Calmodulin Inhibitor W13 Induces Sustained Activation of ERK2 and Expression of p21cip1*

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Cells have evolved signal transduction pathways that allow them to respond to extracellular signals. Those signaling pathways will lead to the regulation of effector proteins that will finally cause cell proliferation or differentiation.

One of the major signaling pathways by which extracellular signals induce cell proliferation and differentiation involves the activation of extracellular signal-regulated kinases (ERKs). Because calmodulin is essential for quiescent cells to enter cell cycle, the role of calmodulin on ERK2 activation was studied in cultured fibroblasts. Serum, phorbol esters, or active Ras induced ERK2 activation in NIH 3T3 fibroblasts. This activation was not inhibited by calmodulin blockade. Surprisingly, inhibition of calmodulin prior to fetal bovine serum addition prolonged activation of ERK2. Furthermore, inactivation of calmodulin in serum-starved cells induced ERK2 phosphorylation that was dependent on MAP kinase kinase (MEK). Inactivation of calmodulin in serum-starved cells also induced activation of Ras, Raf, and MEK. On the contrary, tyrosine phosphorylation of tyrosine kinase receptors was not observed. These results indicate that calmodulin inhibits ERK2 activation pathway at the level of Ras. Calmodulin inhibition induced overexpression of p21cip1 which was dependent on MEK activity. We propose that inhibition of Ras by calmodulin prevents the activation of ERK2 at low serum concentration. Thus, entering into the cell cycle after serum addition would imply the overcoming of the inhibitory effect of calmodulin and consequently ERK2 activation. Furthermore, down-regulation of Ras by calmodulin may be also important to determine the duration of ERK2 activation and to prevent a high p21cip1 expression that would lead to an inhibition of cell proliferation.

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The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; GEF, guanine exchange factor; GAP, GTPase activating protein; CaM, calmodulin; FBS, fetal bovine serum; TPA, 12-0-tetradecanoylphorbol-13-acetate; W12, N-(4-aminoethyl)-2-naphthalenesulfonamide; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RBD, Ras-binding domain of Raf-1; NRK, normal rat kidney cells; GST, glutathione S-transferase; MEK, MAP kinase kinase; SOS, son of sevenless; LPA, lysophosphatidic acid.
nisms leading to ERK phosphorylation by growth factors and oncoproteins, few studies have turned to the question of how the pathway is down-regulated. Several constitutive and inducible ERK phosphatases responsible for its down-regulation have been described (25), including Pyst1 (26), MKP1, 2 (27, 28), and PAC1 (29). Expression of MKP1 has been shown to be ERK- and Ca$^{2+}$-dependent (30). Less is known about the down-regulation of the pathway upstream of ERK. In some cells, ERK mediates the phosphorylation of SOS to terminate Ras-dependent activation of ERK (31).

Ca$^{2+}$ and calmodulin (CaM) are known to act as second messengers in signal transduction pathways and to regulate cell proliferation (32–35). Through the action of CaM-binding-proteins like CaM-dependent kinases II and IV, calcineurin, hnRNP A2, hnRNP C, and others, they regulate a great variety of cellular processes, such as gene expression, protein translation, and protein phosphorylation (36). By using expression vectors capable of inducibly synthesizing CaM sense or antisense mRNAs, it has been shown that progression through G1 and mitosis exit is sensitive to changes in the intracellular concentration of CaM (37). Furthermore, the addition of specific anti-CaM drugs to cell cultures inhibits reentry of growth-arrested cells into the cell cycle (G0/G1 transition), the progression into and through the S phase and the entry and exit from mitosis (35, 38–44). During G1, CaM is essential to activate cdk4 and phosphorylate pRb (44, 45). Moreover CaM participates in the activation of cdc2 during mitosis entry (33) and in its inactivation at the metaphase/anaphase transition (46). Despite the evidence indicating that CaM plays a role in cell cycle entry from quiescence (G0/G1 transition), not much is known about the CaM-dependent steps essential for this transition. CaM has been shown to play a role in the activation of the MAPK signaling pathway (47–51). For example, the Ras-GRF exchange factor of cortical neurons is a CaM-binding-proteins, and a Ca$^{2+}$ influx in these cells is able to activate Ras and ERKs (47). In other cellular types, CaM-dependent kinases have been involved in the MAPK activation pathway (50–51).

We have analyzed here the involvement of CaM in the ERK signaling pathway in cell cultured fibroblast. Surprisingly, results show that CaM is not essential for the activation of the ERK pathway but for its inactivation. This down-regulation of the pathway is due at least in part to an inhibitory effect of CaM on Ras activation. Furthermore, activation of ERK2 by CaM inhibition induces an increase in p21$^{waf1}$ expression together with a cell cycle arrest.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**NIH 3T3 cells (ATCC) or NIH 3T3 cells constitutively expressing active mutant N-Ras (lys61) under a CMV promoter (NIH 3T3RasLys61) (gift of Dr. T. Thompson, Barcelona, Spain) or normal rat kidney cells (NRK) were made quiescent by culturing them in Dulbecco’s minimum essential medium with 0.5% fetal bovine serum (FBS) during 2 days. 10% FBS, 100 µM TPA, 25 ng/ml EGF, 15 µg/ml W13, 15 µg/ml W12, 5 µg KN93, or 2 µg/ml cyclosporin A were added directly to the medium, and cultured for 2 h at 37°C. Then, the samples were washed twice in PBS, coverslips were mounted on glass slides with Mowiol (Calbiochem). To detect ERK1 and ERK2 cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100, 1% BSA in PBS for 10 min at room temperature and were washed twice in PBS, coverslips were mounted on glass slides with Mowiol (Calbiochem). To detect ERK1 and ERK2 cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100, 1% BSA in PBS for 10 min at room temperature. The samples were washed three times (5 min each) in PBS and incubated for 45 min at 37°C with fluorescein-conjugated anti-rabbit antibody (dilution 1:50, Boehringer) in 1% BSA/PBS. After two washes in PBS, coverslips were mounted on glass slides with Mowiol (Calbiochem).

**Measurement of Ras Activation—**The capacity of Ras-GTP to bind to RBD (Ras-binding domain of Raf-1) was used to analyze the amount of active Ras (54, 55). Cells were lysed in the culture dish with 25 mM Tris-HCl, pH 7.5, 5 µg EGF, 150 mM NaCl, 5 mGCa, 1 mM ATP, 1% Triton X-100, 1% N-octyl glucoside, 1 mM PMSF, 1 mM aprotinin, and 20 µM Leupeptin. Cleared (10,000 g) lysates were added to 30 µg GST-ME2 kinase beads for 2 h at 4°C. The beads were washed four times with the lysis buffer. Bound proteins were solubilized by the addition of 0.1% Laemmli loading buffer and run on 12.5% SDS-PAGE gels. Proteins were transferred and immunoblotted as described above using pan-Ras monoclonal antibody (Oncogene Sciences OP40, 1:100 dilution).

**RESULTS**

**Effect of CaM Inhibition on ERK2 Activation—**Stimulation of serum-starved NIH 3T3 fibroblast by 10% PBS, or 100 µM TPA, for 10 min, resulted in an activation of ERK2, as demonstrated by the increase in ERK2 tyrosine phosphorylation analyzed by Western blotting using antiphosphotyrosine antibodies (Fig. 1A) or phospho-specific anti-ERK1 and -ERK2 antibodies (data not shown). To analyze if CaM was essential for the signaling pathways leading to ERK2 phosphorylation, quiescent cells were pretreated with the anti-CaM drug W13 (15 µg/ml) for 20 min prior to stimulation with FBS or TPA. W13 has been shown to be highly specific at the doses used in this work (40, 42, 56, 57). W12 was used as a control because it is a compound chemically similar to W13 but with a much lower affinity for CaM (40). As shown in Fig. 1A, W13-pretreatment did not have any effect on the level of phosphorylated ERK2, determined by Western blotting using antiphosphotyrosine (PY20) antibody.
Calmodulin and ERK2 Activation

The amount of FBS added to the media was higher than 2%, no
0.5% FBS. In agreement with the results in Fig. 1
phosphorylation induced by CaM inhibition was observed at
induce ERK2 phosphorylation. Maximal increase in ERK2
phosphorylation induced by CaM inhibition was observed at
0.5% FBS. In agreement with the results in Fig. 1A, when the
amount of FBS added to the media was higher than 2%, no

FIG. 1. Effect of W13 addition on ERK2 phosphorylation induced by FBS, TPA, or Ras. A, ERK2 phosphorylation in serum- and TPA-stimulated NIH 3T3 fibroblasts after pretreatment with the anti-CaM drug W13. Quiescent NIH 3T3 cells (Q) were stimulated for 10 min with 10% FBS or 100 μM TPA. In the indicated lanes, W12 or W13 (15 μg/ml) was added to the cultures 20 min prior to FBS or TPA addition. B, quiescent NIH 3T3Nras(lys61) (Q) cells, expressing the constitutively active N-ras (lys61) mutant, were treated for 30 min with W12 or W13 (15 μg/ml). In panels A and B, cells were lysed and ERK2 phosphorylation analyzed by Western blotting using the antiphosphotyrosine (PY20) antibody as indicated under "Experimental Procedures." P-ERK2 corresponds to a band of 42 kDa that comigrates with ERK2 using anti-ERK1 and ERK2 antibodies.

The same results were obtained using the antiphospho-ERK2 specific antibodies (data not shown).

ERK2 phosphorylation was also induced at 0.5% FBS by expression of activated N-Ras. Inhibition of CaM in NIH 3T3Nras(lys61)-transformed cells by W13 treatment for 30 min did not have any effect on Ras-induced ERK2 phosphorylation (Fig. 1B). Thus, CaM is not essential for any of the studied signaling pathways leading to ERK2 activation in NIH 3T3.

Effect of CaM Inhibition on ERK2 Down-regulation—Because the duration of ERKs activation is also important for cell response, the effect of CaM inhibition on the timing of ERKs phosphorylation was analyzed. In nontreated cells (data not shown) or in cells preincubated with W12 (20 min prior to 10% FBS addition) (Fig. 2), ERK2 phosphorylation was high at 10 min, started to decrease at 30 min, and was slightly higher than in unstimulated cells by 2 h after FBS addition. Surprisingly, when cells where preincubated with W13, ERK2 phosphorylation still remained high 2 h after the stimulation (Fig. 2). Inhibition of CaM later in G1 (between 4 and 10 h after serum addition), when ERK2 phosphorylation was already decreased, did not lead to a second activation of ERK2 (data not shown). Thus, inhibition of CaM prolonged the phosphorylation of ERK2, suggesting that CaM is involved in its down-regulation.

The effect of W13 on ERK2 phosphorylation in serum-starved cells was also analyzed. When serum-starved cells (0.5% FBS) were incubated with W13 for 30 min, an increase in ERK2 phosphorylation was observed. This increase was similar to that produced by addition of 10% FBS and was not observed with W12 (Fig. 3A). This effect was not mediated by CaM-dependent kinase II or calcineurin because treatment of serum-starved cells with KN93, an inhibitor of CaM-dependent-kinase II, or cyclosporin A, an inhibitor of calcineurin, did not induce ERK2 phosphorylation (Fig. 3A).

Cooperation between FBS addition and CaM inhibition to induce ERK2 phosphorylation was analyzed in quiescent cells that had been maintained for the last 10 h in the complete absence of FBS. Cells were pre-treated with W12 or W13 for 20 min, and ERK2 phosphorylation was studied 10 min after addition of increasing amounts of FBS. As shown in Fig. 3B, W13 addition synergized with low concentrations of FBS to induce ERK2 phosphorylation. Maximal increase in ERK2 phosphorylation induced by CaM inhibition was observed at 0.5% FBS. In agreement with the results in Fig. 1A, when the amount of FBS added to the media was higher than 2%, no additional increase in ERK2 phosphorylation was induced by CaM inhibition (Fig. 3B). Similar results were obtained using NRK cells (data not shown). These results suggest that CaM inhibits any of the signaling pathways by which FBS activates ERK2.

We also analyzed whether ERK2 phosphorylation induced by CaM inhibition in serum-starved cells correlated with a nuclear accumulation of ERKs. As shown in Fig. 4, after 2 days of serum starvation, ERK2 was localized in the cytoplasm. Treatment with W13 for 30 min induced a translocation of ERK2 into the nucleus similar to what occurred with 10% FBS incubation.

FIG. 2. Effect of W13 addition on the duration of ERK2 phosphorylation. Quiescent NIH 3T3 cells were preincubated with either W12 or W13 (15 μg/ml) or without any drug (−) and 20 min later were stimulated with 10% FBS and harvested at the indicated times (t). Cells were lysed and ERK2 phosphorylation was analyzed by Western blotting using the antiphospho-ERK1 and ERK2 antibody as indicated under "Experimental Procedures." A representative experiment of three different experiments is shown in the figure.

Analysis of the Pathway Leading to ERK2 Activation by CaM
Inhibition—The only kinases known to phosphorylate and activate ERK2 are MEK1 and MEK2. Thus, we analyzed whether these kinases were involved in ERK phosphorylation induced by CaM inhibition. As shown in Fig. 5A, ERK2 phosphorylation induced by W13 addition to serum-starved cells was not observed when cells were previously treated with the specific inhibitor of MEK, PD98059 (Calbiochem). Furthermore, W13 addition to serum-starved cells induced an increase in MEK phosphorylation as determined by Western blotting using phospho-MEK-specific antibodies (Fig. 5B). These results indicate that W13 induces ERK2 phosphorylation by MEK and that CaM inhibits the activation of MEK at low serum concentration.

Next, the effect of CaM inhibition on Raf-1, the main kinase involved in MEK1 and MEK2 activation, was analyzed. W13 addition to serum-starved cells induced an increase in Raf-1 activity as determined by immunoprecipitation followed by kinase assay using inactive MEK-GST as substrate, compared with W12-treated cells and quiescent cells (Fig. 6A). CaM inhibition induced also a gel-mobility shift of Raf-1, similar to that induced by FBS (Fig. 6B) that has been related with its phosphorylation and activation. Although Raf activation is not very well understood, the increase in Ras-GTP seems to be an essential event for Raf activation. Thus, the levels of Ras-GTP upon W13 treatment of serum-starved cells were analyzed. As shown in Fig. 7A, CaM inhibition induced an increase in the levels of Ras-GTP that was not observed in W12-treated cells. The levels of Ras-GTP after W13 treatment were as high as those reached upon 10% FBS addition. A mechanism for Ras activation is activation of tyrosine kinase receptors involving autophosphorylation of these receptors and the recruitment of SOS to the plasma membrane through the interaction with the adapter protein GRB2. After 10 min of 10% FBS addition to serum-starved cells, tyrosine phosphorylation was increased in the area of the gel where the EGF receptors and PDGF receptors move, 170–190 kDa (Fig. 7B). On the contrary, no increase in tyrosine phosphorylation in the same area of the gel was observed after the addition of W13 or W12 to serum-starved cells (Fig. 7B). Thus, CaM inhibition induced activation of Ras/Raf/MEK/ERK without any detectable activation of tyrosine kinase receptors.

CaM Inhibition Increased the Levels of the Cell Cycle Inhibitor p21\(^{WAF1/CIP1}\) and Inhibited Cell Proliferation—In agreement with previous reports (38) when W13 was added to quiescent serum-starved NIH 3T3 cells 20 min prior to 10% FBS addition, DNA synthesis at 20 h was inhibited by 80%. As inhibition of CaM prevents entry in S phase and prolongs ERK activation, we looked for a relationship between these two effects. The expression of p21\(^{WAF1/CIP1}\) has been shown to be dependent on ERK1 and ERK2 activity (20). Thus, the levels of this cell cycle inhibitor were analyzed upon CaM inhibition. In W13-pretreated cells, a prolonged expression of p21\(^{WAF1/CIP1}\) was observed upon FBS addition (Fig. 8A), being the p21\(^{WAF1/CIP1}\) protein still present 9 h after serum addition. On the contrary, in control cells (W12-pretreated cells), 10% FBS addition induced a transient increase in p21\(^{WAF1/CIP1}\) that showed a maximum at 2 h (Fig. 8A). Thus, inhibition of CaM induced sustained ERK2 activation...
were lysated and Western blots were performed as indicated under "Experimental Procedures." B, quiescent NIH 3T3 cells (Q) were treated for 10 min with 10% FCS (FBS), 25 ng/ml EGF, or for 30 min with W12 or W13 (15 μg/ml). Cells were lysated and Ras-GTP was determined by precipitating with RBD-Sepharose followed by Western blotting with anti-Ras antibody as indicated under "Experimental Procedures." Both panels A and B are representative results of three different experiments.

DISCUSSION

ERK signaling pathway is very important for the cellular response to extracellular signals. Signaling through this pathway has been shown to mediate differentiation, proliferation, or oncogenic transformation depending on the cellular context and the duration of the activation (19, 58). Thus, in addition to activation of ERK, its down-regulation is also decisive for cell response. Inactivation of the pathway is important to prevent ERK phosphorylation at low serum concentration and to prevent an excessively prolonged peak of ERK activity when cells are stimulated by growth factors. In this sense, constitutive and serum-inducible ERK phosphatases have been described, but inactivation of the pathway upstream of ERK is not well understood. We have analyzed the involvement of CaM in ERK activation and inactivation pathways in cell-cultured fibroblasts.

Ca²⁺ and CaM are essential for the activation of ERK in response to various stimuli in cortical neurons and lymphocytes; however, we have shown here that CaM is not essential to activate ERK2 by serum, TPA or constitutive activation of Ras in cultured fibroblasts (NIH 3T3 or NRK cells). These results agree with the fact that in Rat-1 cells, buffering of Ca²⁺ does not prevent ERK activation induced by LPA or EGF (31). Thus, although CaM is necessary for NIH 3T3 fibroblast to reenter cell cycle from quiescence, it is not essential for ERK2 activation.

We have shown that CaM is involved in the down-regulation of the ERK2 activation pathway. CaM inhibition increases the duration of ERK2 phosphorylation when cells are stimulated with 10% FBS. ERK2 activation has a dual effect on cell proliferation. On one hand it induces the expression of cyclin D1 which is essential for G1 progression (59, 60), and on the other hand it induces p21cip1 expression (20). p21cip1 seems to be necessary at low levels for cdk4/cyclin D1 activation, but at high levels acts as an inhibitor of cdk4/cyclin D1 and cdk2/cyclin E (61, 62). Thus, an intense or highly sustained activation of ERK2 could induce an excessive increase of p21cip1 expression and, as a consequence, cell cycle inhibition. In fact, a high intensity Raf signal has recently been shown to cause a cell cycle arrest mediated by p21cip1 (23, 24). In agreement with that, the sustained activation of ERK2 induced by CaM inhibition in serum-stimulated cells correlates with a lengthened expression of p21cip1 and an inhibition of cell proliferation. At least two reports indicate an inhibitory role of Ca²⁺ on ERK activity. First, expression of the ERK phosphatase, MKP-1, is Ca²⁺-dependent in Rat-1 cells; consequently, a depletion of the intracellular Ca²⁺ in these cells induces a more sustained increase in ERK1 activity (31). Second, Ca²⁺ addition to cultures inhibits EGF-induced stimulation of ERK2 activity in human primary keratinocytes (63). We have also shown that CaM inhibition not only leads to a prolonged activation of ERK2 upon FBS addition but also to an activation of ERK2 at low serum concentration. The fact that, in total absence of FBS, CaM inhibition has almost no effect on ERK phosphorylation, but that a cooperation exists between CaM inhibition and FBS addition for the activation of ERK2, suggests that CaM inhibits any of the signal transduction pathways leading to ERK2 phosphorylation that are activated by FBS. We have analyzed the activation of different elements of the ERK signaling pathway and shown that CaM inhibition leads also to Ras, Raf, and MEK activation. Thus, we conclude that CaM is down-regulating the Ras activation pathway, although a multiple effect of CaM at several levels in the ERK signaling pathway cannot be excluded. We have also proved that CaM-dependent kinase II or the CaM-dependent phosphatase calcineurin are not involved in the inhibitory effect of CaM on the ERK activation pathway.

The rate-limiting step in Ras activation is the exchange of bound GDP for GTP, which is catalyzed by GEFs (16). Several guanine exchange factors are involved in Ras activation in response to different stimuli. In response to tyrosine kinase receptors activation, Ras is activated by the SOS nucleotide-exchange factor. Access of SOS to the membrane where Ras is located is because of the binding of SOS to the receptor through SH2 domain- and SH3 domain-mediated interactions involving
the adapter proteins GRB2 and SHC. Although it has been shown that some tyrosine kinase receptors such as EGF receptor are able to bind CaM (64, 65) we have not observed any increase in tyrosine phosphorylation levels of the EGF receptor or any other protein of 170–190 kDa after CaM inhibition. Other Ras guanine exchange factor have been identified as Ras-GRF/Cdc25Mm (66) and Ras-GRF2 (67). Both factors are most abundant in brain but are also expressed in other tissues and a variety of cell lines (66, 68). Activation of these factors is not very well understood, and they have been suggested to respond to G protein-coupled receptors (69). Both factors contain IQ motifs that allow their binding to CaM, and in both neurons and epithelial cells, its activity seems to be stimulated by Ca$^{2+}$ (47, 67). Those results are controversial because in vitro studies show that full-length Ras-GRF activity is inhibited by CaM (70). Our results agree with the presence of Ras-GRF or a homologous protein in NIH 3T3 cells whose activity is inhibited by CaM. Studies to determine this are underway in our laboratory. Alternatively CaM could activate a GAP protein acting on Ras. In this sense, it has been shown that IQGAP1 binds CaM and that its interaction with cdc42 is inhibited by CaM (71).

We propose two possible physiological roles, which are not exclusive, for the negative regulation of the ERK signaling pathway by CaM. First, CaM is defining a threshold in the Ras/Raf/MEK/ERK signaling pathway to prevent activation of the pathway at low serum concentration because of the basal activity of some components of the pathway upstream of Ras. When growth factor receptors are activated by serum addition, this threshold is overpassed and ERK is activated even in the presence of Ca$^{2+}$ and CaM. Second, CaM down-regulation of Ras is essential to regulate the duration and the intensity of ERK activation. In consequence, CaM inhibition prolongs Ras/Raf/MEK/ERK activation and p21$^{cip}$ expression, and thus induces inhibition of cell cycle progression.

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