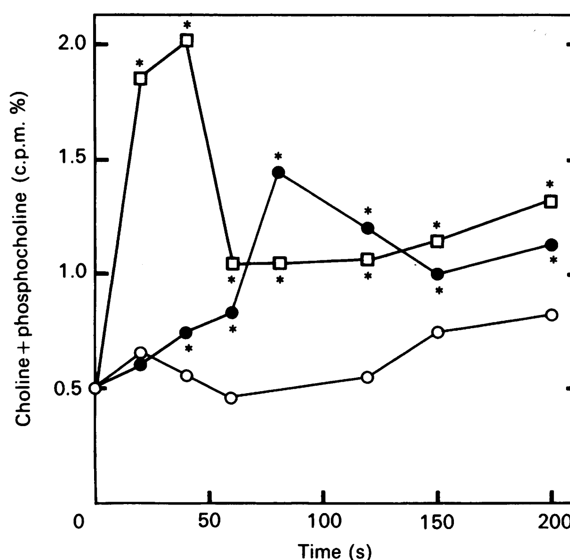

Fig. 3. Effect of PMA and bombesin on PtdCho breakdown over a short time course

Cells were treated as described under 'Methods' and incubated for the appropriate time. After termination of the reaction, the total intracellular choline metabolites (choline + phosphocholine) were measured. Symbols: ○, control; ●, PMA; □, bombesin. * indicates $P < 0.05$ in a two-sided t test (n = 7) compared with controls (○).

phosphocholine levels over the first 10 min of stimulation, and this increase continues at a lower rate for the next 15 min. Elevated levels of phosphocholine can be measured for at least 80 min (results not shown). Less than 5% of the total phosphocholine pool was found in the incubation medium, indicating that phosphocholine is not able to permeate cells. In contrast, stimulation of choline production by bombesin and PMA caused a substantial increase in both intracellular (Fig. 1*b*) and extracellular (Fig. 1*a*) choline. Fig. 1(*b*) shows that intracellular choline increases rapidly over the first 2 min of stimulation, then reaches a peak by 5 min. Extracellular choline (Fig. 1*a*), however, continues to increase linearly. Choline is a freely permeant molecule and presumably equilibrates with the choline-free medium as fast as it is produced. This accounts for the plateau in internal choline (Fig. 1*b*). Neither CDP-choline levels (control, 0.37 ± 0.14% c.p.m.; bombesin, 0.31 ± 0.11% c.p.m.) nor glycerophosphocholine levels (control, 0.38 ± 0.11% c.p.m.; bombesin, 0.41 ± 0.09% c.p.m.) were affected after 20 min stimulation by bombesin. Thus bombesin and PMA both cause a rapid increase in intracellular phosphocholine and choline, followed by efflux of choline from the cell.

We have also investigated the effect of a number of other growth factors on PtdCho breakdown. The results are summarized in Fig. 2. PDGF, vasopressin, bombesin and PMA are all capable of increasing the levels of both intra- and extra-cellular choline and phosphocholine (Fig. 2) to a similar extent. $PGF_{2\alpha}$, bradykinin, insulin and EGF gave little or no stimulation of PtdCho breakdown compared with control cells. None of these growth factors altered the levels of CDP-choline or glycerophosphocholine, except PMA (see below).

The kinetics of release of phosphocholine and choline by vasopressin and PDGF were almost identical with those of bombesin in Fig. 1, suggesting a common mechanism of action for these three growth factors. However, PMA showed some minor differences. Fig. 3 shows a rapid time course of total choline metabolites produced over the first 3 min of ligand stimulation by PMA (●), bombesin (□) or untreated (○) cells. Results are shown as the sum of intracellular choline plus phosphocholine, since these two metabolites were produced with a similar time course. Bombesin stimulated an immediate rise in choline metabolites, which then decreased to a lower rate which was sustained for at least 20 min (see Fig. 1). PMA showed no such rapid rise, but had a short time lag of about 30–40 s before choline metabolite release took place, with a smaller peak after 80 s. Since the decline in intracellular choline plus phosphocholine after the rapid rise in Fig. 3 was not mirrored by increased choline efflux from the cell (results not shown), the decline in choline plus

Table 1. MAG, DAG and TAG levels in stimulated cells

Cells were labelled with [1(3)-^{3}H]glycerol as described under 'Methods', then incubated for 20 min with the appropriate ligand. Cells were washed and the neutral lipids were separated. PMA (100 nM), bombesin (40 nM), PDGF (40 ng/ml) and vasopressin (20 ng/ml) were used at the cited concentrations. Results are means ± S.E.M. (n = 6).

		Level (c.p.m. %*)		
Stimulant	Acylglycerol...	MAG	DAG	TAG
None (control)		0.44±0.06	0.50±0.03	7.39±0.76
Bombesin		0.74±0.16	0.66±0.04	7.45±0.09
PMA		0.38±0.02	0.72±0.03	6.81±0.38
PDGF		0.87±0.03	0.74±0.03	6.67±0.42
Vasopressin		0.84±0.04	0.69±0.03	6.94±0.46

* 'c.p.m. %' is explained in the text.

Table 2. Effect of down-regulation of protein kinase C on ligand-stimulated PtdCho hydrolysis

Cells were pretreated for 24 h with Me_2SO [+ protein kinase C (PKC)] or PMA (400 nM, −PKC) in 10% serum to down-regulate protein kinase C then switched for 16 h to serum-free DMEM containing [*methyl*-^{3}H]choline (4 μCi/ml) and PMA (400 nM) as appropriate. Cells were then treated as in Fig. 2. Choline is the sum of intra- and extra-cellular choline. Results are means ± S.E.M. (n = 8).

	Level (c.p.m. % *)			
	+PKC		−PKC	
Ligand	Choline	Phosphocholine	Choline	Phosphocholine
Control	2.25±0.06	1.16±0.18	2.06±0.10	2.22±0.44
Bombesin	3.45±0.29	2.40±0.06	2.41±0.27	2.51±0.25
PMA	4.22±0.46	1.91±0.36	2.21±0.13	2.15±0.20
PDGF	3.30±0.15	1.99±0.43	2.28±0.32	2.18±0.17
Vasopressin	2.92±0.20	2.06±0.44	2.24±0.08	2.56±0.26

* 'c.p.m. %' is explained in the text.

phosphocholine is probably due to stimulation of PtdCho synthesis. In addition, PMA caused a marked decrease in CDP-choline levels over a 20 min stimulation from 0.37±0.14% c.p.m. (control) to 0.19±0.06% c.p.m. (PMA), whereas bombesin CDP-choline levels (0.31±0.11% c.p.m.) remained unchanged. This plus the data in Fig. 3 suggest that PMA may stimulate PtdCho turnover in a way slightly different from that by which PDGF, bombesin or vasopressin stimulate.

PtdCho hydrolysis by a phospholipase C would give rise to DAG and phosphocholine (Besterman *et al.*, 1986). However, Fig. 2 shows that stimulation of PtdCho hydrolysis by a variety of growth factors generates a large pool of choline. This choline could be generated either by phosphatase action on phosphocholine or by action of a phospholipase D on PtdCho, yielding phosphatidic acid and choline (Cabot *et al.*, 1988*b*). To see whether a phospholipase C was activated by these ligands, DAG levels were monitored in response to PDGF, bombesin and PMA. [1(3)-^{3}H]glycerol-labelled cells were incubated in the presence of growth factors for 20 min. MAG, DAG and TAG were separated and counted for radioactivity. The results are shown in Table 1. PMA, PDGF, vasopressin and bombesin all stimulate DAG production. For bombesin, vasopressin and PDGF there is also a large increase in MAG. This is probably derived from DAG by the action of DAG lipase (Bell *et al.*, 1979). PMA does not cause any increase in MAG, possibly because DAG lipase is inhibited by PMA (Chabbott & Cabot, 1986). TAGs were unchanged by PMA, PDGF, vasopressin or bombesin. For bombesin, vasopressin and PDGF, at least part of the DAG (and MAG) produced will be due to PtdIns(4,5)P_2 hydrolysis. Since PMA does not stimulate PtdIns(4,5)P_2 hydrolysis (Takuwa *et al.*, 1987), the DAG produced by this phorbol ester is presumably derived from PtdCho. So PMA, PDGF, vasopressin and bombesin can apparently stimulate DAG release through activation of a phospholipase C, but we cannot exclude the possibility of activation of a phospholipase D as well.

Ligands such as bombesin and PDGF, which cause PtdIns(4,5)P_2 hydrolysis and generate DAG, may also activate protein kinase C (Nishizuka, 1984). To see whether protein kinase C is involved in the breakdown of PtdCho, cells were pretreated for 40 h with PMA (400 nM) to down-regulate protein kinase C (Rodriguez-Pena & Rozengurt, 1984). Previously we (Morris *et al.*, 1989) have shown that such down-regulated cells lack any immunoprecipitable protein kinase C. PMA, PDGF, vasopressin and bombesin effects on PtdCho turnover in

Table 3. Stimulation of inositol phosphate release by growth factors

Cells were incubated for 18 h in inositol-free DMEM plus 4 μCi of [^{3}H]inositol/ml. Before stimulation, cells were incubated for 15 min in modified Hanks buffer plus 10 mM-LiCl, then stimulated for 20 min with ligands. Reactions were terminated and total inositol phosphates assayed as described by Price *et al.* (1989*a*). The total radioactivity in PtdIns was ~21000 c.p.m./dish. Results ± S.E.M. ($n = 6$).

Ligand	Inositol phosphates (c.p.m.)
Control	527 ± 24
PDGF	2161 ± 110
Bombesin	2008 ± 179
Vasopressin	1707 ± 149
Bradykinin	880 ± 62
$PGF_{2\alpha}$	822 ± 54

such down-regulated cells were then tested. The results are shown in Table 2. Basal levels of phosphocholine are increased in down-regulated cells compared with controls, but choline levels are unchanged. Stimulation of choline and phosphocholine production by PMA, PDGF, bombesin or vasopressin is almost completely abolished in cells in which protein kinase C has been down-regulated (Table 2), although a very small stimulation of choline release by bombesin still occurs. Thus down regulation of protein kinase C blocks the ability of PMA, PDGF, bombesin and vasopressin to stimulate PtdCho breakdown, implying that activation of protein kinase C is required for the stimulation of PtdCho hydrolysis.

Bombesin, PDGF and vasopressin stimulated PtdCho breakdown, but $PGF_{2\alpha}$ and bradykinin did not (Fig. 2). However, all these ligands have been reported to stimulate PtdIns(4,5)P_2 hydrolysis in Swiss-3T3 cells and therefore activate protein kinase C (Hesketh *et al.*, 1988). Thus all these ligands should activate PtdCho turnover. The failure of $PGF_{2\alpha}$ and bradykinin to stimulate PtdCho hydrolysis may relate to their poor stimulation of PtdIns(4,5)P_2 breakdown in our Swiss-3T3 cells. Inositol phosphate production in response to a variety of ligands was assayed as described in Table 3, and the increase in inositol phosphates after 20 min was calculated. PDGF, bombesin and vasopressin gave large increases in inositol phosphates, whereas bradykinin and $PGF_{2\alpha}$ were poor stimulators of PtdInsP_2 turnover. Insulin and EGF had no effect on PtdIns(4,5)P_2 hydrolysis (J. D. H. Morris, unpublished work). Bradykinin and $PGF_{2\alpha}$ may therefore only weakly activate protein kinase C, giving little or no stimulation of PtdCho breakdown.

As well as stimulating the breakdown of PtdCho, some mitogens may also stimulate the synthesis of PtdCho. The ability of a number of mitogens to stimulate [*methyl*-^{3}H]choline uptake into cell lipids was tested. The results are shown in Table 4. PMA, bombesin, vasopressin and PDGF were all capable of stimulating synthesis of PtdCho when cells were supplied with exogenous choline. These growth factors can therefore stimulate both the breakdown and resynthesis of PtdCho. Surprisingly,

Table 4. Effect of growth factors on the incorporation of [*methyl*-^{3}H]choline into cell lipids

Protein kinase C (PKC) was down-regulated as described in Table 2. Cells were then incubated for 1 h in choline-free DMEM (2 ml), washed in PBS and then incubated in modified Hanks (without choline) plus [*methyl*-^{3}H]choline (0.5 μCi/ml). Growth factors or buffer (for control) were added 15 min later, and cells were incubated for a further 30 min. Lipids were extracted as described under 'Methods', the chloroform phase washed with 3 × 900 μl of PBS and an aliquot counted for radioactivity. More than 95% of the lipid radioactivity was in PtdCho when analysed by t.l.c. Values in parentheses are stimulations expressed as a percentage of the control value. Results are means ± S.E.M. ($n = 8$).

Ligand	Radioactivity (c.p.m./dish)	
	+PKC	−PKC
Control	1042 ± 35 (0)	820 ± 48 (0)
PMA	2501 ± 310 (140)	794 ± 37 (0)
Bombesin	1872 ± 272 (80)	883 ± 61 (8)
PDGF	2342 ± 225 (125)	914 ± 128 (11)
Vasopressin	2028 ± 101 (95)	947 ± 61 (16)
$PGF_{2\alpha}$	1831 ± 172 (76)	1298 ± 141 (59)
Insulin	1582 ± 59 (52)	1197 ± 33 (46)
Bradykinin	941 ± 163 (0)	–
EGF	1063 ± 175 (0)	–

$PGF_{2\alpha}$ and insulin were also able to stimulate PtdCho synthesis, although they had little or no effect on breakdown (Fig. 2). EGF and bradykinin were without effect. In cells lacking protein kinase C, the basal rate of PtdCho synthesis is decreased, and PMA, bombesin, PDGF and vasopressin can no longer stimulate PtdCho synthesis to any appreciable extent (Table 4). These four ligands probably increase PtdCho synthesis through activation of protein kinase C. In contrast, the percentage stimulation of PtdCho synthesis by $PGF_{2\alpha}$ or insulin was only slightly decreased by loss of protein kinase C. Thus there are two pathways by which ligands can stimulate PtdCho synthesis, a protein kinase C-dependent pathway (stimulated by PMA, bombesin, PDGF and vasopressin) and a protein kinase C-independent pathway (stimulated by insulin and $PGF_{2\alpha}$).

DISCUSSION

These results show that PDGF, bombesin, vasopressin and PMA can rapidly stimulate both the breakdown (within 40 s) and resynthesis of PtdCho. $PGF_{2\alpha}$ and insulin can activate PtdCho synthesis, but neither EGF nor bradykinin had any effect on PtdCho turnover. Similar results with bombesin (Muir & Murray, 1987) and PMA (Takuwa *et al.*, 1987) have been shown in Swiss-3T3 cells. However, we have also shown that down-regulation of protein kinase C abolished stimulation, by bombesin, PDGF, vasopressin and PMA, of phosphocholine and choline release. This effect is not due to a loss of receptors or changes in binding affinity during down-regulation of protein kinase C, since we have previously demonstrated that long-term PMA treatment does not block bombesin- or PDGF-stimulated PtdIns(4,5)P_2 hydrolysis (Price *et al.*, 1989*a*). It is also

possible that down-regulation activates PtdCho hydrolysis, thus depleting the ligand sensitive pool of PtdCho and rendering cells unresponsive to further stimulation. Indeed, Table 3 shows that phosphocholine levels are increased in down-regulated cells, but since neither DAG levels (control, 0.50±0.06% c.p.m.; down regulated, 0.53±0.08% c.p.m.), choline levels (Table 3) or the amount of label incorporated into the PtdCho pool (control, 101855±2845 c.p.m./dish; down-regulated, 113992±4455 c.p.m./dish) are affected, this explanation is unlikely. We conclude that activation of protein kinase C is required for PtdCho hydrolysis in Swiss-3T3 cells.

Since we can detect the products of both phospholipase C (phosphocholine and DAG) and phospholipase D (choline), both of these phospholipases may be activated by growth factors. However, Takuwa *et al.* (1987) found no evidence for activation of phospholipase D after PMA stimulation of PtdCho hydrolysis in Swiss-3T3 cells.

PMA and bombesin differed qualitatively in their ability to stimulate PtdCho turnover, since (i) PtdCho hydrolysis occurred approx. 40 s after PMA addition, whereas bombesin stimulation was almost instantaneous, (ii) PMA decreased CDP-choline levels, but bombesin did not. These differences may reflect the fact that PMA acts directly on protein kinase C, whereas the other ligands act indirectly via elevated PtdIns(4,5)P_2 hydrolysis to activate protein kinase C.

Bombesin, PDGF, vasopressin, PMA, $PGF_{2\alpha}$ and insulin all stimulated synthesis of PtdCho. Warden & Friedkin (1984) also demonstrated that insulin and PMA could stimulate PtdCho synthesis in Swiss-3T3 cells. In HeLa cells, PtdCho synthesis appears to be controlled by protein kinase C at the level of cytidyltransferase (Paddon & Vance, 1980). However, our results show that down-regulation of protein kinase C blocks the stimulation of PtdCho synthesis by bombesin, PDGF, PMA and vasopressin. These four ligands activate protein kinase C by increasing PtdIns(4,5)P_2 hydrolysis (see Table 3). Conversely, stimulation of PtdCho synthesis by insulin and $PGF_{2\alpha}$ [which do not affect PtdIns(4,5)P_2 hydrolysis] was unaffected by down-regulation of protein kinase C. Thus there are at least two independent pathways for activation of PtdCho synthesis, one via protein kinase C and one by some other mechanism, perhaps involving receptor kinases.

Production of DAG is probably the most important consequence of elevated PtdCho turnover. Stimulation of PtdIns(4,5)P_2 turnover causes release of DAG and activation of protein kinase C, which can itself rapidly down-regulate bombesin and PGDF-stimulated PtdIns-(4,5)P_2 hydrolysis (Brown *et al.*, 1987; Price *et al.*, 1989*a*). This feedback inhibition by protein kinase C would limit PtdIns(4,5)P_2 breakdown and therefore lower DAG levels. To maintain the elevated DAG levels and activated protein kinase C required for some proliferative signals (e.g. PMA, *ras* oncogene; Lloyd *et al.*, 1989), protein kinase C may itself promote PtdCho turnover, keeping up DAG levels in the absence of PtdIns(4,5)P_2 turnover. Regulation of this system could be provided by metabolism of DAG by DAG kinase (McDonald *et al.*, 1988) or DAG lipase (Bishop & Bell, 1988). In addition, since the fatty acid composition of PtdIns differs from that of PtdCho (Esko & Raetz 1983), the DAGs produced will also differ, especially as PtdCho hydrolysis has been demonstrated to generate 1-alkyl-2-acylglycerols as well as diacylglycerols (e.g. Daniel *et al.*, 1986). It is possible that, given the multiplicity of forms of protein kinase C (Coussens *et al.*, 1986), a wider range of DAG types may be required to activate fully the various types of protein kinase C than can be provided by PtdIns hydrolysis.

In conclusion, we have demonstrated that a number of growth factors which are known to stimulate PtdIns(4,5)-P_2 hydrolysis can also stimulate PtdCho breakdown and synthesis. These effects are mediated predominantly by activation of protein kinase C, although insulin and $PGF_{2\alpha}$ appear to act independently of protein kinase C. Stimulated breakdown of PtdCho may occur through activation of both phospholipase C and phospholipase D. Both of these enzymes may be targets for protein kinase C. PtdCho turnover may therefore function to maintain DAG levels (and activation of protein kinase C) over the 12–15 h period that precedes initiation of DNA synthesis.

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