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Generation of Phosphorylcholine as an Essential Event in the Activation of Raf-1 and MAP-Kinases in Growth Factors-Induced Mitogenic Stimulation

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Abstract Cell proliferation is regulated by an appropriate combination of intracellular signals involving activation of kinases and the generation of phospholipid metabolites. We report here that growth factors induce a biphasic generation of phosphorylcholine (*P*Cho) in quiescent NIH 3T3 cells, resulting in an early and transient increase at 100 s and a larger and sustained increase after 3 h of stimulation. Generation of *P*Cho at both early and late times of growth factors stimulation results from the consecutive activation of phospholipase D (PLD) and choline kinase (ChoK). Production of *P*Cho by specific growth factors seems an essential requirement for the early signals associated to activation of Raf-1 and MAP kinases, since blockage of choline kinase completely inhibited activation of Raf-1 and MAP kinases by PDGF or FGF. Both the transient early increase and the late sustained increase in *P*Cho are required for the induction of DNA-synthesis, besides completion of the activation of the serine/threonine kinases cascade. Thus, our results strongly suggest that generation of *P*Cho by the PLD/choline kinase pathway is one of the critical steps in regulating cell growth in NIH 3T3 stimulated by growth factors. © 1995 Wiley-Liss, Inc.

Key words: Growth factors, cell growth, phospholipase D, hemicholinium-3, phosphorylcholine, choline kinase, Raf-1, MAP kinase

Reinitiation of DNA synthesis in quiescent cells is the result of cooperation among multiple signalling pathways [reviewed in Rozengurzt, 1986; Cantley et al., 1991; Pouyssegur and Seuwen, 1992]. Potent fibroblast mitogens like PDGF or FGF are capable of activating several distinct signal transducing elements. However, a complete scheme of all intracellular pathways activated by PDGF or FGF has not been drawn yet. One of the important signals is the rapid activation of the Ser/Thr kinases cascade, involving Raf-1, MEK, and MAP kinases [reviewed in Pelech and Sanghera, 1991, 1992]. Also, the generation of several lipid-derived intracellular messengers, both at early and at late times of stimulation, seems to play a role in this complex cascade of events that finally leads to mitogenesis [reviewed in Warden and Friedkin, 1985; Exton, 1990].

An important concept for the understanding of the control of cell proliferation is the existence of common early key steps for integrating signalling pathways. There is evidence supporting the notion that Raf-1 and MAP kinases are good candidates for integrating and transmitting growth factor signals to the nucleus [Janknecht et al., 1993; Gille et al., 1992; Pulverer et al., 1991]. In this regard, several recent reports have unravelled the mechanism of activation of intracellular serine/threonine kinases [Howe et al., 1992; Kyriakis et al., 1992]. These kinases are connected in a linear mitogenic cascade

Receptor tyrosine kinase
↓
ras-p21 → Raf-1 → MEK → MAP kinase
→ phosphorylation and activation of
transcription factors and p90^{rsk} kinase

that seems to be essential for the Go to S phase transition induced by growth factors. A similar scheme is observed in oncogenes-transformed cells, and in particular in *ras*-transformed cells, since *ras*-p21 associates to the Raf-1 kinase, although the precise mechanism of activation of

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Raf-1 by *ras*-p21 is not fully understood [Kolch et al., 1991].

Phosphatidylcholine (PC) metabolism has been proposed as an essential late event in the regulation of cell growth. This hypothesis is based on the sustained elevated levels of DAG and *P*Cho at late times (after 3 h) of stimulation of quiescent cells by growth factors [Warden and Friedkin, 1985; Exton, 1990; Cuadrado et al., 1993], and the finding of constitutively elevated levels of DAG and *P*Cho in *ras*-transformed cells [Lacal et al., 1987]. Two hypotheses have been proposed. On one hand, a specific PC-PLC activity has been suggested as the target for PDGF and *ras*-p21 pathways [Larrodera et al., 1990]. Alternatively, generation of *P*Cho by the consecutive activation of a PC-PLD and choline kinase has been proposed to be the critical enzymes involved in this process [Carnero et al., 1994; Cuadrado et al., 1993].

Growth factors and oncogenes induce the activation of a PC-PLD, generating phosphatidic acid (PA) and choline, which are further converted into DAG and *P*Cho by the PA-hydrolase and choline kinase respectively [Nishizuka, 1992; Exton, 1990; Warden and Friedkin, 1985]. Both PA and *P*Cho have been shown to be mitogenic to NIH 3T3 cells [Moolenar et al., 1986; Corven et al., 1989; Cuadrado et al., 1993]. Moreover, we have recently demonstrated that the generation of DAG and *P*Cho as a late event in normal cells stimulated by growth factors, and constitutively in *ras*-transformed cells, is a consequence of the activation of a PC-PLD rather than a PC-PLC [Carnero et al., 1994a].

Most growth factors studied can induce the activation of both a PI-PLC in fibroblasts as well as a PC-PLD as an early event (less than 10 min). Moreover, activation of the Ser/Thr kinases cascade takes places within this early time window, indicating that some of the most critical triggering signals should be placed at early times of stimulation. However, this seems to be not the only requirement, since growth factors need to be present for at least 6–8 h in order to efficiently induce DNA synthesis [Pledger et al., 1977, 1978].

In order to study the role of phosphorylcholine as a second messenger involved in the complex network that controls cell proliferation, we have investigated whether the generation of *P*Cho is required for the early and late events associated to cell proliferation. We show that inhibition of ChoK was sufficient to completely block growth factors-induced activation of Raf-1 kinase, p44 and p42 MAP kinases, as well as DNA synthesis. However no effect was observed in any of these parameters under the same conditions of ChoK inhibition when cells were stimulated by serum. Thus, generation of *P*Cho induced by growth factors is an essential requirement for both early and late events of mitogenic signalling pathways.

MATERIALS AND METHODS

Cell Culture and Reagents

NIH/3T3 mouse fibroblasts were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (Gibco, Grand Island, NY) under standard conditions of temperature (37°C), humidity (95%), and carbon dioxide (5%). Commercially available reagents were hemicholinium-3 from Sigma Chemical Co. (St. Louis, MO), homodimer BB platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) from Upstate Biotechnology, Inc. (Lake Placid, NY).

Analysis of Phosphorylcholine Production

Cells were seeded on 6-well plates and grown to confluence. Then, cells were serum starved for 48 h in DMEM supplemented with 10 μCi/ml methyl[^{3}H]choline (50–60 Ci/mmol, Amersham International, Amersham, UK). Cells were then stimulated with the indicated growth factors. After stimulation, the cells were rinsed in ice-cold phosphate buffered solution (PBS) and fixed with 16% ice cold trichloracetic acid (TCA). TCA-soluble material containing phosphorylcholine was resolved in thin layer chromatography (TLC) plates using as liquid phase 0.9% NaCl:methanol: ammonium hydroxide (50:70:5) (V:V:V). The plates were exposed to X-ray film and determination of radioactive phosphorylcholine was conducted by scraping radioactive spots and scintillation counting. When indicated, cells were treated with 6 mM HC-3 for 2 h before treatment and maintained during the stimulation period.

Mitogenic Assays

Cells were seeded on 24-well plates and grown to confluence. They were serum-starved for 24 h and then stimulated for 24 additional h with the indicated mitogens. Four hours before processing 1 μCi/ml methyl[^{3}H]thymidine (45 Ci/mmol, Amersham International) was added and cells incubated under standard conditions of temperature, humidity, and CO_2 atmosphere.

[^{3}H]thymidine incorporation into DNA replicating cells was determined as the amount of radioactivity present in trichloracetic acid (TCA) insoluble material. The cells were rinsed twice with PBS and twice with ice cold 16% TCA. Then, TCA insoluble material was solubilized in 1 ml of 0.25 N sodium hydroxide for 1 h. The solubilized solution was scintillation counted.

Western Blot Analysis of MAP-1 K and MAP-2 K

A peptide corresponding to the C-terminus of MAP-1 K (KERLKELIFQETAR) was conjugated to tyroglobulin by crosslinking with glutaraldehyde. The conjugated peptide was used to raise a rabbit polyclonal antibody by standard procedures. The antiserum was analyzed by Western blotting and found to recognize in whole cell lysates only two bands (p44 and p42) corresponding to MAP-1 K and MAP-2 K.

All the experiments shown were performed using 10 cm dishes of confluent, quiescent NIH 3T3 cells which had been serum-starved for 48 h in DMEM supplemented with 0.5% fetal calf serum. Medium was replaced with 2 ml of serum-free DMEM containing the indicated growth factors or treatments, and stimulations proceeded at 37°C for indicated times. Incubations were terminated by aspirating off media, washing with TD buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM Tris, pH 7.4) and addition of 300 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mM $Na_4P_2O_7$, 50 mM FNa, 0.1 mM Na_3VO_4,20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM PMSF). Nuclei and detergent-insoluble material were removed by centrifugation at 10,000*g* for 10 min. The resulting supernatants were assayed for estimation of total cell protein (Bio-Rad, Rockville Centre, NY) and equal amounts of cell lysate (tipically 40 μg) were boiled at 95°C for 5 min in SDS-PAGE sample buffer. For Western blot analysis, proteins were electrophoresed onto 10% SDS-PAGE gels poured in 20 × 20 cm glasses. Separated proteins were transfered to nitrocellulose and blots were blocked for 2 h in 2% nonfat dried milk in T-TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.005% Tween 20). Blots were washed once in T-TBS and incubated 4 h with a 1:1,000 dilution of the polyclonal anti-MAPK antibody. Blots were washed 3 times for 10 min. in T-TBS, incubated 1 h with 1:1,000 anti-rabbit Ig biotinylated (Amersham), washed 3 times for 10 min with T-TBS, and incubated 30 min with streptavidin-horseradish peroxidase (Amersham) 1:1,000 in T-TBS. After washing 3 times with T-TBS 10 min MAP-1-K and MAP-2-K were detected by the ECL protocol (Amersham). The activation of MAP kinases in response to mitogens was assessed by the mobility shift produced as a consequence of the hyperphosphorylation of these kinases.

Western Blot Analysis of Raf-1

Raf-1 was detected using a polyclonal antibody raised against the Raf-1 peptide CTLTTSPRLLPVF, essentially as described for the MAPK antibody. Western blot analysis was carried out as indicated for MAP-kinases, except that protein extracts were resolved in 8% SDS-PAGE gels.

In Situ MAP-kinase Assay

For the in-gel kinase assay, the polyacrylamide gel was polymerized with 0.25 mg/ml MBP and after electrophoresis, denatured in 6 M guanidine-HCl, washed as described by Kameshita and Fujisawa [1989], and the kinase activity assayed with 50–150 μCi of [γ-^{32}P]-ATP and 10 μM ATP.

Determination of Tyrosine Phosphorylation

Cells were serum starved overnight and incubated with HC-3 at the indicated concentrations for over 30 min. Prior to lysis, they were stimulated with 10 ng/ml of PDGF-BB for 10 min. Lysis was made in buffer containing 50 mM Hepes, pH 7.5, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na_2 VO_3, and 1 mM PMSF. Total cell lysate (100 g) was resolved in 7.5% PAGE and blotted to nitrocellulose. Filters were blocked in buffer containing 25 mM Tris-HCl, pH 7.5, 0.05% Tween, 150 mM NaCl, and 5% BSA for 2 h at 50°C. Phosphotyrosine containing bands were detected by incubating the blot for 2 h in the same buffer with antiphosphotyrosine specific antibody (Upstate Biotechnology, Inc.) followed by additional incubation of 1 h [125 I] protein A.

Analysis of PI3K Activity

Cells were serum starved overnight, incubated 1 h with 6 mM of HC-3, and stimulated with 10 ng/ml PDGF for 5 min. Cells were washed with cold phosphate buffer saline, rinsed with 137 mM NaCL, 20 mM Tris pH:8, 1 mM MgCl, 1 mM $CaCl_2$ (Buffer A), and lysed in buffer A containing 1% NP40 and 1 mM PMSF at 4°C for 20 min. Insoluble material was re-

moved by centrifugation. The supernatants were incubated with 10 μl of a mouse monoclonal antiphosphotyrosine (RPN 138, Amersham) overnight at 4°C. Sepharose Cl4B protein A (Sigma) was added, and incubation continued for additional 1 h. The immunoprecipitates were collected and washed once with PBS, twice with 0.5 M LiCl, 0.1 M Tris pH:7.4 and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. Immunoprecipitates were then assayed for phosphatidyl inositol phosphorylation activity, as described by Varticovski et al. [1991].

RESULTS

PDGF Induce the Generation of *P*Cho as an Early Event

Growth factors induce the rapid generation of choline and *P*Cho after stimulation of quiescent cells [Price et al., 1989]. Generation of these metabolites may be a consequence of activation of PC-specific phosholipases D and C, respectively [Price et al., 1989; Exton, 1990]. Choline kinase (ChoK), the first step in the route of phosphatidylcholine synthesis, is the enzyme responsible for *P*Cho production in the anabolic pathway [Warden and Friedkin, 1985]. Hemicholinium-3, a ChoK inhibitor with no effect on known phospholipases, can be used in vivo as a rather specific inhibitor of choline kinase, therefore making possible to distinguish between PLC- from PLD-induced generation of *P*Cho [Cuadrado et al., 1993; Carnero et al., 1994a]. We have analyzed the generation of both choline and *P*Cho as an early event in PDGF-stimulated NIH 3T3 cells and shown that both metabolites are generated within 2 min of treatment with the growth factor (Fig. 1). While increases of up to 90% over basal levels of choline could be observed, the *P*Cho levels increase was less pronounced, reaching up to 35% of basal levels. When the generation of both metabolites was analyzed in the presence of HC-3, *P*Cho production was completely inhibited while there was no inhibition on the generation of choline, an indication of the lack of effect of HC-3 on PLD activity, in agreement with previous reports [Carnero et al., 1994a,b; Cuadrado et al., 1993]. Similar results were also observed in bFGF-stimulated cells (data not shown), suggesting that this effect may be general to mitogenic growth factors in NIH-3T3 cells. These results indicate that the early production of *P*Cho induced by growth factors is generated as a consequence of the activation of PLD with the generation of choline and its further conversion to *P*Cho by ChoK.

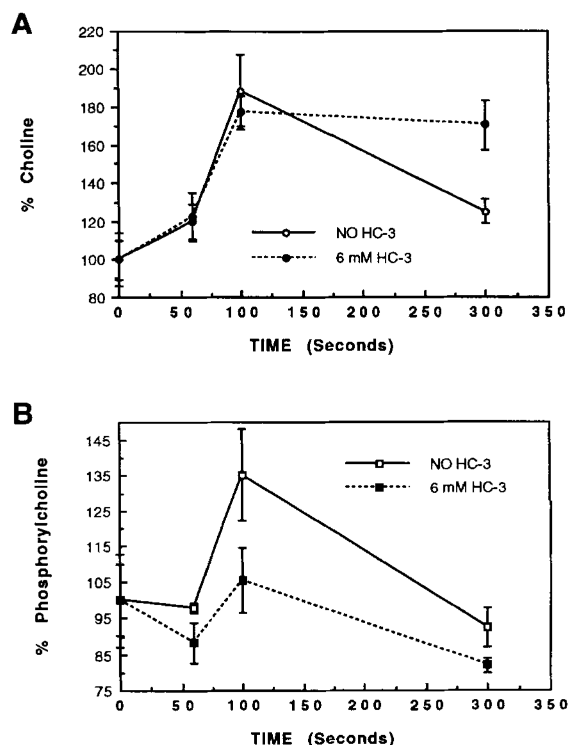


Fig. 1. Effect of HC-3 on the early induction of Cho and *P*Cho by PDGF-BB. Cells were labelled with 10 μCi/ml methyl[^{3}H]choline for 48 h in low serum, and then stimulated with 20 ng/ml of PDGF-BB. At the indicated times, cells were processed for choline and *P*Cho determination as described in Materials and Methods. Where indicated, cells were treated with 6 mM HC-3 2 h before stimulation and maintained during the stimulation period. **A:** Intracellular choline; **B:** intracellular phosphorylcholine. Each point represents the mean ± S.E.M. of three determinations. Data represent percentage over basal levels in unstimulated cells.

Effect of Inhibition of *P*Cho Production on Raf-1 and MAP-Kinase Activation

Since an increase in *P*Cho production can be observed after growth-factors stimulation, and *P*Cho itself has mitogenic activity [Cuadrado et al., 1993], we have used HC-3 to assess the role of this metabolite in the regulatory network involved in the control of mitogenesis induced by growth factors. When NIH 3T3 cells were treated with bFGF or PDGF in the presence of HC-3, their mitogenic activity was drastically reduced, while serum addition was not affected under similar conditions (Fig. 2). We have previously shown that HC-3 does not affect phospholipases activities [Cuadrado et al., 1993; Carnero et al., 1994a,b]. Figure 3 shows that HC-3 does not inhibit the ligand-receptor interaction, the

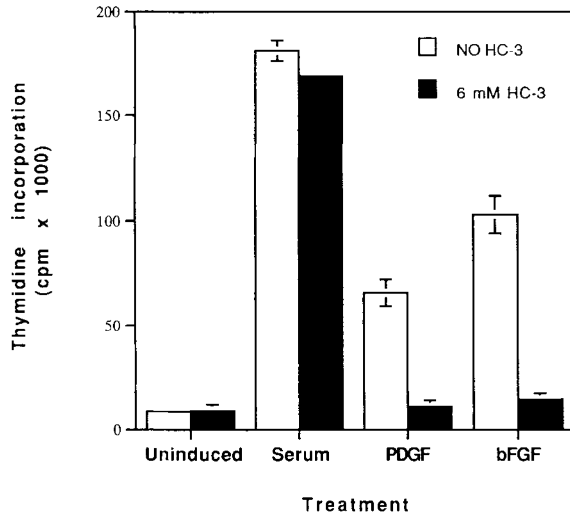


Fig. 2. HC-3 effect on DNA synthesis. Quiescent cells were stimulated for 24 h with 10% FCS, 20 ng/ml PDGF-BB, 20 ng/ml bFGF, or left untreated and incubated in the presence of [^{3}H]thymidine as described in Materials and Methods for DNA synthesis estimation. Where indicated, 6 mM HC-3 was present in the culture media during stimulation. Each point represents mean ± S.E.M. of three determinations.

receptor's tyrosine kinase activity or the PI3 kinase activation by PDGF receptor. These results suggest the involvement of choline kinase in the mitogenic signalling. However, the fact that serum was not affected by HC-3 indicates that other mitogens present in serum can signal through pathways that do not involve choline kinase activity.

Raf-1 and MAP-kinases have been proposed to be regulatory kinases for integrating a wide variety of mitogenic signals [Pelech and Sanghera, 1991, 1992]. Thus, we analyzed the effect of HC-3 on the activation of these kinases induced by stimulation with growth factors. Figure 3A shows that in vivo, treatment of quiescent NIH 3T3 cells with HC-3 completely blocked the ability of PDGF or bFGF to induce the mobility shift of Raf-1, whereas serum activation was not sensitive to this inhibitor (Fig. 4A). The mobility shift observed in p42 and p44 MAPK after treatment with PDGF or bFGF was also blocked in vivo by treatment with HC-3 (Fig. 4B). As observed for Raf-1, serum stimulation of MAPK was not affected by HC-3 treatment. These results strongly indicate that HC-3, a choline ki-

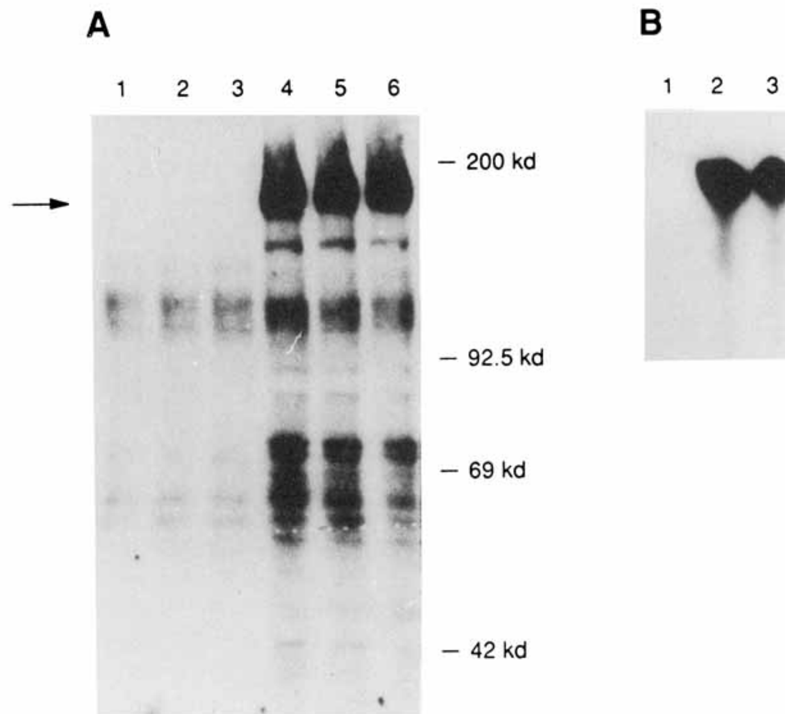
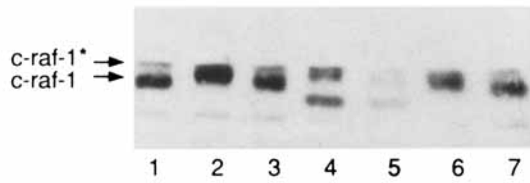


Fig. 3. Effect of HC-3 on activation of the PDGF receptor or PI3 kinase. **A:** Quiescent cells were preincubated 30 min with 0 mM (lanes 1, 4) 2 mM (lanes 2, 5), and 6 mM (lanes 3, 6) of HC-3 and then stimulated with 10 ng/ml of PDGF (lanes 4–6). The arrow indicates the size of the PDGF receptor. **B:** Effect of HC-3 on PI3 kinase activity. Cells were incubated with 0 mM (lanes 1, 2) or 6 mM of HC-3 (lane 3), and then stimulated with 0 ng/ml (lane 1) or 10 ng/ml (lanes 2, 3) of PDGF-BB.

A



B

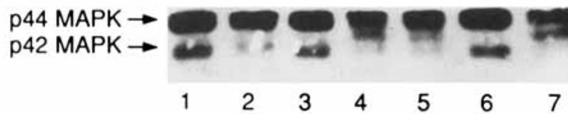


Fig. 4. Effect of HC-3 treatment on the activation of Raf-1 and MAPK by mitogens. Cells were grown and treated as described in Materials and Methods for Raf-1 and MAPK activation, determined as the mobility shift in SDS/PAGE and Western blotting. **A:** Effect of HC-3 treatment on the mobility shift of Raf-1 induced by PDGF, FGF, and serum. Lane 1: quiescent cells. Lane 2: quiescent cells stimulated 10 min with 10 ng/ml bFGF. Lane 3: quiescent cells preincubated 2 h with 6 mM HC-3 and then stimulated 10 min with 10 ng/ml bFGF. Lane 4: quiescent cells stimulated 10 min with 20% fetal calf serum. Lane 5: quiescent cells were preincubated 2 h with 6 mM HC-3 and then stimulated 10 min with 20% FCS. Lane 6: quiescent cells stimulated 10 min with 10 ng/ml PDGF. Lane 7: quiescent cells, preincubated 2 h with 6 mM HC-3, were stimulated 10 min with 10 ng/ml PDGF. **B:** Effect of HC-3 treatment on the mobility shift of MAPK induced by PDGF, FGF, and serum. Lanes as in A except lane 6, HC-3 treated cells and lane 7, HC-3 untreated cells.

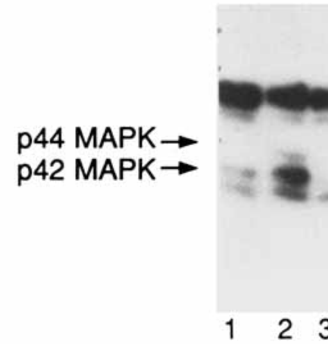


Fig. 5. Effect of HC-3 on the activation of MAP kinases by PDGF using an "in situ" kinase assay. The "in situ" kinase assay was performed as described in Materials and Methods using 0.5 mg/ml MBP as substrate. **Lane 1:** quiescent cells. **Lane 2:** cells stimulated with 10 ng/ml PDGF for 10 min. **Lane 3:** cells were pretreated 1 h with 6 mM HC-3 and then stimulated 10 min with 10 ng/ml PDGF in the presence of 6 mM HC-3.

nase inhibitor, was sufficient to block Raf-1 and MAP kinases activation induced by growth factors under in vivo conditions.

To further strengthen our conclussion, we next investigated the effect of HC-3 treatment on the in vivo activation of MAP kinases by PDGF treatment as determined by their ability to phosphorylate MBP in an SDS-polyacrylamide gel. Figure 5 shows that the activity of both p44 and p42 MAPK were drastically reduced when the cells were stimulated in the presence of HC-3, indicating that generation of *P*Cho by PDGF is essential for MAPK activation. Thus, these results are consistent with the inhibitory effects of HC-3 treatment on the mitogenic activity of growth factors and support a critical role of choline kinase in the generation of phosphorylcholine as a second messenger essential for mitogenicity.

Requirement of Choline Kinase for Entry in the S Phase of the Cell Cycle

NIH 3T3 fibroblasts can be made quiescent by incubation in serum-free medium, and released from this blockage by addition of diverse specific growth factors or serum. In the presence of HC-3, entry into the S phase induced by specific growth factors is completely blocked, while there is no effect on either serum-stimulated DNA-synthesis (Fig. 2) [Cuadrado et al., 1993].

Production of *P*Cho following PDGF or FGF stimulation reaches a 130% increase over basal levels at 6 h and remains for at least 6 additional h [Cuadrado et al., 1993; Carnero et al., 1994a]. When cells were challenged with PDGF or bFGF in the presence of 6 mM HC-3, a concentration sufficient to drastically inhibit the endogenous choline kinase activity [Cuadrado et al., 1993], a complete inhibition of the late *P*Cho production was observed (Fig. 6), indicating that *P*Cho produced at late times is also the result of choline kinase activation. Similar results were also obtained when serum was used as mitogen [Cuadrado et al., 1993; Carnero et al., 1994a], indicating that growth factors induce *P*Cho production by specific activation of choline kinase.

As indicated before, some of the early events associated to cell growth stimulation induced by b-FGF (Raf-1 and MAP-K activation) are blocked by HC-3 treatment. Thus, we wondered whether the early and late generation of *P*Cho were relevant for the Go to S phase transition. For this purpose we examined the ability of HC-3 to block DNA synthesis when added at different times during the cell cycle after stimulation with FGF.

Figure 7 shows that HC-3 is able to block efficiently (about 90%) the entry into the S phase when added up to 4 h of stimulation with the mitogen. However, when HC-3 is added at 6–8

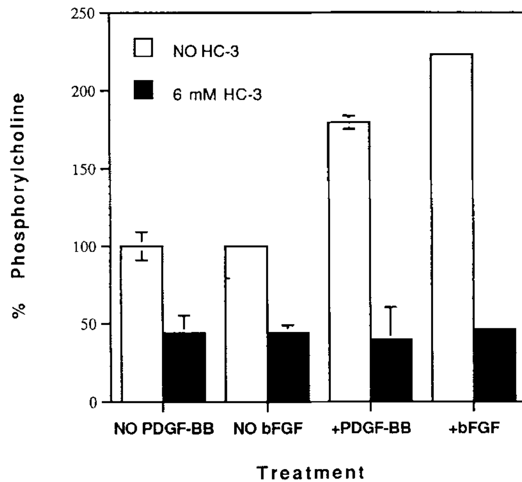


Fig. 6. Effect of HC-3 on the late induction of *P*Cho generation. Cells were labelled with 10 μCi/ml methyl[^{3}H]choline for 48 h in low serum and then stimulated with 20 ng/ml of PDGF-BB, bFGF, or left untreated for 6 h. At this time, cells were washed with PBS and fixed in 1 ml of ice-cold 16% TCA. Where indicated, cells were treated with 6 mM HC-3 2 h before stimulation and HC-3 was maintained during the 6 h stimulation period. Each point represents the mean ± S.E.M. of three determinations for the PDGF and two for bFGF.

h, only a partial inhibition (about 50%) was observed. Finally, when HC-3 was added at later times, during a 24-h incubation period of bFGF stimulation, it was unable to block DNA synthesis. Similar results were also obtained when PDGF was used as mitogen (results not shown). Thus, these results indicate that HC-3 treatment does not result in a toxic effect for NIH 3T3 fibroblasts, and points to a specific effect of this drug in the blockage of critical early and late events of the cell cycle.

DISCUSSION

During the last few years there has been a great advance in the identification of the elements that are controlling the activation of Raf-1 and MAP kinases induced by growth factors [reviewed by Pouyssegur and Seuwen, 1992]. As a consequence we can visualize a linear pathway connecting the following signalling molecules:

tyrosine kinase receptors → GRB-2

→ SOS → Ras → Raf-1 → MEK → MAPK.

Ras proteins are one of the key elements in the control of this mitogenic transducing pathway. However, this linear pathway is not the only one controlled by *ras* proteins. Although less known at the molecular level, there is abundant information demonstrating that *ras* proteins are coupled to other pathways where PKC or phospholipases are involved [Lacal et al., 1987], and there is evidence that *Ras* may be activating an alternative pathway where JNK is involved [Devary et al., 1993]. Furthermore, although a physical interaction between *ras* and Raf-1 has been reported, the fact that this interaction does not result in a direct activation of Raf-1, implies the involvement of additional elements for a full activation of this pathway [Zhang et al., 1993]. In addition, other independent or parallel pathways may exist, since MAPK can be activated following different routes [Lange-Carter et al., 1993].

Mitogenic stimulation of NIH 3T3 fibroblasts with growth factors or *ras* oncogenes is associated with an increase in the level of diacylglicerol and phosphorylcholine [Lacal et al., 1987]. We have demonstrated that generation of these signaling molecules is a consequence of the activation of a type D phospholipase (that renders phosphatidic acid and choline), followed by the activation of choline kinase (which phosphorylates choline to phosphorylcholine) and a phosphatidic acid hydrolase (which converts PA to DAG) [Carnero et al., 1994a]. This complex pathway, alternative to a direct involvement of a putative type C phospholipase, suggests an important role of choline kinase in the transduction of mitogenic signals. Using hemicholinium-3 (HC-3) to block the in vivo activity of choline kinase, we have demonstrated that this enzyme is required for the entry in the S phase of the cell cycle induced by some mitogenic factors [Cuadrado et al., 1993]. Here, we show that HC-3 is able to block the activation induced by bFGF and PDGF of two key kinases for the integration of growth promoting signals, Raf-1 and MAPK. These results suggest that HC-3 inhibits a signal transducing element upstream of Raf-1 and MAPK, whose function is essential for the regulation of the activity of these kinases.

The inhibitory effect of HC-3 is a consequence of the blockage of the generation of *P*Cho that takes place at less than 2 min of stimulation. This early peak of *P*Cho generation, although not as large as the late increase, may be localized to a specific compartment which would make it more effective. Moreover, the existence of a late increase in phosphorylcholine associated to the mitogenic activity of PDGF and bFGF supports a role of this intracellular signaling molecule at different points in the cell cycle. Finally, there is evidence that CTP-phosphocholine cytidyltrans-

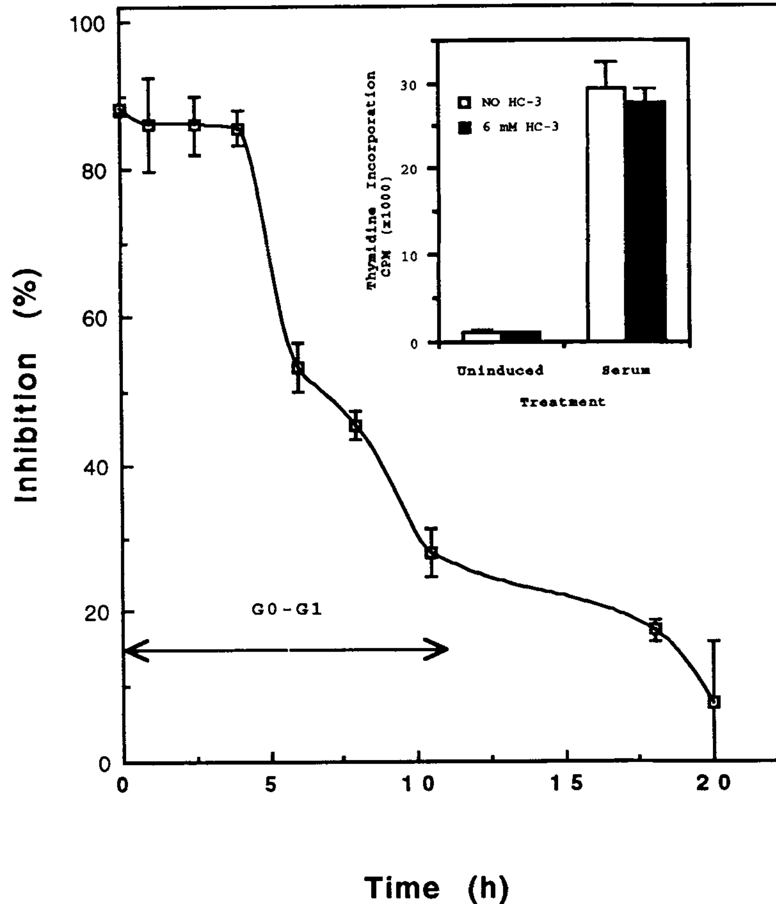


Fig. 7. Effect of HC-3 treatment on the entry into the S phase. Quiescent NIH 3T3 cells were stimulated during a period of 24 h with 10 ng/ml of bFGF. HC-3 was added to a final concentration of 6 mM at 0, 1, 2.5, 4, 6, 8, 10.5, 18, or 20 h after the addition of the growth factor. (^{3}H)-Thymidine was added for the last 4 h in all cases and cells were processed as indicated in Materials and Methods. Data represents the percentage of HC-3 inhibition relative to the (^{3}H)-Thymidine incorporation of control cells, stimulated by bFGF. The inset shows that the (^{3}H)-Thymidine incorporation of fetal serum stimulated cells was not affected by the treatment with HC-3 for the 24 h of the stimulation.

ferase (CT), the enzyme that is responsible for the conversion of *P*Cho into CDP-Cho, is phosphorylated by $p34^{cdc2}$, casein kinase II, and MAPK (Cornell and Wieprecht, personal communication). Although the physiological meaning of these phosphorylations is still unknown, these results suggest that CT may be also regulated by the Ser/Thr kinases cascade with a rapid shutoff of the early increase in *P*Cho.

There are several significant results that indicate that HC-3 inhibits specific steps in signal transduction rather that being a non-specific inhibitor: (1) HC-3 does not interfere with the interaction of growth-factors with their receptors, nor their kinase activity or substrates activation; (2) HC-3 inhibits neither Raf-1 nor MAPK activation by serum; (3) HC-3 is not active in blocking DNA synthesis when added after 8 h of stimulation; (4) HC-3 inhibition can be bypassed by *P*Cho addition; (5) insuline can supplement the inhibitory effect of HC-3 imposed to FGF, PDGF, and other growth factors; and (6) serum contains a combination of mitogenic factors that do not rely on choline kinase for their mitogenicity. Thus, production of *P*Cho by choline kinase seems to be a critical step in mitogenic stimulation of growth factors but it is dispensable and redundant as all other important messages such as PLCγ or PI3K activation. In addition, the existence of two peaks of *P*Cho after mitogenic stimulation, implies the presence of a complicated network of intracellular signals with the putative activation of multiple pathways.

We have demonstrated a clear connection among production of *P*Cho and early events (activation of Raf-1 and MAPK), and late events (DNA synthesis) of growth factors stimulation. While the precise mechanism of modulation of

these events by *P*Cho remains to be identified, the recent observation of a direct interaction between *ras* and Raf-1, and the fact that this interaction is not sufficient to activate Raf-1, opens the possibility for the contribution of other signaling molecules activated by *ras* in this pathway. *P*Cho is an obvious candidate to be involved in the activation of Raf-1 as a consequence of direct interaction with this kinase or through an as yet unidentified novel mechanism. The determination of the precise system by which *P*Cho activates Raf-1 and MAPK will constitute a critical issue in understanding the role of *P*Cho and choline kinase in cell growth regulation.

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