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# Neuronal survival induced by neurotrophins requires calmodulin

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It has been reported that phosphoinositide 3-kinase (PI 3-kinase) and its downstream target, protein kinase B (PKB), play a central role in the signaling of cell survival triggered by neurotrophins (NTs). In this report, we have analyzed the involvement of $Ca^{2+}$ and calmodulin (CaM) in the activation of the PKB induced by NTs. We have found that reduction of intracellular $Ca^{2+}$ concentration or functional blockade of CaM abolished NGF-induced activation of PKB in PC12 cells. Similar results were obtained in cultures of chicken spinal cord motoneurons treated with brain-derived neurotrophic factor (BDNF). Moreover, CaM inhibition prevented the cell survival triggered by NGF or BDNF. This effect was counteracted by the transient expression of constitutive active forms of the PKB, indicating that CaM regulates NT-induced cell survival through the activation of the PKB. We have investigated the mechanisms whereby CaM regulates the activation of the PKB, and we have found that CaM was necessary for the proper generation and/or accumulation of the products of the PI 3-kinase in intact cells.

## Introduction

Neurotrophins (NTs)* and their tyrosine kinase receptor, Trk, are involved in the development, maintenance, and repair of the nervous system (Lewin and Barde, 1996). NTs include NGF, brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5 (Davies, 1994). There is growing evidences indicating that phosphoinositide 3-kinase (PI 3-kinase), one of the intracellular effectors activated by these factors, plays a central role in the regulation of cell survival in a wide variety of neuronal cell populations (Kaplan and Miller, 2000).

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*Abbreviations used in this paper: anti–P-Tyr, antiphosphotyrosine; BAPTA, 1,2 bis(2-aminophenoxy) ethene N,N,N′,N′-tetraacetic acid; BDNF, brain-derived neurotrophic factor; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; CaM, calmodulin; CaMKK, CaM-dependent protein kinase kinase; EGFP, enhanced green fluorescence protein; ERK, extracellular signal–regulated kinase; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; MTN, motoneuron; NT, neurotrophin; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PtdIns, phosphoinositides; RBD, Ras-binding domain; TFP, trifluoperazine dimaleate; TUNEL, TdT-mediated dUTP nick end labeling.

Key words: calmodulin; neurotrophin; cell survival; PKB; motoneuron

The pathway that leads to the activation of the PI 3-kinase after Trk activation begins with the autophosphorylation of the receptor at the residue $Tyr^{490}$ and the phosphorylation of Shc (Obermeier et al., 1993; Hallberg et al., 1998). This allows the recruitment to the membrane of several adaptor proteins, which upon tyrosine phosphorylation interact with and activate PI 3-kinase (Holgado-Madruga et al., 1997; Yamada et al., 1997). Furthermore, it has been reported that Ras cooperates in the activation of the PI 3-kinase, since dominant negative forms of this GTPase inhibit PI 3-kinase activity (Rodriguez-Viciana et al., 1994). PI 3-kinase catalyzes the phosphorylation of the D3 hydroxyl group of the inositol ring of phosphoinositides (PtdIns) (Czech, 2000). PtdIns-3,4-$P_2$ and PtdIns-3,4,5-$P_3$ are the main products generated and provide docking sites for pleckstrin homology (PH) domains of effector proteins such as the Ser/Thr protein kinase B (PKB), the cellular homologue of the transforming oncogene *v-Akt* (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991). The interaction of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ with PKB allows the translocation of the protein to the plasma membrane where it becomes fully activated upon phosphorylation at two residues, $Thr^{308}$ and $Ser^{473}$ (Alessi et al., 1996).

In a variety of cell systems, including neuronal cells, PKB mediates an important part of the trophic signal derived

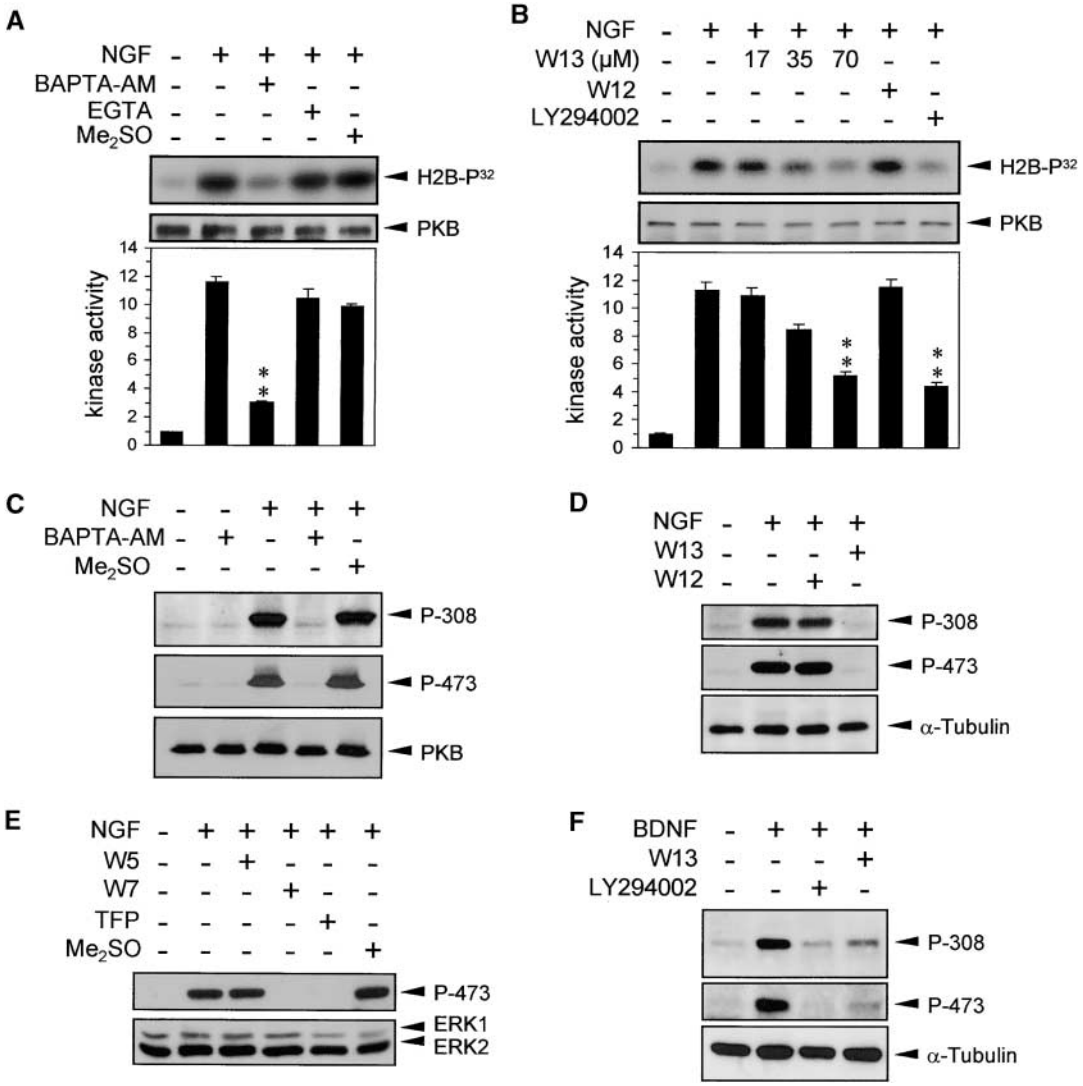


Figure 1. **Activation of PKB by NGF requires both $Ca^{2+}$ and CaM.** PC12 cells (A–E) or MTNs (F) were treated with BAPTA-AM (50 μM), EGTA (5 mM), LY294002 (50 μM), W5 or W7 (100 μM), TFP (50 μM), W13 or W12 (70 μM, unless otherwise indicated), or with vehicle ($Me_2SO$) and then stimulated for 5 min with NGF (50 ng/ml; A–E) or with BDNF (50 ng/ml; F) as indicated. (A and B) PKB was immunoprecipitated and kinase activity assayed with histone H2B as substrate (top panels and graphs). Equal amounts of immunoprecipitated PKB were checked by Western blot using a specific antibody against PKB (bottom panels). Radiolabeled spots were quantified and kinase activity expressed as fold induction over basal and represented as the mean ± SEM of two independent experiments. $^{**}P$ value using the Student's $t$ test was <0.01 relative to the treatment with NGF alone. (C–F) Phosphorylation of the residues $Thr^{308}$ (top panels) and $Ser^{473}$ (middle panels) of PKB was analyzed by Western blot using specific phospho-antibodies. Protein loading was checked, reprobing the filters with specific antibodies against total PKB, ERK, or α-tubulin as indicated (bottom panels).

from PI 3-kinase activation (Dudek et al., 1997; Philpott et al., 1997; Crowder and Freeman, 1998). Several studies have reported that PKB interferes with the cell death machinery phosphorylating and inactivating proteins that are directly involved in the induction of apoptosis such as GSK3β, BAD (a member of the Bcl-2 family of proteins), or members of the Forkhead family of transcription factors involved in the transcription of Fas ligand (Datta et al., 1999).

Bioelectrical activity cooperates with NTs in promoting neuronal survival during development (Franklin and Johnson, 1992). Neuronal activity exerts its trophic effects by moderately increasing the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). $Ca^{2+}$ triggers the activation of similar signaling pathways to those activated by NTs, mainly through the $Ca^{2+}$ receptor protein calmodulin (CaM) (Finkbeiner and Greenberg, 1996). Moreover, it has been reported that activation of Trk leads to a small and rapid increase of $[Ca^{2+}]_i$ (Pandiella-Alonso et al., 1986; Jiang and Guroff, 1997). However, the involvement of $Ca^{2+}$ in the response of the cells to the NTs has been poorly characterized. In the present work, we show that CaM is necessary for the promotion of cell survival triggered by NTs in PC12 cells and in chicken spinal cord motoneurons (MTNs). Our results demonstrate that this effect is mainly due to the regulation of PKB activity. We provide evidence that CaM is necessary to detect PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in the plasma membrane of live cells thus providing a possible mechanism by which CaM regulates PKB activity and cell survival.

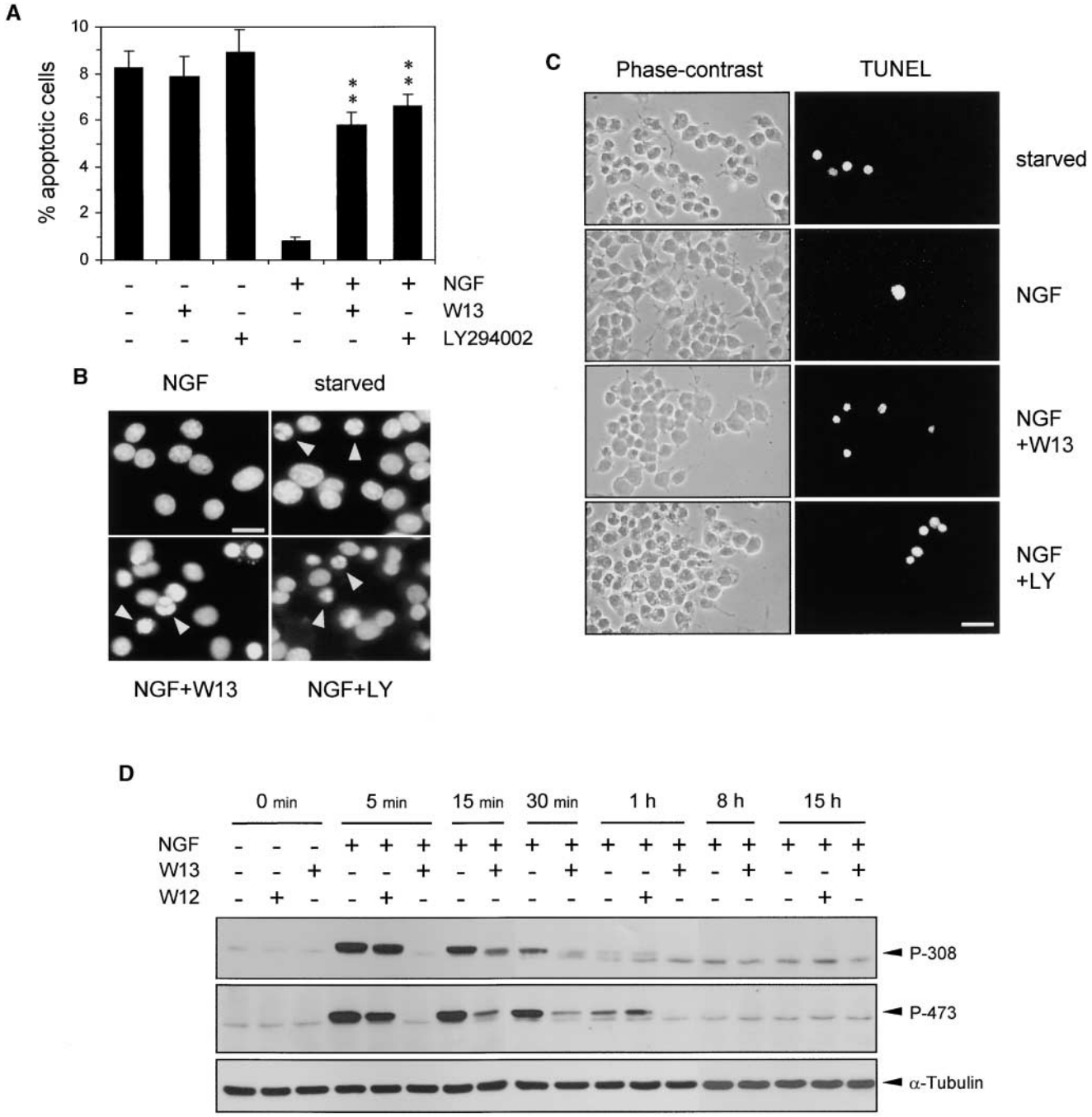


Figure 2. **NGF requires CaM to promote cell survival in PC12 cells.** PC12 cells were serum starved and treated with NGF (10 ng/ml), LY294002 (20 μM), W13 (30 μM), or left untreated as indicated. After 15 h, cells were fixed, stained with Hoechst 33258, or subjected to a TUNEL assay. (A) Percentage of cells displaying typical nuclear apoptotic morphology. The values represent the mean ± SEM of three independent experiments. $^{**}P$ value using the Student's $t$ test was $<0.01$ relative to the treatment with NGF alone. (B) Representative photomicrographs showing the morphology of the nuclei of the cells in the different treatments. Arrowheads indicate the apoptotic nuclei. (C) Representative phase–contrast micrographs and TUNEL reaction of the same field of the cultures treated above. (D) PC12 cells were treated with W13 (30 μM) and then stimulated for the indicated times with NGF (10 ng/ml). Phosphorylation of the residues $Thr^{308}$ (top panel) and $Ser^{473}$ (middle panel) of PKB was analyzed by Western blot using specific phospho-antibodies. Protein loading was checked, reprobing the filters with a specific antibody against α-tubulin (bottom panel). Bars: (B) 10 μm; (C) 20 μm.

## Results

### NT-induced PKB activation requires $Ca^{2+}$ and CaM

PKB is activated by NGF in PC12 cells through a mechanism involving PI 3-kinase (Park et al., 1996; Andjelkovic et al., 1998). We wanted to analyze the involvement of $Ca^{2+}$ and CaM in this activation. For this, we chelated the intracellular $Ca^{2+}$ using 1,2 bis(2-aminophenoxy) ethene N,N,N′,N′-tetraacetic acid (BAPTA) or the extracellular $Ca^{2+}$ using EGTA, and then we analyzed the activation of PKB after NGF stimulation. NGF induced a strong increase

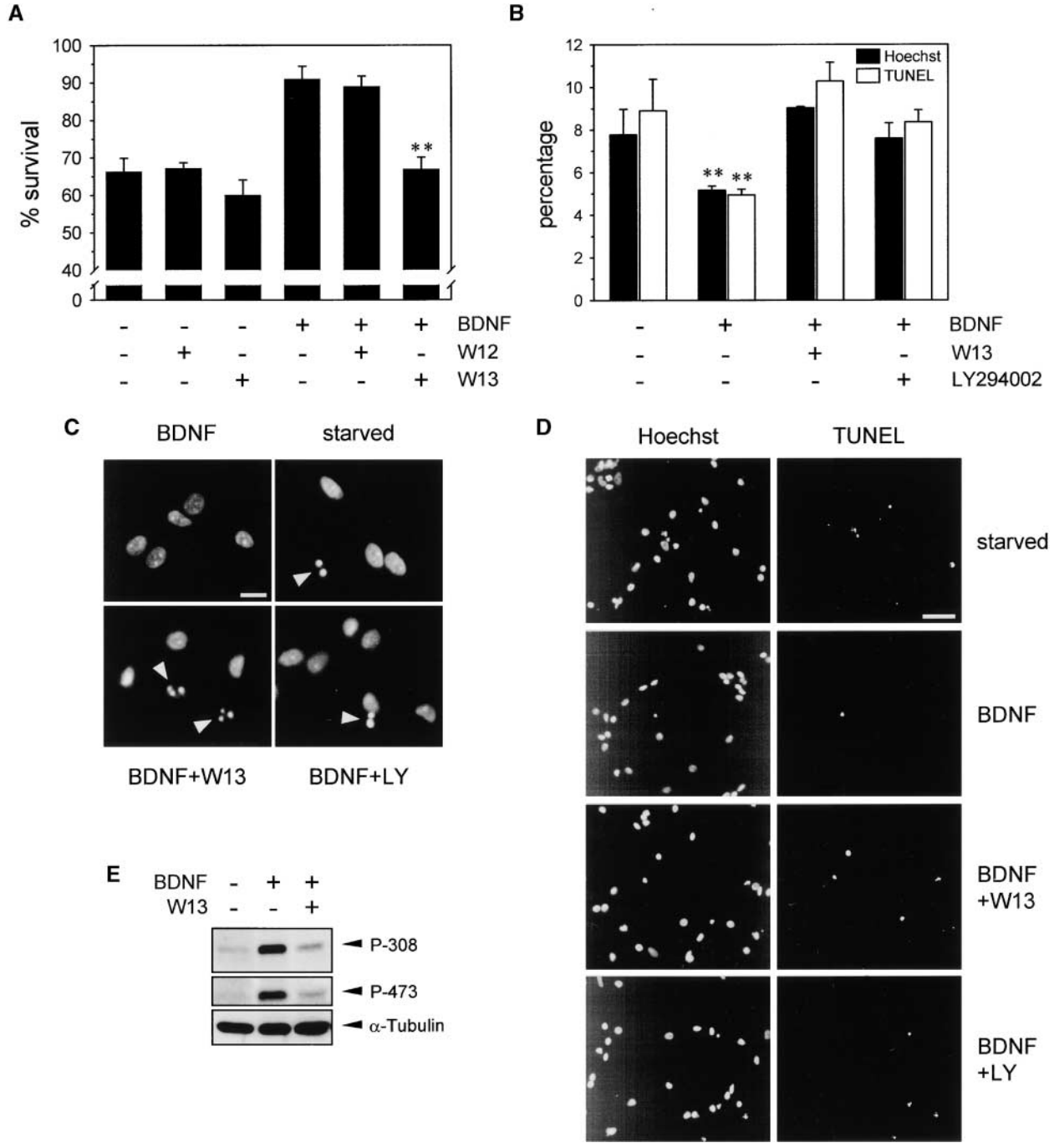


Figure 3. **BDNF requires CaM to promote cell survival in MTNs.** MTNs were starved of muscle extract and were treated with BDNF (10 ng/ml), W13 (30 mM), W12 (30 mM), or left untreated as indicated. (A) After 24 h, cells were counted, and survival was expressed as the percentage of cells remaining in each treatment. Graph shows the mean ± SEM of three independent experiments. ***P* value using the Student's *t* test was <0.01 relative to the treatment with BDNF alone. (B) After 15 h, cells were fixed and subjected to a TUNEL assay and stained with Hoechst 33258. The percentage of cells displaying typical nuclear apoptotic morphology and the percentage of TUNEL-positive cells were evaluated and represented as the mean ± SEM of two independent experiments. ***P* value using the Student's *t* test was <0.01 relative to the treatment with BDNF alone. (C) Representative photomicrographs showing the morphology of the nuclei of the cells treated in B and stained with Hoechst 33258. Arrowheads indicate the apoptotic nuclei. (D) Representative photomicrographs showing TUNEL reaction and Hoechst staining of the same field of the cultures treated in B. (E) MTNs were treated with W13 (30 μM) and then stimulated for 5 min with BDNF (10 ng/ml) as indicated. Phosphorylation of the residues $Thr^{308}$ (top panel) and $Ser^{473}$ (middle panel) of PKB was analyzed by Western blot using specific phospho-antibodies. Protein loading was checked, reprobing the filters with a specific antibody against α-tubulin (bottom panel). Bars: (C) 10 μm; (D) 40 μm.

in PKB activity (~11-fold over basal) that was almost completely prevented by BAPTA (Fig. 1 A). In contrast, concentrations of EGTA that effectively block depolarization-induced activation of extracellular signal–regulated kinase (ERK) mitogen-activated protein (MAP) kinases (Egea et al., 1999) did not significantly affect the activation of PKB

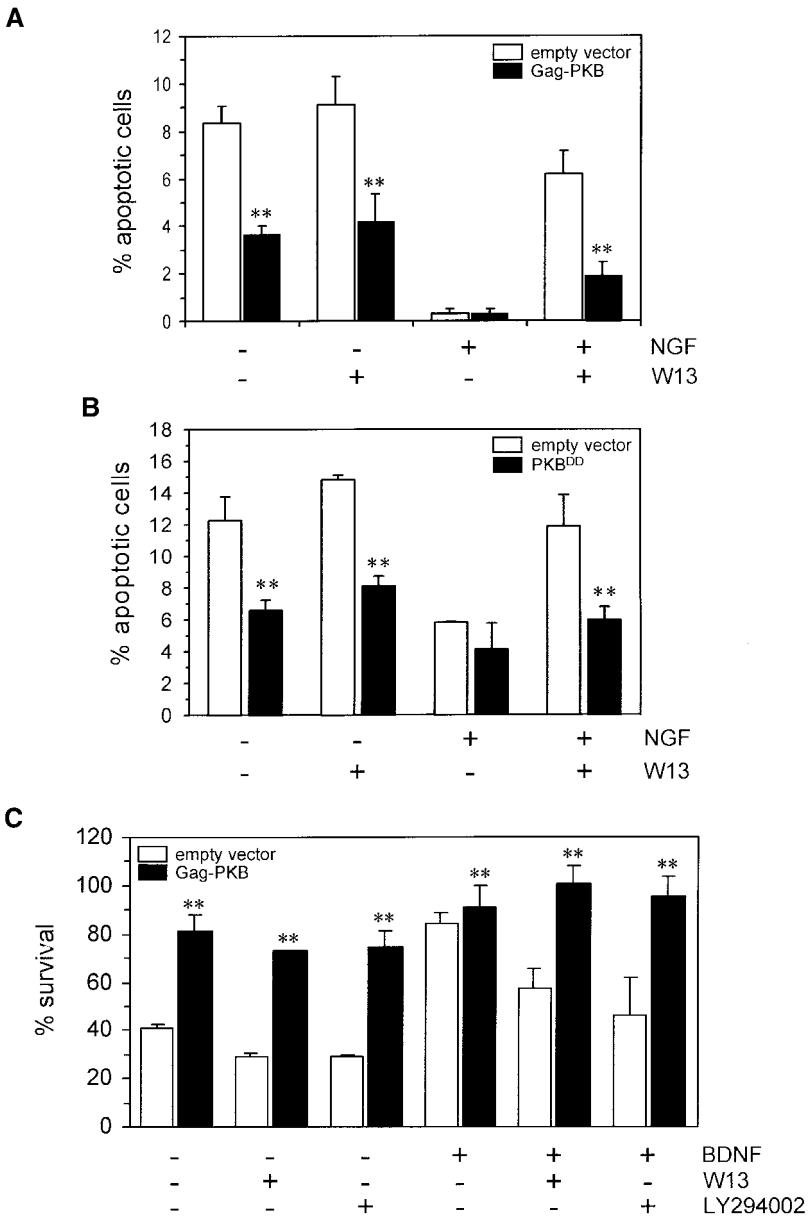


Figure 4. **Constitutively active forms of PKB prevent the cell death induced by CaM antagonists in NT-maintained cultures.** PC12 cells (A and B) or MTNs (C) were transiently cotransfected with pEGFP and pCMV5-HA-PKB$^{T308D/S473D}$ (PKBDD) or pSG5-Gag-PKB or the empty vector. (A and B) PC12 cells were serum starved and treated with NGF (10 ng/ml), W13 (30 μM), or left untreated as indicated. After 15 h, cells were fixed and stained with Hoechst 33258. The percentage of apoptotic cells scored into the EGFP-positive cell population was evaluated and represented as the mean ± SEM of three independent experiments. (C) MTNs were treated with BDNF (10 ng/ml), W13 (30 μM), LY294002 (20 μM), or left untreated as indicated. After 24 h, EGFP-positive cells were counted, and survival was expressed as the percentage remaining of EGFP-positive cells in each treatment. The graph shows the mean ± SEM of three independent experiments. ***P* value using the Student's *t* test was <0.01 when comparing the cultures transfected with pCMV5-HA-PKB$^{T308D/S473D}$ or pSG5-Gag-PKB with those transfected with the empty vector in each treatment (A–C).

(Fig. 1 A). In parallel experiments, we observed that the CaM antagonist W13 mimicked the effect of BAPTA on NGF-induced PKB activity. As shown in Fig. 1 B, increasing concentrations of W13 blocked the activation of PKB in a dose-dependent manner. At 70 mM, W13 reached an inhibitory effect similar to that observed with the specific PI 3-kinase inhibitor LY294002 (Vlahos et al., 1994) (Fig. 1 B). At this concentration, the effect of W13 was specific, since the same concentration of W12, a less active structural analogue ($W13_{IC50}$ = 68 μM versus $W12_{IC50}$ = 260 μM; Hidaka and Tanaka, 1983), did not affect NGF-induced PKB activity (Fig. 1 B). Moreover, 70 μM of W13 effectively inhibits the autophosphorylation of CaMKII induced by ionomycin in PC12 cells, a well-known $Ca^{2+}$/CaM-dependent process (unpublished data; Egea et al., 2000).

PKB activity is mainly induced by phosphorylation of the residues $Thr^{308}$ and $Ser^{473}$ (Alessi et al., 1996). We used specific phospho-antibodies against each of these two residues to check the phosphorylation of PKB upon NGF stimulation in the presence of $Ca^{2+}$ chelators or CaM antagonists. According to the experiments of kinase activity shown above, BAPTA (Fig. 1 C) and W13 but not W12 (Fig. 1 D) blocked the phosphorylation of both residues. Moreover, the inhibition exerted by W13 was sustained over the time of treatment (Fig. 2 D). Other CaM inhibitors, such as W7 (100 μM) or the W13 structurally unrelated trifluoperazine dimaleate (TFP; 50 μM), displayed similar effects as those observed with W13 (Fig. 1 E). In these experiments, we also included the CaM inhibitor W5 as a control of the unspecific effects of W7 ($W7_{IC50}$ = 28 μM versus $W5_{IC50}$ = 240 μM; Hidaka and Tanaka, 1983). As shown in Fig. 1 E, W5 did not significantly affect PKB phosphorylation, confirming the specificity of W7 effects. Finally, as expected from the kinase activity assay shown in Fig. 1 A, EGTA did not modify the phosphorylation of the PKB induced by NGF (unpublished data).

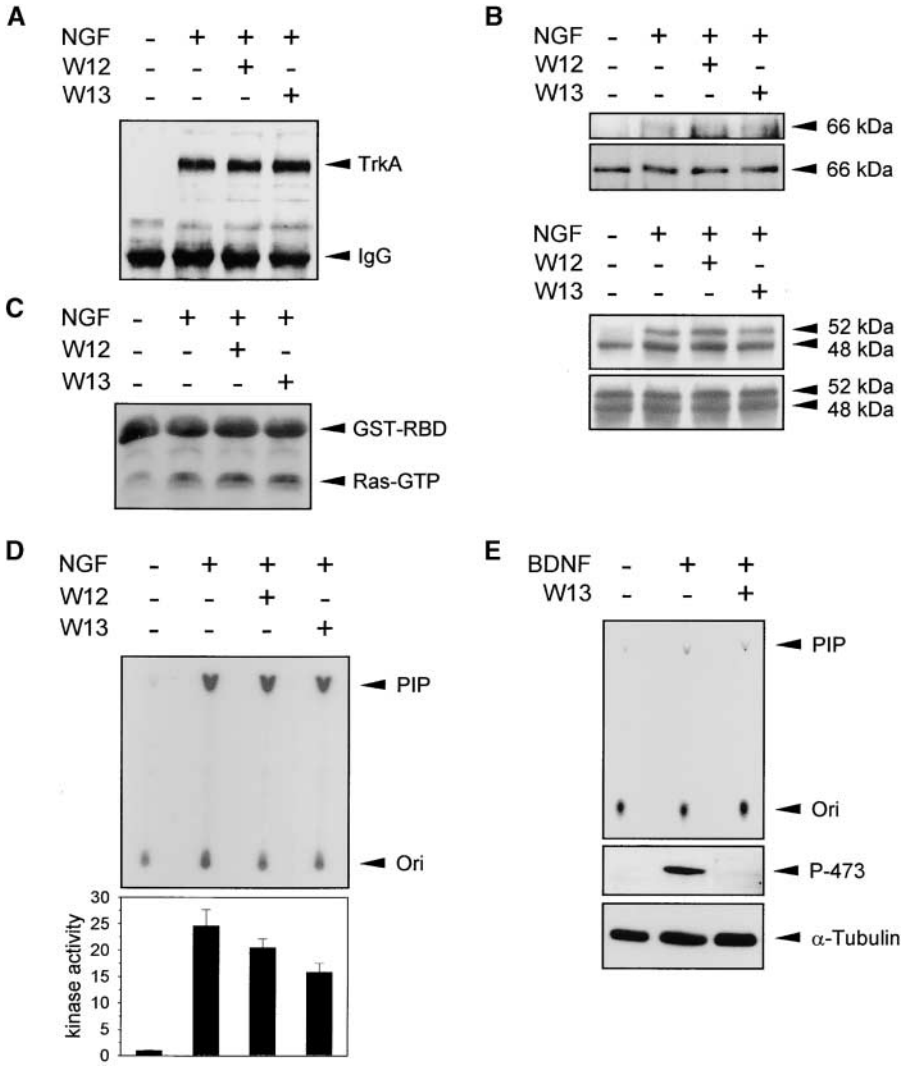


Figure 5. **Regulation by CaM of the NT-induced activation of PKB occurs downstream of PI 3-kinase.** PC12 cells were treated with 70 mM of W12 or W13 and then stimulated for 5 (A–C) or 2 min (D) with NGF (50 ng/ml) as indicated. (A) TrkA was immunoprecipitated and tyrosine phosphorylation of the receptor analyzed by Western blot using an anti–P-Tyr antibody. (B) Shc proteins were immunoprecipitated and analyzed by Western blot with an anti–P-Tyr antibody (top panels). Position of the 66-, 52-, and 48-kD isoforms of Shc is indicated. Protein loading was checked, reprobing the filters with a specific antibody against total Shc (bottom panels). (C) Active Ras (Ras-GTP) was precipitated with GST–RBD coupled to glutathione-Sepharose, and Ras was detected by Western blot using a pan-Ras antibody. (D) PI 3-kinase activity was assayed in P-Tyr immunoprecipitates using L-α-phosphatidylinositol as substrate (top panel). Radiolabeled spots were quantified and kinase activity expressed as fold induction over basal. Bottom graph represents the mean ± SEM of two independent experiments. (E) MTNs were treated with 70 μM of W13, stimulated for 2 min with BDNF (50 ng/ml), and PI 3-kinase activity assayed as in D (top panel). The same cell extracts were analyzed by Western blot using a specific phospho-antibody against the residue $Ser^{473}$ of PKB (middle panel). Protein loading was checked reprobing the filter with a specific antibody against α-tubulin (bottom panel).

We have reported previously that chicken spinal cord MTNs can be maintained in culture in the presence of BDNF (Becker et al., 1998). Like PC12 cells treated with NGF, the stimulation of cultured MTNs with BDNF phosphorylates and activates PKB in a PI 3-kinase–dependent manner (Dolcet et al., 1999). In this context, we analyzed whether MTNs also required CaM to activate PKB. As shown in Fig. 1 F, W13 inhibited both $Thr^{308}$ and $Ser^{473}$ phosphorylations to a similar extent to that observed with LY294002 (Fig. 1 F).

## CaM antagonists block NT-induced cell survival

The survival induced by NGF in PC12 cells can be strongly attenuated by inhibitors of PI 3-kinase (Yao and Cooper, 1995) or by treatments that block PKB activity (Weihl et al., 1999; Salinas et al., 2000). Therefore, we wanted to analyze whether the inhibition of PKB induced by CaM antagonists had any effect on cell survival. For this purpose, we maintained PC12 cell cultures in the presence of NGF (10 ng/ml) plus W13 (30 mM) or LY294002 (20 mM) for 15 h. At the end of the treatments, cells were fixed and stained with Hoechst 33258 or subjected to a TdT-mediated dUTP nick end labeling (TUNEL) assay. As shown in Fig. 2 A, the percentage of cells that display typical morphological apoptotic nuclei in the cultures treated with NGF plus W13 increased significantly when compared with cultures treated with NGF alone. Values were similar to those obtained in cultures maintained without any trophic support or in cultures treated with NGF plus LY294002. The values of apoptotic nuclei in W13 treatments were indistinguishable from those observed in starved cultures or in cultures treated with NGF plus LY294002 (Fig. 2 B). Similarly, W13 increased the number of TUNEL-positive cells in cultures maintained with NGF (5.98 ± 1.04%) when compared with cells treated with NGF alone (3.26 ± 0.37%), reaching similar values to those observed in cultures treated with NGF plus LY294002 (7.01 ± 0.84%). (In these experiments, the percentage of TUNEL-positive cells in cultures without trophic support was 9.96 ± 1.17) [Fig. 2 C].) The concentration of W13 used in these experiments did not increase the cell death observed in trophic support-starved cultures, suggesting that W13 was specifically interfering with the intracellular signaling pathway involved in NGF-induced cell survival (Fig. 2 A). As a control, we checked whether the concentration of W13 used in the experiments of survival (30 μM) inhibited the activation of PKB induced by 10 ng/ml NGF. As

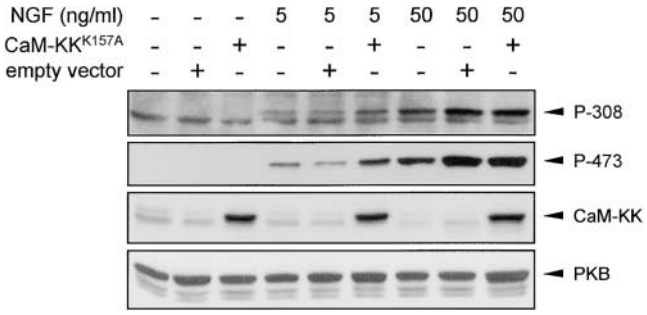


Figure 6. **CaMKK is not required for the activation of PKB induced by NGF.** PC12 cells were transiently transfected with pcDNA3-CaMKK$^{K157A}$ (CaMKK$^{K157A}$), empty vector, or left untransfected and then stimulated for 5 min with 5 or 50 ng/ml of NGF as indicated. Phosphorylation of Thr$^{308}$ and Ser$^{473}$ (top panels) of PKB was analyzed by Western blot using specific phospho-antibodies. Expression of CaMKK$^{K157A}$ in the same protein lysates was analyzed reprobing the filter with a specific antibody against CaMKK (middle panel). Protein loading was checked with a specific antibody against PKB (bottom panel).

shown in Fig. 2 D, this concentration of the drug induced an effective and sustained inhibition of both Thr$^{308}$ and Ser$^{473}$ phosphorylations of PKB.

We next analyzed the involvement of CaM in cell survival in a primary culture of neurons, and for this we used chicken MTNs maintained with BDNF. This experimental paradigm has several similarities with PC12 cells maintained with NGF. For example, we have demonstrated previously that BDNF-induced MTN survival is prevented by LY294002 (Dolcet et al., 1999), and here we have shown that the activation of PKB triggered by BDNF requires $Ca^{2+}$ and CaM (Fig. 1 F). Therefore, MTNs were treated with BDNF (10 ng/ml) plus W13 (30 μM), and cell survival was analyzed after 24 h. Our experiments showed that W13 but not W12 prevented the BDNF-induced MTN survival without increasing the cell death of parallel control cultures maintained in basal media without BDNF (Fig. 3 A). This effect correlated with a significative increase in the number of TUNEL-positive cells (Fig. 3, B and D) and in the number of the cells displaying typical morphological apoptotic nuclei (Fig. 3 B). As shown in Fig. 3 C, the morphology of the apoptotic nuclei in W13 treatments was indistinguishable from that observed in starved cultures or in cultures treated with BDNF plus LY294002. Fig. 3 E shows that 30 μM of W13 effectively blocked the phosphorylation of PKB induced by 10 ng/ml BDNF.

## Constitutive active forms of PKB prevent the cell death induced by CaM antagonists

The results above show a good correlation between inhibition of PKB and inhibition of cell survival induced by CaM antagonists. To test whether the prevention of cell survival triggered by these inhibitors was due to the inhibition of PKB, we transfected PC12 cells and MTNs with one of the two types of constitutive active forms: Gag-PKB, a constitutive plasma membrane-bound protein, and HA-PKB$^{T308D/S473D}$, a tagged form of the protein that carries a mutational acidic charge in addition to its main regulatory phosphorylation sites (Burgering and Coffer, 1995; Alessi et al., 1996). The transfected cultures were then treated with the corresponding NT plus W13, and at the end of the treatments cell survival was evaluated and compared with control cultures transfected with the empty vector. Results showed that Gag-PKB protected both PC12 cells and MTNs from the cell death induced by trophic factor withdrawal (Fig. 4, A and C, untreated cultures). Similar effects displayed HA-PKB$^{T308D/S473D}$ in serum-starved PC12 cells (Fig. 4 B, untreated cultures). Interestingly, Gag-PKB and HA-PKB$^{T308D/S473D}$ were also able to prevent the cell death triggered by W13 in cultures of PC12 maintained with NGF (Fig. 4, A and B, respectively). Accordingly, the cell death induced by W13 in BDNF-maintained MTNs was strongly reduced in Gag-PKB– (Fig. 4 C) and in HA-PKB$^{T308D/S473D}$-transfected cultures (unpublished data) when compared with the empty vector–transfected cultures. These results indicate that CaM antagonists exert their effect on NT-induced survival, mainly inhibiting the activation of PKB.

## CaM does not modulate early signaling events involved in PKB activation

The results presented in Fig. 1 indicate that $Ca^{2+}$ and CaM regulate upstream event(s) involved in the phosphorylation and activation of PKB. We have shown previously that inhibition of CaM does not affect the phosphorylation of TrkA and Shc, the association of Shc to Grb2, or the activation of Ras (measured by glutathione *S*-transferase–Ras-binding domain [GST–RBD] pull-down) induced by NGF in PC12 cells (Fig. 5, A–C; Egea et al., 2000). In this work, we also analyzed whether NGF-induced PI 3-kinase activity could require CaM. PC12 cells were treated with W12 or W13, stimulated for 2 min with NGF, and the PI 3-kinase activity was measured in antiphosphotyrosine (anti–P-Tyr) immunoprecipitates. As shown in Fig. 5 D, PI 3-kinase displayed an ~25-fold activation after NGF stimulation. However, W13 did not significantly inhibit PI 3-kinase activity when compared with parallel cultures treated with similar concentrations of W12 (Fig. 5 D, graph). Since W13 but not W12 effectively blocked PKB phosphorylation in the same cell lysates (unpublished data; Fig. 1 B), we therefore conclude that W13 inhibits PKB phosphorylation at some step downstream of PI 3-kinase. These experiments were also performed in cultures of MTNs, obtaining similar results. As shown in Fig. 5 E, W13 did not inhibit the PI 3-kinase activity induced by BDNF in MTNs (Fig. 5 E, top panel), despite that in the same cell extracts, the BDNF-induced PKB phosphorylation was completely prevented by the inhibitor (Fig. 5 E, bottom panels). Finally, to rule out a direct effect of W13 on the PI 3-kinase activity of intact cells, we included W13 directly in the kinase buffer of PI 3-kinase assays. In these experiments, we did not detect any significant effect of CaM antagonists on the PI 3-kinase activity induced by NGF (unpublished data).

## NGF does not require CaM-dependent protein kinase kinase to phosphorylate PKB

A recent study reported that the $Ca^{2+}$/CaM-dependent protein kinase kinase (CaMKK) is able to mediate cell survival by directly phosphorylating and activating PKB (Yano et al., 1998). To address the involvement of CaMKK in the activa-

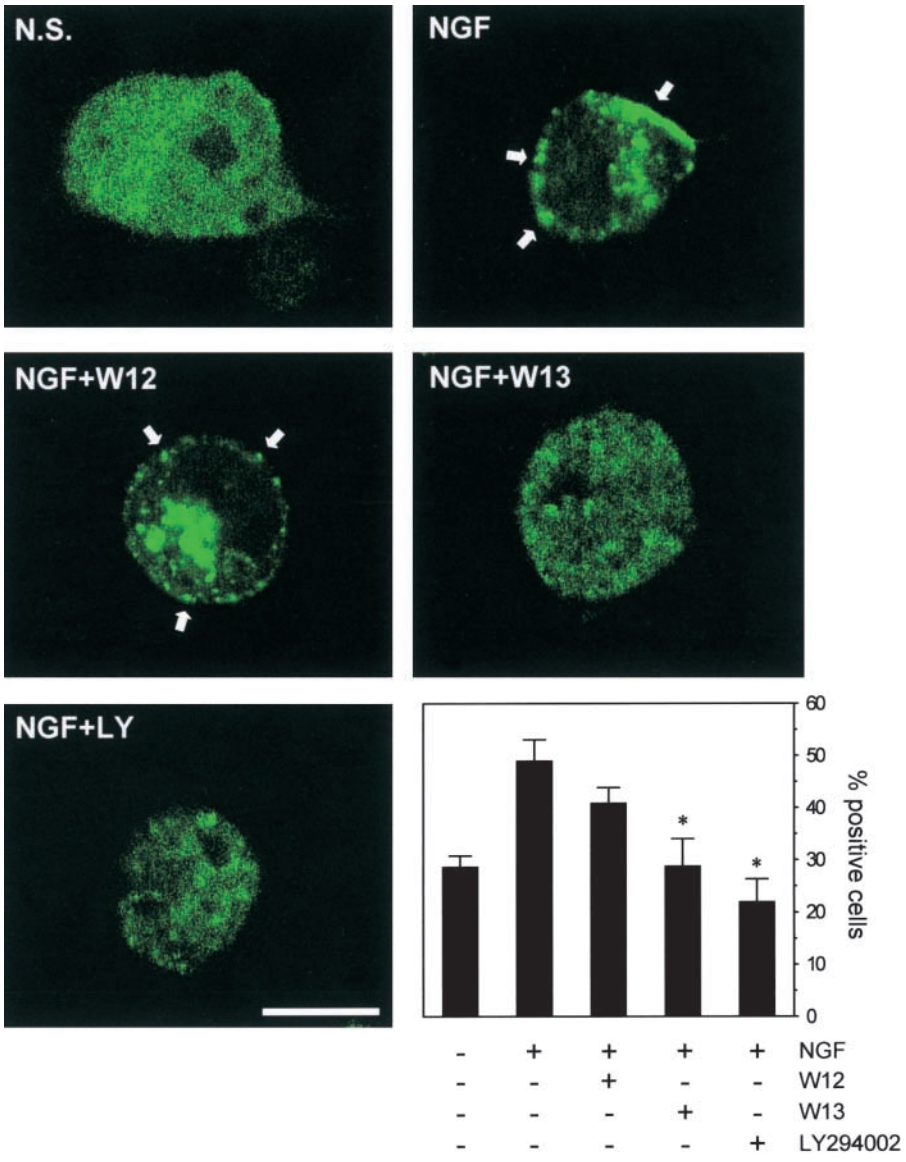


Figure 7. **CaM antagonists prevent the in vivo generation of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in PC12 cells.** PC12 cells were transiently transfected with EGFP–PKB–PH, treated with W12 (70 μM), W13 (70 μM), or LY294002 (50 μM), and then stimulated for 20 min with NGF (50 ng/ml) as indicated. N.S. indicates EGFP-PH-PKB transfected cells without any treatment. At the end of the treatments, cells were fixed, and EGFP distribution was visualized using a laser confocal microscope. Laser confocal sections through the middle of representative cells in each treatment are shown. Arrows indicate the peripheral redistribution of the EGFP–PKB–PH. The lower graph represents the percentage of cells displaying plasma membrane fluorescence (% positive cells) in each treatment. The values represent the mean ± SEM of two independent experiments. **P* value using the Student's *t* test was <0.05 relative to the treatment with NGF alone or relative to the treatment with NGF plus W12. Bar, 10 mM.

tion of PKB induced by NGF, PC12 cells were transfected with $CaMKK^{K157A}$, a dominant negative form of CaMKK that carries a point mutation in the ATP-binding site (Yano et al., 1998). These cultures were stimulated for 5 min with 5 or 50 ng/ml of NGF, and PKB activation was analyzed by Western blot using specific phospho-antibodies. As shown in Fig. 6, phosphorylation of PKB at the residues $Thr^{308}$ and $Ser^{473}$ in cultures transfected with the mutant $CaMKK^{K157A}$ was not significantly lower than that observed in control cultures transfected with the empty vector or in nontransfected cultures. Expression of $CaMKK^{K157A}$ in transfected cultures was confirmed by Western blot using an antibody against CaMKK (Fig. 6). PC12 cells were also transfected with $CaMKK^{1–413}$, a constitutive active form of CaMKK that lacks the regulatory CaM-binding domain (Enslen et al., 1996). $CaMKK^{1–413}$ did not induced a significant increase in the phosphorylation of residues $Thr^{308}$ or $Ser^{473}$ of Akt/PKB (unpublished data).

## CaM antagonists block plasma membrane localization of EGFP-PH-PKB

We have shown that CaM antagonists block NT-induced PKB phosphorylation without affecting the in vitro PI 3-kinase activity. It has been suggested previously that CaM may be required to detect the products generated by the PI 3-kinase into the plasma membrane of intact cells (Yang et al., 2000). To analyze this possibility, we transiently transfected PC12 cells with a construct encoding EGFP fused in frame with the PH domain of PKBα (EGFP–PKB–PH). After 36 h, cells were treated with W12, W13, or LY294002, stimulated for 20 min with NGF, fixed, and the distribution of the fluorescence assessed with a confocal microscope. A typical result is shown in Fig. 7. EGFP–PKB–PH showed a diffuse cytoplasmatic localization in nonstimulated PC12 cells (Fig. 7, N.S.). However, NGF induced a significant redistribution of the EGFP–PKB–PH fusion protein from the cytosol to the periphery of the cell (Fig. 7, NGF and graph).

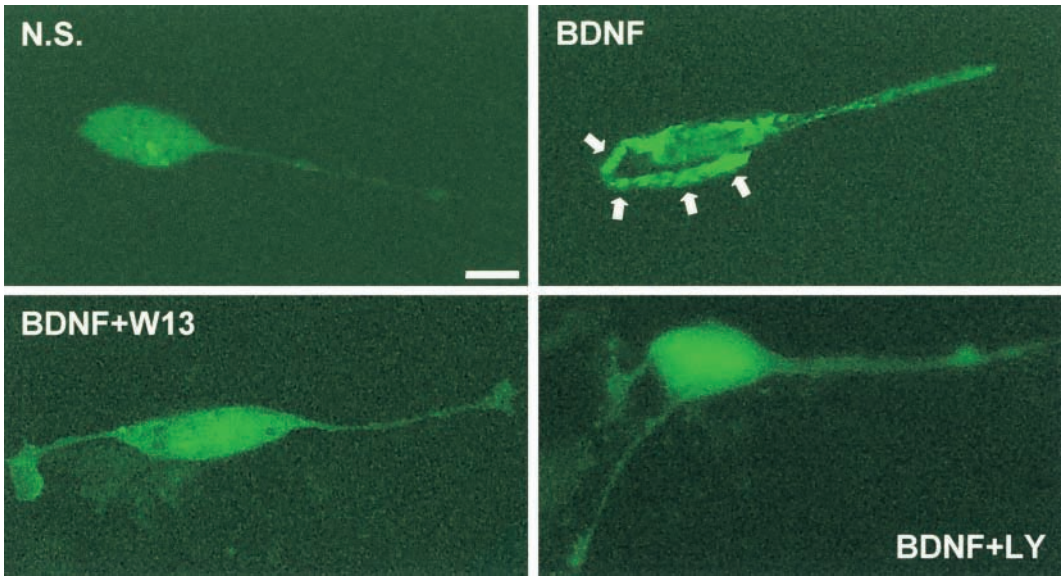


Figure 8. **CaM antagonists prevent the in vivo generation of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in MTNs.** MTNs cells were transiently transfected with EGFP–PKB–PH, treated with W13 (70 μM) or LY294002 (50 μM), and then stimulated for 20 min with BDNF (50 ng/ml) as indicated. N.S. indicates EGFP-PH-PKB–transfected cells without any treatment. At the end of the treatments, cells were fixed, and EGFP distribution was visualized using a laser confocal microscope. Laser confocal sections through the middle of representative cells in each treatment are shown. Arrows indicate the peripheral redistribution of the EGFP–PKB–PH. Bar, 10 mM.

This effect was due to the activation of the PI 3-kinase and the subsequent generation of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in the plasma membrane, since NGF did not induce any redistribution of the fluorescence in PC12 cells transfected with EGFP alone (unpublished data) and treatment with LY294002 completely abolished the peripheral distribution of the EGFP–PKB–PH fusion protein induced by NGF (Fig. 7, NGF + LY and graph). Cells stimulated with NGF that have been pretreated with W13 displayed a similar pattern of fluorescence to that observed in nonstimulated cells or in cells treated with NGF plus LY294002 (Fig. 7, NGF + W13), reducing significantly the percentage of cells displaying peripheral fluorescence (Fig. 7, graph). This effect was specific, since W12 did not significantly modify the peripheral pattern of fluorescence induced by NGF (Fig. 7, NGF + W12 and graph). Similar experiments were also performed in EGFP–PKB–PH–transfected MTNs. Confocal sections of representative cells treated with BDNF, BDNF plus W13, BDNF plus LY294002, or left untreated are shown in Fig. 8. As seen, W13 also abolished the redistribution of the fluorescence to the plasma membrane in MTNs stimulated with BDNF; this effect is similar to that observed using LY294002. Therefore, these results suggest that CaM is involved in the generation and/or stabilization of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in the plasma membrane of intact cells.

## Discussion

In this study, we report that CaM is necessary for the induction of cell survival triggered by NGF or BDNF in PC12 cells or MTNs, respectively. Our results demonstrate that this effect on cell survival is mainly due to the regulation of PKB activation. Moreover, we have shown that CaM regulates the presence of detectable amounts of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in the plasma membrane of intact cells thus providing a possible mechanism whereby CaM regulates the activation of PKB and the promotion of cell survival.

We have observed that extracellular $Ca^{2+}$ blockade did not affect the activation of PKB induced by NGF in PC12 cells thus confirming previous observations obtained in Balb/c-3T3 fibroblasts stimulated with EGF (Conus et al., 1998). In contrast, our results point out that intracellular $Ca^{2+}$ is completely necessary for the activation of PKB. This effect seems to be mediated by CaM, since CaM inhibition mimicked the effects of intracellular $Ca^{2+}$ blockade on PKB activation. It has been reported previously that Trk activation induces a small and rapid increase of $[Ca^{2+}]_i$ (Jiang and Guroff, 1997). This is probably achieved through the activation of PLCγ and the subsequent release of $Ca^{2+}$ from intracellular stores through inositol 1,3,4-triphosphate receptors (Obermeier et al., 1996). This mechanism seems to participate in the activation of the ERK MAP kinases, since TrkA receptors that carry a point mutation in the tyrosine that binds PLCγ ($TrkA^{Y785F}$) fail to completely activate these kinases (Stephens et al., 1994). However, it is less clear whether this $IP_3$-dependent $Ca^{2+}$ release could participate in the activation of PKB induced by Trk. For instance, NGF effectively activates PKB in Rat-1 cells expressing $TrkA^{Y785F}$ (Ulrich et al., 1998). Furthermore, the inhibition of $IP_3$ receptors with the specific inhibitor 2-APB (Maruyama et al., 1997) does not block the activation of PKB induced by NGF in PC12 cells (unpublished data). On the basis of these observations, it is possible that an $IP_3$-independent mechanism of intracellular $Ca^{2+}$ release induced by Trk could be involved in the activation of PKB. Alternatively, it could be also possible that basal levels of $[Ca^{2+}]_i$ are required to activate PKB.

It has been reported that membrane depolarization promotes cell survival in rat sympathetic neurons and granule neurons through a mechanism involving CaMKII and PI 3-kinase (Hack et al., 1993; Vaillant et al., 1999; Ikegami and Koike, 2000). However, we have not observed any effect of KN-62, a specific inhibitor of CaMKII, on the activation of PKB induced by NGF in PC12 cells (unpublished data). According to this result, it has been reported that KN-62 does not block cell survival of sympathetic neurons maintained with NGF (Vaillant et al., 1999; Ikegami and Koike, 2000). Results obtained in depolarized cerebellar granule neurons have provided contradictory conclusions regarding the participation of the PI 3-kinase in the depolarization-induced cell survival (D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997). However, it seems clear that depolarization-induced survival is a CaM-dependent PI 3-kinase-independent phenomenon in chicken MTNs (Soler et al., 1998). These CaM-dependent PI 3-kinase-independent mechanisms have been attributed recently to the CaMKK. It has been reported that *N*-methyl-D-aspartate–induced increase of $[Ca^{2+}]_i$ in NG108 neuroblastoma cells activates CaMKK, which in turns directly phosphorylates and activates PKB (Yano et al., 1998). In this study, we have analyzed the participation of CaMKK in PKB activation, and we have observed that $CaMKK^{K157A}$, a dominant negative form of the enzyme, did not abolish the activation of PKB induced by NGF, even at low nonsaturant concentrations of the NT (that is, 5 ng/ml). Moreover, $CaMKK^{K157A}$ did not have any effect on NGF-induced PC12 cell survival (unpublished data). According to these results, the promotion of cell survival induced by insulin-like growth factor in NG108 neuroblastoma cells was not affected by $CaMKK^{K157A}$ (Yano et al., 1998). Together, these results suggest that the CaM-dependent mechanisms involved in the promotion of cell survival induced by increases of $[Ca^{2+}]_i$ (for example, due to membrane depolarization or $Ca^{2+}$-mobilizing agonists) are distinct from those involved in cell survival promoted by NTs.

To ascertain the mechanism(s) whereby CaM modulates PKB activity in NT-treated cells, we explored some of the upstream steps involved in its activation. Neither the phosphorylation of Trk or Shc, the interaction of Shc with Grb2, nor the activation of Ras induced by NGF in PC12 cells required CaM (Egea et al., 2000). In the present work, we have also analyzed the activation of PI 3-kinase in anti–P-Tyr immunoprecipitates in NGF-stimulated PC12 cells and in BDNF-stimulated MTNs, concluding that it was not affected specifically by the treatment with CaM antagonists. However, as judged by the lack of peripheral distribution of the EGFP–PKB–PH fusion protein CaM antagonists did not allow to detect PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in the plasma membrane of intact cells after NGF and BDNF stimulation. These apparently contradictory results obtained using in vitro and in vivo assays have also been observed in 3T3L1 adipocytes. In these cells, CaM antagonists prevent the presence of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ in the plasma membrane induced by insulin or PDGF without affecting the PI 3-kinase activity measured in anti–P-Tyr immunoprecipitates (Yang et al., 2000). Therefore, these results seem to indicate that CaM is necessary for the generation and/or stabilization of the PI 3-kinase products. Several mechanisms can account for the role of CaM in this effect. For example, it can be possible that CaM exerts a regulation of PI 3-kinase activity in intact cells that is undetectable in the in vitro kinase assays. Such an explanation has been reported in the regulation of the PI 3-kinase by Ras. Dominant negative forms of this GTPase interact with and inhibit the activity of the PI 3-kinase, but this is only detectable in in vivo assays. In anti–P-Tyr immunoprecipitates, the interaction is lost, and therefore the inhibitory effect of the dominant negative forms of Ras cannot be observed (Rodriguez-Viciana et al., 1994). Another explanation could be that CaM can be required for the localization of the PI 3-kinase close to its substrates in intact cells. Indeed, CaM has been involved in the appropriate intracellular targeting of several proteins such as Rad or p21(Cip1) (Moyers et al., 1997; Taules et al., 1999). These hypothesis are supported by recent findings, showing that both the p85 and p110 subunits of the PI 3-kinase are able to interact with CaM in a $Ca^{2+}$-dependent manner (Joyal et al., 1997; Fischer et al., 1998). Finally, the possibility that CaM could negatively regulate the activity of the phosphatase(s) involved in the downregulation of the products generated by the PI 3-kinase cannot be ruled out. In this case, the inhibition of CaM would accelerate the disappearance of PtdIns-3,4-$P_2$/PtdIns-3,4,5-$P_3$ from the plasma membrane.

Our findings have relevant physiological implications in the developing nervous system. It is well known that increases of $[Ca^{2+}]_i$ underlying neuronal activity cooperate with NTs to promote neuronal survival. This phenomenon ensures that the correct and functional connections will be selected over the aberrant and nonfunctional ones (Franklin and Johnson, 1992). This phenomenon can be reproduced in vitro. For instance, levels of membrane depolarization that by themselves do not have any trophic effect synergize with limited amounts of NGF to promote cell survival in cultures of sympathetic neurons (Vaillant et al., 1999). Our results suggest a mechanism that could explain the synergistic effect of membrane depolarization on NT-induced cell survival in which $Ca^{2+}$ and CaM would sensitize the pathway involved in the activation of PKB and in the promotion of cell survival. Moreover, since $Ca^{2+}$ and CaM are also necessary for the activation of the ERK/MAP kinases induced by NTs (Egea et al., 2000) we propose that $Ca^{2+}$ and CaM play a central role in the regulation of the intracellular signaling pathways activated by NTs.

## Materials and methods

### Cell culture and cell lysates

PC12 cells were cultured as described (Egea et al., 1999). Chicken spinal cord MTNs were purified from 5.5-d-old chick embryos (Comella et al., 1994) with minor modifications described previously (Soler et al., 1998) and cultured for 48 h in the presence of muscle extract before the treatment with BDNF. For acute stimulations, PC12 cells were serum starved for 12–15 h, and MTNs were starved of muscle extract for 3–5 h. Inhibitors were added within the last hour of serum starvation.

Total cell lysates were obtained solubilizing the cells in 2% SDS and 125 mM Tris, pH 6.8, and sonicated. For immunoprecipitations, kinase assays, or Ras activity, cells were lysed at 4°C in the adequate lysis buffer (see below), and nuclei and cellular debris were removed by microfuge centrifugation. Protein concentration in cell lysates was quantified using the Bio-Rad Laboratories Dc protein assay.

### Plasmids and cell transfection

CaMKK and PKBα cDNAs were cloned from PC12 total RNA using the RobusT reverse transcription-PCR kit (Finnzymes). The dominant negative form ($CaMKK^{K157A}$) and the constitutive active form ($CaMKK^{1–413}$) of CaMKK have been described previously (Enslen et al., 1996; Yano et al., 1998). $CaMKK^{K157A}$ point mutant was generated using the QuickChange Site-directed mutagenesis kit (Stratagene) and sequenced to confirm the mutation. $CaMKK^{1–413}$ was generated by PCR, amplifying the sequence that codifies for the first 413 amino acids of the protein using the DyNAzyme EXT DNA polymerase (Finnzymes). $NH_2$-terminally tagged green fluorescent protein EGFP–PKB–PH was constructed incorporating a fragment of 750 bp, encoding the first 250 amino acids of PKBα (containing the PH domain) with the EGFP as described previously (Currie et al., 1999). All of the constructs were subcloned into the mammalian expression vector pcDNA3 (Invitrogen). DNA purification for cell transfection was performed using the QIAGEN Plasmid Maxi kit.

PC12 cells were transfected by electroporation as described in Espinet et al. (2000). MTNs were transfected 3 h after purification using the Lipofectamine 2000 reagent as suggested by the manufacturer (Life Technologies). When indicated, PC12 cells or MTNs were cotransfected with pEGFP (CLONTECH) and pSG5-Gag-PKB, pCMV5-HA-$PKB^{T308D/S473D}$, or the empty vector, using a one to four molar ratio.

### Evaluation of cell survival

PC12 cells cultured in serum-containing medium were changed to a serum-free medium containing NGF (10 ng/ml) plus the indicated inhibitors. After 15 h, cells were fixed and stained with the DNA dye Hoechst 33258 as described previously (Dolcet et al., 1999). When indicated, cells were also subjected to a TUNEL assay using the in situ cell death detection kit, TMR red (Roche Diagnostics GmbH). Cell death was expressed as the percentage of cells displaying typical nuclear apoptotic morphology or as the percentage of TUNEL-positive cells. In the EGFP/Gag-PKB or EGFP/HA-$PKB^{T308D/S473D}$ cotransfection experiments, treatments started after 36 h of transfection, and the percentage of apoptotic nuclei in each treatment was scored in the EGFP-positive cell population.

MTNs maintained for 48 h in the presence of muscle extract were washed and treated for an additional 24 h in basal medium containing BDNF (10 ng/ml) plus the indicated inhibitors. At the end of the treatments, cell survival was evaluated as described previously (Soler et al., 1998) (in the EGFP/Gag-PKB cotransfection experiments, only the EGFP-positive cells were counted). Alternatively, MTNs were fixed after 15 h of treatment and subjected to a TUNEL assay and to a Hoechst staining as described above.

### Western blot and immunoprecipitation

Western blot and immunoprecipitation were performed as described (Egea et al., 2000). Anti–phospho-Akt-$Thr^{308}$ and anti–phospho-Akt-$Ser^{473}$ antibodies (New England Biolabs, Inc.), anti-pan ERK, anti-Shc, and anti-CaMKK (Transduction Laboratories), anti-PKB C-20 (Santa Cruz Biotechnology, Inc.), anti–P-Tyr (clone 4G10) (Upstate Biotechnology), and anti–α-tubulin (Sigma-Aldrich) were used as suggested by the manufacturer. TrkA immunoprecipitation was performed using the antipan–Trk antibody (203) as described previously (Becker et al., 1998).

### PI 3-kinase assay

PI 3-kinase activity was measured in anti–P-Tyr immunoprecipitates using L-α-phosphatidylinositol as substrate and [γ-$^{32}$P] ATP (Amersham Pharmacia Biotech) as described previously (Egea et al., 1999). Phosphorylated lipids were resolved in a thin layer chromatography, detected by autoradiography, and quantified in a PhosphorImager (Boehringer).

### Ras activity assay

Activation of Ras was evaluated by a nonradioactive method using the GST–RBD (de Rooij and Bos, 1997) as described previously (Egea et al., 2000). Ras was detected using an antipan Ras antibody (Oncogene Research Products).

### PKB kinase assay

At the end of treatments, cells were lysed in a buffer containing 1% Triton X-100, 50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin, 2 mM benzamidine, 20 mg/ml leupeptin, 1 mM $Na_3VO_4$, 40 mM β-glycerophosphate, and 25 mM NaF. PKB was immunoprecipitated with the C-20 anti-PKB antibody (Santa Cruz Biotechnology, Inc.) and protein G–Sepharose. Immunocomplexes were sequentially washed three times with lysis buffer, two times with 50 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, two times with 0.5 M LiCl, 100 mM Tris, pH 8.0, 1 mM EDTA, and once with 50 mM Tris, pH 7.4, 10 mM $MgCl_2$. Kinase reaction was performed at room temperature for 30 min in a kinase buffer containing 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT supplemented with 2.5 mg of histone H2B (Boehringer) and 3 mCi of [γ-$^{32}$P] ATP (Amersham Pharmacia Biotech). The reaction was stopped by adding SDS-PAGE sample buffer. Histone H2B was resolved by SDS-PAGE. Radioactive spots were detected by autoradiography and quantified in a PhosphorImager (Boehringer).

### Fluorescence microscopy

PC12 cells or MTNs were plated into poliornitine-coated coverslips or into poliornitine/laminine-coated plates, respectively. Cells were transiently transfected with the EGFP–PKB–PH construct, and after 36–48 h cells were starved of trophic support and treated as indicated. At the end of stimulations, the medium was removed, and the cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (pH 7.4) for 30 min. Cells were washed twice with PBS and mounted using Vectashield (Vector Laboratories). Images were scanned on a Leica TCS 4D (PC12 cells) or on a ZEISS LSM 310 (MTNs) laser confocal microscope using an oil immersion objective of 63×. Percentage of cells displaying cell surface fluorescence was obtained counting 50–100 cells per coverslip using an epifluorescence microscope with an objective of 40×.

### Materials

BAPTA-AM was from Molecular Probes. W5, W7, TFP, and LY294002 were from Calbiochem-Novabiochem. BDNF was from Alomone Laboratories. All other reagents were from Sigma-Aldrich. Antipan–Trk (203) and PC12 cells were a gift from D. Martin-Zanca (CSIC-Universidad de Salamanca, Salamanca, Spain). The construct encoding the GST–RBD fusion protein was a gift from F. McKenzie, (State University of New York, Stony Brook, NY). pSG5-Gag-PKB was a gift from J. Downward (Imperial Cancer Research Foundation, London, UK). pCMV5-HA-$PKB^{T308D/S473D}$ was a gift from D.R. Alessi (University of Dundee, Scotland, UK) and B.A. Hemmings (Friedrich Miescher Institute, Basel, Switzerland). 7S NGF was prepared in our laboratory as described previously (Mobley et al., 1976).

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