Akt Activation Is Necessary for Growth Factor–Induced Trafficking of Functional K_{Ca} Channels in Developing Parasympathetic Neurons

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Chae, Kwon-Seok, Miguel Martin-Caraballo, Marc Anderson, and Stuart E. Dryer. Akt activation is necessary for growth factor–induced trafficking of functional K_{Ca} channels in developing parasympathetic neurons. J Neurophysiol 93: 1174–1182, 2005; doi: 10.1152/jn.00796.2004. 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 on 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, overexpression 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 (Brazill et al. 2002; Vanhaesebroeck and Alessi 2000). 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 (Brunet et al. 2001; Kaplan and Miller 2000).

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 (Hajduch et al. 2001; Summers et al. 1999).

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 et al. (1999) reported that Akt activation is necessary and sufficient to mediate a rapid modulation of L-type Ca^{2+} channels in cerebellar granule neurons by IGF-1. That effect contributes to IGF-1 regulation of granule cell survival. In addition, there is a recent report indicating that Akt can phosphorylate GABA 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^{2+}-activated K^{+} channels (K_{Ca}) during the normal development of chick ciliary ganglion (CG) neurons (Cameron et al. 1998, 2001; Lhuillier and Dryer 2002; Subramony and Dryer 1997). Both factors seem to act on a pool of pre-existing K_{Ca} channels and/or auxiliary proteins, because their effects persist following complete inhibition of protein synthesis (Cameron et al. 1998; Subramony and Dryer 1997; Subramony et al. 1996). Moreover, there are significant immunohistochemically detectable intracellular pools of SLO α-subunits, which are essential components of functional K_{Ca} channels (Lhuillier and Dryer 2002). TGFβ1 stimulation of K_{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 show a direct role for Akt in regulation of K_{Ca}, or in any other process in CG neurons.

The purpose of this 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 KCa. More significantly, we show that Akt activation is necessary for mobilization of functional KCa 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 seems to be sufficient to initiate all of the processes needed to evoke trafficking of KCa channels and to thereby assure developmental acquisition of an appropriate electrophysiological phenotype.

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 (Cameron et al. 1998, 1999; Lhuillier and Dryer 2000, 2002; Subramony et al. 1996). In pharmacological experiments, cultures were pretreated for 1 h with the PI3K inhibitors LY294002 (Sigma, St. Louis, MO) or wortmannin (Calbiochem, San Diego, CA), or the Akt inhibitor 1L6-hydroxymethyl-chiro-inositol 2→(2')-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 biotin staining procedures 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-isomers NRG1 (RandD Systems) or recombinant TGFβ1 (RandD Systems) was added to pretreated and control cells as indicated.

Plasmids and transfections

A plasmid-encoding modified Renilla green fluorescent protein (GFP) was produced 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 cathepsin-β (CTMP) was provided by Dr. Brian Hemmings (Frederich Meiser Institute, Basel, Switzerland). All of these plasmids and transfections are commercially available (Amersham, Piscataway, NJ) and were used to co-transfect 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 delivery (Frederich Meiser Institute, Basel, Switzerland). All of these methods used a BioRad model PDS-100/He apparatus 12 h after cells were dissociated. After transfection, cell morphology was examined and used to select transfected and nontransfected cells.

Electrophysiology

Macroscopic KCa, and voltage-activated Ca2+ currents were measured and normalized for cell size as described previously (Cameron et al. 1998, 2001; Dourado and Dryer 1992; Lhuillier and Dryer 2000, 2002, 2003; Subramony et al. 1996). Briefly, 25-ms depolarizing steps to 0 mV were applied from a holding potential of −40 mV in normal and nominally Ca2+-free salines containing 250 mM TTX, and the net Ca2+-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 MgCl2, 10 HEPES-KOH, and 10 EGTA, pH 7.2. Normal external salines for measurements of KCa channels contained (in mM) 145 NaCl, 5.4 KCl, 0.8 MgCl2,5 0.5 CaCl2, 5 glucose, and 13 HEPES-NaOH, pH 7.4. Voltage-activated Ca2+ 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 SE. 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

Immunoblot analyses and cell-surface biotinylation assays

For immunoblot analyses of Akt phosphorylation, E9 CG neurons were treated with 1 or 10 nM NRG1 or vehicle, for varying durations, as indicated in the figures and figure legends. Cells were washed in ice-cold PBS and 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 KCa channels). Briefly, each treatment group (control, NRG1-treated, and TGFβ1-treated) was comprised of neurons dissociated from 30 E9 ciliary ganglia plated onto a 60-mm poly-d-lysine-coated plastic culture dish. After growth factor treatment (10 nM NRG1 for 3 h or 1 nM TGFβ1 for 6 h), the cells were exposed to 0.5 mg/ml of the membrane-impermeable biotinylated reagent sulfo-NHS-LC-Biotin for 1 h, 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 NaPO4, 1 mM sodium orthovanadate, 1 mM PMSF, and Sigma protease inhibitor cocktail, and the lysates were centrifuged for 10 min at 14,000g 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 h. 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 three to four times, and the error bars represent SE.
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 this 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 h of treatment but that returned to baseline levels over the course of 12 h of continuous exposure to 1 nM NRG1 (Fig. 1, A and C). Note that in a previous study, NRG1 actions on $K_{Ca}$ were only examined with a 12-h exposure, and therefore we did not observe stimulation at 1 nM (Cameron et al. 2001). In 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 ≥24 h of continuous treatment (Fig. 1, B and 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 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 ≥30 min, but returned to close to baseline after 3 h of continuous exposure to NRG1 (Fig. 2A). In contrast, application of 10 nM NRG1 evoked an increase in Akt phosphorylation, at least a component of which persisted for ≥24 h 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, because 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 $K_{Ca}$ evoked by 1 or 10 nM NRG1 measured at 3 or 12 h, respectively, after the onset of treatment (Fig. 3, A and B). Consistent with our previous studies on TGFβ1, these PI3K inhibitors had no effect on the expression of macroscopic voltage-activated $Ca^{2+}$ currents (Fig. 3C). In addition, there was no change in the kinetics of $Ca^{2+}$ current activation or deactivation (data not shown). In summary, NRG1 evokes an increase in Akt phosphorylation and the functional expression of $K_{Ca}$, the duration of which depends on NRG1 concentration. Both effects require activation of PI3K.

Akt activation is required for NRG1 and TGFβ1 stimulation of $K_{Ca}$

Akt is one of many downstream effectors of PI3K. Is this enzyme necessary for the stimulation of $K_{Ca}$ 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 overexpression 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 overexpressing DN-Akt together with GFP failed to exhibit a significant increase in the functional expression of $K_{Ca}$ after treatment with either 1 or 10 nM NRG1 or after...
treatment with 1 nM TGF-β1 (Fig. 4A). Cells overexpressing GFP alone showed normal responses to both growth factors. Overexpression 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 overexpression of dominant-negative forms of Akt, because 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, 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 μM HIMO reduced the ability of NRG1 and TGF-β1 (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 overexpression 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-h time-points rather more difficult, more so with DN-Akt than with HIMO. Caspase inhibition seemed to be effective at reducing apoptosis, because 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 overexpression of DN-Akt continued to block NRG1 and TGF-β1 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 overexpression 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 373 and threonine 308 (Brazil et al. 2002; Knobbe et al. 2004; Maira et al. 2001). We found that overexpression of GFP-tagged CTMP blocked stimulation of KC\textsubscript{a} evoked by either NRG1 or TGF\beta1, but had no effect on expression of voltage-activated Ca\textsuperscript{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 KC\textsubscript{a}? To test this hypothesis, we examined KC\textsubscript{a} expression in E9 ciliary neurons overexpressing a constitutively active form of Akt (CA-Akt). This form of Akt has been engineered to contain a myristoyl-regulatory domain of Akt and reduces its activity at the plasma membrane-impermeable reagent (sulfo-NHS-LC-Biotin). Cells were lysed, and the biotinylated proteins were isolated using a cell-surface biotinylation assay. Briefly, E9 CG neurons were treated with vehicle, NRG1, or caspase inhibitor. A similar pattern is also observed in the responses to TGF\beta1, because short-term effects on macroscopic KC\textsubscript{a} require microtubular transport and Golgi processing (Lhuillier and Dryer 2002).

We have previously suggested that growth factors induce movement of pre-existing KC\textsubscript{a} 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 directly show that NRG1 and TGF\beta1 cause Akt-dependent trafficking of KC\textsubscript{a} channels to the plasma membrane by means of a cell-surface biotinylation assay. Briefly, E9 CG neurons were treated with vehicle, NRG1 (3 h at 10 nM), or TGF\beta1 (6 h at 1 nM), at which times cell surface proteins were biotinylated by a 1-h exposure to a membrane-impermeable reagent (sulfo-NHS-LC-Biotin). Cells were lysed, and the biotinylated proteins were isolated using streptavidin-agarose, separated by PAGE, and analyzed by immunoblot using a commercially available antibody against SLO \alpha-subunits (Fig. 8). An antibody against \beta-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 TGF\beta1 caused a robust increase in the surface expression of SLO compared with untreated controls (Fig. 8, A and C), fully consistent with the results of more indirect electrophysiological experiments. Moreover, this effect was nearly abolished in CG neurons pretreated with the Akt inhibitor HIMO (5 \muM; Fig. 8, B and C), providing additional evidence that Akt is essential for growth factor-evoked movement of functional KC\textsubscript{a} channels from intracellular pools into the plasma membrane.
Akt is a downstream effector of PI3K whose activation seems 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 amount of literature documenting an essential role for receptor-regulated PI3Ks in the regulation of many forms of neuronal plasticity (Izzo et al. 2002; Lin et al. 2001; 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 long-term potentiation (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 KCa 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 KCa channels.

Endogenous NRG1 and TGFβ1 are required for the normal developmental expression of macroscopic KCa 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 KCa (Dourado et al. 1994). The effects of NRG1 and TGFβ1 persist when protein synthesis is blocked (Cameron et al. 1998; Subramony et al. 1997), indicating that both factors act on pre-existing pools of channels. Intracellular pools of KCa chan-
nels can be visualized by immunofluorescence microscopy using antibodies directed against SLO α-subunits (Lhuillier and Dryer 2002). One detail of this study worth noting is that NRG1 is able to cause a much more rapid mobilization of KCa 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 KCa are blocked by pretreatment with PI3K inhibitors (Lhuillier and Dryer 2002). In this 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 24 h). 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, because lower concentrations of NRG1 evoked a transient increase in macroscopic KCa expression in ciliary cells that peaked in 3 h and returned to baseline in <12 h, even in the continuous presence of NRG1. In contrast, application of a 10-fold higher concentration of NRG1 induced a sustained increase in KCa expression that could persist for at least 2 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 KCa channels. As already noted, NRG1 and TGFβ1 increase phosphorylation of Akt, and the ability of both factors to increase functional expression of macroscopic KCa and phosphorylation of Akt is blocked by the PI3K inhibitors LY294002 and wortmannin. More importantly, stimulation of macroscopic KCa by growth factors is blocked by overexpression of DN-Akt. Stimulation of KCa is also blocked by overexpression of CTMP, an Akt-binding partner that negatively regulates its activity (Brazil et al. 2002; Maira et al. 2001), as well as by pretreatment with HIMO, a newly developed selective Akt

FIG. 7. CA-Akt stimulation of macroscopic KCa requires intact microtubular transport and Golgi processing systems. A: stimulation of macroscopic KCa by overexpression 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 mM brefeldin-A, which causes disruption of the Golgi apparatus. B: these procedures had no effect on voltage-activated Ca2+ currents.

FIG. 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 h 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: 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 4 repetitions of these experiments. Ordinate is the SLO α/β-actin ratio, a measure of the amount of cell-surface SLO expression.
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 show that Akt activation is necessary for growth factor–evoked mobilization of K\(_{\text{Ca}}\) in ciliary neurons. In addition, overexpression of CA-Akt caused an increase in K\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) and does not require even basal levels of class I PI3K to proceed.

On the other hand, the effect of CA-Akt on K\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) mobilization evoked by TGF\(\beta\)1 (Lhuillier and Dryer 2002) and, more recently, for NRG1 (K. S. Oh, K. S. Chae, and S. E. Dryer, unpublished observations). Moreover, in direct experiments, we observed here that NRG1 and TGF\(\beta\)1 increase the number of SLO \(\alpha\)-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\(_{\text{Ca}}\) channels. It bears noting that these effects cannot be explained as a nonspecific effect on membrane structure, general excitability, or ion channel expression, because 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 showed that TGF\(\beta\)1 causes an increase in the number of ciliary neuron K\(_{\text{Ca}}\) 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 TGF\(\beta\)1. 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 K\(_{\text{Ca}}\) channels rather than decreasing degradation of the channels. Thus we previously showed that TGF\(\beta\)1 increases in K\(_{\text{Ca}}\) 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 insertion 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 \(<30\) s of IGF-1 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 seems 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 K\(_{\text{Ca}}\) channels is also related to apoptosis or survival of ciliary neurons. As discussed previously (Cameron et al. 1999; Dryer 1998), 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 (Maderdrut et al. 1988; Meriney et al. 1987; Subramony and Dryer 1996; Wright 1981). Functional plasma membrane K\(_{\text{Ca}}\) channels contribute to the late phases of spike repolarization of ciliary neurons, and spike duration in ciliary neurons decreases once functional K\(_{\text{Ca}}\) channels are present (Dryer et al. 1991). Therefore the appearance of K\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) expression should occur at the same time, or slightly before, the onset of apoptosis, and that treatments that inhibit expression of K\(_{\text{Ca}}\) should reduce the extent of apoptosis. Published studies support both of these predictions. Thus the appearance of functional K\(_{\text{Ca}}\) channels coincides with apoptosis in chick CG (Dourado and Dryer 1992; Landmesser and Pilar 1974), and immunoneutralization of TGF\(\beta\) in chick embryos, which markedly reduces the developmental expression of K\(_{\text{Ca}}\) (Cameron et al. 1998), also reduces developmental cell death in chick CG (Krieglstein et al. 2000).

In summary, we have shown that Akt plays a central role in regulating plasma membrane mobilization of functional K\(_{\text{Ca}}\) channels in response to NRG1 and TGF\(\beta\)1, 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.

A C K N O W L E D G M E N T S

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