Development/Plasticity/Repair

p75 Neurotrophin Receptor Mediates Neuronal Cell Death by Activating GIRK Channels through Phosphatidylinositol 4,5-Bisphosphate

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The pan neurotrophin receptor p75^{NTR} signals programmed cell death both during nervous system development and after neural trauma and disease in the adult. However, the molecular pathways by which death is mediated remain poorly understood. Here, we show that this cell death is initiated by activation of G-protein-coupled inwardly rectifying potassium (GIRK/Kir3) channels and a consequent potassium efflux. Death signals stimulated by neurotrophin-mediated cleavage of p75^{NTR} activate GIRK channels through the generation and binding of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2/PIP2] to GIRK channels. Both GIRK channel activity and p75^{NTR}-mediated neuronal death are inhibited by sequestration of PtdIns(4,5)P_2 and application of GIRK channel inhibitors, whereas pertussis toxin treatment has no effect. Thus, p75^{NTR} activates GIRK channels without the need for $G_{i/o}$-proteins. Our results demonstrate a novel mode of activation of GIRK channels, representing an early step in the p75^{NTR}-mediated cell death pathway and suggesting a function for these channels during nervous system development.

Key words: **nerve growth factor (NGF); regulated intramembrane proteolysis (RIP); apoptosis; Kir channel; neuronal death; phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]; signal transduction**

Introduction

Neurotrophins play various roles in the nervous system, regulating diverse outcomes from neuronal survival and death to synaptic plasticity (Dechant and Barde, 2002; Kalb, 2005). Programmed cell death, a process that occurs during the refinement and maturation of the developing nervous system, is mediated by the pan neurotrophin receptor p75^{NTR} (Frade et al., 1996; Kalb, 2005; Kenchappa et al., 2006). p75^{NTR} is widely expressed during development but is subsequently downregulated. However, it is also re-expressed in the adult in response to injury, where it has consistently been shown to be responsible for the cell death accompanying neurodegeneration (Miller and Kaplan, 2001; Kalb, 2005; Nykjaer et al., 2005).

Neuronal cell death signals mediated by p75^{NTR} are initiated by ligand binding, resulting in cleavage of p75^{NTR} to generate a transmembrane-linked C-terminal fragment (CTF) (see Fig. 1*A*) (Kanning et al., 2003; Kenchappa et al., 2006) containing the cytoplasmic juxtamembrane death domain (or "Chopper" domain) that triggers cell death (see Fig. 1*A*) (Coulson et al., 2000; Underwood et al., 2008). A second cleavage event mediated by γ-secretase releases the intracellular domain from the membrane and can also regulate cell death (see Fig. 1*A*) (Coulson et al., 2000; Kenchappa et al., 2006). p75^{NTR}-induced death signals use the downstream cell death machinery known as the apoptosome (caspase-9/apoptosis protein activating factor) (Troy et al., 2002; Coulson et al., 2004), which is activated by many cell death cascades. However, although a range of downstream effectors are known to be recruited by p75^{NTR} (Roux and Barker, 2002; Reichardt, 2006) and can regulate gene transcription resulting in cell death (Kenchappa et al., 2006), the mechanisms that underlie recruitment of the apoptosome to initiate cell death remain poorly understood (Miller and Kaplan, 2001; Coulson et al., 2004). One factor that has been widely implicated in the early apoptotic response is potassium efflux. This efflux contributes to formation of the apoptosome complex and is a prerequisite for DNA laddering and cleavage of pro-caspase 3 (Hughes et al., 1997; Maeno et al., 2000; Yu and Choi, 2000; Cain et al., 2001). We therefore hypothesized that regulation of ionic flux could be the key to elucidating this death pathway.

G-protein-coupled inwardly rectifying potassium (GIRK) channels are potassium-selective ion channels (Clapham, 1997; Stanfield et al., 2002) that are widely expressed in the developing and adult nervous system (Chen et al., 1997; Karschin and Karschin, 1997; Signorini et al., 1997; Wickman et al., 2000). In adult neurons, activation of GIRK channels by a range of neurotransmitters modulates neuronal excitability. Gating of these channels by neurotransmitters traditionally requires activation of

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pertussis-sensitive heterotrimeric G-proteins and release of $\beta\gamma$ subunits that bind to and activate the channels (Clapham, 1997; Stanfield et al., 2002). However, $\beta\gamma$ subunits alone are unable to cause gating, and it is now clear that the membrane phospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is a necessary cofactor that is alone sufficient for activation of GIRK channels (Huang et al., 1998; Kobrinsky et al., 2000; Sadja et al., 2003). PtdIns(4,5)P_2 levels are regulated by the balance between its kinase-mediated generation from less-phosphated phosphatidylinositol forms, primarily PtdIns(4)P, and its breakdown by phospholipase C (or phosphatidyl inositol 3-kinase), which hydrolyzes PtdIns(4,5)P_2 to inositol 1,4,5-triphosphate and diacylglycerol (Lee and Rhee, 1995). Indeed, closure of GIRK channels can be mediated by hydrolysis of PtdIns(4,5)P_2 (Kobrinsky et al., 2000; Sohn et al., 2007). Here, we show that ligand-stimulated generation of the p75^{NTR} C-terminal fragment stimulates production of PtdIns(4,5)P_2. This in turn gates GIRK channels, resulting in potassium efflux and cell death.

Materials and Methods

Neuronal culture. The killing Chopper peptide (PalmF29pen) and an unrelated control peptide (Palm gp130pen) were synthesized as described previously (Coulson et al., 2000). Dorsal root ganglion (DRG) neurons were dissected from postnatal day zero (P0) C57Bl/6 mice and cultured at low density in complete medium [Monomed II medium (CSL, Melbourne, Australia; JRH Biosciences, Melbourne, Australia) containing 1% fetal bovine serum (FBS) (JRH Biosciences) and nerve growth factor (2.5S NGF 50 ng/ml; Chemicon, Melbourne, Australia)] in the presence or absence of pertussis toxin (10, 33.3, or 100 ng/ml; List Biological Laboratories, Campbell, CA). After overnight incubation, the cultures were transferred into complete medium containing peptides at a final concentration of 2 μM, together with tetraethylammonium chloride (TEA; Sigma, Sydney, Australia), bupivacaine (Sigma), 4-aminopyridine (0.1–3 mM; Sigma), δ-dendrotoxin (30–300 nM; Alomone Labs, Jerusalem, Israel), apamin (10–1000 nM; Sigma), iberiotoxin (10–100 nM; Alomone Labs), or a stable form of tertiapin [Tertiapin-Q, synthesized by Auspep as described previously (Jin and Lu, 1999)]. The number of live cells was immediately counted and then recounted 2 or 24 h later. Data were analyzed by Student's *t* test.

Expression constructs. p75^{NTR} plasmids were constructed from rat cDNA and were made as described previously (Coulson et al., 2000) in the enhanced green fluorescent protein (EGFP)-internal ribosomal entry site (IRES) (Clontech, Mountain View, CA) vector. The extracellular noncleavable form of p75^{NTR} (p75-Nglycos) contained a point mutation Val243Asn to create an N-linked glycan attachment sequence, which we have shown prevents extracellular and intracellular cleavage (Underwood et al., 2008). The C-terminal fragment mimic construct (p75-CTF) contained the signal peptide, transmembrane, and entire intracellular domain (also called sptc152) (Coulson et al., 2000) and was resistant to intracellular cleavage (Underwood et al., 2008). pCMV-GIRK2–182-IRK1 (Lewohl et al., 1999) was a kind gift from A. Harris (University of Texas, Austin, TX). pcDNA3.1-GIRK2-AAA was a kind gift from P. A. Slesinger (The Salk Institute, La Jolla, CA). G-protein β_1 and γ_2 expression constructs in pCDNA3.1 vectors were supplied by Guthrie cDNA Resource Center. pβARK (Touhara et al., 1995) was a kind gift from P. Poronnik (The University of Queensland, Brisbane, Australia). The pleckstrin homology domain of phospholipase Cδ (PH-PLCδ) (Falasca et al., 1998) in pEGFP-C1 was kindly supplied by A. Yap (The University of Queensland, Brisbane, Australia), and PH-PLCδ fused to enhanced yellow fluorescent protein (EYFP) (Várnai et al., 2002) was a kind gift from T. Balla (National Institute for Child Health and Human Disease, Bethesda, MD).

Human embryonic kidney cell culture. Human embryonic kidney 293 T (HEK293T) cells, which have a high level of constitutive cleavage, or HEK293 cells, in which basal p75^{NTR} cleavage is low but can be induced, were plated in Roswell Park Memorial Institute (RPMI) medium in 10% FBS on glass coverslips at 50,000 cells per well of a 24-well plate. Cells were transfected with Fugene 6 (Roche, Basel, Switzerland) according to the manufacturer instructions, with 0.5 μg of each plasmid to a total concentration of 2 μg of DNA per well. All transfections contained 0.25–0.5 μg of pIRES-EGFP for transfected-cell identification, and a β-galactosidase plasmid (Coulson et al., 2000) was used to keep the total DNA received consistent across all conditions. Consistent levels of expression of proteins between transfection conditions was determined by Western blotting (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). After 48 h, the cells were lysed (10 mM Tris, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% NP-40 with addition of 1× Roche Complete Inhibitors). Approximately 30 μg of each lysate was separated on 4–20% Tris-glycine gels (Invitrogen, San Diego, CA), Western blotted, and immunostained using anti-intracellular p75^{NTR} antibody (1:2000; Promega, Sydney, Australia), HRP-conjugated anti-GFP (1:20,000; Abcam, Cambridge, MA), or anti-GIRK2 (1:200; Alomone Labs), and reprobed with α-tubulin (1:1000; MP Biochemicals, Sydney, Australia). Band densitometry of experimental replica blots were quantified using NIH ImageJ and analyzed by ANOVA.

HEK293 cell caspase assay. HEK293 cells were cultured and transfected as described above. After 48 h, cells were transferred to serum-free medium and were treated with phorbolester (PMA; 400 nM) for 16 h to promote p75^{NTR} proteolytic processing. They were then lysed for 20 min on ice in 25 μl of caspase lysis buffer, after which the lysates were mixed with assay buffer containing Ac-DEVD-AMC (16 μM; Sigma) caspase substrate. Caspase activity (cleavage of the substrate) was measured by Fluostar Optima plate reader (excitation filter, 380 ± 10 nm; emission filter, 460 nm) over 90 min.

HEK293T cell death assay. HEK293T cells were cultured and transfected as described above. After 72 h, cells were stained for viability with propidium iodide and analyzed on an LSRII Flow Cytometer (Becton Dickinson, Sydney, Australia). The level of cell death was determined by subtracting the percentage of viable EGFP-expressing cells in each condition from the percentage of viable EGFP-expressing cells in an EGFP-only transfected population. Data were analyzed by ANOVA.

In vitro *apoptosome assay.* The soluble fractions of cell lysates were obtained after osmotic shock and physical sheering using a Duall homogenizer and centrifugation at 100,000 × *g*. Cells were lysed in hypotonic extraction buffer (5 mM EGTA, 50 mM PIPES, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, pH 7) containing potassium at a range of concentrations (20, 50, 80, 110, or 140 mM). Protein (100 μg) from each lysate was incubated (in 50 μl reaction mix at 37°C) with 2 μM cytochrome *c* (Sigma) and 0.5 μM dATPs (Sigma) and the fluorescent caspase substrate Ac-DEVD-AMC (100 μM; Sigma). Caspase activity was measured by Fluostar Optima plate reader (excitation filter, 380 ± 10 nm; emission filter, 460 nm) every 5 min for 1 h, and emissions were standardized to identical reactions lacking dATPs (Genini et al., 2000; Cain et al., 2001).

Potassium efflux assay. HEK293T cells plated at 120,000 cells per well (24-well plate) and transfected for 36 h were incubated with 2.5 μCi/ml ^{86}Rb for 6 h at 37°C in 5% CO_2. The medium was then removed, the cells were washed thoroughly, and fresh medium was added to each well. Supernatant and cell lysates (resuspended in 300 μl PBS containing 1% Triton X-100) were then collected into separate tubes at various time points and counted using scintillant (Starscint; Packard, Meriden, CT). ^{86}Rb efflux (counts in the medium) was calculated as a percentage of the total radioactivity (medium plus cell lysate).

Electrophysiological recordings. HEK293T cells were transfected as described above. Cells were superfused with a solution containing the following (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.2 with KOH, or 110 NaCl, 30 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.2. Whole-cell recordings were obtained 36–48 h after transfection. Whole-cell pipettes were fabricated from thick-walled borosilicate glass and had an impedance of 2–3 MΩ when filled with an internal solution consisting of the following (in mM): 135 $KMeSO_4$, 8 NaCl, 0.3 EGTA, 10 HEPES, 2 Mg_2ATP, and 0.3 Na_3GTP, pH 7.2 with KOH, osmolarity 300 mOsm/kg. Signals were recorded using a Multiclamp 700A amplifier (Molecular Devices, Union City, CA). Currents were filtered at 2 kHz and sampled and analyzed using Axograph (Molecular Devices). All cells were voltage clamped at −60 mV and hyperpolarizing voltage steps applied in 10 mV increments. When high

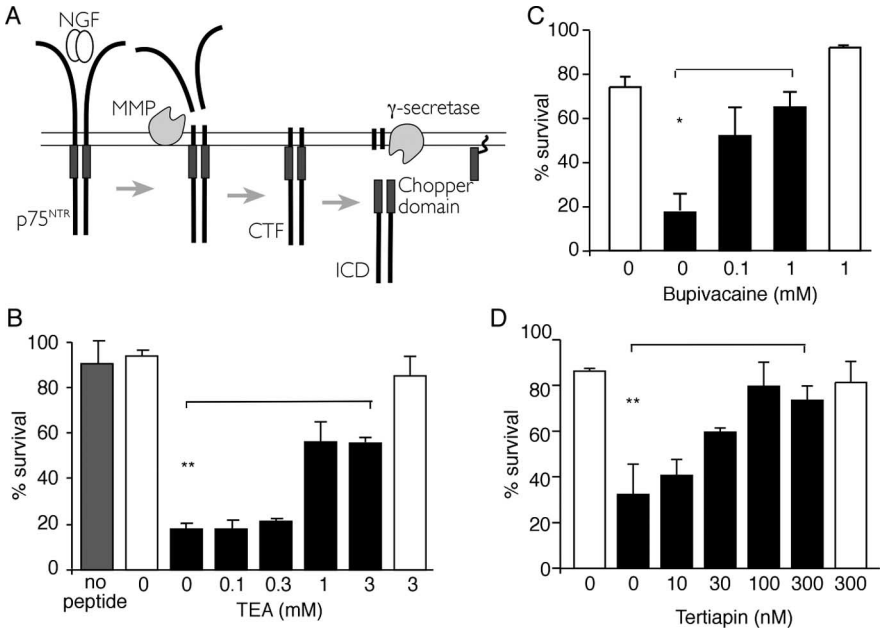

Figure 1. Chopper domain peptide-induced cell death is inhibited by potassium channel blockers ***A***, p75^{NTR} undergoes regulated intramembrane proteolysis, whereby the full-length p75^{NTR} is cleaved by metalloprotease (MMP) activity within the extracellular domain. This cleavage can be induced by neurotrophic ligands such as NGF. After extracellular cleavage, the remaining CTF of p75^{NTR} is cleaved intracellularly by presenilin-dependent γ-secretase activity, releasing the soluble intracellular domain fragment (ICD). A membrane-linked peptide corresponding to the intracellular juxtamembrane Chopper domain was used to constitutively induce neuronal death. Neuronal survival after 2 h of treatment with the death-inducing Chopper domain (black bars) or control (white bars) peptides in the presence of the K^+ channel inhibitors TEA (***B***) and bupivacaine (***C***). The gray bar indicates untreated neurons ($n = 3$ replica wells; mean ± SD; representative of 3 and 2 experiments, respectively). ***D***, Neuronal survival was determined after 2 h of treatment with Chopper domain or control peptides in the presence of tertiapin ($n = 3$ replica wells; mean ± SD; representative of 4 experiments). $*p < 0.05$, $**p < 0.01$, relative to vehicle-treated condition.

external potassium solution was used, cells were voltage clamped at −30 mV. Capacitance and leak conductances were subtracted using a P/4 procedure. Values for peak GIRK currents were measured from the steady state current at −90 mV with cells voltage clamped at −30 mV, and at −120 mV when cells were voltage clamped at −60 mV. Freshly made tertiapin was used at a concentration of 100, 200, and 300 nM in the superfusing solution. Pertussis toxin (10, 33.3, or 100 ng/ml) was added to the culture medium 18 h before cell recordings.

Neurotrophin cleavage assay. HEK293 cells were transfected with full-length p75^{NTR} and 24 h later cells were transferred into serum-free RPMI with or without 200 ng/ml 7S NGF (Chemicon). After 48 h, the cells underwent whole-cell recordings or were lysed, and 20 μl of each lysate was separated on 4–20% Tris-glycine gels, Western blotted, and immunostained using anti-intracellular p75^{NTR} antibody. Band densitometry was quantified using NIH ImageJ and analyzed by one-tailed Student's *t* test.

Confocal analysis. HEK293T or Cos7 cells plated on coverslips were transfected with PH-PLCδ-EYFP together with full-length p75^{NTR} or β-galactosidase expression plasmid. After 24 h, the cells were fixed in 4% paraformaldehyde in PBS and mounted on slides in mounting medium (Dako, High Wycombe, UK). Photomicrographs of cells in each condition were taken using a Zeiss (Oberkochen, Germany) Meta confocal microscope, keeping exposure times and depth of optical sections identical. Cell images were then analyzed by Image-pro plus software. Statistical analysis was performed using a Student's *t* test (significance, $p < 0.01$).

PtdIns(4,5)P_2 ^{32}P assay. Cos7 cells plated in 15 cm dishes were grown to 80% confluency and infected with ~10^9 PFU of p75^{NTR} or dominant-negative Rac virus (a kind gift from S. Yoon, Ohio State University, Columbus, OH). Cells were incubated for 3 h in phosphate-free Krebs/Ringer's solution (117.5 mM NaCl, 1.2 mM $CaCl_2$, 3.6 mM KCl, 0.8 mM $MgS0_4$, 5 mM $NaHC0_3$, 20 mM HEPES-NaOH, pH 7.4, 10 mM glucose, 0.1% BSA) before labeling with phosphate-free Krebs/Ringer's solution containing 1% FBS, 100 μM Na_xP_i, 1 mCi [γ-^{32}P]-orthophosphate. Cells were scraped in ice-cold PBS, pelleted by centrifugation, and cellular phosphoinositides extracted using acidified $CHCl_3$:MeOH. Lipids were loaded onto an oxalate pretreated Silica 60 TLC plate (Whatman, Brentford, UK) and developed in $CHCl_3$:MeOH:H_20:NH_4OH (90:90:20:7). PtdIns(4,5)P_2 was identified by comparison with unlabeled PtdIns(4,5)P_2 (Cell Signaling Technology, Danvers, MA) visualized using an acid molybdate spray.

DRG death assay. Plasmids were microinjected into DRG neurons (~300 cells per condition) as described previously (Coulson et al., 1999, 2000) with DNA at 100 μg/ml, except in the case of β_1 and γ_2 subunits, which were injected together at 50 μg/ml each. DNA for a β-galactosidase expression plasmid (Coulson et al., 1999) was used to make up the total DNA content to be equivalent between conditions. The percentage of survival was determined by counting live injected cells, identified by coinjected Fluororuby (1:1000; Invitrogen) 2 h after injection and again 16–18 h later.

In vitro *caspase activity assay.* The right eye vitreous of embryonic day 4.5 (E4.5) chicks was injected with 1 μl of water containing 10 or 50 pmol of the potassium channel inhibitor tertiapin. In each of three experiments, at least six eyes were injected per condition, but some retinas were not assayed because of embryo death or dissection damage. After 20 h, the retinal ganglion cell layer was dissected, dissociated in 200 μl of PBS by trituration through a 25-G needle, and assayed for endogenous caspase activity by cleavage of a fluorescent caspase substrate according to the manufacturer instructions (#3005372; Roche), except that all 200 μl was used in the reaction with 100 μl of substrate solution. Relative fluorescence unit values (RFUs) were within recommended levels. Correction of RFUs for the weight of the eyes did not affect the percentage reduction between vehicle and tertiapin treatments.

Results

p75^{NTR}-mediated death requires GIRK channel activity

We have shown previously that transfection of the juxtamembrane domain (referred to as the Chopper domain) (Fig. 1*A*) of p75^{NTR} into DRG neurons leads to significant cell death. The rate of death is significantly faster than that mediated by full-length p75^{NTR} (Coulson et al., 2000) but occurs through the same pathway (Coulson et al., 2004). We initially screened the effect of a wide range of potassium channel blockers to investigate whether Chopper domain-mediated death requires potassium efflux. Chopper domain-induced cell death was significantly inhibited by the broad spectrum potassium channel blocker TEA (Fig. 1*B*), the GIRK and leak channel inhibitor bupivacaine (Fig. 1*C*), and the GIRK and ROMK1 (Kir1.1) channel inhibitor tertiapin (Fig. 1*D*). Although these potassium channel blockers are relatively nonspecific, each of them has been shown to block GIRK channel activity (Jin and Lu, 1998). In contrast, the more specific potassium channels blockers that we tested (i.e., apamin, iberiotoxin, 4-aminopyridine, and δ-dendrotoxin) (Coetzee et al., 1999) had no effect on Chopper-induced cell death (data not shown). Thus, GIRK channel inhibition was considered the most parsimonious explanation for the reduced neuronal death induced by TEA, bupivicaine, and tertiapin.

To test whether p75^{NTR}-induced cell death requires GIRK

channels, we coexpressed Kir3.1 and Kir3.2 (GIRK1/2) subunits together with p75^{NTR} in HEK293 cells, which do not endogenously express either GIRK channel subunits or p75^{NTR} (supplemental Fig. 1*C*, available at www.jneurosci.org as supplemental material). When either p75^{NTR} or GIRK1/2 channels was expressed alone, there was no activation of death signaling (Fig. 2*A*). In contrast, coexpression of p75^{NTR} with GIRK1/2 channels led to a significant increase in caspase activation (Fig. 2*A*).

Because p75^{NTR} death signaling can be activated by extracellular cleavage (Kenchappa et al., 2006; Underwood et al., 2008), we next determined whether cleavage of p75^{NTR} was required for caspase activation using a mutant form of p75^{NTR} (p75-Nglycos) that cannot be extracellularly cleaved because of the insertion of an N-glycosolation sequence at the cleavage site and that does not induce cell death when overexpressed in DRG neurons (Underwood et al., 2008). In contrast to wild-type p75^{NTR}, p75-Nglycos did not induce caspase activity above control levels. We next used a truncated form of p75^{NTR} that mimics the endogenously generated, extracellularly cleaved, transmembrane CTF form of p75^{NTR} (Fig. 1*A*). This protein is not cleaved intracellularly (Fig. 3*E*) and induces significantly more cell death than full-length p75^{NTR} when overexpressed in DRG neurons (Coulson et al., 2000). When p75-CTF was coexpressed with GIRK1/2 channels, the level of caspase activity increased above that induced by full-length p75^{NTR} (Fig. 2*A*). Importantly, GIRK1/2 channels were required for p75-CTF to induce the death of HEK293T cells (Fig. 2*B*). These results indicate that extracellular (but not intracellular) cleavage of p75^{NTR}, in addition to coexpression of GIRK channel subunits, are required for induction of caspase activation.

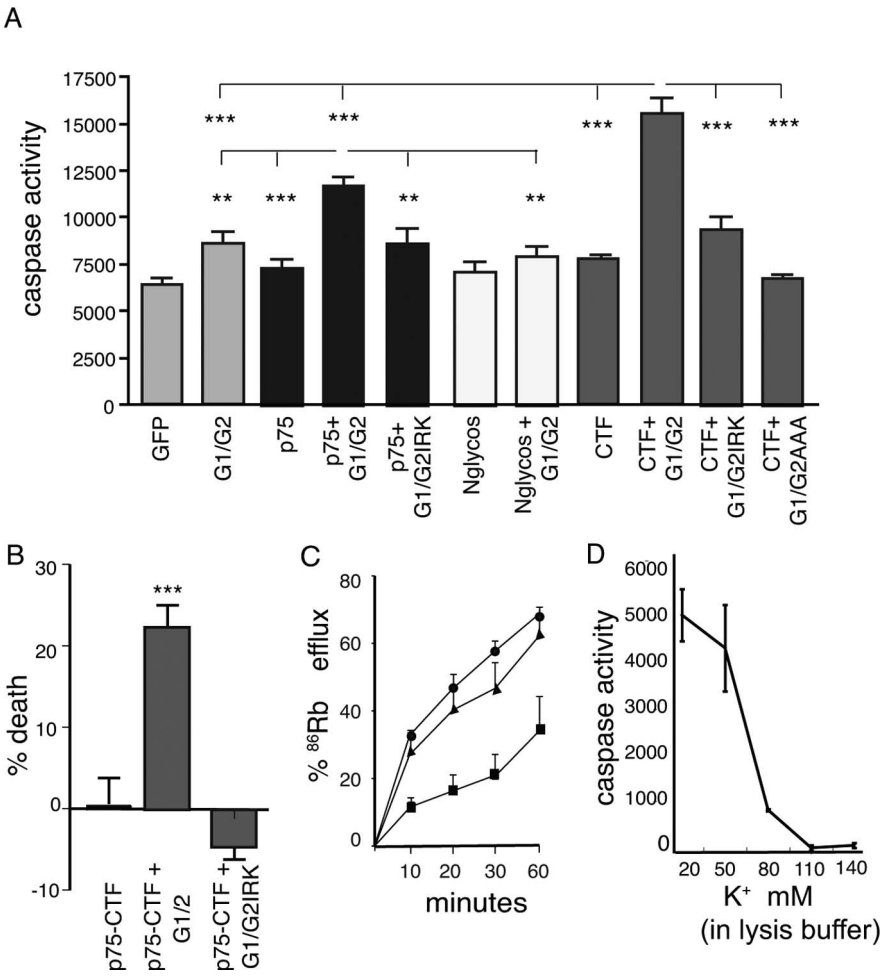

Figure 2. Extracellular cleavage of p75^{NTR} and potassium efflux through GIRK channels is necessary for cell death. ***A***, Summary data showing caspase activity (see Materials and Methods) in HEK293 cells transfected as indicated and treated overnight with 400 nM PMA to induce extracellular cleavage. Coexpression of functional GIRK channels with full-length p75^{NTR} induces caspase activity above that of controls. Coexpression of the nonfunctional chimeric GIRK2-IRK1 subunit (G2IRK) instead of wild-type GIRK2 precludes the increase in caspase activity. Coexpression of the p75-Nglycos mutant (which cannot be cleaved extracellularly) with GIRK1/2 channels similarly fails to stimulate caspase activity. The CTF form of p75^{NTR} induced caspase activity above that induced by the control or wild-type p75^{NTR} only when coexpressed with functional GIRK1/2 channels (mean ± SEM; $n = 3$ replica wells; representative of 3 independent experiments). $^{**}p < 0.01$, $^{***}p < 0.001$. ***B***, Summary data showing death of HEK293T cells transfected with p75-CTF alone, or p75-CTF together with GIRK1 and either GIRK2 or a chimeric GIRK2-182-IRK1 channel (G2IRK) relative to that of cells transfected with EGFP alone (0%) (mean ± SEM; $n \geq 8$). $^{***}p < 0.001$, relative to both p75-CTF alone and GIRK1/2 alone (data not shown). ***C***, The amount of ^{86}Rb released from transfected HEK293T cells over 60 min was measured to determine the relative efflux of potassium. Activation of GIRK channels by either $\beta\gamma$ subunits (circles, top plot) or p75-CTF (triangles, middle plot) caused a similar level of efflux, which was significantly greater than that seen with EGFP-transfected cells (squares, bottom plot) (mean ± SD; $n = 3$). ***D***, Effect of potassium concentration on apoptosome activation was measured in HEK293T cell lysates by the relative extent of caspase activity as described in Materials and Methods. Potassium concentrations >110 mM significantly inhibited apoptosome activity ($n = 3$ experiments; $p < 0.05$).

To confirm that GIRK channel activity is required for death signaling, we used chimeric GIRK2 subunits, in which the terminal 245 amino acids of GIRK2 were replaced with those from IRK1 (GIRK182-IRK) (Lewohl et al., 1999). This chimeric subunit lacks binding domains for $\beta\gamma$ subunits and PtdIns(4,5)P_2, and channels containing these subunits are nonfunctional (Lewohl et al., 1999; Sadja et al., 2003). Both p75^{NTR}- and p75-CTF-induced caspase activity and p75-CTF-induced death signaling were prevented in cells expressing the chimeric GIRK1/GIRK182-IRK channels (Fig. 2*A*,*B*). Similarly, p75-CTF-induced caspase activity was precluded when a pore mutant GIRK 2 subunit (pGIRK2-AAA), which cannot pass potassium, was substituted for wild-type GIRK2 in this assay (Fig. 2*A*). These results indicate that p75^{NTR}-mediated caspase activity and cell death require activation and potassium flux through GIRK channels.

To determine whether potassium flux accompanied the initiation of cell death, the movement of the potassium analog ^{86}Rb was compared between cells coexpressing GIRK1/2 channel subunits and control GFP-expressing cells. Cells in which either p75-CTF or $\beta\gamma$ subunits were coexpressed with GIRK channels had increased potassium efflux compared with that which occurred in control cells (Fig. 2*C*), suggesting that the p75-CTF-mediated cell death was caused by efflux of intracellular potassium ions through GIRK channels.

We therefore tested the relationship between potassium concentration and activation of the apoptosome. The components of the apoptosome were reconstituted *in vitro* in buffers containing potassium at concentrations ranging from 0 to normal intracellular levels (140 mM), and caspase activity was determined. These

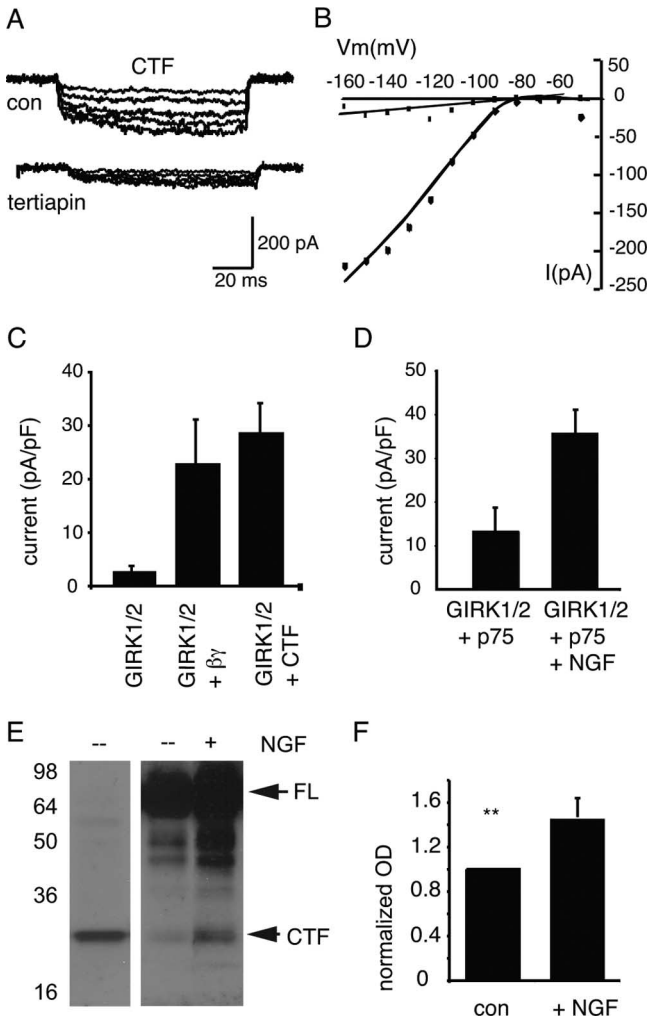

Figure 3. The C-terminal fragment of p75^{NTR} activates GIRK channels. HEK293T cells were transfected with combinations of GIRK 1 and 2 subunits, $\beta\gamma$ subunits, and CTF-p75 together with GFP for identification of positive cells (see Materials and Methods). Forty-eight hours after transfection, whole-cell recordings were made from GFP-positive HEK293T cells to ascertain GIRK channel activity. ***A***, Inward currents evoked by hyperpolarizing voltage steps from a holding potential of −60 mV were present in HEK293T cells transfected with GIRK1/2 and p75-CTF. The p75-CTF activated inward current was blocked by tertiapin (200 nM). The steady state current–voltage relationship before and after addition of tertiapin is shown in ***B***. ***C***, Summary of data from currents recorded at −140 mV in cells expressing only GIRK1/2, GIRK1/2, and $\beta\gamma$ subunits or GIRK1/2 and p75-CTF. ***D***, Summary of data from currents recorded in HEK293 cells expressing GIRK1/2 together with full-length p75^{NTR} with or without overnight treatment with NGF. NGF treatment stimulated a threefold increase in p75^{NTR}-mediated GIRK channel activity. ***E***, Representative Western blot of HEK293 cells expressing CTF (left lane), full-length p75^{NTR} with (right lane) or without (middle lane) overnight treatment with NGF. ***F***, Significant accumulation of the C-terminal fragment generated from full-length p75^{NTR} was promoted by NGF treatment (mean ± SD; $n = 4$; $^{**}p < 0.01$). con, Control.

experiments showed that at 50 and 80 mM potassium, there was significant caspase activity, whereas 110 and 140 mM potassium blocked caspase activity completely (Fig. 2*D*). This indicates that potassium efflux via GIRK channels is necessary for cell death initiated by the p75^{NTR} receptor.

The C-terminal fragment of p75^{NTR} activates GIRK channels

To directly test whether p75^{NTR} can gate GIRK channel activity, we made whole-cell recordings from HEK293T cells transfected with GIRK1/2 and p75-CTF. In cells expressing GIRK1/2 alone, GIRK channel activity was negligible, with an average current density of 2.9 ± 0.9 pA/pF ($n = 4$) (Fig. 3*C*). As expected (Clapham, 1997), expression of GIRK1/2 subunits together with $\beta\gamma$ subunits evoked a robust inward current (23.1 ± 8.9 pA/pF; $n = 8$) (Fig. 3*C*) that was blocked by 100–300 nM tertiapin ($n = 4$). Expression of GIRK1/2 together with p75-CTF also resulted in a prominent inward current (28.9 ± 5.4 pA/pF; $n = 12$) that activated near the potassium reversal potential (Fig. 3*A–C*) and was blocked by tertiapin (Fig. 3*A*,*B*) ($n = 4$), confirming that GIRK channels mediate this current.

We next tested whether full-length p75^{NTR}, either alone or after neurotrophin treatment, could activate GIRK channel activity. HEK293 cells expressing GIRK1 and GIRK2 together with full-length p75^{NTR} also displayed GIRK currents (13.6 ± 5.2 pA/pF; $n = 9$), although these were significantly smaller than those evoked by p75-CTF. However, treatment of these cultures with NGF led to a threefold increase in channel activity to 36.0 ± 8.2 pA/pF ($n = 4$; $p < 0.02$) (Fig. 3*D*).

To determine whether the NGF-stimulated increase in p75^{NTR}-mediated GIRK activity correlated with increased cleavage of p75^{NTR}, cell lysates after NGF treatment were examined for p75^{NTR} cleavage products. The amount of C-terminal fragment produced by cells expressing full-length p75^{NTR}, as determined by Western blotting, was clearly increased by exposure to NGF (Fig. 3*E*,*F*) ($n = 4$). These results show that p75^{NTR} undergoes a level of endogenous cleavage that is significantly increased by NGF treatment. The demonstration of ligand-induced increases in both the production of the C-terminal fragment from full-length p75^{NTR} and GIRK channel activity provides evidence that it is the cleaved form of p75^{NTR} that mediates GIRK channel activity and death signaling.

p75^{NTR} activates GIRK channels independent of G-proteins

G-protein-coupled receptor stimulation of GIRK channel activity by $\beta\gamma$ subunits and PtdIns(4,5)P_2 requires their binding to the cytoplasmic C-terminal domain of the channels (Mark and Herlitze, 2000; Sadja et al., 2003). GIRK channel activity initiated by p75-CTF may also require the C-terminal domain of GIRK channels, because cell death initiated by p75^{NTR} is inhibited by expression of chimeric GIRK182-IRK subunits (Fig. 2*B*) To confirm that GIRK182-IRK channels were not activated by p75^{NTR}, we made whole-cell recordings from HEK293T cells expressing GIRK1 and GIRK182-IRK subunits. Under these conditions, expression of p75-CTF or $\beta\gamma$ subunits failed to stimulate GIRK channel activity ($n = 5$). Thus, p75^{NTR}-mediated activation of GIRK channels requires the presence of the GIRK2 C-terminal domain. Because this domain contains binding sites for both $\beta\gamma$ subunits and PtdIns(4,5)P_2 (Mark and Herlitze, 2000; Sadja et al., 2003), this result suggests that p75^{NTR}-mediated activation of GIRK channels requires either $\beta\gamma$ subunits or PtdIns(4,5)P_2. We therefore coexpressed the carboxyl tail of the β-adrenergic receptor kinase (pβARK), which binds to and removes both PtdIns(4,5)P_2 and $\beta\gamma$ subunits (Touhara et al., 1995), together with GIRK1/2 subunits and p75-CTF in HEK293T cells. Cells coexpressing pβARK had a complete inhibition of p75^{NTR}-stimulated GIRK channel activity ($n = 3$), indicating that p75^{NTR} stimulates generation of either $\beta\gamma$ subunits or PtdIns(4,5)P_2 (or both). However, p75^{NTR} receptors are not coupled to G-proteins, and treatment of transfected HEK293T cells with pertussis toxin to inactivate $G_{i/o}$-proteins had no effect on either p75^{NTR}-stimulated GIRK channel activity ($n = 3$) or cell death (Fig. 4*A*), suggesting that activation of GIRK channels by p75^{NTR} is unlikely to result from the generation of $\beta\gamma$ subunits.

We therefore asked whether PtdIns(4,5)P_2 may be a required cofactor for p75^{NTR}-stimulated gating. To selectively sequester PtdIns(4,5)P_2, we coexpressed PH-PLCδ together with p75-CTF and GIRK1 and GIRK2. Expression of the PH-PLCδ domain resulted in a large reduction in p75-CTF-stimulated GIRK channel activity (6.8 ± 1.7 pA/pF; $n = 7$) compared with that observed in cells coexpressing GIRK1/2 and p75-CTF (28.9 ± 5.4 pA/pF; $n = 12$) (Fig. 4*B*,*C*). These results show that the C-terminal fragment of p75^{NTR} activates GIRK channels via a PtdIns(4,5)P_2-mediated mechanism.

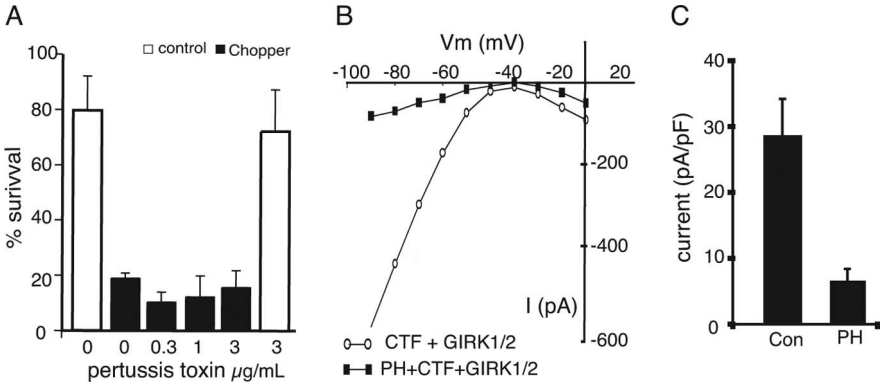

Figure 4. p75^{NTR} activates GIRK channels independent of G-proteins. ***A***, Survival of neurons pretreated overnight with pertussis toxin and subsequently treated for 2 h with Chopper or control peptides. No inhibition of Chopper-mediated cell death was observed (mean ± SD; $n = 3$). ***B***, Representative current–voltage relationship of HEK293T cells transfected with or without PH-PLCδ domain plasmid (PH) in combination with GIRK 1 and 2 subunits and CTF. Forty-eight hours after transfection, whole-cell recordings were made from EGFP-positive HEK293T cells in 30 mM extracellular potassium solution. The PH-PLCδ domain significantly reduced the CTF-mediated GIRK channel activity. ***C***, Summary data for steady-state currents evoked at −90 mV for control (Con) ($n = 12$) cells and those transfected with PH-PLCδ (PH; $n = 7$).

p75^{NTR} stimulates increased production of PtdIns(4,5)P_2 through Rac

To test whether p75^{NTR} regulates PtdIns(4,5)P_2 levels, we compared the levels of PtdIns(4,5)P_2 in cells expressing either p75^{NTR} or β-galactosidase. We first examined recruitment to the plasma membrane of PH-PLCδ fused to an EYFP marker (Stauffer et al., 1998). p75^{NTR}-expressing HEK293T (Fig. 5*A*,*B*) and Cos7 cells (data not shown) had significantly more membrane-localized PH-PLCδ-EYFP than control plasmid-transfected cells, indicating that p75^{NTR} upregulates PtdIns(4,5)P_2 generation. To determine whether full-length p75^{NTR} or its endogenously produced C-terminal fragment increased PtdIns(4,5)P_2 generation, we tested the mutant p75-Nglycos that is resistant to extracellular cleavage. Cells expressing PH-PLCδ-EYFP together with p75-Nglycos had significantly less PtdIns(4,5)P_2 than those expressing wild-type p75^{NTR} (Fig. 5*B*), indicating that both PtdIns(4,5)P_2 generation and GIRK channel activity are mediated by the C-terminal fragment of p75^{NTR}. PtdIns(4,5)P_2 levels were next measured in adenovirus-infected control and p75^{NTR}-expressing Cos7 cells by radiolabeling with ^{32}P-orthophosphate before lipid extraction and analysis by thin-layer chromatography. p75^{NTR}-expressing cells were again found to generate significantly more PtdIns(4,5)P_2 than control virus-infected cells (Fig. 5*C*,*D*).

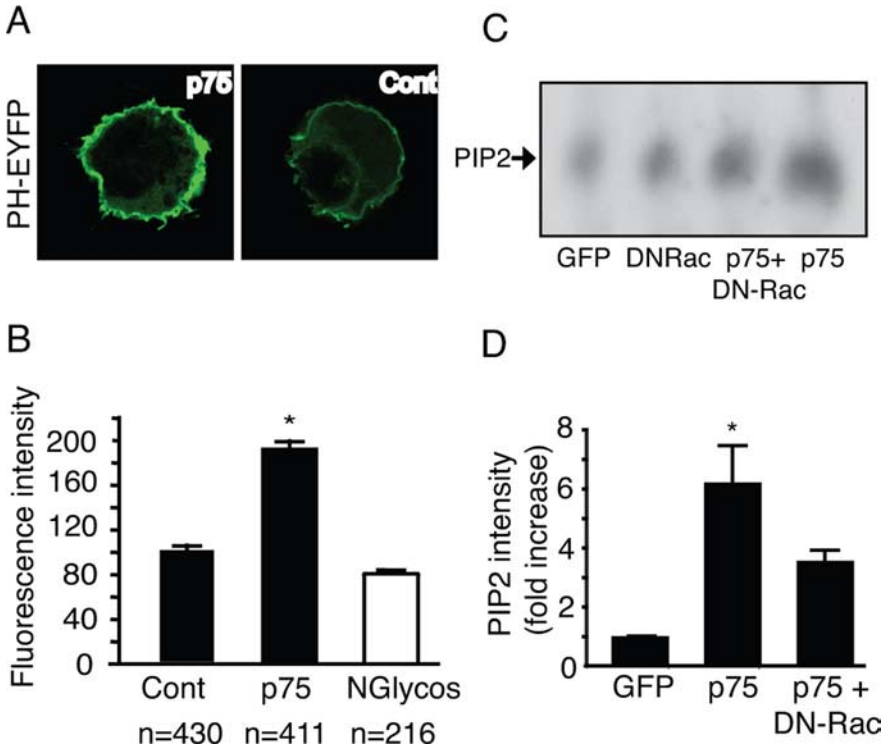

Figure 5. p75^{NTR} stimulates increased production of PtdIns(4,5)P_2. ***A***, Confocal images of HEK293T cells coexpressing PH-PLCδ-EYFP and either β-galactosidase (right) or p75^{NTR} (left). ***B***, HEK293T cells expressing p75^{NTR} had significantly higher levels of membrane-localized EYFP. HEK293T cells expressing a noncleavable form of p75^{NTR} (Nglycos) had significantly lower levels of membrane-localized EYFP (mean ± SD; $*p < 0.01$). ***C***, Cos7 cells expressing EGFP, DN-Rac, p75^{NTR}, or p75^{NTR} and DN-Rac were labeled with [γ-^{32}P]-orthophosphate, and cellular lipids were extracted and separated on oxalate-treated TLC plates before exposure to x-ray film or phosphoimage plates. ***D***, The significant increase in generation of PtdIns(4,5)P_2 (PIP2) by p75^{NTR} was inhibited by blocking Rac activity (mean ± SD; $n = 3$; $*p < 0.05$).

Because neurotrophin-activated p75^{NTR} death signaling activates and requires the GTPase Rac (Harrington et al., 2002), which in turn can stimulate PI5K (type I phosphatidylinositol 5 kinases) activity and thereby increase PtdIns(4,5)P_2 levels (Hinchliffe, 2000; Oude Weernink et al., 2004), we next investigated the role of Rac in p75^{NTR}-mediated PtdIns(4,5)P_2 generation. We found that, although expression of dominant-negative Rac (DN-Rac) had little effect on basal PtdIns(4,5)P_2 levels, Cos7 cells infected with DN-Rac together with p75^{NTR} had intermediate PtdIns(4,5)P_2 levels (Fig. 5*C*,*D*). Moreover, DN-Rac significantly reduced GIRK channel activity stimulated by p75-CTF-expressing cells (DN-Rac/p75-CTF/GIRK1/2, 16.9 ± 8.03 pA/pF; $n = 7$ cf. β-gal/p75-CTF/GIRK1/2). These results indicate that p75^{NTR} signals regulate PtdIns(4,5)P_2 and GIRK channel activity via Rac.

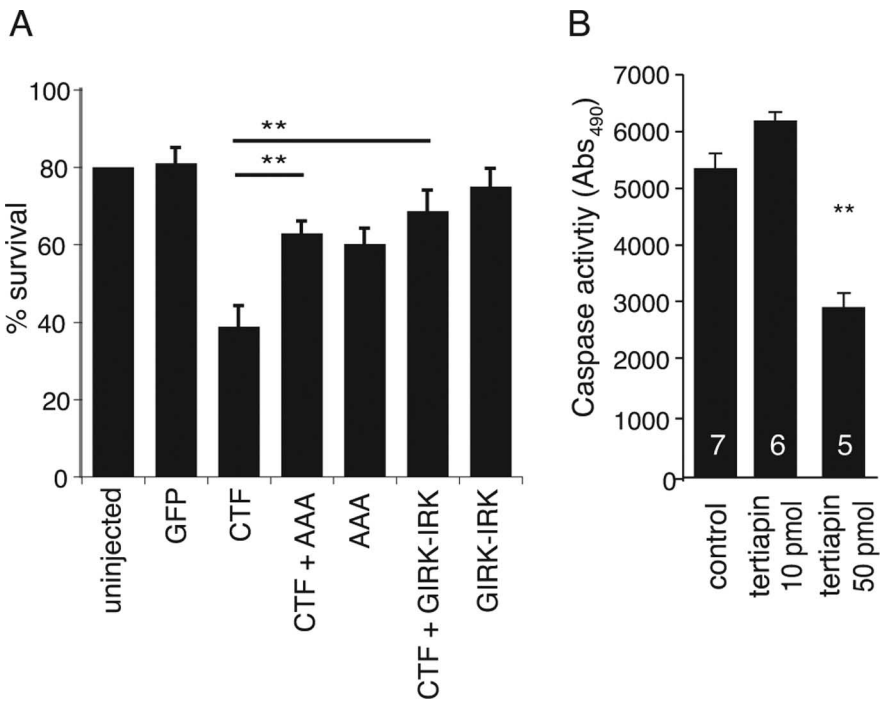

Figure 6. p75^{NTR}-mediated DRG neuronal death requires potassium flux though GIRK channels. ***A***, Survival of DRG neurons 16 h after microinjection with GFP or p75-CTF alone or together with pGIRK2-AAA or pGIRK2/IRK expression constructs after 24 h in NGF. Expression of these subunits in the absence of death signaling had no effect on survival but prevented p75-CTF-mediated neuronal death (mean ± SD; $n \geq 3$; $^{**}p < 0.01$). ***B***, Eyes of E4.5 chicks were injected with 1 μl of water containing the potassium channel inhibitor tertiapin. After 20 h, the retinal ganglion cell layer was assayed for caspase activity (see Materials and Methods). Tertiapin (50 pmol) reduced the activity significantly ($^{**}p = 0.0011$). The number of retinas is indicated per condition (representative of 3 experiments).

GIRK channel activity is required for p75^{NTR}-mediated neuronal death

We have shown that the death sequence activated by neurotrophin stimulation of full-length p75^{NTR} or its C-terminal fragment requires activation of GIRK channels and efflux of cytosolic potassium. To test whether this pathway mediates neuronal cell death, we used the pGIRK-182-IRK chimeric subunit as well as the pore mutant GIRK 2 subunit (pGIRK2-AAA), neither of which could be activated by p75^{NTR} ($n = 4$). Both subunits act in a dominant-negative manner when incorporated with other subunits to form a channel (Kuzhikandathil and Oxford, 2000). Expression of these mutant GIRK2 subunits individually in cultured DRG neurons had no effect on neuronal survival (Fig. 6*A*). In contrast, activation of p75^{NTR} death signaling by overexpression of p75-CTF in DRG neurons resulted in significant cell death (Fig. 6*A*), which was inhibited by coexpression of either of the GIRK2 mutant constructs (Fig. 6*A*), presumably by sequestering functional GIRK subunits expressed by these neurons (supplemental Figs. 3, 4, available at www.jneurosci.org as supplemental material). These results show that preventing activation of and/or ion flux through endogenous GIRK channels can inhibit p75^{NTR}-mediated neuronal death.

To further investigate the physiological significance of our findings *in vivo*, we tested a robust model, the developing chick retina, where neurotrophin activation of p75^{NTR} (Frade et al., 1996) has been shown to mediate cell death through the Chopper domain (Coulson et al., 2000). We found that injection of the GIRK channel antagonist tertiapin into the developing chick eye significantly reduced cell death (Fig. 6*B*). These results suggest that efflux of potassium through endogenous GIRK channels is required for p75^{NTR}-mediated neuronal death *in vitro* and *in vivo*.

Discussion

p75^{NTR} is widely expressed during the developmental period of programmed cell death and is re-expressed in the adult in response to injury, where it has consistently been shown to be responsible for the cell death accompanying neurodegeneration. In this study, we defined a cellular pathway initiated by p75^{NTR} that leads to cell death. Our results point to a previously unidentified involvement of GIRK channels in the p75^{NTR}-mediated death signaling pathway and a novel mechanism of GIRK channel activation through the generation of PtdIns(4,5)P_2.

p75^{NTR}-mediated cell death requires GIRK channel activity

The present study has demonstrated that overexpression of either full-length p75^{NTR} or its C-terminal fragment induces caspase activation and death of HEK293 cells by a mechanism that requires functional GIRK channels and is accompanied by significant potassium efflux. Similarly, death of DRG neurons induced *in vitro* by the C-terminal fragment of p75^{NTR} was also prevented by expression of dominant-negative GIRK2 subunit plasmids (Lewohl et al., 1999; Sadja et al., 2003). Using electrophysiological recordings, we have also shown that coexpression of p75^{NTR} activates GIRK1/2 channels in HEK293 cells. These results suggest that p75^{NTR} activates GIRK channels and that cell death is initiated by the resultant potassium efflux.

Functionally, tertiapin blocked both Chopper domain-induced DRG neuron death and naturally occurring developmental retinal ganglion cell death signaling *in vivo*, which is neurotrophin dependent and requires p75^{NTR} (Frade et al., 1996). Although this does not definitively demonstrate the specificity of the actions of tertiapin on NGF-mediated cell death, the inhibition was of a similar magnitude to that seen with anti-p75^{NTR} antibodies, anti-NGF hybridoma cells (Frade et al., 1996), or Chopper domain-blocking peptides (Coulson et al., 2000). Notably, whereas both full-length p75^{NTR} as well its C-terminal fragment promote cell death, the actions of the full-length receptor are significantly less than that of the C-terminal fragment (Coulson et al., 2000). Our demonstration that the amplitude of GIRK currents evoked by full-length p75^{NTR} are smaller than those evoked by the C-terminal fragment, and that both cell death and GIRK channel activity are enhanced by NGF treatment is consistent with initiation of cell death by GIRK channel activity. Together, our results support the idea that binding of neurotrophin to p75^{NTR} leads to activation of GIRK channels through second messengers that are activated by the juxtamembrane (Chopper) region of the p75^{NTR} C-terminal fragment. This GIRK channel activity is a necessary first step in initiating the death cascade.

Despite debate over the last decade, the p75^{NTR} death signal is thought to be principally ligand activated (Frade et al., 1996; Bamji et al., 1998; Lee et al., 2001; Kenchappa et al., 2006). More

recently, it has been proposed that p75^{NTR} is activated by pro-neurotrophins (Lee et al., 2001). However, this model cannot fully account for the range of ligand and receptor combinations though which p75^{NTR}-mediated cell death can occur. We suggest that the activation step is likely to involve cleavage of p75^{NTR} (Fig. 7) (Coulson et al., 2004), a process that can occur after stimulation by either mature or pro-neurotrophin ligands (Kenchappa et al., 2006; our study). Consistent with this, stimulation of metalloprotease cleavage of full-length p75^{NTR} by neurotrophin treatment correlated with significantly increased GIRK activity, and the noncleavable form of full-length p75^{NTR} failed to increase PtdIns(4,5)P_2 levels or caspase activity.

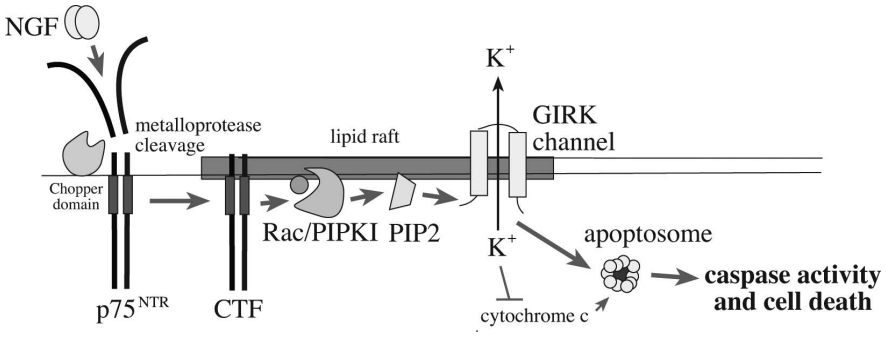

Figure 7. Model of the proposed p75^{NTR}- and GIRK channel-mediated cell death signaling pathway. Death signaling is activated by metalloprotease cleavage of p75^{NTR} after stimulation by mature or proneurotrophins (NGF). The C-terminal fragment of p75^{NTR} activates Rac, which in turn activates PIPKI, with signal transduction assisted by colocalization of signaling molecules within lipid raft-like membrane domains. The locally generated PtdIns(4,5)P_2 (PIP2) then binds to the C-terminal domain of neighboring GIRK2 subunits, activating GIRK1/2 heterotrimeric channels. The resulting efflux of potassium through the GIRK channels lowers the intracellular potassium concentration, releasing inhibition of the apoptosome, and resulting in capsase activation and ultimately cell death.

Although extracellular cleavage of p75^{NTR} precedes release of the intracellular domain by γ-secretase cleavage (Kanning et al., 2003; Underwood et al., 2008), the C-terminal fragment protein used herein is not a substrate for γ-secretase (Fig. 3*E*). Consequently, the GIRK channel-dependent cell death pathway is expected to be activated by metalloprotease cleavage of p75^{NTR} and mediated by the resultant C-terminal fragment before γ-secretase cleavage (Fig. 7). This is in contrast to a previous report showing that generation of the intracellular domain fragment of p75^{NTR} is required for cell death (Kenchappa et al., 2008). However, this study used neurons cultured in a high extracellular concentration of potassium, which could have protected against C-terminal fragment-induced death (Underwood et al., 2008) by preventing potassium efflux through GIRK channels. Nonetheless, because there were also other differences between the culture conditions, the exact reason for the contrasting results remains unclear.

Based on our findings, we propose that GIRK channel activity is an essential step in initiating the cell death cascade. In this model, proneurotrophins (Lee et al., 2001; Kenchappa et al., 2006), or any stimulus that increases generation of the C-terminal fragment of p75^{NTR} (Underwood et al., 2008), would similarly activate this death pathway.

p75^{NTR} activates GIRK channels through PtdIns(4,5)P_2 binding

Neurotransmitter receptor-mediated gating of GIRK channels is thought to be a membrane-delimited pathway that requires activation of pertussis-sensitive G-proteins and release of βγ subunits that bind to and activate the channel (Clapham, 1997; Stanfield et al., 2002). Although GIRK channels can also be activated by PtdIns(4,5)P_2 alone, their activation by βγ subunits is believed to have a mandatory requirement for PtdIns(4,5)P_2 (Huang et al., 1998; Stanfield et al., 2002). Consistent with this, a number of studies have shown that hydrolysis of PtdIns(4,5)P_2 by receptor-mediated activation of phospholipase C underlies inhibition of these channels by a range of neurotransmitters (Kobrinsky et al., 2000; Stanfield et al., 2002; Sohn et al., 2007). Our demonstration that p75^{NTR} can effectively activate GIRK channels via a βγ subunit-independent mechanism challenges this view. Molecular interference (pGIRK2-IRK; pβARK) with the PtdIns(4,5)P_2-binding domain and sequestration of PtdIns(4,5)P_2 (PH-PLCδ) inhibited both channel activity and p75^{NTR}-mediated cell death. Furthermore, expression of wild-type p75^{NTR} but not a nonmetalloprotease-cleavable form of p75^{NTR} resulted in significantly increased cellular levels of PtdIns(4,5)P_2.

PtdIns(4,5)P_2 generation was mediated through Rac1 GTPase activity, a necessary downstream component of the p75^{NTR} cell death pathway (Harrington et al., 2002). It has been shown previously that PIPKI, which is responsible for the majority of cellular PtdIns(4,5)P_2 generation from PtdIns(4)P, are recruited to the plasma membrane and directly activated by Rac (Carpenter et al., 1999; Hinchliffe, 2000; Osborne et al., 2001; Oude Weernink et al., 2004). Given the affinity of activated Rac, PtdIns(4,5)P_2, and GIRK channels for cholesterol-rich membrane domains (Hinchliffe, 2000; Koyrakh et al., 2005), it is possible that this signaling cascade occurs locally within subregions of the plasma membrane of the cell (Fig. 7). Although, the pathway by which p75^{NTR} activates Rac has not been characterized, p75^{NTR} can be palmitoylated (Barker et al., 1994), a posttranslational modification that can play an important role in regulating signaling by spatially segregating proteins into cholesterol-rich membrane domains. Furthermore, nonpalmitoylatable mutant forms of p75^{NTR} fail to induce neuronal death (Coulson et al., 2000; Underwood et al., 2008) and do not activate GIRK channel activity ($n = 5$) (data not shown), supporting a model whereby the p75^{NTR}-mediated signaling pathway that activates GIRK channels is membrane delimited (Fig. 7).

Intracellular potassium levels regulate cell death

The role of cytosolic potassium in regulating cell survival and death has been widely reported, but rarely has the flux been linked directly to the activation of a specific potassium channel. We show that potassium efflux through activated GIRK channels and the resultant reduction in cytosolic potassium promotes formation of the apoptosome and activation of caspases, because maintenance of physiological levels of potassium, either exogenously *in vitro* or through use of the GIRK channel inhibitor tertiapin *in vivo*, prevented caspase activity. Others have similarly found that efflux of potassium is an early and key event regulating apoptosis in neuronal (Yu et al., 2001) and nonneuronal (Hughes et al., 1997; Maeno et al., 2000) cells through regulation of the apoptosome (Cain et al., 2001). It is also coincident with the appearance of the apoptotic marker annexinV on the extracellular lipid membrane (Dallaporta et al., 1998), is associated with activation of jun kinase phosphorylation pathways (Wang et al., 1999), and promotes DNA laddering and cleavage of pro-caspase

3 to its active form (Hughes et al., 1997; Maeno et al., 2000). Because all these events are known to occur in cells undergoing p75^{NTR}-mediated death (Coulson et al., 2004), the most likely action of GIRK channel activity in the p75^{NTR} death signaling pathway is mediation of the release of apoptosome inhibition.

A new function for GIRK channels controlling p75^{NTR}-mediated signaling

Although GIRK channels have a relatively well characterized role in controlling neuronal excitability by mediating the actions of inhibitory neurotransmitters in adult neurons, a role for GIRK channels before the development of mature neuronal circuitry has not been investigated widely. It has been suggested that GIRK channel action during early postnatal development regulates maturation of functional circuits at a time when activity is primarily excitatory (Karschin and Karschin, 1997; Sickmann and Alzheimer, 2002). We propose that GIRK channels participate in the fundamental process of neuronal selection that occurs both in development and in neurodegenerative disease, as a checkpoint in the p75^{NTR}-mediated death pathway. Refinement of neurite connectivity is another process in which p75^{NTR} is involved, and the p75^{NTR}-mediated inhibition of axon growth is also prevented by excitatory activity and depolarization (Singh and Miller, 2005). Therefore, p75^{NTR}-mediated axonal growth inhibition by neuronal excitation may also involve GIRK channel activation. Our demonstration that p75^{NTR} activates and requires potassium flux through GIRK channels in order for neuronal death to occur provides the first evidence of a molecular link between factors that control neuronal excitability and growth factor signaling in the regulation of neuronal selection, and identifies a new role of GIRK channel activity.

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