Ethanolamine analogues stimulate DNA synthesis by a mechanism not involving phosphatidylethanolamine synthesis

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Abstract Dimethylethanolamine (0.5–1 mM), added to serum-starved NIH 3T3 fibroblasts, stimulated DNA synthesis 11–32-fold, and it also greatly enhanced the relatively modest (15–20-fold) mitogenic effect of insulin. Ethanolamine and momomethylethanolamine alone had no effects on DNA synthesis, but they also enhanced the stimulatory effect of insulin, although less effectively than dimethylethanolamine did. Lower concentrations (2.5–5 μg/ml) of compound D 609 (tricyclo-9-yl-xanthogenate), which had no effects on phospholipase activities, synergistically enhanced the combined effects of ethanolamine analogs and insulin on DNA synthesis without affecting the synthesis of ethanolamine phospholipids. These results suggest that ethanolamine and its analogues, formed by phospholipase D-mediated hydrolysis of ethanolamine phospholipids, may have growth regulatory functions independent of their role as phospholipid precursors.

Key words: Ethanolamine analogue; Insulin; Compound D 609; DNA synthesis

1. Introduction

Recent reports from another [1] and our laboratory [2,3] demonstrated that in NIH 3T3 fibroblasts externally added choline phosphate can greatly enhance DNA synthesis particularly in the presence of insulin. Although the significance of this phenomenon still remains to be elucidated, it would be important to know whether other intermediates of phospholipid metabolism can also regulate cell growth, and if yes, how it is related to phospholipid synthesis. Thus, a major goal of this work was to examine the possible mitogenic effects of ethanolamine and its analogs, which can be formed by phospholipase D-mediated hydrolysis of ethanolamine phospholipids [4,5].

The current dogma is that phospholipid precursors are essential for cell growth solely because of the increased need to synthesize phospholipids, important components of biomembranes. Since increased phospholipid synthesis indeed accompanies stimulated cell growth [6], it is difficult to provide evidence that water-soluble intermediates of phospholipid metabolism also affect mitogenesis by an independent mechanism. Such evidence would include the demonstration that a portion of mitogenic activity of phospholipid intermediates is not accompanied by increased phospholipid synthesis.

High (15–35 μg/ml) concentrations of tricyclodecan-9-yl-xanthogenate (compound D 609) has recently been used to inhibit phospholipase C-mediated hydro-lysis of phosphatidylcholine (PtdCho) [7–11]. We have shown that at these concentrations compound D 609 also inhibits phospholipase D activity [12]. However, when we began to use compund D 609 to study the role of phospholipase activities in cell growth regulation, it turned out that at lower concentrations this compound exhibits mitogenic activity. As we will show here, in NIH 3T3 fibroblasts dimethylethanolamine and, to less extents, monomethylethanolamine and ethanolamine each can stimulate DNA synthesis, and compound D 609 is capable of enhancing the mitogenic effects of ethanolamine analogues without altering the rate of synthesis of phosphatidylethanolamine (PtdEtn).

2. Experimental procedures

2.1. Materials

Ethanolamine, monomethylethanolamine, dimethylethanolamine, choline phosphate and Dowex-50W(H^+) were purchased from Sigma; compound D 609 was from Kamiya Biomedical Company; insulin was bought from Boehringer Mannheim; [2-^{14}C]ethanolamine (50 mCi/mmol), [methyl-^{14}C]choline chloride (50 mCi/mmol), and [methyl-^{3}H]thymidine (85 Ci/mmol) were from Amersham; and tissue culture reagents were purchased from GIBCO-BRL.

2.2. Cell culture

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

2.3. Labeling of cellular DNA with [^{3}H]thymidine

NIH 3T3 fibroblasts were grown in 12-well tissue culture dishes to about 30% confluency in the presence of 10% serum, followed by incubation of fibroblasts in serum-free medium for 24 h. Subsequently, fibroblasts were washed and then were treated (in serum-free medium) first with ethanolamine analogues and/or compound D 609 for 20 min, followed by treatments with insulin (500 nM) in the continuous presence of the other agents for 16 h. Finally, incubations were continued in the presence of [methyl-^{3}H]thymidine (1.5 μCi/well) for 60 min. Fibroblasts were washed twice with phosphate-buffered saline and then four times with 5% trichloroacetic acid. The acid-insoluble material was redissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated ^{3}H activity in a liquid scintillation counter.

2.4. Measurement of the synthesis of PtdCho and PtdEtn

NIH 3T3 fibroblasts were grown as above, followed by treatments in serum-free medium with compound D 609 and insulin for 2 h in the presence of [^{14}C]choline (1.12×10^6 dpm/well; 50 μM choline) or [^{14}C]ethanolamine (1.22×10^6 dpm/well; 50 μM ethanolamine). Phospholipids were separated as described earlier [13]. CDP-choline and CDP-ethanolamine were separated from other products on Silica Gel G thin-layer plates using the solvent system of 0.5% NaCl/methanol/concentrated NH_3 (50:50:5, v/v/v). Labeled choline, ethanolamine and their phosphorylated derivatives were separated on Dowex 50W(H^+) columns as indicated earlier [14].

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Abbreviations: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylthanolamine.

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3. Results

We have previously reported [2] that in NIH 3T3 fibroblasts ethanolamine alone has no mitogenic effect, but it can significantly enhance the relatively modest stimulatory effect of insulin on DNA synthesis. As shown in Fig. 1, monomethylethanolamine alone was also an ineffective inducer of DNA synthesis but it potentiated the mitogenic effect of insulin to an even greater extent than ethanolamine did. In contrast, 0.5 and 1 mM concentrations of dimethylethanolamine enhanced DNA synthesis 12- and 33-fold, respectively, even in the absence of insulin (Fig. 1). In addition, dimethylethanolamine more effectively potentiated the mitogenic effect of insulin than the other ethanolamine compounds (Fig. 1). Interestingly, despite the observed differences in their mitogenic potencies, each ethanolamine analogue was maximally effective at 1 mM concentration. Using these compounds at 1.5 or 2 mM concentrations resulted in slightly less mitogenic activity, while 5 mM monomethylethanolamine or diethylethanolamine strongly inhibited the mitogenic effect of insulin (data not shown). We suspect that high concentrations of ethanolamine analogs shift the cellular pH upward to a point which is unfavorable for the mitogenic action of insulin. However, this point remains to be verified.

The combined mitogenic effects of insulin and ethanolamine analogues were inhibited by both 100 nM wortmannin (55–60% inhibition) and 0.5 mM 8-bromo cyclic AMP (~90% inhibition), but not by the protein kinase C inhibitor GF 109203X (data not shown). In contrast, the combined mitogenic effects of choline phosphate and insulin were not inhibited by wortmannin [3]. Thus, ethanolamine analogues and

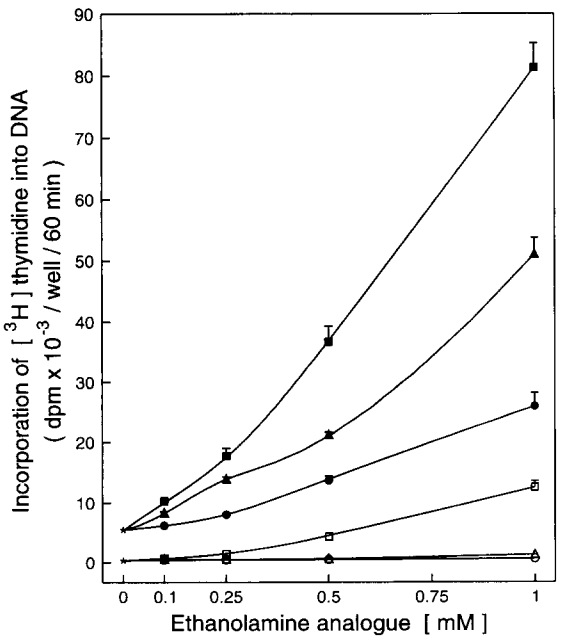

Fig. 1. Concentration-dependent effects of ethanolamine analogues on DNA synthesis in NIH 3T3 fibroblasts. Serum-starved (24 h) subconfluent fibroblasts were treated for 16 h with 0.1–1 mM concentrations of ethanolamine (○–●), monomethylethanolamine (△–▲), or dimethylethanolamine (□–■) in the absence (open symbols) or presence (closed symbols) of 500 nM insulin. Each point represents the mean ± S.E. of six experiments performed with the same passage of cells. These data were confirmed in nine other experiments.

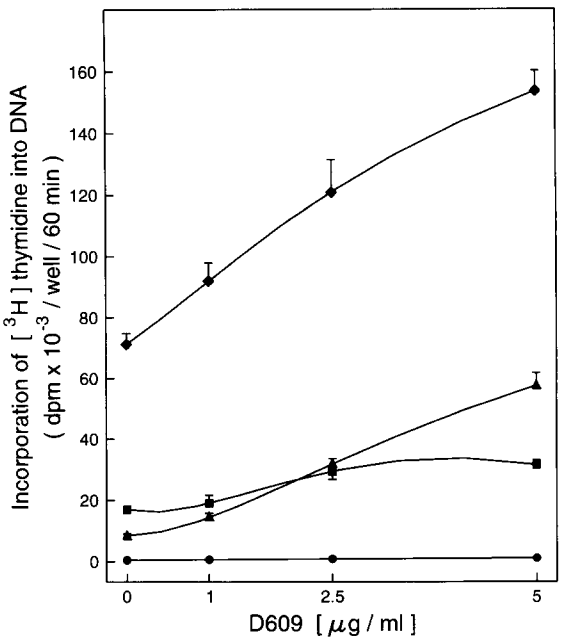

Fig. 2. Concentration-dependent effects of compound D 609 on DNA synthesis induced by choline phosphate and insulin. Serum-starved subconfluent NIH 3T3 fibroblasts were treated with 1–5 μg/ml concentrations of compound D 609 for 16 h in the absence (●) or presence of 500 nM insulin (▲), 1 mM choline phosphate (■), or insulin plus choline phosphate (◆). Each point represents the mean ± S.E. of six experiments performed with the same passage of cells. These potentiating effects of D 609 were confirmed in 15 additional experiments.

choline phosphate potentiate the mitogenic effects of insulin through wortmannin-sensitive and- insensitive mechanisms, respectively.

During this study we found that low (1–5 μg/ml) concentrations of D 609, which do not modify phospholipase activities [12], can greatly enhance the mitogenic effect of insulin; this is shown in Fig. 2. In addition, compound D 609 was also found to potentiate the effect of choline phosphate and the synergistic effects of insulin and choline phosphate on DNA synthesis (Fig. 2). Parallel to their synergistic actions on DNA synthesis, insulin and compound D 609 (5 μg/ml) also enhanced, in a slightly synergistic manner, the synthesis of CDP-choline (Fig. 3A) and PtdCho (Fig. 3B). Since PtdCho synthesis is required for cell cycle progression [15], these latter data may be interpreted to mean that compound D 609 enhanced the mitogenic activity of insulin by increasing PtdCho synthesis. However, this point remains to be verified.

In contrast to PtdCho synthesis, the synthesis of PtdEtn was not affected by compound D 609; this compund actually inhibited the small stimulatory effect of insulin (Fig. 3D). Compound D 609 also failed to modify the formation of PtdCho from PtdEtn through the methylation pathway which was examined in [^{14}C]ethanolamine-labeled fibroblasts (data not shown). Thus, it was of interest to determine whether compound D 609, despite its inability to stimulate PtdEtn synthesis or methylation of PtdEtn, was able to enhance the mitogenic effects of ethanolamine analogues. As shown in Fig. 4, a lower (2.5 μg/ml) concentration of compound D 609 clearly synergistically enhanced the mitogenic effects of each ethanolamine analogue. Similar potentiating effects were also observeable, although they were somewhat less evident, at the

higher (5 μg/ml) concentration of compound D 609 used (Fig. 4). Importantly, in the absence of insulin, 5 μg/ml of compound D 609 also enhanced the mitogenic effects of 0.5 and 1 mM concentrations of dimethylethanolamine 3.2- and 4.1-fold, respectively. Thus, potentiation of the mitogenic effects of this ethanolamine analogue by compound D 609 does not require the presence of insulin.

4. Discussion

A major finding of this paper is that addition of dimethylethanolamine to fibroblasts induced DNA synthesis on its own, and it also greatly enhanced the mitogenic effect of insulin. Many physiological stimuli increase methylation of PtdEtn (reviewed in ref. [16]), and phosphatidyl-*N,N*-dimethylethanolamine is a more effective phospholipase D substrate than PtdCho [17]. This raises the testable possibility that concomitant activation of PtdEtn methyltransferase and phospholipase D can result in the formation of sufficient amounts of dimethylethanolamine to stimulate cell growth. As an example, the protein kinase C activator phorbol 12-myristate 13-acetate has been shown to stimulate the activity of both PtdEtn methyltransferase [18–20] and phospholipase D [4] in fibroblasts and other cell types. Preliminary experiments in this laboratory indicate that potentiation of the mitogenic effect of insulin by phorbol ester [3] can be inhibited by 1 mM 3-deazaadenosine, a well known inhibitor of methyl transferase [21], implying the role of methylated ethanolamine analogs in the mediation of co-mitogenic effects of phorbol ester.

Since ethanolamine and its analogues are incorporated into phospholipids, it would be tempting to ascribe their mitogenic effects to increased phospholipid synthesis. However, compound D 609 was able to potentiate the mitogenic effects of ethanolamine analogues without affecting the synthesis of PtdEtn and its methylated forms. This indicates that ethanolamine analogues can enhance DNA synthesis by a mechanism which is not mediated by increased synthesis of PtdEtn and its methylated derivatives. Collectively, these observations justify further examination of the possible growth regulatory role of dimethylethanolamine in vivo.

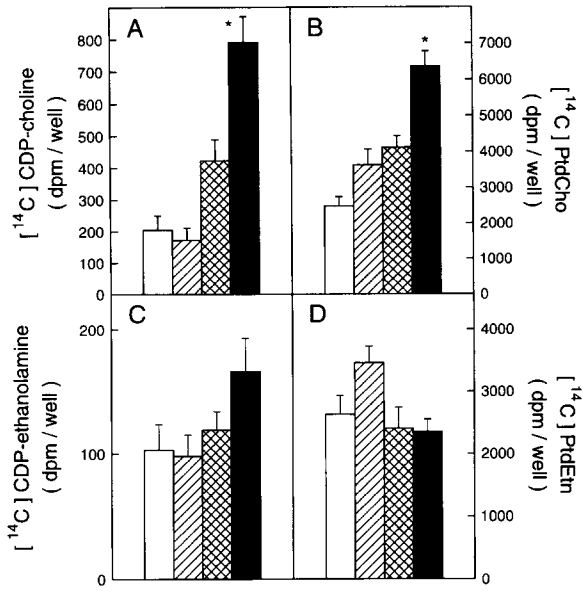

Fig. 3. Effects of D 609 and insulin on the synthesis of PtdCho and PtdEtn in NIH 3T3 fibroblasts. Serum-starved fibroblasts, grown in 12-well culture dishes, were incubated with [^{14}C]choline (A and B) or [^{14}C]ethanolamine (C and D) for 2 h in the absence (open bar) or presence of 500 nM insulin (striped bar), 5 μg/ml of D 609 (cross hatched bar), or insulin plus D 609 (solid bar). Data represent the mean ± S.E.M. of nine experiments performed with the same passage of cells. *P (P<0.01) different from the respective control value (Student's t-test).

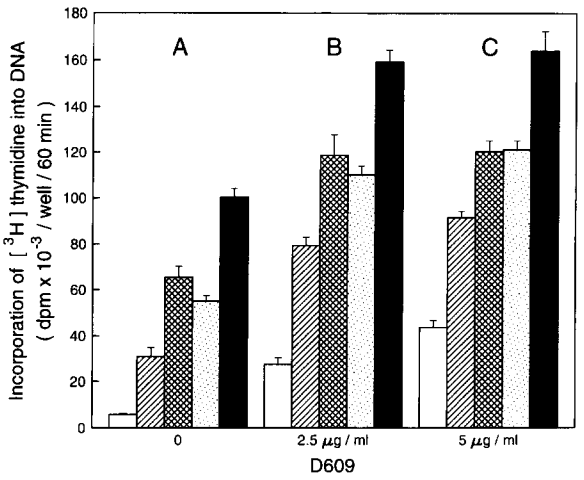

Fig. 4. Combined synergistic effects of ethanolamine analogues, insulin, and compound D 609 on DNA synthesis in NIH 3T3 cells. Serum-starved fibroblasts were treated with 500 nM insulin for 16 h in the absence (open bar) or presence of 1 mM ethanolamine (striped bar), 1 mM monomethylethanolamine (cross hatched bar), 0.5 mM dimethylethanolamine (stippled bar), or 1 mM dimethylethanolamine (solid bar). Compound D 609 was either absent (A), or it was present at a concentration of 2.5 μg/ml (B) or 5 μg/ml. Data represent the mean ± SE of six experiments performed with the same passage of cells. These results were confirmed in six additional experiments.

The mechanism by which ethanolamine analogues enhance DNA synthesis is presently not known. However, considering that growth factor effects are extremely sensitive to changes in intracellular pH, ethanolamine analogues might act by slightly enhancing intracellular pH. We are presently examining this and other possibilities.

Recently, we have demonstrated that concentrations of compund D 609 which inhibit PtdCho-specific phospholipase C activity also inhibit phospholipase D activity [12]. Here we showed that lower concentrations of compound 609 exhibit co-mitogenic effects, which reinforces our previous conclusion that compound D 609 cannot be used as a specific inhibitor of phospholipase C [12]. However, the use of this compound in this work helped to clarify the absence of causal relationship between the mitogenic effects of ethanolamine analogues and phospholipid synthesis. It remains to be seen whether this compound will be similarly useful in the study of other aspects of signal transduction.

In summary, we showed that dimethylethanolamine, which can be formed by the concerted actions of activated methyltransferase and phospholipase D, is a mitogen in NIH 3T3 fibroblasts. Evidence is also presented to indicate that the mitogenic actions of ethanolamine analogues are unrelated, at least in part, to their role as precursors for phospholipid synthesis. Further work is required to determine the possible role of dimethylethanolamine in the regulation of cell growth in vivo.

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