Phosphocholine and sphingosine-1-phosphate synergistically stimulate DNA synthesis by a MAP kinase-dependent mechanism

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Abstract We have previously shown that in NIH 3T3 fibroblasts phosphocholine (PCho) potentiates sphingosine-1-phosphate (S1P)-induced mitogenesis. Here we report that PCho and S1P also synergistically stimulate DNA synthesis in mouse Swiss 3T3 fibroblasts and in mouse JB6 epidermal cells. The combined actions of PCho and S1P on DNA synthesis were associated with synergistic activation of the p42/p44 mitogen-activated protein (MAP) kinases. Ethanolamine (50–100 μM) further enhanced the synergistic effects of PCho and S1P on DNA synthesis but not on MAP kinase activity. The results indicate that the synergistic mitogenic effects of PCho and S1P (i) are not restricted to NIH 3T3 fibroblasts, (ii) are predominantly mediated by the MAP kinase-dependent signal transduction pathway, and (iii) are enhanced by ethanolamine via a MAP kinase-independent mechanism.

Key words: Phosphocholine; Sphingosine-1-phosphate; MAP kinase; DNA synthesis

1. Introduction

Phosphocholine (PCho), an intermediate of phosphatidylcholine biosynthesis, has recently been shown by others [1,2] and by us [3–5] to stimulate DNA synthesis in NIH 3T3 fibroblast cultures. These findings, coupled with other observations that PCho levels are regulated by growth factors [6], oncogenes [7–9], and chemical carcinogens [10–12], suggest that PCho may be involved in the regulation of cell growth, and perhaps in carcinogenesis, in vivo. It is, therefore, important to determine how widespread the growth regulatory effects of PCho are, and which signal transduction mechanisms are involved.

Work in our laboratory has so far established that in NIH 3T3 fibroblasts PCho acts on mitogenesis through an extracellular site, and that its relatively small effect on DNA synthesis is greatly potentiated by ATP, insulin, or sphingosine-1-phosphate (S1P) [5]. While the combined effects of PCho and ATP as well as PCho and insulin appeared to be predominantly mediated by a pp70 S6 kinase-dependent mechanism [5], the signal transduction pathway mediating the combined effects of PCho and S1P remained to be clarified. Thus, an important goal of this work was to determine the mechanism of combined mitogenic actions of PCho and S1P.

The overall significance of the observed mitogenic effects of PCho depends on, among others, how widespread these effects are. Accordingly, another important goal was to test the possible synergistic mitogenic effects of PCho and S1P in additional cell lines. For this purpose, in this study we chose Swiss 3T3 fibroblasts and JB6 epidermal cells. It was of interest to examine Swiss 3T3 fibroblasts, because the mitogenic effect of S1P on DNA synthesis was first detected in this cell line [13,14]. JB6 cells were chosen because we have a parallel ongoing investigation in this laboratory testing the role of PCho in induced transformation of these cells. Our data show that PCho and S1P exert synergistic stimulatory effects on DNA synthesis in both Swiss 3T3 fibroblasts and JB6 cells. Furthermore, we demonstrate that the mitogenic actions of PCho and S1P are associated with synergistic activation of the p42/p44 MAP kinases.

2. Materials and methods

2.1. Materials

PCho and ethanolamine were purchased from Sigma; S1P was bought from Biomol; the MAP kinase assay kit was obtained from New England Biolabs; and [*methyl*-^{3}H]thymidine (85 Ci/mmol) was purchased from Amersham.

2.2. Cell lines

The promotion-sensitive JB6 clone-41 cell line was kindly supplied by Dr. Nancy H. Colburn (National Institutes of Health, Frederick, MD, USA); the NIH 3T3 clone-7 cell line was donated by Dr. Douglas R. Lowy (National Institutes of Health, Bethesda, MD, USA); and the Swiss 3T3 cell line was from the American Type Culture Collection (Rockville, MD, USA). Each cell line was continuously cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin/streptomycin/neomycin (50 μg/ml, 50 μg/ml and 100 μg/ml, respectively) and glutamine (2 mM).

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

In each case, cells were grown in 12-well tissue culture dishes to about 40% confluence in the presence of 10% serum, washed, and then incubated in serum-free medium for 24 h. Cells (~80% confluent) were washed again and then treated (in serum-free medium) for 16 h with PCho and/or S1P in the absence or presence of ethanolamine as indicated in the text. S1P was added 10 min prior to PCho, while PCho and ethanolamine were added to the medium simultaneously. 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, then four times with 5% trichloroacetic acid, and finally twice with absolute ethanol. 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. Determination of MAP kinase activity

This was performed as described previously [5]. Briefly, serum-starved NIH 3T3 cells in 6-well tissue culture dishes were first treated for 10 min with S1P, and then for 15 min with PCho (in the continuous presence of S1P) in the absence or presence of ethanolamine as indicated in the legend to Fig. 4. The activity state of MAP kinase was determined by immunoblot analysis [5]. The phosphospecific MAP kinase antibody used here recognizes the tyrosine 204 phosphorylation site in the activated forms of p42 and p44 MAP kinases.

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Abbreviations: PCho, phosphocholine; S1P, sphingosine-1-phosphate; MAP, mitogen-activated protein

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3. Results and discussion

3.1. Mitogenic effects of PCho and S1P in Swiss 3T3 and JB6 cells

Both in serum-starved Swiss 3T3 cells (Fig. 1A) and JB6 cells (Fig. 1B), 5 μM S1P appreciably stimulated DNA synthesis, but the rate of S1P-stimulated incorporation of radiolabeled thymidine into DNA was only about 10% of that observed upon readdition of 10% serum (not shown). Similarly, PCho alone, even when added at a maximally effective concentration (1 mM), had only very small effects on DNA synthesis in both cell lines. However, both in Swiss cells (Fig. 1A) and JB6 cells (Fig. 1B) PCho was able to greatly enhance S1P-induced DNA synthesis, and to sensitize the cells to the actions of lower concentrations of S1P. Thus, in PCho-treated cells S1P at a concentration as low as 0.25 μM became a potent inducer of DNA synthesis. Importantly, in the presence of PCho the mitogenic effect of 5 μM S1P was roughly equivalent with that of 10% serum.

For a comparison, we also determined the combined effects of S1P and PCho on DNA synthesis in NIH 3T3 cells (Fig. 1C). Of the three cell lines examined, S1P and PCho, both alone and in combination, had the largest mitogenic effects in NIH 3T3 cells (Fig. 1C compared to Fig. 1A and B). However, in additional experiments we found that in comparison to the two other cell lines, in NIH 3T3 cells serum was also about twice more effective in stimulating DNA synthesis. Taking all these findings into account, it seems fair to conclude that PCho and S1P have comparable synergistic effects on DNA synthesis in the three cell lines used in these experiments.

At an optimal (5 μM) concentration of S1P, 0.25 mM PCho detectably enhanced the mitogenic effect of S1P in both Swiss 3T3 cells (Fig. 2A) and JB6 cells (Fig. 2B). Maximal potentiating effects required 1 mM PCho; elevation of PCho concentration to 2 mM failed to yield larger effects.

3.2. Potentiation of the combined stimulatory effects of PCho and S1P by ethanolamine

Ethanolamine is present in the blood at concentrations ranging from 5 to 50 μM in various species ([15], and references therein), which can be further enhanced under certain conditions, including liver regeneration [15] and renal failure [16]. In addition, phospholipase D-mediated hydrolysis of phosphatidylethanolamine, which has been shown to be stimulated by several agents in numerous cell types (reviewed in ref. [17]), is also likely to elevate ethanolamine levels in the extracellular space. For these reasons, it was of interest to

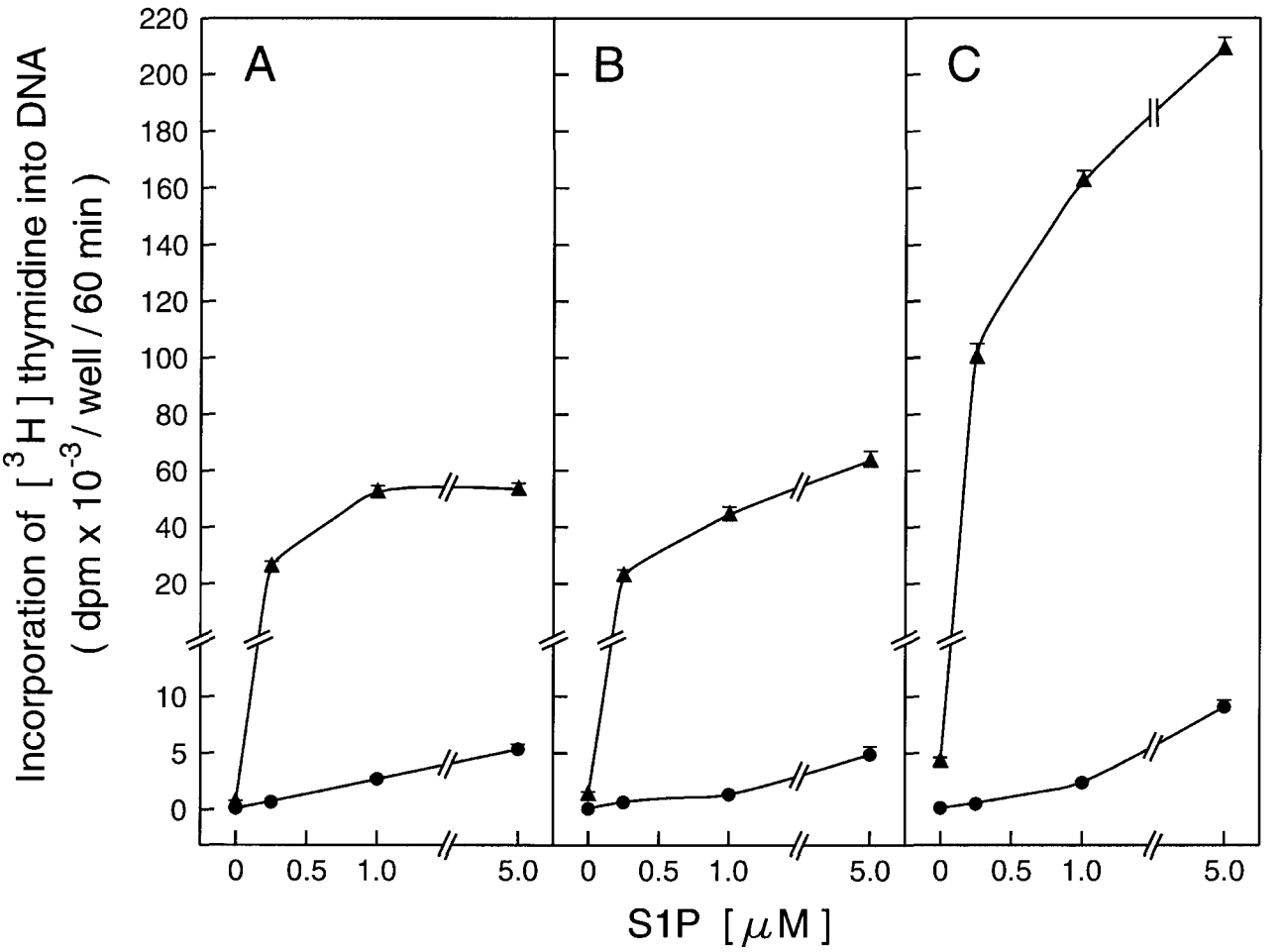

Fig. 1. PCho and S1P synergistically stimulate DNA synthesis in Swiss 3T3, JB6, and NIH 3T3 cells. Serum-starved (24 h) Swiss 3T3 (A), JB6 (B) and NIH 3T3 (C) cells were treated for 16 h with 0–5 μM concentrations of S1P in the absence (●) or presence (▲) of 1 mM PCho, followed by incubation in the presence of [*methyl*-^{3}H]thymidine for 1 h. Each point represents the mean ± S.D. ($n = 6$). Similar results were obtained in two other experiments each performed in triplicate.

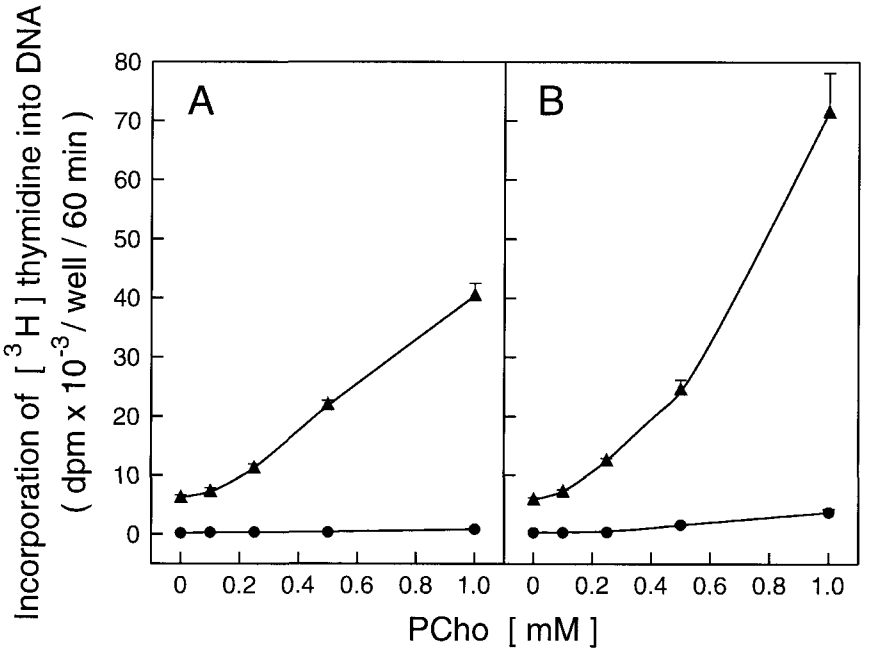

Fig. 2. Concentration-dependent effect of PCho on S1P-induced DNA synthesis in Swiss 3T3 and JB6 cells. Serum-starved Swiss 3T3 (A) and JB6 (B) cells were treated for 16 h with 0–1 mM concentrations of PCho in the absence (●) or presence (▲) of 5 μM S1P, followed by incubation in the presence of [*methyl*-^{3}H]thymidine for 1 h. Each point represents the mean ± S.D. ($n = 3$). Similar results were obtained in two other experiments each performed in triplicate.

determine how ethanolamine might affect the combined stimulatory effects of S1P and PCho on DNA synthesis. As shown in Fig. 3, addition of ethanolamine at 50 and 100 μM concentrations to NIH 3T3 cells further enhanced the synergistic mitogenic effects of PCho and S1P even when S1P was used at a maximally effective (5 μM) concentration. Ethanolamine had no potentiating effects when used at 20 μM concentration, and its effects were not further enhanced by elevating its concentration above 100 μM. When 1 mM PCho and 5 μM S1P were added alone, 100 μM ethanolamine enhanced only the effect of PCho (about 3-fold; data not shown).

3.3. *Combined effects of PCho and S1P on MAP kinase activity*

Possible activation of p42/p44 MAP kinases (also known as extracellular signal-regulated kinases, or ERKs) by S1P and PCho was examined in NIH 3T3, because these agents had the largest effects on DNA synthesis in this cell line. A previous study has demonstrated that in Swiss 3T3 cells S1P alone can detectably stimulate MAP kinase activity [18]. As shown in Fig. 4, in NIH 3T3 fibroblasts as well 0.5 and 5 μM concentrations of S1P alone induced relatively weak, but detectable, activation of MAP kinase (Fig. 4). Similarly, PCho alone had only a small effect on MAP kinase activity. However, simultaneous treatments of cells with S1P (0.5 or 5 μM) and PCho resulted in strong activation of MAP kinase (Fig. 4). Interestingly, the combined effects of S1P and PCho on MAP kinase activity were not enhanced by ethanolamine (Fig. 4), despite the ability of ethanolamine to increase the synergistic effects of S1P and PCho on DNA synthesis (Fig. 3). It still should be noted that 0.5 and 5 μM concentrations of S1P were equally effective in stimulating MAP kinase activity (Fig. 4), although in the experiment shown in Fig. 1 maximal stimulation of DNA synthesis required 5 μM S1P. The only explanation we can offer to explain this apparent discrepancy is that maximal stimulation of DNA synthesis may require the presence of S1P for a longer than 15 min period (the length of incubation time used for the MAP kinase assay). Since S1P is known to be metabolically unstable, in case of DNA synthesis assay a higher starting concentration of S1P may be needed.

In conclusion, we showed that in each of the three cell lines examined here, simultaneous treatments with maximally effec-

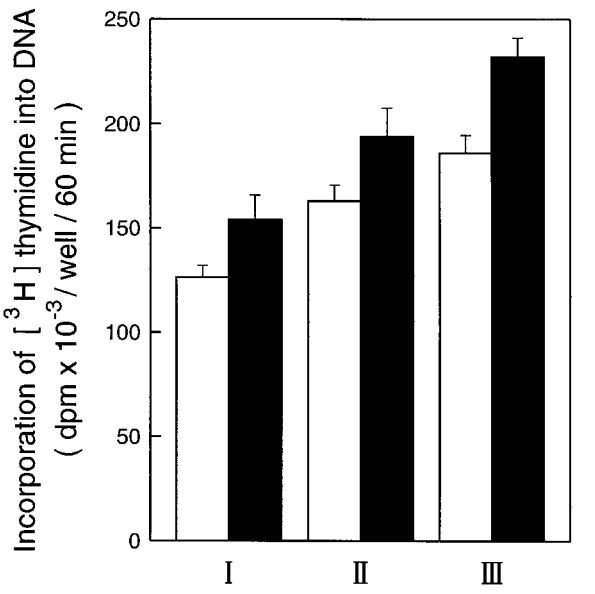

Fig. 3. Potentiating effect of ethanolamine on PCho plus S1P-induced DNA synthesis in NIH 3T3 fibroblasts. Serum-starved NIH 3T3 fibroblasts were incubated for 16 h in the presence of 1 mM PCho plus 0.5 μM S1P (□) or PCho plus 5 μM S1P (■) in the absence (I) or presence of 50 μM ethanolamine (II) or 100 μM ethanolamine (III). Data are the mean ± S.D. ($n = 6$). Similar results were obtained in three other experiments each performed in triplicate.

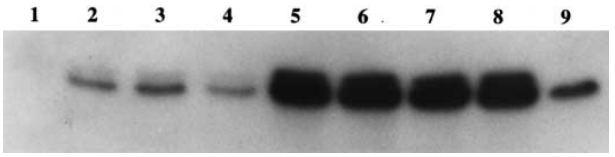

Fig. 4. Synergistic stimulation of MAP kinase activity by PCho and S1P in NIH 3T3 cells. Serum-starved cells were first treated with S1P for 10 min, followed by incubations in the presence of 1 mM PCho and 100 μM ethanolamine for an additional 15 min period as indicated. Additions were as follows: none (lane 1), 0.5 μM S1P (lane 2), 5 μM S1P (lane 3), PCho (lane 4), 0.5 μM S1P plus PCho (lane 5), 5 μM S1P plus PCho (lane 6), 0.5 μM S1P plus PCho plus ethanolamine (lane 7), 5 μM S1P plus PCho plus ethanolamine (lane 8). Lane 9 represents the MAP kinase standard. The lower and upper bands represent the p42 and p44 MAP kinases, respectively.

tive concentrations of S1P and PCho induced DNA synthesis to about the same level as 10% serum. In contrast, both agents were poor mitogens when applied alone. These data indicate that synergistic stimulation of DNA synthesis by S1P and PCho is not restricted to NIH 3T3 cells; in fact, these agents in combination may be part of a widespread regulatory mechanism. Interestingly, two previous studies implicated, separately, both PCho [1] and S1P [19] in the mediation of mitogenic effects of certain growth factors, including platelet-derived growth factor and fibroblast growth factor. Our results now suggest that PCho and S1P can serve as effective mediators of the growth factor effects only if they act simultaneously. Considering that (i) PCho acts through an extracellular target [5], (ii) cells express receptors for S1P [20–22], and (iii) S1P (being a phosphate-containing compound) may not be taken up by the cells efficiently, it seems likely that both compounds need to be released from cells for their mitogenic actions to occur. This possibility is presently being studied in our laboratory.

S1P and PCho also synergistically activated the p42/p44 MAP kinases. This suggests that these enzymes play an important role in the mediation of mitogenic effects of S1P and PCho. Interestingly, ethanolamine was able to further increase the synergistic effects S1P and PCho on DNA synthesis, but not on MAP kinase activity. This indicates that the mitogenic activities of S1P and PCho can be enhanced by a MAP kinase-independent mechanism. The experiments with ethanolamine also suggest the interesting possibility that activation of phosphatidylethanolamine-specific phospholipase D [18] may lead to the potentiation of mitogenic effects of S1P and PCho. This possibility as well as the physiological significance of the synergistic mitogenic effects of S1P and PCho remains to be determined.

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