Activation of protein kinase CβI constitutes a new neurotrophic pathway for deafferented spiral ganglion neurons

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Summary

In mammals, degeneration of peripheral auditory neurons constitutes one of the main causes of sensorineural hearing loss. Unfortunately, to date, pharmacological interventions aimed at counteracting this condition have not presented complete effectiveness in protecting the integrity of cochlear neural elements. In this context, the protein kinase C (PKC) family of enzymes are important signalling molecules that play a role in preventing neurodegeneration after nervous system injury. The present study demonstrates, for the first time, that the PKC signalling pathway is directly neurotrophic to axotomised spiral ganglion neurons (SGNs). We found that PKCβI was strictly expressed by postnatal and adult SGNs both in situ and in vitro. In cultures of SGNs, we observed that activators of PKC, such as phorbol esters and bryostatin 1, induced neuronal survival and neurite regrowth in a manner dependent on the activation of PKCβI. The neuroprotective effects of PKC activators were suppressed by pre-treatment with LY294002 (a PI3K inhibitor) and with U0126 (a MEK inhibitor), indicating that PKC activators promote the survival and neurite outgrowth of SGNs by both PI3K/Akt and MEK/ERK-dependent mechanisms. In addition, whereas combining the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) was shown to provide only an additive effect on SGN survival, the interaction between PKC and neurotrophin signalling gave rise to a synergistic increase in SGN survival. Taken together, the data indicate that PKCβI activation represents a key factor for the protection of the integrity of neural elements in the cochlea.

Key words: Cochlea, Peripheral nervous system, PKC, Neuroprotection, Signal transduction, Neuritogenesis

Introduction

Primary afferent auditory neurons reside in the spiral ganglion (SG) and are bipolar neurons that extend a peripheral process to the hair cells in the organ of Corti and a central process to the cochlear nucleus. Damage or loss of these spiral ganglion neurons (SGNs) is an important component of hearing impairment, and their long-term survival is critical for the success of cochlear prostheses. Over the past decade, a variety of molecules have been experimentally applied to protect SGNs, including neurotrophins (Ernfors et al., 1996; Shinohara et al., 2002; Zheng et al., 1995), depolarising agents (Hegarty et al., 1997; Kanzaki et al., 2002), caspase inhibitors (Lallemend et al., 2003; Liu et al., 1998) and JNK antagonists (Pirvola et al., 2000). However, until now, the clinical use of these trophic agents has remained difficult, with limited performance. This may be essentially due to the fact that all these studies have concentrated on blocking just one of the individual death-related phenomena leaving the door open for other processes to produce cellular death. Accordingly, the challenge is to identify additional pharmacological agents that interact with other useful therapeutic targets and, as a priority, to improve the advances in our understanding of the basic molecular mechanisms involved in the control of cochlear neurons survival.

In this context, regarding the extensive evidence supporting a specific role in influencing neuronal survival, protein kinase C (PKC) activation may be relevant as a candidate for SGN survival regulation. In mammals, the PKC enzyme family is heterogeneous and comprises at least twelve known isoforms divided into three major subsets according to their sensitivity to the second messengers Ca^{2+} and diacylglycerol (DAG) (Poole et al., 2004). The conventional PKCs (α, βI, βII and γ) are regulated by both Ca^{2+} and DAG, the novel PKCs (δ, ϵ, η, θ, μ and ν) are insensitive to Ca^{2+} but respond to DAG, and the atypical PKCs (ζ, λ and PKMζ) are regulated by neither DAG nor Ca^{2+}. Several PKC isoforms are commonly co-expressed in the same cell, where they are believed to have different functions. Subcellular distribution, enzyme-protein interactions of the inactive isoforms and different activation requirements are general concepts used to explain the functional specificity of these isoforms. The specific role of PKCs in influencing neuronal death is complex. PKC activation promotes neuronal survival of chick sympathetic and sensory neurons (Bhave et al., 1990; Wakade et al., 1988) and

reduces serum-deprivation-induced death of cerebellar granule neurons (Zirrgiebel et al., 1995). Moreover, PKC inhibitors induce apoptosis in cortical (Koh et al., 1995) and cerebellar granule neurons (Zirrgiebel et al., 1995), as well as in neuronal cell lines (Behrens et al., 1999; Zhang et al., 1995). However, PKC activation does not attenuate apoptosis of rat sympathetic ganglion neurons induced by serum or nerve growth factor (NGF) deprivation (Creedon et al., 1996; Martin et al., 1992).

One of the most ubiquitous families of growth factors involved in SGN survival is the neurotrophin family, which signals through the Trk family of receptor tyrosine kinases. Binding of neurotrophins to their respective Trk receptors can virtually activate at least three major signalling pathways in neurons: the mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase (MEK/ERK), phosphoinositide 3-kinase (PI3K)/Akt and phospholipase Cγ1 (PLCγ1) pathways (Huang and Reichardt, 2003; Kaplan and Miller, 2000), whose the relative functional contribution can differ according to the neuronal cell type. In the cochlea, conventional PKCs (cPKCs) have also been hypothesised to be involved in synaptic repair of auditory neuron dendrites after overstimulation of glutamate receptors, an in vivo model of sudden deafness after acoustic trauma or ischemia (Lerner-Natoli et al., 1997). In the experiments presented here, we investigated the role of PKC activation in SGN survival and neuritogenesis. We found that the PKCβI isoform is exclusively expressed in neural elements of the cochlea and that PKC activators, such as phorbol esters and bryostatin 1, a new safe anti-cancer agent (Clamp and Jayson, 2002), rescue SGNs from cell death and enhance neuritic outgrowth in vitro via the activation of PKCβI.

On studying the intracellular pathways involved in these trophic actions, we showed that PI3K and MEK/ERK are required for the survival-promoting effect of both neurotrophins and PKC activators, whereas PKCβI is only involved in PKC-activator-induced neuroprotection. Moreover, when assuming the additive effect on SGN survival using PKC activators and neurotrophins, of particular relevance was the first demonstration that these two extrinsic cues actually synergise to regulate neuronal survival in the cochlea.

Materials and Methods

Chemicals

Poly-L-ornithine, DNAse, U0126, LY294002, 4β-phorbol 12-myristate 13-acetate (PMA), 4α-PMA, phorbol 12,13-dibutyrate (PDBu), Gö6976, genistein, U73122, BAY 11-7082, sulfasalazine, LiCl, cytosine arabinoside (AraC), fluorodeoxyuridine (FUDR) and bryostatin 1 (bryo) were purchased from Sigma (St Louis, MO). Papain and ovalbumin (ovomucoid with bovin serum albumin) were obtained from Worthington Biochemical (Lakewood, NJ). GF109203X and laminin were from ICN Pharmaceuticals (Costa Mesa, CA). Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies (Rockville, MD). LY333531 was purchased from AG Scientific (San Diego, CA). Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT3) were from Peprotech (Rocky Hill, NJ).

Primary cultures of spiral ganglion neurons

Treatment of animals was in accordance with Belgian Ministry of Agriculture guidelines in agreement with EEC laboratory animal care and use regulations. Experiments were performed on Wistar rat spiral ganglion neurons (SGNs) from postnatal day (P) 5-6 or adult rats. Rats obtained from our rodent breeding facility were euthanised and decapitated. Mandibles were removed, bullae exposed and temporal bones excised and transferred into Petri dishes containing phosphate buffered saline (PBS) supplemented with glucose (6 g/l). With the aid of a dissecting microscope and watchmaker's forceps, the cochlea was isolated and its spiral ganglion excised. Spiral ganglia were incubated for 45 minutes at 37°C in a papain-DNAse solution (1.5 ml of papain at 1 mg/ml, 0.5 ml of DNAse at 0.1%; 20 spiral ganglia/2 ml solution). Enzymatic activity was terminated by adding ovalbumin (0.5 ml of a 10 mg/ml solution). The explants were then washed with DMEM supplemented with N1 additives (Bottenstein and Sato, 1979) and glucose (6 g/l, final concentration). Mechanical dissociation of the spiral ganglia tissue was achieved by trituration with a siliconised Pasteur pipette. For cell survival experiments, the resulting ganglion cell suspension was seeded in 96-well plates (Nunc, Rockside, Denmark; 50 μl per well; 5000 neurons/well for the P5-6 ganglia) previously sequentially coated with poly-L-ornithine (0.1 ng/ml in 15 mM borate buffer) for 1 hour and laminin (10 μg/ml in PBS) for 2 hours at 37°C. For determination of DNA fragmentation, proliferation assays and the PKC activation analyzes, cells were plated on coverslips in 4-well chamber slides (Nunc). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Immunostainings

For immunohistochemistry, rat cochleae were fixed in 4% paraformaldehyde at 4°C overnight and transferred into a solution of 30% sucrose in PBS for an additionnal overnight incubation at 4°C. Cochleae were then embedded in Tissue Tek (Sakura, the Netherlands) and frozen at –80°C. Frozen 10-20 μm sections were prepared using a 2800 Frigocut cryostat (Reichert-Jung, Cambridge Instruments, Germany). Neuronal cultures or cryostat sections were fixed with 4% paraformaldehyde (10 minutes at 20°C). The preparations were then incubated overnight at 4°C with primary antibodies, i.e. anti-βIII tubulin (1/1500; clone TUJ1, Babco, USA), a specific marker for neuronal lineage (Fanarraga et al., 1999), and anti-PKC (1/100, Santa Cruz Inc., CA, USA): anti-PKCα (C-20, sc-208), anti-PKCβI (C-16, sc-209), anti-PKCβII (C-18, sc-210) and anti-PKCγ (C-19, sc-211). Three washes in PBS were performed before incubation for 1 hr at 20°C, with a secondary antibody conjugated to either peroxidase (Dako, Glostrup, Denmark), FITC (fluorescein isothiocyanate) or TRITC (tetramethylrhodamine B isothiocyanate) (Jackson ImmunoResearch Laboratories, West Grove, PA) fluorophores. After three rinses in PBS, the immunofluorescent preparations were counterstained with the nuclear dye Topro-3 (Molecular Probes, Leiden, The Netherlands) and were mounted on microscope slides, mounted in Vectashield (Vector, Burlingame, CA), and imaged using a Bio-Rad MRC 1024 laser scanning confocal microscope. Peroxidase-labelled cells were revealed using the DAKO Liquid DAB+ kit, a high sensitivity substrate-chromogen system (DAKO Diagnostics, Heverlee, Belgium), and then examined with an inverted microscope (Zeiss, Axiovert 100, Germany). Negative controls were carried out by omission of the primary antibody or by using the blocking peptide for each antibody (data not shown).

Neuronal survival and neuritogenesis determination

At the end of the culture period, cells were fixed and immunostained for TUJ1 and peroxidase secondary antibody as described above. Criteria used to determine neuronal viability were: (1) TUJ1 positivity, with a large labelled cytoplasm; and (2) an absence of nuclear pyknosis. The measure of the extent of neuritogenesis was achieved as previously described (Lefebvre et al., 1994). Briefly, the number of neurons bearing neuritic processes that were of a defined length of at least three neuronal cell body diameters were counted. Neurites were counted only if they had an obvious attachment to the

neuronal soma. The neuritic index was calculated as the ratio of neurons bearing neurites of the defined length or greater per total number of neurons.

Semi-quantitative RT-PCR

Total RNAs from P5 Wistar rat spiral ganglia (20 spiral ganglia per sample) or brain were extracted and purified using the RNAgents Total RNA Isolation System kit (Promega, Leiden, The Netherlands). First strand cDNA synthesis was performed under the following conditions: 1 μg of total RNA was incubated with 1 μl oligo(dT) (0.5 mg/ml, Invitrogen) at 65°C for 10 minutes, then at 4°C for 5 minutes. A reverse transcription reaction mixture was then added to the RNA-oligo (dT) sample for a final volume of 20 μl, containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 500 μM dNTPs, 10 mM DTT and 200 U superscript II reverse transcriptase (Invitrogen). The sample was incubated at 42°C for 1 hour. Two microlitres of the first-strand cDNA synthesis reaction were used as a template and added to 50 μl of PCR reaction mixture containing 0.5 μM of both forward and reverse primers synthesised by Eurogentec (Seraing, Belgium), 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, and 1.25 U of Taq Polymerase (Invitrogen). The PCR program was run with an MJ Research PTC 200 instrument. The thermal cycling protocol started with a 3 minute pre-incubation at 94°C followed by 31 cycles made (1) 30 seconds at 94°C, (2) 30 seconds at the optimal annealing temperature, and (3) 30 seconds at 72°C. The protocol was finally completed by an extension step at 72°C for 9 minutes. We used 60°C for the annealing of PKCα and γ and 58°C for PKCβI and βII. Five microlitres of the PCR reaction was analysed in a 2% agarose gel in Tris-acetic acid-EDTA (TAE) buffer. To exclude possible PCR contaminations, negative controls for amplification consisted of RNA processed without the addition of reverse transcriptase. The sequences of the primer pairs that were used were as follows: rat PKCα (product size, 323 bp): forward, 5′-TGAACCCTCAGTGGAATGAGTCCT, reverse, 5′-ATGGCTGCTTCCTGTCTTCTGAAG (Dwivedi and Pandey, 1999); rat PKCβI (product size, 454 bp): forward 5′-ATCTGGGATGGGGTGACAAC (Yoshimura et al., 1997) reverse 5′-GGAGTCAGTTCCACAGGCTG; rat PKCβII (product size, 446 bp): forward 5′-ATCTGGGATGGGGTGACAAC, reverse 5′-TAGG-ACTGGTGGATGGCGGG (Yoshimura et al., 1997); rat PKCγ (product size 305 bp): forward, 5′-CGGGCTCCCACATCAGA-TGAG; reverse, 5′-AGTAGCTCTGAGACACCAAAG (Dwivedi and Pandey, 1999). The co-amplification of an internal control housekeeping β-actin protein mRNA was performed by using a forward primer (5′-GATCTTGATCTTCATGGTGCTAGG) and a reverse primer (TTGTAACCAACTGGGACGATATGG) that amplified a 730 bp cDNA fragment. The gels were scanned using a Typhoon 9200 Scanner (Amersham Biosciences, The Netherlands) and subsequent analyses were performed with ImageQuant Software (Amersham Biosciences). Values presented were derived from densitometry arbitrary units.

Western blot analysis

About 20 spiral ganglia (representing one sample) from P5-6 rat pups were lysed on ice using 100 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM DTT, 1 mM sodium orthovanadate and protease inhibitor cocktail (Roche)]. After 30 minutes, the lysate was cleared of insoluble debris by centrifugation for 10 minutes at 11,000 ***g***. The supernatant was collected, and the protein concentration was determined using the Bradford method, as previously described (Bradford, 1976). Protein lysates (30 μg of proteins) were then mixed with an equal volume of gel loading buffer (glycerol 20%, SDS 4%, Tris 100 mM, β-mercaptoethanol 5% and Bromophenol Blue) before being boiled for 3 minutes. After boiling, proteins were loaded onto a 10% SDS-PAGE. The proteins were then transferred on a polyvinylidene difluoride membrane (Amersham, Roosendaal, The Netherlands) by semi-dry electroblotting in transfer buffer (glycine 192 mM, Tris 25 mM and methanol 20%). Blots were then blocked for 1 hour at room temperature in blocking buffer [0.2% I-BLOCK (Tropix, Bedford, MA) diluted in TBS supplemented with 0.05% Tween 20 (Bio-Rad, Nazareth, Belgium) (TTBS)]. The primary antibodies directed against cPKC isoforms (Santa Cruz Inc., CA) were incubated for 1 hour at room temperature in the blocking buffer. Peroxidase-conjugated monoclonal anti-rabbit antibodies (clone RG-16, Sigma Aldrich, 1:3000) were incubated 1 hour at room temperature. Blots were then washed extensively and developed by using enhanced chemoluminescence (Pierce, Aalst, Belgium).

Analysis of DNA fragmentation

Internucleosomal DNA cleavage was assessed by the conventional terminal deoxynucleotidyltransferase-mediated UTP nick end-labelling (TUNEL staining) assay, according to the manufacturer's instructions (Intergen, Oxford, UK). Briefly, cell cultures were fixed with 4% paraformaldehyde for 10 minutes at 20°C. Equilibration buffer was then applied for 5 minutes at 20°C and the cultures were incubated with working strength TdT enzyme for 1 hour at 37°C. This reaction was stopped with working strength stop/wash buffer for 10 minutes and, after washing, cultures were immunostained for βIII tubulin and observed using a confocal microscope, as previously described.

Bromodeoxyuridine incorporation assays

At the beginning of the culture, bromodeoxyuridine (BrdU) (Sigma), which is an S-phase marker, was added to the cultures (at a final concentration of 20 μM) for 15 or 24 hours before fixation and staining. All treatments were performed simultaneously with the addition of BrdU. At the end of the culture period, coverslips were fixed for 10 minutes in 4% (v/v) paraformaldehyde, permeabilised in 0.1% Triton X-100 for 10 minutes and incubated in HCl 2 N for 15 minutes at 37°C. Next, cultures were incubated in borate buffer (0.1 M; pH 9) for 2 minutes before being rinsed in PBS. Cultures were then incubated with an anti-BrdU FITC-conjugated antibody for 45 minutes (1:3, v/v; Becton-Dickinson). After three rinses in PBS, cells were counterstained with propidium iodide (1/200, 3 minutes; Sigma) in order to visualise all nuclei for the determination of the total cell number. The preparations were mounted in Vectashield and imaged using a Bio-Rad MRC1024 laser scanning confocal microscope. The fraction of cells that incorporated BrdU was determined by counting five non-overlapping microscopic fields (Axiovert 135 fluorescence microscope, 20× objective, Zeiss) for each coverslip. Each condition was applied in duplicate and repeated on three different occasions.

Statistical analysis

Statistical analyses were performed by one-way ANOVA using a GraphPad Prism program (GraphPad, San Diego, CA). Each experiment was performed in triplicate and repeated on at least three different occasions. Individual comparisons were performed using Dunnett's post hoc tests. Data were expressed as the mean±s.d. and results were considered statistically significant if $P<0.05$ for both the one-way ANOVA and the Dunnett's test.

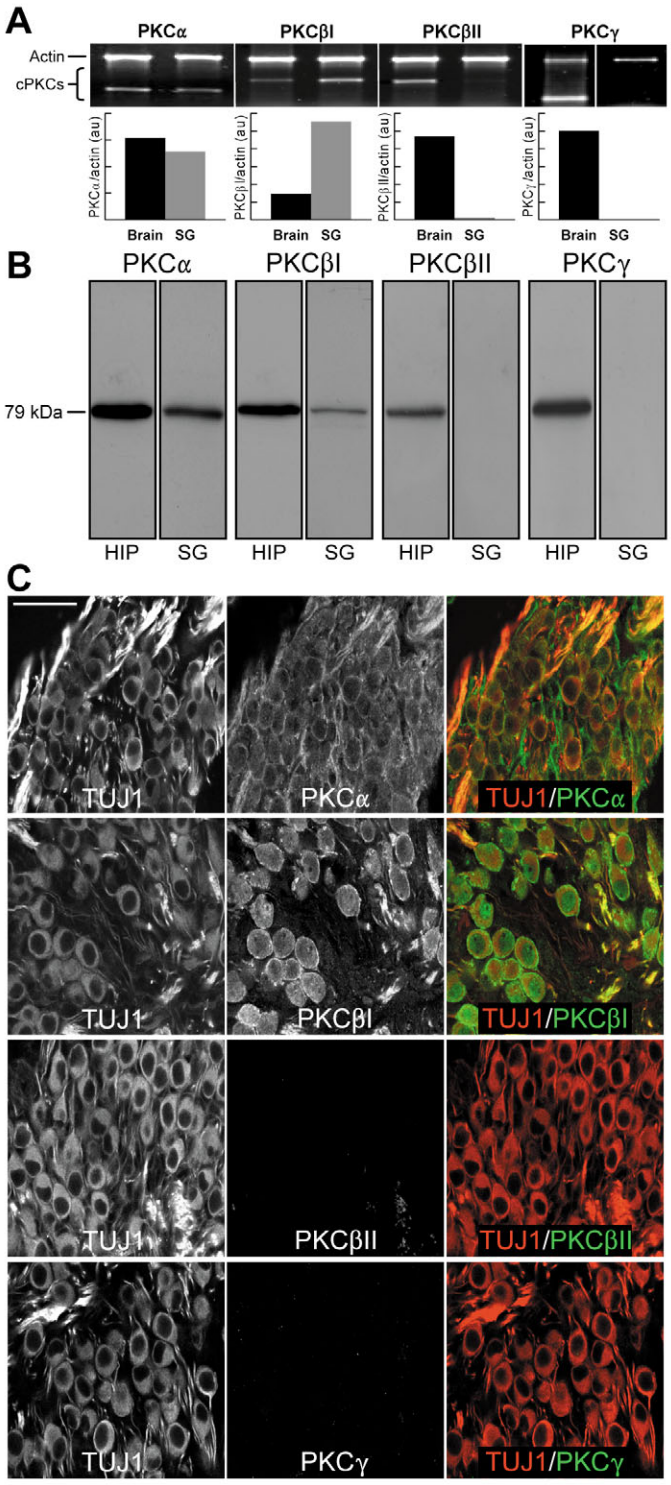
Non-linear regressions were generated with the GraphPad Prism program using the Hill equation, allowing the calculation of the inhibitor concentration that yields half-maximum inhibition, i.e. IC_{50}.

Results

Conventional PKC expression in spiral ganglion neurons

Using semi-quantitative RT-PCR, we detected only PKCα and PKCβI of the cPKCs in P5 SG tissue, whereas all four cPKC

transcripts were found to be expressed in whole brain extracts from P5 rats (Fig. 1A). To confirm this pattern of expression, cPKC protein expression was checked by western blot (Fig. 1B). Using specific antibodies, we showed the presence of PKCα and PKCβI in tissue homogenates from postnatal rat SG, while no immunoreactivity for PKCβII or PKCγ was observed on western blots in the same protein extracts. To map the specific expression pattern of these PKCs, the presence of each isoform in SG cells was revealed by double labelling with neuronal (anti-βIII tubulin, TUJ1) and cPKC immunostaining (Fig. 1C). Observation of postnatal cochlear sections with confocal microscopy showed an apparent ubiquitous localisation of PKCα immunoreactivity, including SGNs and non-neuronal cells, whereas a strong immunostaining for PKCβI was specifically confined to neurons, including soma and neurites. As predicted, no immunoreactivity for PKCβII or PKCγ was detected in any SG cell type. Analyses of cPKC immunostaining in cochlear sections of adult rats showed a similar pattern of cPKC expression in SG (data not shown).



PKCβI activation rescues spiral ganglion neurons from cell death in vitro

Previous studies have shown that SGNs undergo cell death after deafferentation in vitro and in vivo because of the loss of trophic support coming from their central target (Sekiya et al., 2003b; Spoendlin and Suter, 1976), from cells of the organ of Corti (Webster and Wester, 1981), including hair cells (Ernfors et al., 1996; Shinohara et al., 2002) and supporting cells (Stankovic et al., 2004). To test the ability of PKC activation to protect SGNs from cell death in that condition, primary SGNs were cultured for 24 hours in a definite medium containing the phorbol ester PMA. PMA, as with all members of the phorbol ester family, mimics the action of DAG on PKC to induce membrane translocation and activation of the enzyme (Oancea et al., 1998), with a higher efficacy and specificity than the alternative PKC activators (Kazanietz et al., 1992). As shown in Fig. 2A, PMA treatment of SG cultures significantly increases neuronal survival, compared with the survival level of SGNs in untreated control conditions. This effect is quantitatively comparable with the effect of neurotrophins (control, 100±13%; PMA 100 nM, 266±37%; BDNF 20 ng/ml, 259±43%; NT3 20 ng/ml, 256±43%, n=9) and saturates at 100 nM. Our previous studies have demonstrated that cultured postnatal SGNs undergo cell death after deafferentation through an apoptotic mechanism observed by TUNEL staining after 15 hours in vitro, the time of culture at which a maximum

Fig. 1. cPKC expression and cellular distribution in the spiral ganglion (SG). (A) mRNA levels of cPKCs in P5 rat brain and SG. Semi-quantitative RT-PCR was performed using primers specific for PKCα, PKCβI, PKCβII, PKCγ and β-actin genes. Densitometric analysis was performed and results are expressed as the ratio of cPKC gene/β-actin in arbitrary units (au). (B) Immunoblotting for PKCα, PKCβI, PKCβII and PKCγ from P5 rat SG or adult rat hippocampus total protein extracts. All four isoforms of cPKCs are recognised by their specific antibody at the expected molecular weight of 80 kDa in hippocampus protein extracts, whereas only PKCα and PKCβI isoforms are present in SG extracts. Each western blot was repeated on at least three different occasions, and with protein extracts from distinct SG samples. (C) Representative confocal micrographs of P5 rat SG sections selected in the medial cochlear turn and double stained for βIII tubulin (TUJ1, neuronal marker) and different cPKC isoforms detected with the antibodies used in western blot experiments. Anti-PKCα antibody reveals a ubiquitous expression towards SG cells whereas anti-PKCβI antibody stains SGNs exclusively. No PKCβII or PKCγ were detected in SG sections. The experiments were performed in triplicate and repeated on at least three independent occasions, and observations were made in all cochlear turns, with similar results. Bar, 40 μm.

of neurons show intranuclear DNA fragmentation (Lallemend et al., 2003). This implies that PMA exerts a prosurvival effect by the prevention of apoptosis. To test this hypothesis, we quantified the number of TUNEL-positive neurons after 15 hours in culture in the presence or absence of PMA (100 nM). In PMA-treated cultures, the number of TUNEL-positive neurons was significantly reduced (Fig. 2B), ascribing the neuroprotective action of PMA, at least in part, to an anti-apoptotic action in the neurons.

We further determined whether PMA neuroprotection is due to PKC activation. SG cultures were incubated for 24 hours

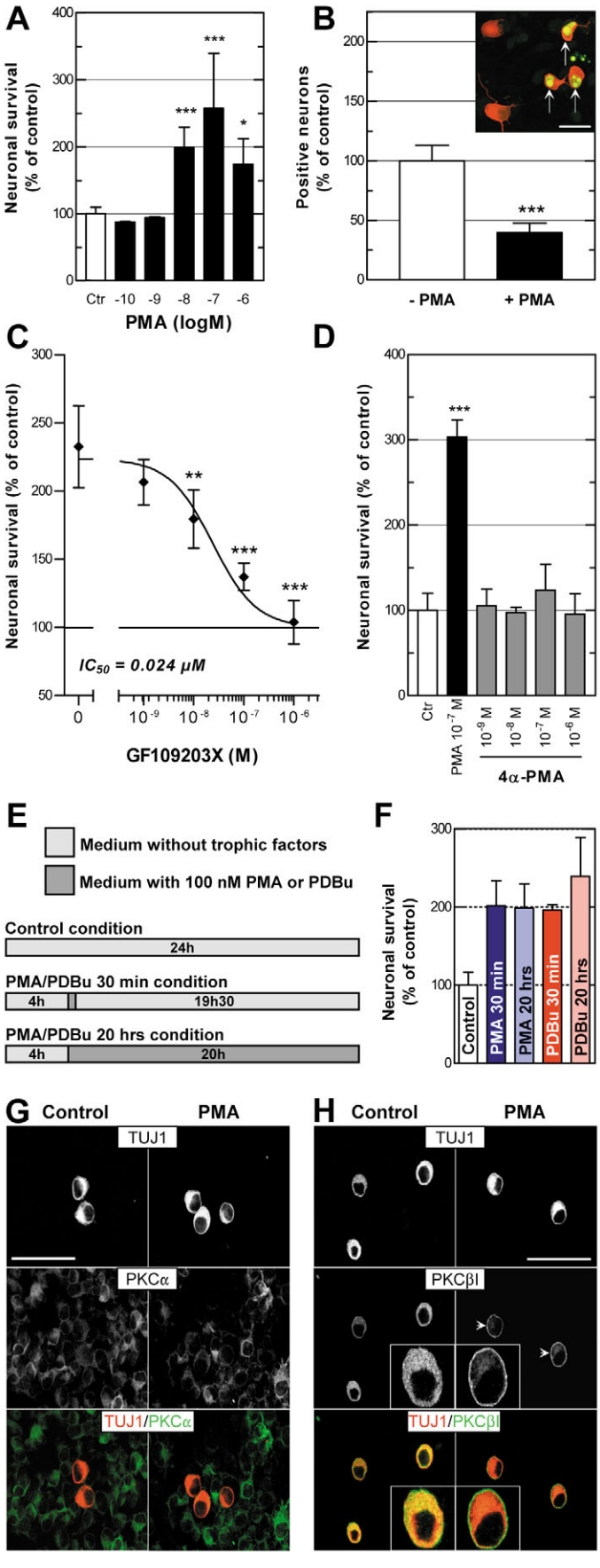


Fig. 2. Specific neuronal-PKCβI activation can rescue SGNs from apoptosis induced by TFD. (A) SGNs deprived of trophic factors were cultured for 24 hours in the presence or absence of increasing concentrations of PMA. The neurons were fixed and immunostained with anti-βIII tubulin antibody (TUJ1), as described in Materials and Methods, and the remaining number of viable neurons as a percentage of neurons in the untreated control condition was determined. All TUJ1-positive neurons were included in these counts, regardless of neurite length. Each such determination was performed in triplicate and repeated in three independent experiments. The mean value is shown in the figure; error bars in this and all subsequent figures indicate standard deviation (n=9; *P<0.05; ***P<0.001). (B) SGN cultures were treated or not with 100 nM PMA and neuronal apoptosis was determined by coupling the TUNEL bioassay to TUJ1 immunostaining 15 hours after completion of the spiral ganglia dissection. One hundred percent TUNEL-positive neurons was defined as the number of neurons presenting a TUNEL stain per total number of neurons in control cultures (n=6; ***P<0.001). The inset shows a representative confocal micrograph of TUJ1 (red)/TUNEL (green) double-stained P5 SGNs in control condition (–PMA) in which three out of five neurons are dying (arrows). Bar, 20 μm. (C) SGNs were cultured for 24 hours in the presence of 100 nM PMA and increasing concentrations of GF109203X, a specific inhibitor of PKC with high affinity for conventional isoforms. Cells were fixed and neuron viability was quantified, as determined in A (n=10; **P<0.01; ***P<0.001). (D) SGNs were incubated for 24 hours with 100 nM PMA (as a positive control) or increasing concentrations of the PMA inactive analogue, 4α-PMA. Neuronal cell counts were obtained as described in A (n=6; ***P<0.001). (E) Schematic representation of the neuroprotection paradigm performed in F. (F) SGNs were cultured in the presence or absence of PMA or PDBu (each at 100 nM) in conditions presented in E. SGNs were deprived of trophic factors for 4 hours. The neurons were then subjected to a PMA or PDBu treatment for 30 minutes or 20 hours. For each medium change, wells were rinsed three times in definite control medium, i.e. without trophic factor, to ensure the total elimination of agents from wells. Neuron viability was measured as determined in A (n=6-12; *P<0.05). (G,H) Representative confocal micrographs of expression and cellular redistribution of PKCα and PKCβI in SG cells before and after stimulation with a 30 minute PMA treatment. SG cells were cultured for 4 hours in a definite control medium. At this time, cultures were treated with 100 nM PMA or vehicle (control condition) for 30 minutes. Next, the cells were fixed and immunostained with TUJ1 and PKCα or PKCβI antibodies. In H, PKCβI is uniformly located exclusively in the cytoplasm of neurons in untreated cultures, whereas a 30 minute treatment with PMA induces its redistribution to the cell membrane (arrowheads). Insets show two TUJ1-positive neurons at higher magnification with a cytoplasmic (control condition) or a membranous (with PMA) PKCβI staining. The experiments were performed in duplicate and repeated on at least three different occasions, with similar results. Bars, 50 μm.

Table 1. IC_{50} values (nM) for isoform-selective PKC inhibitors*

Agent	IC_{50} SGNs	IC_{50} values for PKC isoform†							
		α	βI	βII	γ	δ	ϵ	ζ	η
GF109203X	24	8.4	18	ND	ND	210	132	5800	ND
Gö6976	1	2.3	6	ND	ND	–	–	–	ND
LY333531	8	360	4.7	5.9	400	250	600	$>10^5$	52

*Comparison with our results on inhibition of PMA-induced neuroprotection on SGNs using these inhibitors.

Abbreviations: ND, not determined; –, no effect; IC_{50} SGNs, [agent] (nM) providing 50% inhibition on the neuroprotective action afforded by PMA treatment on SGNs.

†Way et al., 2000.

with PMA and increasing concentrations of GF109203X (GF), a specific PKC inhibitor that exhibits high affinity for PKCα and PKCβI (Martiny-Baron et al., 1993). As expected, GF completely inhibited the neuronal survival-promoting effect of PMA treatment (Fig. 2C and Table 1), with concentrations already effective in the nanomolar range. The calculated IC_{50} of 24 nM was very close to the one described in the literature for the βI isoform (18 nM, Table 1) (Way et al., 2000). Previous studies have shown that prolonged PMA application can lead to PKC inhibition. Indeed, a prolonged phorbol ester exposure of SGN cultures induces PKCβI downregulation (data not shown). However, survival of SGNs under basal conditions in this study was not modified by PKC inhibition (control: 100±15%; GF 1 μM: 116±25%, n=10, P>0.05), an observation which indicates that the response observed after PMA treatment is due to activation and not downregulation of PKC. In support of a specific effect of PMA, the inactive PMA analogue on PKC activation, 4α-PMA, was unable to stimulate SGN survival at any concentration tested (Fig. 2D). To ensure that the observed effect of PMA on SGN survival was the result of a direct activation of the cPKCs, we analysed the survival of these neurons in the presence of the indolocarbazole Gö6976. This compound shows very good selectivity for PKC over other protein kinases and discriminates between cPKC isoforms and the other ones, targeting specifically PKCα and PKCβI, without affecting nPKC, even at micromolar concentrations (Martiny-Baron et al., 1993). Gö6976 at 1 nM, a concentration used to inhibit nearly 50% of the activity of cPKCs, reduced the PMA (100 nM)-induced neuroprotective action on SGNs by 49% (n=5, Table 1). To confirm the involvement of the βI isoform in the neuroprotective action of PMA, we used a very selective inhibitor of PKCβ, i.e. LY333531 (Way et al., 2000). Treatment of SG cultures with PMA (100 nM) and LY333531 (1 μM) caused a complete inhibition of PMA-mediated neuronal survival (control, 100±9%; PMA 100 nM, 279±58%; PMA 100 nM + LY333531, 118±20; LY333531, 113±29%; n=6), with an IC_{50} of ~8 nM (using 0.1 nM-1 μM concentrations; data not shown), while the IC_{50} described in the literature for the βI isoform was about 5 nM (Table 1).

To assess the dynamics of this PMA signal and the subsequent PKC activation in SGNs, cells were pulsed with PMA at 100 nM for 30 minutes (4 hours after plating to avoid cell detachment) and SGN survival was quantified after 24 hours (Fig. 2E,F). In this condition, the PMA treatment resulted in an increase in SGN survival significantly higher than in control conditions. Moreover, the effectiveness of the protection was as high as that observed in the corresponding positive control condition, i.e. where PMA treatment was maintained for 20 hours. Similar results were seen using another phorbol ester, phorbol 12,13-dibutyrate (PDBu, 100 nM) (Fig. 2F). These results indicate that PKC activators have the capacity to signal a trophic effect within 30 minutes of treatment.

The activation and, consequently, the subcellular redistribution of PKC in response to PMA treatment, was further analysed to determine and confirm which of the cPKC isoforms is involved in the PMA effect. Indeed, PKC translocation to the membrane upon activation and membrane localisation are currently used as markers for PKC activation (Newton, 1995). Because the protective effect of PMA is already effective after a 30 minute treatment, we used a short-term PMA treatment to visualise direct activation of cPKCs in neurons. We first analysed the expression of cPKC isoforms in basal culture conditions, i.e. without any trophic stimulus. Double labelling with neuronal (TUJ1) and PKC antibodies in 4 hour control cultures showed a ubiquitous PKCα immunoreactivity in SGNs and non-neuronal cells (Fig. 2G), whereas the βI isoform was strictly restricted to the neurons (Fig. 2H), as seen on cochlear sections. Exposure of SG cultures to PMA for 30 minutes (4 hours after plating) markedly induced membrane redistribution of PKCβI in SGNs (arrowhead, Fig. 2H), whereas there was no induction of PKCβI expression in non-neuronal cells, even after 24 hours of culture, either in the presence or absence of PMA (data not shown). Conversely, stimulation with PMA at a concentration of 100 nM did not change PKCα subcellular localisation, in comparison with the control condition (Fig. 2G). Regarding the βII and γ isoforms, in all conditions, no staining (i.e. no induction of protein expression) was observed in SG culture (data not shown). Taken together, these data indicate that PKC activation is responsible for the neuroprotective effect of PMA and that the neuronal-specific PKCβI isoform mediates this protection.

PKC-activation-induced neuroprotection is not mediated by non-neuronal cells

To examine the influence of non-neuronal cells on PKC-activator-induced neuronal survival, freshly dissociated SGNs were cultured for 24 hours on enriched non-neuronal SG cells (i.e. fewer than 5 neurons/well) pre-cultured for 96 hours. A 30 minute pre-treatment of the pre-cultured SG cells with PDBu (100 nM) did not increase the number of SGNs in comparison with control cultures (Fig. 3A,B). By contrast, sister cultures treated with PDBu for the last 24 hours showed a pronounced increase in neuronal survival (Fig. 3A,B, P<0.001). Similar results were obtained using PMA (100 nM; data not shown).

The mechanism by which PKC activators enhance SGN survival may also involve an increase in the proliferation of non-neuronal cells and subsequently an increase in the secreted endogenous neurotrophic factors in culture (Chen et al., 2003; Trupp et al., 1997). To investigate whether PMA is mitogenic for SG cells, we performed a BrdU incorporation assay for 15 or 24 hours. After 15 hours in culture, no mitogenic effect of PMA was detected, whereas foetal bovine serum (FBS, 10%)

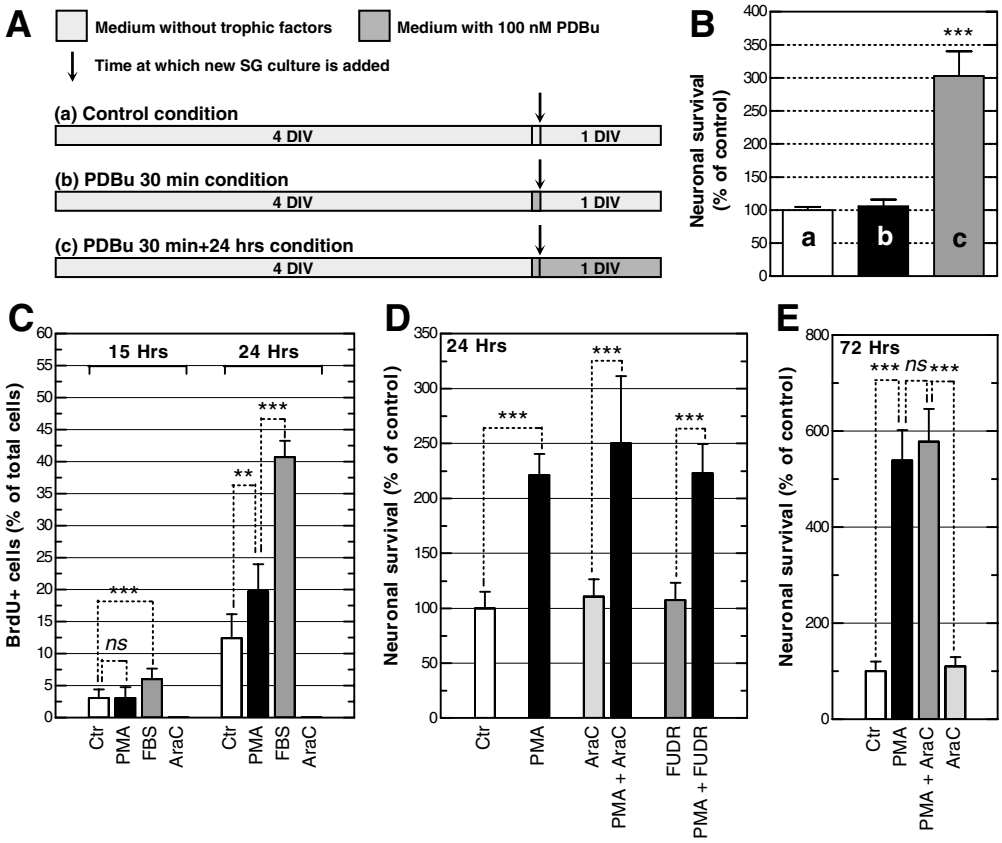


Fig. 3. Non-neuronal cells do not mediate PKC-activator-induced neuroprotection. (A) Schematic representation of the neuroprotection paradigm performed in B. (B) SGNs were cultured in the presence or absence of PMA or PDBu (each at 100 nM) on 96-hour SG cultures activated or not by PMA or PDBu for 30 minutes (see conditions presented in A). The number of viable neurons in the new 24-hour cultures as a percentage of neurons in the untreated control condition (a) was determined (n=6; ***P<0.001). (C) SG cells were cultured in medium containing BrdU and either vehicle or one of the tested compounds (100 nM PMA, 10% FBS, 10 μM AraC). After 15 or 24 hours, cells were fixed and processed for immunocytochemistry and propidium iodide staining to identify nuclei. The proliferative level in culture was evaluated as the number of BrdU-positive cells per total cell number in the culture. Each determination was performed in duplicate, and repeated in three independent experiments. The mean value is shown (n=6; *ns*, not significant; **P<0.01; ***P<0.001). (D) SG cells were cultured for 24 hours in the presence or absence of 100 nM PMA and specific anti-proliferative agents, i.e. AraC (10 μM) and FUDR (20 μM). Average numbers of TUJ1-positive SGNs under different experimental conditions were counted, as previously described in Fig. 2A (n=9; ***P<0.001). (E) AraC was used to block proliferation in culture of SGNs in the presence or absence of 100 nM PMA and, after 72 hours, cells were fixed and neuronal viability was expressed as the percentage of neurons present in the untreated control condition (n=6; *ns*, not significant; ***P<0.001).

induced a significant increase in the number of proliferating cells compared with control cultures (Fig. 3C). Conversely, after 24 hours in culture, PMA induced a significant increase in the level of BrdU incorporation in comparison with control cultures (Fig. 3C). To be sure that the neurotrophic effect of PMA after 24 hours in culture was not related to its proliferative effect, we quantified SGN survival in the presence of PMA with or without antimitotic drugs, i.e. AraC (10 μM) and fluorodeoxyuridine (FudR, 20 μM). We found that AraC and FudR totally inhibit proliferation of SG cells in culture for 24 hours, in the presence or the absence of trophic factors, i.e. PMA or FBS (data not shown), and had no effect on neuronal survival (Fig. 3D). In these cultures, PMA-induced neuronal survival was unaffected. In addition, SG cells were grown for 72 hours in the presence of PMA with or without AraC. The neuronal survival effect of PMA was still significantly higher than in the control culture after 72 hours (Fig. 3E) and the presence of AraC did not change it. Altogether, these results demonstrate that non-neuronal cells are not involved in the neurotrophic effect of PKC activators.

Intracellular pathways involved in the neurotrophic effect of PMA

To characterise the PKC signalling implicated in neuronal survival, we focused on intracellular mediators that are activated by PKC and are essential components in the survival of other neuronal types. One of these pathways implicates the mitogen-activated protein kinase kinase (MEK/ERKs), whose activation has been largely involved in protection from neuronal cell death (Ishikawa and Kitamura, 1999; Xia et al., 1995). Accordingly, we examined the effect of U0126, a specific inhibitor of MEK, on the ability of PMA to protect SGNs from deafferentation-induced cell death. As shown in Fig. 4, U0126 significantly reduced the neuroprotective effect afforded by the PMA treatment. Another pathway reported to act as a critical neuronal survival component involves PI3K/Akt activation (Brunet et al., 1999; Brunet et al., 2001; Yamaguchi et al., 2001). We therefore added the specific PI3K inhibitor LY294002 at different concentrations to SG cultures in the presence of PMA (100 nM). The addition of LY294002 significantly inhibited the survival-promoting effect of PMA (Fig. 4), implying that PI3K is also required for the survival of SGNs in the presence of PMA. U0126 and LY294002 used alone did not change the survival of SGNs (control, 100±11%; 20 μM U0126, 123±25%; 20 μM LY294002, 98±25%; n=6-15). To determine whether PI3K and ERK1/2 cooperate to promote the survival of SGNs, PMA-maintained SGNs were treated with both U0126 and LY294002 at their calculated IC_{50} (1.5 μM and 2.5 μM, respectively) and the number of

surviving neurons was determined. The combined inhibition of both PI3K and ERK1/2 caused the death of SGNs to a similar extent to the withdrawal of trophic factors (control, 100±11%; 100 nM PMA, 304±25%; PMA + 1 μM U0126, 190±28%; PMA + 1 μM LY294002, 180±19%; PMA + U0126 + LY294002, 109±12%, *n*=6-9). If the role of the MEK/ERK pathway is well established in the downstream cascade of PKC-activation-induced cell survival (Maher, 2001), an involvement of PI3K/Akt in mediating PKC activation is not classical. As reported by others, the missing link in PKC-PI3K/Akt signalling could be the proline-rich tyrosine kinase 2 (PYK2), also known as RAFTK (related adhesion focal tyrosine kinase) (Girault et al., 1999), which can be tyrosine phosphorylated and activated by PKC in neurons (Lev et al., 1995). When genistein (20 μM), a tyrosine-specific kinase inhibitor (Akiyama et al., 1987), was included in the SG culture medium, the ability of PMA (100 nM) to increase SGN survival was significantly inhibited (control, 100±26%; PMA, 277±77%; genistein, 94±22%; PMA+genistein, 140±37%; *n*=9, *P*<0.001), a result that is consistent with previously described PKC/PYK2/PI3K signalling cascades (Sarkar et al., 2002; Sayed et al., 2000; Shi and Kehrl, 2001).

As PKCβ has been described as a component of the NF-κB signalling axis (Saijo et al., 2002) and NF-κB activity is of primary importance to sensory neuron survival (Fernyhough et al., 2005), we next sought to examine whether the Rel/NF-κB family of transcription factors could play a role in PMA-induced survival of deafferented SGNs using two specific NF-κB inhibitors, BAY 11-7082 (BAY) (Pierce et al., 1997) and sulfasalazine (SZ) (Wahl et al., 1998). Our experiments revealed that there was no difference (*P*>0.05) in the number of SGNs surviving after 24 hours in the presence of PMA (100 nM), either alone or with BAY (2 μM) or SZ (1 mM) (Fig. 4). In addition, the number of TUNEL-positive neurons in the PMA condition (100 nM, 15 hours) was not modified (*P*>0.05) in the presence of SZ (1 mM) (control, 100±13%; 100 nM PMA, 39.4±8%; SZ, 104.7±39%; PMA + SZ, 51±17%, *n*=3-6).

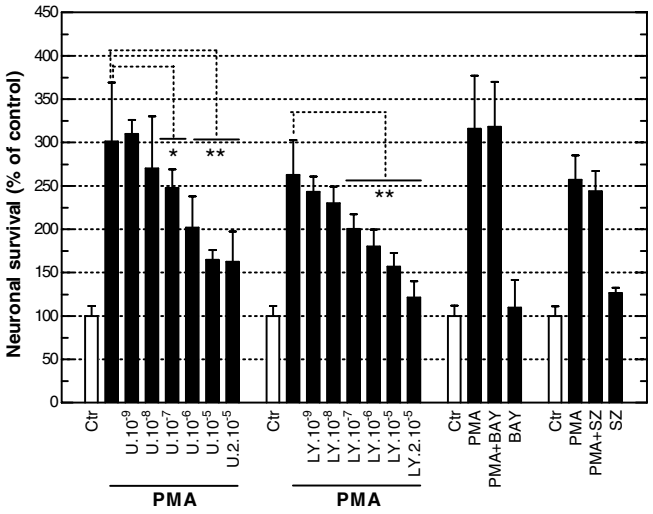


Fig. 4. MEK/ERK and PI3K/Akt, but not NF-κB, are key components in the PMA-induced survival of SGNs. SGNs were treated for 24 hours with or without PMA at 100 nM and increasing concentrations of U0126 (U), LY294002 (LY) or 2 μM BAY 11-7082 (BAY) or 1 mM sulfasalazine (SZ) to reduce, respectively, the enzymatic activity of MEK, PI3K or NF-κB complex. Neuronal survival was quantified, as determined in Fig. 2A (*n*=8; **P*<0.05; ***P*<0.01).

Enhanced regeneration of SGN neurites by PKC activation

We explored the effects of PMA on neurite formation by SGNs in culture, a useful parameter for studying axonal regeneration. To evaluate neurite outgrowth in SGN cultures, only neurons bearing neurites of a defined length of at least three neuronal cell body diameters were considered, and the neuritic index was calculated as described in Materials and Methods. SGNs cultured for 24 hours in the presence of increasing concentrations of PMA (10 nM-1 μM) exhibited greater observable neurite extension (Fig. 5A) than in control condition, i.e. without trophic factors. At 100 nM, PMA significantly increased neurite outgrowth (*P*<0.001; Fig. 5B) in a manner similar to the one observed in the presence of neurotrophins (control, 100±19.3%; 100 nM PMA, 308±76%; BDNF 20 ng/ml, 315±59%; NT3 20 ng/ml, 298±58%; *n*=6).

Treatment of SGN cultures with GF (1 μM) led to an inhibition (Fig. 5B) of the PMA-dependent neuritogenic effect, whereas sister cultures receiving GF alone showed neurite extension comparable with that observed in control cultures (control, 100±23%; GF 1 μM: 99±58%; *n*=6). In addition, exposure of SGN cultures to PMA (100 nM) for 30 minutes markedly induced redistribution of PKCβI to growing neurites (data not shown). Taken together, our results strongly suggest that, in SGN cultures, PMA-induced neuritic growth is mediated directly through the activation of PKCβI. As for the

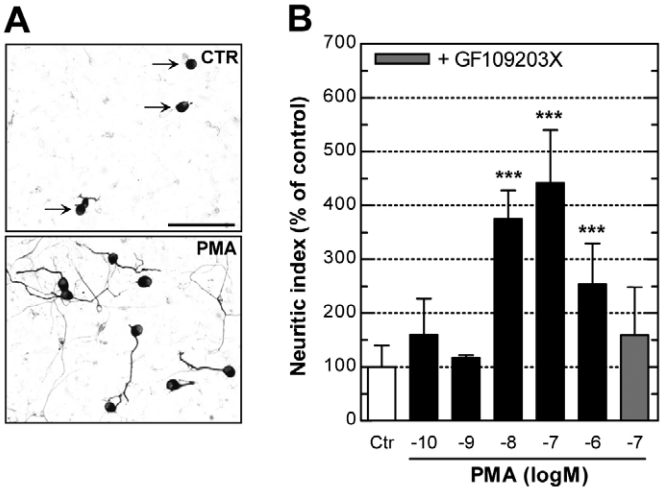


Fig. 5. PKC activation increases neurite outgrowth in SGN cultures. (A) Representative photomicrographs of TUJ1-stained SGNs in 24 hour culture treated without (CTR) or with 100 nM PMA (PMA). Arrows in the control condition show aneuritic neurons usually found in 24 hour control cultures. Bar, 100 μm. (B) SGNs were treated for 24 hours with or without increasing concentrations of PMA and in combination or not with the specific PKC inhibitor, i.e. GF (1 μM). Subsequently, cells were fixed and immunostained with TUJ1 antibody and the neuritic index in each condition as a percentage of the neuritic index in the untreated control condition was determined (*n*=6; ****P*<0.001).

survival assay, we studied neurite outgrowth in the presence of two antimitotic drugs, i.e. AraC (10 μM) and FudR (20 μM). We found that the observed increase in neurite extension in the presence of PMA (100 nM) was not inhibited by either AraC or FudR (control, 100±33%; 100 nM PMA, 426±92%; PMA + AraC, 403±98%; PMA + FudR, 441±77%; *n*=6), and that these two agents had no effect themselves on neurite extension (control, 100±33%; AraC, 88±31%; FudR, 131±48%; *n*=6), indicating that neurite extension in SGN cultures does not depend on cell layer density.

To define the signalling molecules involved in PMA-induced neuritogenesis, we used the two kinase inhibitors LY294002 and U0126 at their optimal concentrations (i.e. 20 μM). The addition of LY294002 or U0126 concomitantly with PMA (100 nM) completely blocked PMA-induced neurite outgrowth (control, 100±16%; PMA, 327±66%; PMA + LY294002, 104±34%; PMA + U0126, 100±29%; *n*=6-10).

PKC versus neurotrophin signalling

It has been demonstrated that BDNF and NT3 and their corresponding high-affinity receptors TrkB and TrkC are responsible, to a certain extent, for the survival of SGNs both in vitro and in vivo (Fritzsch et al., 1997; Hansen et al., 2001; Shinohara et al., 2002). Conversely, the nerve growth factor (NGF) and its high-affinity receptor TrkA are not involved (Fritzsch et al., 2004; Zheng et al., 1995). The intracellular regulatory mechanisms that control neuronal survival afforded by neurotrophins could comprise the MAPK/ERK and the PI3K/Akt signalling pathways as previously shown (Yuan and Yankner, 2000). In our culture conditions, the addition of LY294002 or U0126 inhibited the survival-promoting action of BDNF and NT3 (Fig. 6A).

We next examined whether PKC activation functioned downstream of Trk receptor activation in neurotrophin-dependent survival signalling. Blockade of PKC signalling with GF at 1 μM had no effect on SGN survival attributable to BDNF and NT3, whereas, as expected, GF (1 μM) completely inhibited the survival action afforded by PMA treatment in sister cultures (Fig. 6B). To confirm these results, we used immunocytochemistry to study the effect of BDNF and NT3 on PKCβI distribution. In contrast to the PMA treatment, no PKCβI membrane redistribution was observed in the presence of BDNF or NT3 (data not shown). Taken together, these results clearly show that two different pathways mediate SGN survival in vitro, one mediated by PKCβI and another being totally PKC independent.

Enhancing SGN survival by combining PKC activation and neurotrophins

Given that PKC activation and neurotrophin signalling define parallel survival pathways in SGNs, we tested whether the combinatorial actions of neurotrophins and PMA (each factor used at a level providing its maximal trophic effect) could produce additive effects on neuronal survival. For that purpose, we used 96 hour long-term cultures in which neuronal survival is very low (fewer than 5 neurons/well) in the absence of any trophic support. In these conditions, it is easier to appreciate the different levels of neurotrophic factor-induced survival and to discriminate between additive and synergistic effects. After 96 hours in culture, BDNF, NT3 and PMA significantly promoted the survival of SGNs in comparison with control cultures (Fig. 7), whereas NGF had no effect (control, 100±32.7%; NGF 100 ng/ml, 138.7±31.9%, *n*=6, *P*>0.05). Simultaneous BDNF and NT3 exposure resulted in an increase in SGN survival similar to the arithmetic sum of the survival effect of each factor alone, whereas the addition of NGF to neurotrophins did not modify (*P*>0.05; data not shown) their survival-promoting action. This was consistent with additive trophic effects of BDNF and NT3 on their respective high-affinity receptors, i.e. TrkB and TrkC, rather than an effect on their low affinity receptor (p75NGFR). Interestingly, PKC signalling and neurotrophins were seen to interact synergistically: the addition of PMA in combination with BDNF or NT3 led to a greater SGN survival than might be expected by assuming strictly additive effects (Fig. 7, compare theoretical values to experimental values). Furthermore, even

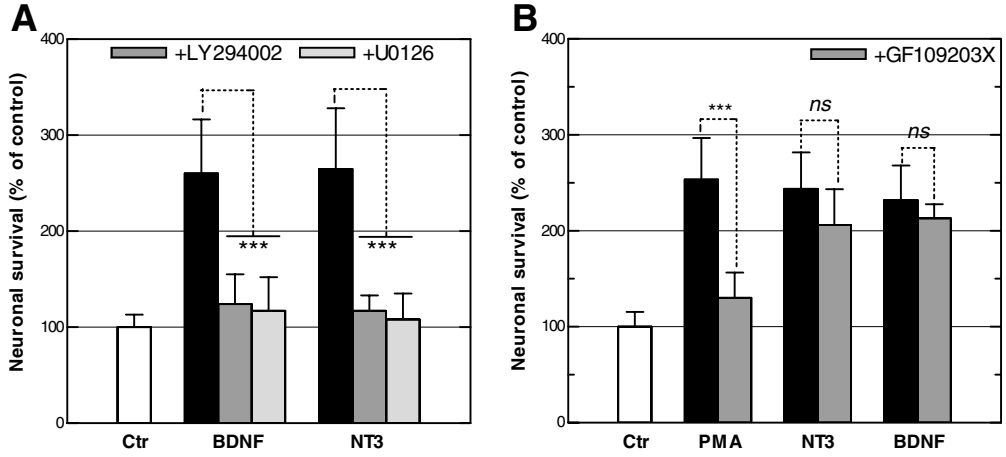


Fig. 6. The survival-promoting action of neurotrophins in SGNs depends on PI3K and MEK/ERK signalling but is independent of PKC activity. (A) SGNs were cultured in the presence of BDNF or NT3 (each at 20 ng/ml) plus LY294002 or U0126 (each at 20 μM). Cells were fixed 24 hours later and the remaining number of viable neurons were counted, as determined in Fig. 2A (*n*=6; ***P<0.001). (B) SGNs were treated with 100 nM PMA, NT3 or BDNF (each at 20 ng/ml) in the presence or absence of 1 μM GF109203X to block the enzymatic activity of PKC. 24 hours later, cells were fixed and neuronal survival was scored, as described in Fig. 2A (*n*=9; *ns*, not significant; ***P<0.001).

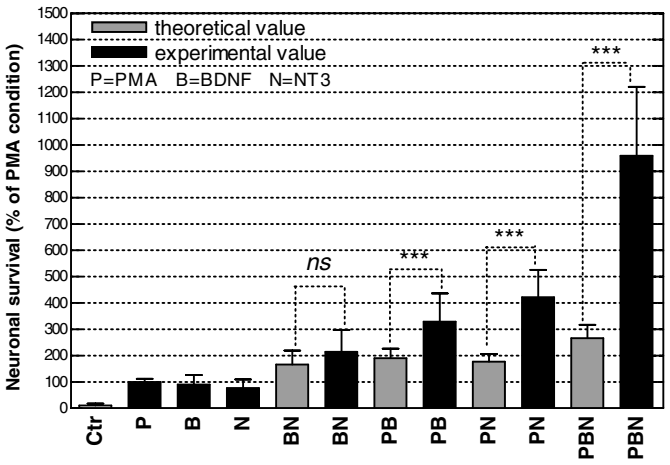


Fig. 7. PKC activation and neurotrophins synergistically enhance survival of SGNs. SGNs were cultured for 96 hours in the presence of different combinations of PMA (P, 100 nM), BDNF (B, 100 ng/ml) and NT3 (N, 100 ng/ml). Cells were fixed and neuronal viability in the different experimental conditions was expressed as a percentage of the average number of TUJ1-positive neurons in PMA condition. Black bars represent measurements observed in experimental conditions and grey bars represent theoretical values calculated by considering the experimental survival effect of each factor alone (n=15; *ns*, not significant; ***P<0.001).

low concentrations of BDNF or NT3 (1-5-10 ng/ml) were sufficient to produce a significant synergistic effect on neuronal survival in the presence of PMA (data not shown). In the presence of NGF, which is not itself trophic, the neuroprotective effect of PMA was not increased (PMA 100 nM, 100±22.6%; PMA 100 nM + NGF 100 ng/ml, 101±31.3%, n=6), which suggests that the synergistic effect observed with BDNF or NT3 together with PMA treatment occurs via a specific intracellular pathway involving activation of TrkB or TrkC as well as PKC. The most noteworthy observation was the large synergistic effect induced by the combination of the three different trophic factors. The number of surviving neurons in the presence of PMA plus BDNF and NT3 was increased by tenfold in comparison with conditions in which each factor was applied alone, and was increased by ~100-fold compared with control conditions. These data indicate that PKC and neurotrophin signalling pathways are relatively distinct but can converge synergistically to increase SGN survival.

The effect of bryostatin on SGN survival

Phorbol esters are tumour promoters and, therefore, are not viable options for drug development. Novel PKC activators may offer an alternative, but their safety for eventual clinical use remains to be demonstrated. A compound that activates PKC and lacks tumour-promoting activity is the natural product bryostatin 1 (bryo). Bryo is being actively investigated in humans (Phases I and II) as an anti-cancer agent (Clamp and Jayson, 2002). In our experiments, we showed that treatment of SG cultures with bryo at a concentration range of 1-100 nM for 24 hours significantly increased the number of surviving SGNs (Fig. 8A). The bryo concentration necessary to obtain maximal neuronal survival was found to be tenfold less than that of PMA. Concomitantly with its neuronal survival effect, bryo markedly increased neurite outgrowth in SGN cultures (control, 100±27%; 100 nM PMA, 450±93%; 10 nM Bryo, 408±55%; n=9) (Fig. 8A, inset). We also observed that at 10 nM, similarly to PMA, short-term treatment with bryo (30 minutes, 10 nM) was as efficient as long-term treatment (P>0.05; 24 hours) in promoting neuronal survival (data not shown). Bryo caused PKCβI redistribution to the plasma membrane in SGNs (Fig. 8B), without any effect on the α isoform at the tested concentration (data not shown). To

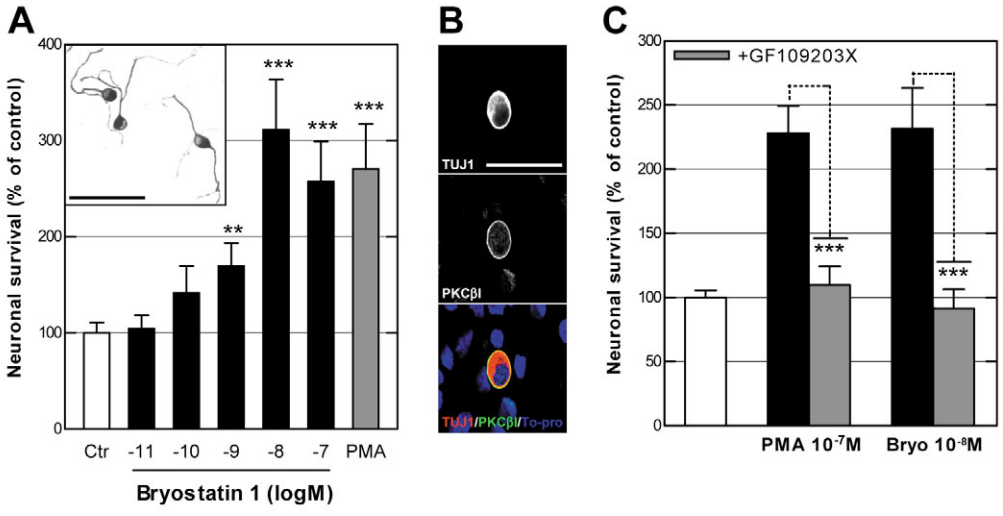


Fig. 8. Bryostatin 1, a non-tumour-promoting PKC activator, rescues SGNs from cell death. (A) SGNs were deprived of trophic factor in the presence of increasing concentrations of bryo or 100 nM PMA for 24 hours. Neurons were fixed and the remaining number of viable neurons were counted, as determined in Fig. 2A (n=6; **P<0.01; ***P<0.001). The inset shows a representative micrograph of TUJ1-stained SGNs in a 24 hour culture treated with bryo (10 nM). Bryo treatment strongly enhanced neurite outgrowth, in comparison with the control condition in Fig. 5B. Bar, 80 μm. (B) Representative photomicrographs of PKCβI membrane redistribution in SGNs upon 30 minute bryo exposure. SGNs were cultured for 4 hours in control medium. Next, cultures were treated for 30 minutes with 10 nM bryo. Cells were then fixed and immunostained with PKCβI and TUJ1 antibodies; nuclear staining with TO-PRO-3 was used to appreciate cell density. Before the bryo treatment, PKCβI had a uniform cytoplasmic distribution, as seen in the control condition (Fig. 2H). The experiments were performed in duplicate and repeated on two different occasions, with similar results. Bar, 40 μm. (C) SGNs were treated with 100 nM PMA or 10 nM bryo in the presence or absence of 1 μM GF109203X. After 24 hours, cells were fixed and neuronal viability was examined, as described in Fig. 2A (n=6; ***P<0.001).

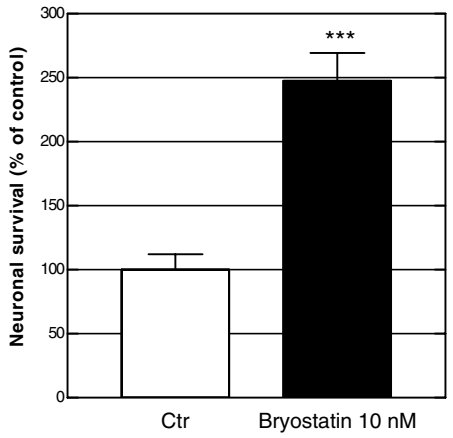


Fig. 9. PKC activation increases survival of adult SGNs. Adult SGNs were cultured in the presence or absence of bryo (10 nM) for 24 hours. Numbers of viable neurons in cultures were counted, as determined previously in Fig. 2A (n=6; ***P<0.001).

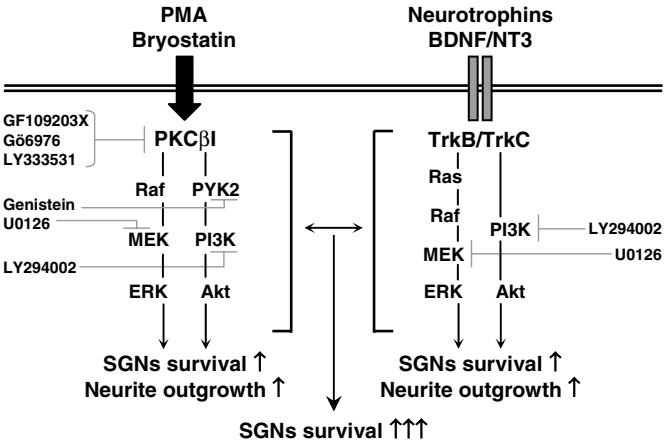


Fig. 10. A model showing the multiple actions of PKC activators (PMA/bryostatin) and neurotrophins (BDNF/NT3) on intracellular signalling pathways that lead to protection and neuritogenesis of SGNs after deafferentation in vitro. Upon activation of PKCβI and TrkB/TrkC, both the PI3K/Akt and MAPK/ERK pathways are required for protection and neurite extension of SGNs, as demonstrated by using specific inhibitors of PKC (GF109203X, Gö6976, LY333531), PI3K (LY294002) and MEK (U0126). The combination of neurotrophins with PMA treatment leads to a synergistic action on SGN survival. PYK2 represents an intermediary kinase, which could be involved in the signal pathway linking PKCβI activity to PI3K signalling (see Discussion for details).

confirm the involvement of PKC activation in bryo neuroprotection, SG cultures were treated with bryo in the presence or absence of GF at 1 μM (Fig. 8C). The neuroprotection afforded by bryo (10 nM) or PMA (100 nM) was totally abolished in the presence of GF. Moreover, LY333531 at 10 nM, a concentration previously reported to inhibit 50% of the activity of PKCβ without affecting other PKC isoforms, reduced the bryo (10 nM)-induced neuroprotective action on SGNs by 48% (control, 100±13.5%; bryo 10 nM, 230±29%; bryo 10 nM + LY333531 10 nM, 168±10.7%; n=8; P<0.01), considering 230 (bryo)–100 (Ctr)=130 as the bryo action, and 230 (bryo)–168 (bryo + LY333531)=62 as the LY333531 inhibitory effect at the concentration of 10 nM. Taken together, these results confirm the specific requirement of neuronal PKCβI activation for neuroprotection and define bryo as a potential therapeutic agent for deafferentation-induced SGN death.

PKC activation protects adult SGNs

Finally, we asked whether PKC activation could increase adult SGN survival in vitro. Fig. 9 shows that treatment of adult SGNs with 10 nM bryo for 24 hours resulted in efficient neuroprotection, in comparison with the survival level of SGNs in the untreated, control conditions.

Discussion

The loss of contact of SGNs with their peripheral and central targets results in progressive neuronal cell death both in vitro and in vivo. Most of the studies devoted to understanding and counteracting the cell death of these neurons after deafferentation have focused mainly on the protective role of peptidic growth factors, such as neurotrophins (Ernfors et al., 1996; Shinohara et al., 2002), glial-cell-line-derived neurotrophic factor (Kanzaki et al., 2002; Ylikoski et al., 1998), fibroblast growth factor (Altschuler et al., 1999; Sekiya et al., 2003a) or macrophage colony-stimulating factor (Yagihashi et al., 2005). However, if these peptides are efficient in promoting SGN survival, they are not sufficient to protect them fully. In this context, the results of our study provide the first demonstration that the PKCβI pathway is involved in SGN survival after trophic factor deprivation (TFD). In addition, PMA and neurotrophins can interact synergistically to enhance each other's action on SGN survival, opening new ways to treat inner ear injury.

PKCβI activity is required for neuronal survival and neuritogenesis

The enzymes of the PKC family have been implicated in the regulation of cell death in a number of cell types and several studies suggest that different isoforms may have unique or even opposite effects on cell survival. Indeed, it has been shown that PKCα, PKCβ, PKCϵ, PKCλ and PKCζ play a role in inhibiting cell death (Gubina et al., 1998; Huang et al., 2001; Lin et al., 1997; Maher, 2001; Weinreb et al., 2004), while PKCδ acts in a pro-apoptotic manner, even in neuronal cells (Anantharam et al., 2002; Bright et al., 2004). In the present study, we have shown evidence that PKCα and PKCβI are present in the SG, confirming previous reports describing cPKC expression in the SG (Garcia and Harlan, 1997; Lerner-Natoli et al., 1997). The finding that PKCβI expression is restricted to SGNs suggests a specific neuronal function for this isoform. Administration of PMA, a specific activator of cPKCs and nPKCs, to neuronal cultures was shown to abolish the appearance of TUNEL-positive neurons and to increase neuronal survival, consistent with the inhibition of the apoptotic pathway engaged in trophic-factor-deprived neurons. Inhibition of the PMA survival effect on SGNs in culture was noted with GF109203X, Gö6976 and LY333531 at nanomolar concentrations, with IC_{50} corresponding to an implication of PKCβI (see Table 1). In

addition, PMA specifically induced the translocation of PKCβI in SGNs, whereas no PKCα redistribution was observed in the same conditions. Together, these data are consistent with a primary role for PKCβI in SGN survival and neuritogenesis.

Intracellular signalling pathways recruited by PKC activation and responsible for SGN survival

In neurons, as in other cell types, two main intracellular signalling pathways may be implicated in the promotion of survival: the MEK/ERK pathway (Bonni et al., 1999; Xia et al., 1995) and the PI3K/Akt pathway (Datta et al., 1997; Dudek et al., 1997). In our experiments, blocking the MEK/ERK pathway using the pharmacological agent U0126 was shown to reduce PMA-induced neuroprotection against TFD. Similarly, inhibition of the PI3K/Akt pathway by LY294002 was shown to be effective in reversing the neuroprotection of PMA. However, in both cases, high concentrations (20 μM) of U0126 or LY294002 were necessary to exert maximal inhibition on the survival-promoting action of PMA without, however, completely reversing its effect (PMA vs PMA+LY or PMA+U0126: $P<0.05$, Fig. 4). Simultaneous blockade of both PI3K/Akt and MEK/ERK pathways by combining the two drugs (U0126 and LY294002) at more specific concentrations (1 μM) caused the complete reversal of the neuroprotective action afforded by PMA treatment. Thus, it can be concluded that the cooperation of these two pathways accounts for the majority of the survival-promoting effect of PKC activation on SGNs. Although the role of the MEK/ERK pathway is well established in the downstream cascade of PKC-activation-induced cell survival (Maher, 2001), the involvement of PI3K/Akt in mediating PKC activation is not classical. The molecular link between PKC and PI3K could be the non-receptor tyrosine kinase PYK2 (Girault et al., 1999), which can be tyrosine phosphorylated and activated by PKC in neurons (Lev et al., 1995), and which thereafter could activate the PI3K/Akt pathway (Sarkar et al., 2002; Sayed et al., 2000; Shi and Kehrl, 2001). Indeed, we have shown here that genistein, a selective tyrosine-kinase inibitor (Akiyama et al., 1987), inhibits the PMA-induced neuronal survival. Taken together, our data suggest that, in SGNs, activation of PKC stimulates both MEK/ERK and PI3K/Akt cascades, and that these pathways work in concert to modulate SGN survival.

PKC versus neurotrophin signalling

Binding of neurotrophins to their respective Trk receptors gives rise to autophosphorylation of specific tyrosines, which induces the binding of different adaptor proteins, including PLCγ1 and Shc, which become phosphorylated by the Trk. Active Shc leads to the activation of the MEK/ERK and PI3K pathways, whereas the association of PLCγ1 with Trk regulates intracellular calcium levels and PKC activity through the cleavage of the substrate phosphatidylinositol (4,5)-bisphosphate into DAG and inositol (1,4,5)-trisphosphate. Trk-activation-induced survival and axonal growth responses in mammalian peripheral neurons are dependent upon the integrity of the Shc and on MEK/ERK and PI3K activity, but not on the PLCγ1 pathway (Huang and Reichardt, 2003; Yuan and Yankner, 2000). Here, on exploring the downstream signalling events underlying the effect of BDNF and NT-3 on SGN survival, we found that both MEK/ERK and PI3K/Akt pathways were required, as previously described (Hansen et al., 2001). In contrast, we showed that neurotrophin-induced survival of SGNs is not compromised when PKC activity is inhibited and that neurotrophin application to SGN cultures does not result in any translocation of cPKCs. Moreover, we observed that U73122, a specific inhibitor of PLC pathway, does not affect neurotrophin action (data not shown). Thus, our data provide conclusive evidence that neurotrophin signalling acts independently of PKC activity to promote SGN survival, and that signalling initiated at the PLCγ docking site on Trk receptors plays a minor role in the neurotrophin-survival effect, as previously described for peripheral neurons (Atwal et al., 2000).

We also observed that combinations of neurotrophins (BDNF and/or NT-3) with PMA synergistically enhance SGN survival (Fig. 10). PMA could synergise with neurotrophins to result in an increased and prolonged activation of the involved intracellular common pathways, i.e. MEK/ERK and PI3K/Akt pathways (Marshall, 1995). This type of convergent effect can occur in at least two different ways: (1) synergy could be of the magnitude of phosphorylation or in the half-life of the phosphorylated kinase involved; and (2) PMA and neurotrophins activate or inhibit a common target molecule, e.g. anti- or pro-apoptotic factors leading to synergistic induction or inhibition of factors involved in neuronal survival. Indeed, PKC and neurotrophins can phosphorylate the proapoptotic factor BAD (Cory and Adams, 2002), whose inactivation has been shown to ameliorate the survival of SGNs in TFD conditions (Bok et al., 2003). Another possibility would be a direct crosstalk between PKC and Trk signalling pathways. In this context, PKC has been shown to phosphorylate the phosphotyrosine phosphatase SHP-1 (Brumell et al., 1997; Shen et al., 1991), inhibiting its catalytic activity. SHP-1 can function as a Trk receptor phosphatase, controlling the level of Trk activity and limiting subsequent neurotrophin-induced neuronal survival (Marsh et al., 2003). PMA could also directly interfere with Trk signalling by inducing the expression of Trk(s) and making the neurons more responsive to neurotrophins (Frank et al., 1996; Sommerfeld et al., 2000). In support of this model, activation of PKC has been shown to upregulate the density of the cell surface tyrosine kinase receptor of insulin via transcriptional and translational events (Yamamoto et al., 2000).

Bryostatin 1, a safe PKC activator molecule, protects SGNs through the activation of neuron-specific PKCβI

It is of note that, if PMA, by activating PKCβI in SGNs, acts as a potent trophic stimulus against TFD-induced cell death of these neurons, it does not represent a viable option for therapeutic drug development because of its tumour-promoting activity. The bryostatins are a structurally novel family of marine natural products that are in clinical evaluation as anti-cancer agents (Clamp and Jayson, 2002). Bryostatins, like phorbol esters and the endogenous ligand DAG, modulate PKC activity by binding to the C1 domains of the protein and inducing translocation from the cytosol to cellular membranes (Wender et al., 1988). The recent data showing that bryo has neuroprotective properties in an Alzheimer's disease mouse model (Etcheberrigaray et al., 2004) prompted us to explore its use as a promoter of SGN survival. Bryo, at nanomolar

concentrations, significantly reduces SGN cell death induced by TFD. The use of specific PKC inhibitors (GF and LY333531) and studies on the localisation (consistent translocation) of cPKC isoforms following bryo treatment, indicate that activation of PKCβI is necessary for the neuroprotective action of bryo. More importantly, our data demonstrate the capacity for bryo to enhance survival of deafferented adult SGNs, confirming PKC activation as an important means of improving SGN survival.

Pathophysiological considerations

Previous analyses aimed at defining ways to reduce neural degeneration in the peripheral auditory system have centred primarily on neurotrophins as survival factors (Ernfors et al., 1996; Shinohara et al., 2002). However, although attempts to improve SGN survival by neurotrophins have demonstrated positive results, in clinical practice such interventions with neurotrophins are difficult due to several drawbacks that generally affect large polypeptides when used as drugs. Neurotrophins are prone to rapid proteolytic degradation in vivo and cause undesired pleiotropic effects. Our finding that, by activating neuron-specific PKCβI, bryo significantly improved the survival of deafferented SGNs in vitro, shows promise for use in cochlear neuroprotection. Indeed, this small molecule, given its lack of tumour-promoting activity, relatively low toxicity when applied systemically, and current use in humans, constitutes a suitable candidate for further drug development in vivo.

It has previously been shown that cPKC expression is upregulated in SGNs after AMPA injury, a pertinent model to disrupt contact between SGNs and hair cells and to mimic neurodegeneration following noise trauma (Lerner-Natoli et al., 1997). In this context, the treatment of injured SGNs with a PKC activator, such as bryo, should lead to a more pronounced neurotrophic effect. Future studies are needed to determine whether cPKC activation can lead to enhanced SGN protection and regeneration in vivo. In parallel to increased SGN survival, one major focus in hearing research is on developing mechanisms that stimulate neuritogenesis or axonal regeneration, especially in cochlear implant treatment, where closer contact between SGNs and the electrode is necessary. The present work is of particular interest in this regard, because we show increased neurite regeneration in axotomised SGNs after PKC activation. This is consistent with previous reports that PKC activity is involved in the mechanisms behind the early phases of axonal regeneration (Geddis and Rehder, 2003; Wiklund et al., 1996; Wu et al., 2003) and with the assumption that, in the injured cochlea, PKC activation might represent a key signal for maintaining the integrity of neural elements.

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