**ATP-dependent Choline Phosphate-induced Mitogenesis in Fibroblasts Involves Activation of pp70 S6 Kinase and Phosphatidylinositol 3’-Kinase through an Extracellular Site**

**SYNERGISTIC MITOGENIC EFFECTS OF CHOLINE PHOSPHATE AND SPHINGOSINE 1-PHOSPHATE***

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In serum-starved NIH 3T3 clone 7 fibroblasts, choline phosphate (ChoP) (0.5–1 mM) and insulin synergistically stimulate DNA synthesis. Here we report that ATP also greatly enhanced the mitogenic effects of ChoP (0.1–1 mM) both in the absence and presence of insulin; maximal potentiating effects required 50–100 μM ATP. The co-mitogenic effects of ATP were mimicked by adenosine 5’-O-(3-thiotriphosphate), adenosine 5’-O-(2-thiodiphosphate), ADP, and UTP, but not by AMP or adenosine, indicating the mediatory role of a purinergic P2 receptor. Externally added ChoP acted on DNA synthesis without its detectable uptake into fibroblasts, indicating that ChoP can be a mitogen only if it is released from cells. Extracellular ATP (10–100 μM) induced extensive release of ChoP from fibroblasts. ChoP had negligible effects, even in the presence of ATP or insulin, on the activity state of p42/p44 mitogen-activated protein kinases, while in combination these agents stimulated the activity of phosphatidylinositol 3’-kinase (PI 3'-kinase). Expression of a dominant negative mutant of the p85 subunit of PI 3’-kinase or treatments with the PI 3’-kinase inhibitor wortmannin only partially (40–50%) reduced the combined effects of ChoP, ATP, and insulin on DNA synthesis; in contrast, the pp70 S6 kinase inhibitor rapamycin almost completely inhibited these effects. ATP and insulin also potentiated, while rapamycin strongly inhibited, the mitogenic effects of sphingosine 1-phosphate (S1P). Furthermore, even maximally effective concentrations of ChoP and S1P synergistically stimulated DNA synthesis. The results indicate that in the presence of extracellular ATP and/or S1P, ChoP induces mitogenesis through an extracellular site by mechanisms involving the activation of pp70 S6 kinase and, to a lesser extent, PI 3’-kinase.

Stimulation of a phosphatidylcholine-hydrolyzing phospholipase C activity has been implicated in the actions of several growth factors; however, in practically all cases 1,2-diacylglycerol was thought to be the growth-regulatory molecule (1–5). Recently, it has been reported that in NIH 3T3 fibroblasts externally added choline phosphate (ChoP) stimulated DNA synthesis (6). However, a maximal effect required 20 mM ChoP. These authors (6) also reported some limited uptake of ChoP by these fibroblasts, implying that ChoP acted through an intracellular target. Thus, it may appear logical to assume that a high concentration of external ChoP is needed to elicit mitogenic effects because of its poor penetration through the cell membrane.

Recently, using NIH 3T3 clone 7 cells, we found that the addition of 1 mM ChoP to the incubation medium was sufficient to induce maximal effects on DNA synthesis both in the absence and presence of insulin (7). Since the intracellular concentration of ChoP is already about 0.5 mM (6), it was difficult to envision how extracellular ChoP (at 1 mM level) could further increase it. In an attempt to solve this issue, it was necessary to reexamine, as we did in this work, the uptake of ChoP into NIH 3T3 fibroblast cultures, which are available in our laboratory.

The NIH 3T3 cell line, originally selected from NIH Swiss mouse embryo cultures (8), shows considerable heterogeneity. Recently, Wang's laboratory (9) isolated two genetically stable subclones, termed P-3T3 and N-3T3 cells, which apparently make up the major portion of the original NIH 3T3 cell line. The two subclones are different in many respects; most importantly, the growth of P-3T3 cells is stimulated, while that of N-3T3 cells is inhibited, by phorbol 12-myristate 13-acetate (PMA) as well as by v-Abl, v-Src, and Bcr-Abl tyrosine kinases (9, 10). It is conceivable that since the original NIH 3T3 line was established, a considerable degree of heterogeneity among the existing NIH 3T3 lines has developed. Taking these findings into account, it appeared necessary to examine how these subclones respond to the presence of ChoP in the incubation medium. We therefore extended the studies on the mitogenic actions of ChoP to the P-3T3 and N-3T3 subclones as well.

Should ChoP act through an extracellular target, then it clearly becomes important to determine how this compound can reach an effective concentration in the extracellular space. This led us to investigate possible potentiation of both the efflux and mitogenic action of ChoP by an extracellular effector. Among others, extracellular ATP was considered such a potentiating agent because of its known ability to enhance, although usually only modestly, the mitogenic actions of a variety of growth factors (Ref. 11 and references therein).

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1 The abbreviations used are: ChoP, choline phosphate; PMA, phorbol 12-myristate 13-acetate; ATPγS, adenosine 5’-O-(3-thiotriphosphate); ADPβS, adenosine 5’-O-(2-thiodiphosphate); S1P, sphingosine 1-phosphate; PI, phosphatidylinositol; pp70sv, pp70 S6 kinase; BAPTA/AM, (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl)ester; MAP, mitogen-activated protein; RT-PCR, reverse transcription-polymerase chain reaction.
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In fibroblasts, extracellular sphingosine 1-phosphate (SIP) was shown to stimulate cell proliferation (12, 13) and also to mobilize calcium from internal stores via an inositol triphosphate-independent mechanism (12, 14). In several cellular systems SIP can also activate specific G protein-coupled cell surface receptors (15, 16), which is often associated with transient increases in cytoplasmic calcium concentration (15). Although ChoP has no effects on the distribution of cellular calcium (17), its mitogenic effects may be modified by calcium. Furthermore, ChoP could affect the function of SIP receptor. For these reasons, it was of interest to determine possible interaction between the mitogenic effects of ChoP and SIP.

Here we report that in NIH 3T3 fibroblasts both extracellular ATP and SIP greatly enhanced the mitogenic effects of ChoP. Cellular uptake of ChoP was not detectable. However, ATP caused quantitatively significant release of cellular ChoP into the medium. The mitogenic effects of ChoP appear to involve pp70 S6 kinase (pp70^S6K) and phosphatidylethanolamine kinase (PI 3'-kinase) (18). These results suggest that in the presence of extracellular ATP and/or SIP, ChoP can be an important positive regulator of cell growth.

**EXPERIMENTAL PROCEDURES**

**Materials**—ChoP, choline kinase, ATP, ADP, ADP-β-S, AMP, adenosine, UTP, rapamycin, thapsigargin, ionomycin, and Dowex-50W-H' were bought from Sigma; 2-methylthio-ATP was from RBI Research Biochemicals International; insulin and bombesin were bought from Boehringer-Mannheim; p-tyrosine 



Mitochondria isolated from NIH 3T3 Fibroblasts—This was determined as described above, except that fibroblasts were incubated with [14C]choline (1.23 × 10^6 dpm/well; 50 mCi/mmol) and [14C]ChoP from NIH 3T3 Fibroblasts—This was determined as described above, except that fibroblasts were incubated with [14C]choline (1.23 × 10^6 dpm/well; 50 mCi/mmol) for 3 h.

**Determination of MAP Kinase Activity**—Cells in 6-well tissue culture dishes were treated for first 10 min, when appropriate, with ATP, and then for an additional 5, 15, or 30 min with ChoP, insulin, or PMA in combinations and at concentrations indicated in the legend to Fig. 6. Samples for immunoblot analysis were prepared as described previously (21). Phosphospecific MAP kinase antibody (Biolabs), which recognizes the tyrosine 204 phosphorylation site, was used to detect the activated (phosphorylated) forms of p42 and p44 MAP kinases. The Western immunoblotting protocol was performed according to the instructions provided by the manufacturer.

**Stable Overexpression of Dominant Negative Mutant of p85 Subunit PI 3'-Kinase**—The plasmid SR-ap85, which contains the dominant negative mutant of the p85 subunit of the PI 3'-kinase, was kindly provided by Dr. Masato Kasuga (Kobe University, Japan); the details for the construction of the plasmid have been described elsewhere (22). NIH 3T3 clone 7 cells were co-transfected with pRBK, a plasmid conferring hygromycin B resistance, and SR-ap85 (or the empty vector) using Lipofectin (Life Technologies, Inc.) as described previously (23). Selection was started by the addition of 220 μg/ml hygromycin B to the culture medium. After 14–16 days in selection medium, single clones were subcloned by limited dilution and examined for the presence of dominant negative mutant p85 mRNA and the protein subunit by RT-PCR and Western blotting, respectively. For RT-PCR, the total cellular RNA was isolated from transfected NIH 3T3 cells grown in the hygromycin B selection medium using the STAT 60 RNA isolation kit (Tel-Test, Inc.) and a Gen-RNA RT-PCR System (Promega) following the procedure recommended by the manufacturer. Each reaction mixture contained 1 μg of RNA template and 50 pmol of upstream and downstream primers, which had the sequence 5’-CCC GAA CTT CCC AGG AAA TCC-3’ and 5’-GTC AAT CTC ACG ATA CTC AGC-3’. Two control reactions, one with RNA from untransfected NIH 3T3 cells and the other with cDNA template isolated from the 85B, were included. The primers were designed to hybridize the flanking sequences of the deletion in Δap85. After 40 cycles of polymerase chain reaction, the amplified cDNA products were analyzed on a 1.5% agarose gel.

**PI 3'-Kinase Assay**—Serum-starved (24 h) fibroblasts, grown in 100-mm plastic dishes, were treated for 20 min with agents as indicated in Fig. 8. PI 3'-kinase activity was determined in anti-phosphotyrosine immunoprecipitates by the method described by Okada et al. (24, 25) with several modifications. Washed cells were scraped into ice-cold lysing buffer (1 ml) containing 137 mM NaCl, 20 mM Tris/HCl, pH 7.4, 1.4 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 1% Nonident P-40. After solubilization for 10 min, insoluble material was removed by centrifugation at 15,000 × g for 15 min. PI 3'-kinase enzyme was immunoprecipitated by incubating the lysates with 40 μl of P-20-agarose conjugate overnight at 4°C. After successive washings (24), the pellets (on ice) were redissolved in a buffer (20 μl) containing 10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, and 1 mM Na3VO4, followed by the addition of 30 μl of PI 3'-kinase reaction mixture containing 0.5 mg/ml phosphatidylinositol (dispersed by sonication in a solution containing 50 mM Hepes, 1 mM EDTA, and 1 mM NaH2PO4), 5 μM MgCl2, and (γ-32P)ATP (100 μCi, 5 μCi). The reactions were carried out in a water bath at 30°C for 5 min and then stopped by successive addition of 15 μl of 4× HCl and 0.13 ml of chloroform/methanol (1:1, v/v). After phase separation, the product phosphatidylinositol 3-phosphate was separated on silica gel G plates impregnated with 1% potassium oxalate as described (25). After autoradiography, the spots were evaluated by densitometry or by determining 32P activity in the spots in a liquid scintillation counter.

**RESULTS**

Potentiating Effects of ATP on the Mitogenic and Co-mitogenic Effects of ChoP in Clone 7/3T3 Fibroblasts—The addition
of ATP (10–100 μM) alone to serum-starved clone 7/3T3 fibroblasts failed to stimulate DNA synthesis over a 16-h incubation period, but it enhanced, although less than 2-fold, the relatively small stimulatory effect of insulin (Fig. 1). Since 100 μM ATP was certainly maximally effective, in most of the following experiments this concentration was used to determine its possible effects on ChoP and insulin-induced mitogenesis.

Next we examined the ability of ATP, alone and in combination with insulin, to reduce the effective mitogenic concentration of ChoP. In the absence of other agents, 1 mM ChoP enhanced DNA synthesis about 10-fold, while 0.5 mM or lower concentrations of ChoP had much smaller effects or no effects (Fig. 2). In the presence of ATP or ATP plus insulin (500 nM), 1 mM ChoP was capable of stimulating DNA synthesis about 45- or 215-fold, respectively (Fig. 2). It should be noted that in the same experiment 10% serum stimulated DNA synthesis about 160-fold (not shown). Importantly, in the presence of ATP and insulin even 0.1 mM ChoP, a concentration that may be physiologically quite relevant (see below), stimulated DNA synthesis 26-fold (Fig. 2). A similar effect in the presence of ATP alone or insulin alone required about 0.25 or 0.5 mM ChoP, respectively (Fig. 2).

To have a better idea of the mechanism of co-mitogenic ATP effects, next we determined the effects of several ATP analogues and other nucleotides on insulin and/or ChoP-induced DNA synthesis; each compound was used at a 100 μM concentration. AMP and adenosine were totally ineffective, and 2-methylthio-ATP was about 45% as effective as ATP, while ATPβS, ADP, ADPβS, and UTP were equally effective, or even more effective, than ATP (Table I).

Comparison of the Synergistic Effects of ChoP, ATP, and Insulin on DNA Synthesis in the P-3T3 and N-3T3 subclones—It was known that only P-type, and not N-type, cells respond to the cell growth stimulatory effects of PMA and tyrosine kinase activators (9, 10). Data in Fig. 3 show that while the responses of these cells to insulin alone were similar, insulin and ChoP had significant synergistic effects on DNA synthesis only in the P-3T3 subclone.

ATP was able to significantly enhance the mitogenic effects of both ChoP alone and ChoP plus insulin in both subclones, although the three agents together were still at least 3–4 times more effective inducers of DNA synthesis in the P-3T3 than in the N-3T3 cells (Fig. 4). However, these differences appeared to be mainly due to the poor response of N-3T3 cells to ChoP plus insulin but not to ATP. The finding that the combined effects of ChoP, ATP, and insulin were only quantitatively different in the two subclones suggested that the use of clone 7/3T3 cell population for additional studies on the combined effects of ChoP and ATP will not lead to misinterpretation of data.
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ATP in the presence of 1 mM ChoP in N-3T3 cells. Similar differences were found in six other experiments using 1 mM ChoP and insulin (open symbols) or presence (closed symbols) of 500 nM insulin. Each point represents the mean ± S.E. of three experiments each performed in triplicate with the same passage of cells. Similar differences were found in six other experiments using 1 mM ChoP.

ATP Stimulates the Release but Not the Cellular Uptake of ChoP in Various Fibroblast Cultures—Previous data reported by another laboratory (6) indicated incorporation of a limited amount of ChoP into NIH 3T3 fibroblasts when it was added to the medium at a high (20 mM) concentration. This is an important issue, because if ChoP acts through an intracellular target, the use of this compound even at 20 mM concentration would be justified. However, should ChoP act through an extracellular target, then questions arise of where extracellular ChoP is derived from and what concentration range this compound might reach in the extracellular space. For these uptake studies, the lowest mitogenically active concentration (0.1 mM) of [14C]ChoP was used, because this is likely to be physiologically relevant, and we also tried to maintain the specific activity of ChoP as high as possible. During a 10-h incubation period, we could detect neither the uptake of [14C]ChoP into the clone 7/3T3, P-3T3, and N-3T3 cultures nor its incorporation into cellular phosphatidylcholine (Table II). Similarly, no uptake of labeled ChoP into clone 7 fibroblasts was observed after incubations for 2, 4, or 6 h (data not shown). In contrast, Ha-Ras-transformed fibroblasts incorporated significant amounts of labeled ChoP, which then was used for phosphatidylcholine synthesis (Table II). Interestingly, in the transformed cells both the uptake and incorporation of [14C]ChoP into phosphatidylcholine were inhibited by ATP (Table II). These results with the transformed cells also indicated that the experimental design used here was suitable to detect incorporation of labeled ChoP into fibroblasts.

It is important to add that, both in the absence and presence of ATP, added labeled ChoP remained metabolically very stable in the incubation medium; in the three untransformed cell populations we could not observe any statistically significant degradation of labeled ChoP during a 10-h incubation period (data not shown).

The results shown above made it clear that ChoP must induce mitogenesis through an extracellular site. Therefore, if ChoP plays a role in mitogenesis, it should be able to exit the cells in a probably highly regulated manner. To see if this is the case, next we tested the possible effect of ATP on the release of ChoP from fibroblasts into the medium. When clone 7/3T3 fibroblasts were incubated in the presence of 1 mM ChoP (open symbols) or ChoP plus 500 nM insulin (closed symbols). Each point represents the mean ± S.E. of three experiments each performed in duplicate with the same passage of cells. 100 μM ATP was found to exert similar effects in six other experiments.
were obtained in two other experiments each performed in triplicate with the same passage of cells. Similar results were obtained in three incubations performed with the same passage of cells. Similar results were obtained in these experiments performed in triplicate.

Effects of ChoP, Insulin, and ATP on MAP Kinase Activity—Initially, insulin stimulates several growth-regulatory mechanisms involving Shc-Grb2-Sos trimers as well as the various complexes of insulin receptor substrate 1 with Grb2, PI 3'-kinase, Syk, and Nck. In many cell lines the Ras — MAP kinase signal transduction pathway appears to be a major downstream converging point for these mechanisms (26–28). In contrast, in Swiss 3T3 (29) and Balb/c 3T3 cells (30), insulin alone did not stimulate MAP kinase activity. Similarly, when we assessed MAP kinase activity by measuring its rate of tyrosine phosphorylation (31, 32), we observed that in NIH 3T3 fibroblasts insulin had only a very small, nearly undetectable, transient effect on this enzyme activity between 5 and 15 min of incubation. At these time intervals, ChoP in combination with either insulin or ATP also had only very small effects (data not shown). Reproducible, but still very small, effects of ChoP in combination with insulin or ATP were observed (Fig. 6). In contrast, PMA, which is a less efficient inducer of DNA synthesis than ChoP plus insulin or ChoP plus ATP (Table III), greatly stimulated MAP kinase activity (Fig. 6). Furthermore, similar to Balb/c 3T3 fibroblasts (28), PMA and insulin synergistically stimulated MAP kinase activity (Fig. 6). Thus, in these fibroblasts only the protein kinase C-dependent mitogenic actions of insulin appear to involve the MAP kinase pathway.

The Role of pp70s6k and PI 3'-Kinase in the Mitogenic Effects of ChoP and ATP—Similar to many other mitogens, insulin also stimulates ribosomal S6 kinases (pp70s6k and pp90rsk). It is believed that activation of MAP kinase and pp90rsk are on the same pathway, while activation of pp70s6k by insulin occurs by a MAP kinase-independent (33) and PI 3'-kinase-dependent mechanism (34). Rapamycin (5 mM), an apparently specific inhibitor of pp70s6k (34, 35), strongly inhibited the combined mitogenic effects of ChoP plus ATP and/or insulin (Fig. 7). Rapamycin also effectively inhibited the mitogenic effects of platelet-derived growth factor and insulin-like growth factor I (data not shown). In comparison, a concentration of wortmannin (200 nM) that should have maximal inhibitory effect on PI 3'-kinase (36, 37), had clearly smaller inhibitory effects on ChoP plus ATP and/or insulin-induced DNA synthesis (Fig. 7). None of these inhibitors had detectable effects on cell viability as determined by the trypan blue exclusion assay.

Recent studies suggest that wortmannin may not be a specific inhibitor of PI 3'-kinase (38, 39). To further examine the role of PI 3'-kinase in the mediation of ChoP effects, we expressed the dominant negative mutant of the p85 subunit of this enzyme. In each of the five clones isolated, the ratio between the wild type (0.268 kilobase pairs) and mutant (0.169 kilobase pairs) subunits was about 1:1, based on the detection of amplified cDNA. Most studies were performed with one clone, but in an additional experiment the results were confirmed with the four other clones. As shown in Table IV, in the mutant p85 expressor cells the effects of ChoP plus insulin and ChoP plus ATP as well as the combined effects of the three agents on DNA synthesis were similarly, but only partially (~50%), reduced compared with the vector control cells. We should note here that for presently unknown reasons a previous exposure of cells to hygromycin B decreased the mitogenic effect of ChoP alone, although such treatment did not seem to affect the mitogenic activities of ATP and insulin in the presence of ChoP. This effect of hygromycin B is unlikely to affect interpretation of data, because both the vector control and the mutant expressor cells were exposed to hygromycin B for the
same period of time. As a positive control, in the mutant p85 subunit expressing cells the mitogenic effect of PMA was almost completely blocked (Table IV). This result was expected, because previously we showed (40) that in NIH 3T3 cells wortmannin strongly inhibited PMA-induced DNA synthesis.

If the mitogenic effects of ChoP plus ATP and ChoP plus insulin involved PI 3'-kinase activity, as suggested by data in Table IV, then these agents would be expected to increase the lipid kinase activity of this enzyme. As shown in Fig. 8, insulin alone, and particularly in combination with PMA or ChoP, significantly enhanced the formation of phosphatidylinositol 3-phosphate by PI 3'-kinase; these stimulatory effects ranged from -3.5- to 6-fold, as determined by densitometry. Formation of phosphatidylinositol 4-phosphate was not observed. ChoP and ATP alone had no effects (not shown), but in combination they enhanced PI 3'-kinase activity nearly 2-fold (Fig. 8). The combined stimulatory effects of ChoP and insulin were not enhanced by ATP (Fig. 8). Overall, these data indicated that the ATP- and insulin-dependent stimulation of DNA synthesis by ChoP is associated with increased PI 3'-kinase activity.

Synergistic Mitogenic Effects of ChoP and S1P—Since extracellular S1P stimulates proliferation of fibroblasts (12, 13), it was of interest to examine a possible relationship between the mitogenic actions of SIP and ChoP. In NIH 3T3 fibroblasts, 0.5 M S1P caused an approximately 3-fold increase in DNA synthesis, and a maximal (-13-fold) stimulatory effect required -5 M S1P (Fig. 9). Similar to the mitogenic effect of ChoP, the stimulatory effects of both lower and higher concentrations of S1P on DNA synthesis were greatly enhanced by both ATP and insulin (Fig. 9). Furthermore, S1P-induced DNA synthesis was also more effectively inhibited by rapamycin than by wortmannin (Fig. 10). More surprisingly, ChoP and S1P synergistically enhanced, even when both presented at maximally effective concentrations, DNA synthesis (Fig. 11). Extracellular ATP was able to further increase the synergistic effects of ChoP and S1P (Fig. 11). It should be noted here that other phosphate-containing compounds, including ethanolamine phosphate (0.1–2 mM) and creatine phosphate (0.1–5 mM) had no mitogenic effects.

Possible Role of Calcium in ChoP-Induced Mitogenesis—Both S1P (12, 14) and ATP (Ref. 41 and references therein) can cause transient increases in cytoplasmic calcium levels. Although ChoP does not mobilize calcium (17), an increase in intracellular calcium by ATP or S1P may enhance the mitogenic effects of ChoP. If this is the case, then other calcium-elevating agents would also be expected to enhance the mitogenic effects of ChoP. In fibroblasts, bombesin has been shown to raise the level of cytoplasmic calcium as a consequence of increased hydrolysis of inositol phospholipids (42–44). In agreement with data in a previous report (45), a maximally effective concentration (100 nM) of bombesin alone had only a relatively small (-2.5-fold) stimulatory effect on DNA synthesis (Table V). However, bombesin clearly potentiated, although less efficiently than ATP, the mitogenic effect of ChoP (Table V). It is of interest to note that in the presence of ATP, bombesin had no such potentiating effect (Table V), suggesting that ATP and bombesin used, at least in part, the same mechanism to enhance the mitogenic effect of ChoP. A short (10-min) pretreatment with the calcium-mobilizing agents thapsigargin (1 M) and ionomycin (1 M), followed by treatment of washed cells with ChoP for 16 h, also led to 1.6–1.8-fold increases in the mitogenic effects of ChoP (data not shown). In another experiment, initially performed in a calcium-free medium, 10 M
BAPTA/AM (a cell-permeable calcium chelator) was added to cells 20 min prior to ATP (100 μM); after an additional 10-min incubation in the presence of ATP, cells were washed and then incubated further in a calcium-containing medium for 16 h in the presence of ATP and ChoP. Under these conditions, ATP enhanced the effect of ChoP (1 mM) on DNA synthesis about 2.8-fold, and BAPTA/AM inhibited the potentiating effect of ATP, but not the effect of ChoP alone, by ~40% (data not shown).

**DISCUSSION**

In this work we have shown that extracellular ATP could greatly enhance the mitogenic and co-mitogenic effects of ChoP, even when the latter was present at a maximally effective concentration. Since a number of P2 receptor agonists, but not AMP or adenosine, mimicked the co-mitogenic effects of ATP, it seems clear that the effects of ATP were mediated by one of the P2 receptors. Furthermore, the finding that UTP was able to enhance the effect of ChoP suggests the specific role of the P2u receptor, which is uniquely stimulated by UTP, in addition to ATP and ATPγS (46, 47).

Perhaps the most important aspect of the co-mitogenic ATP effect is that it decreased the mitogenically active concentration of ChoP from about 1 mM to 0.1–0.25 mM. Since in normal fibroblasts the concentration of ChoP is around 0.5 mM (6), it is reasonable to assume that a substantial release of ChoP from these cells could elevate the concentration of ChoP in the extracellular space to the 0.1–0.25 mM range. In this context, the ability of ATP to induce extensive release of ChoP from cells becomes a very important issue, because presently this is the only known mechanism by which ChoP can become a mitogen. Preliminary studies in our laboratory show that after a 4-h treatment period ATP induces massive ChoP release (15–35% of the total cellular pool) in each cell type examined so far, including C3H/10T1/2 fibroblasts, Rat1 fibroblasts, MCF-7 human breast carcinoma cells, and epidermal JB6 cells. Thus, it appears that if once the concentration of ATP in the extracellular space reaches a certain value (10–50 μM), then a sufficient amount of ChoP from cells is probably released to enhance cell growth, an action that is also potentiated by ATP. Accordingly, extracellular ATP is clearly an agent that not only increases the release of ChoP, but at the same time also potentiates the action of ChoP through an extracellular target. Interestingly, despite the large number of papers describing the effects of extracellular ATP, the issues of how much ATP is present in the extracellular space and in the circulation and how ATP molecules can get there are much less studied. However, it is known that ATP can be liberated from cells at the site of a wound, during platelet activation, and by the necrosis of cells during tumor growth (reviewed in Ref. 48). Clearly, more information is needed about the regulation of ATP concentration in the extracellular medium before the true physiological significance of the combined mitogenic effects of ChoP and ATP can be assessed.

While the p42/p44 MAP kinases appear to play key roles in the mediation of mitogenic effects of insulin in many cellular systems (26–28), in NIH 3T3 cells, as in other 3T3 lines (29, 30), these enzymes were significantly activated by insulin only in the presence of PMA. Although in the presence of ChoP insulin slightly activated MAP kinase, these effects were much smaller than that obtained with PMA alone. Collectively, these observations indicate that the mitogenic effects of ChoP, both in the presence of insulin and ATP, are predominantly mediated by a MAP kinase-independent pathway and that only the protein kinase C-dependent mitogenic effects of insulin involve a MAP kinase-dependent pathway.

Previous reports suggested that in fibroblasts p42/p44 MAP kinases are required for mitogenesis (49, 50). However, in
pp70s6k (34), which directs site-specific phosphorylation of Thr252 in the catalytic domain of pp70s6k (57). Thus, the PI 3'-kinase-dependent effects of ChoP may also involve pp70s6k.

Similar to ChoP, extracellular S1P also enhanced DNA synthesis in a strongly ATP- and insulin-dependent manner. While S1P has been shown to stimulate DNA synthesis in fibroblasts (12, 13), this is the first study to reveal its synergistic interaction with ATP and insulin. However, even maximally effective concentrations of ChoP and S1P had strong synergistic effects, indicating important differences in their actions. One clear difference is that S1P, in contrast to ChoP, is an activator of MAP kinases (58). Collectively, these findings suggest three possible mechanisms to explain the synergistic actions of ChoP and S1P. (i) ChoP and S1P may act through separate sites. In this case, their individual effects may be amplified by a common target along the signal transduction pathway, or the effect of ChoP may be amplified by calcium generated by S1P (as well as by ATP, bombesin, and possibly other calcium-elevating agents). (ii) ChoP and S1P may act through separate sites on pp70s6k (both ChoP and S1P) and MAP kinase (only S1P) activities, resulting in synergistic activation of mitogenesis. (iii) Finally, ChoP may bind to and enhance the function of the recently described S1P receptors (15, 16). Further experiments are required to distinguish between these possibilities. Also, the exact contribution by calcium to the potentiation of ChoP effects by ATP and S1P remains to be clarified.

An interesting question is how ChoP and ATP may affect the actions of other growth factors. Cuadrado et al. reported (6) that ChoP was also required for the actions of other growth factors such as platelet-derived growth factor and fibroblast growth factor. Although we could repeat their findings (6) that 5 mM hemicholinium-3, an inhibitor of choline kinase, inhibited the actions of these growth factors both on choline kinase activation and DNA synthesis, we also found that this drug has apparently nonspecific effects on DNA synthesis; *e.g.*, this inhibitor was found to actually enhance the mitogenic effects of both insulin and ChoP. Thus, this agent may not be used for the study of the role of choline kinase in the regulation of mitogenesis. Most recently we expressed choline kinase in NIH 3T3 fibroblasts; these overexpressors are presently being used to clarify the role of ChoP in the mitogenic actions of various growth factors.

A potential problem of using the NIH 3T3 cell line is the apparent heterogeneity of cells in their response to growth regulatory agents. This prompted us to extend the studies on the mitogenic effects of ChoP, ATP, and insulin to the P-3T3 and N-3T3 subclones, which make up the major fraction of the NIH 3T3 line (9, 10). Evidently, growth regulation in the two subclones by the above agents is quite different, although the primary reason for the observed differences was the greatly reduced mitogenic activity of ChoP in the N-3T3 population. The fact that ATP restored the response of these cells to ChoP to a significant extent, particularly in the presence of insulin, indicated that using the clone 7 (original) NIH 3T3 cell population for these studies will not lead to misinterpretation of the data. In addition, N-3T3 cells may be used (since the base line of ChoP action is low) to identify other potentiating agents and to determine the exact mechanism(s) by which ChoP interacts

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**TABLE V**

**Bombesin potentiates the mitogenic effect of ChoP in the absence of ATP**

| Addition | Incorporation of [3H]thymidine into DNA |
|----------|---------------------------------------|
|          | Without bombesin | With bombesin |
| None     | 1110 ± 160        | 2780 ± 210    |
| ChoP     | 20,690 ± 1630     | 38,340 ± 2520 |
| ChoP + ATP | 64,770 ± 3010 | 67,460 ± 3650 |

Swiss 3T3 fibroblasts, these MAP kinases were less important for the mitogenesis induced by bombesin plus insulin (29). Our results further indicate that stimulation of DNA synthesis through combined treatments with ChoP, ATP, and insulin is possible without involving the MAP kinase-dependent mitogenic pathway.

ChoP, when added in combination with insulin or ATP, stimulated PI 3'-kinase activity. In addition, the expression of a dominant negative mutant of the p85 subunit of PI 3'-kinase or the addition of wortmannin led to partial inhibition of the combined mitogenic effects of ChoP, ATP, and insulin. These data indicate that while maximal increase in DNA synthesis by these agents requires PI 3'-kinase, treatments with ChoP, ATP, and insulin can also induce mitogenesis by a PI 3'-kinase-independent pathway.

In contrast to wortmannin, rapamycin, a specific inhibitor of pp70s6k (34, 35), strongly inhibited the mitogenic effects of ChoP both in the absence and presence of ATP and insulin. These observations implicate pp70s6k in the mediation of mitogenic effects of ChoP. The pp70s6k enzyme is a ubiquitous Ser/Thr kinase that is activated by virtually all known mitogens and that plays a key role in the progression of cells from G1 to S phase of the cell cycle (51–54). Full activation of pp70s6k requires several independent stimuli resulting in multiple phosphorylation of the enzyme (Ref. 55 and references therein). Activated pp70s6k phosphorylates ribosomal S6 protein, resulting in increased translation of mRNAs containing a polypyrrolidine tract (56). PI 3'-kinase is an important regulator of pp70s6k (34), which plays a key role in the progression of cells from G1 to S phase of the cell cycle (51–54). Full activation of pp70s6k requires several independent stimuli resulting in multiple phosphorylation of the enzyme (Ref. 55 and references therein). Activated pp70s6k phosphorylates ribosomal S6 protein, resulting in increased translation of mRNAs containing a polypyrrolidine tract (56). PI 3'-kinase is an important regulator of pp70s6k (34), which directs site-specific phosphorylation of Thr252 in the catalytic domain of pp70s6k (57). Thus, the PI 3'-kinase-dependent effects of ChoP may also involve pp70s6k.
with ATP, insulin, and SIP.

The major features of the combinatorial effects of ChoP, ATP, and SIP are illustrated in the scheme shown in Fig. 12. Accordingly, ChoP, whose formation is stimulated by growth factors (59), carcinogens (60–62), and oncogenes (63, 64), is secreted from cells by a process that is activated by extracellular ATP through a purinergic P<sub>2</sub>-type cell surface receptor. The effect of ChoP through an extracellular site is amplified by ATP and S1P by mechanisms probably involving receptor. The effect of ChoP through an extracellular site is dependent cell growth-regulatory mechanisms.

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**REFERENCES**

1. Larrodera, P., Cornet, M. E., Diaz-Meco, M. T., Lopez-Barabona, M., Diaz-Laviada, I., Guddal, P. H., Johansen, T., and Moscat, J. (1990) Cell 61, 1113–1120.

2. de Herreros, A. G., Dominguez, I., Diaz-Meco, M. T., Graziani, G., Cornet, M. E., Guddal, P. H., Johansen, T., and Moscat, J. (1991) J. Biol. Chem. 266, 6825–6829.

3. Choudhury, G. G., Sylvia, V. L., and Sakaguchi, A. Y. (1991) J. Cell Biol. 116, 21471–21477.

4. Lee, J. J., and Pitch, P. F. (1994) Am. J. Physiol. 266, C319–C334.

5. Cheatham, B., and Kahn, C. R. (1995) Endocrine Rev. 16, 117–142.

6. Saltiel, A. R. (1996) Am. J. Physiol. 270, E375–E385.

7. Seufferlein, T., Witthers, D. J., and Rozengurt, E. (1996) J. Biol. Chem. 271, 21411–21417.

8. Payne, D. M., Rossomando, A., Martino, P., Erickson, A. K., Her, J.-H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1992) Nature 356, 651–653.

9. Blenis, J., Chung, J., Eirikson, E., Alcotra, D. A., and Eirikson, R. L. (1991) Cell Growth & Differ. 2, 279–285.

10. Chung, J., Grammer, T. C., Lemon, K. F., Kazlauskas, A., and Blenis, J. (1994) Nature 370, 71–75.

11. Chung, J., Koo, C. J., Crabtree, G. R., and Blenis, J. (1992) Cell 70, 1227–1236.

12. Arace, A., and Wyman, M. P. (1993) Biochem. J. 296, 297–301.

13. Fry, M. J. (1994) Biochim. Biophys. Acta 1226, 2629–2668.

14. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. O. (1995) J. Biol. Chem. 270, 25352–25355.

15. Ferby, I. M., Waga, I., Hoshino, M., Kame, K., and Shimizu, T. (1996) J. Biol. Chem. 271, 11684–11688.

16. Kiss, Z., and Tomono, M. (1995) FEBS Lett. 371, 185–187.

17. Gonzalez, F. A., Rozengurt, E., and Heppel, L. A. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4530–4534.

18. Takawa, N., Takawa, Y., Bollag, W. E., and Rasmussen, H. (1987) J. Biol. Chem. 262, 182–188.

19. Arace, A., and Wyman, M. P. (1993) Biochem. J. 296, 297–301.

20. Kiss, Z., and Tomono, M. (1995) Biochem. Biophys. Res. Commun. 214, 1226–1236.

21. Kiss, Z., and Anderson, W. B. (1989) Oncogene 4, 7415–7419.

22. Kiss, Z., and Anderson, W. H. (1994) EMBO J. 13, 885–892.

23. Bierman, A. J., Koenderman, L., Tool, A. J., and de Laat, S. W. (1990) J. Cell Biol. 116, 21484–21498.

24. Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S., and Takenawa, T. (1988) Science 239, 640–643.

25. Erb, L., Lustig, K. D., Sullivan, D. M., Turner, J. T., and Weissman, A. G. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10449–10453.

26. Ferry, C. E., Sullivan, D. M., Paradiso, A. M., Lazarowski, E. R., Burch, L. H., Olsen, J. C., Erb, C., Weissman, G. A., Bouer, R. C., and Turner, J. T. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3275–3279.

27. Gordon, J. L. (1986) Biochem. J. 233, 309–319.

28. Pages, G., Lenormand, P., L’Allais, G., Chambard, J.-C., Melcione, S., and Pousségur, J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8319–8323.

29. Bronedello, J. M., McKenzie, P. R., Sun, H., and Pousségur, J. (1995) Oncogene 10, 1895–1904.

30. Lane, H. A., Fernandez, A., Lamb, N. J. C., and Thomas, G. (1993) Nature 363, 170–172.

31. Ming, X. F., Burgering, B. M., Wannenst, S., Claesson, W. L., Heldin, C. H., and Avruch, J. (1995) Curr. Opin. Cell Biol. 7, 806–814.

32. Han, J.-W., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) J. Biol. Chem. 270, 21396–21403.

33. Kiss, Z., and Tomono, M. (1995) Biochem. Biophys. Res. Commun. 214, 1895–1904.

34. Ferreris, H. B., Reinhard, C., Kozma, S. C., and Thomas, G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4441–4445.

35. Weng, Q.-P., Andrabi, K., Klippel, A., Kozlowski, M. T., Williams, L. T., and Avruch, J. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 5744–5748.

36. Wax, J., Spiegel, S., and Sturgill, T. W. (1996) J. Biol. Chem. 271, 11484–11488.

37. Warden, C. H., and Friedkin, M. (1985) J. Biol. Chem. 260, 6006–6011.

38. Itoh, G., Tsurumaki, M., and Nakaiki, Y. (1980) Biochem. Biophys. Res. Commun. 196, 946–952.

39. Paulson, B. K., Porter, J. T., and Kent, C. (1989) Biochim. Biophys. Acta 1004, 274–277.

40. Kiss, Z., and Tomono, M. (1995) FEBS Lett. 358, 243–246.

41. Ratnam, S., and Kent, C. (1996) Arch. Biochem. Biophys. 323, 313–322.

42. Kiss, Z., and Krilly, K. S. (1995) FEBS Lett. 357, 279–282.