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Joaquim Egea, Carme Espinet and Joan X. Comella

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Calcium Influx Activates Extracellular-regulated Kinase/Mitogen-activated Protein Kinase Pathway through a Calmodulin-sensitive Mechanism in PC12 Cells*

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Joaquim Egea‡, Carme Espinet, and Joan X. Comella§

From the Grup de Neurobiologia Molecular, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, 25198 Lleida, Catalonia, Spain

Evidence suggests that membrane depolarization is able to promote neuronal survival through a sustained, although moderate, increase in the intracellular calcium. We have used the PC12 cell line to study the possible intracellular pathways that can be activated by calcium influx. Previously, we observed that membrane depolarization-induced calcium influx was able to activate the extracellular-regulated kinase (ERK)/mitogen-activated protein kinase pathway and most of this activation was calmodulin-dependent. We demonstrated that a part of the ERK activation is due to the phosphorylation of the epidermal growth factor receptor. Here, we show that both the epidermal growth factor receptor phosphorylation and the Shc-Grb2-Ras activation are not calmodulin-modulated. Moreover, dominant negative mutant Ha-*ras* (Asn-17) prevents the activation on ERKs by membrane depolarization, suggesting that Ras and calmodulin are both necessaries to activate ERKs by membrane depolarization. We failed to observe any significant induction and/or modulation of the A-Raf, B-Raf or c-Raf-1 kinase activities, thus suggesting the existence of a MEK kinase different from the classical Raf kinases that directly or indirectly can be modulated by Ca^{2+}/calmodulin.

Several studies have reported that chronic depolarization of plasma membrane prevent the cell death that occurs after deprivation of neurotrophic factors in many populations of neurons in culture. This effect is mediated by a sustained increase of the intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$)[1] that enters the cell through voltage-gated calcium channels (VGCC) (1–4). E. M. Johnson's laboratory conceptualized this phenomenon in the "Ca^{2+} set-point hypothesis," which postulates that moderate increases in the $[Ca^{2+}]_i$ (less than 100 nM above the basal values) can promote the survival of neurons in culture even in the absence of trophic support (for review, see Ref. 5). However, the molecular basis relevant for the depolarization-induced neuronal survival have been shown to be controversial and remain to be elucidated (6–8). One possible mechanism by which membrane depolarization could promote survival is by activating signaling pathways similar to those activated by neurotrophic factors. Among these, the Ras/MAP kinase and the phosphatidylinositol 3-kinase (PI 3-kinase) pathways have been shown to be the most relevant (9, 10).

Although membrane depolarization and subsequent increase in $[Ca^{2+}]_i$ is not able to promote cell survival or differentiation in PC12, in low serum medium some effects of increased $[Ca^{2+}]_i$ have been described. For example, membrane depolarization induced by adding KCl (high-K^+) to the culture medium is able to preserve priming and preexisting neurites induced by NGF treatment (11). It can also induce differentiation in cells sensitized with levels of NGF, which alone are insufficient to induce morphological differentiation (12). Finally, it has been reported that potassium combined with Bay K induces long term survival and differentiation of PC12 cells (13).

Variations in the $[Ca^{2+}]_i$ has been shown to be determinant in the regulation of the Ras/MAP kinase pathway through mechanisms not completely understood (14, 15). Rusanescu *et al.* (13) demonstrated that an increase in $[Ca^{2+}]_i$ directly or indirectly induces Shc tyrosine phosphorylation, which in turn associates with Grb2 and Sos, resulting in the activation of Ras. Moreover, membrane depolarization is able to induce tyrosine phosphorylation of the EGFR to a sufficient extent to activate the ERK/MAP kinase pathway (16–18) and this activation seems to be necessary to activate this signaling pathway (18). Furthermore, PYK2, an intracellular tyrosine kinase related to the focal adhesion kinase, is also activated by increases in the $[Ca^{2+}]_i$ that can in turn activate Ras through a Src-dependent pathway (19).

As Ca^{2+} ions enter the cytosol, they encounter a number of proteins that regulate their biochemical effects. Central among them is calmodulin (CaM), a small Ca^{2+}-binding protein, which can bind up to four Ca^{2+} ions. After binding to Ca^{2+}, CaM changes its conformation and it is able to regulate the activity of many different proteins. In several cell types, CaM has been shown to modulate Ras either directly or indirectly. Farnsworth *et al.* (20) have demonstrated that Ca^{2+} stimulation of Ras can be mediated by CaM through a Ras-GTP exchange

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The authors dedicate this paper to Julia.

‡ Predoctoral fellow of Generalitat de Catalunya.

§ To whom correspondence should be addressed: Grup de Neurobiologia Molecular, Departament de Ciències Mèdiques Bàsiques, Universitat de Lleida, Rovira Roure 44, E-25198 Lleida, Catalonia, Spain. Tel.: 34-973-70-24-14; Fax: 34-973-70-24-26; E-mail: Joan.Comella@cmb.UdL.es.

[1] The abbreviations used are: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; VGCC, voltage-gated Ca^{2+} channel; MAP, mitogen-activated protein; PI 3-kinase, phosphatidylinositol 3-kinase; high-K^+, high level of K^+; NGF, nerve growth factor; EGF, epidermal growth factor; EGFR, EGF receptor; BIM I, bisindolylmaleimide I; PMA, phorbol 12-myristate 13-acetate; ERK, extracellular-regulated kinase; MEK, MAPK/ERK kinase; CaM, calmodulin; CaM-K, CaM-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; anti-Tyr(P), anti-phosphotyrosine; GST, glutathione *S*-transferase; PI, L-α-phosphatidylinositol; Trk, tropomyosin receptor kinase; PKC, protein kinase C; PLC, phospholipase C; MOPS, 4-morpholinepropanesulfonic acid.

factor which contains an IQ motif, referred to as Ras-GRF (20). Moreover, some CaM-binding Ras-like GTPases have been described (21, 22), even though its functional activity on MAP kinase activation has not yet been tested. These include RIT, which is widely distributed in human tissues, and RIN, whose expression is unusually confined to the nervous system. However, none of these proteins has been shown to be present in the PC12 cell line. Additionally, CaM has been shown to be able to regulate several CaM-dependent protein kinases (CaM-K) being CaM K II and IV the best characterized. CaM-K IV has been linked to the activation of several MAP kinases (including JNK-1, p38, and to a lesser extent ERK2) (23). However, CaM-K IV has been shown to be absent in PC12 cells and therefore the involvement of this kinase in the activation of ERK MAP kinase is controversial (23).

Recently, it has been suggested that PI 3-kinase can be involved in the regulation of the ERK MAP kinase pathway although its implication seems to be cell type- and ligand-specific (24). For example, in Swiss 3T3 fibroblasts, PI 3-kinase seems to regulate the prolonged activation of the ERK MAP kinases (25). Additionally, it has been demonstrated that integrin-dependent activation of the ERK MAP kinases is reverted by wortmannin and LY294002, two selective PI 3-kinase inhibitors (26). Moreover, a report from the laboratory of Sacks (27) has shown that CaM is able to bind and modulate the activity of the PI 3-kinase. The relevance of these results in the PC12 cell system remains to be proved.

Our laboratory has previously shown that membrane depolarization of PC12 cells (17) and chicken motoneurons (8) is able to activate the ERK MAP kinase pathway through a Ca^{2+}/CaM-dependent mechanism. In the present work, we have investigated more precisely the level at which CaM is acting on the Ras/MAP kinase pathway. We show here that this modulation is located lower to Ras but upstream of MEK. Moreover, our results suggest that CaM action is independent of the classical forms of Raf (c-Raf-1, A-Raf, or B-Raf) indicating the existence of a MEK kinase, different from Raf and activated by a Ras-dependent mechanism after membrane depolarization, that would be regulated directly or indirectly by Ca^{2+}/CaM.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Stimulation and Cell Lysates—PC12 cells were grown on 75-cm^2 tissue culture dishes (Corning) in Dulbecco's modified Eagle's medium supplemented with 6% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 6% heat-inactivated horse serum (Life Technologies, Inc.). Medium was further supplemented with 10 mM Hepes. The M-M17–26 PC12 subline was grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 10% heat-inactivated horse serum (Life Technologies, Inc.). Culture medium from two cell lines was further supplemented with 20 units/ml penicillin and 20 μg/ml streptomycin. Cells were maintained at 37 °C in a saturating humidified atmosphere of 95% air and 5% CO_2.

For experiments, PC12 and M-M17–26 cells were allowed to proliferate in polyornithine precoated tissue culture dishes (Corning) until they reached 80% confluence. Before acute stimulation with NGF (100 ng/ml), KCl (75 mM), or PMA (1.6 μM), cells were washed three times and cultured for an additional 15–20 h in serum-free medium. Before acute stimulations, the indicated cultures were exposed to different protein inhibitors: the CaM inhibitors calmidazolium chloride (Calbiochem-Novabiochem Corp., San Diego, CA), trifluoperazine dimaleate (Calbiochem-Novabiochem Corp.), W5 and W7 (Sigma), and W12 and W13 (Sigma); the PKC inhibitor BIM I (Calbiochem-Novabiochem Corp.); the MEK inhibitor PD98059 (Calbiochem-Novabiochem Corp.); or the PI 3-kinase inhibitor LY 294002 (Calbiochem-Novabiochem Corp.). After stimulation, cells were rinsed rapidly in ice-cold phosphate-buffered saline at pH 7.2 and solubilized at 4 °C in 0.4 ml of lysis buffer (see below). After 15 min of incubation on ice, cells were scraped from the dishes and cell lysates were orbitally rotated for 30 min at 4 °C. Nuclei and cellular debris were removed by microcentrifuge centrifugation at 10,000 × *g* and 4 °C for 15 min. Protein concentration in the supernatant was quantified by a modified Lowry assay as described by the provider (Bio-Rad DC protein assay).

Western Blot—Western blot assay was performed with immunoprecipitates or cell lysates by resolving the proteins in SDS-polyacrylamide gels. The proteins were transferred onto polyvinylidene difluoride Immobilon-P transfer membrane filters (Millipore, Bedford, MA) using a Pharmacia semidry Trans-Blot (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Antibodies against the phosphorylated forms of ERK1 and ERK2, MEK1/2 and Akt (New England Biolabs, Inc., Beverly, MA), pan-ERK and c-Raf-1 (Transduction Laboratories, Lexington, KY), pan-MEK (New England Biolabs, Inc.), MEK1 (UBI, Lake Placid, NY), PLCγ (Transduction Laboratories), EGFR, A-Raf and B-Raf (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or pan-Shc (Transduction Laboratories) were used according to the supplier instructions. After incubation with specific peroxidase-conjugated secondary antibodies, membranes were developed with an enhanced chemiluminescence Western blotting detection system (Pierce)

Immunoprecipitation—Immunoprecipitation of Shc, PLCγ, or c-Raf-1 (Transduction Laboratories) and EGFR, A-Raf, or B-Raf (Santa Cruz Biotechnology Inc.) was performed with specific antibodies according to the supplier's instructions. Immunoprecipitated proteins were electrophoresed, transferred, and detected essentially as described above. To detect Grb2 association in Shc immunoprecipitates, membranes were blocked with TBS-T20 (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat milk, probed with a polyclonal anti-Grb2 antibody diluted in TBS-T20 containing 0.2% nonfat milk and, finally, incubated with a specific peroxidase-conjugated secondary antibody. To detect tyrosine-phosphorylated PLCγ and EGFR, membranes were blocked with TBS-T20 containing 5% bovine serum albumin, probed with the 4G10 anti-phosphotyrosine monoclonal antibody (anti-Tyr(P)) and incubated with a specific peroxidase-conjugated secondary antibody. To immunoprecipitate the p85 subunit of the PI 3-kinase, extracts from NGF-treated or depolarized PC12 cells were subjected to immunoprecipitation overnight at 4 °C with the anti-Tyr(P) antibody 4G10 (1/100). Immunocomplexes were precipitated with protein A-Sepharose coupled to rabbit anti-mouse polyclonal antibody. p85 was detected using an specific anti-p85 antibody (UBI) as described by the supplier.

MEK and Raf Kinase Activity Assay—MEK *in vitro* kinase assay was performed in MEK immunoprecipitates by using recombinant GST-ERK2 (UBI) and [γ-^{32}P]ATP (Amersham Pharmacia Biotech) as substrates. After treatment, cells were lysed with lysis buffer (1% Nonidet P-40, 0.25% deoxycholate, 50 mM Tris, pH 7.5, 1 mM EGTA, 50 mM β-glycerophosphate, 150 mM NaCl, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 mM benzamidine, and 20 μg/ml leupeptin). After the removal of nuclei and cellular debris, cell lysates were precleared for 1 h at 4 °C with 20 μl (v/v) of protein A-Sepharose. Five hundred μg of protein of the supernatant was transferred to a new tube, and the anti-MEK1 antibody was added (1/250) (UBI). After 2 h at 4 °C, immunocomplexes were precipitated with 40 μl (v/v) of protein A-Sepharose for an additional 1 h at 4 °C. Precipitates were washed three times with lysis buffer and three more times with assay kinase buffer (20 mM MOPS, pH 7.2, 1 mM dithiothreitol, 5 mM EGTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate). Precipitates were resuspended in 50 μl (final volume) of assay kinase buffer supplemented with 100 μM ATP, 15 mM $MgCl_2$, 6 μCi of [γ-^{32}P] ATP (3000 Ci/mmol) (Amersham Pharmacia Biotech), and 400 ng of GST-ERK2 (UBI). Kinase assay was allowed to proceed for 30 min at 30 °C. Reaction was stopped with 5× SDS-PAGE sample buffer, and products were separated by SDS-PAGE. After drying the gel, the phosphorylation signal was quantified on a PhosphorImager (Boehringer Mannheim). Radioactive spots were also detected by autoradiography by exposing the TLC plate to Fuji medical x-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan) overnight at –70 °C.

Raf kinase activity was measured by using wild-type MEK1 (Santa Cruz Biotechnology Inc.) and [γ-^{32}P] ATP (Amersham Pharmacia Biotech) as substrates. After treatments, cells were lysed with lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 2 mM EDTA, 50 mM β-glycerophosphate, 137 mM NaCl, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 mM benzamidine, and 20 μg/ml leupeptin). After the removal of nuclei and cellular debris, cell lysates were precleared for 1 h at 4 °C with 20 μl (v/v) of protein G-Sepharose. Seven hundred and fifty μg of protein of the supernatant was transferred to a new tube, and 1 μg of anti-c-Raf-1 antibody (Transduction Laboratories), anti-B-Raf antibody, or anti-A-Raf antibody (Santa Cruz Biotechnology Inc.) was added. After 2 h at 4 °C, immunocomplexes were precipitated with 40 μl (v/v) of protein

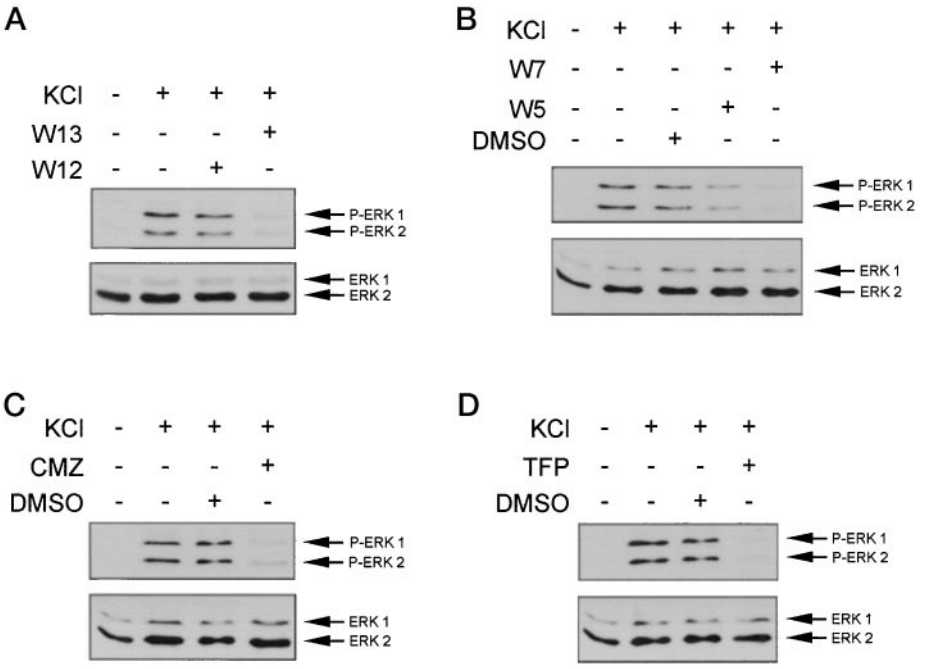

FIG. 1. **Inhibition of depolarization-induced ERK phosphorylation after pretreatment with several CaM antagonists.** PC12 cells were serum-starved, and pretreated (+) or not (−) for 1 h with different CaM antagonists: 70 μM W12 or W13 (*A*), 100 μM W5 or W7 (*B*), 25 μM calmidazolium (*CMZ*) (*C*), 50 μM trifluoperazine (*TFP*) (*D*), or with the vehicle control (0.1% Me_2SO) (*B–D*) and then stimulated (+) or not (−) for 5 min with 75 mM KCl. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody (*upper panels*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panels*) as a control of the protein content per lane. *Arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively.

G-Sepharose for an additional 1 h at 4 °C. Precipitates were washed three times with lysis buffer and three more times with assay kinase buffer (25 mM Hepes, pH 7.5, 1 mM dithiothreitol, 1 mM EGTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate). Precipitates were resuspended in 25 μl (final volume) of assay kinase buffer supplemented with 100 μM ATP, 50 mM $MgCl_2$, 6 μCi of [γ-^{32}P] ATP (3000 Ci/mmol) (Amersham Pharmacia Biotech), and 120 ng of wild type MEK1 (Santa Cruz Biotechnology Inc.). Kinase assay was allowed to proceed for 20 min at 30 °C. Reaction was stopped with 5× SDS-PAGE sample buffer, and products were separated by SDS-PAGE. After drying the gel, the phosphorylation signal was quantified and detected as described above.

PI 3-Kinase Activity Assay—After stimulation, cells were solubilized in 1% Nonidet P-40 buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 mM benzamidine, and 20 μg/ml leupeptin). Nuclei and debris were removed by centrifugation, and approximately 750 μg of protein were subjected to immunoprecipitation overnight at 4 °C with the anti-Tyr(P) antibody, 4G10. Immunocomplexes were collected with protein A-Sepharose preconjugated with a rabbit anti-mouse IgG antibody and sequentially washed with lysis buffer, LiCl buffer (100 mM Tris, pH 7.5, 0.5 M LiCl, 1 mM EDTA, and 1 mM sodium orthovanadate), and TNE buffer (25 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA). Immunocomplexes were incubated with a mixture of L-α-phosphatidylinositol and L-α-phosphatidyl-L-serine (final concentration 0.5 mg/ml each) and 10 μCi of [γ-^{32}P] ATP. Incubation was allowed to proceed for 20 min at room temperature. Phosphorylated lipids were then extracted and resolved by TLC using *n*-propanol:H_2O: acetic acid (66:33:2, v:v:v) as solvent. Radioactive spots were detected by autoradiography by exposing the TLC plate to Fuji medical x-ray film (Fuji Photo Film Co. Ltd.) overnight at –70 °C.

Ras Activity—Ras activity was measured with a non-radioactive method as described previously (28). Briefly, treated cells were solubilized for 15 min in lysis buffer containing 25 mM Tris, pH 7.5, 5 mM EGTA, 15 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 1% *N*-octyl-β-D-glucopyranoside, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 mM benzamidine, and 20 μg/ml leupeptin. Nuclei and cellular debris were removed, and 50 μg of the recombinant GST-RBD protein previously coupled to glutathione-Sepharose (Amersham Pharmacia Biotech) were added to approximately 750 μg of protein. Protein complexes were allowed to form for 2 h at 4 °C. Precipitates were washed three times with lysis buffer without *N*-octyl-β-D-glucopyranoside and once with phosphate-buffered saline. Finally precipitates were resuspended with SDS-PAGE loading buffer and denatured proteins were loaded in a 12% SDS-PAGE. Immunodetection was done using an anti-pan-Ras antibody (Oncogene Research Products, Cambridge, MA) and a anti-mouse IgG coupled to horseradish peroxidase as a secondary antibody. Blots were developed with the enhanced chemiluminescence Western blotting detection system described above.

Materials—The rest of biochemicals were obtained from Sigma. Anti-Grb2 was a gift from Dr. J. Ureña (University of Barcelona, Barcelona, Spain), anti-pan-Ras was from Dr. O. Bachs and Dr. N. Agell (University of Barcelona, Barcelona, Spain), anti-EGFR was from Dr. G. Capellà and Dr. C. García (Hospital de Sant Pau, Barcelona, Spain), and anti-Tyr(P) (4G10) were from Dr. D. Martin-Zanca (CSIC-University of Salamanca, Salamanca, Spain). The GST-RBD construct was obtained from Dr. F. McKenzie (State University of New York, Stony Brook, NY) through Dr. O. Bachs and Dr. N. Agell. The PC12 subline M-M17–26, kindly provided by Dr. G. M. Cooper (Harvard Medical School, Boston, MA) through Dr. A. Aranda (CSIC, Madrid, Spain), was obtained after transfection with the dominant negative mutant Ha-*ras* (Asn-17). 7 S NGF was prepared in our laboratory from salivary glands as described previously (29).

RESULTS

Functional Inhibitors of CaM Can Prevent the Depolarization-induced Activation of the ERK MAP Kinase—We have previously reported that ERK MAP kinase activation induced by high-K^+ in PC12 cells is specifically blocked by the CaM antagonist W13, but not by its structural analogue W12, which is about 5 times less potent (17, 30, 31) (Fig. 1*A*). In the present report, this result has been extended to other CaM inhibitors that include W7/W5 (30, 32) (Fig. 1*B*), calmidazolium (33, 34) (Fig. 1*C*), and trifluoperazine (35, 36) (Fig. 1*D*). In all cases, these inhibitors showed a similar effect to that elicited by W13, *i.e.* when PC12 cell were pretreated with the inhibitors, they prevented the activation of the ERK MAP kinase induced by high-K^+ (Fig. 1). The blockade of high-K^+-induced ERK activation was dose-dependent (data not shown) and specific, since structural homologues (W5 and W12) of active inhibitors (W7 and W13, respectively) were not able to block the ERK activation (Fig. 1, *A* and *B*). Moreover, prevention of ERK activation exerted by the CaM inhibitors was not mediated by an alteration of the calcium currents after membrane depolarization, *i.e.* these drugs did not alter the kinetics of Ca^{2+} entry into the cytoplasm (data not shown) (8, 17).

CaM Inhibitors Do Not Prevent Tyrosine Phosphorylation of the EGFR Induced by Membrane Depolarization—It has been previously reported that transactivation of receptors with tyrosine kinase activity such the EGFR in PC12 cells by membrane depolarization seems to be necessary to reach a complete activation of the ERK MAP kinase pathway (16–18). These data suggested us the possibility that CaM inhibitors may

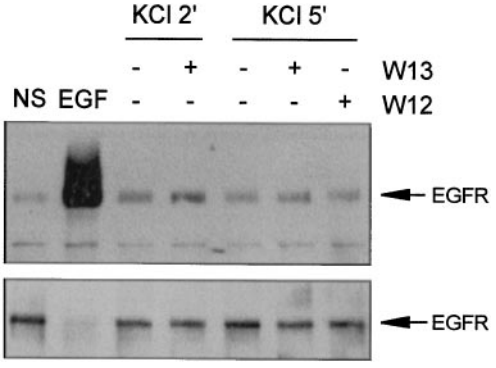

FIG. 2. **Induction of EGFR tyrosine phosphorylation following membrane depolarization and after pretreatment with W13.** PC12 cells were serum-starved, and pretreated (+) or not (−), for 1 h with 70 μM W12 or W13 and then stimulated (+) or not (−), for 2 or 5 min with 75 mM KCl, for 5 min with 100 ng/ml NGF or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with an anti-EGFR antibody and immunoprecipitates were analyzed on Western blot with the 4G10 anti-Tyr(P) antibody (*upper panel*) and stripped and reprobed with an anti-EGFR antibody (*lower panel*) as a control of the protein content per lane. *EGFR*-labeled *arrows* indicate the position of the EGFR protein.

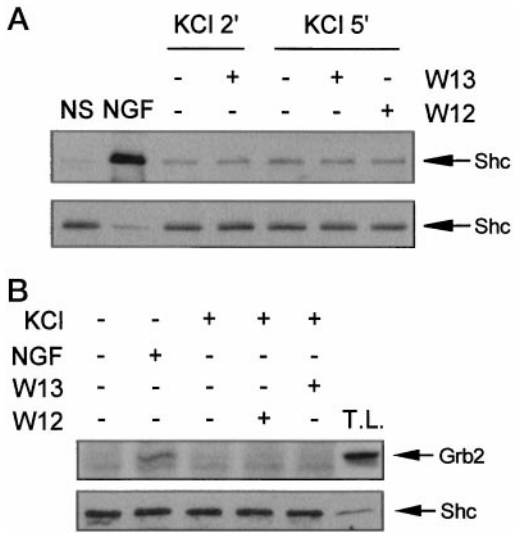

FIG. 3. **Induction of Shc tyrosine phosphorylation and Grb2-Shc association following membrane depolarization and after pretreatment with W13.** *A*, PC12 cells were serum-starved, and pretreated (+) or not (−), for 1 h with 70 μM W12 or W13 and then stimulated (+) or not (−), for 2 or 5 min with 75 mM KCl, for 5 min with 100 ng/ml NGF or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with an anti-Shc antibody and immunoprecipitates were analyzed on Western blot with the 4G10 anti-Tyr(P) antibody (*upper panel*) and stripped and reprobed with an anti-Shc antibody (*lower panel*) as a control of the protein content per lane. Shc labeled arrows indicate the position of the 66-kDa Shc protein. *B*, PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with 70 μM W12 or W13 and then stimulated (+) or not (−), for 5 min with 75 mM KCl, with 100 ng/ml NGF or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with an anti-Shc antibody and immunoprecipitates were analyzed on Western blot with an anti-Grb2 antibody (*upper panel*) and stripped and reprobed with an anti-Shc antibody (*lower panel*) as a control of the protein content per lane. *Grb2*- and *Shc*-labeled arrows indicate the position of the Grb2 and 66-kDa Shc proteins, respectively. *T.L.*, total cell lysate.

exert its effects on the high-K^+-induced activation of ERKs indirectly through the blockade of the kinase activity of the EGFR. To test this hypothesis, the EGFR was immunoprecipitated from 2- and 5-min depolarized cells pretreated or not with the CaM antagonists W12 and W13. To study the kinase activity of the receptor, immunoprecipitates were analyzed on Western blot with an anti-Tyr(P) antibody. As shown in Fig. 2, high-K^+ was able to activate the tyrosine kinase activity of the EGFR although to a much lesser extent than EGF. However, in cell lysates where the ERK phosphorylation due to membrane depolarization was completely prevented by W13 (data not shown), neither W13 nor W12 pretreatment was able to modify the level of tyrosine phosphorylation of the receptor (Fig. 2). Therefore, the observed lack of ERK phosphorylation in the W13-treated cultures after membrane depolarization could not be attributed to an inhibition of the kinase activity of the EGFR.

CaM Inhibitors Modulate neither the Tyrosine Phosphorylation of Shc nor the Association of Shc to Grb2—The ERK MAP kinase cascade is usually initiated by the interaction of trophic factors with their corresponding tyrosine kinase receptors resulting in the autophosphorylation of the receptor. Phosphorylated tyrosine kinase receptors activate Ras by a mechanism that requires the tyrosine phosphorylation of Shc (for review, see Ref. 9). The ability of Shc to activate Ras is mediated by the association of Shc to Grb2 and Sos. It has been previously reported that depolarization-induced ERK activation can result from a direct depolarization-induced phosphorylation of tyrosine kinase receptors such as the EGFR (18). Nevertheless, the activation of EGFR was not sensitive to W13 (see above). Shc has also been reported to play a central role in the Ca^{2+}-induced ERK activation after membrane depolarization (15). We have analyzed the possibility that the W13 CaM antagonist can modulate the ERK activity through the function of Shc. To test this possibility, we have studied the effects of CaM antagonists over the tyrosine phosphorylation of Shc and its association to Grb2 after membrane depolarization. For this, PC12 cells were pretreated with W13 and then the cells were depolarized for 2 and 5 min with 75 mM KCl. The structurally related W12 homologue was included in the experiments to assess the specificity of the W13 effects. Extracts were subjected to immunoprecipitation with an anti-Shc antibody that recognizes the 66-, 52-, and 46-kDa isoforms of this protein. Immunoprecipitates were resolved in SDS-PAGE, blotted, and probed with an anti-Tyr(P) antibody or, alternatively, with an anti-Grb2 antibody. As shown in Fig. 3*A*, high-K^+ induced the tyrosine phosphorylation of the 66-kDa isoform of the Shc protein, although to a much lesser extent than NGF. The same pattern of tyrosine phosphorylation was observed for the 52- and 48-kDa isoforms of Shc protein (data not shown). W13 treatment did not affect the level of tyrosine phosphorylation of the 66-kDa (Fig. 3*A*), 52- and 46-kDa isoforms (data not shown) of Shc protein at any time tested after membrane depolarization. On the other hand, at 5 min, high-K^+ allowed the co-immunoprecipitation of Shc with Grb2, although the amount of co-immunoprecipitated Grb2 was by far lower to that obtained after NGF stimulation (Fig. 3*B*). Nevertheless, as seen for the tyrosine phosphorylation of Shc, in high-K^+-stimulated W13-pretreated cultures, the amount of Grb2 co-immunoprecipitated with Shc was not apparently decreased when compared with cultures stimulated with 75 mM KCl alone (Fig. 3*B*). When protein extracts from the different experimental conditions described above were analyzed to assess the state of ERK phosphorylation, it was found that membrane-depolarized, W13-pretreated cultures, showed a significant reduction in the level of ERK phosphorylation when compared with the extracts from depolarized cultures without any drug pretreatment or pretreated with W12 (data not shown). Therefore, it seems that W13 effects on ERK activity are not attributable to an alteration in the function of the Shc proteins because this drug modulates neither the tyrosine phosphorylation of Shc proteins nor their association with Grb2 after membrane depolarization.

Depolarization-induced PLCγ Tyrosine Phosphorylation Is Not Prevented by CaM Inhibitors: Involvement of PKC—Besides the tyrosine phosphorylation of Shc it has been suggested that activated tyrosine kinase receptors were able to activate ERKs through a PLCγ-dependent mechanism (9, 37). PLCγ activity generates diacylglycerol that, together with Ca^{2+} ions, act as activators of PKC (38). PKC activity can be the mechanism by which PLCγ activates Ras-ERK MAP kinase pathway since phorbol esters, which mimic diacylglycerol, are able to activate ERKs (39–42). Since Shc-Grb2 association is not affected by W13, we have explored whether or not membrane depolarization is able to activate PLCγ. In order to address this issue, protein extracts similar to those obtained for the study of the Shc-Grb2 interaction were subjected to PLCγ immunoprecipitation with a specific antibody and immunoprecipitates were probed with an anti-Tyr(P) antibody. The results of this assay showed that PLCγ becomes tyrosine-phosphorylated after high-K^+ treatment (Fig. 4*A*), although to a lesser extent to that obtained after NGF stimulation (Fig. 4*A*). This result indicates that membrane depolarization can induce a redundant ERK activation through a PLCγ- and Shc-dependent mechanism. We then further assayed whether or not CaM inhibitor W13 was able to prevent depolarization-induced PLCγ tyrosine phosphorylation. As shown in Fig. 4*A*, W13 is not able to significantly alter the degree of tyrosine phosphorylation of PLCγ when compared with that induced by high-K^+ on cultures treated with W12 or in non-pretreated cultures. Therefore, the W13 effects on depolarization-induced ERK activity cannot be explained by a blockade of the PLCγ activation. The limited activation of PLCγ observed after membrane depolarization suggests that this enzyme is not involved in the ERK activation found in depolarized cells. However, to further analyze the relevance of this PLCγ activity on the activation of ERKs, we assessed the involvement of PKC. To approach this issue, we used the specific PKC inhibitor BIM I (43) and we studied whether or not this drug was able to prevent the activation of ERKs after membrane depolarization. As shown in Fig. 4*B*, concentrations of the inhibitor from 1 to 5 μM failed to inhibit the depolarization-induced ERK phosphorylation. As a control, we used the phorbol ester PMA (1.6 μM), which induces ERK phosphorylation through a PKC-dependent mechanism. As shown in Fig. 4*C*, PMA induced a strong ERK phosphorylation that was totally prevented by BIM I. These results, together with the poor activation of PLCγ, suggest that the hypothetical contribution of the PLCγ-PKC pathway to the activation of ERKs after membrane depolarization seems not very relevant.

Modulation of the ERK MAP Kinase Pathway by CaM Inhibitors Occurs Downstream of p21^{ras}—Rosen *et al.* (16) have demonstrated that the Ca^{2+} influx induced by membrane depolarization is able to activate ERK MAP kinases by a mechanism that involves the small G protein p21^{ras} in PC12 cells and in primary cultures of cortical neurons (14). Most of the signaling pathways that have been proposed to be involved in the activation of the ERK MAP kinases after increasing the $[Ca^{2+}]_i$ converge on p21^{ras}, thus indicating a key role of this protein in this process (15). In this context, we were interested into know whether the W13 CaM inhibitor can explain its inhibitory effect on ERK activity through the inhibition of the Ras activity. We approached this issue using a method that has been previously reported by de Rooij and Bos (28). We found that a 2- or 5-min depolarizing stimulus was able to moderately activate p21^{ras} (Fig. 5*A*). However, W13 pretreatment of cultures did not alter the level Ras activity induced by high-K^+ at any of the times tested. As an additional control, the same extracts used in the Ras activity were analyzed by Western blot with an

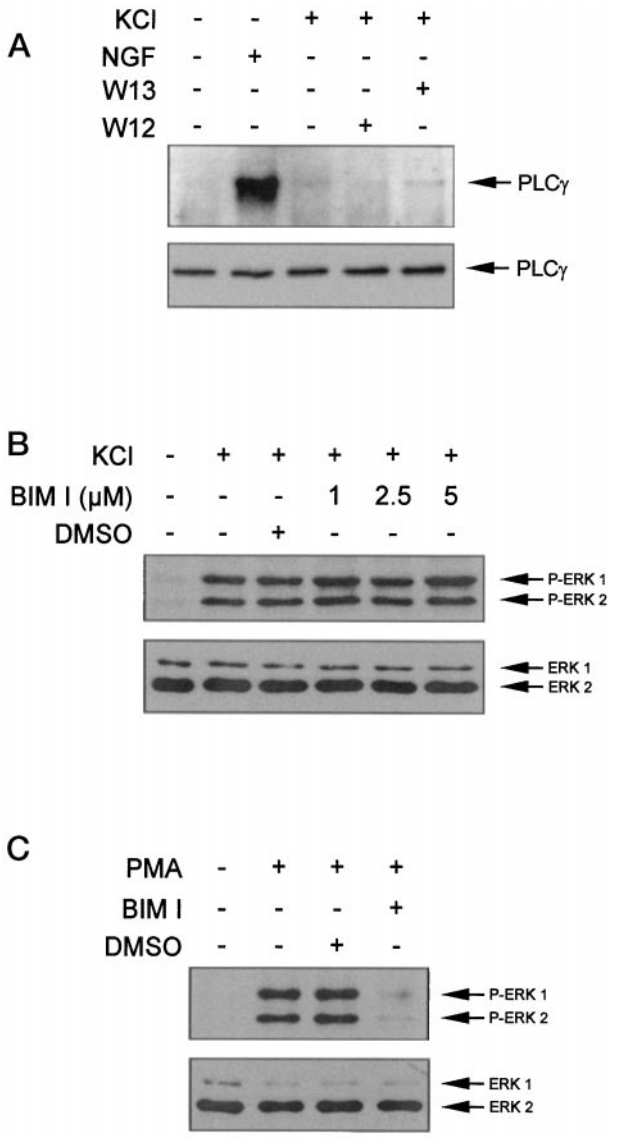

FIG. 4. **Induction of PLCγ tyrosine phosphorylation following membrane depolarization and after pretreatment with W13: involvement of PKC in the induction of ERK activity after membrane depolarization.** *A*, PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with 70 μM W12 or W13 and then stimulated (+) or not (−), for 5 min with 75 mM KCl or with 100 ng/ml NGF. After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with an anti-PLCγ antibody and immunoprecipitates were analyzed by Western blot with the 4G10 anti-Tyr(P) antibody (*upper panel*) and reprobed with an anti-PLCγ antibody (*lower panel*) as a control of the protein content per lane. *PLCγ*-labeled *arrows* indicate the position of PLCγ protein. *B*, PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with the indicated concentrations of the PKC inhibitor BIM I or with the vehicle (0.17% Me_2SO), and then stimulated (+) or not (−), for 5 min with 75 mM KCl. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *Arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively. *C*, PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with the indicated concentrations of the PKC inhibitor BIM I or with the vehicle (0.17% Me_2SO), and then stimulated (+) or not (−), for 5 min with 1.6 μM PMA. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *Arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively.

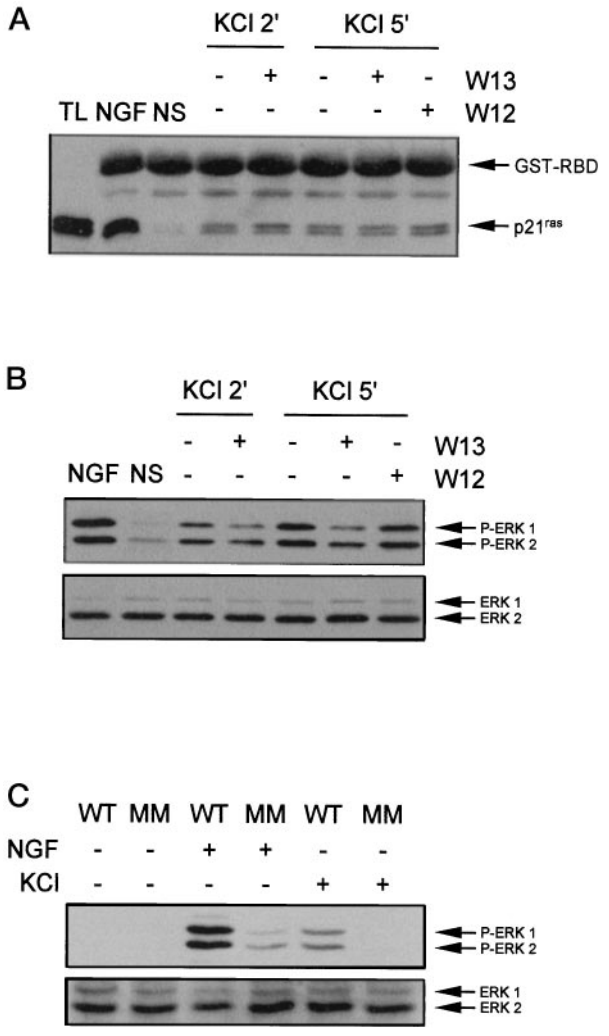

FIG. 5. **Profile of p21^{ras} activation following membrane depolarization after pretreatment with W13.** PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with 70 μM W13 or W12, and then stimulated (+) or not (−), for 2 or 5 min with 75 mM KCl or for 5 min with 100 ng/ml NGF. After treatment, cells were lysed and protein extracts were obtained. *A*, protein extracts were subjected to precipitation with 50 μg of the recombinant GST-RBD protein precoupled to protein G-Sepharose (see "Experimental Procedures"). Precipitates were analyzed by Western blot with an anti-pan-Ras antibody. *TL*, total cell lysate. *GST-RBD-* and *Ras*-labeled *arrows* indicate the position of the recombinant GST-RBD protein and the p21^{ras} protein, respectively. *B*, protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *Arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively. *C*, wild type PC12 cells (*WT*) and M-M17–26 cells (*MM*), a PC12 subline that constitutively expresses the dominant inhibitory Ha-*ras* (Asn-17) mutant, were serum-starved and then stimulated (+) or not (−), for 5 min with 75 mM KCl or with 100 ng/ml NGF. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *Arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively.

anti-phospho-ERK antibody. Results shown in Fig. 5*B* demonstrate that the level of ERK phosphorylation in high-K^+-stimulated, W13-pretreated cultures was significantly reduced than those stimulated with high-K^+ without W13 pretreatment. These results indicate that the W13 modulation of the ERK activation does not depend on an effect of CaM inhibitors on the classical form of Ras.

To determine whether endogenous Ras is necessary for high-K^+ to activate the ERK MAP kinase pathway we studied the ability of membrane depolarization to activate ERKs in a PC12 subline (M-M17–26) that constitutively expresses the dominant inhibitory Ha-*ras* (Asn-17) mutant (44). These cells respond to membrane depolarization with a normal Ca^{2+} influx as measured by using the fluorescent dye fura-2 (data not shown). When we depolarized these cells, we observed that ERK phosphorylation was strongly reduced in the M-M17–26 cells when compared with the level of ERK phosphorylation observed in the wild type PC12 cells after membrane depolarization (Fig. 5*C*). This result suggest that high-K^+ requires a functional p21^{ras} to activate the ERK MAP kinases.

The PI 3-Kinase/Akt Pathway Is Not Required for Depolarization-dependent ERK Activation—Another protein that becomes tyrosine-phosphorylated and activated after trophic factor receptor stimulation (*i.e.* Trk or EGFR) is the PI 3-kinase. Recently, it has been suggested that PI 3-kinase can contribute to the activation of ERK MAP kinases (24–26). Moreover, it has been suggested that Ras may modulate the PI 3-kinase activity (45–49). Interestingly, a recent study by Joyal *et al.* (27) has shown that CaM is able to bind and modulate the activity of the PI 3-kinase. These studies suggest that PI 3-kinase can be a key element in the mechanism by which CaM antagonists exert its inhibitory effect over the activation of the ERK after membrane depolarization. To study the contribution of the PI 3-kinase on the activation of ERK MAP kinases after high-K^+ treatment, we have used several approaches. We first studied the activation of this enzyme by assessing the state of tyrosine phosphorylation of its p85 subunit that has been shown to be a good criterion of its state of activation (6). Cells were stimulated with high-K^+ or NGF (used as a positive control) for 1 or 5 min. Phosphoproteins from these cultures were immunoprecipitated with an anti-Tyr(P) antibody and submitted to Western blot analysis to detect the p85 subunit of PI 3-kinase using a polyclonal antibody. This assay showed that membrane depolarization failed to activate the PI 3-kinase (Fig. 6*A*). However, Western blot from cells treated with NGF showed a clear band corresponding to p85 thus indicating the activation of this enzyme after NGF treatment (Fig. 6*A*). We have also measured the activity of PI 3-kinase in different experimental conditions by immunoprecipitating phosphoproteins and assaying the immunoprecipitates for their ability to generate L-α-phosphatidylinositol 3-phosphate from L-α-phosphatidylinositol (PI). The results obtained with the different experimental conditions were essentially the same as those described for the p85 precipitation, *i.e.* membrane depolarization was unable to activate the PI 3-kinase whereas NGF induces a strong activation (Fig. 6*B*). Consistent with this result, when the state of phosphorylation of Akt, a well known downstream element of the PI 3-kinase pathway (50–52), was tested with an anti-phospho-Akt-specific antibody, we found that NGF was able to phosphorylate Akt whereas membrane depolarization was not (Fig. 6*C*). Finally, LY295002, a specific inhibitor of the PI 3-kinase activity (53), was not able to prevent the high-K^+-induced ERK phosphorylation in PC12 cultures (Fig. 6*D*). Nevertheless, at the dose of 25 μM, LY295002 was able to prevent the Akt phosphorylation induced by NGF thus demonstrating that the drug was effective to inhibit PI 3-kinase activity (Fig. 6*E*).

Taken together, these results demonstrate that activation of ERK MAP kinases after membrane depolarization does not depend on the activation of PI 3-kinase/Akt pathway.

Depolarization-induced MEK Phosphorylation and MEK Activity Are Inhibited by CaM Antagonists—It has been reported that ERKs are activated by phosphorylation on threonine and

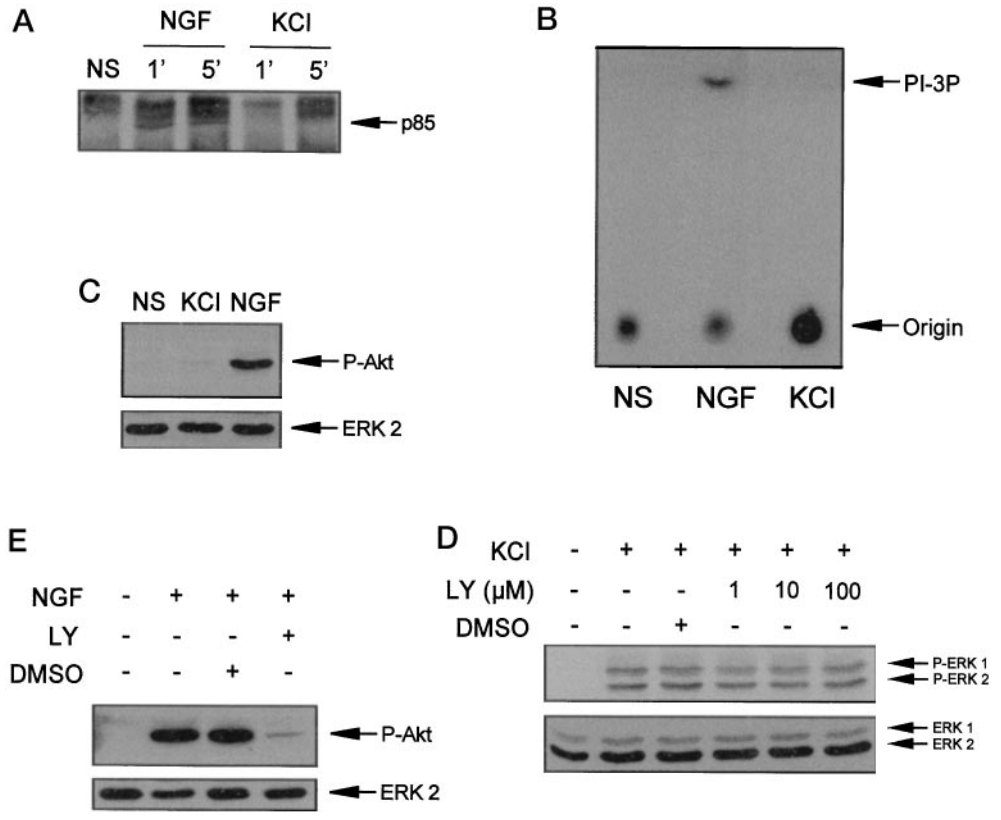

FIG. 6. **Effect of membrane depolarization on PI 3-kinase/Akt pathway.** *A*, PC12 cells were serum-starved and then stimulated for 1 or 5 min with 75 mM KCl, 100 ng/ml NGF, or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with the 4G10 anti-phosphotyrosine antibody. Immunoprecipitates were analyzed on Western blot with an anti-p85 antibody. *p85*-labeled *arrow* indicates position of the p85 protein. *B*, PC12 cells were serum-starved and then stimulated for 1 min with 75 mM KCl, 100 ng/ml NGF, or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with the 4G10 anti-phosphotyrosine antibody. PI 3-kinase activity was assayed in the immunoprecipitates as described under "Experimental Procedures" with L-α-phosphatidylinositol as substrate. *PI-3P*-labeled *arrow* indicates de position of the L-α-phosphatidylinositol 3-phosphate. *Origin*-labeled *arrow* indicates the position of the point of sample application. *C*, PC12 cells were serum-starved and then stimulated for 5 min with 75 mM KCl, 100 ng/ml NGF, or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were analyzed on Western blot using an anti-phospho-Akt antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *P-Akt-1*- and *ERK2*-labeled *arrows* indicate the position of the phosphorylated form of Akt-1 and ERK2 proteins, respectively. *D*, PC12 cells were serum-starved, pretreated or not (−), for 30 min with the indicated concentrations of LY 294002 or with the vehicle (0.2% Me_2SO) and then stimulated (+), or not (−), for 5 min with 75 mM KCl. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *Arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively. *E*, PC12 cells were serum-starved, pretreated (+) or not (−), for 30 min with 25 μM LY 294002 or with the vehicle (0.05% Me_2SO), and then stimulated (+) or not (−), for 5 min with 100 ng/ml NGF. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-Akt antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *P-Akt-1*- and *ERK2*-labeled *arrows* indicate the position of the phosphorylated form of Akt-1 and ERK2 proteins, respectively.

tyrosine residues by the dual specificity MAPK/ERK kinases MEK1 and MEK2 (54–56). In PC12 cells, the implication of MEK1 in the activation of ERKs after membrane depolarization was first reported by Rosen *et al.* (14). We have corroborated this result using the selective MEK kinase inhibitor PD98059 (57, 58), which, when used at 10–100 μM, blocked almost completely the depolarization-induced ERK phosphorylation (Fig. 7*A*). The central role of MEK in translating membrane depolarization into ERK activation, together with the observation that CaM antagonists block ERK phosphorylation (Fig. 1*A*), suggest that CaM inhibitors may exert its inhibitory effect on ERK activity by blocking MEK activity. To examine this possibility, we performed MEK kinase assays in which the ability of the W13 CaM inhibitor to block MEK activity was tested. The increase in MEK kinase activity observed in depolarized cultures (Fig. 7*B*) was almost completely abolished when cultures were pretreated with W13 (Fig. 7*B*). These results seem to be specific since W12 was found to be ineffective in preventing depolarization-induced MEK activity increase (data not shown). Thus, these results suggest that the lack of phosphorylation on ERKs after membrane depolarization in cultures pretreated with the W13 calmodulin antagonist requires a functionally active MEK.

Although MEK is a dual specific serine/tyrosine kinase, it is itself regulated by phosphorylation mainly on specific serines in response to trophic factor stimulation by the Raf family of kinases (59, 60). On the basis of our previous results, the question that arose was whether the CaM antagonists block directly MEK activity or this inhibitory effect occurs at some upstream element in the MAP kinase cascade. To approach this issue, we have used an antibody that is able to detect MEK1/2 only when phosphorylated at Ser-217/221, a situation that reflects its functional activation. As shown in Fig. 7*C*, the W13 calmodulin antagonist showed a dose-dependent inhibition on depolarization-induced MEK phosphorylation. At 70 μM W13, the maximal inhibitory effect is achieved. At this concentration, pretreatment with W12 did not have any significant effect, supporting the specificity of the inhibitory effect observed with W13 (Fig. 7*D*). Moreover, the kinetics of phosphorylation blockade induced by W13 on MEK, *i.e.* when measured after 2

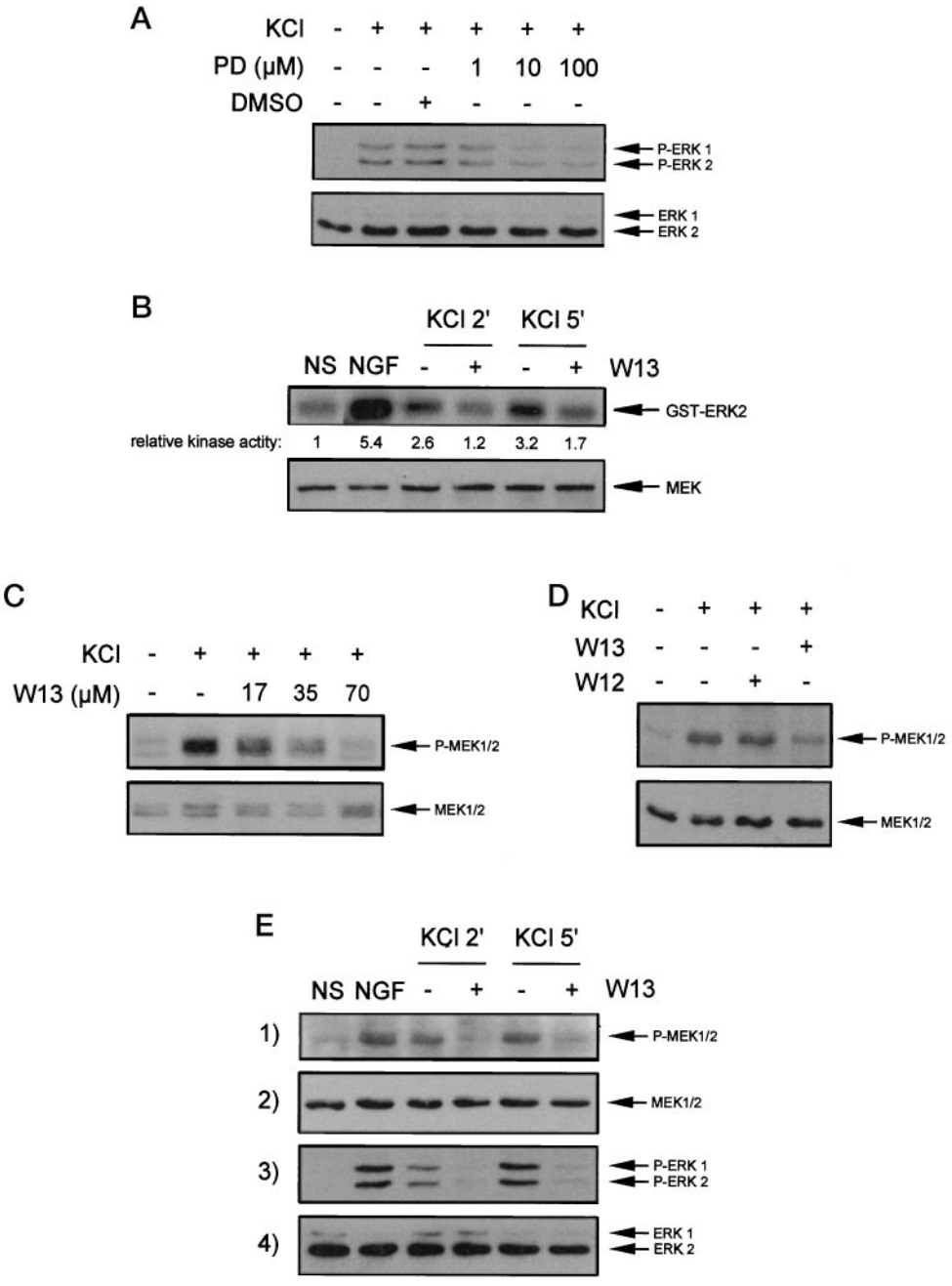

FIG. 7. **Inhibition of depolarization-induced MEK activity and MEK phosphorylation after pretreatment with W13.** *A*, PC12 cells were serum-starved, pretreated or not (−), for 30 min with the indicated concentrations of the MEK inhibitor PD 98059 or with the vehicle (0.2% Me_2SO), and then stimulated (+) or not (−), for 5 min with 75 mM KCl. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-ERK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-ERK antibody (*lower panel*) as a control of the protein content per lane. *Arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively. *B*, PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with 70 μM W13 or W12, and then stimulated for 2 or 5 min with 75 mM KCl, for 5 min with 100 ng/ml NGF, or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with an anti-MEK antibody. Immunoprecipitates were used to determine the kinase activity of MEK (see "Experimental Procedures") using recombinant GST-ERK2 as substrate (*upper panel*) and were analyzed on Western blot with the same antibody used in the immunoprecipitation step (*lower panel*) as a control of the enzyme content in the immunoprecipitates. MEK activation was quantitated with a Phosphor Imager and is expressed as the relative kinase activity where the activity of untreated cells was taken as equal to unity. *C*, PC12 cells were serum-starved, pretreated or not (−), for 1 h at the indicated concentrations of W13, and then stimulated (+) or not (−), for 5 min with 75 mM KCl. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-MEK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-MEK1/2 antibody (*lower panel*) as a control of the protein content per lane. *D*, PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with 70 μM W13 or W12, and then stimulated (+) or not (−), for 5 min with 75 mM KCl. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospho-MEK1/2 antibody (*upper panel*) and stripped and reprobed with an anti-pan-MEK1/2 antibody (*lower panel*) as a control of the protein content per lane. *E*, PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with 70 μM W13, and then stimulated for 2 or 5 min with 75 mM KCl, for 5 min with 100 ng/ml NGF or left unstimulated (*NS*). After treatment, cells were lysed and protein extracts were analyzed on Western blot sequentially with an anti-phospho-MEK1/2 antibody (*1*; *P-MEK1/2*), stripped and reprobed with an anti-pan-MEK1/2 antibody (*2*; *MEK1/2*), stripped and reprobed with an anti-phospho-ERK1/2 (*3*; *P-ERK1/2*), and stripped and reprobed with an anti-pan-ERK antibody (*4*; *ERK*). The Western blots with the anti-pan-MEK1/2 antibody (*2*; *MEK1/2*) and the anti-pan-ERK antibody (*4*; *pan-ERK*) were used as a control of the protein content per lane. In *C–E*, *P-MEK1/2*- and *MEK1/2*-labeled *arrows* indicate the position of phosphorylated and non-phosphorylated forms of MEK1/2 proteins, respectively. In *E*, *arrows* labeled *P-ERK1* and *P-ERK2* or *ERK1* and *ERK2* indicate the position of the phosphorylated and non-phosphorylated forms of ERK1 and ERK2 proteins, respectively.

or 5 min after depolarization, strongly correlated with the kinetics of phosphorylation blockade of ERK (Fig. 7*E*), thus suggesting that MEK and ERK are in the same CaM-dependent pathway.

A-Raf, B-Raf, and c-Raf-1 Activation after Membrane Depolarization—Our results show that W13 inhibits MEK activity and MEK phosphorylation (Fig. 7, *C* and *D*). We have also shown that Ras plays a critical role in the activation of ERKs after membrane depolarization although W13 does not inhibits Ras activity. These results argue for the existence of MEK kinases, activated by Ras, that would be modulated directly or indirectly by Ca^{2+}-CaM. The best characterized kinases that are able to activate MEK are the Raf family members, which include c-Raf-1, B-Raf, and A-Raf (55, 61). All of them have been reported to be expressed in PC12 cells (54, 62). Moreover, the three Raf members are activated in response to growth factors such as EGF or NGF, thus suggesting that they can contribute to the activation of ERK MAP kinases after growth factor stimulation (62). On the basis of our previous results, we wanted to know whether W13 blockade of MEK phosphorylation after membrane depolarization is due to an inhibitory effect of W13 over any of the Raf isozymes. To approach this issue, we depolarized PC12 cells for 5 min and we performed kinase assays in the immunoprecipitates obtained with specific antibodies against each of the Raf isoforms. A-Raf showed an undetectable kinase activity after NGF or high-K^+ treatments, suggesting that in our PC12 cell model, none of these stimuli are able to activate this enzyme (Fig. 8*A*). On the other hand, B-Raf showed a slight increase in its kinase activity after high-K^+ or NGF treatment (Fig. 8*B*), whereas c-Raf-1 activity was poorly stimulated after high-K^+ treatment when compared with the activity obtained after NGF treatment (Fig. 8*C*). The high-K^+-induced kinase activity in B-Raf and c-Raf-1 immunocomplexes was not blocked significantly by the pretreatment of cultures with the W13 CaM antagonist (Fig. 8, *B* and *C*). In all Raf activity studies, the state of ERK phosphorylation was verified using specific anti-phospho-ERK antibodies as described in Figs. 5*B* and 7*E*. In all cases, high-K^+ induced a significant increment of ERK phosphorylation that was specifically prevented by W13 pretreatment (data not shown). Therefore, we conclude that the regulation of ERK activity by the W13-sensitive pathway is not due to the inhibition of any of the Raf activities.

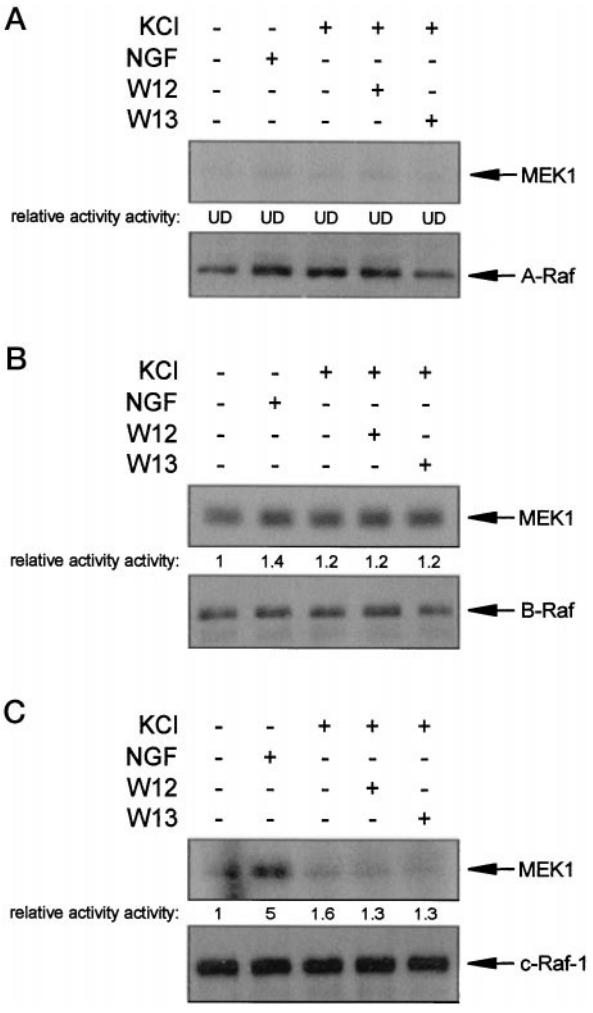

FIG. 8. **Activation of A-Raf, B-Raf, and c-Raf-1 following membrane depolarization.** PC12 cells were serum-starved, pretreated (+) or not (−), for 1 h with 70 μM W12 or W13, and then stimulated (+), or not (−), for 5 min with 75 mM KCl or with 100 ng/ml NGF. After treatment, cells were lysed and protein extracts were subjected to immunoprecipitation with an anti-A-Raf (*A*), an anti-B-Raf (*B*), or an anti-c-Raf-1 antibody (*C*). Immunoprecipitates were used to determine the kinase activity of the different Raf proteins (see "Experimental Procedures") using wild type MEK1 as substrate (*upper panels*) and were analyzed on Western blot with the same antibody used in the immunoprecipitation step (*lower panels*) as a control of the enzyme content in the immunoprecipitates. A-Raf, B-Raf, and c-Raf-1 activation was quantitated with a Phosphor Imager and is expressed as the relative kinase activity where the activity of untreated cells was taken as equal to unity. *UD*, unquantifiable activity.

DISCUSSION

We have previously reported that CaM is involved in the mediation of ERK activation after membrane depolarization in chicken spinal cord motoneurons (8) and PC12 cells (17). We observed this phenomenon by using the W13 CaM antagonist. In the present work, we have broadened this analysis in PC12 cells using other CaM inhibitors that include W7, calmidazolium, and trifluoperazine. In all cases, the CaM antagonist prevents the activation of ERK MAP kinases after membrane depolarization. The aim of this work was to find the mechanism by which W13 exerts its inhibitory effect.

Activation of the Ras-ERK MAP kinase pathway by membrane depolarization was first reported by Rosen *et al.* (14). In that report, it was demonstrated that $p21^{ras}$ has an important role in mediating ERK MAP kinase activation after membrane depolarization (14). The mechanism by which Ras is activated after membrane depolarization has not been clearly defined. To date, different Ca^{2+}-dependent pathways have been involved in this activation (15). Thus, it has been suggested that membrane depolarization needs the activation of the EGFR, in a ligand-independent manner, to activate ERKs (18). However, on the basis of the results presented here (see also Ref. 17), the inhibition of ERK activity by CaM inhibitors could not be explained by a modulation of the EGFR phosphorylation by W13, since this inhibitor does not modify the phosphorylation state of the receptor induced by membrane depolarization (Fig. 2).

Shc plays a central role in most of the pathways proposed to activate the Ras-ERK MAP kinase pathway after Ca^{2+} influx (15). As proposed by Zwick *et al.* (18), the Shc tyrosine phosphorylation observed after membrane depolarization seems to be dependent on the activation of the EGFR. Other authors have suggested that activation of Shc after membrane depolarization is also dependent on the activity of the intracellular tyrosine kinase Src (13). Alternatively, the protein-tyrosine kinase PYK2 has been proposed to play a key role in the activation of the Ras-ERK MAP kinase pathway after Ca^{2+} influx through a Shc-dependent mechanism (19). Our results demonstrate that CaM antagonists do not modulate the Shc function since they do not modify the Shc tyrosine phosphoryl-

ation or the association of Shc to Grb2 after membrane depolarization, thus suggesting that CaM modulation occurs downstream of Shc.

The activation of the PLCγ by tyrosine kinase receptors has been proposed to be a relevant mechanism by which ERK MAP kinases can be activated (37). Such mechanism seems to involve, probably, some specific isoforms of PKC (39). Moreover, in PC12 cells activated PKC is able to activate Ras and thereafter the ERK MAP kinase pathway (42). Our results demonstrate that membrane depolarization is able to activate the tyrosine phosphorylation of PLCγ, being a possible pathway that connects Ca^{2+} influx to Ras activation. The activation of PLCγ as a consequence of the EGFR activation has been previously reported (63, 64), and this could be the mechanism that will operate in our system. However, the PKC-specific antagonist BIM I, at concentrations that completely prevent the activation of ERKs after PMA treatment, was not able to prevent the activation of ERKs after membrane depolarization. This result suggests that the hypothetical PLCγ-PKC pathway does not make an important contribution to the activation of ERK MAP kinases by depolarizing stimuli.

The most important point in the present analysis is to test the relevance of the activation of Ras observed after membrane depolarization in the activation of ERK MAP kinases. For this, we used the M-M17–26 PC12 cell line, which constitutively express a dominant negative form of Ras. Moreover, we have used this cell line since these cells responds with an normal entry of Ca^{2+} after membrane depolarization, contrary to what it has been reported for other PC12 cell lines that express inducible forms of dominant negative Ras such as GsrasDN6 PC12 cells (14). In M-M17–26 cells, depolarization is unable to activate ERKs, suggesting that Ras activity is necessary for membrane depolarization-induced ERK activation, as has been reported for similar systems (13, 14). This result discards the possibility that parallel pathways to Ras can be operative in depolarized PC12 cells to activate ERKs. When we studied the level of Ras activity in depolarized wild type PC12 cells, a significant increase in Ras activity was found, as measured by the method described by de Rooij and Bos using the GST-RBD tool (28). However, pretreatment of cells with W13 does not significantly modify the Ras activity. This is a relevant result since it suggest that proteins with a GTP exchange activity that contains CaM binding domains, such as Ras-GRF, are not probably involved in the regulation of Ras activity after membrane depolarization in PC12 cells (20).

Recent studies have shown that PI 3-kinase could be a good candidate to explain the CaM-dependent effects on the ERK MAP kinase pathway activation after membrane depolarization. First, the kinase activity of this enzyme has been shown to be modulated by CaM *in vitro* (27). Second, PI 3-kinase has been reported to become activated after Ca^{2+} influx in cerebellar granule neurons (7). Third, it has been demonstrated that PI 3-kinase may be a regulable molecule downstream of Ras in some cellular systems (45–49). Forth, and more important, it has been reported that PI 3-kinase activity is able to modulate the ERK MAP kinase pathway in some cellular models (25, 26). However, our results clearly demonstrate that, after membrane depolarization, neither PI 3-kinase activity nor Akt phosphorylation, a downstream element of the PI 3-kinase, can be detected. Moreover, the selective PI 3-kinase inhibitor LY 294002 was not able no prevent the activation of the ERK MAP kinases after membrane depolarization at doses that are effective to inhibit the Akt phosphorylation after NGF stimulation. Therefore, these results suggest that the PI 3-kinase does not mediate the activation of the ERK MAP kinases after membrane depolarization in PC12 cells. These observations are in agreement with reports that depolarization-induced survival is not dependent on the PI 3-kinase activity in some neuronal populations (6, 8).[2]

Functionally active MEK has been reported to be necessary to reach a complete activation of ERK MAP kinases after membrane depolarization (14). Here we provide further evidence on this phenomenon by using the selective MEK inhibitor, PD 98059. When used at the appropriate concentrations, PD 98059 is able to prevent ERK phosphorylation after membrane depolarization. Moreover, the CaM inhibitor W13 is able to modulate both the MEK activity and its state of phosphorylation to a sufficient extent to explain the absence of ERK phosphorylation and activity in high-K^+-stimulated PC12 cells. This observation suggests that CaM regulation of ERK activity occurs upstream of MEK. PC12 cells possess the three isoforms of the MEK kinase Raf, *i.e.* A-, B-, and c-Raf-1 (54, 62). We addressed the study of the involvement of these enzymes in the MEK activation after membrane depolarization. Our results demonstrate that the activity of Raf isozymes in the activation of MEKs after membrane depolarization is not very relevant. First, we were unable to detect any significant A-Raf activation after high-K^+-stimulation in PC12 cell cultures. Second, B-Raf activation after membrane depolarized was comparable to that obtained after NGF-stimulation. However the total level of activation is very low (~20–30% of increase respect to the unstimulated cells). More important, the CaM inhibitor W13 is poorly effective in preventing the activation of B-Raf after membrane depolarization. Moreover, B-Raf activity has been involved in sustained, rather than transient activation of the ERK MAP kinases (62, 65, 66). Therefore, membrane depolarization, which promotes a rapid and transient activation of ERKs (17), will not probably use B-Raf as an effector system. Finally, c-Raf-1 has been involved in the acute stimulation of the ERK MAP kinases after NGF or EGF stimuli in PC12 cells (62). In our hands, c-Raf-1 is only moderately activated (~60%) after membrane depolarization. In the parallel assays, we have found 500–600% activation when PC12 cells were stimulated with NGF. It is then clear that the rapid activation of ERKs observed after membrane depolarization could not be attributable the moderate activation of c-Raf-1. The controls established in order to assess the amount of immunoprecipitated enzyme in each assay demonstrated that the three Raf isozymes are present in our PC12 cells. Therefore, the poor or undetectable Raf activity obtained in high-K^+ treated cultures can be attributable neither to a lack of the presence of these enzymes in our cells nor to a inefficiency of the antibody to immunoprecipitate them. Therefore, these results indicate the existence of alternative forms of MEK kinases, different from the Raf family members, that would be more relevant in the translation of the raising in $[Ca^{2+}]_i$ into the ERK MAP kinase pathway activation.

Taken together, our results suggest that Ras and CaM are both necessary to activate the ERK MAP kinases after membrane depolarization. This activation seems to be independent of the PI 3-kinase/Akt pathway and of the PKC activity. Moreover, our results point out that CaM regulates, directly or indirectly, the activity of a MAP kinase kinase kinase, activated by Ras and different from Raf isozymes, that would be the main element involved in the activation of MEK and ERK MAP kinases after membrane depolarization. Activation of MEK and ERK MAP kinases independent of Raf has been previously reported in other cell systems. For example in Swiss-3T3 and COS 7 cells, cAMP, which is able to block the activation of c-Raf-1, is not able to prevent the activation of

[2] J. X. Comella, unpublished results.

MEK and ERK activities (67). On the other hand, dominant positive mutants of the atypical PKCζ seem to activate ERKs in a MEK-dependent manner without increasing c-Raf-1 activity (68, 69). Finally, other MAP kinase kinase kinases have been identified, different from Raf isozymes that potentially could activate ERKs (see Refs. 55 and 61). The mechanisms by which these kinases are regulated are unclear, which opens the possibility that some of them could explain the phenomena reported here.

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