Phorbol ester stimulates choline uptake in Swiss 3T3 fibroblasts following introduction of the gene encoding protein kinase C α

Barbara E. SLACK,* $\ddagger$ Jeffrey BREU,* Etta LIVNEH, $\dagger$ Hagit ELDAR $\dagger$ and Richard J. WURTMAN*

*Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Bldg. E25-604, Cambridge, MA 02139, U.S.A.,

and $\dagger$ Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Phorbol 12-myristate 13-acetate (PMA) stimulated radiolabelled choline uptake and incorporation into phosphatidylcholine (PtdCho) in a time- and concentration-dependent manner in wild-type NIH 3T3 fibroblasts. The accumulation of labelled choline induced by PMA was paralleled by an increase in choline mass. The results implicate protein kinase C (PKC) in the regulation of choline uptake. In order to address the PKC-subtype specificity of this response, a study was undertaken in Swiss 3T3 fibroblast cells, which normally express very low levels of PKC α . A retroviral expression system was used to introduce the genes for PKC α and neomycin resistance (used for selection) into the cells. Two resulting lines expressed PKC α at levels that were 20-fold higher than those found in the control (neomycin-resistant) line, or in the wild-type cells. In control Swiss 3T3

fibroblasts, 1 μ M PMA elevated choline levels by only 30%, whereas, in Swiss 3T3 cell lines that stably over-expressed PKC α , PMA caused a 5-fold enhancement in [14 C]choline accumulation. This concentration of PMA significantly increased [14 C]PtdCho levels in both control and PKC α -over-expressing lines, although the effect in the latter was significantly greater. The effects of PMA were inhibited by the PKC antagonist sphingosine. These results implicate PKC α in the regulation of choline accumulation and phospholipid synthesis in fibroblasts. Although additional PKC subtypes appear to participate in the control of PtdCho synthesis in these cells, PMA-stimulated choline uptake in Swiss 3T3 fibroblasts is almost entirely dependent on the presence of PKC α .

INTRODUCTION

Since the recognition that multiple subtypes of protein kinase C (PKC) exist, a great deal of interest has focused on the role played by the different isoenzymes in cell function. Recently, reports have appeared linking PKC α , the most widely distributed of the isoforms (Nishizuka, 1988), to regulation of a variety of transporters, including P-glycoprotein, an energy-dependent pump associated with the multi-drug-resistance phenotype (Blobe et al., 1993), and a calcium transporter (Riedel et al., 1993). In the present study, the role of PKC α in regulation of choline transport was examined in mammalian fibroblasts.

Phorbol esters are direct activators of PKC, and are known to stimulate the incorporation of choline into phosphatidylcholine (PtdCho) in a variety of mammalian cell types (Kreibich et al., 1971; Kinzel et al., 1979; Paddon and Vance, 1980; Pelech et al., 1984; Liscovitch et al., 1987; Kolesnick, 1987; Kolesnick and Paley, 1987; Cook et al., 1989); an effect that is attributable to activation of CTP:phosphocholine cytidyltransferase (Paddon and Vance, 1980; Pelech et al., 1984; Utal et al., 1991), and can be inhibited by treatment with PKC antagonists or prior down-regulation of PKC (Liscovitch et al., 1987; Kolesnick and Paley, 1987; Kolesnick, 1987; Cook et al., 1989). In addition to increasing cytidyltransferase activity, phorbol esters and diacylglycerol (endogenous activators of PKC) have been reported to stimulate choline uptake (Lijian et al., 1988; Cook et al., 1989; Slack et al., 1992; Tronchère et al., 1993), although this effect has been less consistently observed (Paddon and Vance, 1980; Jones and Kent, 1991; Kiss et al., 1991).

In the present paper, the regulation of choline uptake by

activators of PKC was compared in two fibroblast cell lines. In wild-type NIH 3T3 fibroblasts, which normally express high levels of PKC α (Mischak et al., 1993), phorbol 12-myristate 13-acetate (PMA) markedly enhanced choline uptake. PMA did not stimulate choline uptake in control Swiss 3T3 fibroblasts, which express little endogenous PKC α . However, after the introduction into these cells of DNA coding for the PKC α subtype, PMA caused a striking, dose-dependent, increase in the accumulation of choline. The results show that PKC α specifically mediates the stimulation of choline uptake by phorbol esters in mammalian cells induced to over-express this isotype stably. Moreover, they demonstrate that, in cells that normally express comparable levels of PKC α , choline uptake is stimulated in a similar fashion by PKC activators.

MATERIALS AND METHODS

Materials

Media and sera for cell culture, and anti-PKC α polyclonal antibody, were from Gibco BRL (Grand Island, NY, U.S.A.). Sterile plasticware was supplied by Falcon Plastics (Los Angeles, CA, U.S.A.). PMA, dimethyl sulphoxide, sphingosine, protease inhibitors and goat anti-rabbit peroxidase-linked secondary antibody were from Sigma (St. Louis, MO, U.S.A.). 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7) was purchased from Seikagaku America Inc. (Rockville, MD, U.S.A.). PMA was dissolved in dimethyl sulphoxide and kept at -20°C . Sphingosine was dissolved in ethanol, and H-7

in water. Stock solutions of all compounds were further diluted in media immediately before an experiment. Control media always contained the appropriate concentrations of vehicle. [*methyl*- ^{14}C]Choline chloride was from New England Nuclear (Boston, MA, U.S.A.). Materials for SDS/PAGE were from Bio-Rad Laboratories (Richmond, CA, U.S.A.), and polyvinylidene difluoride membranes were from Millipore Corp. (Bedford, MA, U.S.A.).

Cell culture

NIH 3T3 fibroblasts (kindly provided by Dr. Seymour Garte, Department of Environmental Medicine, New York University Medical Center, New York, NY, U.S.A.) were maintained in Dulbecco's modified Eagle Medium (D-MEM) containing 10% horse serum and 2.5% fetal-calf serum. Swiss 3T3 fibroblasts were grown in D-MEM containing 10% calf serum. Growth media were buffered with NaHCO_3 , and cultures were maintained in an incubator at 37°C in an atmosphere of 5% CO_2 . A retroviral expression system was used to introduce the genes for PKC α and neomycin resistance (used for clonal selection) into Swiss 3T3 cells. Two of the resulting lines (designated lines SF1.4 and SF3.2) were used in this study, and compared with a control line (SC1) carrying an integrated vector coding for the neomycin-resistance gene alone (Eldar et al., 1990), and with wild-type Swiss 3T3 fibroblasts. All cells were subcultured on to 60 mm plastic culture dishes before experiments, and grown to confluent density.

Labelling and extraction of PtdCho and metabolites

Cells were labelled with [*methyl*- ^{14}C]choline chloride in N2 medium (Bottenstein and Sato, 1979) from which choline had been omitted. Incubations were carried out in N2 medium buffered with HEPES and maintained at 37°C in room air. PMA or vehicle (0.1% dimethyl sulphoxide) was added to the medium along with labelled choline for 1 h labelling experiments. Alternatively, cells were pretreated for 60 min with PMA or vehicle, and then labelled for 5 min. When antagonists were tested, cells were first pretreated for 30 min with the inhibitor, then incubated with or without PMA in the presence or absence of the inhibitor. The cells were washed twice with phosphate-buffered saline, and extracted with successive additions of chloroform, methanol and water (Slack et al., 1991). Choline-containing phospholipids (in the organic phase) were separated by t.l.c., and their radioactivity was determined by liquid-scintillation counting. Radioactive choline and phosphocholine (in the aqueous phase) were separated by h.p.l.c. and radioactivity was measured with an on-line detector (Berthold Systems, Pittsburgh, PA, U.S.A.). Choline mass in aqueous extracts was analysed by a h.p.l.c. system (Bioanalytical Systems, West Lafayette, IN, U.S.A.) consisting of a polymeric analytical column in series with a choline oxidase column. The reaction product generated by the latter (H_2O_2) was measured by electrochemical detection as described by Slack et al. (1992).

Immunoblotting analysis

Cells were collected in phosphate-buffered saline, sedimented, and the pellet was extracted in a buffer containing 50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 2 mM phenylmethanesulphonyl fluoride, 1 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 0.1 $\mu\text{g}/\text{ml}$ pepstatin, and 1 $\mu\text{g}/\text{ml}$ *N* $^{\alpha}$ -tosyl-lysylchloromethane. The insoluble material was sedimented by centrifugation and the supernatant diluted 1:1 in SDS sample buffer

and separated on 12% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes, which were then blocked in Tris-buffered saline containing 0.05% Tween and 5% Carnation low-fat milk powder, and incubated overnight with a 1:1000 dilution of antibodies to PKC α (Gibco BRL), or a 1:250 dilution of anti-PKC δ (Transduction Laboratories, Lexington, KY, U.S.A.) or anti-PKC ζ (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Membranes were washed, incubated with a horseradish-peroxidase-linked secondary antibody, and the bands were detected by using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, U.S.A.). The resulting bands were quantified by scanning densitometry as described by Slack et al. (1993).

RESULTS

Immunoblot analysis of PKC subtypes in NIH and Swiss 3T3 fibroblast lines

Levels of PKC α were measured by immunoblot in NIH and Swiss 3T3 fibroblasts (Figure 1a). All lines expressed an immunoreactive protein of approx. 80 kDa in size. This band did not appear on membranes incubated with the antibody in the presence of the peptide against which it was raised (results not shown). Scanning densitometric analysis indicated that the level of PKC α in NIH 3T3 cells was approx. 9 times that occurring in control Swiss 3T3 fibroblasts, whereas retroviral introduction of the PKC α gene resulted in a 20-fold increase in PKC α protein relative to control Swiss 3T3 cells. Comparable increases in

Figure 1 Western-blot analysis of PKC subtypes in lysates of NIH 3T3 and/or Swiss 3T3 fibroblast cell lines

(a) Lysates from wild-type NIH 3T3 fibroblasts (NIH; lanes 1, 2), control Swiss fibroblasts (SC1; lanes 3, 4) and Swiss 3T3 fibroblast lines over-expressing the gene for PKC α (SF1.4; lanes 5, 6) were separated on SDS gels, transferred to membranes and probed with an antibody directed against PKC α . PKC δ (b) and PKC ζ (c) in lysates from SC1 (lane 1) and SF1.4 (lane 2) cells. Approximate locations of molecular-size markers (kDa) are shown on the left.

Figure 2 Concentration-dependence of stimulation of [^{14}C]choline uptake by PMA in NIH 3T3 fibroblasts

Cells were pretreated for 55 min with 30 nM PMA in serum-free N2 medium, then labelled for 5 min with [^{14}C]choline (0.5 $\mu\text{Ci}/\text{ml}$; 8.5 μM). Labelled choline and phosphocholine in the aqueous phase were separated and quantified as described in the Materials and methods section. Data represent means $\pm$ S.E.M. from three separate experiments.

PKC α were observed in both SF1.4 (Figure 1a) and SF3.2 (results not shown) over-expressing lines. Swiss 3T3 fibroblasts also express PKC δ , PKC ϵ and PKC ζ (Olivier and Parker, 1992). Immunoblot analysis showed that over-expression of PKC α in SF1.4 cells was not accompanied by increased levels of PKC ϵ (Eldar et al., 1993) or PKC δ (Figure 1b). However, an increase in PKC ζ was observed in SF1.4 cells relative to controls (Figure 1c).

Regulation of choline uptake and metabolism in wild-type NIH 3T3 fibroblasts

The ability of PMA to stimulate choline uptake was first examined in wild-type NIH 3T3 fibroblasts pulse-labelled with [^{14}C]choline for 5 min following pretreatment for 55 min with PMA. The results showed that PMA increased radiolabelled-choline uptake in a dose-dependent fashion (Figure 2). After 5 min of labelling, [^{14}C]phosphocholine levels were approximately double those of labelled choline in untreated cultures, indicating a rapid conversion of choline into phosphocholine. In contrast, in cells pretreated with PMA, labelled-phosphocholine levels were decreased by 50%, whereas choline levels were approx. 5-fold higher with respect to controls. The magnitude of the decrease in phosphocholine was not sufficient to account for the observed increase in choline levels, leading to the conclusion that the increased accumulation of radioactive choline in cells treated with PMA resulted from direct stimulation of choline uptake in these cells (Figure 2).

In order to determine if the striking effect of PMA on [^{14}C]choline uptake reflected an increase in the size of the choline pool, cultures were exposed to various concentrations of PMA for 1 h, in the presence of equivalent concentrations of labelled or unlabelled choline, and [^{14}C]choline levels and choline mass, respectively, were determined. PMA increased choline mass and radioactive-choline levels in a dose-dependent manner. When these two measures were plotted as a function of PMA concentration, the resulting curves were almost superimposable (Figure 3a). In cells labelled for 1 h, measurable amounts of [^{14}C]PtdCho were synthesized from the radioactive precursor phosphocholine. The levels of [^{14}C]PtdCho increased in parallel with labelled choline and choline mass in response to increasing

Figure 3 Effects of PMA on choline mass, [^{14}C]choline levels and [^{14}C]PtdCho synthesis in NIH 3T3 fibroblasts

Cells were incubated with [^{14}C]choline (0.5 $\mu\text{Ci}/\text{ml}$; 8.5 μM), or with an equivalent concentration of unlabelled choline for 1 h in the presence of various concentrations of PMA, and assayed for (a) radioactive choline ($\blacktriangle$) or choline mass ($\bullet$), or (b) [^{14}C]PtdCho content. Data represent means $\pm$ S.E.M. from 4–7 separate experiments.

concentrations of PMA (Figure 3b). The mean estimated EC₅₀ values for PMA's effects on choline mass, [^{14}C]choline content and [^{14}C]PtdCho content were 40, 32 and 36 nM respectively, and were within the same range as the EC₅₀ for PMA-mediated stimulation of choline uptake in cells labelled for 5 min (18 nM; Figure 2).

Regulation of choline uptake and metabolism in Swiss 3T3 fibroblasts stably over-expressing the gene for PKC α and in control fibroblasts

PKC α is the major isoform present in wild-type NIH 3T3 cells (Mischak et al., 1993), raising the possibility that it mediates the observed effect of PMA on choline uptake. In contrast, Swiss 3T3 fibroblasts normally express very low levels of PKC α (Eldar et al., 1990). To test directly the involvement of PKC α in the regulation of choline metabolism, the experiments described above were repeated in two Swiss 3T3 fibroblast cell lines stably over-expressing the genes for PKC α and neomycin resistance (designated lines SF1.4 and SF3.2) and in a control line expressing the neomycin-resistance gene alone (designated SC1). A retroviral expression system was used to introduce the genes for PKC α and/or neomycin resistance (used for clonal selection) into wild-type Swiss 3T3 cells (Eldar et al., 1990). In PKC α -over-expressing cells (SF1.4), PMA increased [^{14}C]choline uptake in a concentration-dependent manner, whereas PMA did not stimulate choline uptake in control (SC1) cells (Figure 4a) or in wild-type Swiss 3T3 fibroblasts (results not shown). As in NIH 3T3 fibroblasts, the increase in choline labelling was paralleled by increases in choline mass (Figure 4a). Moreover, although PMA increased the formation of [^{14}C]PtdCho in both control and PKC α -over-expressing cells, the degree of stimulation was signifi-

Figure 4 Effects of PMA on $[^{14}\text{C}]$ choline, choline mass and $[^{14}\text{C}]\text{PtdCho}$ in Swiss 3T3 fibroblasts

Cells over-expressing the retrovirally introduced PKC α gene (SF1.4, white symbols) or control cells (SC1, black symbols) were incubated for 1 h in $[^{14}\text{C}]$ choline or an equivalent concentration of unlabelled choline in the presence of various concentrations of PMA, then extracted and assayed for (a) choline mass (circles) or radioactive choline (triangles) and (b) radioactive PtdCho. Values for labelled metabolites represent means $\pm$ S.E.M. from four separate experiments. Values for choline mass represent means $\pm$ S.D. from a single experiment performed twice with similar results. Error bars that lie within the boundaries of the symbol are not shown. * $P < 0.05$, values are significantly different from those in corresponding control cultures.

cantly greater in cells that over-expressed PKC α (Figure 4b). The EC $_{50}$ values for the effects of PMA ranged from 22 to 28 nM, and were very similar to those observed in NIH 3T3 fibroblasts. PMA also caused a striking increase in $[^{14}\text{C}]$ choline accumulation in a second PKC α -over-expressing line (SF3.2) (results not shown). Although these results were obtained in cells labelled for 1 h, stimulation of $[^{14}\text{C}]$ choline uptake in cells pretreated with PMA was apparent after only 5 min of labelling, consistent with an effect on choline uptake (results not shown).

Effects of PKC inhibitors on PMA-induced stimulation of choline uptake and PtdCho synthesis

The PKC antagonist sphingosine (100 μM) significantly decreased the stimulation of $[^{14}\text{C}]$ choline uptake and labelled-PtdCho formation in control and PKC α -over-expressing Swiss 3T3 cells (Table 1). Similarly, in NIH 3T3 fibroblasts, sphingosine decreased the stimulation of choline uptake by PMA from 330 ± 52 to $111 \pm 24\%$ of control, and decreased the stimulation of PtdCho labelling by PMA from 454 ± 48 to $75 \pm 14\%$ of control values. The PKC antagonist H-7 was less effective than sphingosine, causing a smaller but significant decrease in the stimulation of choline uptake and PtdCho synthesis by PMA in Swiss 3T3 fibroblasts (results not shown).

Table 1 Effect of sphingosine on stimulation of accumulation of labelled choline and PtdCho

Cells were pretreated with control medium or with medium containing sphingosine (Sph) for 30 min, then labelled with $[^{14}\text{C}]$ choline in the presence of PMA or dimethyl sulphoxide for 1 h with or without sphingosine. Cells were extracted and assayed for radioactive free choline and PtdCho. Values represent means $\pm$ S.E.M. from four separate experiments performed in triplicate, and are expressed as a percentage of control values. Mean control values for choline were 15509 ± 4196 and 11631 ± 1995 d.p.m./mg of protein for SC1 and SF1.4 cell lines respectively. Mean control values for labelled PtdCho were 24954 ± 14048 and 21284 ± 7908 d.p.m./mg protein respectively (means $\pm$ S.E.M., $n = 4$).

Cell line	Treatment	$[^{14}\text{C}]\text{Choline}$ (% of control)	$[^{14}\text{C}]\text{PtdCho}$ (% of control)
SC1	Control	100	100
	PMA (100 nM)	$181 \pm 27^*$	$786 \pm 201^*$
	Sph (100 μM)	54 ± 9	77 ± 22
	Sph + PMA	$72 \pm 16^\dagger$	$402 \pm 85^\dagger$
SF1.4	Control	100	100
	PMA (100 nM)	$1088 \pm 251^*$	$882 \pm 140^*$
	Sph (100 μM)	75 ± 18	87 ± 15
	Sph + PMA	$320 \pm 138^\dagger$	$354 \pm 95^\dagger$

* $P < 0.05$, significantly different from control cultures, by analysis of variance.

† $P < 0.05$, significantly different from PMA-treated cultures, by analysis of variance.

DISCUSSION

Although much interest has recently been focused on the possibility that individual PKC subtypes may phosphorylate specific substrates, few of these substrates have been identified (for a recent review, see Dekker and Parker, 1994).

The present paper implicates PKC α in the activation of choline uptake and its incorporation into PtdCho. In NIH 3T3 wild-type fibroblasts, PMA increased $[^{14}\text{C}]$ choline accumulation and total free choline in a dose-dependent manner. The initial manifestation of this effect was a concentration-dependent increase in the uptake of $[^{14}\text{C}]$ choline after only 5 min of labelling in cells pretreated with PMA. A similar effect was reported by Lijian et al. (1988), who observed that PMA caused a 2-fold increase in $[^{14}\text{C}]$ choline uptake in C3H10T1/2 fibroblasts labelled for 2.5 min with radioactive choline. PKC α is the major PKC subtype present in NIH 3T3 fibroblasts (Mischak et al., 1993). To test directly the ability of PKC α to regulate choline uptake, the genes for PKC α and neomycin resistance (used for selection) were introduced into Swiss 3T3 fibroblasts (which normally express low levels of this PKC subtype). Cells expressing the neomycin resistance gene alone were used for controls. The methods used to generate these cell lines have been previously described (Eldar et al., 1990). The two PKC α -over-expressing lines (SF1.4 and SF3.2) used in the present study exhibited high levels of PKC α immunoreactivity and PKC activity (assayed as histone phosphorylation *in vitro*) relative to controls (Eldar et al., 1990). The growth rates of the PKC α -over-expressing lines were similar to those of control cells in 10% calf serum, but were markedly increased relative to controls in 1% serum-containing medium (Eldar et al., 1990). Both control (neomycin resistant) and PKC α -over-expressing lines reached higher saturation density than the parental cells, but none of these lines showed growth in soft agar (Eldar et al., 1990).

In cells over-expressing PKC α , but not in control lines, PMA caused a marked stimulation of choline uptake and accumulation, implicating PKC α in the response. Since Swiss 3T3 fibroblasts also express PKC δ , PKC ϵ and PKC ζ (Olivier and Parker, 1992),

we tested the possibility that PKC α over-expression might alter levels of one or more of these subtypes. It was previously shown that PKC ϵ levels were unchanged in SF1.4 cells relative to controls (Eldar et al., 1993). Immunoblot analysis with antibodies to PKC δ and PKC ζ revealed that levels of the former were unchanged by PKC α over-expression, whereas PKC ζ levels were somewhat increased (Figure 1). The rabbit anti-PKC ζ antibody (obtained from Boehringer Mannheim) was raised against a peptide sequence from the C-terminal region of the molecule. This sequence has been previously used to generate antibodies that do not cross-react with PKC α (Ono et al., 1989); therefore it is likely that the results described here reflect a genuine increase in PKC ζ levels in cells over-expressing PKC α . However, PKC ζ is unlikely to mediate the response to PMA observed in PKC α -over-expressing lines, because it lacks a phorbol-ester-binding site (Ono et al., 1989), and is not stimulated by diacylglycerol or by phorbol esters (Nakanishi and Exton, 1992). Because no stimulation of choline uptake by phorbol esters was observed in control Swiss 3T3 fibroblasts, it is probable that neither PKC δ nor PKC ϵ is capable of mediating this effect. However, the results do not exclude other PKC subtypes not present in Swiss 3T3 fibroblasts as possible regulators of choline uptake in other cell types.

In addition to stimulating choline uptake, PMA increased the conversion of phosphocholine into PtdCho. In time-course experiments this was manifested as a decrease in labelling of the phosphocholine pool, relative to control cells, with a concomitant increase in radioactivity in PtdCho (results not shown). This effect of PMA has been observed by others (Paddon and Vance, 1980; Pelech et al., 1984; Cook et al., 1989; Jones and Kent, 1991), and, like the effect of PMA on choline uptake described here, was significantly greater in Swiss 3T3 fibroblasts expressing high levels of PKC α . However, a 4–7-fold increase in PtdCho synthesis was induced by PMA even in control Swiss 3T3 fibroblasts (Figure 4 and Table 1), suggesting that other PKC subtypes also participate in the regulation of PtdCho synthesis. Additional effects associated with PKC α over-expression in these Swiss 3T3 cell lines include decreases in epidermal-growth-factor receptor number (Eldar et al., 1990), up-regulation of phospholipase D activity (Eldar et al., 1993) and increased sensitivity to the stimulatory effect of PMA on secretory cleavage of the amyloid precursor protein (Slack et al., 1993).

The high-affinity choline transporter expressed by cholinergic neurons has been intensively studied, principally because this uptake mechanism is believed to be closely coupled to the synthesis of the neurotransmitter acetylcholine (reviewed by Blusztajn and Wurtman, 1983). The choline-transport mechanism found in other mammalian cell types, including fibroblasts, has a lower affinity for choline, and most of the choline that is taken up is converted into phosphocholine (Pelech and Vance, 1984; Sunga et al., 1988). In heart (Sunga et al., 1988) and liver cells (Pelech et al., 1981, 1982), short-term exposure to analogues of cyclic AMP inhibits both choline uptake and PtdCho synthesis. In liver cells, this appears to be due to inhibition both of choline uptake and of PtdCho-synthetic enzymes. Similarly, in yeast (Nikawa et al., 1990), choline uptake and phospholipid-synthetic enzymes tend to be regulated in a co-ordinated manner by substrate availability and by growth phase. Our results indicate that in mammalian fibroblasts, as well, activation of choline uptake and of PtdCho synthesis can be regulated in concert by the PKC activator PMA, and that this co-ordinated enhancement is dependent on the presence of PKC α . It has yet to be determined whether this low-affinity choline transporter is a direct substrate for PKC α , or is regulated by an unidentified intermediate. Although the cDNA sequence of a putative high-affinity choline

transporter was recently published (Mayser et al., 1992), it was subsequently demonstrated that this sequence encodes a creatine transporter (Bradley et al., 1993; Schloss et al., 1994). Resolution of this issue awaits isolation and purification of the low-affinity choline transporter.

The participation of PKC in the regulation of choline uptake and membrane phospholipid synthesis suggests that growth factors, hormones and neurotransmitters that generate diacylglycerol (an endogenous activator of PKC) via activation of receptors coupled to phospholipase C might stimulate choline metabolism in addition to their other signaling functions. If a similar mechanism regulates the high-affinity choline transporter in cholinergic neurons, then the activation by released acetylcholine of muscarinic receptors coupled to phospholipase C would increase the supply of choline for resynthesis of both neurotransmitter and membrane. Receptor-mediated enhancement of choline uptake and PtdCho synthesis, together with stimulation of phospholipid hydrolysis (Liscovitch, 1992, and references therein) may represent additional mechanisms contributing to membrane remodelling; a necessary cellular response to mitogenic or differentiating stimuli, and to synaptic transmission.

This work was supported in part by a grant from the National Institute of Mental Health (MH-28783). The gift of NIH 3T3 cells from Dr. Seymour Garte is gratefully acknowledged.

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Received 28 March 1994/30 August 1994; accepted 7 September 1994