Phorbol myristate acetate stimulates [³H]choline incorporation into phosphatidylcholine independently of the ‘*de novo*’ pathway in Krebs-II ascitic cells: a unique effect of phorbol ester on choline uptake

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The effect of phorbol 12-myristate 13-acetate (PMA) on [³H]choline incorporation into phosphatidylcholine (PtdCho) and on the ‘*de novo*’ pathway of PtdCho synthesis has been investigated, compared with that of oleic acid, in ascitic-strain Krebs-II cells. Both compounds stimulated [³H]choline incorporation into PtdCho, but the PMA-induced incorporation was saturable at concentrations of the agonist around 100 nM, whereas no saturation was noticed with oleic acid up to 1 mM. Chase experiments showed no effect of PMA on the conversion of phosphocholine into CDP-choline. The phorbol ester did not stimulate any of the enzyme activities of the ‘*de novo*’ pathway, whereas oleic acid increased specifically by 2.5-fold the CTP:phosphocholine cytidyltransferase (CT, EC 2.7.7.15) activity. In addition, no change in the subcellular distribution of CT was observed upon incubation with PMA, in contrast with oleic acid treatment. Cells challenged with oleic acid showed a

25-fold increase in diradylglycerol (DG) content, which was not modified upon incubation with 200 nM PMA, the most effective concentration of phorbol ester promoting choline incorporation. Subcellular fractionation of Krebs-II cells on Percoll gradients revealed that [³H]PMA and 1-radyl-2-[³H]oleoyl-glycerol, derived from exogenously supplied [³H]oleic acid, both exhibited the same enrichment in the endoplasmic reticulum. We have previously shown that the labelled fatty acid also accumulated in the endoplasmic reticulum [Tercé, Record, Tronchère, Ribbes and Chap (1992) *Biochem. J.* **282**, 333–338]. However, PMA induced a stimulation of choline uptake, which was not provoked by PMA 4-*O*-methyl ether, which interacts poorly with protein kinase C. Our data provide evidence that the enhancement of [³H]choline incorporation into PtdCho triggered by PMA and oleic acid proceeds via completely distinct mechanism(s).

INTRODUCTION

The regulation of phosphatidylcholine (PtdCho) biosynthesis through the ‘*de novo*’ pathway, which is in large part controlled by the translocation of CTP:phosphocholine cytidyltransferase (CT, EC 2.7.7.15), has been intensively studied during the past decade in a variety of cell types [1–3]. It has been established that activation of CT is provoked upon translocation from the cytosol to the endoplasmic reticulum [1,3]. Many different stimuli have been found to increase PtdCho synthesis, including fatty acids [4–8], exogenous phospholipase C [9–12], phospholipase A₂ [13] or phorbol ester [14,15]. However, the physiological factors which regulate PtdCho synthesis, trigger the translocation process and determine the target membrane are not yet clearly defined. CT translocation can be modulated by fatty acids [4–6], PtdCho concentration [16], and recently diradylglycerols (DGs) have been implicated in PtdCho synthesis and CT translocation [17,18].

We have already shown that the translocation process involves two different aspects: the targeting of CT to the endoplasmic reticulum and the binding of the enzyme on the target membrane [19]. We have also shown that this binding is regulated by the amount of oleic acid incorporated in the endoplasmic reticulum [19]. In order to investigate further the CT translocation process, we have analysed the effect of phorbol 12-myristate 13-acetate (PMA), another activator of PtdCho synthesis.

Triggering CT translocation by phorbol ester has been a matter of debate [20]. However, a recent report demonstrated PMA-induced CT translocation in HeLa cells [18]. Besides

protein kinase C translocation [21], this phorbol ester also induces the translocation of diacylglycerol kinase [22] and of some subtypes of glucose transporter [23].

We present evidence here that PMA is ineffective in triggering CT translocation to the endoplasmic reticulum in Krebs-II cells. Moreover, PMA has no effect on the cell content of DGs, which were tremendously increased upon cell challenge with oleic acid. Altogether, our data support the idea that DG is required to induce CT translocation, whereas PMA acts independently of the ‘*de novo*’ pathway in Krebs-II cells. Actually, the unique effect of PMA in Krebs-II cells was to stimulate choline uptake.

MATERIALS AND METHODS

Chemicals and products

CTP, oleic acid, phosphocholine, CDP-choline, choline, diolein, dibutyl phthalate, PMA 4-*O*-methyl ether (4-*O*-methyl-PMA) and PMA were obtained from Sigma (St. Louis, MO, U.S.A.). [*methyl*-³H]Choline chloride [2.89 TBq (78 Ci)/mmol], phospho[*methyl*-¹⁴C]choline ammonium salt [2.22 GBq (60 mCi)/mmol], CDP-[*methyl*-¹⁴C]choline ammonium salt [2.26 GBq (50 mCi)/mmol], [9,10-(*n*)-³H]oleic acid [185 GBq (5 Ci)/mmol] and [*γ*-³²P]ATP [110 TBq (3000 Ci)/mmol] were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). [20-³H(*n*)]PMA [469.9 GBq (12.7 Ci)/mmol] and [U-¹⁴C]sucrose [17.6 GBq (475 mCi)/mmol] were obtained from NEN. Eagle’s minimum essential medium and Hepes were from Seromed (Lille, France), and Dulbecco’s PBS was from GIBCO. Lumasolve was purchased from Lumac (Landgraaf, The Nether-

Abbreviations used: CT, CTP:phosphocholine cytidyltransferase; DG, *sn*-1,2-diradylglycerol; [³H]DG, 1-radyl-2-[³H]oleoyl-glycerol; PMA, phorbol 12-myristate 13-acetate; 4-*O*-methyl-PMA, PMA 4-*O*-methyl ether; PtdCho, phosphatidylcholine.

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lands). A stock solution of 100 mM oleic acid was prepared as previously described [4] by dissolving the fatty acid in 0.12 M KOH in 95% (v/v) ethanol, and stored at -20°C . Oleic acid from the stock solution was dried and resuspended at the required concentration in Eagle's medium by stirring vigorously and by sonication before experiments. Phorbol ester stock solutions were prepared in dimethyl sulphoxide, whose final concentration in the assays never exceeded 0.05% (v/v).

Cell incubations

Krebs-II cells were harvested from mice, washed in TKM buffer (25 mM Tris/HCl, pH 7.4, 100 mM KCl, 5 mM MgCl_2) and resuspended in Eagle's medium containing 40 mM Hepes, pH 7.4, as previously described [8,11]. Incorporation of choline into phospholipids was performed on cells incubated for various periods with $2\text{ }\mu\text{Ci/ml}$ [^3H]choline (sp. radioactivity $280\text{ }\mu\text{Ci/mmol}$, taking into account the choline concentration into Eagle's medium) in the presence of various concentrations of the agonist (oleic acid or PMA). At each incubation time, 2×10^6 cells were harvested and pelleted (13000 g for 30 s). Then the pellet was extracted as described by Bligh and Dyer [24], and the radioactivity of the chloroform phase was determined by liquid-scintillation counting.

Pulse-chase experiments

Cells were labelled with [^3H]choline (sp. radioactivity $280\text{ }\mu\text{Ci/mmol}$) in Eagle's medium, buffered with 40 mM Hepes, pH 7.4, during 2 h at 37°C and pelleted. The cell pellet was washed twice with unlabelled Eagle's medium and resuspended in the same medium at a concentration of 4×10^6 cells/ml. Labelled cells were then incubated in the absence or presence of the agonists up to 1 h. Samples ($500\text{ }\mu\text{l}$) were harvested at various times and pelleted (2800 g for 1 min) by using an MSE microfuge (Kontron Instruments). The cell pellet was extracted as described by Bligh and Dyer [24], and the radioactivity from the organic phase was determined. The products from the aqueous phases were separated by t.l.c. as described by Vance et al. [25] and the radioactivity was determined after scraping.

Choline transport assay

The transport assay was performed as described by Baxter et al. [26] with slight modifications. Briefly, cells (4×10^6 cells/ml) were incubated at 37°C in Eagle's medium, plus 40 mM Hepes, pH 7.4, with [^3H]choline ($2\text{ }\mu\text{Ci/ml}$; sp. radioactivity $280\text{ }\mu\text{Ci/mmol}$) and the different agonists. Samples ($500\text{ }\mu\text{l}$) were taken at various times up to 40 min and the reaction was stopped by dilution in $600\text{ }\mu\text{l}$ of ice-cold PBS. Cells were recovered from the medium by centrifugation in a MSE microfuge (9000 g for 1 min) through a cushion of dibutyl phthalate/oil (11:4, v/v), which was then frozen in liquid nitrogen, and the tip of the microfuge tube was excised with a micro-tube cutter. Tips were transferred to scintillation vials containing 1 ml of Lumasolve and 10 ml of Picofluor 40. Total ^3H radioactivity represented intracellular [^3H]choline and extracellular [^3H]choline trapped in the cell pellet. The volume of extracellular fluid trapped with the pellet was estimated by using [^{14}C]sucrose and never exceeded 0.2% of the total radioactivity.

Cellular fractionation

Cells were incubated for 30 min in Eagle's medium (4×10^6 cells/ml) in the presence of the labelled agonists, washed

three times in cold TKM buffer and resuspended to 10^7 cells/ml in lysis buffer (TKM buffer containing 1 mM ATP). Cell disruption, differential centrifugation and subcellular fractionation on a Percoll gradient were performed as reported previously [8,11]. Fractions were collected from the top of the gradient and stored for up to 1 h at 4°C before enzyme assays. Lipids were directly extracted from gradient fractions as reported previously [8].

In cell-free-system experiments, a postnuclear supernatant was incubated for 5 min in the presence of [^3H]PMA (200 nM , $0.32\text{ }\mu\text{Ci/nmol}$) and further submitted to fractionation as described above. Samples ($200\text{ }\mu\text{l}$) of each gradient fraction were counted by liquid scintillation.

Enzyme assays

Choline kinase (EC 2.7.1.32), CT and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) were determined as previously reported [11,25,27].

Quantification of *sn*-1,2-diradylglycerols (DGs)

Krebs-II cells were incubated with the agonists and extracted by the method of Bligh and Dyer [24]. The lipid fraction was dried under nitrogen and resolubilized with 7.5% (w/v) octyl β -D-glucoside, 5 mM cardiolipin and 1 mM-diethylenetriaminepentacetic acid (final volume $20\text{ }\mu\text{l}$). DG levels were then quantified as described by Wright et al. [28], which is a modification of the method of Preiss et al. [29], by using *Escherichia coli* DG kinase (Lipidex) and [γ - ^{32}P]ATP.

Lipid analysis

Unesterified [^3H]oleic acid or [^3H]DG were separated on silica gel G with hexane/diethyl ether/formic acid (55:45:1, by vol.) as solvent [8].

Radioactivity from each spot was determined by scanning plates with an automatic t.l.c. linear analyser (Berthold LB 2842), followed by liquid-scintillation counting.

Miscellaneous

Protein was determined by the method of Lowry et al. [30], with BSA as a standard. Radioactivity was determined on a Kontron analytical Intertechnic counter (type SL 4000) with Ultimagold or PicoFluor 40 (Packard Instrument Co.) as scintillation fluid. Student's *t* test was used for statistical analysis.

RESULTS

Effect of PMA and oleic acid on the incorporation of [^3H]choline into choline glycerophospholipids

Cells were incubated with [^3H]choline in the presence of increasing concentrations of oleic acid or PMA (Figure 1). Oleic acid (0.1 – 1 mM) stimulated the incorporation of [^3H]choline into choline glycerophospholipids (Figure 1a). Cell lysis did not increase in this concentration range, but higher concentrations of the fatty acid (5 mM) induced complete lysis of the cells [8]. By contrast, the effects of PMA on [^3H]choline incorporation occurred in a 1000-fold lower concentration range (Figure 1b). Stimulated labelling of phospholipids was already detected at 10 nM PMA, but rapidly reached a maximum at 50 nM . Higher concentrations of PMA (50 nM – $1\text{ }\mu\text{M}$) had no additional effect on [^3H]choline incorporation. Cell lysis was never above the control levels, even at a PMA concentration of $1\text{ }\mu\text{M}$ (results not

Figure 1 Time- and concentration-dependent effect of oleic acid and PMA on $[^3\text{H}]$ choline incorporation into phospholipids

Cells were incubated with $[^3\text{H}]$ choline (2 $\mu\text{Ci/ml}$) in the absence or in the presence of increasing concentrations of oleic acid (a) or PMA (b). At each incubation time, radioactivity present in the lipid phase was determined as described in the Materials and methods section. (a) Control ($\circ$); oleic acid 10 μM ($\blacklozenge$); 100 μM ($\triangle$); 500 μM ($\blacktriangle$); 1 mM ($\square$). (b) Control ($\circ$); PMA 10 nM ($\bullet$); 50 nM ($\triangle$); 100 nM ($\blacktriangle$); 500 nM ($\square$); 1 μM ($\blacksquare$). Results represent means $\pm$ S.E.M. of three separate experiments.

shown). The maximal increase in $[^3\text{H}]$ choline incorporation observed after 30 min of incubation was 10-fold at 1 mM oleic acid, but only 3.3-fold at 50 nM PMA. Concentrations of 500 μM oleic acid and 200 nM PMA were used in further experiments and are in the same concentration range as in other reports [7,8,14,31,32].

Chase experiments with PMA

Chase experiments were then performed to analyse the level of the various choline derivatives. The results are presented in Figure 2 and show that 200 nM PMA did not modify the conversion of phosphocholine and CDP-choline derivatives (Figures 2b and 2c) as compared with control cells, therefore suggesting the lack of CT activation. Consistent with that observation, the chase experiment had no effect on PtdCho labelling, at least during the first 30 min (Figure 2d). Thus the increase in PtdCho labelling induced by PMA during the pulse of $[^3\text{H}]$ choline as reported in Figure 1(b) suggested a mechanism distinct from the 'de novo' pathway. During the chase experiment reported in Figure 2, we noticed a higher amount of internalized choline (Figure 2a). We also checked the effect of oleic acid as a positive control. The fatty acid induced a rapid and strong decrease in phosphocholine labelling (Figure 2b) and a concomitant increase in CDP-choline labelling (Figure 2c) compared with untreated cells. Radioactivity in PtdCho increased rapidly and continuously upon addition of oleic acid (Figure 2d). As previously reported [8], these results demonstrate that CT is the regulatory enzyme of the CDP-choline pathway in Krebs-II cells activated with oleic acid.

Effect of PMA and oleic acid on the enzymes of the 'de novo' pathway for PtdCho synthesis

We compared the effects of oleic acid and PMA on the enzymes of the 'de novo' pathway (Table 1). We found a 2.5-fold increase

Figure 2 Pulse-chase experiment in the presence of oleic acid or PMA

Krebs-II cells (4×10^6 cells/ml) were prelabelled for 2 h with $[^3\text{H}]$ choline (2 $\mu\text{Ci/ml}$), rinsed twice and incubated in the absence ($\circ$) or in the presence of 500 μM oleic acid ($\blacklozenge$) or 200 nM PMA ($\blacksquare$). At the times indicated, cells were harvested, lipids were extracted and radioactivity was determined in free internalized choline (a), phosphocholine (b), CDP-choline (c) and PtdCho (d). Similar results were obtained in two other experiments.

Table 1 Effect of oleic acid and PMA on the activity of enzymes of the 'de novo' pathway

Cells were incubated for 30 min in the presence of 500 μ M oleic acid or 200 nM PMA. Activity of choline kinase, CT and choline phosphotransferase was assayed in the homogenate as described in the Materials and methods section. Results represent means $\pm$ S.E.M. of at least three independent experiments: * $P < 0.01$.

Cell treatment...	Activity (nmol/min per mg of protein)		
	Control	Oleic acid	PMA
Choline kinase	1.29 $\pm$ 0.36	1.39 $\pm$ 0.38	1.39 $\pm$ 0.42
CT	0.99 $\pm$ 0.28	2.55 $\pm$ 0.46*	0.88 $\pm$ 0.33
Cholinephosphotransferase	0.135 $\pm$ 0.05	0.082 $\pm$ 0.03	0.12 $\pm$ 0.03

Table 2 DG levels in Krebs-II cells in response to PMA and oleate

Cells were incubated in the absence or presence of 500 μ M oleate or 200 nM PMA. After lipid extraction, DGs were measured in the lipid phase as described in the Materials and methods section. Results represent means $\pm$ S.E.M. of four separate experiments: * $P < 0.05$.

	DG amount (pmol/10 ⁷ cells)
Control	33.5 $\pm$ 7
Oleic acid	854 $\pm$ 270*
PMA	34.3 $\pm$ 8

in CT activity after oleic acid treatment, whereas choline kinase and cholinephosphotransferase were not affected. Upon treatment of cells with PMA, no significant differences were observed for either enzyme of the 'de novo' pathway (Table 1). Oleic acid treatment induced an increased activity of CT in the membrane fraction compared with control cells (5.74 $\pm$ 1.40 versus 1.07 $\pm$ 0.48 nmol/min per mg of protein) at the expense of the cytosolic fraction (0.78 $\pm$ 0.40 versus 4.74 $\pm$ 0.87 nmol/min per mg of protein). PMA did not change the specific activity of CT in any cell compartment, which was 1.32 $\pm$ 0.2 and 4.60 $\pm$ 0.8 nmol/min per mg of protein in membrane and cytosol fractions respectively.

In order to understand the role of DG as a regulator of PtdCho synthesis, we have analysed the DG content of Krebs-II cells (Table 2). No changes were noticed in cells challenged with PMA, whereas oleic acid increased by 25 times the mass of this neutral lipid. This observation suggested that different pathways were activated by oleic acid and PMA.

Comparative subcellular distribution of CT with [³H]PMA, [³H]oleic acid and [³H]DG

We investigated the subcellular distribution of CT activity compared with that of oleic acid or PMA (Table 3). As suggested above, CT activity was mainly localized in the membrane fraction only in the presence of oleic acid, whereas 90 % of [³H]oleic acid but also 90 % of [³H]PMA were associated with membranes.

Plasma membranes and endoplasmic reticulum were further separated. The distribution of [³H]oleic acid across a Percoll gradient has already been reported [19], and it was similarly distributed between the two types of membranes when added to a homogenate. Interestingly, addition of [³H]PMA directly to a

Table 3 Distribution of CT, free [³H]oleic acid and [³H]PMA between particulate and cytosol

Cells were incubated for 30 min in the absence or presence of [³H]oleic acid (500 μ M, 3.4 μ Ci/ μ mol) or PMA (200 nM, 0.32 μ Ci/nmol). CT activity and free [³H]oleic acid or [³H]PMA were measured in the particulate and cytosolic cell fractions as described in the Materials and methods section. Results are expressed as percentages of total activity in the cytosol and particulate fractions for CT and represent the mean of three separate experiments. Values in parentheses represent the distribution of [³H]oleic acid and [³H]PMA. Results are representative of a typical experiment.

Cell treatment...	Distribution (% of total)		
	Control	PMA	Oleic acid
Particulate	19.1	19.6 (89)	80.1 (93)
Cytosol	80.9	80.4 (11)	19.9 (7)

Figure 3 Subcellular distribution of CT, [³H]PMA and [³H]DG

Lysed cells were incubated for 5 min in the absence (○) or presence (●) of [³H]PMA (200 nM; 0.32 μ Ci/nmol). Subcellular distribution of CT activity (a) and [³H]PMA (b) was measured in each fraction from the Percoll gradient as described in the Materials and methods section. (c) Cells were incubated for 30 min in the presence of 500 μ M [³H]oleic acid (3.4 μ Ci/ μ mol), lysed, and the particulate fraction was further fractionated on a Percoll gradient. Subcellular distribution of [³H]DG was analysed after extraction of gradient fractions followed by lipid separation as described in the Materials and methods section. Results represent means of two experiments and are expressed in nmol/min per 10⁶ cells (a) or as a percentage of total gradient content (b and c).

cell-free system did not change the cellular distribution of the basal amount of CT located in the endoplasmic reticulum (Figure 3a), whereas [³H]PMA was mainly associated with this fraction (Figure 3b). Oleic acid added to the cells was able to provoke CT translocation on to the endoplasmic reticulum [8], and triggered

Figure 4 Effect of PMA on choline transport

Cells were incubated at 37 °C in Eagle's medium/40 mM Hepes, pH 7.4, with [3 H]choline (2 μ Ci/ml) in the absence (○) or in the presence of 200 nM PMA (●) or 200 nM 4-O-methyl-PMA (▼). At the times indicated, samples were harvested and treated as described in the Materials and methods section. Results represent the means $\pm$ S.E.M. of four experiments: * $P < 0.05$, ** $P < 0.02$ for difference between control and PMA-treated cells.

the formation of DG, which was also mainly located on the endoplasmic reticulum (Figure 3c). These observations demonstrate that oleic acid-derived DG and PMA displayed similar subcellular localizations, but that oleic acid treatment only was able to induce CT translocation to the endoplasmic reticulum (Table 3).

PMA stimulates choline transport

In order to explore further the stimulatory effect of PMA on choline incorporation we also measured choline uptake. From 5 min, PMA induced a significant increase of choline transport compared with control cells (Figure 4). At 40 min the rate of choline uptake was $32 \pm 12\%$ higher in the presence of phorbol ester. Interestingly, 4-O-methyl-PMA, a structural analogue of PMA which exhibits poor affinity towards protein kinase C [33], showed no effect on choline uptake. This result suggests that enhancement by PMA of choline uptake is protein kinase C-mediated.

DISCUSSION

In the present work we have addressed the question of the effect of PMA on PtdCho synthesis in Krebs-II cells, compared with oleic acid as a positive control. Oleic acid is one major activator of PtdCho synthesis through the 'de novo' pathway [1,2]. As we found recently, oleic acid stimulates PtdCho synthesis in Krebs-II cells through the 'de novo' pathway [8]. In the present work we compared the effect of both oleic acid and PMA on the turnover of the polar head group.

It is clear from our results that these two agents display different effects on [3 H]choline incorporation into choline glycerophospholipids. Activation of [3 H]choline incorporation by PMA required a 1000-fold lower concentration than for oleic acid (Figure 1), comparable with that observed on neural cells [7]. The rapid metabolism of unesterified oleic acid into phospholipids or neutral lipids observed in Krebs-II cells [8] could explain the non-saturable effect of oleic acid.

The major difference between oleic acid and PMA was observed on the pathways involved in PtdCho synthesis. The activation of the 'de novo' pathway by oleic acid was demonstrated by chase experiments and measurement of enzyme activities of the 'de novo' pathway in Figure 2 and Table 1, which showed a selective activation and translocation of CT from cytosol to membranes. In contrast with oleic acid, we never detected any activation of the 'de novo' pathway after PMA treatment (Figure 2 and Table 1). Indeed, PMA did not induce significant translocation of CT from the cytosol to membranes in our experimental conditions. This was rather unexpected, since PMA was already found to activate the 'de novo' pathway and CT translocation in glioma cells [7], HeLa cells [14,34] and GH3 cells [15]. We showed previously [19] that the proportion of CT bound *in vivo* to membrane was correlated with the amount of unesterified oleic acid in the endoplasmic reticulum. However, the intracellular localization of free oleic acid could not explain by itself the selectivity of the endoplasmic reticulum as the target membrane [19].

Measuring distribution of CT activity between particulate and cytosol fractions, we observed no PMA-induced translocation of CT. The lack of translocation of CT observed in Table 3 could not be related to a lack of incorporation of PMA into the cell, since we could detect about 90% of [3 H]PMA associated with the particulate fraction. In addition, [3 H]PMA added directly to a cell-free system was almost entirely incorporated into the endoplasmic reticulum, but no increase in CT activity could be detected in this membrane (Figures 3a and 3b). There is no direct relationship between membrane-bound PMA and CT, in contrast with oleic acid and CT.

The cellular effects of PMA are usually related to the activation of protein kinase C. However, CT has not been found to be a substrate for protein kinase C [1], and PMA-induced phosphorylation of the enzyme *in vivo* did not occur [20], suggesting that phosphorylation of CT is not a regulatory pathway modulated by PMA activation. The effect of PMA observed on glioma cells could be attributed to the 'de novo' pathway, and, interestingly, in that work PMA was mainly localized in the plasma membrane [7]. The lack of activation of the 'de novo' pathway observed in our conditions could be related to the presence of PMA mainly in the endoplasmic reticulum *in vitro* (Figure 3b) and *in vivo* (results not shown). We have therefore investigated the intracellular distribution of DGs.

Our data demonstrate that PMA enhances [3 H]choline incorporation without any increase in DG content and CT translocation, in contrast with oleic acid treatment (Table 2). In HeLa cells, PMA stimulates DG formation [18], probably through the phospholipase D/phosphatidate phosphohydrolase pathway [35]. Results from Tables 2 and 3 support the idea that DG induces CT translocation [18] in our cell model. However, [3 H]DG displays a subcellular distribution similar to that of [3 H]PMA (Figures 3b and 3c).

Most of the intracellular PMA is membrane-bound (Figure 3), although the effective part of the phorbol ester appears to be the cytosolic one, because of possible interaction with the protein kinase C stimulating choline uptake. Effectively our results with 4-O-methyl-PMA indicate that the stimulation of the uptake is not just a consequence of a lipidic effect of the phorbol ester PMA on membranes, but might require protein kinase C activation. Our results differ from those reported by Fjose et al. [32], who observed no effect of PMA on choline uptake.

Stimulation of transporters by agonists is poorly documented, especially in the case of choline. Instead, PMA has been shown to enhance glucose uptake by mediating translocation of the transporter [23]. Carrier-mediated choline uptake in Krebs-II

cells belongs to a cyclic type of transport mechanism [36], and further experiments would be required to determine whether PMA acts on the translocation process of the choline transporter in our cell model.

Although stimulated (Figure 4), the choline uptake is probably saturable at longer times than those we investigated, as observed around 2 h in untreated cells [36]. Saturation of choline internalization certainly accounts for the dose-dependent maximum effect of PMA on [³H]choline incorporation into PtdCho (Figure 1b). This incorporation operates through the basal amount of membrane-bound CT that we demonstrate earlier [19], since no additional supply of CT is triggered by PMA (Figure 2, Table 3). The basal membrane-bound CT is another rate-limiting factor which can explain the results reported in Figure 1(b).

We have also considered the possible involvement of the base-exchange reaction, which appears to be stimulated by PMA in a neuroblastoma cell line [37]. The activity of the enzymic reaction is relatively high in Krebs-II cells (0.8 nmol of PtdCho/min per mg of protein), measured as described by Siddiqui and Exton [38], but no change could be noticed upon cell treatment with the phorbol ester (results not shown).

Altogether, our results evidence a stimulation of [³H]choline incorporation by PMA independently of DG, formed either by synthesis 'de novo' [18] or by PtdCho degradation. Enhanced PtdCho synthesis not related to PtdCho degradation has been noticed in NIH 3T3 cells [39]. Several reports indicate distinct intracellular mechanism(s) for either oleic acid or PMA [7] and for diacylglycerols or PMA [17,40]. The effect of PMA in these former studies has not been clearly elucidated. In the present work we establish that PMA-induced [³H]choline incorporation into Krebs-II cells operates through a route quite distinct from stimulation of the 'de novo' pathway.

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