Moderate increases of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), induced by either the activation of tropomyosin receptor kinase (Trk) receptors for neurotrophins or by neuronal activity, regulate different intracellular pathways and neuronal survival. In the present report we demonstrate that glial cell line-derived neurotrophic factor (GDNF) treatment also induces [Ca\(^{2+}\)]\(_i\), elevation by mobilizing this cation from internal stores. The effects of [Ca\(^{2+}\)]\(_i\) increase after membrane depolarization are mainly mediated by calmodulin (CaM). However, the way in which CaM exerts its effects after tyrosine kinase receptor activation remains poorly characterized. It has been reported that phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream target protein kinase B (PKB) play a central role in cell survival induced by neurotrophic factors; in fact, GDNF promotes neuronal survival through the activation of the PI 3-kinase/PKB pathway. We show that CaM antagonists inhibit PI 3-kinase and PKB activation as well as motoneuron survival induced by GDNF. We also demonstrate that endogenous Ca\(^{2+}\)/CaM associates with the 85-kDa regulatory subunit of PI 3-kinase (p85). We conclude that changes of [Ca\(^{2+}\)]\(_i\) induced by GDNF, promote neuronal survival through a mechanism that involves a direct regulation of PI 3-kinase/PKB pathway activity by CaM. GDNF promotes neuronal survival mediated by the Ca\(^{2+}\)/CaM complex. This study suggests that CaM promotes neuronal survival through a mechanism that involves a direct regulation of PI 3-kinase/PKB pathway activity by CaM.
and MTN survival, we studied the ability of GDNF to induce changes in the [Ca^{2+}]. The effect of Ca^{2+} chelators and CaM inhibitors on PI 3-kinase/PKB activation and GDNF-induced MTN survival was also studied. Here we report that GDNF stimulation induces an increase in [Ca^{2+}], from the intracellular Ca^{2+} stores. This increase regulates the activation of both PI 3-kinase and PKB, and therefore regulates MTN survival through a mechanism that involves CaM. Finally, we present evidence that CaM associates with the endogenous PI 3-kinase in a Ca^{2+}-dependent manner.

MATERIALS AND METHODS

MTN Isolation, Survival Evaluation, and Cell Death Characterization—Sperm cord MTNs were purified from embryonic day 5.5 (E5.5) chick embryos according to Comella et al. (21), with minor modifications. Survival evaluation was performed as described by Soler et al. (22). Presented values are the means ± S.E. of eight wells from a representative experiment that was repeated at least three times. Cell death characterization was evaluated by estimating the percentage of apoptotic cells with respect to the total number of cells counted in each condition using the Hoechst 33258 dye (Sigma, Madrid, Spain) as described in Soler et al. (10). To assess the caspase-3 activation in dying MTNs, immunocytochemistry using an antibody against cleaved caspase-3 (Cell Signaling, Servicios Hospitalarios, Barcelona, Spain) was performed. After 18 h of treatment with the indicated culture medium, cells were fixed in 4% paraformaldehyde for 20 min, rinsed in PBS, and blocked for 1 h at room temperature in 5% fetal calf serum, 0.1% Triton X-100 in PBS. The primary antibody was used at a concentration of 1:50 in blocking solution for 24 h at 4 °C. RRX-conjugated donkey anti-rabbit antibody was from Jackson ImmunoResearch (Baltimore, MD) and either pCMV5-HA-PKB(A) or the empty vector, using a one to three molar ratio. For survival experiments, only EGFP-positive cells were counted as described previously by Biswas and Greene (23), with modifications. MTNs were co-transfected after plating and washed 48 h later, and the different experimental conditions were established. Cell survival was expressed as the percentage of fluorescent cells remaining in the culture well after 24 h of treatment, with respect to the fluorescent cells present at the beginning of treatment.

Neurotrophic factors were obtained from Alomone (Jerusalem, Israel); LY294002, N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7), and N-(6-aminohexyl)-1-naphthalenesulphonamide (W5) from Calbiochem (San Diego, CA); EGTA, N-(4-aminobuty)-5-chloro-2-naphthalenesulphonamide (W13), N-(4-aminobuty)-2-naphthalenesulphonamide (W12), trifluoperazine dimaleate (TFP), ionomycin, dantrolene, and thapsigargin from Sigma; and 1,2-bis-(2-aminophenoxy)ethene N,N,N,N'-tetraacetic acid acetylomethyl ester (BAPTA-AM) from Molecular Probes (La Jolla, CA). Intracellular Ca^{2+} Determination—Isolated MTNs, plated on laminin-coated coverslips, were washed with Krebs solution with the following millimolar composition: NaCl, 145; KCl, 5; CaCl_2, 2; MgCl_2, 1; Hepes, 10; glucose 11; pH 7.4 and incubated in this medium with Fluo-4 acetoxyethyl ester (5 μM, Molecular Probes) for 1 h at 37 °C. Coverslips were mounted on the stage of a Nikon TE-200 inverted microscope equipped with a Nikon 40×, 1.5 numerical aperture, epifluorescent objective. Samples were stimulated with a laser line at 488 nm (Melles Griot, Carlsbad, CA), and the resultant fluorescence was recorded at 535 nm. Calcium concentration was calculated as described in Kao et al. (24) using K_C for Fluo-4 of 400 nm. F_0, max values were obtained in the presence of 50 μM iomycin, and F_0, max was determined after exposure to 10 μM ionomycin. All experiments were performed at room temperature. Dantrolene (40 μM), thapsigargin (1 μM), and Ca^{2+} (250 μM) were added 10 min before and maintained throughout the experiment. For confocal studies, images were taken at each 1-μm planar interval from the top of the cell using an Ultraview confocal module (PerkinElmer Life Sciences, Barcelona). Images were recorded every 4 s using a charged coumaride camera and analyzed using commercial software (Life Sciences Ltd., England).

Immunoprecipitation, Western Blot Analysis, and Kinase Assays—For detection of the phosphorylated forms of PKB in total cell lysates, 30 μg of total protein was resolved in SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride Immobilon-P transfer membrane filters (Millipore, Huesca-Erless, Barcelona) using an Amersham Biosciences semidyem Trans-Blot (Barcelona) according to the manufacturer’s instructions. The membranes were blotted with anti-phospho-PKB-specific antibodies: anti-phospho-Ser-473 or anti-phospho-Thr-308 (New England Biolabs, Servicios Hospitalarios, Barcelona) following the instructions of the provider. To control the content of the specific protein per lane, membranes were reprobed with a monoclonal anti-tubulin antibody (Sigma) or anti-PKB antibody (C-20) (Santa Cruz Biotechnology, Quimigra, Barcelona) as described by the providers. Blots were developed using the Super Signal chemiluminescent substrate (Pierce, Cullink, Barcelona).

PI 3-kinase activity was measured as previously described by Dolcet et al. (1). Briefly, 150 μg of total protein was subjected to immunoprecipitation overnight at 4 °C with an anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology, Reactiva, Barcelona). Immunocomplexes were collected with protein A-Sepharose preconjugated with a rabbit anti-mouse IgG antibody (Sigma), washed, and incubated with a mixture of [γ-32P]ATP (Amersham Biosciences) and histone H2B (Sigma) as substrates. The reaction was performed for 30 min at room temperature and stopped by adding sample buffer and boiling for 5 min. Immunocomplexes were resolved by SDS-PAGE, and the radioactive spots were detected by autoradiography, and quantified in the PhosphorImager (Roche Diagnostics, Barcelona).

PKB activity was performed as described by Soler et al. (10). Briefly, 150 μg of total protein was immunoprecipitated with 1 μg of anti-PKB antibody (C-20) (Santa Cruz Biotechnology) for 1 h at 4 °C. Immunocomplexes were collected with protein G (Sigma), washed, and incubated with 3 μCi of [γ-32P]ATP (Amersham Biosciences) and histone H2B (Sigma) as substrates. The reaction was performed for 30 min at room temperature and stopped by adding sample buffer and boiling for 5 min. Immunocomplexes were resolved by SDS-PAGE, and the radioactive spots were detected by autoradiography and quantified in the PhosphorImager.

To detect immunoprecipitation assays MTNs were lysed in a Nonidet P-40 buffer (20 mM Tris, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.1% SDS) and 10 μg/ml of anti-PKB antibody (BD Transduction Laboratories) in the presence of 0.1 mM CaCl_2 or 2 mM EGTA. Samples were incubated then with protein G for 2 h at 4 °C. Immunocomplexes were washed three times with ice-cold lysis buffer containing CaCl_2 or EGTA, suspended with sample buffer, boiled, resolved in SDS-polyacrylamide gel, and transferred onto polyvinylidene difluoride Immobilon-P transfer membrane filters. Blots were probed with an anti-p85 monoclonal antibody and P85 (Amersham Biosciences) and histone H2B (Sigma) as substrates. The reaction was performed for 30 min at room temperature and stopped by adding sample buffer and boiling for 5 min. Immunocomplexes were resolved by SDS-PAGE, and the radioactive spots were detected by autoradiography and quantified in the PhosphorImager.

C. RRX-conjugated complexes were collected with protein G (Sigma), washed, and incubated with 3 μCi of [γ-32P]ATP (Amersham Biosciences) and histone H2B (Sigma) as substrates. The reaction was performed for 30 min at room temperature and stopped by adding sample buffer and boiling for 5 min. Immunocomplexes were resolved by SDS-PAGE, and the radioactive spots were detected by autoradiography and quantified in the PhosphorImager.

CaM-Sepharose Pull-down Experiments—CaM-Sepharose pull-down was performed as described in Ref. 26 with minor modifications. Cells were lysed in a Nonidet P-40 buffer, and 800 μg of protein extract was supplemented with a final concentration of either 0.1 mM CaCl_2 or 2 mM EGTA. Then 20 μl of CaM-Sepharose 4B beads (Amersham Biosciences) or Sepharose alone, pre-blocked with 1% bovine serum albumin/PBS, was added to the corresponding lysate and incubated overnight at 4 °C. Beads were washed three times with lysis buffer containing CaCl_2 or EGTA. Precipitation with uncoated Sepharose beads in the presence of CaCl_2 was performed as a negative control. Complexes were analyzed by Western blot as described above with the anti-p85 antibody.

RESULTS

GDNF Increases Intracellular Ca^{2+} Concentration—It has previously been reported that the stimulation of receptors with intrinsic tyrosine kinase activity, such as TrkA, induces intracellular Ca^{2+} mobilization (5, 6). To analyze whether GDNF treatment and the consequent Ret activation are able to induce changes in [Ca^{2+}], MTNs were loaded with Fluo-4, and the resultant changes in intracellular Ca^{2+} or fluorescence levels were measured in individual cell bodies. When cells were treated with 100 ng/ml GDNF intracellular Ca^{2+} increased (620 ± 101 nm) with respect to the basal level (142 ± 14 nm; mean ± S.E.) (Fig. 1A). This indicated that [Ca^{2+}],
Cytosolic Ca\(^{2+}\) Increase and PI 3-Kinase Activation by GDNF

A

![Graph showing intracellular Ca\(^{2+}\) levels with and without Dantrolene following GDNF treatment.]

B

![Bar graph showing A.F.U. Net Increase with different treatments: GDNF, Cd\(^{2+}\), Dan, TG with and without GDNF.]

C

![Images showing cell morphology under different conditions with GDNF.]
after GDNF treatment. This Ca\textsuperscript{2+} response to GDNF is a rapid phenomenon, reaching maximum levels within the first minute of GDNF application. Thereafter \([\text{Ca}^{2+}]_i\), remains elevated (340 mM) above the basal levels (140 mM) for the next minutes of measurement (Fig. 1A).

To investigate whether the GDNF-evoked \([\text{Ca}^{2+}]_i\), increase was of intracellular or extracellular origin, cells were stimulated with 100 ng/ml GDNF in the presence, or absence, of the non-selective voltage-dependent Ca\textsuperscript{2+} channel blocker Cd\textsuperscript{2+}. Increases in Ca\textsuperscript{2+} were expressed as changes in fluorescence intensity above basal levels (\(F_0 - F\)). When the cells were pre-treated with 250 \(\mu\)M Cd\textsuperscript{2+} for 15 min and then exposed to GDNF in the presence of Cd\textsuperscript{2+}, the peak of intracellular fluorescence was minimally affected (from an average of \(F_0 - F = 67.3 \pm 6.8\) AFU in the absence of Cd\textsuperscript{2+} to \(F_0 - F = 76.3 \pm 11.2\) AFU in the presence of Cd\textsuperscript{2+}, Fig. 1B). These data indicate that the GDNF-evoked \([\text{Ca}^{2+}]_i\), rise primarily involved the mobilization of Ca\textsuperscript{2+} from intracellular stores, rather than Ca\textsuperscript{2+} influx through voltage-dependent Ca\textsuperscript{2+} channels. To further analyze this hypothesis, we used two different pharmacological approaches: depletion of Ca\textsuperscript{2+} from internal stores using the microsomal Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin (TG) (27) or blockade of Ca\textsuperscript{2+} release from the stores with dantrolene, a muscle relaxant used as an inhibitor of intracellular Ca\textsuperscript{2+} mobilization (28). Cells were pre-treated for 30 min with 1 \(\mu\)M TG, then stimulated with GDNF (100 ng/ml) with an identical dose of TG, changes in intracellular fluorescence were then measured. In the presence of TG only a slight increase in intracellular fluorescence (\(F_0 - F = 19.7 \pm 5.1\) AFU) was observed when compared with only GDNF-stimulated MTNs (\(F_0 - F = 67.3 \pm 6.8\) AFU) (Fig. 1B) indicating that post TG treatment, the \([\text{Ca}^{2+}]_i\), increase was 3.4-fold smaller than GDNF-treated cells. On the other hand, when MTNs were pre-treated for 30 min with 40 \(\mu\)M dantrolene and then stimulated with GDNF (100 ng/ml) plus dantrolene, no increase in \([\text{Ca}^{2+}]_i\), was observed (150 mM and \(F_0 - F = 4.9 \pm 1.2\) AFU, in Fig. 1A and 1B, respectively). Together, these results provide strong evidence that GDNF increases \([\text{Ca}^{2+}]_i\), by mobilizing the cation from the intracellular stores, rather than promoting Ca\textsuperscript{2+} influx from the extracellular compartment. Moreover, confocal images of intracellular fluorescence distribution after GDNF stimulation clearly indicated a moderate increase in the fluorescence intensity distributed in compartments along the MTN soma and neurites (Fig. 1C). This is in clear contrast with the diffuse fluorescence pattern observed after MTN depolarization using high K\textsuperscript{+} medium (data not shown). Thus, these results suggest that GDNF stimulation of cultured MTNs induces an increase of \([\text{Ca}^{2+}]_i\), and that the intracellular Ca\textsuperscript{2+} stores are the main source for the peak response to this neurotrophic factor.

**Ca\textsuperscript{2+} and CaM Are Involved in GDNF-induced PKB Activation**—GDNF and its GFLs relatives induce in vitro neuronal survival through a mechanism involving PI 3-kinase and PKB activation (10, 29). PKB is a well known downstream PI 3-kinase effector, and its activation is mainly induced by the phosphorylation of residues Ser-473 or Thr-308 (17). Because of the role of PKB in neuronal survival, and the involvement of Ca\textsuperscript{2+} and CaM in the activation of PKB induced by the neurotrophins (3), we wanted to analyze the involvement of Ca\textsuperscript{2+} and CaM in GDNF-induced PKB activation and neuronal survival. To this end, cells were treated with different calcium chelators (EGTA and BAPTA-AM) and CaM antagonists (W13, W7, and trifluoperazine dimaleate, TFP), and after GDNF stimulation the PKB phosphorylation was analyzed. MTNs were cultured for 48 h in the presence of muscle extract (MEX), and these cells were then serum- and MEX-deprived for 12 h and preincubated for 0, 15, or 30 min with 2.5 mM EGTA or 30 min with 25 \(\mu\)M BAPTA-AM to chelate either extracellular or intracellular Ca\textsuperscript{2+}, respectively. Cells were then washed and stimulated for 7 min with 100 ng/ml GDNF in the presence of identical doses of EGTA or BAPTA-AM. MTNs were lysed, and cell lysates were analyzed by immunoblotting using anti-phospho-Ser-473 (\(\alpha\)-P-Ser-473) or anti-phospho-Thr-308 (\(\alpha\)-P-Thr-308) antibodies. An increase in Ser-473 and Thr-308 phosphorylation in GDNF-stimulated cells were observed when directly compared with non-stimulated cells. However, when cultures were pre-treated and stimulated in the presence of Ca\textsuperscript{2+} chelators, the level of PKB phosphorylation was similar to the non-stimulated cultures (Fig. 2A). Thus intracellular and extracellular Ca\textsuperscript{2+} were both required for PKB phosphorylation after GDNF treatment. To exclude the possibility that extracellular Ca\textsuperscript{2+} was entering the cell through voltage-gated Ca\textsuperscript{2+} channels, nifedipine or amiloride (L-type and T-type voltage-gated Ca\textsuperscript{2+} channel antagonists, respectively) were used and the PKB phosphorylation subsequently analyzed. The presence of these antagonists did not affect Ser-473 (Fig. 2B) or Thr-308 phosphorylation (data not shown) after GDNF stimulation. However, similar doses of nifedipine, but not amiloride, did block the ERK MAP kinase phosphorylation after membrane depolarization using a high K\textsuperscript{+} medium as expected due to previous results published by our laboratory (22) (Fig. 2B). The Ca\textsuperscript{2+} measurements described above also reinforce the hypothesis that Ret activation does not require Ca\textsuperscript{2+} influx from the extracellular space through Ca\textsuperscript{2+} channels. Thus the requirement of the extracellular Ca\textsuperscript{2+} for PKB phosphorylation is probably due to the presence of a cadherin-like motif in the Ret extracellular domain as reported previously (13, 14) (see “Discussion”).

To determine whether CaM was mediating the Ca\textsuperscript{2+} effect on PKB phosphorylation, cells were treated with different CaM antagonists. MTNs were cultured under similar conditions as described above, except that they were pre-treated with 7 \(\mu\)M W13, or an identical dose of W12, its less active structural analogue (W13 IC\textsubscript{50} = 68 \(\mu\)M versus W12 IC\textsubscript{50} = 260 \(\mu\)M) as a control of the nonspecific effects of W13 (30). After this, cells were stimulated with 100 ng/ml GDNF for 7 min in the presence of the same doses of W13 or W12 and PKB phosphorylation was then analyzed. GDNF induced an increase in Ser-473 and Thr-308 phosphorylation with respect to the non-stimulated cultures; however, W13, but not W12, prevented PKB phosphorylation (Fig. 3). This suggests that CaM is required for this effect. We also analyzed other CaM antagonists such as W7 and its less active structural analogue W5 (W7 IC\textsubscript{50} = 28 \(\mu\)M versus W5 IC\textsubscript{50} = 240 \(\mu\)M) (30) and a CaM antagonist structurally unrelated to W13 and W7, called TFP. Fig. 3 shows that the presence of 10 \(\mu\)M W7, or 5 \(\mu\)M TFP, inhibited Ser-473 and Thr-308 phosphorylation induced by GDNF. However, 10 \(\mu\)M W5 did not significantly inhibit this phosphorylation, which indicates the specific effect of W7. Thus these results, obtained using different CaM antagonists that exert their effects...
through different molecular mechanisms (30), strongly suggests that CaM is required for PKB phosphorylation after GDNF stimulation of cultured spinal cord MTNs.

To further analyze the effect of Ca\(^{2+}\)/H11001 and CaM on GDNF-induced PKB activation, kinase activity assays were performed in PKB immunoprecipitates from MTNs treated with Ca\(^{2+}\)/H11001 chelators or CaM antagonists. In accordance with the results described above, GDNF-induced PKB kinase activity was prevented by the Ca\(^{2+}\)/H11001 chelators BAPTA-AM or EGTA, as well as by CaM antagonist W13 or the PI 3-kinase inhibitor LY 294002 (50 \(/\mu\)M) (Fig. 3B). These results indicate that GDNF promotes PKB phosphorylation and activation in cultured MTNs through a mechanism involving both Ca\(^{2+}\) and CaM.

CaM Antagonist W13 Prevented GDNF-induced MTN Survival—GDNF induces in vitro MTN survival through the activation of the PI 3-kinase/PKB pathway. It is known that the PI 3-kinase inhibitor LY 294002 blocks GDNF-induced PKB activation and MTN survival (10). To further analyze whether PKB inhibition, induced by CaM antagonists, had any effect on MTN survival, MTNs were cultured in the presence of MEX for 48 h, then washed and the different conditions established. Cell survival was then evaluated 24 h later as the percentage of surviving cells with respect to the total cell number present in the same microscopic field at the beginning of the experiment. Isolated MTNs survive in culture in the presence of saturating concentrations of MEX (100%) (21), however, in the absence of this extract (NE) 40–50% of cells were found to die after 24 h. In the presence of a medium containing 10 ng/ml GDNF, ~80% of the MTNs remained alive during a similar period of time, however, when 7 \(/\mu\)M W13 was added to the medium the level of MTN survival was similar to that of NE-treated cells (50%) (Fig. 4A). The same dose of W12 had no effect on GDNF-induced MTN survival.

To determine whether CaM antagonists suppress GDNF-induced survival through an apoptotic cell death process, as observed after trophic factor withdrawal, the percentage of apoptotic cells was quantified. Experiments were performed with the DNA binding dye Hoechst 33258, and cells displaying apoptotic morphology (highly condensed DNA, fragmented into two or more chromatin aggregates) were counted. In cultures grown in the presence of GDNF, the percentage of cells displaying this feature was 4.4 ± 0.1%. However, after GDNF deprivation, or after 7 \(/\mu\)M W13 or 50 \(/\mu\)M LY 294002 treatment, the percentage of apoptotic cells was significantly increased (12.3 ± 0.2%, 12.6 ± 0.1%, and 14.7 ± 0.2%, respectively) (Fig. 4B). Similar results were obtained using an antibody directed against fragmented
caspase-3 (which is an indication of an apoptotic process) (Fig. 4C). The percentage of fragmented caspase-3 positive cells increased after W13 or LY 294002 treatments when compared with GDNF-supplemented cultures (data not shown). Both parameters indicate that CaM inhibition of GDNF-treated cultures induces an apoptotic MTN cell death process at the same dose that blocks PKB phosphorylation.

**Constitutively Active Forms of PKB Prevent the Cell Death Induced by CaM Antagonists**—In the present work we suggest a correlation between PKB inhibition and prevention of MTN neuronal survival induced by CaM antagonists. To test whether the inhibition of neuronal survival induced by these antagonists was due to the blockade of PKB activation, we transfected MTNs with the constitutively active form PKBT308D/S473D, a PKB that carries a mutational acidic charge, which increases its activity 20-fold in non-stimulated cells, in addition to its main regulatory phosphorylation sites (17). MTNs were then co-transfected with pEGFP and PKBT308D/S473D or the empty vector 30 min after plating. Cells were washed 48 h after transfection, the culture medium was replaced, and the different experimental conditions were established. Cell survival was evaluated 24 h after treatment as the percentage of fluorescent cells remaining in the culture well with respect to the fluorescent cells present at treatment initiation. Results showed that PKBT308D/S473D protected MTNs from the cell death induced after neurotrophic factor withdrawal (Fig. 5). Twenty-four hours after neurotrophic factor deprivation, cultures transfected with the empty vector showed ~50% of surviving cells, however, when cells were transfected with PKBT308D/S473D, the percentage of surviving cells after a similar period of deprivation was ~80%. On the other hand, 7 μM W13 caused 50% of cell death in GDNF-treated MTNs transfected with the empty vector, whereas the same treatment on PKBT308D/S473D transfected cultures was unable to reduce cell survival (~78%). These results indicate that PKB T308D/S473D protects MTNs from the cell death induced by W13, which further suggests that CaM antagonists block GDNF-promoted MTN survival through the inhibition of PKB activation.

**Ca2+ and CaM Are Involved in GDNF-induced PI 3-Kinase Activation**—The results presented above indicate that Ca2+ and CaM could regulate an upstream event involved in the phosphorylation and activation of PKB. Because PI 3-kinase is one of the main upstream activators of PKB (31), we analyzed whether PI 3-kinase activation by GDNF also required Ca2+ and CaM. MTNs were pretreated with Ca2+ chelators (2.5 mM EGTA or 25 μM BAPTA-AM) or CaM antagonists (7 μM W13 or 7 μM W12) and then stimulated for 7 min with GDNF (100 ng/ml). PKB was immunoprecipitated and kinase activity assayed with H2B as substrate. Efficiency of PKB immunoprecipitation in the different conditions was checked by Western blot using a specific PKB antibody (α-PKB).
PKB phosphorylation (Fig. 6). These results suggest that GDNF induces PI 3-kinase activation and neuronal survival through a mechanism involving Ca\textsuperscript{2+}/H11001 and CaM.

**CaM Associates with the 85-kDa Regulatory Subunit of PI 3-Kinase**—To support the hypothesis of a physiological regulation of PI 3-kinase activation by CaM we evaluated the possible endogenous interaction between these two proteins in MTN cell lysates. MTNs were cultured 48 h in the presence of MEX, then washed and deprived of both serum and MEX for 12 h. The cultures were then either stimulated with 100 ng/ml GDNF or left untreated. Cell lysates were incubated with CaM-Sepharose to co-precipitate PI 3-kinase. To detect this protein in the CaM-Sepharose pull-down we analyzed them by Western blot using a specific monoclonal antibody against the 85-kDa regulatory subunit of the PI 3-kinase (p85) (32, 33). We found that p85 was able to co-precipitate with CaM-Sepharose but not with Sepharose beads without CaM. Moreover, this interaction was only present when the cell lysates were supplemented with Ca\textsuperscript{2+}, and binding was abolished when the Ca\textsuperscript{2+} of the cell lysates was chelated with EGTA, thus indicating that CaM and p85 interaction is Ca\textsuperscript{2+}-dependent (Fig. 7A). Interestingly, GDNF stimulation did not modify the interaction between CaM and p85.

We also analyzed the endogenous association of CaM and
p85 using a co-immunoprecipitation strategy. MTNs were cultured under similar conditions as described above, and cell lysates were immunoprecipitated with the anti-p85 antibody. The immunoprecipitates were analyzed by Western blot, and the immunoblots were probed with an anti-CaM antibody. CaM was found to co-immunoprecipitate with p85 in both non-stimulated and GDNF-stimulated MTNs, thus indicating the endogenous association of both proteins independently of PI 3-kinase activation (Fig. 7B). As previously observed in the pull-down experiments, CaM and p85 interaction was found to be Ca2+-dependent. When 7 μM W13 was added during the immunoprecipitation process the association was strongly reduced, indicating that W13 displaced CaM binding to PI 3-kinase in vitro (Fig. 7B).

DISCUSSION

Intracellular Ca2+ elevation is a well known element of signaling pathways implicated in activity-dependent neuronal survival (1) and the synaptic plasticity of long term potentiation (34). Some neurotrophic factors induce a small and rapid survival (1) and the synaptic plasticity of long term potentiation pathways implicated in activity-dependent neuronal cell death (3). De Bernardi et al. (6) concluded that NGF elicits a Ca2+ rise by causing intracellular Ca2+ mobilization rather than by extracellular Ca2+ influx. Our study shows that GDNF stimulation also induces an [Ca2+]i increase and that this Ca2+ increase is not prevented by the non-selective voltage-dependent Ca2+ channel blocker Cd2+, which prevents Ca2+ influx from the extracellular space. Ca2+ increase is prevented, however, when intracellular Ca2+ cannot be mobilized by depletion of the stores with TG, or by blocking the release from the stores with dantrolene. Thus, our results demonstrate that the [Ca2+]i increase induced by GDNF originates from internal stores in agreement with De Bernardi. Previous findings in PC12 and bovine chromaffin cells, however, suggest that the increase in [Ca2+]i found after NGF stimulation requires both extracellular Ca2+ influx, through a pathway different from the voltage-gated Ca2+ channels, and intracellular Ca2+ redistribution (5). In this study the authors observed that EGTA application, previous to NGF treatment, blocked the increase of [Ca2+]i. However, when EGTA was applied after NGF, an [Ca2+]i rise occurred but was smaller than that induced in the Ca2+-containing medium. In the present study this observation could be substantially different because the GDNF receptor Ret has a peculiar extracellular domain. The Ret extracellular domain is unique when compared with other receptor tyrosine kinases because of the presence of a cadherin-like domain with Ca2+-binding sites (13, 37, 38). It has been demonstrated previously that extracellular Ca2+ ions are required for the Ret and GDNF complex formation that induces Ret oligomerization and autophosphorylation (14). Thus EGTA treatment in our system prevents GDNF binding to Ret and therefore its activation. If Ret activation is prevented, all of the intracellular pathways activated by this receptor are also inhibited, including those related to the Ca2+ influx from the extracellular space. In fact, our results demonstrate that PI 3-kinase and PKB activation are inhibited when MTNs are treated with GDNF plus EGTA, indicating that extracellular Ca2+ is required for the activation of this pathway induced by GDNF. However, PKB phosphorylation is not inhibited in the presence of GDNF plus antagonists of the voltage-gated Ca2+ channels, indicating that Ca2+ influx through these channels is not required for PKB activation. In conclusion, extracellular Ca2+ is required for the activation of intracellular pathways induced by GDNF because of its role in mediating Ret binding to GDNF. Nevertheless, extracellular Ca2+ is not required for the [Ca2+]i increase observed after GDNF stimulation, because preventing Ca2+ influx from the extracellular space with Cd2+ or voltage-gated Ca2+ antagonists does not block the [Ca2+]i increase or PKB activation.

Our results demonstrate that intracellular Ca2+ is required for PI 3-kinase and PKB activation after GDNF stimulation, and we have shown that CaM is responsible for mediating this effect. Previous results have demonstrated that intracellular Ca2+ and CaM are also required for PKB activation after BDNF stimulation in cultured MTNs (3). Thus, CaM antagonists prevent PKB activation and cell survival under GDNF or BDNF treatment. In both paradigms, cell survival is mainly mediated by the activation of the PI 3-kinase/PKB pathway (10, 25). We therefore suggest that CaM has a critical role as a mediator of cell survival by regulating the activation of this pathway by survival factors. Our results are in accordance with another report (4) showing the same central role of CaM mediating cell survival in embryonic neocortical neurons. One important point that we additionally demonstrate in the present work is the direct interaction between CaM and the p85 subunit of PI 3-kinase. Using pull-down and co-immunoprecipi-
tation experiments we show that CaM associates with the p85 regulatory subunit of PI 3-kinase and that this association is Ca$^{2+}$-dependent. These results indicate that CaM-regulated proteins are not involved in this mechanism and that CaM directly regulates PI 3-kinase activation in a Ca$^{2+}$-dependent manner. The same conclusion has been published by Joyal et al. (39) in other cell systems. A high affinity CaM target sequence within the 110-kDa catalytic subunit of PI 3-kinase (p110) has also been described, which is able to bind CaM in a Ca$^{2+}$-dependent manner (40). Thus, these reports (39, 40) and ours reflect a possible modulation between CaM and both subunits of PI 3-kinase at least for the results obtained using in vitro strategies. This complex regulation of PI 3-kinase is also reinforced by previous data obtained using the neurotrophin family of neurotrophic factors (3). In this report, CaM antagonists did not significantly inhibit PI 3-kinase activity in immunoprecipitates in vitro but did prevent phosphatidylinositol generation or stabilization in vivo. Thus, CaM regulates PI 3-kinase either by modulating its kinase activity level or by regulating the generation and/or stabilization of its products. The final consequence of both actions is the activation of PKB and the promotion of neuronal survival. Ca$^{2+}$ and CaM are also involved in the survival promoting effect induced by membrane depolarization. Nevertheless, the intracellular pathways involved in this survival effect are not the same, because PI 3-kinase inhibitors did not block cell survival and PI 3-kinase is not activated by a high K$^{+}$ medium (22). In this model Ca$^{2+}$/CaM are probably activating a CaM-binding protein different from PI 3-kinase (41).

GDNF is expressed in embryonic muscle and Schwann cells and promotes MTN survival during development (8, 42, 43). The presence of GDNF in target tissues of MTNs suggest that the MEX could also induce measurable changes in [Ca$^{2+}$]$_i$ and promote MTN survival in vitro by activating the same pathways as GDNF. However, MEX stimulation did not induce an increase in the intracellular Ca$^{2+}$ level, and CaM antagonists did not block the neuronal survival induced by MEX (22), suggesting the main component of MEX that induces MTN survival in our culture system is not GDNF. This hypothesis is also reinforced by previous results from our laboratory where we described that the survival-promoting effect of MEX is

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**Fig. 6.** Ca$^{2+}$ and CaM are involved in GDNF-induced PI3K activation. MTNs were treated with 7 μM W12 or W13, 2.5 mM EGTA, 25 mM BAPTA-AM, or 50 μM LY 294002 and then stimulated for 7 min with GDNF (100 ng/m). PI3K activity was assayed in P-Tyr immunoprecipitates using L-phosphatidylinositol as substrate (top panel). Radiolabeled spots were quantified, and kinase activity was expressed as fold induction over basal. The arrow labeled “Origin” indicates the point of sample application. The arrow labeled “PI-3P” indicates the position of PI-3P generated as the consequence of PI 3-kinase activity. The same cell extracts were analyzed by Western blot using the α-P-Ser-473 (middle panel) and re-probed with α-tubulin (bottom panel). The bottom graph represents the quantification of the PI 3-kinase activity (mean ± S.E.) of three independent experiments. Asterisks indicate significant differences when comparing cultures treated with W13, LY 294002, BAPTA-AM, or EGTA to GDNF alone stimulate cultures using the Student t test ($p < 0.05$).
mainly mediated by the activation of the Jak pathway (44). The Jak pathway is activated by cytokines suggesting that these neurotrophic factors are the most important elements in mediating the survival effect of MEX. However, the possibility cannot be ruled out that other factors, such as GDNF, which are present in the MEX at a lower concentration, also contribute to the survival and electrophysiological differentiation of MTNs (45).

In conclusion, our findings provide evidence that GDNF induces an [Ca\(^{2+}\)]\(_{i}\) increase from internal stores, which is required for the promotion of MTN survival by GDNF and that the activation of the survival-promoting pathway involving PI 3-kinase/PKB could be regulated by the direct binding of CaM to p85 regulatory subunit of PI 3-kinase (39). Recent reports also show the involvement of Ca\(^{2+}\), CaM-regulated kinases, cAMP, and ERK signaling pathways mediating other neuronal functions such as synaptic plasticity, learning, and memory (reviewed in Ref. 46). Further research of the intracellular pathways, and the mechanisms involved in their regulation, implicated in all these processes will lead to a better understanding of the molecular basis of neuronal development and function.

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