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Analysis of the water-soluble products of phosphatidylcholine breakdown by ion-exchange chromatography

Bombesin and TPA (12-*O*-tetradecanoylphorbol 13-acetate) stimulate choline generation in Swiss 3T3 cells by a common mechanism

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A method for the rapid and quantitative separation of glycerophosphocholine, choline phosphate and choline upon ion-exchange columns is described. The method has been utilized to examine the stimulation of phosphatidylcholine breakdown in quiescent Swiss 3T3 cells in response to bombesin and 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The stimulated generation of choline is shown to precede that of choline phosphate, with no effect upon glycerophosphocholine levels; but was attenuated in cells in which protein kinase C activity was down-regulated. The results thus suggest that stimulation of the cells with either bombesin or TPA activates phospholipase D-catalysed phosphatidylcholine breakdown by a common mechanism involving the activation of protein kinase C.

INTRODUCTION

A range of fibroblast mitogens stimulate the receptor-activated breakdown of PtdIns(4,5)P_2, generating the two second messenger molecules, Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol (DAG) (Berridge, 1987). It has been proposed that this is the means whereby agonists, such as bombesin, stimulate cell proliferation (Berridge, 1987). However, a number of observations question this being the sole mechanism of action of such growth factors. In particular, agonist-stimulated PtdIns(4,5)P_2 hydrolysis is a transient process which is rapidly desensitized (see e.g. Brown *et al.*, 1987), whilst the cellular level of DAG has been demonstrated to remain elevated in the absence of significant amplified PtdIns(4,5)P_2 breakdown (S. J. Cook & M. J. O. Wakelam, unpublished work).

A partial explanation to this paradox has come from the demonstration that a range of agonists can, in addition to stimulating inositol phospholipid metabolism, activate the hydrolysis of phosphatidylcholine (PtdCho) in various cell types (see Pelech & Vance, 1989, for a review). Several mechanisms have been put forward to account for this response. Both phospholipase C- (Slivka *et al.*, 1987) and phospholipase D- (Bocckino *et al.*, 1987) catalysed hydrolysis of PtdCho have been proposed, and it has been suggested that the lipase activation can be both a direct agonist–receptor-stimulated event or secondary to the activation of protein kinase C.

Whilst there has been an increase in the investigation of PtdCho metabolism, many of these studies have failed to analyse the individual water-soluble metabolites generated following PtdCho hydrolysis. This omission makes it difficult in many cases to interpret the data obtained in terms of a precise hydrolytic pathway. Metabolites of PtdCho have been measured using t.l.c. (Yavin, 1976), but these methods are time-consuming and have proved unreliable. Consequently we have developed an ion-exchange procedure to separate choline (Cho), choline phosphate (Cho*P*) and glycerophosphocholine (Gro*P*Cho). This method is rapid and reproducible and has been employed to analyse bombesin- and TPA-stimulated PtdCho breakdown in Swiss 3T3 cells. In agreement with the proposal of Cabot *et al.* (1988), from studies upon REF-52 cells, the data suggest that agonist-stimulated PtdCho breakdown is a phospholipase D-catalysed process, which in Swiss 3T3 cells is dependent upon functional C-kinase activity.

MATERIALS AND METHODS

Cell culture and stimulations

Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) donor calf serum at 37 °C in a 5% CO_2 atmosphere at 95% humidity. Cells were routinely passaged when subconfluent. For experiments, cells were seeded on to 24-well plates, and 24 h later the medium was changed to one containing 1% (v/v) donor calf serum and 1.5 μCi of [*methyl*-^{3}H]choline chloride/ml (specific radioactivity 76 Ci/mmol). Experiments were performed 48 h later on the cells which were then confluent and quiescent. Preliminary experiments demonstrated that equilibrium labelling of the Cho-containing lipids was achieved following a 36 h incubation with [^{3}H]Cho (results not shown).

After removal of the labelling medium, the cells were

Abbreviations used: Cho, choline; Cho*P*, choline phosphate; DAG, *sn*-1,2-diacylglycerol; Gro*P*Cho, glycerophosphocholine; HBG, Hanks buffered saline, pH 7.4, containing 2% (w/v) bovine serum albumin and 10 mM-glucose; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

washed by incubation with 0.5 ml of Hanks buffered saline for 5 min, with 0.5 ml of Hanks buffered saline containing 10 mM-glucose and 1% (w/v) bovine serum albumin (HBG) for 10 min, and then with fresh HBG for a further 30 min; all incubations were performed at 37 °C. Stimulations were initiated by the addition of 0.15 ml of HBG or agonists in HBG and terminated by the direct addition of 0.5 ml of ice-cold methanol, except in those experiments in which intracellular Cho levels were examined, where the incubation medium was aspirated immediately prior to the addition of methanol. The plates were stood on ice for 10 min and the contents of each well were scraped and transferred to tubes to which chloroform was added. Samples were extracted for 60 min before chloroform and water were added to a final ratio of 1:1:0.9 ($CHCl_3$/MeOH/H_2O). The tubes were centrifuged at 14000 *g* for 5 min and the upper aqueous methanolic phase was taken for analysis of the water-soluble Cho-containing metabolites. An aliquot of the lower chloroform phase was dried down and the radioactivity associated with the Cho-containing lipids measured by scintillation counting following the addition of 3 ml of Ecoscint (National Diagnostics, Manville, NJ, U.S.A.).

Ion-exchange chromatography

Dowex-50-WH$^+$ was extensively washed in 1 M-HCl followed by distilled water until the washings were of constant pH (approx. 5.5). Columns (1 ml) were prepared in glass wool plugged Pasteur pipettes. The methanolic aqueous phase of samples (see above) was diluted to 5 ml with water and loaded on to the columns. The run-through and a 4 ml water wash were collected together and made up the Gro*P*Cho fraction. Cho*P* was eluted by the further addition of 20 ml of water and Cho by the addition of 20 ml of 1 M-HCl. Aliquots of each fraction were taken and the associated radioactivity was determined by scintillating counting. Elution profiles were checked in each experiment by the inclusion of a separate column upon which standards were loaded. [*methyl*-^{14}C]Cho*P* and [*methyl*-^{3}H]Cho chloride were from Amersham International, Amersham, Bucks., U.K. [*N-methyl*-^{3}H]Gro*P*Cho was prepared from labelled PtdCho (Amersham) by transacylation with monomethylamine by the method of Clarke & Dawson (1981).

Separation of the water-soluble Cho metabolites by t.l.c.

Standards and aqueous cell extracts were separated on heat-activated silica gel G plates by the method of Yavin (1976) as modified by Kolesnick & Paley (1987). The plate was dried and divided into 0.5 cm sections which were excised and mixed with 3 ml of Ecoscint. The associated radioactivity was determined by liquid scintillation counting.

Determination of lipid breakdown

The chloroform-soluble cellular extract, prepared as above, was chromatographed upon silica gel G plates by the method of Skipski *et al.* (1964); Cho-containing lipids were located by visualizing standards, run on the same plates, by iodine staining. Loss of label from PtdCho and sphingomyelin was determined by scintillation counting following excision of the appropriate spots.

Results are presented as means ± S.D.; statistical analysis was performed using Student's unpaired *t*-test.

Tissue culture materials were from Gibco, Paisley, Scotland, U.K.; bombesin was from CRB, Cambridge, U.K.; Dowex 50X8-400 H$^+$ cation-exchange resin, phorbol esters and unlabelled lipids were from Sigma, Poole, Dorset, U.K. All other reagents of the highest grade available were from previously reported sources (Gardner *et al.*, 1989; Palmer & Wakelam, 1989).

RESULTS

The separation of Cho from Cho*P* by ion-exchange chromatography on Dowex H$^+$ columns was first reported by Dowdall *et al.* (1972). We have adapted this method to separate Cho, Cho*P* and Gro*P*Cho present in cellular extracts. Fig. 1 shows the elution profile obtained when [^{3}H]Gro*P*Cho, [^{14}C]Cho*P* and [^{3}H]Cho, in the presence of a non-radioactive aqueous methanolic phase of a Swiss 3T3 cell extract, were separated on a 1 ml Dowex 50-W-H$^+$ column. The recovery of each metabolite and cross-contamination of the samples were calculated. These results are shown in Table 1 and demonstrate greater than 90% recovery of each standard. Table 1 also shows that there was essentially no cross-contamination of [^{3}H]Cho with [^{3}H]Gro*P*Cho or [^{14}C]Cho*P*. A small (6%) cross-over between [^{3}H]-Gro*P*Cho and [^{14}C]Cho*P* was detected, but this was not of a significant enough magnitude to affect the results.

This separation method was applied to aqueous methanolic extracts prepared from control and bombesin-stimulated [^{3}H]Cho-labelled cells as shown in Fig. 2. The profile shows three distinct peaks of ^{3}H radioactivity of identical mobility to the corresponding standards shown in Fig. 1; i.e. from left to right, Gro*P*Cho, Cho*P* and Cho. Eluates from the Dowex 50-W-H$^+$ separation of a cell extract and of standards were freeze-dried, dissolved in water and separated by t.l.c. Scraping the plates and determining the radioactivity

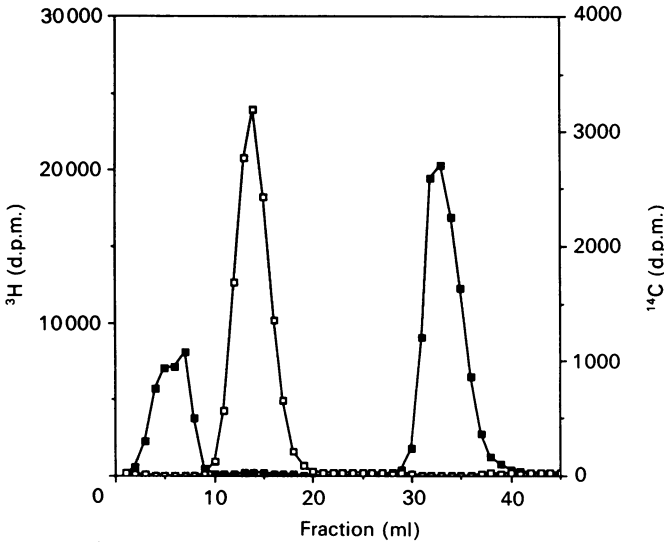

Fig. 1. The separation of Gro*P*Cho, Cho*P* and Cho by ion-exchange chromatography on Dowex-H$^+$ columns

A mixture of [^{3}H]Gro*P*Cho, [^{14}C]Cho*P* and [^{3}H]Cho was added to an aqueous methanolic extract prepared from Swiss 3T3 cells and separated on a 1 ml Dowex-50-H$^+$ column as described in the Materials and methods section. ■, ^{3}H; □, ^{14}C.

Table 1. Analysis of recoveries of and cross-contamination between [^{3}H]Cho-labelled metabolites separated on Dowex columns

Standard samples of [^{3}H]Gro*P*Cho, [^{14}C]Cho*P* and [^{3}H]Cho were separated, in the presence of an aqueous methanolic extract prepared from Swiss 3T3 cells, on Dowex columns. Recoveries and cross-over were determined by dual-label liquid scintillation counting and by t.l.c. of freeze-dried fractions from the columns. The results are means calculated from eight separate experiments. Other details are as in the Materials and methods section.

		% Contamination with:		
Fraction	Recovery (%)	Cho	Cho*P*	Gro*P*Cho
Cho	94.8±8.4	–	2.51±4.0	0
Cho*P*	92.6±9.1	2.5±4.0	–	2.9±2.8
Gro*P*Cho	91.5±7.5	0	6.1±2.8	–

associated with each fraction demonstrated that the Cho*P* and Cho fractions were uncontaminated with other Cho-containing metabolites but that the Gro*P*Cho fraction was contaminated with 8% Cho*P*.

Since the [^{3}H]Cho utilized in this study was labelled in the methyl position, additional labelled metabolites could be being generated in the cells, in particular betaine aldehyde and betaine through the action of choline dehydrogenase and betaine aldehyde dehydrogenase. In order to eliminate these metabolites as potential contaminants, the generation of labelled compounds other than [^{3}H]Gro*P*Cho, [^{3}H]Cho*P* and [^{3}H]Cho in cells

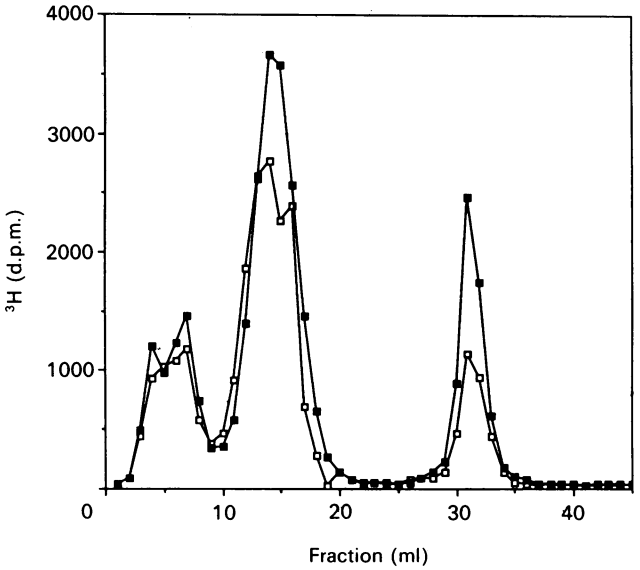

Fig. 2. Analysis of [^{3}H]Cho-labelled metabolites from Swiss 3T3 cells by ion-exchange chromatography

Quiescent Swiss 3T3 cells were grown on 24-well plates and labelled with 1.5 μCi of [^{3}H]Cho/ml for 48 h. Aqueous methanolic extracts were prepared from control and bombesin (617 nM)-stimulated (5 min) cells. These were then separated on Dowex columns as described in the Materials and methods section. □, Control cells; ■, bombesin-stimulated cells.

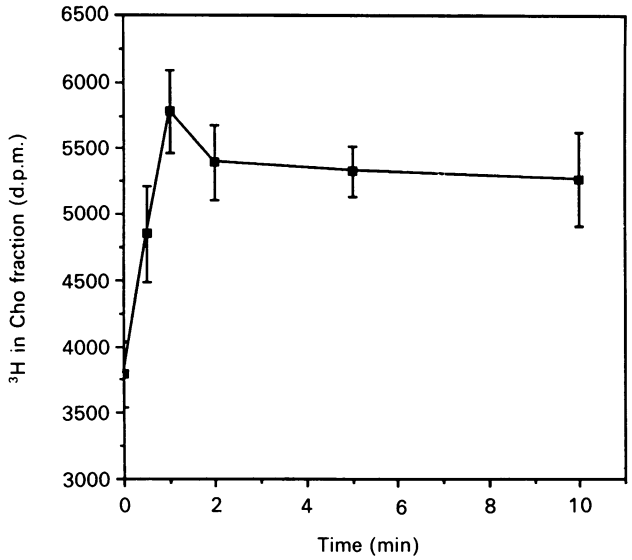

Fig. 3. Bombesin-stimulated increases in cell-associated [^{3}H]Cho

Quiescent Swiss 3T3 cells were labelled for 48 h with 1.5 μCi of [^{3}H]Cho/ml as described in the Materials and methods section. The cells were washed and stimulated with 617 nM-bombesin for the stated times, the medium was aspirated and incubations quenched by the addition of methanol. Aqueous methanolic extracts were prepared and the [^{3}H]Cho-containing fractions isolated on Dowex columns; all other details are described in the Materials and methods section. No significant change was observed in the d.p.m. associated with Cho in unstimulated cells. Results are expressed as means±S.D. and are from one typical experiment where $n = 3$.

was monitored. Separation of an aqueous extract prepared from labelled cells by t.l.c. demonstrated only minimal labelling (less than 100 d.p.m.) of betaine or betaine aldehyde in control, bombesin-stimulated and TPA-stimulated cells. Stimulation by the agonist had no significant effect (less than 5%) upon the radioactivity associated with these metabolites. Extension of the elution of the Dowex columns with water up to 45 ml and sequential elution with 0.25, 0.5 or 0.75 M-HCl did not demonstrate any radiolabelled peaks in addition to those shown in Figs. 1 and 2. The separation of the water-soluble metabolites upon Dowex columns was thus concluded to be valid.

Stimulation of Swiss 3T3 cells with 617 nM-bombesin caused the rapid generation of both intracellular and total Cho (Figs. 3 and 4). The increase in total [^{3}H]Cho was apparent at 10 s, significant after 1 min ($P = 0.002$) and typically doubled by 2 min. However, the rate of [^{3}H]Cho production was reduced between 2 and 5 min, after which it paralleled the basal increase in [^{3}H]Cho (Fig. 4). The precise details of the time course varied between individual experiments and in some instances the labelling of [^{3}H]Cho was clearly significant by 10 s. Intracellular [^{3}H]Cho mirrored this rise in total Cho levels up to 2 min; thereafter, however, intracellular [^{3}H]Cho levelled off (Fig. 3). A significant increase in [^{3}H]Cho*P* was only observed after 15 min of stimulation ($P < 0.001$) and varied in magnitude from 20 to 58% above basal between individual experiments (Fig. 4). No significant change in the radioactivity associated with

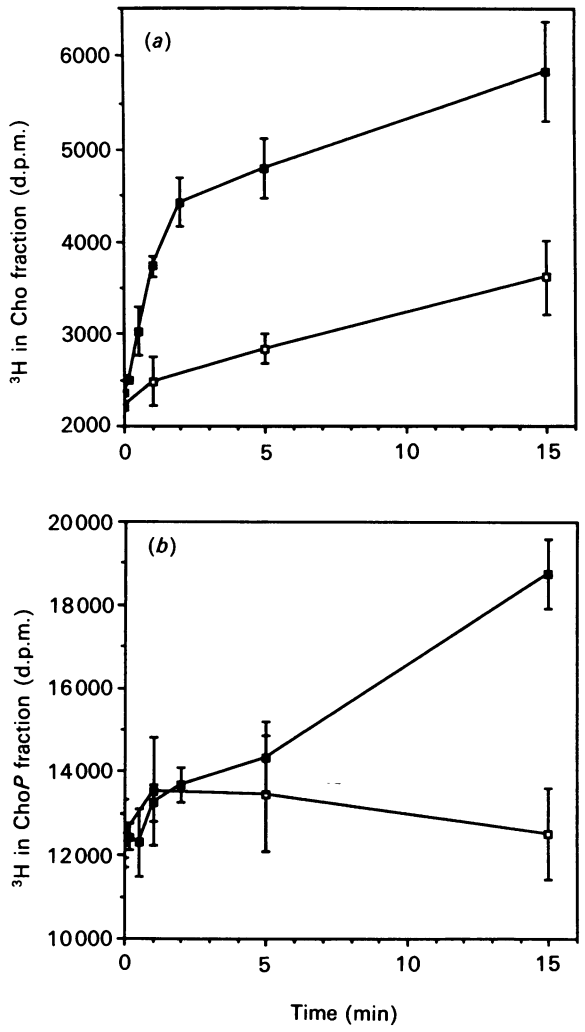

Fig. 4. Time course of bombesin-stimulated changes in Cho and Cho*P*

[^{3}H]Cho-labelled Swiss 3T3 cells were stimulated for the stated times with 617 nM-bombesin, and [^{3}H]Cho, [^{3}H]Cho*P* and [^{3}H]Gro*P*Cho were separated as described in the Materials and methods section. The results are means ± S.D. from one typical experiment where $n = 3$. □, Control cells; ■, bombesin-stimulated cells. (*a*) Cho, (*b*) Cho*P*.

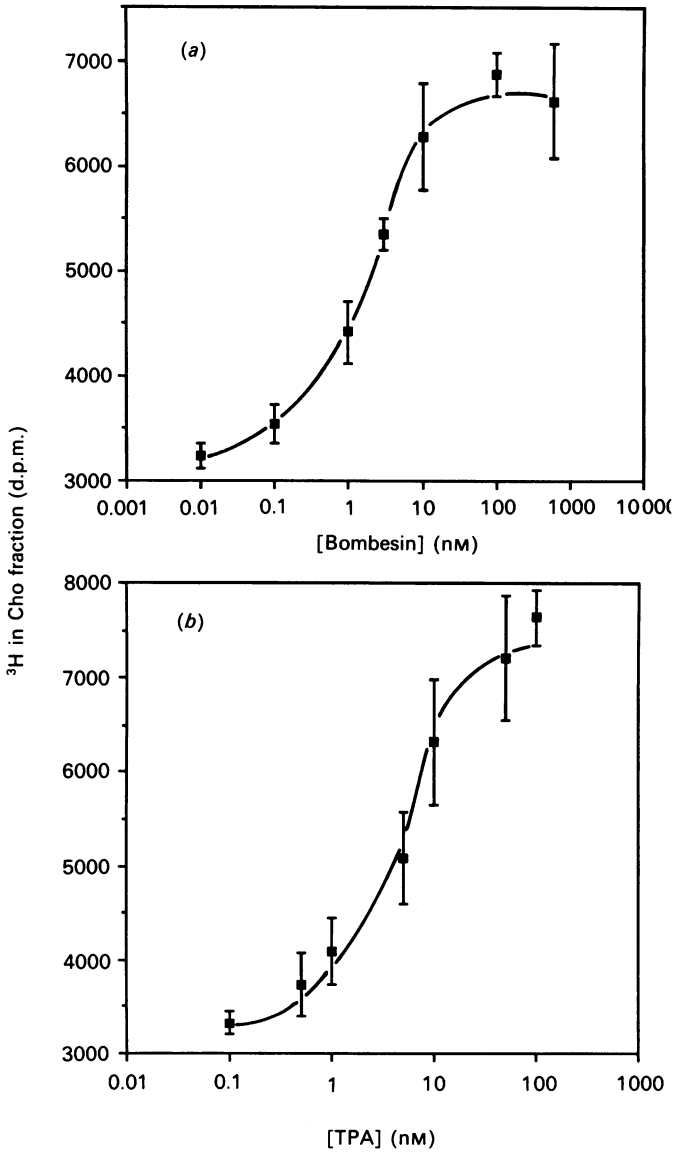

Fig. 5. Dose dependence of bombesin- and TPA-stimulated [^{3}H]Cho generation.

[^{3}H]Cho-labelled Swiss 3T3 cells were stimulated with increasing concentrations of (*a*) bombesin (for 2 min), or (*b*) TPA (for 5 min), and the radioactivity associated with the Cho fraction was determined as described in the Materials and methods section. The results are means ± S.D. from single typical experiments where $n = 3$.

[^{3}H]Gro*P*Cho was observed in response to bombesin (results not shown, but see Fig. 2). The increase in [^{3}H]Cho generation in response to bombesin was dose dependent (Fig. 5*a*); the bombesin concentration giving half-maximal stimulation of [^{3}H]Cho generation (EC_{50}) was 2.30 ± 0.57 nM (mean ± S.D. of three separate experiments).

The generation of [^{3}H]Cho in Swiss 3T3 cells in response to stimulation with the tumour-promoting phorbol ester TPA was also both time- and dose-dependent (Figs. 5*b* and 6) with an EC_{50} of 3.1 ± 1.6 nM (mean of three experiments). In contrast to stimulation with bombesin, a significant increase [^{3}H]Cho was observed only after 2 min of stimulation ($P = 0.004$); however, as with bombesin stimulation, Cho generation continued to rise for the 60 min duration of the timecourse (results not shown). Elevation of [^{3}H]Cho*P* was not significant until 15–30 min after addition of the phorbol ester (Fig. 6). The non-C-kinase activating β-phorbol did not elevate [^{3}H]Cho levels above the vehicle control (2286 ± 116 d.p.m. in control; 2330 ± 153 d.p.m. with 100 nM-β-phorbol; 5931 ± 53 d.p.m. with 100 nM-TPA; results from a single typical experiment, $n = 3$, 5 min stimulation).

Treatment of Swiss 3T3 cells with 400 nM-TPA for 48 h has been demonstrated to down-regulate protein kinase C activity in this cell type (Rodriguez-Pena & Rozengurt, 1984) with no loss in the ability of bombesin to stimulate inositol phosphate production (Brown *et al.*,

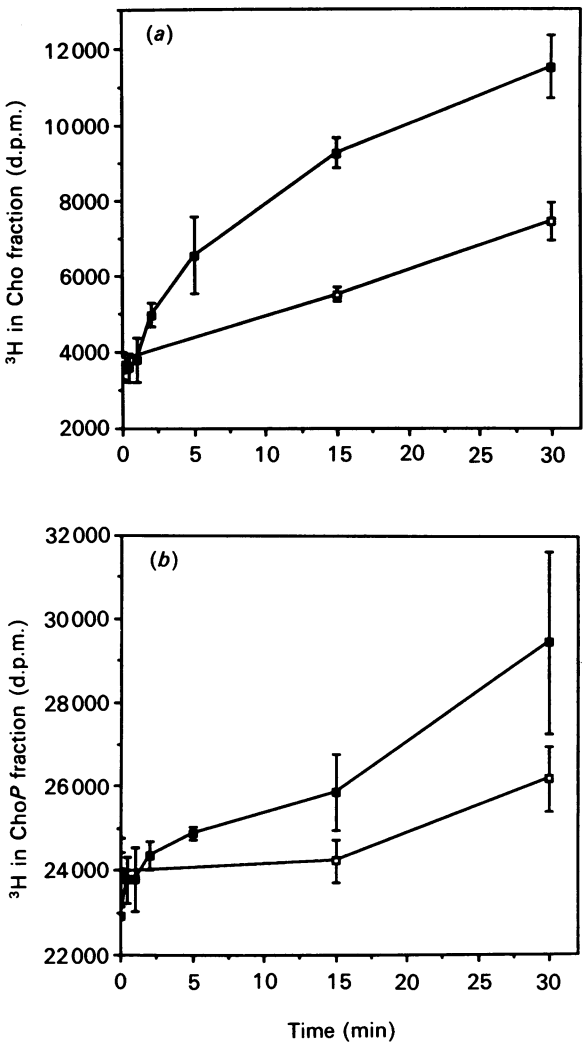

Fig. 6. Time course of TPA-stimulated changes in Cho and Cho*P*

[^{3}H]Cho-labelled Swiss 3T3 cells were stimulated for the stated times with 100 nM-TPA, and [^{3}H]Cho, [^{3}H]Cho*P* and [^{3}H] Gro*P*Cho were separated as described in the Materials and methods section. The results are means ± S.D. from one typical experiment where $n = 3$. □, Control cells; ■, TPA-stimulated cells. (*a*) Cho, (*b*) Cho*P*.

1987). When cells were so treated, the stimulation of [^{3}H]Cho generation in response to both bombesin and TPA was attenuated (Table 2). A 15 min pre-treatment of normal cells with 1 μM-staurosporine (a proposed C-kinase inhibitor) also reduced the stimulation of [^{3}H]Cho generation in response to both bombesin and TPA, though to a lesser extent (results not shown).

Analysis of the Cho-containing lipids by t.l.c. demonstrated that radioactivity was being lost from PtdCho. A 2 min stimulation with 617 nM-bombesin reduced the radioactivity associated with PtdCho from 76961 ± 5195 d.p.m./well to 66205 ± 1176 d.p.m., whereas 100 nM-TPA reduced PtdCho from 80802 ± 363 to 75735 ± 1926 d.p.m. (results are from single typical experiments where $n = 3$ or 4); no change was observed in the radioactivity associated with sphingomyelin in response to stimulation with either bombesin or TPA.

Table 2. Bombesin- and TPA-stimulated [^{3}H]Cho generation in normal and C-kinase down-regulated cells

Swiss 3T3 cells were labelled with [^{3}H]Cho for 48 h, as described in the legend to Fig. 3, in the presence or absence of 400 nM-TPA. After washing, the cells were stimulated with 617 nM-bombesin or 100 nM-TPA as described and the amount of [^{3}H]Cho generated was normalized to the labelling of the lipids. Results are means ± S.D. for a single typical experiment where $n = 3$ in each case. The stimulated values in the down-regulated cells are not significantly different from the appropriate controls as determined by Student's *t*-test. Other details are as in the legend to Fig. 3.

TPA pretreatment	Stimulation	^{3}H in Cho fraction (d.p.m)
−	None	2776 ± 396
−	Bombesin, 2 min	3682 ± 398
−	TPA, 15 min	5203 ± 231
−	None, 15 min	3273 ± 358
+	None	2238 ± 186
+	Bombesin, 2 min	2572 ± 176
+	TPA, 15 min	2740 ± 214
+	None, 15 min	2660 ± 197

DISCUSSION

The analysis of PtdCho turnover has been hampered by the lack of a suitable assay method. Much of the work in the literature has used variations of the t.l.c. separation method of Yavin (1976) which is time-consuming and in our hands often unreliable in separating Cho from Cho*P*. Many studies (e.g. Muir & Murray, 1987; Welsh *et al.*, 1988) have simply measured the radioactivity associated with the total water-soluble metabolites without identifying the primary product and therefore the route of hydrolysis; an important point if PtdCho is being viewed as a potential source of DAG. Recently, an ion-exchange separation of the Cho metabolites was described by Liscovitch *et al.* (1987), but this procedure involved the use of h.p.l.c. The method we describe in this paper is quick and reproducible and yields quantitative separation and recovery of Cho, Cho*P* and Gro*P*Cho. We cannot say if the method will also separate betaine, but in the cell system we have examined this metabolite is such a minor component that it did not interfere with the analysis. Similar extremely low levels of betaine have also been observed in Swiss 3T3 cells (Warden & Friedkin, 1984), NG108-15 cells (Liscovitch *et al.*, 1987) and brain cells (Yavin, 1976). The analysis of PtdCho breakdown by this Dowex ion-exchange method should therefore be applicable to a wide range of cell types.

Stimulation of Swiss 3T3 cells with bombesin and TPA resulted in the release of Cho-containing metabolites from PtdCho but not from sphingomyelin, the other major Cho-containing lipid in the cells (see the Results section). Stimulation of the cells with maximal concentrations of either bombesin or TPA caused the loss of only about 5% of the label from the total PtdCho fraction. This is in agreement with the finding of Liscovitch *et al.* (1987) using TPA-stimulated NG108-15 cells and is probably due to the relatively small generation of Cho (see e.g. Fig. 4) as compared with the large size of the cellular PtdCho pool.

The breakdown of PtdCho to generate [^{3}H]Cho in response to bombesin stimulation was both rapid and dose dependent (Figs. 4 and 5*a*); the EC_{50} obtained (approx. 2 nM) is of a similar order to that obtained for bombesin-stimulated incorporation of [^{32}P]P_i into PtdCho in Swiss 3T3 cells (Muir & Murray, 1987). The data presented in Figs. 4 and 6 strongly suggest that the primary water-soluble product generated in response to stimulation with both bombesin and TPA was Cho, with an increase in Cho*P* only being observed at later time points. Thus it appears that the generation of Cho in these cells occurs as a consequence of phospholipase D-catalysed breakdown of PtdCho.

The slower increase in Cho*P* in response to bombesin may be due to the activation of a PtdCho phospholipase C enzyme, but could also be due to phosphorylation of liberated Cho by choline kinase. The ratio of Cho to Cho*P* in equilibrium-labelled resting cells is of the order of 1:5 or 6 (see Figs. 4 and 6). Phosphorylation by choline kinase following a rapid doubling in the level of free Cho as seen in Fig. 4 might simply reflect the activation of the PtdCho synthetic pathway in order to replenish the parent lipid. We are at present unable to predict which of these two sources of Cho*P* is the more likely, due to the lack of selective inhibitors of choline kinase and the difficulties of comparing the inter-relationships between the Cho and Cho*P* pools in a stimulated cells system. Indeed a 2-fold increase in choline kinase activity and PtdCho biosynthesis have been shown to follow stimulation of Swiss 3T3 cells with TPA and serum (Warden & Friedkin, 1984, 1985). The metabolic fate of liberated Cho is further complicated by the exit of [^{3}H]Cho from the cell. Fig. 3 demonstrates that the level of cell-associated Cho plateaus after 2 min, whereas the [^{3}H]Cho associated with both the cells and the incubation medium continues to increase (Fig. 4). A similar plateau in cell-associated [^{3}H]Cho was observed in TPA-stimulated cells (results not shown). Therefore, whilst some of the liberated Cho is presumably phosphorylated and reincorporated into PtdCho, some equilibrates with the extracellular medium.

Since the intracellular [^{3}H]Cho level rises to a new steady state level, which is maintained over at least 30 min even though the activity of choline kinase is high in Swiss 3T3 cells (Warden & Friedkin, 1984), sustained PtdCho hydrolysis in both bombesin-and TPA-stimulated cells is implied. This is in contrast to the rapidly densensitized bombesin-stimulated generation of inositol phosphates in the same cell line (Brown *et al.*, 1987). The rate of Cho generation at this new 'steady state' level is essentially the same in both the bombesin- and the TPA-stimulated cell, which would suggest a common mechanism of PtdCho breakdown caused by the two stimulants.

Bombesin stimulation causes the sustained generation of DAG in Swiss 3T3 cells (S. J. Cook & M. J. O. Wakelam, unpublished work) and has previously been shown to stimulate the incorporation of [^{32}P]P_i into PtdCho, but with a lag of 20 min (Muir & Murray, 1987). Our results strongly suggest that bombesin stimulates breakdown of PtdCho in the first instance by the activation of phospholipase D, on the basis that the Cho concentration is elevated between 10 and 30 s after stimulation, whilst Cho*P* always appears later. Receptor-regulated phospholipase D activity has been proposed to account for PtdCho hydrolysis in vasopressin-stimulated hepatocytes (Bocckino *et al.*, 1987) and REF-52 cells (Cabot *et al.*, 1988) and in chemotactic-peptide-stimulated HL-60 cells (Pai *et al.*, 1988). Therefore this mechanism appears to operate in many cell types. However, this does not rule out the possibility that phospholipase D activity is being stimulated by the increase in intracellular free Ca^{2+} concentration, or by the stimulation of protein kinase C activity in response to bombesin-stimulated inositol phospholipid hydrolysis.

Stimulation of PtdCho hydrolysis by tumour-promoting phorbol esters has been described in a number of systems (see Pelech & Vance, 1989) and has been tentatively proposed to be involved in providing a second phase of increased DAG levels, through the mediation of protein kinase C (Muir & Murray, 1987). The results presented here suggest that treatment of Swiss 3T3 cells with TPA can elicit rapid breakdown of PtdCho, apparently by the stimulation of phospholipase D activity. That this effect of TPA is mediated by increased C-kinase activity is supported by the lack of effect of the inactive control, β-phorbol. The EC_{50} for the effect of TPA upon [^{3}H]Cho production (approx. 3 nM) is in very close agreement with that observed for the activation of rat brain C kinase (Castagna *et al.*, 1982), the generation of DAG (Takuwa *et al.*, 1987) and the inhibition of bombesin-stimulated inositol phosphate production in Swiss 3T3 cells (Brown *et al.*, 1987). Consequently, activation of C-kinase in Swiss 3T3 cells may lead both to the desensitization of PtdIns(4,5)P_2 hydrolysis and to the breakdown of PtdCho, thus potentiating its own activation whilst reducing the increase in intracellular free Ca^{2+} concentration. Thus it is possible that the stimulation of PtdCho breakdown in response to bombesin is activated by the increase in C-kinase activity as a consequence of PtdIns(4,5)P_2 breakdown rather than being a direct receptor-activated event.

In order to address this possibility, experiments were performed upon cells treated with 400 nM-TPA for 48 h to down-regulate protein kinase C activity. The almost complete abolition of TPA-stimulated [^{3}H]Cho generation in the down-regulated cells (Table 2) confirms the results with β-phorbol, that the phorbol ester exerted its effect via the activation of C-kinase. Additionally, the ability of bombesin to stimulate [^{3}H]Cho generation was almost completely abolished in the down-regulated cells (Table 2). Similar, though less substantial, inhibitions were observed in cells treated with the putative C-kinase inhibitor staurosporine. Therefore, it appears, at least in this cell line, that agonist-stimulated phospholipase D activity is secondary to the activation of protein kinase C.

Our conclusions differ in the precise hydrolytic pathway of PtdCho breakdown from those of Muir & Murray (1987) and Takuwa *et al.* (1987) working with the same cell line; however, the EC_{50} values obtained suggest that the same phenomenon was being analysed. This anomaly can be explained in a number of ways. Firstly, Muir & Murray (1987) examined the incorporation of [^{32}P]P_i into PtdCho, yet this is a secondary event which probably reflects compensatory resynthesis of the lipid and is thus a less sensitive assay of PtdCho breakdown. In determining the time course of release of Cho metabolites from [^{3}H]Cho-labelled cells in response to bombesin (Muir & Murray, 1987) and TPA (Takuwa *et al.*, 1987), no resolution of the individual Cho metabolites was performed. In addition, the earliest time point measured

by Muir & Murray (1987) was 20 min following stimulation with either bombesin or TPA; this would be too late to have observed agonist-stimulated breakdown of PtdCho (see Fig. 3). Furthermore, after 20 min of treatment with either agent, the radioactivity associated with Cho*P* is elevated (Figs. 3 and 5), and while the fold increase may not be as large as that in Cho, the increase in absolute radioactivity may be equivalent, as a consequence of the larger Cho*P* pool size. Therefore this could lead to some confusion as to which metabolite is the initial product. Takuwa *et al.* (1987) have demonstrated that TPA treatment of Swiss 3T3 cells stimulates an increase in DAG levels, an effect they attribute to phospholipase C-catalysed cleavage of PtdCho, since they did not observe an increase in the mass of phosphatidic acid (PtdOH). However, rapid conversion of PtdCho-derived PtdOH to DAG has been demonstrated (Martin, 1988), and this may account for the discrepancy.

In summary, these results suggest that bombesin, a mitogen known to elicit rapid PtdIns(4,5)P_2 breakdown in Swiss 3T3 cells, is also capable of stimulating rapid hydrolysis of PtdCho, apparently by a C-kinase-stimulated phospholipase D-catalysed mechanism. To our knowledge no second-messenger-like functions have been reported for Cho; indeed, it is unlikely that such an abundant metabolite could serve such a role, but the functions and metabolic fate of the generated PtdOH in plasma membranes appear to be diverse. PtdOH has been proposed to function both as a growth factor (Moolenaar *et al.*, 1986) and as a Ca^{2+} ionophore (see e.g. Putney *et al.*, 1980). In addition, PtdOH can be converted to DAG by the action of PtdOH phosphohydrolase (Martin, 1988). This event may be relevant to the elevation of DAG and activation of C kinase in long-term responses of cells to stimuli such as growth factors, since the agonist-stimulated PtdIns(4,5)P_2 hydrolysis is rapidly desensitized (Brown *et al.*, 1987).

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