cAMP-mediated Growth Inhibition in Fibroblasts Is Not Mediated via Mitogen-activated Protein (MAP) Kinase (ERK) Inhibition

Fergus R. McKenzie and Jacques Pouyssegur
From the Centre de Biochimie, CNRS, Parc Valrose, 06108 Nice, France

Growth factors stimulate fibroblast cell division by activating the recently identified mitogen-activated protein kinase (MAP kinase) signaling cascade. In contrast to our previous work (Kahan, K., Seuwen, K., Meloche, S. and Pouyssegur, J. (1992) J. Biol. Chem. 267, 13369–13375), several reports have suggested that an elevation in intracellular cAMP blocks cell proliferation by attenuating MAP kinase activation. Hence we re-examined the effect of a long term increase in intracellular cAMP and therefore cAMP-dependent protein kinase (PKA) activation on the MAP kinase cascade in CCL39 fibroblasts. The concomitant addition of cAMP-elevating agents prostaglandin E, (PGE,) and IBMX did not inhibit the mitogen-mediated activation of p44 MAP kinase. However, a 5 min PGE,/IBMX pretreatment abolished the MAP kinase response, in a manner correlating with the extent of PKA activity. This inhibition was transient in nature, and while modifying the time course of growth factor-mediated p44 MAP kinase, activation did not diminish the magnitude of the response. Thus the major peak of MAP kinase activity normally present 5 min after α-thrombin addition was now evident at 10 min in the presence of PGE, / IBMX. CCL39 cell proliferation is inhibited by elevated cAMP levels. Such an inhibition could reflect either a reduction in the number of cells entering the cell cycle or a delay in the time required to go through the cycle. Bromodeoxyuridine labeling experiments revealed that the CAMP-mediated inhibition of DNA synthesis in CCL39 cells was not due to a delay in S phase entry, but was due to a reduction in the number of cells entering S phase.

Thus we conclude that although PKA activation may slightly modify the time course of MAP kinase activation in response to mitogens in CCL39 cells, the PKA-mediated inhibition of cell division occurs through modulation of an intracellular target, distinct from the p42/p44 MAP kinase cascade.

In normal untransformed fibroblast cell lines, it has long been appreciated (2) that a significant elevation of intracellular cAMP levels may potently inhibit cell growth and division. The presumed mediator of this effect is the cAMP-dependent protein kinase (PKA),1 a widely studied component of a signaling cascade that links extracellular signals to a variety of cellular functions (3). Until recently, the proposed target for the action of PKA in the arrest of cell growth was unknown. As elevated cAMP levels inhibit cell proliferation mediated by either G-protein-coupled receptors or receptors with intrinsic tyrosine kinase activity, it was assumed that the target for PKA was downstream of the initial signaling events and likely to be a central player in the mitogenic response (2).

Virtually all known mitogens stimulate cell division through the activation of the recently described mitogen-activated protein kinase (MAP kinase) cascade (4–6). In a widely studied model of growth factor signaling, the CCL39 fibroblast cell line, a 42- and 44-kDa MAP kinase have been identified (7). Both proteins form part of a signaling complex involving the sequential activation of Ras, a MAP kinase kinase kinase (MAPKKK) or MEKK, a MAP kinase kinase (MAPKK or MEK), with MAP kinase being the final member of this cascade (6, 8). It is now evident that the upstream MAP kinase activators form part of an ever increasing group of kinases that provide input signals into each of the MAP kinase family members including p42/44 MAP kinase and the highly homologous J un/stress (8, 9) and p38 kinases (10). However, an obligate step in the activation of p42 and p44 MAP kinases is the passage of the stimulatory signal from the Ras oncoprotein to the MAPKKK (for review, see Ref. 11). At present, three such MAPKKK have been identified in mammalian cells, including the homologous Raf-1 (also termed cRaf-1) and B-Raf serine/threonine kinases and MEK kinase, which shares little structural similarity to the former two proteins (14–17). An additional Raf family member A-Raf, which has been shown to interact with Ras (18) is also likely to be a MAPKKK.

p44 MAP kinase is a cytoplasmic protein in resting CCL39 cells that becomes activated following its phosphorylation on threonine and tyrosine residues (7). After activation, p44 MAP kinase is subject to redistribution within the cell and can activate target proteins such as phospholipase A, p90 rsk, and p62 TCF in the plasma membrane, cytoplasm, and nucleus, respectively (6, 7). The role of MAP kinase has been highlighted by experiments involving the expression of either dominant-negative MAP kinase mutants (p44 MAPK- TA), p44 MAP kinase antisense, or MAP kinase phosphatase (MKP-1), each of which results in a blockade of the mitogenic response to growth factors (12, 13). Thus MAP kinase can be regarded as an intracellular
messenger and a central component of the mitogenic response. A member of the MAP kinase signaling cascade would thus present itself as a suitable target for the growth inhibitory function of PKA.

To this effect, several publications have recently shown that the ability of Ras to activate Raf-1 was impaired in cells treated with cAMP-elevating agents, leading to a loss in ability to activate MAP kinase (19–23; see Ref. 24 for review). It would therefore appear that PKA prevents growth factor mediated cell division by the attenuation of an obligate step in the activation of MAP kinase.

We have previously demonstrated that increased cAMP levels in CCL39 cells, while blocking the mitogenic response (30), do not inhibit the ability to stimulate p44 MAP kinase (1). In an attempt to resolve this apparent contradiction, we have re-examined the effect of PKA activation on p44 MAP kinase stimulation. We show that long term elevation of cAMP levels induces a temporal shift in the activation of both MAP kinase and its activator MAPKK. However, the magnitude and duration of p44 MAP kinase activation is not mediated by a modification of MAP kinase activation, but by the modification of at least one additional target.

**EXPERIMENTAL PROCEDURES**

**Materials**

Highly purified human α-thrombin and recombinant basic FGF were generous gifts of Dr. J. W. Fenton II (New York, State University of Health, Albany, NY) and Dr. D. Gospodarowicz (University of California, Medical Center, San Francisco, CA), respectively. [3H]Adenine and [γ-32P]ATP was obtained from Amersham Corp. Antiserum (anti-ERK-1), which specifically immunoprecipitates p42 MAP kinase, was as described previously (25). Anti-erbB (anti-ERK-1), which specifically immunoprecipitates p44 MAP kinase, was a kind gift from Dr. E. Van Obberghen (26). Antiserum Kelly #3, which immunoprecipitates both p42 and p44 MAP kinase, will be described in detail elsewhere. Antisera (Kawa), which specifically immunoprecipitates p45 MAPKK, was as described previously (27). Anti-BrdUrd IgG were from Amer- sham. All other materials were obtained from Sigma unless otherwise stated.

**Methods**

Cells and Culture Conditions—CCL39 cells are an established line of Chinese hamster lung fibroblasts (American Type Culture Collection). Cells expressing human muscarinic (m1) receptors (clone M1-B1) were obtained as described previously (28). Cells lacking PKA activity (clone CCL39PKA−) were obtained by transfecting CCL39 cells by the calcium phosphate method with the MT-REV(AB)-neo expression vector (kind gift of G. S. McKnight), which contains the coding region of the RIIα subunit gene of PKA with three point mutations that prevent cAMP binding and activation of the enzyme (29). Transcription of the con- struct is directed by an inducible metallothionein promoter. However, the basal activity was sufficient to produce the kinase deficient phenotype. Following transfection, stable clones were selected in G418 (400 μg/ml) and their PKA activity assessed regularly (see below) to ensure stability of the phenotype.

Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.), supplemented with 7.5% fetal calf serum, antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin), and 25 mM sodium bicarbonate at 37°C in an humid atmosphere (5% CO2, 95% air). Cells were serially passaged upon reaching confluence, and intracellular ATP pools were labeled by incubation in serum free DMEM containing [3H]adenine (2 μCi/ml) for 24 h. The cells were washed three times with HEPES-buffered DMEM (pH 7.4) before being stimulated in HEPES-buffered DMEM (pH 7.4) for the indicated times in the presence of suitable agonist. The incubation was stopped by rapid aspiration of the media, followed by extracting the cells with ice-cold 5% trichloroacetic acid and 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 μM β-glycophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A, 1% Triton X-100). Cells were then removed from the surface of the plate and centrifuged briefly at 12,000 × g in a benchtop centrifuge to pellet nonsed cells. The supernatants were then assayed for PKA activity exactly as described (31) in the presence of 100 μM Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) as substrate. Total PKA activity was determined in the presence of 10 μM cAMP. PKA activity was defined as that sensitive to the inhibitor peptide PKA-inhibitor peptide (1 μM) (32).

Assay of MAP Kinase—Quiescent cells in 12-well plates were incubated in HEPES-buffered DMEM medium prior to stimulation with growth factors for the indicated times at 37°C. The cells were then washed twice with cold PBS and lyzed in 0.5 ml of Triton X-100 lysis buffer (50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 μM β-glycophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A, 1% Triton X-100) for 25 min at 4°C. After clarification with centrifugation at 12,000 × g for 15 min at 4°C, the lysates were precluded for 1 h at 4°C with 1 μM of normal rabbit serum and protein A-Sepharose (Pharmacia Biotech Inc.). The lysates were then incubated for 2 h at 4°C with 4 μl of either antiserum (anti-ERK-1, p42 MAP kinase), or Kelly #5 (p42/p44 MAP kinase), preadsorbed to protein A-Sepharose beads. The supernatants were collected by centrifugation and washed four times with Triton X-100 lysis buffer and once with kinase buffer (20 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 10 mM p-nitrophenyl phosphate). Myelin basic protein kinase activity was assayed by resuspending the final pellet in a total volume of 40 μl of kinase buffer containing 0.25 mg/ml myelin basic protein and 50 μM [γ-32P]ATP (specific activity = 5500 cpm/pmol). Reactions were initiated with ATP and incubated at 30°C for 10 min. Assays were stopped by the addition of 40 μl of 2× Laemmli’s sample buffer. After heating to 95°C for 5 min, the samples were analyzed by SDS-gel electrophoresis on 10% gels. The gels were stained with Coomassie Blue, dried, and subjected to autoradiography.

Measurement of DNA Synthesis Reinitiation—Cells were seeded on glass coverslips at a density of 250,000 cells/well of a six-well plate and rendered quiescent just prior to confluence by serum removal overnight. Cells were then stimulated with the appropriate agonist and BrdUrd was added (10 μM final). After 22 h or suitable time point, the incorpora-

2 F. R. McKenzie and J. Pouysségur, manuscript in preparation.

3 F. R. McKenzie and J. Pouysségur, manuscript in preparation.
Activation of MAP kinase is not inhibited by concomitant addition of cAMP-elevating agents. Quiescent M1-81 cells in 12-well plates were stimulated for 5 min with each of α-thrombin (THR) (1 unit/ml), FGF (25 ng/ml), serum (10%), or carbachol (CCH) (10⁻⁶ M), in the presence (open bars) or absence (closed bars) of 10⁻⁶ M PGE₁, 1 mM IBMX. Cells were then lysed, and the activity of MAP kinase was measured exactly as described under "Experimental Procedures." Data are mean values ± half of the range from three experiments.

Results

Activation of MAP Kinase Is Not Inhibited by Concomitant Addition of cAMP-elevating Agents—In mitogen-stimulated CCL39 hamster lung fibroblasts, a sustained intracellular elevation of cAMP serves as a potent inhibitor of cell cycle re-entry (30). We have previously published that the target(s) for this action does not lie in the p42/p44 MAP kinase-activating cascade (1), in marked contrast to what has since been reported by several groups (19–23). Hence to re-address this question in detail, we examined the ability of a range of growth factors that function through activation of other receptors with intrinsic tyrosine kinase activity or receptors which modify G-protein function through activation of either receptors with intrinsic tyrosine kinase activity or receptors which modify G-protein activity, we examined the time courses of each of these signals.

Growth factor stimulation, cells were lysed as described under "Experimental Procedures" and the lysates divided into two. The activity of p44 MAPK (A) or p45 MAPK (B) was then measured in each half of the cell lysate. The results are expressed as mean values ± half of the range of duplicate measurements from two individual experiments.

In an attempt to define the conditions leading to inhibition of MAP kinase activation, we examined the time courses of each of cAMP production and PKA activation in response to PGE₁/IBMX challenge, together with the ability of PGE₁/IBMX to block MAP kinase activation in response to α-thrombin (Fig. 3). Upon addition of PGE₁/IBMX to quiescent M1-81 cells, the intracellular level of cAMP rapidly increases, with the maximal increase observed after approximately 20 min of agonist addition. Intracellular cAMP remains at 80% of this maximal value for several hours following PGE₁/IBMX addition (not shown). 2 min following addition of PGE₁/IBMX the intracellular cAMP levels reach 70% of the maximal value obtained after 20 min of stimulation (Fig. 3A). This time course is paralleled by the increase in PKA elicted by PGE₁/IBMX. Maximal activity is
of PKA by PGE1/IBMX proceeds with a slight lag when compared with the time course of cAMP production. The accumulation of intracellular cAMP (A), together with the activation of PKA (B), were measured in response to PGE1 (10^{-6} M), 1 mM IBMX at a range of time points indicated, exactly as described under "Experimental Procedures." In a separate experiment (C), quiescent M1-81 cells in 12-well plates were stimulated with either α-thrombin (1 unit/ml) (closed circles) or FGF (25 ng/ml) (open squares). Prior to addition of these growth factors, the cells were pretreated with PGE1 (10^{-6} M), IBMX (1 mM) for the times indicated (from 10 min before growth factor addition to 1 min after growth factor addition). Cells were lysed 5 min after growth factor addition and p44 MAPK activity was determined as described in the legend to Fig. 1. Data are presented as the percentage inhibition of FGF (●) or α-thrombin (●)-stimulated p44 MAPK activity after a 5-min stimulatory period, in the absence of PGE1/IBMX. Data are mean values ± S.E. from either two (A, B) or three (C) experiments. Error bars have been omitted from C for clarity, with error being less than 8% of the mean.

Again obtained after 20 min of agonist addition. As for cAMP, the level of PKA activity remains elevated for several hours following addition of the agonist indicated. However, activation of PKA by PGE1/IBMX proceeds with a slight lag when compared with the time course of cAMP production. At 2 min, the PKA activity is at only 30% of its maximal stimulated activity (Fig. 3B). In an additional range of experiments, PGE1 on its own produced a similar spectrum of activity, although in each case it was less potent than in the presence of IBMX and the duration of the response was significantly shorter (results not shown). Thus it is apparent that the activation status of PKA in M1-81 cells closely follows the level of intracellular cAMP, but with a slight delay. We then examined the time course of inhibition of MAP kinase activation (Fig. 3C) by PGE1/IBMX. As previously noted (Fig. 1), addition of PGE1/IBMX at the same time (time 0) or 1 min after addition of either FGF or α-thrombin fails to attenuate the ability of both agonists to stimulate MAP kinase. The ability of PGE1/IBMX to inhibit agonist-stimulated MAP kinase is time-dependent and starts to be evident only when PGE1/IBMX is added at least 2 min before growth factor addition. Inhibition is maximal after 5 min of pretreatment (Fig. 3C). Therefore the cAMP-mediated inhi-

bition of MAP kinase activation closely correlates with the activation of PKA. However, it appears that the ability of PKA to inhibit growth factor-mediated MAP kinase activation depends on the prior activation of PKA. If the enzyme has not attained a significant level of activity before the addition of MAP kinase activating agent, then it is no longer possible to inhibit MAP kinase activation by PKA. PKA Activation Does Not Completely Block p44 MAP Activation, but Alters the Time Course of Activation—Having established that activation of PKA could indeed attenuate MAP kinase activation in M1-81 fibroblasts under appropriate conditions, we next examined if an increase in PKA activity completely blocked the ability to stimulate MAP kinase or if the effect was transient in nature. MAP kinase activity was determined in parental CCL39 cells in response to α-thrombin at a range of different time points. As noted previously, a 5-min pretreatment with PGE1/IBMX attenuated the ability of α-thrombin to stimulate MAP kinase, when viewed 5 min after α-thrombin addition (Fig. 4A). However, to our surprise, the ability of PGE1/IBMX to exert this inhibition was entirely transient in nature. It may be noted that the α-thrombin-mediated activation of MAP kinase at 10 and 20 min is considerably greater than that seen in the absence of PGE1/IBMX pretreatment. (Fig. 4A, inset). Hence, PKA activation does not inhibit the activation of MAP kinase, but modifies its time course of activation. If the overall activity of MAP kinase is
PKA and MAP Kinase

**TABLE I**
Sustained PKA activation reduces the number of cells entering S phase

| Treatment            | Cells incorporating BrdUrd (%) |
|----------------------|-------------------------------|
| Control              | 5                             |
| PGE$_1$/IBMX         | 7                             |
| PGE$_1$              | 8                             |
| Serum                | 87                            |
| Serum + PGE$_1$/IBMX | 37                            |
| Serum + PGE$_1$      | 40                            |
| Thrombin             | 47                            |
| Thrombin + PGE$_1$/IBMX | 11                        |
| Thrombin + PGE$_1$   | 75                            |
| FGF                  | 42                            |
| FGF + PGE$_1$/IBMX   | 70                            |
| FGF + PGE$_1$        | 66                            |
| Carbachol            | 8                             |
| Carbachol + PGE$_1$/IBMX | 5                       |
| Carbachol + PGE$_1$  | 4                             |

Compared, then it is actually greater following PKA activation. At later time points the PKA-induced modification in the time course of MAP kinase activation is more difficult to quantify. A similar profile is obtained when serum is used as MAP kinase activating agent for each of CCL39 and Rat-1 fibroblasts and normal rat smooth muscle cells (Fig. 4B). In each case following PGE$_1$/IBMX pretreatment, the peak of MAP kinase activity at 5 min in response to serum addition is completely absent. However, MAP kinase activity 1 h following serum addition is comparable for CCL39 cells, Rat-1 cells, and vascular smooth muscle cells, regardless of the presence of cAMP-elevating agents (Fig. 4B). These results indicate that the target for PKA-mediated inhibition of cell division is unlikely to be the MAP kinase cascade.

**TABLE II**
PKA activation does not delay, but blocks, S phase re-entry

| Cells incorporating BrdUrd | 12-14 h | 14-16 h | 16-18 h | 18-20 h |
|---------------------------|---------|---------|---------|---------|
| Control                   | 1       | 1       | 2       | 3       |
| PGE$_1$/IBMX              | 1       | 2       | 2       | 3       |
| Serum                     | 8       | 12      | 31      | 39      |
| Serum + PGE$_1$/IBMX      | 4       | 7       | 15      | 18      |

One of the original indications that cAMP, the first second messenger discovered (39), could act as a growth inhibitory agent was provided by Burk, who demonstrated that the growth of both normal and transformed baby hamster kidney cells was retarded by the addition of reagents that prevented intracellular degradation of cAMP (40). The universality of cAMP’s growth inhibitory effect, at least for cells of fibroblastic origin (41), was later appreciated (2). However, a suitable target for cAMP, exerting its effect presumably through the activation of PKA, has until recently remained elusive. Recently, a number of groups have reported that one likely target for active PKA was the ubiquitous MAP kinase signaling cascade (19-23). Specifically, PKA activation has been shown to attenuate an obligate step in the MAP kinase cascade, the activation of out effect on either basal or carbachol-mediated BrdUrd incorporation.

**DISCUSSION**

One of the original indications that cAMP, the first second messenger discovered (39), could act as a growth inhibitory agent was provided by Burk, who demonstrated that the growth of both normal and transformed baby hamster kidney cells was retarded by the addition of reagents that prevented intracellular degradation of cAMP (40). The universality of cAMP’s growth inhibitory effect, at least for cells of fibroblastic origin (41), was later appreciated (2). However, a suitable target for cAMP, exerting its effect presumably through the activation of PKA, has until recently remained elusive. Recently, a number of groups have reported that one likely target for active PKA was the ubiquitous MAP kinase signaling cascade (19-23). Specifically, PKA activation has been shown to attenuate an obligate step in the MAP kinase cascade, the activation of...
PKA and MAP Kinase 13481

The mechanism of inhibition of Raf-1 by PKA has recently been suggested to involve two separate events (51). In addition to weakening the interaction of Raf-1 with Ras (20), thus preventing the initial translocation of Raf to the plasma membrane, PKA phosphorylates the Raf-1 kinase domain, inhibiting autophosphorylation of Raf-1 kinase (20, 21, 52). The latter explains the ability of PKA to inhibit v-Raf (which does not require Ras to be functionally active) and hence the ability of elevated intracellular cAMP levels to revert v-Raf-transformed NIH 3T3 cells (53). At present, of the three MAPKKK enzymes that have been identified, Raf-1, B-Raf, and MEK kinase, all appear to be inhibited by PKA activation (52, 54, 55). A fourth member of the Raf oncoprotein family, A-Raf, exists which is likely to be a MAPKKK, although data for this functional effect are currently lacking (15). Interestingly, MEK kinase appears to be one member of what may be a large family of protein kinases as at least four genes putatively coding for MEK kinases homologues exist. In addition, a significant number of reports exist that suggest that additional MAPKKK proteins are expressed in both mammalian cells (56–58) and in Xenopus oocytes (59). A surprising recent finding by Moscat and colleagues is that a protein kinase C (PKC) family member, PKC α, may also function as a MAPKKK (68), again increasing the complexity of the system.

The simplest explanation to account for the ability of PKA to modify the time course, but not to inhibit the activation of MAP kinase in CCL39 cells, is that in this cell line, although the presently identified MAPKKK are inhibited by PKA, there exists an as yet unidentified MAPKKK (see above) that is not inhibited by PKA. Such a PKA-insensitive MAPKKK expressed by CCL39 cells would have to have a time course of activation considerably slower than that of the identified PKA sensitive MAPKKK. However, one must add the caveat that the time course of activation of a MAPKKK insensitive to PKA would be identical, irrespective of whether Ras is able to bind to Raf family members which are sensitive to PKA. An additional possibility is that in CCL39 cells, although the presently identified MAPKKK are inhibited by PKA, there exists an as yet unidentified MAPKKK that is stimulated by PKA. This may indeed be the case in PC12 cells, where elevated cAMP has been reported to be (26) or not be able (54) to generate a significant stimulation of MAP kinase. However, this is unlikely to be the case for CCL39 cells as elevated levels of cAMP do not on their own stimulate MAP kinase (Figs. 1 and 2).

In mammalian cells, virtually all of the cAMP effects can be attributed to the activation of PKA. However, protein kinase A-dependent cAMP responses have been described. The ability of cAMP to inhibit the GLUT4 but not the GLUT1 glucose transporter may be mediated by direct binding of cAMP to GLUT4 (34). In addition, a range of different ion channels, some of which contain a putative cyclic nucleotide binding site (60), may be modulated in a stimulatory manner by direct binding of cAMP (35–37). In CCL39 cells, it would appear that the ability of elevated cAMP levels to modify the kinetics of MAP kinase activation are entirely due to the activation of PKA, as the activation of MAP kinase in CCL39 cells lacking PKA activity is completely insensitive to elevated cAMP (Fig. 5).

To the best of our knowledge, in all cell types studied, activation of MAP kinase is rapid, with peak activity occurring approximately 5 min and no later than 10 min after addition of agonist. In contrast to the situation in primary dog thyrocytes, where TSH does not stimulate MAP kinase (61), in primary cultures of human thyroid follicles, one may note an interesting parallel between the time course of growth factor-stimulated MAP kinase in the presence of activated PKA in CCL39 cells.
and the time course of MAP kinase activation shown in response to TSH (62). In this system, peak MAP kinase activity occurs at approximately 20 min after addition of TSH. Interestingly, TSH also stimulates production of cAMP and hence activation of PKA in thyrocytes (63). One may speculate that the reason for the apparently “slow” time course of MAP kinase activation in human thyroid cells in response to TSH is due to the inhibition of PKA sensitive MAPKKK enzymes, with the signal being propagated by PKA insensitive MAPKKK. In addition, Al-Alawi and colleagues have recently shown that although elevation of cAMP in thyroid cells may attenuate Raf-1 activation, TSH induced mitogenesis still proceeds in a Ras-dependent manner (63, 64). Hence it is highly probable that TSH stimulates MAP kinase and cell division in certain species of thyroid cells via an unidentified PKA insensitive MAPKKK.

We had hoped that this report would clarify the situation regarding PKA and MAP kinase, at least for cells of fibroblastic origin (CCL39 and Rat-1 cells). However, reports in the literature show that the activation of MAP kinase can be both stimulated (26, 65) and inhibited (19–23) by an elevation of intracellular cAMP. Additionally, a recent report shows that neuroepithelium modulation of voltage sensitive K+ currents in the body-wall neuromuscular junction of Drosophila larvae requires concomitant elevation of cAMP and activation of Raf (66). A variety of scenarios are therefore available. However, our data is supported from recently published work in PC12 cells (54), where Vaillancourt and colleagues have shown that elevated cAMP levels, although inhibiting the growth factor mediated activation of B-Raf, have no apparent effect on the activation of MAPKK and MAP kinase. As for CCL39 cells, the simplest explanation for this result is that additional MAPKK exist in PC12 cells which are insensitive to PKA (54). Previous studies in the literature are hampered by a total lack of detailed kinetics of MAP kinase activation, hence it is not possible to conclude whether or not previous results are in discordance with our own (20–23). One possible exception is A14 cells where cAMP appears to inhibit MAP kinase in a protracted manner (19).

The two major points raised by this report are: what additional Ras-dependent PKA-insensitive MAPKKK are expressed in fibroblasts and what is (are) the real target(s) for the PKA-mediated inhibition of cell division? Experiments to define the former are in progress, and our attempts to define the latter are centered on the cell cycle machinery. An obiligate step for the mitogen-stimulated passage of quiescent cells through G1 to S phase appears to be the induction of cyclin D1. A4 Mitogen-stimulated induction of cyclin D1 is blocked by the long term elevation of cAMP levels in CCL39 cells. Hence, bypassing this blockade may be a suitable way to overcome the growth inhibitory effect of long term PKA activation. Current experiments are seeking to address this possibility. As has been discussed (see above), the MAP kinase family of serine/threonine kinases can be subdivided into three distinct groups; the classical p42/p44 MAP kinases, the Jnk kinase/stress-activated kinase family, and the p38/osmotically activated kinases (67). The role of the latter two kinase families in mitogenic signaling remains to be determined. It will, thus, be of interest to discover what effect, if any, activation of PKA has on the latter two recently identified MAP kinase family members.

Acknowledgments—We are grateful to Dr. E. Van Obberghen for providing anti-MAP kinase antibodies and Ellen Van Obberghen-Schilling for helpful comments on the manuscript.

REFERENCES
1. Kahan, K., Seuwen, K., Meloche, S., and Pouyssegur, J. (1992) J. Biol. Chem. 267, 13369–13375
2. Pasant, I., Johnson, G. W., and Anderson, W. B. (1975) Annu. Rev. Biochem. 44, 491–522
3. Cohen, P. (1992) Trends Biochem. Sci. 17, 408–413
4. Crees, C. M., Alassandrini, A., and Erikson, R. L. (1992) Cell Growth Differ. 3, 125–142
5. Blenis, J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 5889–5892
6. Nishida, E., and Gotoh, Y. (1993) Trends Biochem Sci. 18, 128–131
7. Lenormand, P., Sardet, C., Pagès, G., L’Allemain, G., Brunet, A., and Pouyssegur, J. (1993) J. Cell Biol. 122, 1079–1088
8. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Blumer, K., and Johnson, G. (1993) Science 260, 315–319
9. Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 77, 1037–1047
10. Han, J., Lee, J. D., Bills, L., and Ulevitch, R. J. (1994) Science 265, 808–811
11. Moodie, S. A., and Wolffman, A. (1994) Trends Genet. 10, 44–48
12. Pagès, G., Lenormand, P., L’Allemain, G., Chambard, J., Meloche, S., and Pouyssegur, J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8319–8323
13. Brandelio, J. M., McKenzie, F. R., Sun, H., Tonks, N. K., and Pouyssegur, J. (1995) Oncogene 10, 1895–1904
14. Jowett, L., Lévesque, P., Nkialéni, S., Cohen, P., and Marshall, C. (1992) Cell 71, 335–342
15. Kyriakis, J., App, H., Zhang, X., Banerjee, P., Brautigan, D., Rapp, U., and Arvuch, J. (1992) Nature 357, 421–427
16. Jaiswal, R. K., Moodie, S. A., Wolffman, A., and Landreth, G. E. (1994) Mol. Cell. Biol. 14, 6944–6953
17. Lange-Carter, C., Pleiman, C., Gardner, A., Blumer, K., and Johnson, G. (1993) Science 260, 315–319
18. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
19. Burress, B. M., Th, Prong, J. G., van Weeren, P. C., Chardin, P., and Bos, J. L. (1993) EMBO J. 12, 3271–3280
20. Wu, J., Dent, P., J. Elnik, T., Wolffman, A., Meijer, J. M., and Sturgl, T. W. (1993) Science 262, 1065–1069
21. Cook, S. J., and McCormick, F. (1993) Science 262, 1069–1072
22. Cook, S. J., and McCormick, F. (1993) Cell 73, 1051–1057
23. Sevetson, B. R., Kong, X., and Lawrence, J. C. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10305–10309
24. Sevigny, J., Cooper, J. A., and Pouyssegur, J. (1994) Biochem. J. 307, 589–594
25. Frödin, M., Perold, P., and Van Obberghen, E. (1994) J. Biol. Chem. 269, 4607–4614
26. Pagès, G., Brunet, A., L’Allemain, G., and Pouyssegur, J. (1994) EMBO J. 13, 3003–3010
27. Seuwen, K., Kahan, C., Hartmann, T., and Pouyssegur, J. (1990) J. Biol. Chem. 265, 22292–22299
28. Rogers, K. V., Golman, P. S., Frizzell, R. A., and McKnight, G. S. (1990) EMBO J. 9, 315–319
29. Nakamura, T., and Gold, G. H. (1987) Nature 325, 442–444
30. Di Francesco, D., and Tortora, P. (1991) Nature 351, 145–147
31. Brüggemann, A., Pardo, L. A., Stöhmer, W., and Pongs, O. (1993) Nature 365, 455–458
32. Barlat, I., Henglein, B., Plet, A., Lamb, N., Fernandez, A., McKenzie, F. R., Calothy, G., and Van Obberghen, E. (1995) J. Biol. Chem. 270, 13709–13716
33. Hänfl, S., Adler, H. S., Mischak, H., Jansch, P., Heidecker, G., Wolffman, A., Pippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994) Mol. Cell. Biol. 14, 6696–6703
34. Perold, P., Frödin, M., Bannier, J.-C., Calleja, V., Scinnea, J. C., Filloux, C., Caldyth, G., and Van Obberghen, E. (1995) FEBS Lett. 357, 290–296

4. G. L’Allemain, J. N. Lavoie, and J. Pouyssegur, manuscript in preparation.
53. Chen, J., and Iyengar, R. (1994) Science 263, 1278–1281
54. Vaillancourt, R. R., Gardner, A., and Johnson, G. L. (1994) Mol. Cell. Biol. 14, 6522–6530
55. Lange-Carter, C. A., and Johnson, G. L. (1994) Science 265, 1458–1461
56. Zheng, C.-F., Ohmichi, M., Saltiel, A. R., and Guan, K.-L. (1994) Biochemistry 33, 5595–5599
57. Haystead, C. M. M., Gregory, P., Shirazi, A., Fadden, P., Mosse, C., Dent, P., and Haystead, T. A. J. (1994) J. Biol. Chem. 269, 12804–12808
58. Reuter, C. W. M., Catling, A. D., Jelinek, T., and Weber, M. J. (1995) J. Biol. Chem. 270, 7644–7655
59. Itoh, T., Kaibuchi, K., Masuda, T., Yamamoto, T., Matsuura, Y., Maeda, A., Shimizu, K., and Takai, Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 975–979
60. Guy, H. R., Durell, S. R., Warmke, J., Drysdale, R., and Ganetzky, B. (1991) Science 254, 730
61. Lamy, F., Wilkin, F., Baptist, M., Posada, J. R., Roger, P. P., and Dumont, J. E. (1993) J. Biol. Chem. 268, 9398–9401
62. Saunier, C., Tournier, C., Jacquelin, C., and Pierre, M. (1995) J. Biol. Chem. 270, 3695–3697
63. Al-Alawi, N., Rose, D. W., Buckmaster, C., Ahn, N., Rapp, U., Ménkoth, J., and Feramisco, J. R. (1995) Mol. Cell. Biol. 15, 1162–1168
64. Kupperman, E., Wen, W., and Meinkoth, J. L. (1993) Mol. Cell. Biol. 13, 4477–4484
65. Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994) J. Biol. Chem. 269, 7851–7854
66. Zhong, Y. (1995) Nature 375, 588–592
67. Cano, E., and Mahadevan, L. (1995) Trends Biochem. Sci. 20, 117–120
68. Berra, E., Diaz-Meco, M. T., Lozano, J., Frutos, S., Munilla, M. M., Sanchez, P., Sanz, L., and Moscat, J. (1995) EMBO J. 14, 6157–6163

PKA and MAP Kinase