1,2-Diacylglycerols overcome cyclic AMP-mediated inhibition of phosphatidylcholine synthesis in GH₃ pituitary cells

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Previous studies showed that phorbol esters and thyrotropin-releasing hormone (TRH) stimulated phosphatidylcholine synthesis via protein kinase C in GH₃ pituitary cells [Kolesnick (1987) *J. Biol. Chem.* **262**, 14525–14530]. In contrast, 1,2-diacylglycerol-stimulated phosphatidylcholine synthesis appeared independent of protein kinase C. The present studies compare phosphatidylcholine synthesis stimulated by these agents with inhibition via the cyclic AMP system. The potent phorbol ester phorbol 12-myristate 13-acetate (PMA, 10 nM) increased [³²P]P_i incorporation into phosphatidylcholine at 30 min to 159 ± 6 % of control. The adenylate cyclase activator cholera toxin (CT; 10 nM) and the cyclic AMP analogue dibutyryl cyclic AMP (1 mM) abolished this effect. CT similarly abolished TRH-induced phosphatidylcholine, but not phosphatidylinositol, synthesis. This is the first report of inhibition of receptor-mediated phosphatidylcholine synthesis by the cyclic AMP system. The 1,2-diacylglycerol 1,2-dioctanoylglycerol (diC₈) also stimulated concentration-dependent phosphatidylcholine synthesis. DiC₈ (3 μg/ml) induced an effect quantitatively similar to that of maximal concentrations of PMA and TRH, whereas a maximal diC₈ concentration (30 μg/ml) stimulated an effect 3–4-fold greater than these other agents. CT decreased the effect of diC₈ (3 μg/ml) by 80 %. Higher diC₈ concentrations overcame the CT inhibition. Similar results were obtained with dibutyryl cyclic AMP. Additional differences were found between low and high concentrations of diC₈. Low concentrations of diC₈ failed to induce additive phosphatidylcholine synthesis with maximal concentrations of PMA, whereas high concentrations were additive. Hence, low concentrations of 1,2-diacylglycerols appear to be regulated similarly to phorbol esters, and higher concentrations appear to act via a pathway unavailable to phorbol esters.

INTRODUCTION

It is now evident that protein kinase C plays a central role in the regulation of a variety of cellular functions (for reviews see [1,2]). Hence the mechanism of protein kinase C action has come under increasing scrutiny. There are two major classes of protein kinase C activators; the purported physiological activators, the 1,2-diacylglycerols, and the tumour-promoting phorbol esters. Originally, these agents were utilized interchangeably to study the biological effects of protein kinase C activation. However, there are now numerous reports that 1,2-diacylglycerols and phorbol esters may stimulate different patterns of cellular activation [3–7]. In fact, it is now clear that 1,2-diacylglycerols even stimulate some events not induced by phorbol esters. These include priming of the respiratory burst in human neutrophils [3], glucose-transporter-mediated glucose uptake in rat adipocytes [4], phosphatidylcholine degradation in neuroblastoma [5] and GH₃ cells [6], and sphingomyelin degradation via a sphingomyelinase in GH₃ cells [7]. The present studies were undertaken to compare further the actions of 1,2-diacylglycerols and phorbol esters and to use phosphatidylcholine synthesis as a model of a 'protein kinase C-mediated' event.

Vance and co-workers [8–13] have demonstrated that the protein kinase C and cyclic AMP systems have opposite effects on phosphatidylcholine synthesis in short-term incubations. Phorbol esters were shown to stimulate phosphatidylcholine synthesis via the CDP-choline pathway in HeLa cells [8–10]. This event was mediated by redistribution of the inactive cytosolic form of the enzyme CTP:phosphocholine cytidyltransferase (EC 2.7.7.15) to the membrane. In contrast, the cyclic AMP system inhibited basal phosphatidylcholine synthesis [11–13].

This resulted from inactivation of the membrane-bound cytidyltransferase by redistribution to the cytosol [14] and appeared to correlate with phosphorylation of the enzyme [14].

Previous studies from my laboratory showed that the potent phorbol ester phorbol 12-myristate 13-acetate (PMA) similarly enhanced phosphatidylcholine synthesis via the cytidyltransferase in GH₃ pituitary cells [15]. Thyrotropin-releasing hormone (TRH), which activates protein kinase C by a receptor-mediated mechanism, also stimulated these events [15]. PMA- and TRH-induced phosphatidylcholine synthesis were abolished in cells down-modulated for protein kinase C. In contrast, down-modulation of protein kinase C failed to inhibit 1,2-dioctanoylglycerol (diC₈)-induced phosphatidylcholine synthesis [6]. Additionally, maximal concentrations of diC₈ and phorbol esters produced additive phosphatidylcholine synthesis. These studies were interpreted as evidence that diC₈ stimulated these effects independently of protein kinase C. Thus, paradoxically, TRH appeared to act more like the tumour promoters than the purported 'physiological' protein kinase C activators.

The present studies further investigate phorbol ester and 1,2-diacylglycerol stimulation in GH₃ cells. These studies show that the cyclic AMP system inhibits phosphatidylcholine synthesis induced by maximal concentrations of phorbol esters and TRH. Similarly, the cyclic AMP system inhibits phosphatidylcholine synthesis induced by low concentrations of 1,2-diacylglycerols. However, higher concentrations of 1,2-diacylglycerols overcome cyclic AMP inhibition. Additional differences were found between low and high concentrations of diC₈. Whereas low concentrations failed to produce additive phosphatidylcholine synthesis with a maximal concentration of phorbol esters, high concentrations were additive. Hence, low concentrations of 1,2-

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; diC₈, 1,2-dioctanoylglycerol; TRH, thyrotropin-releasing hormone; CT, cholera toxin; Bt₂cAMP, dibutyryl cyclic AMP; VIP, vasoactive intestinal peptide.

diacylglycerols appear to be regulated similarly to phorbol esters, and higher concentrations appear to act via a pathway unavailable to phorbol esters.

EXPERIMENTAL

Materials

Phorbol esters, choline standards, phospholipid standards, cyclic AMP analogues, cholera toxin (CT), TRH and vasoactive intestinal peptide (VIP) were purchased from Sigma. DiC_8 was from Sigma or Serdary Research Laboratories. DiC_8 from either source yielded identical results. $[^{32}\text{P}]\text{P}_i$ (carrier free) and $[^3\text{H}]\text{choline chloride}$ (80 Ci/mmol) were from New England Nuclear. Liquid-scintillation-counting solution (Liquiscint) was from National Diagnostics. Silica-gel t.l.c. plates (LK6D) were from Whatman. Reagents were h.p.l.c. grade and from Fisher.

Cell culture

GH_3 cells (American Type Culture Collection) were grown as monolayer cultures in Ham's F-10 medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) fetal bovine serum at 37 °C as described in ref. [16]. For some studies, cells were resuspended in 1% horse serum for 36 h before harvesting. These conditions increased the uniformity of the results.

Phospholipid studies

On the day of the experiment, cells were harvested with 0.02% EDTA, washed and incubated (30×10^6 cells/ml) in a balanced salt solution (135 mM-NaCl, 4.5 mM-KCl, 1.5 mM- CaCl_2 , 0.5 mM- MgCl_2 , 5.6 mM-glucose, 10 mM-Hepes, pH 7.4) containing either $[^{32}\text{P}]\text{P}_i$ (1.0 mCi/ml) or $[^3\text{H}]\text{choline chloride}$ (0.02 mCi/ml). After 45 min, cells were resuspended in buffer without radiolabel (5.5×10^6 cells/ml) containing PMA, TRH or diC_8 . Under these conditions the γ -phosphate of ATP and the cellular phosphocholine pool had reached isotopic steady-state with their respective probes. This protocol obviates any effects of these agents on uptake of radiolabel. Dimethyl sulphoxide was used as diluent and did not affect labelling. For most studies with agents that elevate cyclic AMP levels, cells were preincubated with the cyclic AMP analogue dibutyryl cyclic AMP (Bt_2cAMP) for 15 min or with CT for 30 min before agonist addition. Reactions were stopped by extraction of phospholipids with chloroform/methanol/38% HCl (100:100:1, by vol.) containing 10 mM-EDTA. Phospholipids were isolated from the organic phase by t.l.c. with chloroform/methanol/acetic acid/water (50:30:8:3, by vol.) as solvent as described in ref. [16]. Lipids were detected by staining with iodine vapour and, for experiments with ^{32}P , also by radioautography. Radioactivity was determined by liquid-scintillation spectrometry.

It should be noted that diC_8 appears to be 1–2 orders of magnitude more potent than in previous studies [6]. This resulted from the establishment of optimal conditions for stimulation, based on recognition that the effects of 1,2-diacylglycerols were subject to surface-dilution.

Cell viability was assessed by Trypan Blue exclusion analysis. Incubations containing less than 40 μg of diC_8 /ml and 1 μM -PMA uniformly excluded Trypan Blue.

Statistics

Statistical analysis was performed by Student's *t* test and linear-regression analysis by the method of least squares.

RESULTS

Previous studies [6,7] demonstrated that phorbol esters stimulated $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine in cells

Table 1. Inhibition of PMA-induced $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine by CT

Cells (3×10^7 /ml) were labelled for 45 min with $[^{32}\text{P}]\text{P}_i$ (1 mCi/ml) and resuspended (5.5×10^6 cells/ml) in medium without radiolabel containing CT (10 nM) or diluent. PMA (10 nM) or diluent was added after 30 min (zero time). Incubations were stopped after an additional 30 min, and lipids were extracted and resolved by t.l.c. with chloroform/methanol/acetic acid/water (50:30:8:3, by vol.) as solvent. Radioactivity was quantified by liquid-scintillation counting. The data (means $\pm$ S.E.M.) represent triplicate determinations from six experiments.

	$[^{32}\text{P}]\text{Phosphatidylcholine}$	
	(d.p.m./ 10^6 cells)	(% of control)
Control	3200 ± 64	100 ± 2
CT	3040 ± 160	95 ± 5
PMA	5088 ± 192	159 ± 6
PMA $\pm$ CT	3712 ± 190	116 ± 6

labelled for 45 min and resuspended in medium without radiolabel. Incorporation of label was time- and concentration-dependent. The potent phorbol ester, PMA, enhanced $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine to $112 \pm 4\%$ of control after 5 min and to $189 \pm 27\%$ of control after 1 h; the 50% effective concentration (EC_{50}) ≈ 9 nM. Similar results were obtained with $[^3\text{H}]\text{choline}$.

Initial studies measured the effect of CT, an activator of adenylate cyclase, on PMA-induced incorporation of $[^{32}\text{P}]\text{P}_i$ into phosphatidylcholine. For these studies, cells were incubated with CT for 30 min before phorbol ester stimulation. Under these conditions, CT elevated cyclic AMP from 1.0 to 1.5 pmol/ 10^6 cells. Table 1 demonstrates that a maximal concentration of PMA (10 nM) increased incorporation of $[^{32}\text{P}]\text{P}_i$ into phosphatidylcholine to $159 \pm 6\%$ of control (mean $\pm$ S.E.M., $P < 0.001$) after 30 min. Preincubation with CT (0.1 μM) did not significantly affect basal incorporation into phosphatidylcholine. However, CT decreased PMA-stimulated incorporation by 73%, to $116 \pm 6\%$ of control. As little as 0.1 nM-CT was effective, and a maximal effect was achieved with 10 nM. In contrast, the β -subunit of CT (0.1 nM–0.1 μM) was ineffective.

Additional studies were performed with the cyclic AMP analogue Bt_2cAMP to activate the cyclic AMP-dependent protein kinase system directly. For these studies, cells were preincubated with Bt_2cAMP for 15 min before PMA addition. In three separate studies, Bt_2cAMP induced a concentration-dependent inhibition of the effect of PMA (10 nM) to stimulate $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine. As little as 1 μM - Bt_2cAMP decreased the PMA effect by 28%, and a maximal concentration, 5 mM- Bt_2cAMP , was completely inhibitory. Bt_2cAMP did not affect basal incorporation, nor did additional preincubation time up to 1 h enhance the Bt_2cAMP effect. VIP, which elevates cyclic AMP levels via a receptor-mediated mechanism in GH_3 cells [17], also inhibited PMA-induced $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine. A 10 min preincubation with VIP (1 nM) inhibited the effect of PMA by 50%, and VIP (10 nM) abolished PMA stimulation ($n = 3$). Thus Bt_2cAMP , CT and VIP, agents that activate the cyclic AMP system via separate mechanisms, appear to inhibit phorbol-ester-stimulated phosphatidylcholine metabolism similarly.

To explore further the effect of CT on phosphatidylcholine synthesis, studies were performed in which the order of addition of the agents was reversed. For these studies, cells short-term labelled with $[^3\text{H}]\text{choline}$ were stimulated with PMA for 30 min

Fig. 1. Inhibition of PMA-induced $[^3\text{H}]$ choline incorporation into phosphatidylcholine by treatment with CT after PMA

For these experiments, cells were labelled for 45 min with $[^3\text{H}]$ choline (0.02 mCi/ml) and resuspended in medium without radiolabel. Experiments were performed as described in Table 1, except that PMA (10 nM) was added (zero time) before CT (10 nM). The level of $[^3\text{H}]$ phosphatidylcholine at zero time was 820 d.p.m./ 10^6 cells. These data (means $\pm$ S.E.M.) represent duplicate determinations from two experiments. Symbols: ●, control; ○, CT; ▲, PMA; △, PMA + CT.

before CT addition. This approach assessed the effect of the cyclic AMP system after protein kinase C-induced activation of the CDP-choline pathway had been initiated. Fig. 1 demonstrates that a maximal concentration of PMA (0.1 μM), as in previous studies [6,15], enhanced $[^3\text{H}]$ choline incorporation into phosphatidylcholine at a linear rate ($r = 0.99$) 1.85-fold of control. CT, added after 30 min, inhibited the increase in the rate of $[^3\text{H}]$ choline incorporation after PMA by 60%, but did not affect basal incorporation. Similarly, PMA stimulated $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine at a linear rate 1.82-fold of

Table 2. Differential inhibition of TRH-induced $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine and phosphatidylinositol by CT

These experiments were performed as described in Table 1 with cells preincubated with CT for 30 min before a 30 min incubation with TRH. The concentrations of the agents utilized were: TRH 0.1 mM; PMA 10 nM; CT 10 nM. These data (means $\pm$ S.E.M.) represent triplicate determinations from five experiments: ^asignificantly different from control ($P < 0.001$); ^bsignificantly different from PMA or TRH ($P < 0.001$).

	Incorporation (d.p.m./ 10^6 cells)	
	$[^{32}\text{P}]\text{Phosphatidylcholine}$	$[^{32}\text{P}]\text{Phosphatidylinositol}$
Control	6300 $\pm$ 126	9200 $\pm$ 184
CT	5670 $\pm$ 315	8004 $\pm$ 460
PMA	8820 $\pm$ 504 ^a	8832 $\pm$ 368
PMA + CT	6804 $\pm$ 378 ^b	7820 $\pm$ 368
TRH	9513 $\pm$ 315 ^a	31096 $\pm$ 1932 ^a
TRH + CT	7308 $\pm$ 504 ^b	30728 $\pm$ 2392 ^a

Table 3. Effect of CT on $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine stimulated by maximal concentrations of PMA, TRH and diC_8

These studies were performed as described in Table 1 with cells preincubated for 30 min with CT (10 nM) before a 30 min incubation with PMA (0.1 mM), TRH (0.1 mM) or diC_8 (30 $\mu\text{g}/\text{ml}$). These data (means $\pm$ S.E.M.) represent triplicate determinations from four experiments: ^asignificantly different from PMA ($P < 0.005$); ^bsignificantly different from TRH ($P < 0.005$).

	$[^{32}\text{P}]\text{Phosphatidylcholine}$	
	(d.p.m./ 10^6 cells)	(% of control)
Control	4975 $\pm$ 149	100 $\pm$ 3
CT	4328 $\pm$ 299	87 $\pm$ 6
PMA	7463 $\pm$ 398	150 $\pm$ 8
PMA + CT	5323 $\pm$ 348 ^a	107 $\pm$ 7 ^a
TRH	6965 $\pm$ 348	140 $\pm$ 7
TRH + CT	5274 $\pm$ 398 ^b	106 $\pm$ 8 ^b
diC_8	11293 $\pm$ 846	227 $\pm$ 17
diC_8 + CT	10000 $\pm$ 896	201 $\pm$ 18

control, and CT, added after 30 min, completely abolished this effect ($n = 2$). These studies indicate that protein kinase C-mediated activation of the CDP-choline pathway may be antagonized by the cyclic AMP system even after activation has been completed.

Previous studies had demonstrated that TRH induced phosphatidylcholine, but not phosphatidylinositol, synthesis via protein kinase C [15]. Studies were therefore performed to determine the effect of the cyclic AMP system on these parameters. The left column of Table 2 shows that PMA (10 nM) and TRH (0.1 μM) similarly enhanced $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine at 30 min, to 140 $\pm$ 8% and 149 $\pm$ 5% of control respectively. CT decreased basal incorporation slightly, to 89 $\pm$ 5% of control, and markedly inhibited the effect of PMA

Fig. 2. Effect of CT on $[^{32}\text{P}]\text{P}_i$ incorporation into phosphatidylcholine stimulated by various concentrations of diC_8

These studies were performed as described in Table 3. The level (mean $\pm$ S.E.M.) of $[^{32}\text{P}]\text{phosphatidylcholine}$ in control incubations was 2632 $\pm$ 96 d.p.m./ 10^6 cells. These data represent duplicate determinations from three experiments. Symbols: □, diC_8 ; ▨, diC_8 + CT.

Fig. 3. Effect of Bt₂cAMP on [³²P]P_i incorporation into phosphatidylcholine stimulated by various concentrations of diC₈.

These studies were performed as described in Table 3 with a 5 min preincubation with Bt₂cAMP. The level (mean ± S.E.M.) of [³²P]phosphatidylcholine in control incubations was 4584 ± 578 d.p.m./10⁶ cells. These data represent duplicate determinations from three experiments. Symbols: □, diC₈; ▨, diC₈ + Bt₂cAMP.

($P < 0.001$) and TRH ($P < 0.001$). In contrast, the right column of Table 2 shows that CT did not affect the TRH-induced increase in [³²P]P_i incorporation into phosphatidylinositol to 338 ± 21 % of control. As demonstrated previously, PMA had no effect on incorporation of label into phosphatidylinositol [15]. Hence, inhibition by CT was specific for phosphatidylcholine synthesis.

Subsequent studies ($n = 4$) compared the effects of maximal concentrations (0.1 μM) of PMA and TRH with those of a maximal concentration of the 1,2-diacylglycerol diC₈ (30 μg/ml). Previous studies demonstrated that diC₈, like PMA and TRH, stimulated phosphatidylcholine synthesis via the CDP-choline pathway. This did not result from utilization of the 1,2-diacylglycerol as substrate for the CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) reaction, as no diC₈-phosphatidylcholine was formed [7]. Table 3 demonstrates that PMA and TRH increased [³²P]P_i incorporation into phosphatidylcholine to 1.5- and 1.4-fold of control, respectively, whereas diC₈ stimulated a greater increase, to 2.2-fold of control at 30 min of stimulation. In seven additional studies, diC₈ similarly stimulated a larger effect than TRH (2.7- versus 1.6-fold of control) at 45 min of stimulation. In contrast with the effects of PMA and TRH, this effect was not inhibited by CT. Thus the effect of diC₈ on phosphatidylcholine synthesis contrasts with the effects of PMA and TRH.

Further studies examined whether CT might inhibit the effect of submaximal concentrations of diC₈. In these studies ($n = 3$), cells preincubated with CT (0.1 μM) for 45 min were stimulated with diC₈ (3–30 μg/ml) for an additional 45 min. Fig. 2 demonstrates that diC₈ induced concentration-dependent incorporation of [³²P]P_i into phosphatidylcholine. As little as 3 μg of diC₈/ml increased [³²P]phosphatidylcholine to 150 ± 6 % of control,

Table 4. Lack of additivity of diC₈ and PMA at low concentrations of diC₈.

Cells (3×10^7 cells/ml) were labelled with [³H]choline (20 μCi/ml) in a buffered saline solution for 45 min and resuspended in buffer (5.5×10^6 cells/ml) without radiolabel containing dimethyl sulphoxide, diC₈ or diC₈ with PMA (0.1 μM). After 45 min, cell pellets were extracted with 1 ml of chloroform/methanol/HCl (100:100:1, by vol.) and 0.3 ml of buffered saline solution containing 10 mM-EDTA. Portions of the organic phase were dried under N₂ and resolved by t.l.c. with chloroform/methanol/acetic acid/water (50:30:8:3, by vol.) as solvent. Phosphatidylcholine was identified by iodine-vapour staining and quantified by liquid-scintillation counting. The data (means) represent triplicate determinations from five experiments. The [³H]phosphatidylcholine background values in control and PMA-containing incubations were 6632 ± 580 and 10346 ± 640 d.p.m./10⁶ cells respectively. These means have been subtracted from the respective values within each column. The values in parentheses represent the percentages of the expected recovery (the effect of diC₈ in the PMA column divided by the control column).

DiC ₈ (μg/ml)	[³ H]Phosphatidylcholine (d.p.m. above background/10 ⁶ cells)	
	Control	PMA
0.5	1260	530 (42)
1.0	1990	1061 (53)
3.0	3117	1857 (59)
10.0	6998	5836 (87)
20.0	9348	8091 (87)

an effect quantitatively similar to that induced by maximal concentrations of PMA and TRH. Higher concentrations of diC₈ induced greater increases in radiolabel incorporation. A maximal concentration of diC₈ (30 μg/ml) increased [³²P]phosphatidylcholine to 260 ± 27 % of control. In these studies, CT had no effect on basal incorporation to 102 ± 5 % of control. However, CT inhibited the effect of diC₈ (3 μg/ml) by 80 %, to 109 ± 5 % of control ($P < 0.001$ versus diC₈). Higher concentrations of diC₈ overcame the inhibition by CT except for a portion quantitatively similar to that inhibited at 3 μg of diC₈/ml. These studies demonstrate that CT inhibited the effect of diC₈ at concentrations that mimic the effect of maximal concentrations of PMA and TRH.

Similar studies were performed with Bt₂cAMP (Fig. 3). A maximal concentration of Bt₂cAMP (5 mM) had no effect on basal incorporation of [³²P]P_i into phosphatidylcholine to 103 ± 7 % of control. However, Bt₂cAMP markedly inhibited the effect of 3 μg of diC₈/ml ($P < 0.01$ versus diC₈). Again, this inhibition was overcome by higher diC₈ concentrations, except for a portion quantitatively similar to that inhibited at 3 μg of diC₈/ml. Hence the pattern of inhibition by Bt₂cAMP was similar to that of CT.

Since previous studies that demonstrated that 1,2-diacylglycerols and phorbol esters stimulated additive phosphatidylcholine synthesis were performed with maximal concentrations of diC₈, additional studies were performed to determine whether there might be lack of additivity with low diC₈ concentrations. For these studies, cells were short-term labelled with [³H]choline and treated with diC₈ with or without a maximal concentration of PMA. It was argued that, if low concentrations of diC₈ used the same pathway as PMA, then the effects of these agents would not be additive. Alternately, if these agents acted by separate pathways, additive effects would be expected. The 'Control' column of Table 4 again shows that diC₈ stimulates concentration-dependent incorporation of [³H]choline into phosphatidylcholine.

tidylcholine. The 'PMA' column demonstrates that, in the presence of PMA, diC_8 stimulates a smaller effect at all concentrations. This reduction appears to be near constant at all diC_8 concentrations and is equivalent to 1008 ± 204 d.p.m. (mean $\pm$ S.D.) over the entire range of concentrations. Hence the lack of additivity appears to occur at the lowest concentrations, and thereafter diC_8 and PMA appear additive. Hence by two criteria, cyclic AMP inhibition and lack of additivity, low concentrations of diC_8 appear to be regulated similarly to phorbol esters. In contrast, high diC_8 concentrations appear to act via a separate mechanism.

DISCUSSION

The present study compares and contrasts the effects of two classes of protein kinase C activators, 1,2-diacylglycerols and phorbol esters, on phosphatidylcholine synthesis. Previous studies suggested that PMA and TRH stimulated phosphatidylcholine synthesis via protein kinase C and that 1,2-diacylglycerols utilized a separate pathway. This was based primarily on the apparent additivity of phorbol esters and 1,2-diacylglycerols on phosphatidylcholine synthesis and the persistent effect of 1,2-diacylglycerols in cells down-modulated for protein kinase C. In the present study, differences between these agents were again observed; however, some similarities were also found. These studies show that stimulated phosphatidylcholine synthesis was inhibitable by the cyclic AMP pathway. Three different activators of the cyclic AMP system were utilized: the cyclic AMP analogue Bt_2cAMP , the adenylate cyclase activator CT, and the peptide hormone VIP. All three agents similarly inhibited phorbol-ester- and TRH-stimulated phosphatidylcholine synthesis. The cyclic AMP system also inhibited 1,2-diacylglycerol-induced phosphatidylcholine synthesis at concentrations that produced an effect quantitatively similar to that of PMA and TRH. However, higher concentrations of 1,2-diacylglycerols overcame the cyclic AMP inhibition. Similarly, low concentrations of 1,2-diacylglycerols did not stimulate additive phosphatidylcholine synthesis with phorbol esters, whereas higher concentrations appeared to do so. Hence, low concentrations of 1,2-diacylglycerols appear to act similarly to phorbol esters and TRH, and higher concentrations appear to act via a pathway unavailable to these other agents.

This effect of the cyclic AMP system may be specific for phosphatidylcholine, since phosphatidylinositol synthesis stimulated by TRH was not affected. Phosphatidylinositol synthesis has been shown to be independent of protein kinase C [15]. Imai & Gershengorn demonstrated that a short-term incubation with Bt_2cAMP also failed to inhibit TRH-induced phosphoinositide degradation by a phospholipase C [18]. Hence, both aspects of receptor-stimulated phosphoinositide metabolism, synthesis and degradation, appear to be independent of regulation by the cyclic AMP system. These studies demonstrate that the cyclic AMP system dissociates phosphatidylcholine from phosphatidylinositol synthesis during TRH action. The importance of this observation to receptor-mediated signal transduction via the phosphoinositide pathway is currently unknown. This is the first report of inhibition of receptor-stimulated phosphatidylcholine synthesis via the cyclic AMP pathway.

The cyclic AMP system has been shown to antagonize protein kinase C-mediated events at various different stages in the signal-transduction process (for review see [1]). For instance, inhibition of receptor-mediated activation of protein kinase C in platelets, lymphocytes and mast cells resulted from cyclic AMP-induced down-modulation of specific cell-surface receptors [1]. Hence, inhibition occurred at a step proximal to activation of protein kinase C. In the present study, however, inhibition of phosphatidylcholine synthesis appears to have occurred at a step distal to protein kinase C activation.

This is evident since the cyclic AMP system antagonized phosphatidylcholine synthesis after redistribution of the cytidylyltransferase had been completed [15] and after the majority of the protein kinase C had been down-regulated [19].

Differences in the pattern of events induced by 1,2-diacylglycerols and phorbol esters have been noted previously (for review see [1]). It has been suggested that this reflected the more rapid cellular metabolism of 1,2-diacylglycerols, resulting in attenuation of the 1,2-diacylglycerol effect [1]. However, previous studies from my laboratory demonstrated that 1,2-diacylglycerols induced a different set of events from phorbol esters, despite persistently high cellular diacylglycerol levels [19]. Similarly, 1,2-diacylglycerols failed to mimic phorbol-ester-induced conversion of HL-60 cells into macrophages, despite addition of the 1,2-diacylglycerols as often as every hour [20,21]. One possible explanation for some differences between these agents is the activation by 1,2-diacylglycerols, but not by phorbol esters, of a potential inhibitory pathway for protein kinase C involving the action of a sphingomyelinase [7,19,22,23]. The present studies suggest that 1,2-diacylglycerols stimulate another pattern of events, in contrast with phorbol esters. This involves the stimulation of a single event by two separate mechanisms, one involving protein kinase C and the other presumably independent of protein kinase C. In this regard, preliminary studies (R. N. Kolesnick, unpublished work) suggest that the cytidylyltransferase may be activated by 1,2-diacylglycerols by more than one mechanism.

In sum, phorbol esters, TRH and low concentrations of 1,2-diacylglycerols appear to act via a similar mechanism, presumably protein kinase C, to stimulate phosphatidylcholine synthesis in GH_3 cells. In contrast, high concentrations of 1,2-diacylglycerols appear to act via a separate, as yet undefined, mechanism. How this latter event might relate to conditions of abnormal 1,2-diacylglycerol metabolism, such as might occur during cellular transformation by various oncogenes [24,25], remains to be determined.

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