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# Stimulation of choline release from NG108-15 cells by 12-*O*-tetradecanoylphorbol 13-acetate

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The effects of the potent tumour-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) on phosphatidylcholine (PtdCho) metabolism were investigated in the neuroblastoma × glioma hybrid cell line NG108-15. TPA (100 nM) stimulated by 150–200% the release into the medium of $^3$H radioactivity from cells that had been pre-labelled with [$^3$H]choline. H.p.l.c. analysis of the medium revealed that TPA stimulated the release of only free [$^3$H]choline (212±11% of control), without affecting such other labelled metabolites as [$^3$H]phosphocholine and [$^3$H]glycerophosphocholine. This effect was concentration-dependent, with a half-maximal effect obtained at 27.5±6.8 nM, and was observable as early as 5–10 min after exposure to TPA. The TPA-induced release of [$^3$H]choline into the medium was accompanied by a small and variable decrease in cellular [$^3$H]PtdCho (to 93±4% of control). However, the radioactivity associated with water-soluble cellular choline metabolites (mainly [$^3$H]phosphocholine and [$^3$H]glycerophosphocholine) remained unchanged. TPA also stimulated the release of [$^3$H]choline derived from [$^3$H]PtdCho that had been produced via the methylation pathway from [$^3$H]methionine. These data suggest that phosphatidylcholine may serve as the source of free choline released from the cells in response to TPA. The possible enzymic mechanisms underlying this response are discussed.

## INTRODUCTION

Phorbol esters, a group of diterpenoid tetracyclic compounds isolated from croton oil, exhibit potent tumour-promoting (co-carcinogenic) activity in mouse skin [1], but also induce a wide variety of cellular responses in other cell types (reviewed in [2] and [3]). Castagna *et al.* [4] have presented evidence that the first step in the mechanism by which phorbol esters act involves activation of protein kinase C, a $Ca^{2+}$- and phospholipid-dependent enzyme that is normally activated by diacylglycerol [5]. However, more recent evidence suggest that activation of protein kinase C alone cannot account for all cellular responses to phorbol esters [6,7].

Phosphatidylcholine (PtdCho), the most prevalent lipid present in mammalian cells, serves an important structural function as a constituent of the membrane lipid bilayer. In addition, a growing body of evidence indicates that at least a fraction of total cellular PtdCho represents a rapidly turning-over and metabolically responsive pool (e.g. [8]–[11]). Indeed, among the earliest metabolic responses to phorbol esters is a rapid and quite selective stimulation of PtdCho synthesis ([12]–[15]). Other studies have shown that this increase in the incorporation of [$^3$H]choline into PtdCho is preceded by the release of radioactivity from cells that had been pre-labelled with [$^3$H]choline [16,17]. However, the identities of the released substances as well as their sources were not clearly established. In the present study we have utilized a newly developed h.p.l.c. procedure for the analysis of water-soluble choline metabolites [18] to identify the compounds released from pre-labelled NG108-15 cells in response to the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Parts of this study have previously been published in abstract form [19].

## MATERIALS AND METHODS

### Materials

[*methyl*-$^3$H]Choline chloride (80 Ci/mmol), [*methyl*-$^{14}$C]CDP-choline (42 Ci/mol), [*acetyl*-$^{14}$C]acetylcholine iodide (2.3 Ci/mol) and [$^3$H]methionine (12 Ci/mmol) were purchased from New England Nuclear Co., Boston, MA, U.S.A. [$^{14}$C]Ethanolamine hydrochloride (95 Ci/mol) was obtained from ICN Radiochemicals, Irvine, CA, U.S.A. [$^3$H]Glycerophosphocholine, [$^3$H]betaine and [$^3$H]phosphocholine were prepared in our laboratory as previously described [18]. Levamisole and choline oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dimethylethanolamine, tetraphenylboron and heptan-3-one were obtained from Aldrich Co., Milwaukee, WI, U.S.A. Phorbol esters were purchased from Pharmacia P-L Biochemicals (Piscataway, NJ, U.S.A.) and Sigma. Stock solutions of phorbol esters (10 mM in $Me_2SO$) were kept frozen at −20 °C.

### Cell culture

NG108-15, a neuroblastoma × glioma hybrid cell line, was kindly provided by Dr. M. Nirenberg, National

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; $EC_{50}$, concentration causing a half-maximal effect; FCS, fetal-calf serum; $Me_2SO$, dimethyl sulphoxide; PtdCho, phosphatidylcholine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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Institutes of Health, Bethesda, MD. U.S.A. Cells (passages 17–30) were cultured in Falcon 200 ml tissue-culture flasks under a humidified atmosphere of air/$CO_2$ (9:1) in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, U.S.A.) containing 100 $\mu$M-hypoxanthine, 1 $\mu$M-aminopterine, 16 $\mu$M-thymidine (all from Sigma) and 5% fetal-calf serum (FCS; from GIBCO). For experiments, cells were subcultured in Falcon six-well (35 mm diam.) plates at a concentration of $2.5 \times 10^4$ cells/ml. Routinely, media were changed 3–4 days after plating and daily thereafter, and experiments were performed on the subconfluent and undifferentiated cultures on days 4–6 inclusive. In cells supplemented with dimethylethanolamine, medium was replaced with DMEM containing 2 $\mu$M-choline, 2 mM-dimethylethanolamine and 5% FCS (without hypoxanthine, aminopterin and thymidine) on days 4 and 5, and experiments were performed on day 6.

### Assay of metabolites released from pre-labelled cells

Unless otherwise indicated, cells were pre-labelled by incubation with [$^3$H]choline (10 $\mu$Ci/ml per well; 125 nM) for 4 h at 37 °C, in a choline-free chemically defined N2 medium [20]. The cells were then rinsed twice with warm (37 °C) DMEM (2 ml/well) and incubated for 1 h in growth medium in the presence of the test substance. Media were collected and centrifuged (10 s in a Beckman Microfuge) to precipitate detached cells and debris. Routinely, the [$^3$H]choline in the medium was extracted into tetraphenylboron [1.5% (w/v) in heptan-3-one] essentially as described [21]. All of the radioactivity thus extracted was identified by h.p.l.c. as [$^3$H]choline. For h.p.l.c. analysis of unextracted medium, a sample of the supernatant fluid (450 $\mu$l) was acidified with 1 M-HCl (50 $\mu$l) and stored frozen at −15 °C.

### Cell extraction

After removal of the media, ice-cold methanol (0.7 ml) was added to each well, and the cells were scraped from the plates and sonicated with a Heat Systems sonicator (model 225R; setting 3, ten pulses). Chloroform (2 vol.) and water (1 vol.) were added and vortex-mixed for 10 s. The upper phase, containing the water-soluble choline metabolites, and the lower phase, containing choline glycerophospholipids, were allowed to separate; they were then collected, dried by centrifugation under a vacuum and stored at 4 °C for further analysis. Radioactivity was determined by liquid-scintillation spectrometry in a Beckman LS-7500 spectrometer, and protein was determined in samples of the total sonicated cell preparation as described by Lowry *et al.* [22]. Protein content was unaffected by TPA treatment.

### Analysis of labelled choline metabolites

Labelled water-soluble choline metabolites present in media and cell extracts were analysed by h.p.l.c. on a normal-phase silica column, as described in detail elsewhere [18]. In some experiments the method was modified by substituting ammonium acetate for sodium acetate, to give a completely volatile mobile phase. Briefly, two buffers were used, A (acetonitrile/water/ethanol/acetic acid/0.83 M-ammonium acetate, 800:127:68:2:3, by vol.) and B (same components, 400:400:68:53:79, by vol.). A linear gradient from 0 to 100% buffer B (slope = 5%/min) was started 15 min after injection. Flow rate was 2.7 ml/min and column temperature was 45 °C. The retention times of most metabolites were not significantly affected by this modification, except for that of phosphocholine, which was retarded, being eluted at 44–45 min. Metabolically labelled peaks were routinely identified by comparing their retention times with those of standards chromatographed under identical conditions. T.l.c. of the phospholipid fraction was performed on LK6D plates (Whatman, Clifton, NJ, U.S.A.) with a mobile phase of chloroform/methanol/propan-2-ol/0.25% KCl/triethylamine (30:9:25:6:18, by vol.) as described [23].

## RESULTS AND DISCUSSION

### Phorbol esters stimulate [$^3$H]choline release from cells pre-labelled with [$^3$H]choline

In preliminary experiments [19] we observed that, as previously described for rat embryo fibroblasts [16] and HeLa cells [17], TPA treatment caused neural-derived NG108-15 cells that had been pre-labelled with [$^3$H]choline to release increased amounts of radioactivity into their media. As shown in Fig. 1(*a*), the radioactivity in such medium samples consists primarily of [$^3$H]choline, [$^3$H]glycerophosphocholine and [$^3$H]phosphocholine (a peak eluted at the position of a betaine standard, as well as two unidentified peaks eluted just before and after it, were also detectable.) TPA (100 nM) more than doubled the release of free [$^3$H]choline into the medium (Fig. 1*a*);

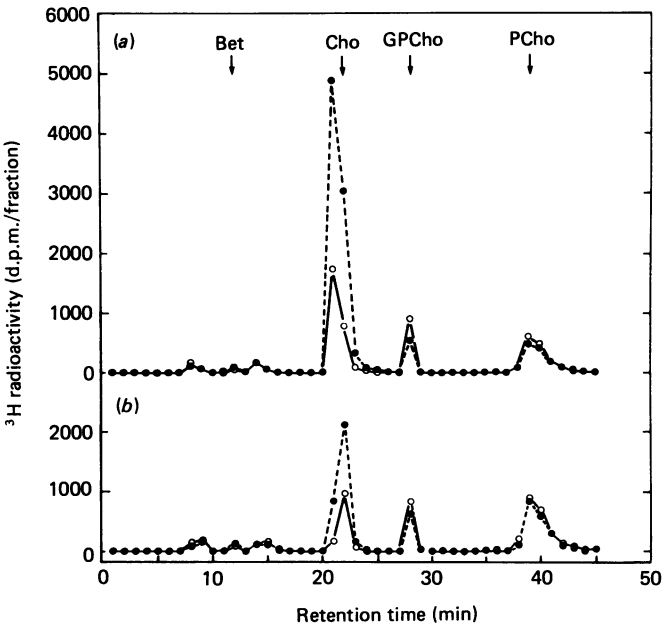


**Fig. 1. H.p.l.c. analysis of media from cells that had been pre-labelled with [$^3$H]choline and challenged with TPA**

Cells were pre-labelled with [$^3$H]choline and then incubated with either TPA (100 nM) or vehicle (0.01% $Me_2SO$) in the absence (*a*) or presence (*b*) of levamisole (1 mM). Samples (20 $\mu$l) of acidified medium were separated by h.p.l.c. as detailed in the Materials and methods section. Representative radiochromatograms are presented from experiments performed in triplicate. Arrows indicate the positions at which betaine (Bet), choline (Cho), glycerophosphocholine (GPCho) and phosphocholine (PCho) standards are eluted. ○, $Me_2SO$; ●, TPA.

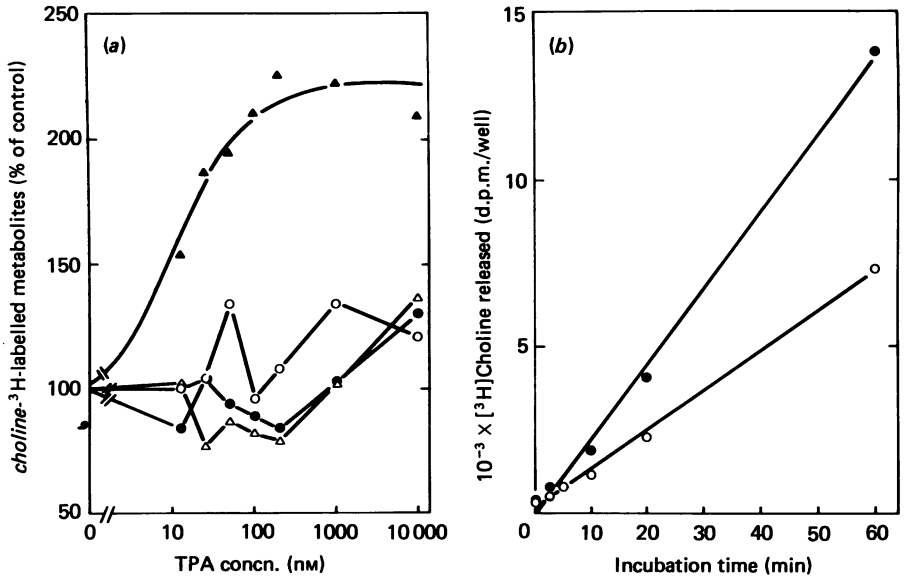


**Fig. 2. Dependence of [$^3$H]choline release from pre-labelled cells on TPA concentration (*a*) and on time of incubation (*b*)**

(*a*) Cells were pre-labelled with [$^3$H]choline and then incubated with the indicated concentrations of TPA. Samples (20 $\mu$l) of acidified medium were separated by h.p.l.c. as detailed in the Materials and methods section, and the radioactivity associated with each metabolite was determined from radiochromatograms like those shown in Fig. 1: ▲, choline; △, glycerophosphocholine; ●, phosphocholine; ○, betaine. Each point represent the mean of triplicate wells. (*b*) Cells were pre-labelled with [$^3$H]choline (1 $\mu$Ci/ml per well) and then incubated with either TPA (100 nM) or vehicle (0.01% $Me_2SO$) for the indicated periods of time. Media were extracted with tetraphenylboron, and samples (250 $\mu$l) were then counted for radioactivity. Each point represents the mean of triplicate wells from a representative experiment. ○, $Me_2SO$; ●, TPA.

in contrast, the release of the other water-soluble metabolites was not significantly affected by TPA, indicating that its effect on choline release was not due to a non-selective increase in membrane permeability.

In previous studies [16,17] it was suggested that TPA increased the release of both [$^3$H]choline and [$^3$H]phosphocholine into the medium. It therefore seemed possible that TPA in our experiments had also stimulated phosphocholine release, but that the phosphocholine was immediately degraded to choline by cell-surface alkaline phosphatases. We examined this possibility by stimulating cells with TPA in the presence of levamisole (1 mM), an inhibitor of alkaline phosphatase [24]. At this concentration levamisole inhibited by 85% the degradation of exogenous phosphocholine by intact NG108-15 cells (results not shown). In the presence of levamisole, but not TPA, concentrations of [$^3$H]choline in the medium decreased, but those of [$^3$H]phosphocholine increased (compare the $Me_2SO$-control chromatogram in Fig. 1*a* with that in Fig. 1*b*), indicating that the drug did indeed inhibit phosphatase activity. However, despite the presence of levamisole, the release of phosphocholine was not stimulated by TPA (Fig. 1*b*). In contrast, [$^3$H]choline release was also stimulated by TPA in the presence of levamisole, though to a lesser extent. The attenuation of the response may be due to non-specific inhibition of the activity of enzymes releasing choline from the cells. These results indicate that it is choline, rather than phosphocholine, whose release is stimulated by TPA from the NG108-15 cells. The apparent disagreement between this conclusion and that of previous studies could reflect the use of different techniques for analysis of choline metabolites (paper chromatography [16] and t.l.c. [17] rather than h.p.l.c.), or differences in the cell lines examined.

### Characteristics of TPA-induced [$^3$H]choline release

The effect of TPA on [$^3$H]choline release was concentration-dependent and saturable (Fig. 2*a*), reaching a maximum of 212±11% (mean±S.E.M., $n = 5$) of control. The TPA concentration causing a half-maximal effect ($EC_{50}$) was 27.6±6.8 nM (mean±S.E.M., $n = 5$). At very high concentrations (e.g. 10 $\mu$M), TPA also increased the release of other water-soluble choline metabolites, probably reflecting its cytotoxicity at these high concentrations [25]. An effect of TPA (100 nM) on [$^3$H]choline release was evident after an apparent lag period of 2.5–5 min and persisted for at least 60 min (Fig. 2*b*). 4$\beta$-Phorbol 12,13-didecanoate, another biologically active phorbol ester, had a similar effect, but its non-tumour-promoting stereoisomer 4$\alpha$-phorbol 12,13-didecanoate was without effect (results not shown).

### TPA also stimulates ethanolamine release

TPA treatment also augmented the release of a radioactive substance exhibiting the retention time of [$^{14}$C]ethanolamine (13 min) from cells that had been pre-labelled with [$^{14}$C]ethanolamine (Fig. 3). No change was noted in the amount of radioactivity of a peak that was eluted at 31 min and identified as phosphoethanolamine by its susceptibility to alkaline phosphatase digestion. Preliminary studies indicate that TPA does not affect the release of radioactivity from cells that had been pre-labelled with [$^{14}$C]serine.

### Effect of TPA on labelled cell-associated choline metabolites

To identify the source of [$^3$H]choline released into the medium in response to TPA, we analysed lipid- and water-soluble extracts from cells that had been pre-

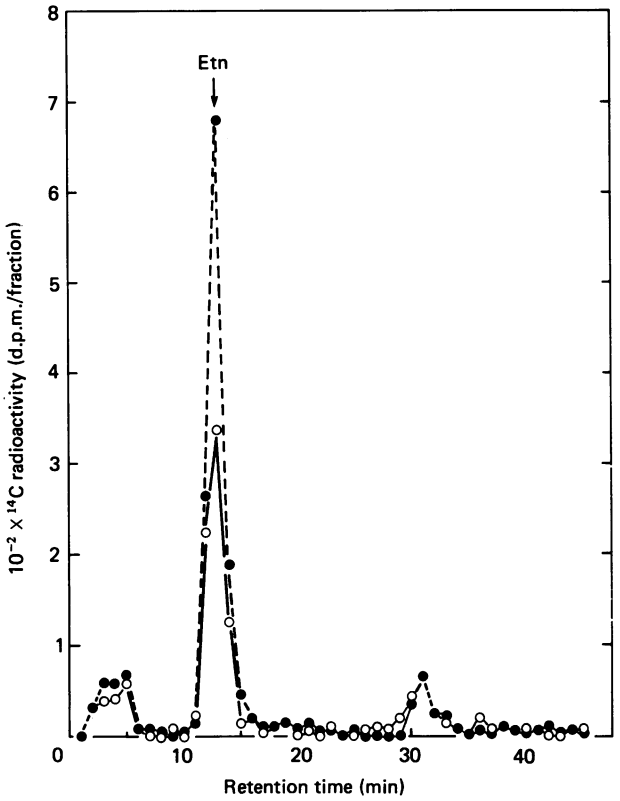


**Fig. 3. H.p.l.c. analysis of media from cells that had been pre-labelled with [$^{14}$C]ethanolamine and challenged with TPA**

Cells were pre-labelled with [$^3$H]ethanolamine (1 $\mu$Ci/ml per well) and then incubated with either TPA (100 nM) or vehicle (0.01% $Me_2SO$). Samples (20 $\mu$l) of acidified medium were separated by h.p.l.c. as detailed in the Materials and methods section. Representative radiochromatograms are presented from experiments performed in triplicates. The arrow indicates the position at which the ethanolamine (Etn) standard is eluted. ○, $Me_2SO$; ●, TPA.

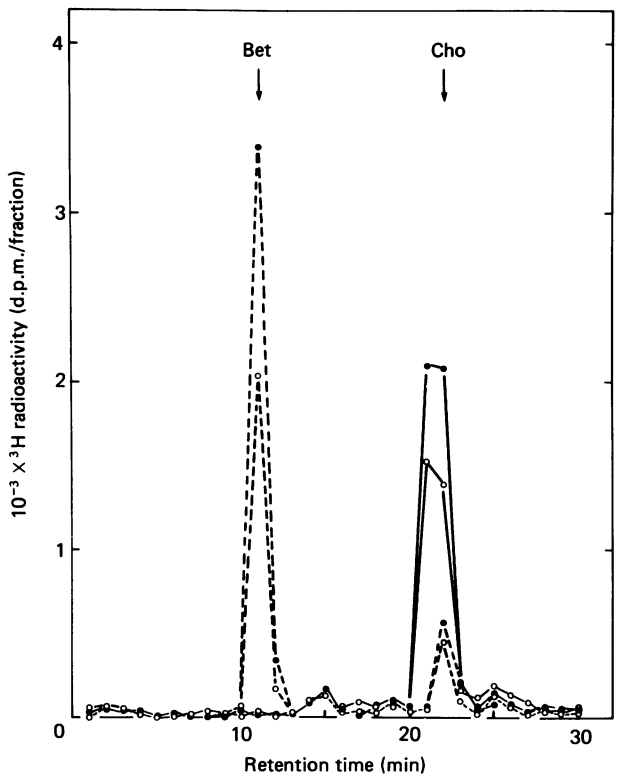


**Fig. 4. Effect of TPA on [$^3$H]choline release from cells pre-labelled with [$^3$H]methionine**

Cells were grown for 40–48 h in modified medium containing dimethylethanolamine (see the Materials and methods section) before being pre-labelled with [$^3$H]methionine (10 $\mu$Ci/ml per well) for 4 h in a dimethylethanolamine-containing medium without unlabelled methionine. Cells were then incubated in the usual growth medium with TPA (100 nM) or vehicle (0.01% $Me_2SO$) for 1 h, and media were collected and extracted with tetraphenylboron. Triplicate extracts from control and TPA-treated cells were combined in 80 $\mu$l of 0.1 M-sodium phosphate buffer (pH 8.0) containing 2 mM-choline chloride, and portions thereof were incubated without (——) or with (----) choline oxidase (1 unit) for 2 h at 37 °C. The reaction was terminated by boiling for 3 min, and the mixture was filtered and separated by h.p.l.c. Radiochromatograms obtained in a representative experiment are presented. Arrows indicate the positions at which betaine (Bet) and choline (Cho) standards are eluted. ○, $Me_2SO$; ●, TPA.

labelled with [$^3$H]choline and then challenged with TPA (Table 1). [$^3$H]Acetylcholine and [$^3$H]CDP-choline were undetectable; the cellular pools of $^3$H-labelled betaine, choline, lysoPtdCho and sphingomyelin were too small to account for the stimulation of choline release by TPA, and moreover were not significantly affected by TPA treatment. Similarly, TPA did not affect the amounts of radioactivity present as [$^3$H]glycerophosphocholine or [$^3$H]phosphocholine. TPA did, however, cause a small and variable decrease in cellular concentrations of [$^3$H]PtdCho (to 93 ± 4% of control; mean ± S.E.M., $n = 6$), suggesting that it may indeed release [$^3$H]choline by stimulating the hydrolysis of a small, responsive, pool of membrane PtdCho. TPA did not affect the rate of [$^3$H]choline uptake in NG108-15 cells (M. Liscovitch, J. K. Blusztajn, A. Freese & R. J. Wurtman, unpublished work).

### TPA stimulates [$^3$H]choline release from cells pre-labelled with [$^3$H]methionine

Neurons are able to convert methione into *S*-adenosylmethionine and use this substance as a donor of methyl groups for the sequential methylation of phosphatidylethanolamine to phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine and finally to PtdCho [26,27]. Incubation of NG108-15 cells with [$^3$H]methionine resulted in the formation, via this pathway, of [$^3$H]choline-labelled PtdCho. To increase the yield of [$^3$H]PtdCho, we preincubated the cells for 40–48 h with dimethylethanolamine (2 mM); this compound is incorporated into membrane phosphatidyldimethylethanolamine (cf. [28]), which is readily transformed to [$^3$H]PtdCho by a single methylation step. H.p.l.c. analysis of media extracted for choline (Fig. 4) revealed that the extracted radioactivity was confined within a single peak exhibiting the retention time of choline (21–22 min); it was also increased by TPA. To identify

**Table 1. Effect of TPA on choline release and cell-associated choline metabolites**

Cells pre-labelled with [$^3$H]choline (1 $\mu$Ci/ml per well) were incubated with either TPA (100 nM) or its vehicle (0.01% $Me_2SO$). Media were collected, centrifuged and extracted with tetraphenylboron. Choline metabolites were extracted from the cells as detailed in the Materials and methods section. Protein content of the cells was 586±31 and 654±47 $\mu$g/well (mean±S.D.) in control and TPA-treated cells respectively. Results are expressed as d.p.m./$\mu$g of protein, and means±S.D. for triplicate wells in a representative experiment are presented. Abbreviation: N.D., not detectable.

| Fraction | $^3$H-labelled metabolite | Treatment... | Radioactivity (d.p.m./$\mu$g of protein) $Me_2SO$ (0.01%) | TPA (100 nM) |
|---|---|---|---|---|
| Medium | Choline | | 22±2 | 48±2 |
| Water-soluble | Betaine | | 2±0.3 | 1.6±0.3 |
| | Acetylcholine | | N.D. | N.D. |
| | Choline | | 8±3 | 9±3 |
| | Glycerophosphocholine | | 125±9 | 130±6 |
| | CDP-choline | | N.D. | N.D. |
| | Phosphocholine | | 116±2 | 118±6 |
| | Other | | 16±1 | 13±1 |
| Phospholipid | PtdCho | | 856±14 | 727±32 |
| | LysoPtdCho | | 9±1 | 7±2 |
| | Spingomyelin | | 14±3 | 11±2 |

this peak further as choline, samples of these extracts were incubated with choline oxidase (which converts choline into betaine in the presence of molecular $O_2$). Most of the radioactivity (80–85%) now exhibited the retention time of betaine (11 min) and hence appears to represent authentic choline. Indeed, TPA caused an increase in this peak while not significantly affecting the minor radioactive fraction whose retention time was not affected by choline oxidase (Fig. 4). Since the incubation of authentic [$^3$H]choline with choline oxidase caused its total (>99%) conversion into betaine, it appears that the contaminating peak is in fact a distinct compound and not merely un-oxidized choline. This conclusion is supported by its unresponsiveness to TPA, as well as by the presence of a large peak of similar properties in extracts of the [$^3$H]methionine-labelled cells themselves (results not shown).

## How is choline release stimulated by TPA?

The amount of choline released by TPA is very small (less than 5% of the total pool size of [$^3$H]PtdCho), making difficult the detection of a corresponding decrease in cellular [$^3$H]PtdCho. Nevertheless, a small and variable decrease in [$^3$H]PtdCho (range 4–15%) did occur in most (5 of 6) experiments. In contrast, [$^3$H]phosphocholine and [$^3$H]glycerophosphocholine were not decreased by TPA, even though they were much less than [$^3$H]PtdCho and therefore more likely to be affected had they been the source of the released [$^3$H]choline. It is thus highly probable that TPA stimulates the release of choline from PtdCho. This conclusion is strongly supported by the experiments in which phospholipids were labelled by [$^3$H]methionine. These experiments confirmed [29] that free [$^3$H]choline can be formed in neurons from [$^3$H]methionine via phospholipid methylation, and further showed that TPA stimulates the release of [$^3$H]choline thus formed. Since this [$^3$H]choline must be derived from [$^3$H]PtdCho, these data are consistent with the hypothesis that TPA stimulates a phospholipase D-mediated hydrolysis of PtdCho (although other enzymic mechanisms, e.g. homologous choline–choline base exchange, cannot be excluded at present). The presence of phospholipase D activity in rat brain synaptosomes has been demonstrated [30]. Although phorbol esters may interact directly with this enzyme, they could also modulate its activity by activating protein kinase C. If stimulation of choline release by TPA is indeed dependent on activation of protein kinase C, then various effector substances may be expected to do the same by receptor-mediated activation of this kinase. Regardless of the mode by which the hypothetical phospholipase is activated, the ensuing changes in the local lipid composition of the membrane may modulate the activities of proteins (e.g. receptors, transporters, and enzymes, such as protein kinase C) whose activities depend on their lipid microenvironment. It is also possible that such lipid products of PtdCho breakdown as phosphatidic acid, an intermediate in phosphoinositide synthesis, may feed into the phosphoinositide cycle, since various stimuli can concurrently affect the metabolism of both PtdCho and phosphoinositides [9–11]. It should be noted, however, that NG108-15 is a hybrid of two tumour cell lines, and therefore its response to TPA may be different from that of normal cells. The possible involvement of choline release in the effects of phorbol esters on cell growth and differentiation awaits clarification.

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