Akt activation is necessary for growth factor-induced trafficking of functional $K_{Ca}$ channels in developing parasympathetic neurons.

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Abstract

The protein kinase Akt is a crucial regulator of neuronal survival and apoptosis. Here we show that Akt activation is necessary for mobilization of large-conductance $K_{Ca}$ channels in ciliary ganglion (CG) neurons evoked by $\beta$-neuregulin-1 (NRG1) and TGF$\beta$1. Application of NRG1 to embryonic day 9 (E9) CG neurons increased Akt phosphorylation, as observed previously for TGF$\beta$1. NRG1- and TGF$\beta$1-evoked stimulation of $K_{Ca}$ is blocked by inhibitors of PI3K, by over-expression of a dominant-negative form of Akt, by over-expression of CTMP, an endogenous negative regulator of Akt, and by application of the Akt inhibitor 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (HIMO). Conversely, over-expression of a constitutively-active form of Akt was sufficient by itself to increase mobilization of functional $K_{Ca}$ channels. NRG1 and TGF$\beta$1 evoked an Akt-dependent increase in cell-surface SLO $\alpha$-subunits. These procedures have no effect on voltage-activated Ca$^{2+}$ currents. Thus, Akt plays an essential role in the developmental regulation of excitability in CG neurons.

Keywords: neuregulin, TGF$\beta$, slowpoke, PI3 kinase
Introduction

The serine/threonine kinase Akt, also known as protein kinase B, is one of many enzymes regulated by the lipid products of class I (receptor-regulated) phosphoinositide-3 kinases (PI3K). In a variety of signal transduction cascades, Akt is recruited to the plasma membrane upon binding of 3’-phosphoinositides to an N-terminal pleckstrin-homology (PH) domain. This brings Akt into sufficient proximity to be activated by membrane-associated 3’-phosphoinositide-dependent kinase-1 (PDK1), and possibly by other membrane-associated PI3K-dependent enzymes (Vanhaesebroeck and Alessi 2000).

Akt acts on a wide variety of substrates. In many cell types, over-expression of active forms of Akt promotes cell survival (Lawlor and Alessi 2001), and several Akt substrates are regulators of apoptosis, including the Bcl-2 family proteins, caspase-9, IκB kinases, and a variety of forkhead transcription factors (Vanhaesebroeck and Alessi 2000; Brazil et al. 2002). There is an extensive literature describing a central role for Akt in the regulation of neuronal survival under a variety of conditions, especially in the context of growth factor signaling (Kaplan and Miller 2000; Brunet et al. 2001).

Recent studies in non-neuronal cells have implicated a role for Akt in the regulation of processes other than apoptosis and cell survival, primarily in the metabolic responses to insulin in adipocytes, myocytes and their derived cell lines (Whiteman et al. 2002). For example, there is evidence that Akt regulates insulin-induced trafficking of glucose transporters such as GLUT4 to the plasma membrane, although this issue has been controversial (Summers et al. 1999; Hajduch et al. 2001).

The possibility of a role for Akt per se -- as opposed to PI3K -- in the regulation of neuronal processes other than cell survival is largely unexplored, although often
assumed. Blair and colleagues reported that Akt activation is necessary and sufficient to mediate a rapid modulation of L-type Ca\textsuperscript{2+} channels in cerebellar granule neurons by IGF-1 (Blair et al. 1999). That effect contributes to IGF-1 regulation of granule cell survival. In addition, there is a recent report indicating that Akt can phosphorylate GABA\textsubscript{A} receptors, leading to a rapid increase in the number of these receptors on the surface of hippocampal neurons, and pointing to a role for Akt in modulation of synaptic strength (Wang et al. 2003). There is also a report that Akt activation is required for dopamine D2 receptor mediated phosphorylation of cAMP response element-binding protein in striatal neurons, suggesting a role for Akt in long-term regulation of neuronal gene expression and plasticity (Brami-Cherrier et al. 2002).

We have previously shown that the growth factors β-neuregulin-1 (NRG1) and transforming growth factor-β1 (TGFβ1) are required for the functional expression of large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (K\textsubscript{Ca}) during the normal development of chick ciliary ganglion (CG) neurons (Subramony and Dryer 1997; Cameron et al. 1998, 2001; Lhuillier and Dryer 2002). Both factors appear to act on a pool of preexisting K\textsubscript{Ca} channels and/or auxiliary proteins, as their effects persist following complete inhibition of protein synthesis (Subramony et al. 1996; Cameron et al. 1998; Subramony and Dryer 1997). Moreover, there are significant immunochemically detectable intracellular pools of SLO α-subunits, which are essential components of functional K\textsubscript{Ca} channels (Lhuillier and Dryer 2002). TGFβ1 stimulation of K\textsubscript{Ca} requires intact PI3K signaling and this factor is able to increase phosphorylation of Akt in CG neurons (Lhuillier and Dryer 2002). However, as noted above, there are a host of downstream effectors of PI3K signaling, and
these data do not demonstrate a direct role for Akt in regulation of $K_{Ca}$, or in any other process in CG neurons.

The purpose of the present study was to examine directly the role of Akt in the regulation of $K_{Ca}$ by the growth factors NRG1 and TGFβ1. We now show that NRG1 evokes robust PI3K-dependent activation Akt, as we have previously shown to occur following TGFβ1 treatment (Lhuillier and Dryer 2002), and that the duration of Akt activation is temporally correlated with stimulation of macroscopic $K_{Ca}$. More significantly, we show that Akt activation is necessary for mobilization of functional $K_{Ca}$ channels to the plasma membrane (as measured physiologically) and for plasma membrane trafficking of SLO α-subunits (measured biochemically) evoked by either NRG1 or TGFβ1. Indeed, movement of Akt to the plasma membrane appears to be sufficient to initiate all of the processes needed to evoke trafficking of $K_{Ca}$ channels, and to thereby assure developmental acquisition of an appropriate electrophysiological phenotype.
Materials and Methods

Cell isolation and culture

CG neurons were dissociated and plated onto poly-D-lysine-coated coverslips or dishes at E9, and in one set of experiments, at E11 or E13, using methods described previously (Subramony et al. 1996; Cameron et al. 1998, 1999; Lhuillier and Dryer 2000, 2002). In pharmacological experiments, cultures were pretreated for 1 hr with the PI3K inhibitors LY294002 (Sigma, St. Louis, MO) or wortmannin (Calbiochem, San Diego, CA), or the Akt inhibitor 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (HIMO) (Calbiochem), prior to growth factor treatment. In a few experiments, the pan-caspase inhibitor Z-VAD-FMK (Calbiochem) was added to the media immediately after biolistic transfection procedures (described further below) to reduce cell death associated with Akt inhibition. In experiments involving growth factors, a recombinant form of human NRG1 comprised of amino acids 177-246 of the EGF domain of β-isoforms NRG1 (R&D Systems), or recombinant TGFβ1 (R&D Systems) was added to pretreated and control cells as indicated.

Plasmids and transfections

A plasmid encoding modified Renilla green fluorescent protein (GFP) was purchased from Stratagene (La Jolla, CA) and was used to allow visualization of transfected cells by fluorescence microscopy, as described previously (Lhuillier and Dryer 2003). Dominant-negative Akt (K179A) (DN-Akt) and a membrane-targeted (myristoylated) and therefore constitutively active Akt (CA-Akt) (Kulik et al. 1997) were generously provided by Dr. George Kulik (Wake Forest University, Winston-Salem, NC). A plasmid encoding GFP-
tagged CTMP was provided by Dr. Brian Hemmings (Frederich Meiscer Institute, Basel, Switzerland). All of these constructs are under the control of the same cytomegalovirus (CMV) promoter, and they were co-transfected into CG neurons along with GFP, except in experiments with CTMP, which carries its own GFP tag. Transfection of E9 CG neurons was accomplished by biolistic methods using a BioRad model PDS-100/He apparatus 12 hr after cells were dissociated, as described previously (Lhuillier and Dryer 2003). The gold particles which carry the constructs were coated with 7 µg of each plasmid. GFP fluorescence is first detectable at 8 hr after transfection, and its expression is very robust by 12 hr. Therefore, in our experimental designs, growth factors were applied 12 hr after transfection with DN-Akt (i.e. in cells containing large quantities of the inhibitory form), whereas microtubular and Golgi disrupting agents were applied 7 hr after transfection with CA-Akt (i.e. before expression of significant amounts of the active form). At various times indicated in the text and figure legends, $K_{Ca}$ or voltage-activated $Ca^{2+}$ currents were assayed by whole-cell recording from GFP-expressing ciliary neurons. Only 1-3 % of CG cells were transfected in any given preparation, a result typical for primary cultures of neurons. Unfortunately, this limitation precludes experiments that entail bulk biochemical assays on transfected cells.

**Immunoblot analyses and cell-surface biotinylation assays**

For immunoblot analyses of Akt phosphorylation, E9 CG neurons were treated with 1 nM or 10 nM NRG1, or vehicle, for varying durations, as indicated in the figures and figure legends. Cells were then washed in ice-cold PBS, lysed in Laemmli buffer, and samples were boiled for 5 min at 95°C and separated by SDS-PAGE on 10% gels. Proteins were
transferred to nitrocellulose membranes, blocked in a Tris-buffered saline solution containing 0.1% Tween-20 and 3% nonfat dried milk, and incubated with antibodies against phospho-Akt (Ser 473) or total Akt (Cell Signaling Technology, Beverly, MA). Blots were analyzed using anti-rabbit secondary antibodies conjugated to horseradish peroxidase and a chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). We used a commercially available surface biotinylation assay (Pierce Biotechnology) to examine cell-surface expression of SLO α-subunits (an essential component of large-conductance K{\text{Ca}} channels). Briefly, each treatment group (control, NRG1-treated, and TGFβ1-treated) was comprised of neurons dissociated from thirty E9 ciliary ganglia plated onto a 60 mm poly-D-lysine-coated plastic culture dish. After growth factor treatment (10 nM NRG1 for 3 hr or 1 nM TGFβ1 for 6 hr), the cells were exposed to 0.5 mg/ml of the membrane-impermeable biotinylation reagent sulfo-NHS-LC-Biotin for 1 hr, washed in ice-cold PBS to remove the biotinylation reagent, and the reaction was terminated by addition of 100 mM glycine in PBS buffer for 10 min on ice. The samples were lysed on ice in a buffer consisting of 20 mM Tris-HCl, pH 7.4, containing 1% NP-40, 10 mM sodium molybdate, 50 mM NaF, 2 mM NaPO_4, 1 mM sodium orthovanadate, 1 mM PMSF, and Sigma protease inhibitor cocktail, and the lysates were centrifuged for 10 min at 14,000 × g at 4°C. A portion of the supernatant was used for measurement of β-actin by immunoblot analysis. The rest was used for determination of cell-surface SLO α-subunits by incubating them with streptavidin-linked agarose beads at 4°C for 1 hr. The beads were collected and washed with lysis buffer, and samples were eluted in Laemmli buffer and assayed for SLO α-subunits by immunoblot analysis. The primary antibody against SLO α-subunits was obtained from Chemicon International (Temecula, CA) and
the antibody against β-actin was from Sigma. Protein bands were quantified using Image J software (National Institutes of Health, Bethesda, MD) and the ratio of biotinylated SLO α-subunit to β-actin taken as the biotinylation index. All experiments were repeated 3-4 times and the error bars represent S.E.M.

**Electrophysiology**

Macroscopic KCa and voltage-activated Ca\(^{2+}\) currents were measured and normalized for cell size as described previously (Dourado and Dryer 1992; Subramony et al. 1996; Cameron et al. 1998, 2001; Lhuillier and Dryer 2000, 2002, 2003). Briefly, 25-ms depolarizing steps to 0 mV were applied from a holding potential of -40 mV in normal and nominally Ca\(^{2+}\)-free salines containing 250 nM tetrodotoxin, and the net Ca\(^{2+}\)-dependent currents were obtained by digital subtraction using PCLAMP software (Axon Instruments). Surface areas were calculated from cell diameters in two orthogonal axes and the diameters were measured using an ocular micrometer. Recording electrodes were made from thin wall borosilicate glass (3-4 MΩ) and filled with a solution consisting of (in mM) 120 KCl, 2 MgCl\(_2\), 10 HEPES-KOH, and 10 EGTA, pH 7.2. Normal external salines for measurements of KCa contained (in mM) 145 NaCl, 5.4 KCl, 0.8 MgCl\(_2\), 5.4 CaCl\(_2\), 5 glucose, and 13 HEPES-NaOH, pH 7.4. Voltage-activated Ca\(^{2+}\) currents were analyzed the same way except that KCl in the recording pipettes was replaced with CsCl as described previously (Dourado and Dryer 1992; Dourado et al. 1994; Lhuillier and Dryer 2000, 2002). Throughout, error bars represent S.E.M. Data were analyzed by one-way ANOVA followed by post hoc analysis using Tukey’s honest significant difference test for unequal n using STATISTICA software, with p < 0.05
regarded as significant. In every experiment, data were collected from a minimum of two platings of ciliary ganglion neurons (i.e. from multiple cultures).
Results

NRG1 increases macroscopic $K_{Ca}$ and Akt phosphorylation in CG neurons

We have previously shown that TGFβ1 causes a sustained increase in Akt phosphorylation that parallels the time-course of $K_{Ca}$ stimulation in ciliary neurons (Lhuillier and Dryer 2002). In the present study, we observed that NRG1 also causes an increase in Akt phosphorylation and stimulation of the functional expression of $K_{Ca}$. However, the duration of the physiological and biochemical effects of NRG1 in CG neurons are concentration-dependent. Thus, application of 1 nM NRG1 evoked a robust stimulation of macroscopic $K_{Ca}$ in ciliary neurons that peaked with 3 hr of treatment but that returned to baseline levels over the course of 12 hr of continuous exposure to 1 nM NRG1 (Fig. 1A, C). Note that in a previous study, NRG1 actions on $K_{Ca}$ were only examined with a 12-hr exposure, and therefore we did not observe stimulation at 1 nM (Cameron et al., 2001). By contrast, application of 10 nM NRG1 evoked a significant increase in $K_{Ca}$ that was seen as early as 30 min after the onset of treatment and that could be sustained for at least 24 hr of continuous treatment (Fig. 1B, C). This observation is consistent with our earlier results (Cameron et al. 2001) and similar to the effects of 1 nM TGFβ1 (Cameron et al. 1998; Lhuillier and Dryer 2000). Application of NRG1 had no effect on the functional expression of voltage-activated Ca$^{2+}$ channels regardless of concentration or treatment duration (data not shown, see also Subramony and Dryer 1997).

A nearly parallel pattern was observed in analyses of Akt activation. In these experiments, NRG1 was applied at concentrations of 1 nM or 10 nM and Akt phosphorylation was assayed by immunoblot analyses at various times after the onset of
treatment (Fig. 2). Application of 1 nM NRG1 caused a transient increase in Akt phosphorylation; a large increase in signal was apparent after a 5-min application, maintained for at least 30 min, but returned to close to baseline after 3 hr of continuous exposure to NRG1 (Fig. 2A). By contrast, application of 10 nM NRG1 evoked an increase in Akt phosphorylation, at least a component of which persisted for at least 24 hr in the continuous presence of the NRG1 (Fig. 2B), similar to the effects that we have previously observed with 1 nM TGFβ1 (Lhuillier and Dryer 2002).

The increase in Akt phosphorylation evoked by NRG1 requires PI3K activation, as it was completely blocked in E9 CG neurons pretreated with either of the mechanistically distinct PI3K inhibitors LY294002 (50 µM) or wortmannin (1 µM) (Fig. 2C). Both inhibitors also abolished the increase in the functional expression of macroscopic KCa evoked by 1 nM or 10 nM NRG1 measured at 3 hr or 12 hr, respectively, after the onset of treatment (Fig. 3A, B). Consistent with our previous studies on TGFβ1, these PI3K inhibitors had no effect on the expression of macroscopic voltage-activated Ca2+ currents (Fig. 3C). In addition, there was no change in the kinetics of Ca2+ current activation or deactivation (data not shown). In summary, NRG1 evokes an increase in Akt phosphorylation and the functional expression of KCa, the duration of which depends on NRG1 concentration. Both effects require activation of PI3K.

**Akt activation is required for NRG1 and TGFβ1 stimulation of KCa.**

Akt is one of many downstream effectors of PI3K. Is this enzyme necessary for the stimulation of KCa evoked by physiologically relevant growth factors? We used three different approaches to test this hypothesis. In the first experiments, we used biolistic
transfection procedures (Lhuillier and Dryer 2003) to induce over-expression in E9 CG neurons of a dominant-negative form of Akt (DN-Akt) in which a lysine residue in the active site is mutated to alanine, thereby eliminating the kinase activity of the enzyme (Kulik et al. 1997). We observed that cells over-expressing DN-Akt together with GFP failed to exhibit a significant increase in the functional expression of $K_{Ca}$ after treatment with either 1 nM or 10 nM NRG1 or after treatment with 1 nM TGF\(\beta\)1 (Fig. 4A). Cells over-expressing GFP alone showed normal responses to both growth factors. Over-expression of DN-Akt had no effect on the density of voltage-activated \(Ca^{2+}\) currents (Fig. 4B).

It is necessary to use caution in interpreting results with over-expression of dominant-negative forms of Akt, as they can potentially bind to and effectively sequester proteins, such as PDK1, that interact with other key molecules (Vanhaesebroeck and Alessi 2000). Therefore, we have also examined the effects of the Akt inhibitor, 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (HIMO) (Hu et al. 2000; Kim et al. 2003; Saeki et al. 2003), which binds with high specificity to the PH domain of Akt. We observed that application of 5 \(\mu\)M HIMO reduced the ability of NRG1 and TGF\(\beta\)1 (Fig. 4C) to cause stimulation of $K_{Ca}$. Application of 200 nM HIMO was not effective (data not shown). As with DN-Akt, application of HIMO had no effect on expression of voltage-activated \(Ca^{2+}\) currents (Fig. 4D). A separate set of experiments was carried out to exclude the possibility that the effects of Akt inhibition on $K_{Ca}$ expression are a secondary consequence of the initiation of apoptosis. We were concerned with this issue because over-expression of DN-Akt caused an increase in the extent of cell death in our cultures. There is nothing surprising about that observation
(Brunet et al. 2001), at this point it could be described as canonical, but it made experiments at the 12-hr time points rather more difficult, more so with DN-Akt than with HIMO. Caspase inhibition appeared to be effective at reducing apoptosis, as we observed a marked increase in the number of cells that expressed GFP and DN-Akt following treatment with the broad spectrum caspase inhibitor Z-VAD-FMK (50 µM) beginning immediately after transfection (data not shown). Given this, it is reassuring that over-expression of DN-Akt continued to block NRG1 and TGFβ1 effects in CG neurons pretreated with Z-VAD-FMK (Fig. 4A) and that Ca^{2+} currents were normal in these cells (Fig. 4B).

An essential role for Akt was also indicated by experiments involving over-expression of an Akt-binding partner known as Carboxy-terminal Modulator Protein (CTMP). CTMP, which is highly expressed in neural tissue, binds to the C-terminal regulatory domain of Akt and reduces its activity at the plasma membrane by preventing phosphorylation at serine 473 and threonine 308 (Maira et al. 2001; Brazil et al. 2002; Knobbe et al. 2004). We found that over-expression of GFP-tagged CTMP blocked stimulation of K_{Ca} evoked by either NRG1 or TGFβ1, but had no effect on expression of voltage-activated Ca^{2+} currents (Fig. 5), consistent with observations made with using treatments that inhibit Akt signaling.

Akt activation is necessary, but is it sufficient to initiate all of the cascades required for mobilization of K_{Ca}? To test this hypothesis, we examined K_{Ca} expression in E9 ciliary neurons over-expressing a constitutively-active form of Akt (CA-Akt). This form of Akt has been engineered to contain a myristoylation motif that causes constitutive targeting to the plasma membrane (Kulik et al. 1997). Compared to cells
expressing GFP alone, E9 cells that also expressed CA-Akt had high densities of macroscopic $K_{Ca}$, even without NRG1 or TGFβ1 treatment (Fig. 6A). Over-expression of CA-Akt had no effect on the expression of voltage-activated Ca$^{2+}$ channels (Fig. 6B).

The stimulation of macroscopic $K_{Ca}$ by CA-Akt was not affected by treatment with the PI3K inhibitors wortmannin or LY294002 (Fig. 6A), indicating, as expected, that Akt is downstream of PI3K. However, the actions of CA-Akt were blocked in cells that were also over-expressing CTMP (Fig. 6A), although Ca$^{2+}$ currents were normal in these cells (Fig. 6B). The effects of over-expression of CA-Akt on $K_{Ca}$ were also blocked by treating CG neurons with microtubular inhibitors (nocodazole and colchicine) or by disruption of the Golgi apparatus (by treating cells with 4 µM brefeldin-A) (Fig. 7A), even though these agents did not affect Ca$^{2+}$ currents (Fig. 7B), as we have noted in a previous study (Lhuillier and Dryer 2002). A similar pattern is also observed in the responses to TGFβ1, as short-term effects on macroscopic $K_{Ca}$ require microtubular transport and Golgi processing (Lhuillier and Dryer 2002).

We have previously suggested that growth factors induce movement of preexisting $K_{Ca}$ channels from intracellular stores into the plasma membrane (Lhuillier and Dryer 2002). However, the evidence for this mechanism was indirect and based primarily on pharmacological experiments, such as those just described. We are now able to demonstrate directly that NRG1 and TGFβ1 cause Akt-dependent trafficking of $K_{Ca}$ channels to the plasma membrane by means of a cell-surface biotinylation assay. Briefly, E9 CG neurons were treated with vehicle, NRG1 (3 hr at 10 nM), or TGFβ1 (6 hr at 1 nM), at which times cell-surface proteins were biotinylated by a 1 hr exposure to a membrane-impermeable reagent (sulfo-NHS-LC-Biotin). Cells were then lysed, and the
biotinylated proteins were isolated using streptavidin-agarose, separated by PAGE, and analyzed by immunoblot using a commercially available antibody against SLO α-subunits (Fig. 8). An antibody against β-actin was used in immunoblot analyses of a portion of the whole-cell lysate to ensure that similar numbers of cells were included in each group. We observed that both NRG1 and TGFβ1 caused a robust increase in the surface expression of SLO compared to untreated controls (Fig. 8A, C), fully consistent with the results of more indirect electrophysiological experiments. Moreover, this effect was nearly abolished in CG neurons pre-treated with the Akt inhibitor HIMO (5 µM) (Fig. 8B, C), providing additional evidence that Akt is essential for growth factor-evoked movement of functional KCa channels from intracellular pools into the plasma membrane.

In summary, Akt is a downstream effector of PI3K whose activation appears to be both necessary and sufficient to initiate all of the steps required for growth factor-evoked insertion of KCa channels into the plasma membrane of ciliary neurons of the chick CG.
Discussion

There is a large literature documenting an essential role for receptor-regulated phosphoinositide-3 kinases (PI3K) in the regulation of many forms of neuronal plasticity (Lin et al. 2001; Izzo et al. 2002; Man et al. 2003), as well as in regulation of neuronal apoptosis and survival (Luo et al. 2003). The principal product of this enzyme, phosphatidylinositol (3,4,5)-triphosphate, binds to PH domains present in many enzymes and typically promotes association of these enzymes to the cytoplasmic face of the plasma membrane (Lemmon and Ferguson 2000). Among other processes, PI3K signaling regulates protein trafficking, for example in insulin-evoked insertion of plasma membrane glucose transporters such as GLUT4 (Tengholm and Meyer 2002) and in insertion of AMPA receptors during LTP in hippocampal neurons (Man et al 2003). Akt is one of the best studied of the many downstream targets of PI3K, but it is by no means the only one. For example, the phosphoinositide products of PI3K also regulate the activity or membrane localization of a diverse group of signaling enzymes, protein kinases and small GTPases important for neural development (Rodgers and Theibert 2002). Consequently, it is inappropriate to assume that Akt is involved in every cascade that entails PI3K activation. Indeed, surprisingly little is known about the role of Akt in regulation of neuronal processes other than apoptosis and survival. Here we have shown that PI3K-dependent Akt activation is required for mobilization of $K_{Ca}$ channels evoked by the growth factors NRG1 and TGFβ1. In addition, we showed that association of Akt with the plasma membrane is sufficient to trigger the cascades that lead to mobilization of $K_{Ca}$ channels.
Endogenous NRG1 and TGFβ1 are required for the normal developmental expression of macroscopic $K_{Ca}$ in ciliary neurons developing in ovo (Cameron et al. 1998, 2001). An avian form of TGFβ1 is present in the target tissues of ciliary neurons (Cameron et al. 1999) whereas NRG1 is expressed in the preganglionic neurons that innervate the CG (Cameron et al. 2001), and both types of cell-cell interactions are required for the normal developmental expression of macroscopic $K_{Ca}$ (Dourado et al. 1994). The effects of NRG1 and TGFβ1 persist when protein synthesis is blocked (Subramony et al. 1997; Cameron et al. 1998) indicating that both factors act on preexisting pools of channels. Intracellular pools of $K_{Ca}$ channels can be visualized by immunofluorescence microscopy using antibodies directed against SLO α-subunits (Lhuillier and Dryer 2002). One detail of the present study worth noting is that NRG1 is able to cause a much more rapid mobilization of $K_{Ca}$ channels than TGFβ1, either because it targets channels located in a different intracellular compartment, or because its intrinsic transduction cascades are faster.

TGFβ1 evokes increases in Akt phosphorylation in CG neurons, and its effects on Akt phosphorylation and $K_{Ca}$ are blocked by pretreatment with PI3K inhibitors (Lhuillier and Dryer 2002). In the present study, we observed that relatively low concentrations of NRG1 cause a robust but transient PI3K-dependent increase in Akt phosphorylation, whereas sustained exposure to higher concentrations of NRG1 evokes a PI3K-dependent increase in Akt phosphorylation, a portion of which remains detectable for as long as the growth factor is present (at least up to 24 hr). Transient activation of Akt evoked by NRG1 and related factors has been observed in non-neuronal, systems (Yarden and Sliwkowski 2001). However, the sustained component of the response described here is
unusual. The physiological responses to NRG1 were temporally correlated with the increase in Akt phosphorylation, as lower concentrations of NRG1 evoked a transient increase in macroscopic $K_{Ca}$ expression in ciliary cells that peaked in 3 hr and returned to baseline in less than 12 hr, even in the continuous presence of NRG1. By contrast, application of a ten-fold higher concentration of NRG1 induced a sustained increase in $K_{Ca}$ expression that could persist for at least two days after the onset of NRG1 treatment, as with Akt activation. One possible explanation for these results is that higher NRG1 concentrations alter ligand-induced internalization of cellular ErbB receptors, thereby allowing for sustained activation of Akt (Waterman et al. 1998; Wiley 2003).

Multiple lines of evidence indicate a crucial role for Akt in growth factor-evoked mobilization of ciliary neuron $K_{Ca}$ channels. As already noted, NRG1 and TGFβ1 increase phosphorylation of Akt, and the ability of both factors to increase functional expression of macroscopic $K_{Ca}$ and phosphorylation of Akt is blocked by the PI3K inhibitors LY294002 and wortmannin. More importantly, stimulation of macroscopic $K_{Ca}$ by growth factors is blocked by over-expression of a dominant-negative form of Akt (DN-Akt). Stimulation of $K_{Ca}$ is also blocked by over-expression of CTMP, an Akt-binding partner that negatively regulates its activity (Maira et al. 2001; Brazil et al. 2002), as well as by pretreatment with HIMO, a newly developed selective Akt inhibitor that binds to PH domains on the enzyme molecule (Hu et al. 2000). CTMP is heavily expressed in neural tissue (Maira et al. 2001), and it is certainly possible that endogenous CTMP plays a role in regulating channel trafficking. In any case, these data collectively demonstrate that Akt activation is necessary for growth factor-evoked mobilization of $K_{Ca}$ in ciliary neurons. In addition, over-expression of a constitutively active form of Akt
(CA-Akt) caused an increase in $K_{Ca}$ expression in E9 CG neurons, even in the absence of growth factors. The effects of CA-Akt were blocked by co-expression of CTMP but persisted in cells concurrently treated with the PI3K inhibitors LY294002 or wortmannin. Thus, Akt activation is also sufficient to trigger the cascades necessary for mobilization of $K_{Ca}$ and does not require even basal levels of class I PI3K to proceed.

On the other hand, the effect of CA-Akt on $K_{Ca}$ activation is blocked by inhibitors of microtubular polymerization, and also by brefeldin-A, an agent that disrupts Golgi processing of membrane proteins. We previously observed the same pattern for $K_{Ca}$ mobilization evoked by TGFβ1 (Lhuillier and Dryer 2002) and, more recently, for NRG1 (Oh, K. S, Chae, K. S., and Dryer, S.E., unpublished observations). Moreover, in direct experiments, we observed here that NRG1 and TGFβ1 increase the number of SLO α-subunits in the plasma membrane. These responses are blocked by HIMO, consistent with the results of electrophysiological analyses, and pointing directly to an essential role for Akt in regulation of the plasma membrane insertion of functional $K_{Ca}$ channels. It bears noting that these effects cannot be explained as a non-specific effect on membrane structure, general excitability, or ion channel expression, as expression of voltage-activated Ca$^{2+}$ channels was not affected by any of the treatments used here that targeted Akt, PI3K, microtubular transport, or the Golgi apparatus in ciliary neurons. In this regard, we previously demonstrated that TGFβ1 causes an increase in the number of ciliary neuron $K_{Ca}$ channels that could be detected in inside-out patches, under conditions where Ca$^{2+}$ concentration is controlled on both sides of the membrane (Cameron et al. 1998). Moreover, there are no obvious differences in average cell size, capacitance, or morphology following treatment with either NRG1 or TGFβ1. However, it bears noting
that the time course of the action of these factors (hours) precludes our measuring changes in cell capacitance from the same cell before and after treatment, and therefore we cannot exclude subtle biophysical changes in membrane properties associated with trafficking of the channels. The available data collectively support that growth factors acting through Akt induce insertion of new KCa channels, rather than decreasing degradation of the channels. Thus, we previously demonstrated that TGFβ1 increases in KCa are blocked completely by botulinum neurotoxins, microtubule inhibitors and brefeldin-A (Lhuillier and Dryer, 2002). Here we observed a similar pattern for NRG1 with respect to microtubules and the Golgi apparatus, and this pattern clearly favors the insertional hypothesis.

Akt has been shown to play an essential role in regulation of voltage-activated Ca^{2+} channels by IGF-1 in cerebellar granule neurons (Blair et al. 1999). That effect occurs with less than 30 sec of IGF1 exposure, which suggests that Akt causes direct phosphorylation of the channels or their auxiliary subunits, rather than stimulation of channel trafficking. In addition, the effect of Akt on Ca^{2+} channels in granule cells appears to be closely related to regulation of neuronal survival (Blair et al. 1999).

Given that, it is interesting to consider whether Akt-dependent regulation of ciliary neuron KCa channels is also related to apoptosis or survival of ciliary neurons. As discussed previously (Dryer 1998; Cameron et al. 1999), survival of developing CG neurons is regulated in part by neuronal activity, as chronic in ovo blockade of synaptic activation of developing CG neurons causes a substantial increase in naturally occurring apoptotic cell death (Wright 1981; Maderdrut et al. 1998; Meriney et al. 1987; Subramony and Dryer 1996). Functional plasma membrane KCa channels contribute to
the late phases of spike repolarization of ciliary neurons, and spike duration in ciliary neurons decreases once functional $K_{Ca}$ channels are present (Dryer et al. 1991). Therefore, the appearance of $K_{Ca}$ is likely to cause changes in intracellular $Ca^{2+}$ dynamics that occur in active CG neurons, and this in turn could regulate neuronal responsiveness to trophic factors as well as other processes related to apoptosis or survival (Johnson et al. 1992). This model predicts that $K_{Ca}$ expression should occur at the same time, or slightly before, the onset of apoptosis, and that treatments that inhibit expression of $K_{Ca}$ should reduce the extent of apoptosis. Published studies support both of these predictions.

Thus, the appearance of functional $K_{Ca}$ channels coincides with apoptosis in chick CG (Landmesser and Pilar 1974; Dourado and Dryer 1992), and immunoneutralization of TGFβ in chick embryos, which markedly reduces the developmental expression of $K_{Ca}$ (Cameron et al. 1998), also reduces developmental cell death in chick CG (Kriegstein et al. 2000).

In summary, we have demonstrated that Akt plays a central role in regulating plasma membrane mobilization of functional $K_{Ca}$ channels in response to NRG1 and TGFβ1, growth factors required for the normal development of the excitable properties of ciliary neurons. This process may also be related to a suite of changes in neuronal properties that coincide with the onset of naturally occurring developmental cell death.
Acknowledgements

We are grateful to Hannah Nguyen for technical assistance and to Dr. Sung-Gil Chi of KyungHee University for advice on signal transduction cascades. Miguel Martin-Caraballo, present address: University of Vermont, Department of Biology, Burlington, VT 05405
Grants

This work was supported by NIH grant NS32748.
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Figure Legends

Figure 1. Acute and sustained stimulation of macroscopic $K_{Ca}$ expression by β-neuregulin-1 (NRG1) in E9 ciliary ganglion neurons. A and B, Traces to the left are typical macroscopic currents evoked by depolarizing voltage steps in normal (control) and Ca$^{2+}$-free (0 Ca$^{2+}$) external saline as indicated. Traces to the right show net Ca$^{2+}$-dependent currents obtained by digital subtraction. Traces in A are from E9 ciliary neurons after 3 hr exposure to medium containing 1 nM NRG1 or normal culture medium, as indicated. Traces in B are from an E9 ciliary neuron after 12 hr exposure to 1 nM or 10 nM NRG1 as indicated. C, Summary of results from many cells. In this and subsequent figures, error bars represent s.e.m. and the number of cells tested are indicated in parentheses above the bars. Note significant (P < 0.05, asterisks) increase in $K_{Ca}$ density in ciliary neurons treated with 1 nM NRG1 for 3 hr but not for 12 hr. By contrast, cells treated with 10 nM NRG1 exhibit a significant increase in $K_{Ca}$ density that occurs within 30 min and that persists for at least 12 hr.

Figure 2. NRG1 evokes concentration- and PI3K-dependent increase in Akt phosphorylation in E9 ciliary ganglion neurons. A, Immunoblot analysis indicating that 1 nM NRG1 causes a transient increase in levels of phospho-Akt (p-Akt) in cultured E9 ciliary ganglion neurons, but has no effect on signals obtained using an antibody insensitive to the phosphorylation state of Akt. Note large increase in Akt phosphorylation that persists up to 30 min, but that returns to close to baseline after 3 hr of continuous exposure to 1 nM NRG1. In this blot, more sample was loaded at the 1 hr, 2 hr and 3 hr time points to ensure that the response was truly transient. B, Application of 10 nM NRG1 causes a robust increase in p-Akt that is still present after 24 hr of continuous exposure to the growth factor. The apparent decrease in total Akt observed at 6 hr and 15 hr reflects uneven loading, but the sustained nature of the response can be clearly discerned. C, NRG1 stimulation of Akt phosphorylation is blocked by pretreatment with the PI3K inhibitors.
Figure 3. PI3K is required for the acute and sustained increases in macroscopic $K_{Ca}$ evoked by 1 or 10 nM NRG1 in E9 ciliary ganglion neurons.  

A, Exposure to 1 or 10 nM NRG1 for 3 hr or 12 hr, respectively, failed to increase $K_{Ca}$ in ciliary ganglion neurons pretreated with the PI3K inhibitor LY294002 (50 µM).  

B, Exposure to 1 or 10 nM NRG1 for 3 hr or 12 hr, respectively, failed to increase $K_{Ca}$ in ciliary ganglion neurons pretreated with the PI3K inhibitor wortmannin (1 µM).  

C, Treatment with wortmannin or LY294002 for 12 hr had no effect on the expression of voltage-activated Ca$^{2+}$ currents, regardless of whether cells were treated with NRG1. In this and subsequent figures, single or multiple asterisks denote $P < 0.05$ compared to the matched controls.

Figure 4. Akt signaling is required for the increase in the functional expression of macroscopic $K_{Ca}$ evoked by NRG1 or TGFβ1.  

A, Transfected E9 ciliary ganglion neurons were examined after 3 hr treatment with 1 nM or 10 nM NRG1. Cells were over-expressing GFP alone or in combination with a dominant-negative form of Akt as indicated. Note that dominant-negative Akt completely blocked both the acute and sustained responses to NRG1, whereas over-expression of GFP alone had no effect. Over-expression of DN-Akt also blocked the increase in macroscopic $K_{Ca}$ evoked by 6 hr exposure of ciliary cells to 1 nM TGFβ1. The effect of DN-Akt on responses to TGFβ1 persisted in cells concurrently treated with a caspase inhibitor (Z-VAD-FMK, 50 µM).  

B, DN-Akt had no effect on expression of voltage-activated Ca$^{2+}$ currents in ciliary
neurons treated with TGFβ1, NRG1, or caspase inhibitor. **C**, Application of the Akt inhibitor HIMO (5 µM) blocked stimulation of K_{Ca} evoked by 10 nM NRG1 and 1 nM TGFβ1, respectively, but had no effect on voltage-activated Ca^{2+} currents (**D**).

**Figure 5.** Over-expression of CTMP, a naturally occurring negative modulator of Akt signaling, eliminates growth factor-evoked stimulation of K_{Ca}. CTMP blocked responses evoked by 3 or 6 hr treatment with 10 nM NRG1 or 1 nM TGFβ1, respectively (**A**), but had no effect on expression of voltage-activated Ca^{2+} currents (**B**).

**Figure 6.** Over-expression of a constitutively-active form of Akt (CA-Akt) stimulates expression of functional plasma membrane K_{Ca} channels. **A**, Concurrent over-expression of CA-Akt and GFP increases macroscopic K_{Ca} expression in ciliary neurons in the absence of growth factors, whereas over-expression of GFP alone has no effect. The effect of CA-Akt over-expression persists in cells pretreated with the PI3K inhibitors LY294002 or wortmannin, but is eliminated in cells concurrently over-expressing CTMP, a negative modulator of Akt signaling. **B**, These treatments have no effect on voltage-activated Ca^{2+} currents.

**Figure 7.** CA-Akt stimulation of macroscopic K_{Ca} requires intact microtubular transport and Golgi processing systems. **A**, Stimulation of macroscopic K_{Ca} by over-expression of CA-Akt does not occur in cells pretreated with nocodazole (20 µM) or colchicine (5 µM), which cause disassembly of microtubules, or in cells treated with 4 µM brefeldin-A,
which causes disruption of the Golgi apparatus. B, These procedures had no effect on voltage-activated Ca$^{2+}$ currents.

Figure 8. NRG1 and TGFβ1 cause an Akt-dependent increase in the plasma membrane expression of SLO α-subunits detected by a cell-surface biotinylation assay. A, Cells were treated for 3 or 6 hr with 10 nM NRG1 or 1 nM TGFβ1, respectively, and cell-surface proteins were biotinylated. Both growth factors increased the number of cell-surface (biotinylated) SLO α-subunits subsequently detected by immunoblot analysis. Expression of β-actin from the whole-cell lysate prepared from each culture was used to control for the number of cells in each sample. B, The effects of NRG1 and TGFβ1 on cell-surface SLO α-subunit expression was blocked in cells pretreated with the Akt inhibitor HIMO (5 µM). C, Densitometric analysis of the results of four repetitions of these experiments. The ordinate is the SLO α / β-actin ratio, a measure of the amount of cell-surface SLO expression.
**Fig. 1**

A

Control

NRG1 (1 nM), 3 hrs

B

NRG1 (1 nM), 12 hrs

NRG1 (10 nM), 12 hrs

C

Control

1 10 10
0.5 3 12

(NRG1, nM) (hr)

K+ current density (mA/cm²)

* p < 0.05

(11) (12) (11) (11)

(57) (10) (12) (10) (10)

(7) (6) (5) (6) (6)
Fig. 2
Fig. 3
**Fig. 4**

A) Graph showing the effects of various treatments on calcium current density (mA/cm²) with error bars for each treatment group.

- **GFP**
  - + + + + + + + + + +

- **DN-Akt**
  - - - - - - - - - -

- **NRG1 (nM)**
  - - - - + + + + + +

- **TGFβ 1 (1 nM)**
  - - - - - - - - - -

- **Caspase inhibitor**
  - - - - + + + + + +

B) Graph showing the effects of various treatments on calcium current density (mA/cm²) with error bars for each treatment group.

- **GFP**
  - + + + + + + + + + +

- **DN-Akt**
  - - - - - - - - - -

- **NRG1 (nM)**
  - - - - + + + + + +

- **TGFβ 1 (1 nM)**
  - - - - - - - - - -

- **Caspase inhibitor**
  - - - - + + + + + +

C) Graph showing the effects of various treatments on potassium current density (mA/cm²) with error bars for each treatment group.

- **HIMO (5 μM)**
  - - + + + + + + + +

- **NRG1 (10 nM)**
  - - - - + + + + + +

- **TGFβ 1 (1 nM)**
  - - + + + + + + + +

D) Graph showing the effects of various treatments on calcium current density (mA/cm²) with error bars for each treatment group.

- **HIMO (5 μM)**
  - - + + + + + + + +

- **NRG1 (10 nM)**
  - - - - + + + + + +

- **TGFβ 1 (1 nM)**
  - - + + + + + + + +

**Notes:**
- The symbol **(*)** indicates statistical significance at the 0.05 level.
- The symbol **(**) indicates statistical significance at the 0.01 level.
Fig. 5
Fig. 6

A

![Graph A]

B

![Graph B]

**Fig. 6**
Fig. 7

A

B

Fig. 7
**Fig. 8**

A

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Fig. 8