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**SHORT COMMUNICATION**

# Stimulation of DNA Synthesis in Untransformed Cells by the Antiviral and Antitumoral Compound Tricyclodecan-9-yl-xanthogenate (D609)

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**ABSTRACT.** The antiviral and antitumor xanthate compound tricyclodecan-9-yl-xanthogenate (D609) is best known for its inhibitory effect on phosphatidylcholine-specific phospholipase C activity. Now we report that in NIH 3T3 cells, but not in several transformed cell types tested, D609 stimulated DNA synthesis when phosphocholine (PCho), insulin, or ATP was also present. Maximal co-mitogenic effects of D609 were observed at 5 μg/mL, a concentration 4–6 times lower than that required to inhibit phospholipase C activity. The synergistic mitogenic effects of D609 and PCho, but not of D609 and insulin, were associated with activation of p42 and, to a lesser extent, p44 mitogen-activated protein (MAP) kinases. The results raise the possibility that the mitogenic activity of D609 in untransformed cells may contribute to its antiviral and antitumor effects. BIOCHEM PHARMACOL **55**;6:915–918, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** compound D609; DNA synthesis; fibroblasts; MAP kinase

D609‡, an antiviral xanthate compound, was characterized initially as an antiviral [1, 2] and antitumoral [3–5] agent that also inhibits protein kinase C activity [6]. More recently, relatively high (20–30 μg/mL) concentrations of D609 were found to inhibit phospholipase C-mediated hydrolysis of phosphatidylcholine as well [5]. Although D609 is also known to inhibit phospholipase D activity [7, 8], several laboratories have used D609 to determine the role of phospholipase C in a variety of cellular mechanisms, including activation of NF-κB [9], transforming growth factor-β signaling [10], Fas/APO-1-induced apoptosis [11], regulation of mitochondrial permeability transition [12], coupling of Ras to Raf-1 activation [13], activation of Raf-1 by tumor necrosis factor-α [14], mitogen-induced hyperphosphorylation of Raf-1 [15], mobilization of cholesterol [16], high-density lipoprotein$_3$-induced signaling [17], and regulation of induction of slow synaptic excitation [18].

Recently, our laboratory found that PCho, the water-soluble product of phospholipase C-mediated phosphatidylcholine hydrolysis, has significant effects on DNA synthesis in NIH 3T3 fibroblasts [19]. While PCho alone is a relatively weak mitogen, it can stimulate DNA synthesis in synergism with insulin, ATP, or sphingosine-1-phosphate [19]. In an effort to determine the possible role of endogenous phospholipase C in cell growth regulation, we have also attempted to use D609 to block this enzyme activity. However, during the course of this work we found that D609 exhibits potent co-mitogenic effects. In particular, as we report here, relatively low (2.5 to 5 μg/mL) concentrations of D609 were found to stimulate DNA synthesis in synergism with PCho and insulin.

## MATERIALS AND METHODS

### *Materials*

D609 was bought from the Kamiya Biomedical Co.; PCho and ATP were purchased from the Sigma Chemical Co.; insulin was from Boehringer Mannheim; a PhosphoPlus MAP kinase kit was bought from New England Biolabs, Inc.; [*methyl*-$^3$H]thymidine (85 Ci/mmol) was purchased from NEN DuPont; and tissue culture reagents were from Life Technologies.

### *Cell Culture*

NIH 3T3 clone-7 fibroblasts, obtained from Dr. Douglas R. Lowy (National Cancer Institute, NIH), were cultured continuously in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, penicillin (50 U/mL), streptomycin (50 μg/mL), and glutamine (2 mM).

### *Labeling of Cellular DNA with [$^3$H]Thymidine*

NIH 3T3 cells were grown in 12-well tissue culture plates in the presence of 10% serum to about 40% confluency,

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‡ *Abbreviations:* D609, tricyclodecan-9-yl-xanthogenate; PCho, phosphocholine; and MAP, mitogen-activated protein.
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followed by incubations in serum-free medium for 24 hr. Cells were washed again, and then treated (in serum-free medium) with D609 for 20 min, with PCho or ATP for 10 min, and then with insulin (in the presence of the other agents, if applicable) for 16 hr. After treatments, incubations were continued in the presence of [*methyl*-$^3$H]thymidine (1 μCi/well) for 60 min. Next, the cells were first washed two times with absolute ethanol, then four times with 5% trichloroacetic acid, and finally with absolute ethanol. The acid-insoluble material was dissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated $^3$H activity in a liquid scintillation counter.

### *Determination of MAP Kinase Activity*

The method to determine the effects of D609 on MAP kinase activity was described previously [19]. Briefly, serum-starved NIH 3T3 cells were first treated with 5 μg/mL of D609 for 20 min, and then with 1 mM PCho and/or 500 nM insulin (in the continuous presence of D609, when applicable) for 10 min. The activity state of p42/p44 MAP kinases was determined by immunoblot analysis [19]. The phosphospecific MAP kinase antibody employed here recognizes the tyrosine 204 phosphorylation site in the activated forms of both MAP kinases.

## RESULTS AND DISCUSSION

DNA synthesis in serum-starved NIH 3T3 cells was enhanced only slightly by 0.5 to 1 mM PCho in the absence of other agents (Fig. 1). Similarly, 2.5 to 5 μg/mL of D609 alone had modest (3- to 12-fold) stimulatory effects on the incorporation of [$^3$H]thymidine into DNA. However, D609 (2.5 to 5 μg/mL) in combination with PCho induced large increases in DNA synthesis (Fig. 1). Detectable (about 2-fold) synergistic effects in the presence of 1 μg/mL of D609 required the addition of 1 mM PCho (Fig. 1). In contrast, in the presence of 5 μg/mL of D609, the addition of a much lower (0.1 mM) concentration of PCho resulted in a markedly greater (~6-fold) stimulation of DNA synthesis (Fig. 1). In fact, in the presence of 5 μg/mL of D609, 0.1 and 1 mM concentrations of PCho were about 55 and 80% as effective as 10% serum in stimulating DNA synthesis (Fig. 1). PCho did not change the stimulatory effect of 10% serum (Fig. 1). Elevation of the D609 concentration to 10 μg/mL resulted in a large decrease in its co-mitogenic effect in the presence of PCho (data not shown). Thus, the concentration range of D609 that elicits stimulatory effects on DNA synthesis is relatively narrow (1–5 μg/mL).

Next we examined the effects of 5 μg/mL of D609 on DNA synthesis in the presence of 0.1 to 1.0 mM PCho as well as ATP or insulin. In the presence of ATP, the stimulatory effect of D609 on DNA synthesis was increased about 4-fold (Fig. 2). In contrast, ATP did not modify significantly the large synergistic effects of PCho plus D609

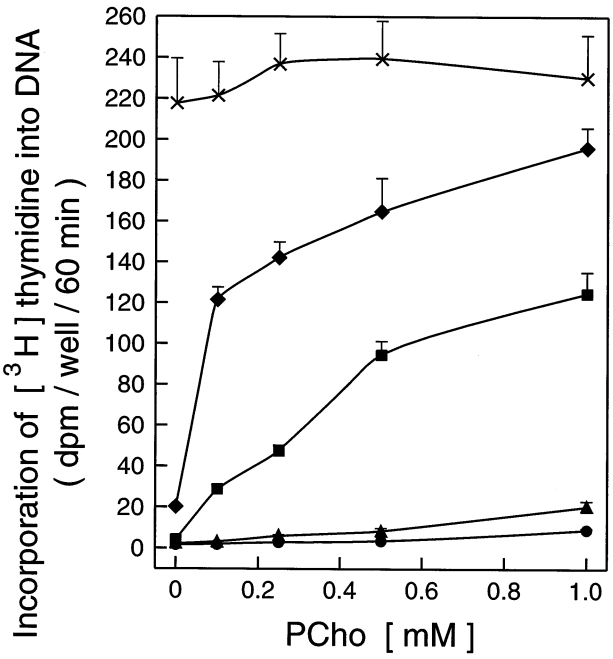


**FIG. 1. Synergistic stimulatory effects of D609 and PCho on DNA synthesis in NIH 3T3 cells. Serum-starved NIH 3T3 cells were untreated (●) or treated with D609 at a 1 μg/mL (▲), 2.5 μg/mL (■), or 5 μg/mL (◆) concentration, or with 10% (v/v) fetal bovine serum (✕) for 20 min, followed by incubations for 16 hr in the presence of 0–1 mM PCho (in the continuous presence of D609, when applicable) as indicated. Values are the means ± SD of six samples in a single experiment. Similar results were obtained in three other experiments, each performed in triplicate.**

(Fig. 2). D609 had equally large stimulatory effects on DNA synthesis in the presence of either insulin or 1 mM PCho; the synergistic effects of D609 plus insulin were enhanced only slightly by co-treatment of cells with 1 mM PCho (Fig. 2). Interestingly, 0.1 to 0.25 mM PCho actually decreased the combined effects of D609 and insulin (Fig. 2), suggesting that D609 enhanced the mitogenic effects of insulin and PCho by different, possibly competing, mechanisms.

Previously, we reported [19] that neither PCho nor insulin, alone or in combination, had significant stimulatory effects on p42/p44 MAP kinase activities (also known as extracellular signal-regulated kinases, or ERKs). Instead, they stimulated DNA synthesis via a pp70 S6 kinase-dependent mechanism [19]. Now we have found that D609 alone also was without effect, but in combination with PCho it greatly enhanced the activity of p42 MAP kinase activity (Fig. 3). D609 and PCho in combination only slightly enhanced p44 MAP kinase activity (Fig. 3). Insulin had practically no effect on MAP kinase activity in the presence of D609, and it also failed to modify the combined effects of D609 and PCho (Fig. 3). In contrast, 5 nM rapamycin, an inhibitor of the function of pp70 S6 kinase [20, 21], had no effect on the actions of D609 and PCho, but it inhibited the combined mitogenic effects of D609 and insulin about 85% (data not shown).

In conclusion, we have shown that D609 can exert large

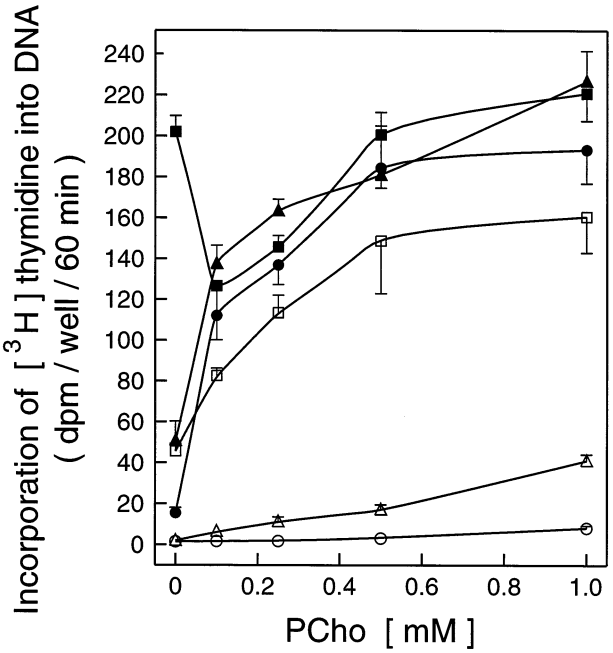


**FIG. 2. Stimulation of DNA synthesis by D609 in the presence of insulin, ATP, and PCho. Serum-starved NIH 3T3 cells were first incubated for 20 min in the absence (open symbols) or presence (closed symbols) of 5 μg/mL of D609, followed by treatments (in the continuous presence [closed symbol] or absence [open symbol] of D609) for 16 hr with 0–1 mM PCho in the absence (○–●) or presence of 100 μM ATP (△–▲), or 500 nM insulin (□–■). Values are the means ± SD of six samples in a single experiment. Similar results were obtained in two other experiments, each performed in triplicate.**

stimulatory effects on DNA synthesis if added to NIH 3T3 cells in combination with PCho or insulin. Only the combined mitogenic effects of D609 and PCho, but not those of D609 plus insulin, are associated with the activation of p42 and, to a lesser extent, p44 MAP kinases. The mechanism of the synergistic effects of D609 and insulin on DNA synthesis remains to be determined, although the observed strong inhibitory effect of rapamycin implicates the role of a pp70 S6 kinase-dependent signal transduction mechanism in the mediation of their effects. In other experiments, D609 in combination with PCho or insulin also had synergistic effects on DNA synthesis in Swiss 3T3

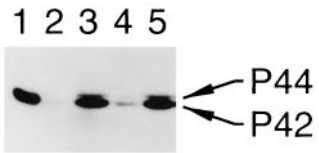


**FIG. 3. Synergistic effects of D609 and PCho on MAP kinase activity. Serum-starved NIH 3T3 cells were first incubated for 20 min in the absence or presence of 5 μg/mL of D609. This was followed by incubations for 10 min in the presence of 1 mM PCho and/or 500 nM insulin. Lane 1 represents the MAP kinase standard, while lanes 2–5 represent treatments with PCho (lane 2), D609 plus PCho (lane 3), D609 plus insulin (lane 4), and D609 plus PCho plus insulin (lane 5). The lower (major) band represents p42 MAP kinase.**

fibroblasts and mouse JB6 epidermal cells but not in Ha-Ras-transformed NIH 3T3 cells or MCF-7 human breast carcinoma cells. This suggests that the co-mitogenic effects of D609 are restricted to untransformed cells. Further experiments are required to examine whether there is a relationship between the co-mitogenic effects of D609 in untransformed cells and their antiviral and antitumoral activities.

Higher (20–50 μg/mL) concentrations of D609, which are required for the inhibition of phospholipase C or D activities [2, 5, 8], exhibited no co-mitogenic effects. Thus, while D609 is clearly not a specific inhibitor of phosphatidylcholine-specific phospholipases, its biological effects observed in the higher concentration range [9–18] may indeed involve inhibition of phospholipid hydrolysis.

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