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INTERRUPTION OF TPA-INDUCED SIGNALS BY AN ANTIVIRAL AND ANTITUMORAL XANTHATE COMPOUND: INHIBITION OF A PHOSPHOLIPASE C-TYPE REACTION

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The effect of tricyclodecan-9-yl-xanthogenate on the phorbolester TPA induced changes in phosphatidylcholine metabolism was investigated. In the simultaneous presence of the xanthate TPA failed to stimulate the metabolic [^{32}P] turnover of the major phospholipids. The precursor molecule [^{3}H] choline was incorporated into phosphatidylcholine after pulse labeling in TPA/D609-treated cells. Thus, the reduction of the [^{32}P] phosphatidylcholine turnover did not appear to result from an inhibition of the TPA-stimulated phosphatidylcholine biosynthesis. However, the xanthate exerted an inhibitory effect on the TPA-stimulated liberation of [^{3}H] phosphorylcholine from [^{3}H] phosphatidylcholine in cells prelabeled with [^{3}H] choline. Furthermore, the TPA-induced rise in the diacylglycerol level was reduced in the presence of the compound. Thus, these results provide evidence that the xanthate inhibits a TPA-induced phospholipase C activity in the intact cell.

The tumor-promoting phorbolester TPA elicits various biological effects in different cell types. TPA is known to bind to the protein kinase C (pkC), thereby activating the enzyme which, in turn, phosphorylates substrate proteins that are involved in growth processes (1, 2). Furthermore, cells respond with a rapid change in their phospholipid metabolism early after the TPA challenge. The rapid acceleration of PC turnover has been noted particularly (3, 4). TPA stimulates both the de novo biosynthesis of PC (5, 6) and phospholipase C (PLC)-catalyzed degradation (6 - 10). Some of the TPA-induced responses have been found to be inhibited by the antivirally (11) and antitumorally active compound, tricyclodecan-9-yl-xanthogenate, coded D609. In vivo, in the initiation promotion carcinogenesis model in the mouse skin (12), D609 treatment resulted in the inhibition of tumor promotion. Both the tumor rate and the yield were reduced by 80% and 97%, respectively (13). In vitro, TPA failed to stimulate the phosphorylation of a number of proteins, while some pkC substrates were still phosphorylated in the presence of the compound (14). This report describes the effect of xanthate on TPA-modulated PC metabolism in cultured cells.

Abbreviations

BME: Eagle's basal medium; CH: choline; CDP-choline: cytidyldiphosphocholine; DAG: diacylglycerol; D600: propylxanthate; D609: tricyclodecan-9-yl-xanthogenate; PA: phosphatidic acid; PC: phosphatidylcholine; PCH: phosphorylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; pkC: protein kinase C; PLC: phospholipase C; PLD: phospholipase D; PS: phosphatidylserine; TPA: 12-O-tetradecanoyl-phorbol-13-acetate.

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MATERIALS AND METHODS

Cell culture and media: Human epidermal carcinoma cells (A431) and primary hamster embryo fibroblasts (HEF) were cultivated as described (11, 14).

Chemicals: 12-O-tretradecanoyl-phorbol-13-acetate was kindly provided by Dr. E. Hecker. Tricyclodecan-9-yl-xanthate and propylxanthate were synthesized according to published methods (15) and kindly provided by Merz & Co., Frankfurt. [^{32}P] orthophosphate ([^{32}P] Pi) and [methyl-^{3}H] choline chloride (specific activity: 80 Ci/mmol) were obtained from Amersham, Buchler, FRG.

Treatment of cells: A431 cells were seeded in Eagle's basal medium, containing 10% fetal bovine serum and 1% each of penicillin and streptomycin at an initial density of 2.5 x 10^5 cells/6 cm dish. 48 h later, the medium was changed and the cells were incubated in BME, 0.5% FBS, pH 7.0, for a further 16-24 h. The initial density of HEF was 4 x 10^5 cells/6 cm dish. 48 h after seeding, incubation of the monolayers with the serum-deprived medium was started for a further 2 days. During the serum-starvation period, the cultures were prelabeled with [^{3}H] choline for either 1, 4 or 16 h (10 µCi/ml). Prelabeling with [^{32}P] Pi was done for 1 h with 20 µCi/ml as described (14). Then the cultures were washed twice prior to stimulation with TPA in fresh BME 0.5% FBS, pH 7.0, for 1 h with or without xanthates. TPA, D609 and D600 were used at final concentrations of 0.1 µM, 30 µg/ml, if not stated otherwise, and 17.8 µg/ml, thus a molarity equal to D609, respectively.

Extraction of phospholipids: For harvest, cells were placed on ice and washed twice with cold phosphate-balanced salt solution. Cells were scraped from the dishes and collected by centrifugation. Total lipids were extracted by the method of Bligh and Dyer (16). The lipids in the combined organic phases were dried in vacuum and resuspended in 50 µl $CHCl_3/CH_3OH$ (1:2, v/v).

Extraction of water-soluble phospholipid metabolites: For analysis of the intracellular phospholipid metabolites, the upper aqueous phases of the total lipid extracts were transferred to fresh tubes, proteins were pelleted after ethanol precipitation, and the supernatants were dried in vacuum. Extracellular phosphorylcholine was recovered from the culture medium. Proteins were precipitated by ethanol and pelleted. The supernatant was harvested and dried in a vacuum. The pellets were dissolved in aqua bidest for chromatography.

Determination of diacylglycérol: A radioenzymatic assay employing diacylglycerol kinase (Amersham) was used for the quantitative determination of DAG under defined mixed micell conditions to solubilize the DAG. The enzyme converts DAG to [^{32}P] phosphatidic acid with [^{32}P-γ] ATP (spec. activity: 0.5 Ci/mmol). The assay was performed according to the description of the supplier. [^{32}P] phosphatidic acid was extracted and subsequently separated from other lipids and residual [^{32}P-γ] ATP by thin-layer chromatography, using phosphatidic acid (Sigma) as reference. The radioactivity attributable to [^{32}P] phosphatidic acid was estimated by radiodensitometry (Linear Analyzer, Berthold) and by liquid scintillation counting.

Thin-layer chromatography: Silica gel plates (60, F254) from Merck were used for separation. Chromatography was carried out as indicated in the figure legends. Radioactive metabolites were detected either by autoradiography or detected and quantitated by radiodensitometry with a Berthold linear analyzer.

RESULTS

Effect of D609 on TPA-induced [^{32}P] phospholipid metabolism

To evaluate whether D609 has any effect on TPA-stimulated metabolic turnover of the major phospholipids, [^{32}P] Pi incorporation in HEF or A431 cells was examined. [^{32}P] Lipid extracts from [^{32}P] Pi prelabeled quiescent cells, TPA-stimulated cells, and TPA/D609-treated cells were compared by performing TLC analysis, autoradiography and radiodensitometric quantitation (Fig. 1). As already documented for other cell types (9), treatment of HEF for 1 h with TPA resulted in an increase in the rate of [^{32}P] incorporation into PC, PE, PI, PA and one unidentified species (lane 2). The factor of stimulation over control values from quiescent cells (lane 1) in the

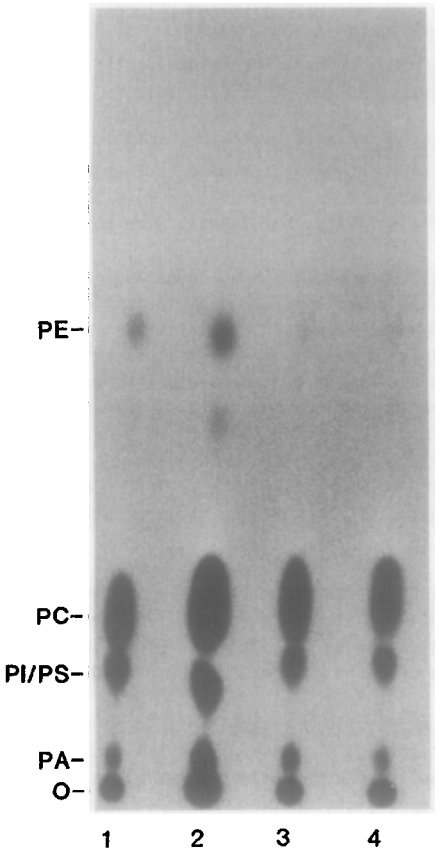

Figure 1: Inhibition of TPA-induced [^{32}P] phospholipid turnover in the presence of D609. Quiescent [^{32}P] Pi prelabeled HEF were treated with 0.1 µM TPA (lane 2), 0.1 µM TPA/15 µg/ml D609 (lane 3) or 0.1 µM TPA/30 µg/ml D609 (lane 4) for 1 h. Untreated cells served as controls (lane 1). A 5-µl volume of each 50 µl lipid extract in $CHCl_3/CH_3OH$ (1:2, v/v) was analyzed by TLC. Five µl of a mixture of known pure phospholipds was chromatographed as reference (5 mg/ml of each PE, PS, PC, PI and PA). To separate PI from PS, TLC was carried out in two dimensions as described earlier (17). $CHCl_3/CH_3OH$/25% NH_3 (65:35:4, v/v/v) served as the developing solvent (17). Lipids were visualized by iodination prior to autoradiography. The origin is indicated by O. The position of standard lipids is indicated at the left.

experiment described here was 2.6, 2.1, 1.4 and 2.2 for PC, PE, PI and PA, respectively, as determined by radiodensitometry. This TPA effect was not observed in D609-exposed cultures (lanes 3, 4). The amount of [^{32}P] orthophosphate incorporated into the phospholipids of xanthate-treated cells was comparable to that of quiescent cells. Similar results were noted for A431 (data not shown).

TPA-stimulated PC biosynthesis in the presence of D609

To characterize the observed xanthate effect on TPA-stimulated PC metabolism further, PC biosynthesis was measured. Quiescent A431 cells were labeled with the specific precursor molecule [^{3}H] choline for 1 h prior to the addition of TPA and D609 in fresh medium. Subsequently, [^{3}H] lipids and the intra- and extracellular water-soluble [^{3}H] choline metabolites were analyzed (Fig. 2). At this time, 93-100% of the [^{3}H] counts in the lipid phase was associated with PC.

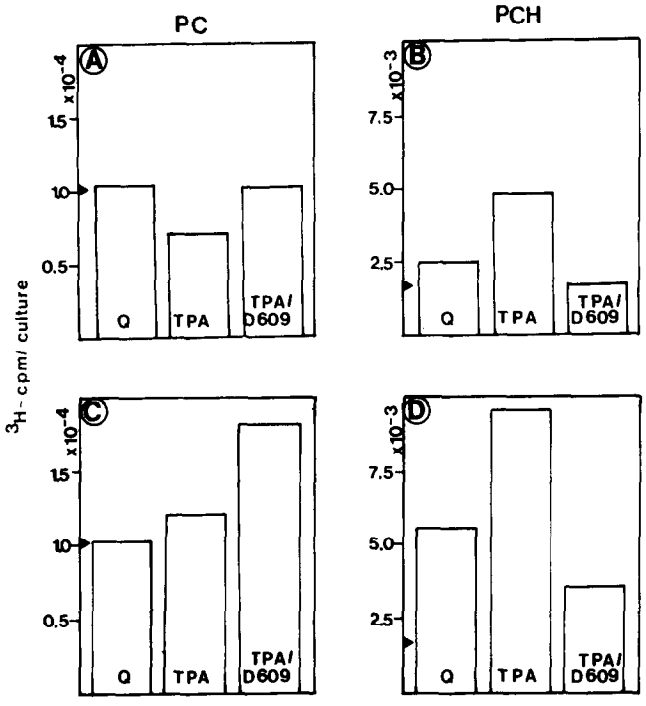

Figure 2: PC synthesis after TPA and TPA/D609 treatment.
Quiescent A431 cells were labeled with 10 µCi/ml [^{3}H] choline in BME, 0.5 % FBS, pH 7.4, washed three times with BME, pH 7.0, and exposed to 0.1 µM TPA or 0.1 µM TPA/30 µg/ml D609. Untreated cultures served as controls (Q). One h (A, B) and 3 h (C, D) after the beginning of treatment, lipids were extracted and analyzed by TLC (A, C). To analyze water-soluble phospholipid metabolites, the culture supernatants (1 ml) were collected (B, D). Components were separated by development in a solvent of either 2.7 M NH_4-acetate/96% ethanol (3:7, v/v) or 0.9 % NaCl/CH_3OH/NH_4OH (50:70:5, v/v/v) as described (6, 17). Choline, phosphorylcholine and CDP-choline (Sigma) served as references and were visualized by iodination. Arrows indicate the cpm at the beginning of treatment.

One h after the beginning of treatment with TPA, the rate of [^{3}H] PC degradation was obviously higher than the rate of de novo biosynthesis, since the level of [^{3}H] PC was reduced compared to that of quiescent and TPA/D609 treated cells (Fig. 2A). In parallel, the amount of extracellular phosphorylcholine (PCH) had increased upon TPA treatment (Fig. 2B). Three h after the beginning of TPA challenge, the degree of [^{3}H] incorporation into both [^{3}H] PC and extracellular [^{3}H] phosphorylcholine was increased compared to the rate in quiescent cells (Fig. 2C, D). Hence, both processes, PC hydrolysis and PC biosynthesis, had taken place simultaneously in TPA-exposed cells. In contrast, simultaneous D609 treatment precluded within the first h the disappearance of label from [^{3}H] PC and its appearance in extracellular phosphorylcholine (Fig. 2A, B). During the next 2 h of exposure to TPA/D609 [^{3}H] PC accumulated. The amount of [^{3}H] radioactivity, either released as phosphorylcholine or incorporated into PC upon TPA stimulation, was nearly equivalent to the sum of radioactivity associated with PC and phosphorylcholine in TPA/D609-treated cells. Thus, D609 did not appear to inhibit [^{3}H] PC synthesis, but apparently PC degradation (resulting in the accumulation of phosphorylcholine) was affected by the xanthate.

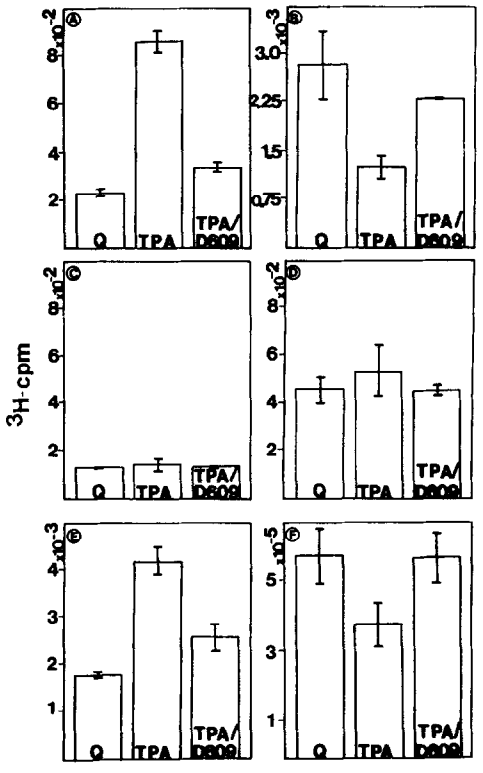

Figure 3: Effect of D609 on the TPA-induced production of [^{3}H] choline metabolites. Quiescent [^{3}H] choline-prelabeled A431 (10 µCi/ml, 4 h, 1.75 x 10^6 cells/6 cm Petri dish) and HEF (5 µCi/ml, 16 h, 0.62 x 10^6 cells/6 cm Petri dish) were washed three times with BME, pH 7.0, prior to 1-h treatment with 0.1 µM TPA or 0.1 µM TPA/30 µg/ml D609 in fresh BME, 0.5% FBS, pH 7.0. Untreated cultures served as controls (Q). A one-tenth volume of the extracts of [^{3}H] lipids and water-soluble intra- and extracellular [^{3}H] choline metabolites was separated by TLC. Detection and quantitation were done by radiodensitometry. The mean values from three independent cultures are given. Bars indicate standard error. A - D: A431; A: PCH extracellular; B: PC; C: CH extracellular; D: PCH intracellular; E, F: HEF; E: PCH extracellular; F: PC. Unlabeled PCH, CH, CDP-choline and PC served as references and were visualized by iodination.

Effect of D609 on the TPA-stimulated release of [^{3}H] phosphorylcholine

The release of [^{3}H] phosphorylcholine from the long-term [^{3}H] choline prelabeled PC pool was next investigated. Again, as described above, after 1 h exposure to TPA or TPA/D609, the [^{3}H] choline metabolites were isolated. TPA stimulated the release of [^{3}H] phosphorylcholine into the medium at a rate of 3.4 times the control value derived from quiescent cultures (Fig. 3A). In addition, the experiment established that TPA did not stimulate the release of [^{3}H] choline significantly (Fig. 3C). Concomitantly, in the corresponding TPA-exposed cultures, [^{3}H] choline radioactivity decreased in the PC fraction by 55% (Fig. 3B). These findings suggest that in A431 cells TPA stimulated PC phoshodiester hydrolysis by phospholipase of the C-type rather than by a phospholipase D-type reaction. In this experiment, simultaneous TPA/D609 treatment again led to marked inhibition of the liberation of [^{3}H] PCH (Fig. 3A). However, no reduction in the level of [^{3}H] choline could be found. These effects corresponded with a reduced rate in [^{3}H] PC degradation (Fig. 3B). Similar results were also obtained with primary hamster embryo fibroblasts (Fig. 3E, F). As in other test systems (13), propylxanthate was found to be inactive

Table 1: DAG production in HEF in response to TPA[a] and TPA/D609[b]

D609 µg/ml	TPA	% picomol [^{32}P] PA/ mg protein[c]
0	0	100[d] (± 13.29)
0	0.1 µM	176 (± 20.98)
10	0.1 µM	170 (± 6.97)
20	0.1 µM	152 (± 16.48)
30	0.1 µM	116 (± 20.96)

a: Treatment was for 15 min.
b: D609 treatment was simultaneous with TPA treatment.
c: Separation of [^{32}P] PA and [^{32}P-γ] ATP: the development mixture consisted of $CHCl_3/CH_3OH$/glacial acetic acid (65:15:5, v/v/v). Lipids were iodinated. Quantitation was done by radiodensitometry. One picomol [^{32}P] PA corresponds to one picomol DAG.
d: Mean value of four parallel cultures in one experiment is given; standard errors are given in brackets.

in this assay (data not shown). The reduced amount of [^{3}H] phosphorylcholine in the supernatant of D609-treated cultures may simply be due to curtailment of the metabolite extrusion process and thus give the false appearance of having an inhibitory effect on the phospholipase C reaction. To rule out this possibility, the concentration of intra- and extracellular phoshorylcholine was compared between D609-treated and untreated cultures (Fig. 3A, D). In cells exposed to D609, [^{3}H] phosphorylcholine did not accumulate intracellularly (Fig. 3D). The level of this cleavage product was nearly equal to that found in quiescent cells.

Effect of D609 on TPA-stimulated production of diacylglycerol

To estimate the DAG levels in the presence and absence of D609 after TPA stimulation, total lipids were extracted. DAG was quantitated in vitro under mixed micelle conditions, using bacterial DAG kinase, which catalyzes the phosphorylation of DAG to phosphatic acid with [^{32}P-γ] ATP as phosphate donor. Compared to the level in quiescent cultures, the DAG concentration was elevated 1.76 times after TPA treatment for 15 min (Table 1). When increasing concentrations of D609 and TPA were added to the cultures, the TPA stimulus effect decreased proportionately. In cultures exposed to 30 µg/ml D609, the level was reduced to nearly that of quiescent cultures. Thus, this experiment provided additional evidence for the inhibition of a phospholipase C-type reaction.

DISCUSSION

As shown in this report, xanthate D609 influences the TPA-induced phosphatidylcholine turnover. In addition to PC degradation by PLC catalysis (7-10, 18), agonist-induced PC catabolism by phospholipase D has been reported to occur (19, 20). In A431 and HEF, TPA seemed to stimulate PC hydrolysis by PLC since the two cleavage products DAG and phosphorylcholine appeared. In the experiments described here and in kinetic studies (data not shown), no clear-cut increases in the choline levels and phosphatidic acid were measured to support a PLD-type reaction (cells were prelabeled with either glycerol, arachidonic acid or lyso-PC). The 2.2-fold and 1.4-fold increase in [^{32}P] incorporation into PA and PI, respectively, found in TPA stimulated cells can

be explained by the recycling of DAG (originating from PC) to PA which, in turn, serves as precursor for the synthesis of PI. This possibility has also been mentioned in other reports (21).

The present study revealed that xanthate inhibited the TPA-stimulated liberation of phosphorylcholine. Moreover, the compound reduced the TPA-stimulated rise in the DAG level. These two effects were accompanied by the concomitant inhibition of the disappearance of label from [^{3}H] PC. Thus, in intact cells D609 presumably inhibits TPA-induced PC-degrading PLC. The compound failed, however, to prevent PC biosynthesis, which is also stimulated by TPA. Moreover, as has been shown recently, in the presence of D609 bacterial phospholipase C does not function when added extraneously to cultured cells. Phospholipase C failed to stimulate the pkC-mediated phosphorylation of proteins (14). Thus, these experiments provide additional support for the inhibitory effect of D609 on phospholipid breakdown catalyzed by a phospholipase C. It cannot be excluded, that D609 may also act on EGF-induced phosphoinositide-specific PLC(s) generating DAG which, in turn, activates pkC (22, 23). D609 interrupts the EGF-induced signal-transmission in A431, thereby leaving the intrinsic tyrosine kinase of the EGF receptor unaffected. However, pkC activation is inhibited while the pkC itself seems to function (14).

Although the biological significance of TPA-stimulated PC degradation by phospholipase C is not yet well understood, it is conceivable that the diacylglycerol arising may serve as a second messenger molecule. It may activate one or more pkC isoenzymes (24) and thus enhance or bifurcate the original signal induced when TPA binds to pkC. The data presented here may explain some of the D609 effects seen in vitro and in vivo in therapeutical approaches, such as the inhibition of TPA-induced promotion in the two-stage carcinogenesis model (12, 13). It might be that xanthate only shows a selective inhibitory activity in TPA-induced processes that depend on prior PC degradation by PLC whereas the processes that occur directly via pkC remain unaffected. The interruption of agonist- (TPA and perhaps growth factor) induced signals at the level of PLC catalyzed PC breakdown appears to be a novel activity mechanism that is displayed by a pharmaceutically active compound, such as xanthate D609 with chemopreventive properties in vivo (13), as well as antiviral (11) and antiproliferative (25) properties.

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