Bombesin and zinc enhance the synergistic mitogenic effects of insulin and phosphocholine by a MAP kinase-dependent mechanism in Swiss 3T3 cells

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Abstract Simultaneous treatment of serum-starved (24 h) Swiss 3T3 cells with insulin (500 nM) and phosphocholine (PCho) (0.25–1 mM) resulted in synergistic stimulation of DNA synthesis via a mitogen activated protein (MAP) kinase-independent rapamycin-sensitive mechanism. Co-treatment of cells with bombesin (10 nM) or zinc (25 μM) enhanced the combined mitogenic effects of insulin and PCho 2–3-fold; however, in the presence of bombesin or zinc the combined effects of insulin and PCho were not inhibited by rapamycin. The potentiating effects of bombesin and zinc on insulin plus PCho-induced DNA synthesis were accompanied by large stimulation of p42 MAP kinase activity. The results indicate that in Swiss 3T3 cell cultures, synergistic stimulation of DNA synthesis by extracellular insulin and PCho via a p42 MAP kinase-dependent mechanism requires the presence of other growth regulatory agents, such as bombesin or zinc.

Key words: Mitogen activated protein; Bombesin; Zinc; Synergistic effect on DNA synthesis; Swiss 3T3 cell

1. Introduction

Insulin alone is a weak mitogen in fibroblasts but it can complement the mitogenic signals initiated by competence factors such as platelet-derived growth factor, fibroblast growth factor, or epidermal growth factor [1]. Bombesin is another growth factor which can enhance the mitogenic activity of insulin in Swiss 3T3 fibroblasts [2,3]. It was reported that bombesin and insulin, in combination, stimulate entry of Swiss 3T3 cells into the S phase of the cell cycle by a mechanism which is independent of pp70 S6 kinase ($pp70^{s6k}$) activity [4] and only partially depends on mitogen activated protein (MAP) kinase activity [5].

We have previously demonstrated that in NIH 3T3 fibroblasts phosphocholine (PCho), an intermediate of phosphatidylcholine synthesis, can also greatly enhance the relatively small stimulatory effect of insulin on DNA synthesis [6–8]. Since the intracellular concentration of PCho is relatively high, and there are mechanisms which can induce the release of cellular PCho to the extracellular space, PCho is likely to be a physiologically relevant regulator of cell growth in the presence of insulin [8]. However, other growth regulatory agents, such as bombesin, could further modify the interdependent mitogenic actions of PCho and insulin. The first goal in this work was to test this possibility.

Zinc, present in the circulation at concentrations of 10–40 μM, is known to be required for growth [9]. Therefore, it was important to determine how zinc might affect the mitogenic effects of insulin and PCho. This was the second important goal of this work. We report that both bombesin and zinc further enhanced the initially MAP kinase-independent synergistic effects of insulin and PCho on DNA synthesis by a mechanism which involved activation of p42 MAP kinase.

2. Materials and methods

2.1. Materials

Insulin and bombesin were purchased from Boehringer Mannheim; PCho and rapamycin were bought from Sigma; the MAP kinase assay kit was obtained from New England Biolabs; tissue culture reagents were from Life Technologies, Inc.; and [*methyl*-^{3}H]thymidine (85 Ci/mmol) was purchased from NEN DuPont.

2.2. Cell culture

The Swiss 3T3 fibroblast line, bought from the American Type Culture Collection (Rockville, MD, USA), was continuously cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin/streptomycin/neomycin (50 U/ml, 50 μg/ml and 100 μg/ml, respectively) and glutamine (2 mM).

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

Swiss 3T3 cells were grown in 12-well tissue culture dishes to about 40% confluency in the presence of 10% serum, washed, and then incubated in serum-free medium for 21 h. The medium was replaced with serum-free fresh medium and cells were incubated with or without 25 μM zinc chloride for 3 h. This was followed by treatments for 16 h in the absence or presence of PCho, bombesin and/or insulin; PCho was added to the incubation medium 5 min prior to bombesin, and bombesin was added to cells 5 min prior to insulin. Finally, incubations were continued in the presence of [*methyl*-^{3}H]thymidine (1 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 [8]. Briefly, serum-starved untreated or zinc-treated (3 h) NIH 3T3 cells in 10 cm diameter tissue culture dishes were first treated with 1 mM PCho for 5 min, then with 10 nM bombesin for 5 min, and finally with 500 nM insulin (in the continuous presence of PCho and bombesin, when applicable) for 10 min. The activity state of p42/p44 MAP kinases was evaluated by immunoblot analysis [8]. The phosphospecific MAP kinase antibody used here recognizes the tyrosine 204 phosphorylation site in the activated forms of MAP kinases.

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Abbreviations: PCho, phosphocholine; MAP, mitogen activated protein; $pp70^{s6k}$, pp70 S6 kinase

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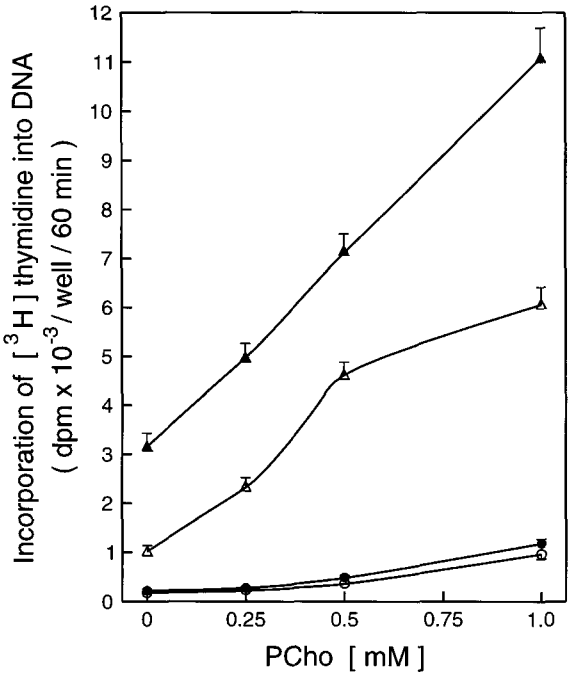

Fig. 1. Concentration-dependent effects of PCho on DNA synthesis in the presence of insulin and bombesin in Swiss 3T3 cells. Serum-starved cells were treated for 16 h with 0–1 mM concentrations of PCho, as indicated, in the absence (○) or presence of 10 nM bombesin (●), 500 nM insulin (△) or bombesin plus insulin (▲). Each point represents the mean ± S.E.M. of six determinations (from separate wells) in one experiment. Similar results were obtained in three other experiments each performed in triplicate.

3. Results

3.1. Synergistic effects of insulin, PCho, and bombesin on DNA synthesis

Addition of bombesin to confluent serum-starved Swiss 3T3 cells has been reported to cause significant stimulation of DNA synthesis [4,5]. In our hands, bombesin alone had mitogenic effects only if the conditioned incubation medium had not been removed prior to the addition of bombesin. In contrast, when after a 21–24 h period of serum starvation the conditioned medium was replaced with fresh serum-free medium shortly (0.5–3 h) before the addition of bombesin, this growth factor alone had no effect on DNA synthesis (Fig. 1). However, under these conditions bombesin clearly retained its ability to potentiate the small mitogenic effect of insulin (Fig. 1). Since we were interested in the potentiating effect of bombesin, we used this protocol throughout this study. In these cells 1 mM PCho alone enhanced DNA synthesis to about the same extent (~7-fold) as insulin did (Fig. 1). However, when added in combination, insulin and 1 mM PCho synergistically (~30-fold) stimulated DNA synthesis (Fig. 1). Furthermore, it should be noted that detectable potentiation of the insulin effect was also observed with 0.25 mM PCho (Fig. 1). Bombesin approximately doubled the combined effects of insulin and PCho on DNA synthesis, while it did not modify the effect of PCho alone (Fig. 1).

Rapamycin, an inhibitor of $pp70^{s6k}$ action [10,11], strongly inhibited the combined effects of bombesin and insulin on DNA synthesis, while the combined effects of insulin, bombesin and PCho remained practically unaffected by rapamycin

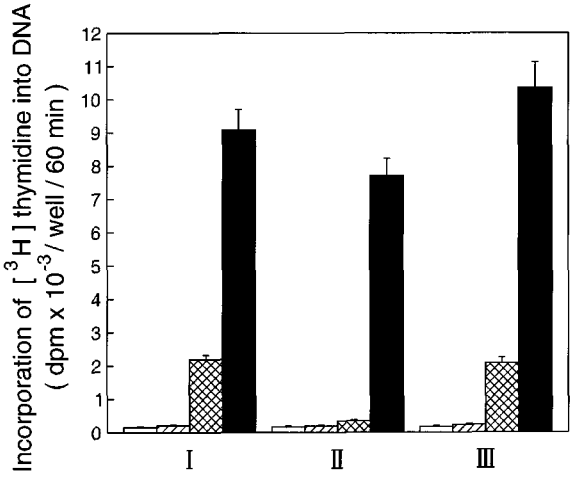

Fig. 2. Effects of rapamycin and wortmannin on DNA synthesis induced by combinations of bombesin, PCho, and insulin. Serum-starved Swiss 3T3 cells were incubated for 16 h in the absence (white bars) or presence of 10 nM bombesin (hatched bars), bombesin plus 500 nM insulin (cross-hatched bars) or bombesin plus insulin plus 1 mM PCho (black bars). There was either no other addition (I), or the incubation medium also contained 10 nM rapamycin (II) or 200 nM wortmannin (III) added 30 min prior to other additions. Values are the means ± S.E.M. of four determinations (from separate wells) in one experiment. Similar results were obtained in two other experiments each performed in triplicate.

(Fig. 2). On the other hand, even a relatively high (200 nM) concentration of wortmannin, an inhibitor of phosphatidylinositol 3′-kinase [12,13], failed to inhibit the synergistic mitogenic effects of insulin and bombesin both in the absence and presence of PCho (Fig. 2).

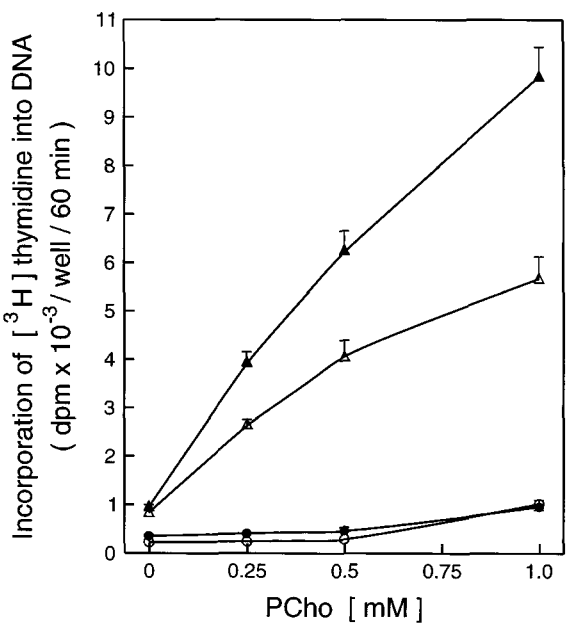

Fig. 3. Potentiating effect of zinc on PCho plus insulin-induced DNA synthesis. Serum-starved Swiss 3T3 cells were treated for 16 h with 0–1 mM concentrations of PCho, as indicated, in the absence (○) or presence of 25 μM zinc (●), 500 nM insulin (△), or zinc plus insulin (▲); zinc was added 3 h prior to other additions. Each point represents the mean ± S.E.M. of six determinations (from separate wells) in one experiment. Similar results were obtained in five other experiments performed in triplicate or duplicate.

3.2. Combined effects of insulin, PCho, and zinc on DNA synthesis

Zinc, present in the circulation at concentrations of 10–40 μM, is an essential micronutrient which is required for normal cell growth [9]. We tested its effects on insulin and PCho-induced DNA synthesis at a physiologically relevant concentration (25 μM). While zinc did not significantly modify the individual effects of PCho or insulin on DNA synthesis, it nearly doubled the combined effects of insulin and PCho at each (0.25–1 mM) concentration of PCho examined (Fig. 3).

In the absence of zinc, a maximally effective (10 nM) concentration of rapamycin inhibited the stimulatory effects of insulin, PCho, and insulin plus PCho by about 80%, 45%, and 63%, respectively (Fig. 4A). In the presence of zinc, rapamycin inhibited the individual effects of insulin and PCho to about the same extent as it did in the absence of zinc; however, in the presence of zinc the synergistic effects of insulin and PCho were inhibited by rapamycin only by about 25% (Fig. 4B).

3.3. Activation of MAP kinase by PCho plus insulin in the presence of bombesin or zinc

The results described so far implied that both bombesin and zinc decreased the dependence of combined mitogenic effects PCho and insulin on pp70^{s6k}. This prompted us to examine activation of MAP kinase by PCho and insulin in the presence of bombesin and zinc. As shown in Fig. 5, PCho and insulin, even in combination, only slightly enhanced p42 MAP kinase activity. Similarly, insulin and bombesin in combination had only a small stimulatory effect on this MAP kinase activity (Fig. 5). However, p42 MAP kinase activity was greatly increased when treatments with PCho plus insulin were performed in the presence of either bombesin or zinc (Fig. 5). In similar studies, zinc failed to enhance the combined effects of PCho, bombesin, and insulin on either DNA synthesis or p42 MAP kinase activity (data not shown). It should be added that while in an earlier work insulin in combination with phorbol ester was found to stimulate both p42 and p44 MAP kinase activities [8], in the present study combinations of insulin with PCho and bombesin or zinc did not cause detectable activation of p44 MAP kinase (Fig. 5).

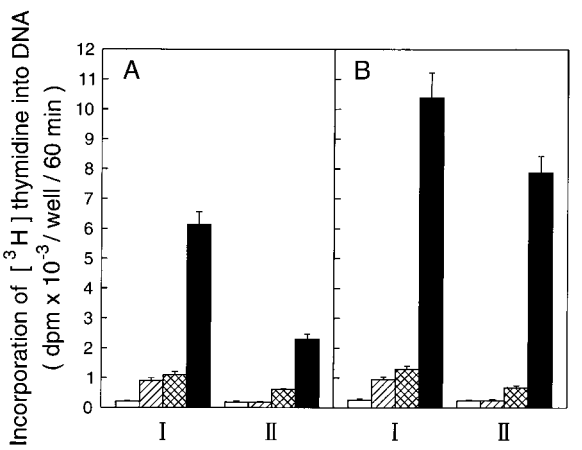

Fig. 4. Effects of rapamycin on DNA synthesis induced by combinations of zinc, PCho and insulin. Serum-starved Swiss 3T3 cells were incubated for 16 h without zinc (A) or with 25 μM zinc (B) in the absence (white bars) or presence of 500 nM insulin (hatched bars), 1 mM PCho (cross-hatched bars) or insulin plus PCho (black bars). Rapamycin (10 nM), either absent (I) or present (II) during the entire treatment period (16 h) was added 30 min prior to other additions. Values represent the means ± S.E.M. of four determinations (from separate wells) in one experiment. Similar results were obtained in two other experiments each performed in triplicate.

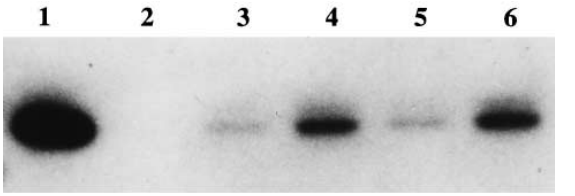

Fig. 5. Potentiating effects of bombesin and zinc on PCho plus insulin-induced MAP kinase activity. Serum-starved Swiss 3T3 cells were either untreated (lane 2), or were treated for a total period of 20 min (except with zinc) with 1 mM PCho plus 500 nM insulin (lane 3), PCho plus insulin plus 25 μM zinc (added 3 h prior to other additions) (lane 4), insulin plus 10 nM bombesin (lane 5), and PCho plus insulin plus bombesin (lane 6). The MAP kinase standard is shown in lane 1. This experiment was repeated once with similar results.

4. Discussion

Bombesin has been reported to stimulate DNA synthesis through several signal transduction pathways. When added to confluent and serum-starved cells without prior change of the conditioned medium, bombesin alone was found to stimulate DNA synthesis by both pp70^{s6k}-dependent [11] and MAP kinase-dependent mechanisms [14]. Interestingly, under the above incubation condition synergistic activation of DNA synthesis by bombesin and insulin showed reduced requirement for both the pp70^{s6k}-dependent [4] and MAP kinase-dependent [5] signal transduction pathways. Thus, in the presence of insulin, bombesin can also stimulate DNA synthesis by a third, presently unidentified, mechanism.

We significantly decreased the complexity of bombesin action by including a washing step shortly before its addition. This step eliminated the effect of bombesin 'alone' (requiring a factor present in the conditioned medium), but preserved its potentiating effect on insulin-induced DNA synthesis. Importantly, while the combined effects of bombesin and insulin as well as that of PCho and insulin on DNA synthesis were strongly inhibited by rapamycin, the combined effects of PCho, bombesin and insulin were much less sensitive to the inhibitory action of rapamycin. The three agents together also induced a much greater activation of p42 MAP kinase than that elicited by insulin in combination with either bombesin or PCho. Collectively, these data imply that bombesin is capable of shifting the combined mitogenic effects of PCho and insulin from a rapamycin-sensitive (presumably pp70^{s6k}-dependent) to a p42 MAP kinase-dependent pathway which is insensitive to rapamycin.

In the periphery, the serum concentration of zinc appears to be in the 10–20 μM range [9] which can be doubled under certain conditions including stress [15]. In view of the multiple roles of zinc in cell growth regulation [9], it was interesting to find that zinc not only permitted but actually further promoted the mitogenic effects of PCho and insulin on DNA synthesis. Furthermore, zinc was also able to divert the combined effects of PCho and insulin from a rapamycin-sensitive to a MAP kinase-dependent mechanism. This suggests that

the level of zinc in the circulation may have a significant impact on mitogenesis induced by extracellular PCho and insulin.

In summary, we have shown that in Swiss 3T3 cells the combined stimulatory effects of PCho and insulin on DNA synthesis are enhanced by both bombesin and zinc. The mechanisms by which bombesin and zinc are able to switch the mitogenic actions of PCho and insulin from a rapamycin-sensitive to a rapamycin-insensitive MAP kinase-dependent mechanism remain to be established.

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