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Calmodulin Prevents Activation of Ras by PKC in 3T3 Fibroblasts*

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We have shown previously (Villalonga, P., López-Alcalá, C., Bosch, M., Chiloeches, A., Rocamora, N., Gil, J., Marais, R., Marshall, C. J., Bachs, O., and Agell, N. (2001) *Mol. Cell. Biol.* 21, 7345–7354) that calmodulin negatively regulates Ras activation in fibroblasts. Hence, anti-calmodulin drugs (such as W13, trifluoroperazine, or W7) are able to induce Ras/ERK pathway activation under low levels of growth factors. We show here that cell treatment with protein kinase C (PKC) inhibitors abolishes W13-induced activation of Ras, Raf-1, and ERK. Consequently, PKC activity is essential for achieving the synergism between calmodulin inhibition and growth factors to activate Ras. Furthermore, whereas the activation of PKC by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) does not induce Ras activation in 3T3 cells, activation is observed if calmodulin is simultaneously inhibited. This indicates that calmodulin is preventing Ras activation by PKC. Treatment of cells with epidermal growth factor receptor or platelet-derived growth factor receptor tyrosine kinase inhibitors does not abrogate the activation of Ras by calmodulin inhibition. This implies that epidermal growth factor receptor and platelet-derived growth factor receptor tyrosine kinase activities are dispensable for the activation of Ras by TPA plus W13, and, therefore, Ras activation is not a consequence of the transactivation of those receptors by the combination of the anti-calmodulin drug plus TPA. Furthermore, K-Ras, the isoform previously shown to bind to calmodulin, is the only one activated by TPA when calmodulin is inhibited. These data suggest that direct interaction between K-Ras and calmodulin may account for the inability of PKC to activate Ras in 3T3 fibroblasts. *In vitro* experiments showed that the phosphorylation of K-Ras by PKC was inhibited by calmodulin, suggesting that calmodulin-dependent modulation of K-Ras phosphorylation by PKC could be the mechanism underlying K-Ras activation in fibroblasts treated with TPA plus W13.

The three members of the Ras family of small GTPases, H-, N-, and K-Ras, are key regulators of signal transduction pathways that control cell proliferation, differentiation, survival, and apoptosis (1–3). The molecular basis for such a great variety of cell responses controlled by Ras proteins relies on the fact that Ras is able to transduce signals from different extracellular stimuli, including growth factors, hormones, and cell-extracellular matrix contacts to many downstream effectors (4, 5). As a molecular switch, Ras cycles between a GTP-bound active and an inactive state when GTP is hydrolyzed to GDP. Active Ras interacts with and modulates the activity of effector proteins. The best characterized Ras effector is the serine/threonine kinase Raf, which leads to the activation of the extracellular signal-regulated kinase (ERK)[1] pathway that plays a major role in cell proliferation and differentiation (6–8). Other effectors for Ras include the lipid kinase phosphatidylinositol-3-kinase (PI3K), which is involved in cell survival, proliferation, and metabolism (9–11), and the nucleotide exchange factors for Ral GTPase, RalGDS, Rlf, and Rlg (12).

Many molecules have been described as influencing the Ras GTP/GDP cycle, mainly through two distinct biochemical activities: 1) the guanine nucleotide exchange factors (GEFs) that regulate the replacement of the nucleotide bound to Ras, favoring the GTP-bound active state; or 2) the GTPase-activating proteins (GAPs), which increase Ras low intrinsic GTPase activity and thereby promote its inactivation. Following extracellular stimulation, GEFs are recruited to the plasma membrane through binding to a set of molecular adaptors, inducing transiently Ras-GTP complexes (13, 14). Distinct signals such as those transduced from tyrosine kinase receptors, G protein-coupled receptors, or integrin-induced cell attachment to the extracellular matrix all lead to Ras activation. In many cases this is achieved through the membrane recruitment of the Sos exchange factor, which in turn depends on the adaptor protein Grb-2. Autophosphorylation of tyrosine kinase receptors or activation of a variety of non-receptor tyrosine kinases such as Src, Pyk-2, or focal adhesion kinase in response to the activation of diverse G protein-coupled receptors or cell-extracellular matrix engagement all create phosphotyrosine residues that allow Grb-2/Sos binding and thus its recruit-

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[1] The abbreviations used are: ERK, extracellular signal-regulated kinase; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; PKC, protein kinase C; CaM, calmodulin; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; EGF, epidermal growth factor; EGFR, EGF receptor; CaMBP, CaM-binding protein; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TBS, Tris-buffered saline; RBD, Ras-binding domain of Raf-1; MBP, myelin basic protein; FCS, fetal calf serum; GST, glutathione-*S*-transferase.

ment to the vicinity of Ras at the plasma membrane, leading to its activation (15, 16).

In addition, other mechanisms are able to link extracellular signals to Ras activation, such as phospholipase C activation and its consequent increase in diacylglycerol and intracellular Ca^{2+}. Ca^{2+} induces the activation of RasGRF, another Ras guanine nucleotide exchange factor present mainly in neurones that lead to Ras activation (17). In addition, together with diacylglycerol, Ca^{2+} is also able to activate RasGRPs, a Ras-GEF family present in lymphocytes and neurones (18). In turn, protein kinase C (PKC) activation by diacylglycerol is also able to activate Ras in some cellular types. The activation of Ras through PKC-dependent GAP inhibition has been reported in T lymphocytes upon T cell receptor activation (19). However, this seems not to be the case for fibroblasts (20), although PKC is able to induce Ras activation in B lymphocytes and COS cells (21). GAP activity can also be regulated by binding to phosphorylated tyrosine kinase receptors (22). In summary, a highly interconnected network of multiple pathways regulates Ras activation in response to extracellular signals.

The Ca^{2+}-binding protein calmodulin (CaM), which acts as a second messenger in cellular signal transduction pathways and regulates cell proliferation (23–26), has been shown to be one of the molecules involved in the modulation of Ras activity. We have shown that CaM down-regulates the Ras/Raf/MEK/ERK pathway in fibroblasts (27, 28), and other groups have shown that CaM is able to inhibit *in vitro* EGFR (29) activity and the shedding of EGF-like growth factors (30). Although low doses of growth factors are not able to induce activation of the Ras/Raf/MEK/ERK pathway in fibroblasts, activation is observed if CaM is simultaneously inhibited, indicating that CaM is preventing the activation of this pathway under basal conditions. Furthermore, CaM inhibition in fibroblasts leads to a more sustained level of ERK activation following serum stimulation. CaM functions are mediated by its association with specific target proteins named CaM-binding proteins (CaMBPs), which are regulated upon CaM binding (31, 32). We have recently demonstrated that K-RasB is a CaMBP and that this Ras isoform is specifically activated when serum-starved NIH3T3 cells are treated with a CaM inhibitor, suggesting a direct relation between CaM binding to Ras and Ras inhibition by CaM (28). However, the activation of Ras by CaM inhibition is low in the absence of growth factors, increasing dramatically in the presence of low levels of mitogens. We have analyzed here the intracellular elements that are cooperating with CaM inhibition to induce Ras activation. Our results show that PKC activity is responsible for this synergism and that CaM is preventing the activation of Ras by PKC in 3T3 fibroblasts.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 cells were grown in Dulbecco's minimum essential medium supplemented with 10% donor calf serum and made quiescent by culturing them for 24 h with media containing 0.5% fetal bovine serum (FBS). Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and made quiescent by incubating 1×10^4 cells/cm^2 until confluence (6–8 days) and keeping them in 0.5% FBS medium overnight during the last day and in serum-free medium for the last 3 h. Purified growth factors (EGF, PDGF or bombesin), 10% FBS, TPA, or drugs (W12, W13, GF109203X, Ro-0432, AG1296, or AG1478) were added directly to the media at the indicated concentrations, and cells were harvested at the time points indicated in the results.

Gel Electrophoresis and Immunoblotting—Cells were lysed in a buffer containing 2% SDS, 67 mM Tris-HCl, pH 6.8, and 10 mM EDTA, and sonicated twice for 10 s. Protein content was measured by the Lowry procedure using bovine serum albumin as standard. These cellular extracts or proteins from pull-down experiments were electrophoresed in SDS-polyacrylamide gels essentially as described (33). After electrophoresis, the proteins were transferred to Immobilon-P strips for 2 h at 60 V. The sheets were preincubated in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), 0.05% Tween 20, and 5% defatted milk powder for 1 h at room temperature and then incubated for 1 h at room temperature in TBS, 0.05% Tween 20, 1% bovine serum albumin and 0.5% defatted milk powder containing the following appropriated antibodies: ERK2 (Upstate Biotechnology, catalog no. 0-157; 1:500 dilution), Pan-Ras (Oncogene Science OP40, 1:100 dilution), N-Ras (Santa Cruz Biotechnology, catalog no. sc-31; 1:100 dilution), K-Ras (Santa Cruz, catalog no. sc-30; 1:100 dilution) monoclonal antibodies and H-Ras (Santa Cruz Biotechnology, catalog no. sc-520, 1:100 dilution), and phospho-ERK1/2 (Cell Signaling Tech, catalog no. 9101; 1:500 dilution) polyclonal antibodies. After washing in TBS with 0.05% Tween 20 (three times, 10 min each), the sheets were incubated with a peroxidase-coupled secondary antibody (1:2000 dilution) (Bio-Rad) for 1 h at room temperature. After incubation, the sheets were washed twice in TBS with 0.05% Tween 20 and once in TBS. The reaction was visualized by ECL (Amersham Biosciences) or Super-Signal (Pierce). Images were then scanned, and intensities were quantified using the Quantity One program (Bio-Rad).

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 (34). Cells ($5–10 \times 10^6$) were lysed in the culture dish with Ras extraction buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 5 mM NaF, 10% (v/v) glycerol, and 0.5% (v/v) 2-mercaptoethanol) plus protease and phosphatase inhibitors. Cleared ($10{,}000 \times g$) lysate was assayed for protein concentration by the Bradford method, and protein-equalized supernatants were incubated for 2 h at 4 °C with glutathione-Sepharose 4B beads pre-coupled with GST-RBD (1 h at 4 °C). The beads were washed four times in the lysis buffer. Bound proteins were solubilized by the addition of 30 μl of Laemmli loading buffer and run on 12.5% SDS-PAGE gels. The amount of Ras in the bound fraction was analyzed by Western blotting.

Raf-1 Kinase Activity Assays—To measure Raf-1 activity, kinase assays following immunoprecipitation were performed essentially as described (35). Briefly, treated cells (2×10^6) were harvested on ice in 40 μl of extraction buffer (20 mM Tris-HCl, pH 7.5, 1.5 M KCl, 1 mM EDTA, 5% (v/v) glycerol, 1% (v/v) Triton X-100, and 5 mM NaF plus protease and phosphatase inhibitors), diluted with 260 μl of dilution buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 5 mM NaF plus protease and phosphatase inhibitors), and clarified by centrifugation ($10{,}000 \times g$). Supernatants (equalized for protein concentration) were then immunoprecipitated for 2 h at 4 °C with 2 μg of anti-Raf-1 (Transduction Laboratories, R-19120) pre-coupled with 20 μl of protein G-Sepharose (Sigma) for 2 h at room temperature. Immunoprecipitates were then washed three times in wash buffer (30 mM Tris, 0.1 mM EDTA, 0.3% 2-mercaptoethanol, 10% glycerol, 1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4) with decreasing amounts of NaCl (high, 1 M; low, 0.1 M and salt-free). Washed immunoprecipitates were incubated for 30 min at 30 °C in 20 μl of MEK buffer (30 mM Tris, 0.1 mM EDTA, 0.3% 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, 0.8 mM ATP, 6.5 μg/ml GST-MEK, and 100 μg/ml GST-ERK2), and the reaction was terminated by the addition of 20 μl of ice-cold Kill buffer (30 mM Tris, 6 mM EDTA, 0.3% 2-mercaptoethanol, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4). Following centrifugation, 6 μl of supernatants (triplicates) were incubated for 15 min at 30 °C with 24 μl of myelin basic protein (MBP) buffer (50 mM Tris, 0.1 mM EDTA, 0.3% 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, 0.1 mM ATP, 50 μCi/ml [γ-^{32}P]ATP, 0.5 μg/μl MBP (Sigma), 0.16 μg/μl bovine serum albumin), and then 24 μl of this reaction mixture were loaded on P81 sheets (Millipore) and washed three times (20 min each) in 75 mM orthophosphoric acid. Remaining radioactivity was then counted on a Cerenkov counter.

In Vitro Phosphorylation—3 μg of purified baculovirus-expressed K-Ras-GST (28) were incubated with a mixture containing 25 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 25 μM ATP in the presence or absence of CaM (7 μg) and in the presence of either $CaCl_2$ (1 μM) or EGTA (1 mM) in a final volume of 30 μl. The reaction was initiated by adding 0.25 μl of rat brain PKC catalytic fragment (Biomol) and [γ-^{32}P]ATP (3000 Ci/mmol) (Amersham Biosciences). Samples were incubated at 30 °C for 30 min, and the reaction was stopped by the addition of Laemmli sample buffer. Samples were electrophoresed on polyacrylamide SDS gel electrophoresis. The gel was stained with Coomassie Blue, destained, and dried. The digital data were obtained with a Bio-Rad phosphorimaging device.

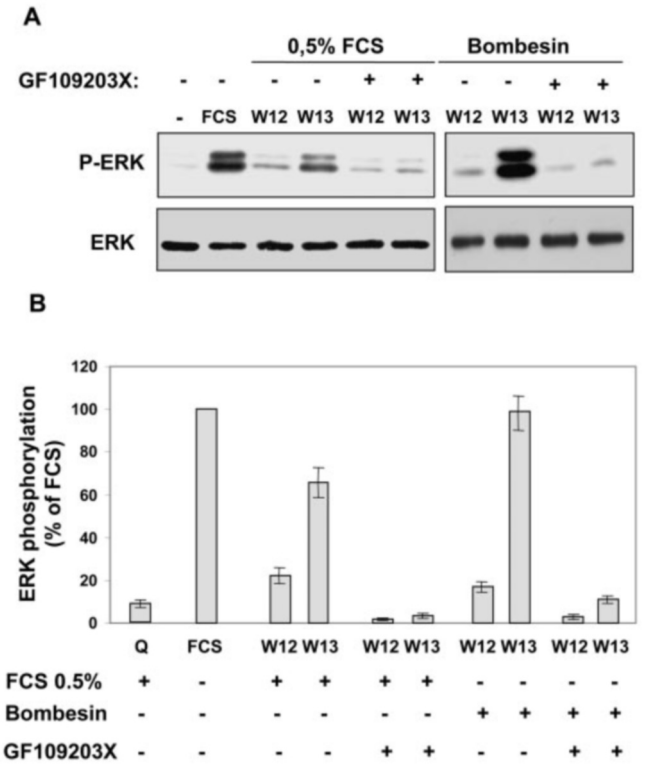

FIG. 1. **ERK activation induced by CaM inhibition in Swiss 3T3 fibroblasts is dependent on PKC activity.** *A*, quiescent Swiss 3T3 fibroblasts were treated for 15 min with 10% FCS and W12 (15 μg/ml) or W13 (15 μg/ml) together with 0.5% FCS or 0.05 nM bombesin in the absence (−) or presence (+) of GF109203X (5 μM). ERK activation was analyzed by Western blotting using anti-phospho-ERK specific antibodies (*P-ERK*; *upper panel*). As a control of the amount of protein, total ERK2 was also analyzed in the same samples (*lower panel*). *B*, quantification of three different experiments as described in *A*. Quantification of ERK phosphorylation by 0.5% FCS is also included.

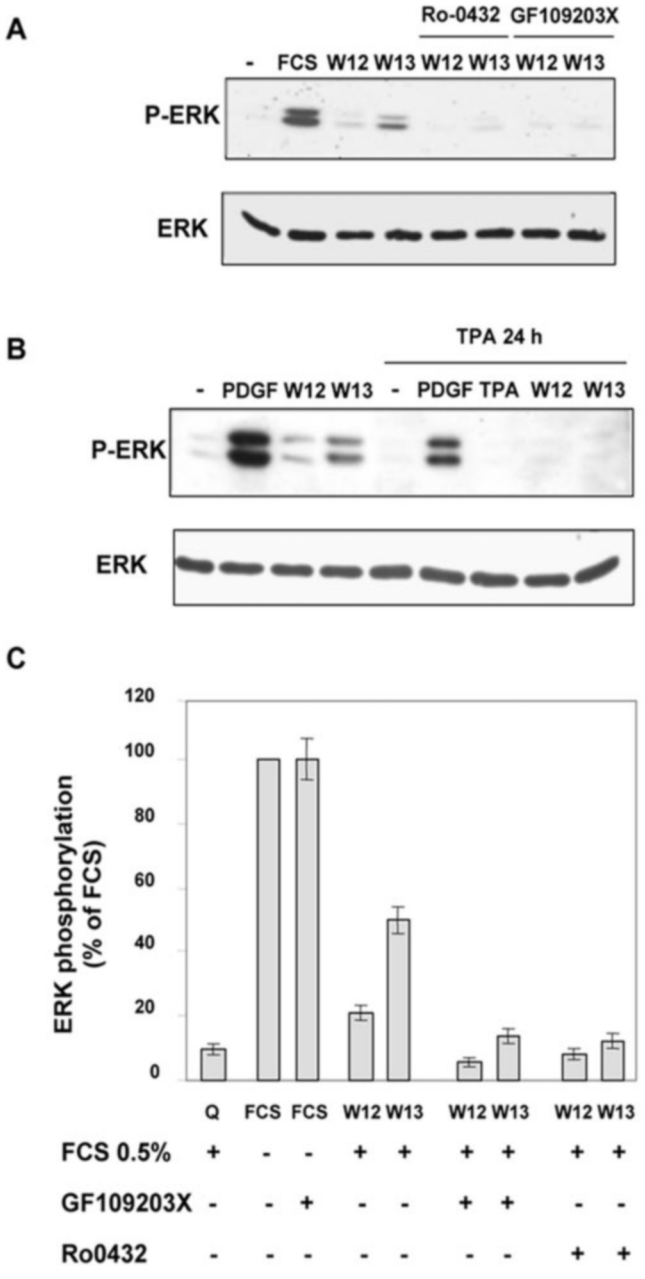

FIG. 2. **ERK activation induced by CaM inhibition in NIH3T3 fibroblasts is dependent on PKC activity.** *A*, quiescent NIH3T3 cells incubated with 0.5% FCS were treated for 15 min with 10% FCS, W12 (15 μg/ml), or W13 (15 μg/ml) in the absence or presence (where indicated) of Ro-0432 (5 μM) or GF109203X (5 μM). *B*, NIH3T3 cells were serum-deprived in 0.5% FCS-containing medium for 24 h in the absence or presence (where indicated) of TPA (500 nM) and then treated for 15 min with PDGF (0.4 nM), W12 (15 μg/ml), W13 (15 μg/ml), or TPA (100 nM). ERK activation was analyzed in the *upper panels* of *A* and *B* by Western blotting using anti-phospho-ERK-specific antibodies. As a control, total ERK2 was also analyzed in those samples (*lower panels*). *C*, the quantification of three different experiments as the ones shown in *A*. Quantification of ERK phosphorylation by 0.5% FCS and the effect of GF109203X on 10% FCS-induced ERK phosphorylation are also included.

RESULTS

ERK Activation Induced by CaM Inhibition in Fibroblasts Is Dependent on PKC Activity—We have shown previously that the addition of anti-CaM drugs synergies with low concentrations of FCS, EGF, PDGF, or bombesin to induce ERK1/2 activation in 3T3 cells (27, 28). The presence of low levels of these factors was essential for observing the activation of ERK by treatment of the cells with CaM inhibitors. We therefore hypothesized that these different stimuli were all able to activate a signaling pathway that was able to cooperate with CaM inhibition to induce ERK activation. One signaling event that is turned on by the majority of growth factors described so far is the activation of isotypes of the PKC family of serine/threonine kinases, especially the conventional PKCs that are activated by diacylglycerol and the phorbol ester class of tumor promoters. We analyzed whether PKC was essential to induce ERK activation by CaM inhibitors. W13 (15 μg/ml) was used to inhibit CaM, and W12 (15 μg/ml) was used as a control drug. We have observed the same effect on ERK activation with other anti-CaM drugs such as trifluoroperazine or W7 (28). As shown in Fig. 1 and in agreement with previous results, neither FCS nor bombesin was able to induce a significant ERK phosphorylation when added at the indicated concentrations to serum-starved Swiss 3T3 under control conditions (W12). When CaM was inhibited by treating the cells at the same time with W13, the activation of ERK was observed. To analyze a possible role for PKC on ERK activation by CaM inhibition, cells were simultaneously treated with a broadly used PKC inhibitor, GF109203X (5 μM). As observed in Fig. 1, the activation of ERK by W13 was completely abolished in the presence of the PKC inhibitor. Similar results were obtained with NIH3T3 cells (Fig. 2, *A* and *C*). Although the addition of W13 but not the control drug W12 to serum-starved (0.5% FCS) cells induced ERK phosphorylation, no phosphorylation was observed when PKC was inhibited by either GF109203X (5 μM) or Ro-4320 (5 μM). In contrast, 10% FCS-induced ERK activation was not

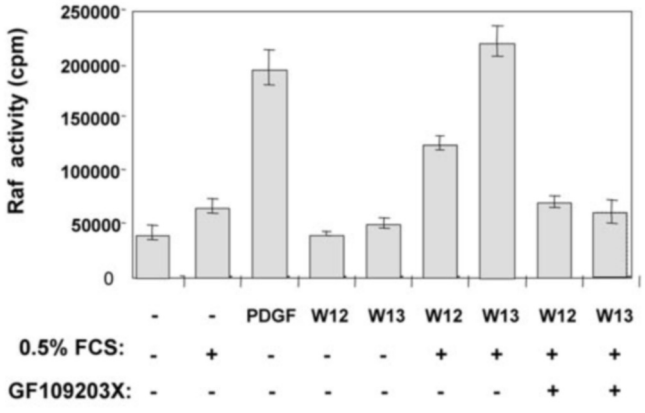

FIG. 3. **Raf-1 activation induced by CaM inhibition in fibroblasts is dependent on PKC activity.** NIH3T3 cells were serum-deprived for 24 h in serum-free medium (−) or 0.5% FCS-containing medium (+) and then treated for 10 min with PDGF (1 nM), W12 (15 μg/ml), or W13 (15 μg/ml) in the absence (−) or presence (+) of GF109203X (5 μM). Cell lysates were immunoprecipitated with anti-Raf-1 antibodies, and the kinase activity of the immunoprecipitates was assayed in a two-step assay against GST-MEK/GST-ERK2 in the first step and MBP in the second one. The mean kinase activity (in cpm) of three replicates for each condition is represented. This is a representative experiment of three independent ones.

inhibited by GF109203X (Fig. 2*C*). To further confirm PKC participation, serum-deprived (0.5% FCS) NIH3T3 cells were treated for 24 h with TPA (500 nM) to induce a depletion of the phorbol ester-sensitive class of PKCs (36). Cells were then treated with PDGF (0.4 nM) as a positive control or with W13 or W12. As shown in Fig. 2*B*, chronic TPA treatment completely prevented the activation of ERK induced by W13. In contrast, the activation induced by PDGF was only partially reduced. Altogether, these results suggested that PKC activity was essential for the activation of ERK by W13 at low doses of growth factors.

Raf-1 and Ras Activation Induced by CaM Inhibition in Fibroblasts Is Dependent on PKC Activity—We also showed previously that the treatment of cultured fibroblasts with W13 induced not only ERK activation but also Ras, Raf-1, and MEK activation (27). The relationship between PKC and the Ras/Raf/MEK/ERK signaling pathway is complex and not completely understood, because different members of the pathway have been shown to be activated by PKCs in distinct cell systems. Consequently, it was difficult to infer directly at what level of the pathway the synergism between PKC activity and CaM inhibition was taking place. To better understand this, the effect of PKC inhibition on the activation of Raf-1 and Ras induced by W13 was analyzed. When serum-deprived (0% FCS) NIH3T3 cells were treated with either W12 or W13, no activation of Raf-1 was observed. As a positive control, cells were treated with PDGF (1 nM) and a 3-fold activation was observed on Raf-1 kinase activity. W13 treatment in the presence of 0.5% FCS induced an increase in Raf-1 activity that was not observed in the complete absence of FCS and was lower in W12-treated cells. This increase in Raf-1 activity was abolished in the presence of GF109203X (Fig. 3). These results indicate that Raf-1 activation induced by CaM inhibition is also PKC-dependent.

We then tested to see whether the previously described activation of Ras by W13 was also sensitive to the presence of PKC inhibitors. To clarify this issue, serum-starved (either 0 or 0.5% FCS) NIH3T3 cells were treated with the anti-CaM drug (W13) or the control drug (W12), and Ras activation was analyzed by the GST-Raf-1-RBD pull-down method. As shown in Fig. 4, *A* and *D*, CaM inhibition induced a low activation of Ras in 0% FCS-starved cells. This activation was clearly higher in cells starved in 0.5% FCS-containing media. Although low, the activation at 0% FCS was reproducible, and the activation at 0.5% FCS was comparable with the one obtained with PDGF (1 nM). Again, this suggested that W13 was cooperating with some factor that was already activated at basal levels in 0% FCS-starved cells and at higher levels in 0.5% FCS-starved cells to allow the activation of Ras. The inhibition of PKC by GF109203X treatment completely blocked the activation of Ras induced by W13 (Fig. 4, *A* and *D*), indicating that Ras activation by W13 was PKC-dependent.

PKC Activation Alone Is Sufficient to Induce Ras Activation When CaM Is Inhibited—These results place PKC activity as a strong candidate to be the signaling event that, in a cooperative fashion with CaM inhibition, is essential for inducing Ras activation at low levels of growth factors. To determine whether PKC was in fact this key signaling element supplied up to now by different growth factors, we tested to see whether PKC activation alone, in a serum-free background, was enough to induce Ras activation when CaM was inhibited. To do this, serum-starved NIH3T3 cells were acutely treated with TPA (100 nM) in the absence or presence of W13 (Fig. 4, *B* and *D*). As described previously (20), TPA treatment of NIH3T3 cells did not change the basal levels of Ras activation significantly. As shown previously, treatment of these cells with W13 alone induced only a slight activation of Ras. Interestingly, when both W13 and TPA were added together a strong synergism was observed, and the levels of active Ras reached values comparable with the ones induced by PDGF treatment (Fig. 4, *B* and *D*). Similar results were obtained using Swiss 3T3 fibroblasts (data not shown). Furthermore, this activation was not observed when cells were simultaneously treated with PKC inhibitors (Ro-0432 or GF109203X) (Fig. 4, *C* and *D*). Altogether, these data point to the fact that CaM is preventing the activation of Ras by PKC in 3T3 fibroblasts.

Activation of Ras by W13 Treatment Does Not Require EGF or PDGF Receptor Activity—Calmodulin inhibitors have been shown to induce the cleavage of membrane-bound proteins leading to tyrosine kinase receptor transactivation, which, in some cases such as the shedding of pro-HB-EGF by metalloprotease disintegrins, is a PKC-dependent process (37–39). We investigated the possibility that TPA, together with W13, was transactivating the EGF or the PDGF receptors and, only as an indirect consequence, inducing Ras activation. To this end, the same experiment depicted in Fig. 3 was performed, but in this case the cells were pre-treated with EGFR or PDGFR tyrosine kinase inhibitors. As shown in Fig. 5, Ras activation induced by TPA (100 nM) plus W13 treatment was not inhibited by pre-incubating the cells with either AG1296 (PDGFR kinase inhibitor) or AG1478 (EGFR kinase inhibitor). As expected, AG1296 was able to inhibit the activation of Ras by PDGF, and AG1478 did so with EGF-induced Ras activation. Genistein (100 μM), a more general tyrosine-kinase inhibitor, had a strong inhibitory effect on EGF-induced ERK activation (70%) but a low inhibitory effect on TPA plus W13-induced ERK activation (30%). These results indicate that the activation of Ras induced by TPA plus W13 was not mainly dependent on EGFR or PDGFR kinase activities.

K-Ras Is the Ras Isoform Activated by CaM Inhibition in the Presence of TPA—We have shown previously that one of the Ras isoforms, K-Ras, is a CaMBP. Furthermore, this isoform is the one specifically activated when CaM is inhibited in serum-starved (0.5% FCS) NIH3T3 cells (28). This favored the hypothesis that the interaction between K-Ras and CaM was, in fact, modulating K-Ras activity. If the element synergizing with W13 inhibition to induce Ras activation was PKC, one should

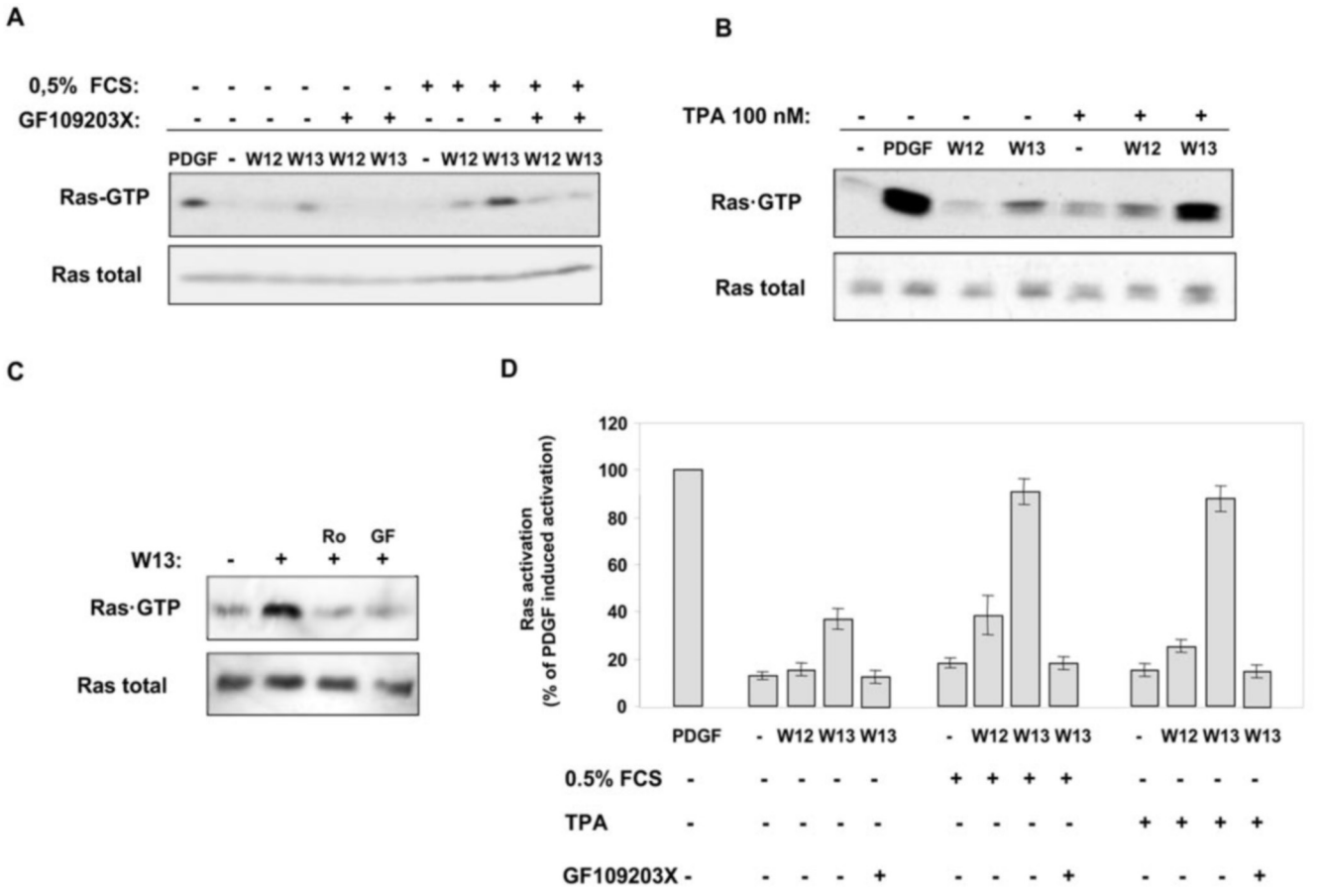

FIG. 4. **Involvement of PKC in Ras activation by W13.** *A*, NIH3T3 cells were serum-deprived for 24 h in serum-free medium (−) or 0.5% FCS-containing medium (+) and then treated for 5 min with PDGF (1 nM), W12 (15 μg/ml), or W13 (15 μg/ml) in the absence (−) or presence (+) of GF109203X (5 μM). *B*, quiescent NIH3T3 cells in a serum-free medium were treated for 5 min with PDGF (1 nM), W12 (15 μg/ml), or W13 (15 μg/ml) in the absence (−) or presence (+) of TPA (100 nM). *C*, cells were treated as described in *B* but with TPA in all cases. Where indicated, 5 μM Ro-0432 (*Ro*) or 5 μM GF109203X (*GF*) were added 15 min prior to the addition of W13 and TPA. In *A*, *B*, and *C*, Ras activation was measured by GST-RBD pull-down followed by Western blot with anti-(pan)-Ras antibodies (*upper panel*). An aliquot of each lysate was also loaded in another gel to analyze total Ras protein levels (*lower panel*). *D*, quantification of three different experiments, including the results shown in *A*, *B*, and *C*.

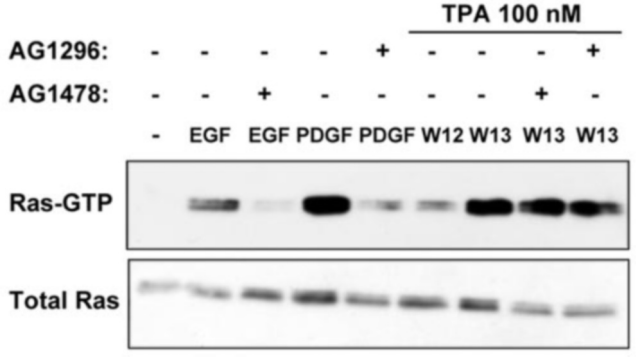

FIG. 5. **Activation of Ras by TPA and W13 treatment does not require EGF or PDGF receptor activity.** Quiescent NIH3T3 cells were incubated in serum-free medium for 2 h and pre-incubated (+) or not pre-incubated (−) with AG1296 or AG1478 for 30 min as indicated. Cells were then treated with EGF (25 ng/ml) or PDGF (1 nM) as controls or with TPA (100 nM) together with W12 (15 μg/ml) or W13 (15 μg/ml). Ras activation was measured by GST-RBD pull-down followed by Western blot with anti-(pan)-Ras antibodies (*upper panel*). An aliquot of each lysate was also loaded in another gel to analyze total Ras protein levels (*lower panel*). The experiment was repeated three times with similar results

expect that treatment with TPA plus W13 would also activate K-Ras specifically. Serum-starved (0% FCS) NIH3T3 cells were treated for 5 min with TPA (100 nM) plus W13 or the control drug W12, and activation of the different Ras isoforms was analyzed by RBD pull-down followed by a Western blot with Ras isoform-specific antibodies. As shown in Fig. 6, the TPA plus W13 treatment induced K-Ras activation specifically.

In Vitro K-Ras Phosphorylation Is Inhibited by CaM—A direct link between K-Ras and PKC was established several years ago when Ballester *et al.* (51) showed that K-Ras could be phosphorylated *in vitro* by PKC. To test whether CaM could modulate this phosphorylation, K-Ras-GST and H-Ras-GST expressed in baculovirus were phosphorylated *in vitro* by the catalytic subunit of PKC in the presence or absence of CaM. As described previously, K-Ras was phosphorylated by PKC, whereas H-Ras phosphorylation was almost undetectable (Fig. 7). Interestingly, phosphorylation was inhibited when CaM was added. Furthermore, the inhibition was reverted if phosphorylation was performed in the absence of Ca^{2+} (plus EGTA), an experimental condition that does not allow binding of CaM to K-Ras (28).

DISCUSSION

An emerging theme in signal transduction is the fact that the activation of a pathway *per se* is not enough to explain its biological effects. To understand these effects, it is crucial to achieve a comprehensive knowledge of the spatiotemporal pattern of pathway activation. A paradigm for this is the activation of the Ras/ERK pathway, which leads to cell proliferation, cell cycle arrest, or differentiation depending on the timing and intensity of its activation (40, 41). Although the molecular

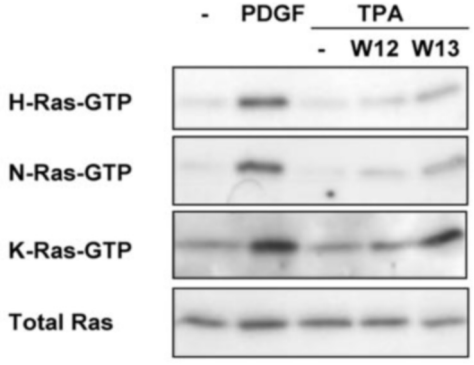

FIG. 6. **Activation of Ras by TPA and W13 is specific for K-Ras.** Quiescent NIH3T3 cells were treated for 5 min with PDGF (1 nM), TPA (100 nM), W12 (15 μg/ml), W13 (15 μg/ml), or TPA plus W12 (15 μg/ml) or W13 (15 μg/ml). Ras activation was measured by GST-RBD pull-down followed by Western blot with specific antibodies for the three Ras isoforms (*upper panel*). An aliquot of each lysate was also loaded in another gel and Western blotted with anti-pan Ras as a control of the amount of Ras per lysate (*lower panel*). The experiment was repeated three times with similar results

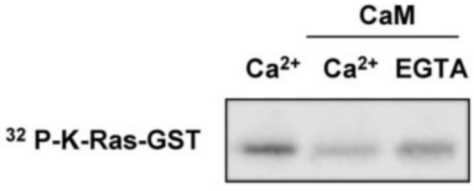

FIG. 7. **Calmodulin inhibits *in vitro* phosphorylation of K-Ras by PKC.** Purified K-Ras-GST expressed in baculovirus was phosphorylated *in vitro* with the catalytic subunit of PKC in the presence of [γ-^{32}P]ATP as indicated under "Experimental Procedures." Where indicated, K-Ras-GST was preincubated (10 min) with CaM (7 μg) in the presence of either Ca^{2+} or EGTA. Digital image obtained with a phosphorimaging device is shown.

mechanisms involved in Ras activation have been intensively studied over the past decade, it is not yet completely understood which mechanisms govern Ras inactivation and, hence, its global signaling output in response to given stimuli. We have previously shown that CaM is essential for the impairment of Ras activation at low concentrations of growth factors in fibroblasts. Furthermore, CaM is important to ensure an appropriate signaling level of the ERK cascade in these cells, as CaM inhibition prior to mitogenic stimulation leads to a more sustained ERK activation and to accumulation of the CDK inhibitor $p21^{cip1}$ (27). CaM is able to specifically impair K-Ras activation but not the activation of the other Ras isoforms, and this is most probably through its direct binding to GTP-bound K-RasB (28). To gain insight into the mechanism of how CaM is able to down-regulate the Ras/ERK pathway, we have analyzed the nature of the signals that are essential to induce the activation of this pathway in cooperation with CaM inhibition.

As we had shown previously, low doses of serum, EGF, PDGF, and bombesin were all able to induce ERK activation in the presence of CaM inhibitors (28). These results suggested that an intracellular regulator of the Ras/ERK pathway, activated by all the above stimuli, was able to activate the Ras/ERK pathway when CaM was inhibited. Protein kinase C has been shown to be a regulator of the Ras/ERK signaling pathway that is able to regulate different levels of this pathway. In many cell types, conventional PKC activation by phorbol ester treatment induces a potent activation of ERK, but there are multiple upstream inputs of PKC in this pathway regarding the cell type. For instance, examples of the activation of Ras, Raf, or MEK by PKC have all been described using different

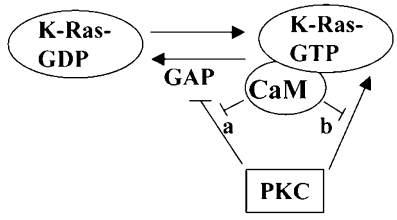

FIG. 8. **Models of CaM and PKC cooperation in Ras activity regulation.** *a*, CaM-bound K-Ras-GTP may be insensitive to or inhibit GAP inhibition by PKC. *b*, PKC may have a positive effect on K-Ras-GTP signaling, but this effect is inhibited in the presence of CaM.

cell systems (20, 21, 42, 43). Therefore, we tested whether PKC was involved in the activation of ERK that CaM inhibitors were able to induce in the presence of low concentrations of growth factors in both NIH3T3 and Swiss 3T3 cells. Incubation with the broad spectrum PKC inhibitor GF109203X blocked ERK activation induced by W13 in Swiss 3T3 cells. Similarly, in NIH3T3 cells treated with either GF109203X, Ro-0432, or, chronically, TPA, the activation of ERK by W13 was also blocked. These results suggested that PKC was necessary for the observed activation of ERK under our experimental conditions.

CaM most probably regulates the Ras/ERK pathway at the level of Ras, because we have shown that CaM binds specifically to GTP-bound K-RasB and that W13 treatment ultimately leads to K-Ras activation. However, the requirement for PKC activity to observe ERK activation in response to CaM inhibition could merely reflect the ability of PKC to activate different levels of this pathway downstream of Ras. To address this issue, we analyzed the effects of PKC inhibition on the activation of Raf-1 and Ras by W13 treatment in NIH3T3. As we had shown previously (27), treatment with W13 induces Raf-1 kinase activity in serum-starved NIH3T3 cells. Interestingly, PKC inhibition completely prevented W13-induced activation of Raf-1. These results indicated that the PKC-dependent step in W13-induced ERK activation was at least at the level of Raf-1. We finally tested whether Ras activation by W13 was also PKC-dependent. PKC inhibition in serum-starved NIH3T3 completely prevented the activation of Ras by W13 under both 0 and 0.5% FCS. This indicated that PKC was necessary for CaM inhibition to induce Ras activation, thereby placing PKC-dependence at the level of Ras.

PKC activity was therefore necessary for the observed activation of the Ras/ERK pathway in response to CaM inhibition under low concentrations of growth factors. Altogether, these results suggested that CaM was preventing Ras activation by PKC in these cells. Thus, under CaM-inhibited conditions, different sources of PKC activity such as the growth factors tested to date were able to induce Ras activation. We therefore analyzed whether PKC activation by TPA treatment alone instead of growth factor treatment was able to cooperate with CaM inhibitors to induce Ras activation in NIH3T3 cells. Interestingly, in these cells PKC activation by TPA is not able to promote Ras activation as measured by the "classic" nucleotide labeling method, although it induces potently ERK activation (20). Using the RBD pull-down method, we confirmed that TPA was unable to induce Ras activation in NIH3T3 cells. The inability of PKC activity alone to promote Ras activation ruled out the possibility that the effect of W13 was to induce PKC activation and, consequently, activation of the Ras/ERK pathway. In sharp contrast, TPA treatment in a CaM-inhibited background induced a robust activation of Ras at levels comparable with those achieved by PDGF treatment. These results suggested that CaM was essential in preventing the activation of Ras by PKC in these cells. Regarding the physiological role of

this effect of CaM on Ras activation by PKC, this would be relevant in circumstances wherein cells need to keep PKC active but Ras should be down-regulated. For instance, after proliferative activation of the cells, PKC activity is more sustained than that of Ras. We hypothesize that CaM is essential for turning off Ras in an environment wherein PKC activity is high, such as in mitogenically stimulated fibroblasts. If PKC activity was not uncoupled to Ras activation in these cells, an excessively sustained ERK activity could lead to cell cycle arrest through $p21^{cip1}$ up-regulation.

It has been reported that CaM inhibitors can induce cleavage of membrane-bound proteins (30), a process that is also influenced by PKC (37, 38, 44), leading to the possibility that TPA, together with W13, was inducing Ras/ERK activation via the shedding of membrane-bound, pro-growth factors. The inhibition of both the EGF and PDGF receptor tyrosine kinases demonstrated that Ras activation by TPA together with W13 was independent of the kinase activity of these tyrosine kinase receptors and thus independent of their extracellular stimulation or intracellular transactivation by a PKC-dependent mechanism.

As we had shown previously, CaM interacts specifically with K-Ras and inhibits its activation (28). We therefore investigated whether PKC activation, together with CaM inhibition, was able to differentially activate Ras isoforms. As expected, TPA plus W13 induced K-Ras activation nicely but did not induce H- or N-Ras activation. This indicated that the interplay between CaM and PKC in regulating Ras at the molecular level was highly specific, as it was restricted only to K-Ras (consistent with our previous observations). Two models for the possible cooperation between CaM inhibition and PKC activity in K-Ras activation that are in concordance with the results presented up to now are shown in Fig. 7.

The mechanistic links lying between PKC and Ras still remain to be characterized in most of the cells types in which Ras is activated by PKC, as this has only been elucidated in T lymphocytes. In these cells, TPA stimulation leads to a very potent activation of Ras, and this has been shown to rely on GAP inhibition (19). It should be noted, however, that lymphocytes express RasGRPs, a novel class of nucleotide exchange factors that are regulated by diacylglycerol, and will probably cooperate in the activation of Ras by phorbol esters in lymphocytes (45). Although there are a few studies of other cell types that suggest that PKC induces GAP inhibition (46–48), there is still no compelling evidence to propose a general role for GAP inhibition in Ras activation by PKC other than in T lymphocytes. Moreover, there is very little information regarding isoform specificity in Ras regulation by PKC, as this has only been investigated in COS cells. In these cells, TPA induces H-, N-, and K-Ras activation (21), highlighting the mechanistic differences that must exist between COS and NIH3T3 cells. In NIH3T3 cells, it has been proposed that TPA induces Ras/ERK pathway activation upstream of Shc phosphorylation and its association with Grb-2 (49). However, these conclusions are made on the basis of the use of dominant negative Ras and Sos proteins, but no measurements of Ras activation are shown. We have clearly demonstrated that TPA does not induce Ras activation in NIH3T3 cells, and the use of RasN17 has proven not to be a reliable tool when studying Ras involvement in a signaling pathway (21, 50).

A strikingly direct link between PKC and K-RasB but not the other Ras isoforms was shown several years ago when PKC induced the direct phosphorylation of K-RasB (51). Our results showed that this phosphorylation could be inhibited *in vitro* by CaM. Consequently, we favor the hypothesis that PKC phosphorylation of K-Ras is somehow activating its downstream signaling and that this effect would be prevented by CaM binding to K-Ras (Fig. 8*b*). However, the physiological consequences of this phosphorylation are not yet clear, and further work is needed to prove this hypothesis.

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