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 Ca2+ and calmodulin (CaM) in the activation of the PKB induced by NTs. We have found that reduction of intracellular Ca2+ 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 evidence 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).

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 Tyr490 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-P2 and PtdIns-3,4,5-P3 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-P2/PtdIns-3,4,5-P3 with PKB allows the translocation of the protein to the plasma membrane where it becomes fully activated upon phosphorylation at two residues, Thr308 and Ser473 (Alessi et al., 1996).

In a variety of cell systems, including neuronal cells, PKB mediates an important part of the trophic signal derived...
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$^{2+}$]). 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$^{2+}$], (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.
**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 in PKB phosphorylation at Thr\(^{308}\) and Ser\(^{473}\), as well as a decrease in apoptosis in PC12 cells.

**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.
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.
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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 Ca2+/CaM-dependent process (unpublished data; Egea et al., 2000).

PKB activity is mainly induced by phosphorylation of the residues Thr308 and Ser473 (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 Ca2+ 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 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).
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 PI3-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 Thr308 and Ser473 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 PI3-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 MTNs also required CaM to activate PKB. As shown in Fig. 1 F, W13 inhibited both Thr308 and Ser473 phosphorylations to a similar extent to that observed with LY294002 (Fig. 1 F).

Figure 5. Regulation by CaM of the NT-induced activation of PKB occurs downstream of PI3-kinase. PC12 cells were treated with 70 μM 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, reexposing 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) PI3-kinase activity was assayed in P-Tyr immunoprecipitates using 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 PI3-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 Ser473 of PKB (middle panel). Protein loading was checked reexposing the filter with a specific antibody against α-tubulin (bottom panel).
membrane-bound protein, and HA-PKBT308D/S473D, a tagged form of constitutive active forms: Gag-PKB, a constitutive plasma membrane-bound protein, and HA-PKBT308D/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-PKBT308D/S473D in serum-starved PC12 cells (Fig. 4 B, untreated cultures). Interestingly, Gag-PKB and HA-PKBT308D/S473D were 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-PKBT308D/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 Ca2+ 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 Ca2+/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 activation 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 Ca2+ 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: Gap-PKB, a constitutive plasma membrane-bound protein, and HA-PKBT308D/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-PKBT308D/S473D in serum-starved PC12 cells (Fig. 4 B, untreated cultures). Interestingly, Gag-PKB and HA-PKBT308D/S473D were 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-PKBT308D/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 antagonists induced by NGF, PC12 cells were transfected with CaMKKK1–413, a constitutive active form of CaMKK that lacks the regulatory CaM-binding domain (Enslen et al., 1996). CaMKKK1–413 did not induced a significant increase in the phosphorylation of residues Thr308 or Ser473 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 antagonists 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 PKB (EGFP–PKB–PH). After 36 h, cells were treated with W12, W13, or LY294002, 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. 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.
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This effect was due to the activation of the PI 3-kinase and the subsequent generation of PtdIns-3,4-P₂/PtdIns-3,4,5-P₃ 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-P₂/PtdIns-3,4,5-P₃ 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-P₂/PtdIns-3,4,5-P₃ 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 Ca²⁺ 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 Ca²⁺ is completely necessary for the activation of PKB. This effect seems to be mediated by CaM, since CaM inhibition mimicked the effects of intracellular Ca²⁺ blockade on PKB activation. It has been reported previously that Trk activation induces a small and rapid increase of [Ca²⁺]ᵢ (Jiang and Guroff, 1997). This is probably achieved through the activation of PLCᵦ and the subsequent release of Ca²⁺ 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 PLCᵦ (TrkA⁷⁸⁵F) fail to completely activate these kinases (Stephens et al., 1994). However, it is less clear whether this IP₃-dependent Ca²⁺ release could participate in the activation of PKB induced by Trk. For instance, NGF effectively activates PKB in Rat-1 cells expressing TrkA⁷⁸⁵F (Ulrich et al., 1998). Furthermore, the inhibition of IP₃ 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 IP₃-independent mechanism of intracellular Ca²⁺ release induced by Trk could be involved in the activation of PKB. Alternatively, it could be also possible that basal levels of [Ca²⁺]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 (Hacker 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\(^{2+}\)], 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 non saturant 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\(^{2+}\)], (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).

Our findings have relevant physiological implications in the developing nervous system. It is well known that increases of [Ca\(^{2+}\)], 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 (Cornella 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 Ro-bustT reverse transcription-PCR kit (Finnzymes). The dominant negative form (CaMKKΔ237°) and the constitutive active form (CaMKKΔ1-415°) of CaMKK have been described previously (Eglen et al., 1996; Yano et al., 1999). CaMKKΔ1-415° point mutant was generated using the QuickChange Site-directed mutagenesis kit (Stratagene) and sequenced to confirm the mutation. CaMKKΔ237° was generated by PCR, amplifying the sequence that codes for the first 413 amino acids of the protein using the DyNAZyme EXT DNA polymerase (Finnzymes). NH2-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 cloned into the mammalian expression vector pcDNA3 (Inviogen). 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 Lipo-fectamine 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-PKBT308D/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 NFG (10 ng/ml) plus the indicated inhibitors. After 15 h, cells were fixed and stained with the DNA dye Hoechst 33258 as described previously (Doi 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 transfection experiments, treatments started after 36 h of transfection. 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., 1999) 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 Hoechst staining as described above.

Western blot and immunoprecipitation
Western blot and immunoprecipitation were performed as described (Egea et al., 2000). Anti-phospho-Akt-Thr208 and anti-phospho-Akt-Ser473 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–a–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 1–e–phosphatidylinositol as substrate and [γ–32P] ATP (Amerham 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–T7 domain of ras (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 phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mM benzamidine, 20 mg/ml leupeptin, 1 mM Na3VO4, 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 MgCl2. Kinase reaction was performed at room temperature for 30 min in a kinase buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl2, and 1 mM DTT supplemented with 2.5 mg of histone H2B (Boehringer) and 3 μCi of [γ–32P] 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).

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. The authors are thankful for the generous gift of the antipan–Trk antibody (203) and PC12 cells were a gift from D. Martin-Zanca (CISC–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–PKBT308D/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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References
Alessi, D.R., M. Andjelkovic, C. Baudewell, P. Cron, N. Morrice, P. Cohen, and B.A. Hemmings. 1996. Mechanism of activation of protein kinase B by inulin and IGF-1. EMBO J. 15:6541–6551.

Andjelkovic, M., H.S. Suidan, R. Meier, M. Frech, D.R. Alessi, and B.A. Hemmings. 1998. Nerve growth factor promotes activation of the alpha, beta and gamma isoforms of protein kinase B in PC12 pheochromocytoma cells. Eur. J. Biochem. 251:193–200.

Becker, E., R.M. Soler, Y.J. Yuse, E. Gine, C. Sanz-Rodriguez, J. Egea, D. Martin-Zanca, and J.X. Comella. 1998. Development of survival responsiveness to brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5, but not to nerve growth factor, in cultured motoneurons from chick embryo spinal cord. J. Neurosci. 18:7903–7911.

Bellacosa, A., J.R. Testa, S.P. Staal, and P.N. Tischkoff. 1991. A retroviral oncogene, Calmodulin modulates neuronal survival | Egea et al. 595
ak, encoding a serine-threonine kinase containing an SH2-like region. Science. 254:274–277.
Burgering, B.M., and P.J. Cofer. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-0H kinase signal transduction. Nature. 376:599–602.
Cofer, P.J., and J.R. Woodgett. 1991. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the CAMP-dependent and protein kinase C families. Eur. J. Biochem. 201:479–481.
Comella, J.X., C. Sanz-Rodriguez, M. Aldea, and J.E. Esquarda. 1999. Skeletal muscle-derived tropomyosin potentiates motoneurons from entering an active cell death program in vitro. J. Neurosci. 14:2674–2686.
Conus, N.M., B.A. Hemmings, and R.B. Pearson. 1998. Differential regulation by calcium reveals distinct signaling requirements for the activation of Akt and p70S6K. J. Biol. Chem. 273:4776–4782.
Crowder, R.J., and R.S. Freeman. 1998. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J. Neurosci. 18:2933–2943.
Currie, R.A., K.S. Walker, A. Gray, M. Deak, A.C. Casamayor, C.P. Downes, P. Cohen, D.R. Alesci, and J. Lucyocq. 1999. Role of phosphorylated 3-4,5-triphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase 1. Biochem. J. 357:575–583.
Czech, M.P. 2000. PI3P and PI3P3: complex roles at the cell surface. Cell. 100:603–606.
Datta, S.R., A. Brunet, and M.E. Greenberg. 1999. Cellular survival: a play in three acts. Genetics 13:2905–2927.
Davies, A.M. 1994. The role of neurotrophins in the developing nervous system. J. Neurobiol. 25:1334–1348.
de Rooij, J., and J.L. Bos. 1997. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. Oncogene. 14:623–625.
D’Mello, S.R., K. Borodezt, and S.P. Soltoff. 1997. Insulin-like growth factor and potassium depolarisation maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J. Neurosci. 17:1548–1560.
Dollet, X., J. Egea, R.M. Soler, D. Martin-Zanca, and J.X. Comella. 1999. Activation of phosphatidylinositol 3-kinase, but not extracellular-regulated kinases, is necessary to mediate brain-derived neurotrophic factor-induced motoneuron survival. J. Neurochem. 73:521–531.
Dudek, H., S.R. Datta, T.F. Franke, M.J. Birnbaum, R. Yan, G.M. Cooper, R.A. Segal, D.R. Kaplan, and M.E. Greenberg. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science. 275:661–665.
Egea, J., C. Espinet, and J.X. Comella. 1999. Calcium influx activates extracellular-regulated kinase/mitogen-activated protein kinase pathway through a calmodulin-sensitive mechanism in PC12 cells. J. Biol. Chem. 274:75–85.
Egea, J., C. Espinet, R.M. Soler, S. Perio, N. Rocamora, and J.X. Comella. 2000. Nerve growth factor activation of the extracellular signal-regulated kinase pathway is modulated by Ca2+ and calmodulin. Mol. Cell. Biol. 20:1931–1946.
Enomoto, H., H. Tokumaru, P.J. Stork, R.J. Davis, and T.R. Sudolering. 1996. Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade. Proc. Natl. Acad. Sci. USA. 93:10803–10808.
Espinet, C., X. Gomez-Arbojes, J. Egea, and J.X. Comella. 2000. Combined use of the green and yellow fluorescent proteins and fluorescence-activated cell sorting to select populations of transiently transfected PC12 cells. J. Neurosci. Methods. 100:63–69.
Finkbeiner, S., and M.E. Greenberg. 1996. Ca2+–dependent–direct routes to Rac mechanisms for neuronal survival, differentiation, and plasticity. Neuron. 16:233–236.
Fischer, R., J. Jullgart, and M.W. Berchtold. 1998. High affinity calmodulin target sequence in the signalling molecule PI 3-kinase. FEBS Lett. 425:175–177.
Franklin, J.L., and E.M. Johnson. 1992. Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. Trends Neurosci. 15:501–508.
Hack, N., H. Hidaka, M.J. Wakefield, and R. Balazs. 1993. Promotion of granule cell survival by high K+ or excitatory amino acid treatment and Ca2+/calmodulin-dependent protein kinase activity. Neuroscience. 57:9–20.
Hallberg, B., M. Ashcroft, D.M. Loeb, D.R. Kaplan, and J. Downward. 1998. Nerve growth factor induced stimulation of Ras requires Trk interaction with Shc and phosphatidylinositol 3′-kinase and formation of a multimeric signaling complex. J. Biol. Chem. 268:22963–22966.
Hallberg, B., A. Timhofer, H.H. Grunnicke, and A. Ullrich. 1996. Transforming potentials of epidermal growth factor and nerve growth factor receptors inversely correlate with their phospholipase C gamma affinity and signal activation. EMBO J. 15:73–82.
Pandilla-Alonso, A., A. Malgaroli, L.M. Vicentini, and J. Meldolesi. 1986. Early rise of cytosolic Ca2+ induced by NGF in PC12 and chromaffin cells. FEBS Lett. 208:48–51.
Park, E.K., S.L. Yang, and S.S. Kang. 1996. Activation of Akt by nerve growth factor via phosphatidylinositol-3 kinase in PC12 pheochromocytoma cells. Mol. Cell. 6:494–498.
Philpott, K.L., M.J. McCarthy, A. Klippel, and L.L. Rubin. 1997. Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical ganglionic neurons. J. Cell Biol. 139:809–815.
Rodriguez-Viciana, P., P.H. Warne, R. Bhand, V. Vanhaesebroeck, I. Gout, M.J. Fry, M.D. Waterfield, and J. Downward. 1994. Phosphatidylinositol 3-0H kinase as a direct target of Ras. Nature. 370:527–532.
Sali, M., H.R. Lopez-Valadado, D. Martin, A. Alvarez, and A. Cuadrado. 2000. Inhibition of PKB/Akt by C2-ceramide involves activation of ceramide-activated protein phosphatase in PC12 cells. Mol. Cell. Neurosci. 15:156–169.
Soler, R.M., J. Egea, G.M. Mintenig, C. Sanz-Rodriguez, M. Iglesias, and J.X. Comella. 1998. Calmodulin is involved in membrane depolarisation-mediated survival of motoneurons by phosphatidylinositol 3-kinase- and MAPK-independent pathways. J. Neurosci. 18:1230–1239.
Stephens, R.M., D.M. Loeb, T.D. Copeland, T. Lawson, A. Greene, and D.R. Kaplan. 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron. 12: 691–705.
Taules, M., A. Rodriguez-Vilarupra, E. Rius, J.M. Estanyol, O. Casanovas, D.B. Sacks, E. Perez-Paya, O. Bachs, and N. Agell. 1999. Calmodulin binds to p21(Cip1) and is involved in the regulation of its nuclear localization. J. Biol. Chem. 274:24444–24448.
Ulrich, E., A. Duwel, A. Kaufmann-Zeh, C. Gilbert, D. Lyon, B. Rudkin, G. Evan, and D. Martin-Zanca. 1998. Specific TrkA survival signals interfere with different apoptotic pathways. Oncogen. 16:825–832.
Vaillant, A.R., I. Mazzoni, C. Tudan, M. Boudreau, D.R. Kaplan, and F.D. Miller. 1999. Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase–Akt pathway to synergistically regulate neuronal survival. J. Cell Biol. 146:955–966.
Vlahos, C.J., W.F. Matter, K.Y. Hui, and R.F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzo[3,6]pyran-4-one (LY294002).
Wehr, C.C., G.D. Ghadge, S.G. Kennedy, N. Hay, R.J. Miller, and R.P. Roob. 1999. Mutant pre senilin-1 induces apoptosis and down regulates Akt/PKB. J. Neurosci. 19:5360–5369.
Calmodulin modulates neuronal survival

Yamada, M., H. Ohnishi, S. Sano, A. Nakazani, T. Ikeuchi, and H. Hatanaka. 1997. Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. *J. Biol. Chem.* 272:30334–30339.

Yang, C., R.T. Watson, J.S. Elmendorf, D.B. Sacks, and J.E. Pessin. 2000. Calmodulin antagonists inhibit insulin-stimulated GLUT4 (glucose transporter 4) translocation by preventing the formation of phosphatidylinositol 3,4,5-trisphosphate in 3T3L1 adipocytes. *Mol. Endocrinol.* 14:317–326.

Yano, S., H. Tokumitsu, and T.R. Soderling. 1998. Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature.* 396:584–587.

Yao, R., and G.M. Cooper. 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science.* 267:2003–2006.