BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **220,** 125–130 (1996)
ARTICLE NO. 0368

Protein Kinase C Inhibitors Enhance the Synergistic Mitogenic Effects of Ethanolamine Analogues and Insulin in NIH 3T3 Fibroblasts

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Received January 22, 1996

Monomethylethanolamine (1 mM) and dimethylethanolamine (1 mM) stimulated DNA synthesis 10- and 15-fold, respectively, in NIH 3T3 fibroblasts. In addition, simultaneous treatments with insulin (500 nM) and methylated ethanolamine analogues (1 mM or less) resulted in synergistic activation of DNA synthesis. The order of mitogenic potency of ethanolamine analogues was dimethylethanolamine > monomethylethanolamine > ethanolamine. Choline (1–5 mM) alone had no effect on DNA synthesis, but it increased the combined effects of lower concentrations of ethanolamine analogues and insulin. The synergistic effects of ethanolamine analogues, choline and insulin were considerably (1.7- to 1.9-fold) enhanced by GF 109203X (3 μM), a specific inhibitor of protein kinase C. The results suggest that ethanolamine analogues enhance insulin-induced DNA synthesis by a mechanism which is inhibited by the protein kinase C system.

In fibroblasts [1] and several other cell types [2–8] activated phospholipase D (PLD) can hydrolyze phosphatidylethanolamine (PtdEtn), in addition to phosphatidylcholine (PtdCho), resulting in the formation of ethanolamine (Etn). In a previous work we have demonstrated that in NIH 3T3 fibroblasts externally added Etn can enhance the mitogenic effect of insulin [9]. Although the potentiating effects of Etn were relatively small, these observations suggested for the first time that Etn may have a direct role in cell growth regulation [9].

Sequential methylation of PtdEtn by *N*-methyltransferases results in the formation of PtdCho as well as two intermediate products, phosphatidyl-*N*,*N*-dimethylethanolamine. Although phospholipid methylation is regulated by many agents [reviewed in ref. 10], the true biological function of these reactions has remained unknown. Importantly, methylated intermediates of PtdEtn can serve as excellent substrates for PLD [11]. Thus, increased phospholipid methylation, followed by the action of activated PLD on methylated PtdEtn species could generate significant amounts of monomethylethanolamine (MMEtn) and dimethylethanolamine (DMEtn). These considerations led us to examine the possible mitogenic activity of MMEtn and DMEtn. Here we show that in NIH 3T3 fibroblasts both compounds stimulate DNA synthesis, and that they also greatly potentiate the mitogenic effect of insulin by a mechanism which is negatively regulated by the protein kinase C (PKC) system.

EXPERIMENTAL PROCEDURES

Materials. [2-^{14}C]Ethanolamine (50 mCi/mmol) and [methyl-^{3}H]thymidine (50 mCi/mmol) were purchased from Amersham; Etn, MMEtn, DMEtn, 8-bromo cyclic AMP, choline phosphate, choline and Dowex-50W[H+] were from Sigma; GF 109203X and staurosporine were from Calbiochem; insulin was bought from Boehringer Mannheim; and tissue culture reagents were purchased from GIBCO-BRL.

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 (DMEM) supplemented with 10% (v/v) fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and glutamine (2 mM).

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Abbreviations used: PLD, phospholipase D; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; Etn, ethanolamine; MMEtn, monomethylethanolamine; DMEtn, dimethylethanolamine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

0006-291X/96 $18.00

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, washed, and then incubated in serum-free medium for 24 h. Then, fibroblasts were treated (in serum-free medium), when applicable, first for 10 min with GF 109203X (3 μM), and then with Etn analogues for 1–40 min, followed by treatments with 500 nM insulin in the continuous presence of other agents for 16 h. Finally, incubations were continued in the presence of [methyl-^{3}H]thymidine (1.0 μCi/well) for 60 min. The cells 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.

Measurement of uptake and metabolism of [^{14}C]Etn in fibroblasts. Fibroblasts were grown in 12-well tissue culture dishes to confluency for about two days in 10% serum-containing medium. This medium was replaced with 700 μl of serum-free (and, thus, Etn-free) and choline-free DMEM and fibroblasts were incubated for 60 min. Finally, wells received unlabeled choline (0.1–20 mM final concentration; applied in 10 μl), and after a 10 min preincubation period incubations were continued in the presence of 50 μM [^{14}C]Etn (10^6 dpm/well; applied in 40 μl) for 30 min. At the conclusion of incubations, the incubation medium was aspirated, then fibroblasts were rapidly (within 20 sec) washed with 5 ml medium. This step was followed by the addition of ice-cold methanol (2 ml) to the wells and by scraping the fibroblasts into methanol. Finally, methanol extracts were rapidly transferred to tubes containing 2-ml chloroform. After phase separation, the water-soluble derivatives of [^{14}C]Etn were separated on Dowex-50W[H+] columns as described earlier [12]. PtdEtn was separated from other phospholipids by thin-layer chromatography [13].

RESULTS

Addition of 1 or 2 mM Etn to serum-starved NIH 3T3 fibroblasts resulted only in a small (~4.5–5-fold) increase in DNA synthesis (Fig. 1A), but 1 mM MMEtn (Fig. 1B) or 1 mM DMEtn (Fig. 1C) stimulated DNA synthesis ~10- and 15-fold, respectively. These compounds exhibited similar differences in their ability to enhance the mitogenic effect of insulin. Thus, while 1 mM Etn enhanced the effect of insulin only ~2.1-fold (Fig. 1A), 1 mM MMEtn (Fig. 1B) and DMEtn (Fig. 1C) increased insulin-induced DNA synthesis ~5.8- and 8.4-fold, respectively. It is noteworthy that well detectable (~2-fold) potentiating effects were obtained with both MMEtn and DMEtn at 0.1 mM concentration.

Choline (1 or 5 mM) itself did not modify the mitogenic effect of insulin, but it considerably enhanced the combined stimulatory effects of both Etn and insulin (Fig. 1A) and MMEtn and insulin (Fig. 1B). Choline also enhanced the combined effects of 0.1–0.5 mM DMEtn and insulin, but it had no consistent effects in the presence of 1 mM DMEtn (Fig. 1C). Choline had no

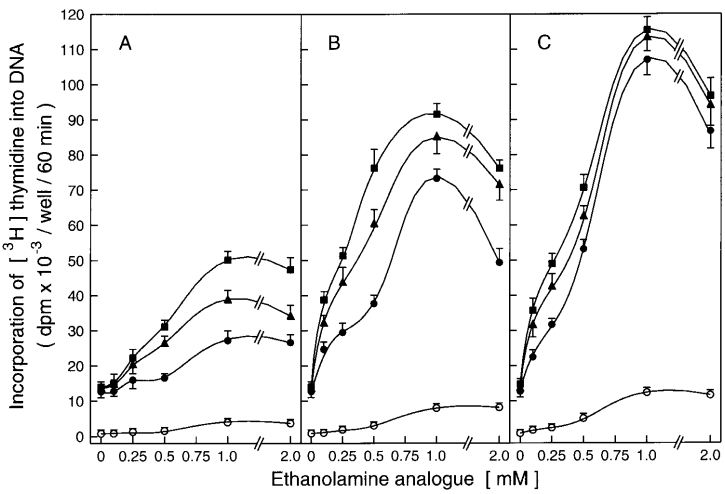

FIG. 1. Combined effects of Etn analogues, insulin and choline on DNA synthesis in NIH 3T3 fibroblasts. Serum-starved (24 h) subconfluent fibroblasts were treated for 16 h with 0–2 mM concentrations of Etn (A), MMEtn (B), or DMEtn (C), in the absence (○) or presence of 500 nM insulin (●), insulin plus 1 mM choline (▲), or insulin plus 5 mM choline (■). Each point represents the mean ± S.E. of six experiments performed with the same passage of cells. Similar results were obtained in six other experiments.

significant effect on DNA synthesis induced by MMEtn or DMEtn in the absence of insulin (data not shown).

Previously, we reported that 1 mM choline phosphate also synergistically enhanced the mitogenic effect of insulin [9]. Despite the fact that both DMEtn and choline phosphate exhibit maximal effects at 1 mM concentration, at this concentration they were able to additively enhance insulin-induced DNA synthesis (Fig. 2). 8-Bromo cyclicAMP, a cell membrane permeable biologically active analogue of cyclicAMP, strongly inhibited the mitogenic effects of both insulin and insulin plus DMEtn (Fig. 2). In contrast, compound GF 109203X, a specific inhibitor of PKC [14], enhanced the combined effects of DMEtn and insulin, but not the effect of insulin alone (Fig. 2). GF 109203X also enhanced (1.6–1.9–fold) the combined effects of Etn or MMEtn and insulin both in the absence and presence of choline (data not shown). Similar potentiating effects were also obtained with staurosporine (1 μM) (not shown), another inhibitor of PKC [15].

Interestingly, 100 nM phorbol 12-myristate 13-acetate (PMA) also synergistically enhanced the effect of insulin on DNA synthesis when the two agents were added together [16], but PMA strongly inhibited the effect of insulin when it was added to the cells 8 h after insulin (data not shown). Presently, PMA plus-insulin induced mitogenesis was slightly enhanced by DMEtn, but the combined mitogenic effects of insulin and DMEtn were almost completely inhibited when PMA was added 8 h after the mitogens (data not shown).

At 5–10 mM concentrations each Etn analogue strongly inhibited insulin-induced DNA synthesis. As shown in Fig. 3, inhibition of the mitogenic insulin effect by 5 mM DMEtn was partially reversed by choline. Choline was the most effective when it was added (together with DMEtn) to cells immediately (1 min) prior to insulin, and it was the least effective when these compounds were added to the cells 40 min before insulin (Fig. 3).

In the absence of choline, GF 109203X was unable to decrease the inhibitory effect of 5 mM DMEtn (Fig. 3). However, co-addition of choline and GF 109203X resulted not only in complete reversal of inhibitory DMEtn effect, but also in a modest increase in insulin-induced DNA synthesis (Fig. 3).

Choline and Etn have been reported to inhibit each other's transport in other cell types [17]. In fibroblasts, high molar excess of choline inhibited [^{14}C]Etn uptake by almost 50%, but choline had

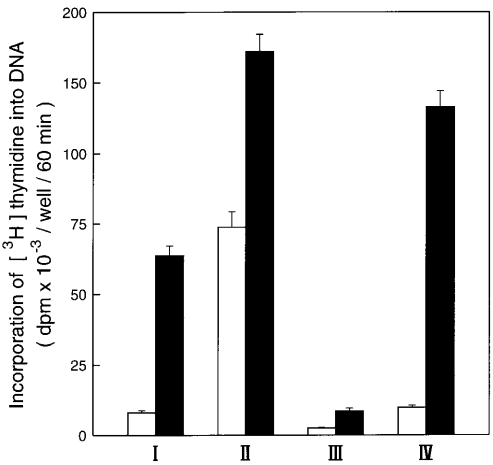

FIG. 2. Effects of choline phosphate, 8-bromo cyclic AMP and GF 109203X on insulin plus DMEtn-induced DNA synthesis. Serum-starved (24 h) subconfluent fibroblasts were treated for 16 h with 500 nM insulin (□) or insulin plus 1 mM DMEtn (■) in the absence (I) or presence of 1 mM choline phosphate (II), 0.5 mM 8-bromo cyclic AMP (III), or 3 μM GF 109203X (IV). Data represent the mean ± S.E. of three experiments performed with the same passage of cells. Similar results were obtained in three other experiments.

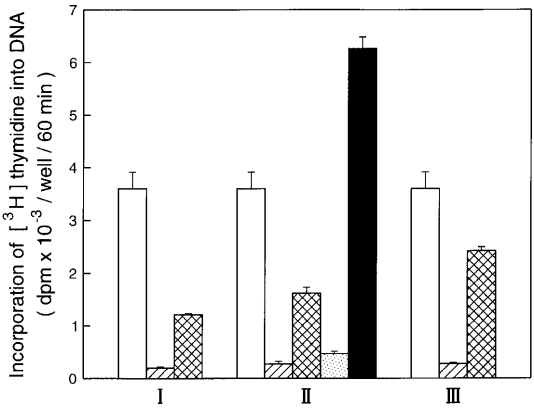

FIG. 3. Reversal of inhibitory DMEtn effect on insulin-induced DNA synthesis by choline and GF 109203X. Serum-starved fibroblasts were treated for 16 h with 500 nM insulin in the absence (□) or presence of 5 mM DMEtn (▨), insulin plus 5 mM DMEtn plus 5 mM choline (▩), insulin plus 5 mM DMEtn plus 3 μM GF 109203X (⬚), or insulin plus 5 mM DMEtn plus 5 mM choline plus 3 μM GF 109203X (■). GF 109203X was added 10 min prior to other additions. DMEtn and choline were added at the same time 40 min (I), 20 min (II), or 1 min (III) prior to the addition of insulin. Data represent the mean ± S.E. of three experiments performed with the same passage of cells. Similar results were obtained in three other experiments.

little, if any, effects on the phosphorylation of Etn and on the synthesis of PtdEtn (Fig. 4). In similar experiments, GF 109203X had no effect on the cellular uptake and/or metabolism of [^{14}C]Etn (data not shown).

DISCUSSION

DMEtn and MMEtn can be formed by the simultaneous actions of activated PtdEtn-*N*-methyltransferase and PLD, while Etn is produced by PLD-mediated hydrolysis of PtdEtn, the second major cellular phospholipid. Because the activity of both methyltransferase [10] and PLD

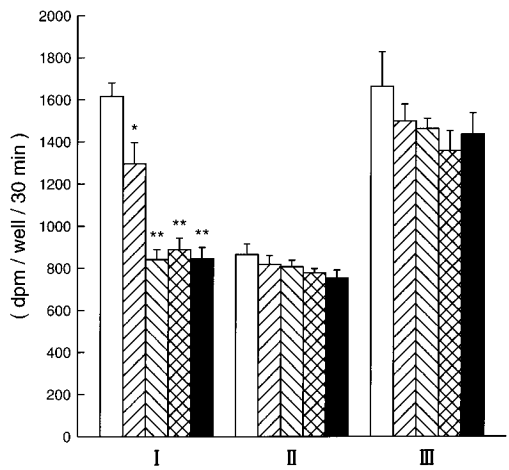

FIG. 4. Concentration-dependent effects of choline on the metabolism of [^{14}C]ethanolamine in NIH 3T3 fibroblasts. Fibroblasts, grown in 12-well culture dishes, were labeled with [^{14}C]ethanolamine for 30 min in the absence (□) or presence of choline added at 0.1 (▨), 1.0 (▧), 5 (▩), or 20 mM (■) concentrations. The cellular content of [^{14}C]ethanolamine (I), [^{14}C]ethanolamine phosphate (II), and [^{14}C]PtdEtn (III) was determined as indicated under Experimental Procedures. Data are the mean ± S.E. of six independent incubations performed with the same passage of cells. Similar results were obtained in two other experiments each performed in triplicate. *,**Significantly ($P < 0.05$*−0.01**) different from the value obtained in the absence of choline (Student's t-test).

[18–20] is affected by many physiological agents, it is of considerable interest that Etn, and particularly its methylated analogues, exhibit mitogenic properties. Presently, the molecular basis for the mitogenic effects of Etn analogues is unknown. However, the ability of choline to enhance the effects of Etn analogues on DNA synthesis without affecting PtdEtn synthesis is a strong indication that increased phospholipid synthesis is not involved in the mediation of mitogenic effects of Etn analogues.

Higher concentrations of Etn analogues inhibited insulin-induced DNA synthesis. The ability of choline to partially prevent this inhibitory effect suggests, although does not prove, that choline might potentiate the combined mitogenic effects of Etn analogues and insulin by counteracting a co-existing smaller inhibitory phase of Etn analogue actions. It is not clear how choline could do this. One possibility is that a cellular pool of Etn may specifically inhibit mitogenesis. Selective decrease of Etn uptake into this pool by choline could then act to prevent the anti-mitogenic effect of Etn. Although data in Fig. 4 provides some evidence that choline inhibits the uptake of Etn into a cellular pool which is not involved in PtdEtn synthesis, further experiments are required to prove the mechanism of potentiating choline effect. An alternative explanation is that the potentiating effects of choline were mediated by choline phosphate, which can also enhance the effect of insulin on DNA synthesis [9].

Somewhat unexpectedly, inhibitors of PKC enhanced the combined mitogenic effects of Etn analogues and insulin, indicating that the signal transduction pathway induced by these mitogens is inhibited by PKC. PMA also inhibited the combined effects of Etn analogues and insulin, but only if it was added 8 h after the mitogens. These data are in accordance with a recent report that in most cell lines examined, PMA exerted antagonistic effects on mitogenesis [21]. Importantly, PMA strongly inhibited growth factor-induced mitogenesis but only when it was added 6–8 h after growth factors [21]. The implication of these data [21] and our results is that in the absence of added PKC activators, only the PKC isoform which is responsible for the inhibitory mitogenic action is active in NIH 3T3 fibroblasts. This is why PKC inhibitors can enhance the combined effects of mitogens. However, it is of interest to note that the effect of insulin or the combined effects of insulin and choline phosphate were not affected by GF 109203X [16]. This is a further strong indication that Etn analogues and choline phosphate enhance insulin-induced DNA synthesis by PKC-regulated and -unregulated mechanisms, respectively.

ACKNOWLEDGMENTS

This work was supported by NIH Grant AA09292 and the Hormel Foundation. We are grateful to Ms. C. Perleberg for secretarial assistance.

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