Developmental Regulation of Neuronal K$_{Ca}$ Channels by TGFβ1: An Essential Role for PI3 Kinase Signaling and Membrane Insertion

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Lhuillier, Loic, and Stuart E. Dryer. Developmental regulation of neuronal K$_{Ca}$ channels by TGFβ1: an essential role for PI3 kinase signaling and membrane insertion. J Neurophysiol 88: 954–964, 2002; 10.1152/jn.00976.2001. TGFβ1 is a target-derived factor responsible for the developmental expression of large-conductance Ca$^{2+}$-activated K$^+$ (K$_{Ca}$) channels in ciliary neurons of the chick ciliary ganglion. The acute effects of TGFβ1 on K$_{Ca}$ channels are blocked by inhibitors of the Erk signaling cascade. Application of the MEK1 inhibitor PD98059 blocked TGFβ1 effects on Erk but had no effect on Akt/PKB phosphorylation. These results indicate that PI3K and Erk represent parallel signaling cascades activated by TGFβ1 in ciliary neurons. The effects of TGFβ1 on functional expression of K$_{Ca}$ are blocked by the microtubule inhibitors colchicine and nocodazole, by botulinum toxins A and E, and by brefeldin-A, an agent that disrupts the Golgi apparatus. These data indicate that translocation of a membrane protein, possibly Slowpoke (SLO), is required for the acute posttranslational effects of TGFβ1 on K$_{Ca}$ channels. Confocal immunofluorescence studies with three different SLO antisera showed robust expression of SLO in multiple intracellular compartments of embryonic day 9–13 ciliary neurons, including the cell nucleus. These data suggest that TGFβ1 evokes insertion of SLO channels into the plasma membrane as a result of signaling cascades that entail activation of Erk and PI3K.

INTRODUCTION

Different neuronal populations often exhibit markedly divergent electrophysiological properties owing to differences in the expression of voltage- and ligand-gated ion channels. These specialized properties of vertebrate neurons emerge gradually during development (Spitzer and Ribera 1998) in part as a result of inductive interactions with other cell types (Dryer 1998). Large-conductance Ca$^{2+}$-activated K$^+$ channels (K$_{Ca}$) play a significant role in the regulation of spike waveform and temporal firing patterns in many neuronal cell types (Fettiplace and Fuchs 1999; Golding et al. 1999; Lang et al. 1997; Shao et al. 1999; Wang et al. 1999). To understand the differentiation of excitability, we have studied the developmental regulation of K$_{Ca}$ channels in autonomic ganglion neurons (reviewed in Dryer 1998).

Functional plasma membrane K$_{Ca}$ channels on ciliary neurons of the parasympathetic chick ciliary ganglion (CG) are first detectable at embryonic day 9 (E9) and reach maximal density by E13, coinciding with the stages at which these neurons form synapses with target tissues in the eye (Dourado and Dryer 1992; Dourado et al. 1994). Ciliary neurons that develop in vivo or in vitro in the absence of target tissues or target-derived factors fail to express normal densities of functional plasma membrane K$_{Ca}$ channels (Cameron et al. 1998, 2001; Dourado and Dryer 1992; Dourado et al. 1994; Subramony et al. 1996). This trophic effect of target tissues is mediated by an avian ortholog of TGFβ1 secreted from intracocular striated muscle cells; this evokes an increase in K$_{Ca}$ density in the plasma membrane as accessed by both whole cell and single-channel measurements (Cameron et al. 1998, 1999; Lhuillier and Dryer 2000).

The intracellular mechanisms that underlie stimulation of ion channels by trophic factors are not well understood. However, the effects of TGFβ1 on ciliary neurons are mediated at least in part by posttranslational processes (Cameron et al. 1998, 2001; Dourado and Dryer 2000; Subramony et al. 1996). We have recently shown that TGFβ1 actions in ciliary neurons require activation of the MAP kinase Erk (Lhuillier and Dryer 2000). Thus TGFβ1 evokes a transient increase in Erk activation that occurs within 5 min and that returns to baseline within 1 h, and the effects of TGFβ1 on stimulation of K$_{Ca}$ are blocked by inhibitors of the Erk signaling cascade. Although most studies of TGFβ signal transduction have focused on Smad proteins (Massague and Chen 2000), there is now evidence that activation of phosphatidylinositol 3-0H kinase (PI3K) is required for some of the actions of TGFβ in nonneuronal cells (e.g., Bakin et al. 2000; Peron et al. 2000). This is of interest because the phosphoinositide products of PI3K, principally phosphatidylinositol-3,4,5-trisphosphate (PtdIns[3,4,5]P$_3$) and PtdIns[3,4]P$_2$, can regulate proteins involved in the targeting and insertion of membrane proteins (Cantrell 2001; Corvera and Czech 1998; Rameh and Cantley 1999) including neuronal ion channels (Blair and Marshall 1997; Melnikova and Gardner 2001; Wu et al. 1998). Here we show that activation of PI3K is an essential step in the TGFβ1-evoked stimulation of macroscopic K$_{Ca}$ in ciliary neurons and that this effect is associated with translocation of proteins to the plasma membrane. Finally, we show that PI3K and Erk repre-

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sent parallel as opposed to serial outputs of the TGFβ transduction pathway in these cells.

METHODS

Cell isolation and culture

These procedures have been described in detail previously (Cameron et al. 1998, 1999; Lhuillier and Dryer 2000; Subramony et al. 1996). Briefly, ciliary ganglion neurons were dissociated at E9 or E13, plated onto poly-d-lysine-coated glass coverslips, and cultured for varying lengths of time as indicated. Whole cell recordings from acutely isolated E13 cells were performed within 3 h of cell dissociation. Recombinant human TGFβ1 was obtained from R&D Systems (Minneapolis, MN). The PI3K inhibitors LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4) and wortmannin were obtained from Sigma (St. Louis, MO). The microtubule-disrupting agents, colchicine and nocodazole (methyl N-(5-thenoyl-2-benzimidazolyl) carbamate), as well as botulinum toxins (serotypes A and E; BoNT/A and BoNT/E), and brefeldin-A were obtained from Calbiochem (San Diego, CA). Cells were incubated with these agents for 30 min before the addition of TGFβ1 and grown for 6 or 12 h as indicated in the text, before KCa was measured by whole cell recording. To test for direct block of KCa channels by these agents, E13 neurons were treated with indicated pharmacological agents for 3 or 6 h and KCa was measured by whole cell recording.

Electrophysiology

Whole cell recordings were made using standard methods as described previously (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Dourado et al. 1994). 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 500 mM tetrodotoxin, and the net Ca2+-dependent currents were obtained by digital subtraction using Pclamp software (Axon Instruments, Foster City, CA). Currents were normalized for cell size by computing the soma surface area as described previously (Cameron et al. 1998, 1999; Dourado and Dryer 1992; Subramony et al. 1996). Similar protocols were used to analyze voltage-activated Ca2+ currents except that KCl in the recording pipettes was replaced with CsCl as described previously (Cameron et al. 1999; Dourado and Dryer 1992; Dourado et al. 1994). Throughout this paper, error bars represent SE. Data were analyzed by one-way ANOVA followed by Scheffe’s multiple range tests using Statistica software (Statsoft, Tulsa, OK), with P < 0.05 regarded as significant.

Immunoblot analyses

For measurements of Akt/PKB phosphorylation, 10 E9 ciliary ganglia were plated onto each coverslip. TGFβ1 (1 nM) was applied to cultures 3 h after plating and maintained for varying lengths of time as indicated. Control coverslips did not receive trophic factors. Cells were then washed in ice-cold PBS and lysed in 2× Laemmli sample buffer. Samples were boiled for 5 min and separated by SDS-PAGE on 12% gels. Proteins were transferred to nitrocellulose membranes, which were then blocked in a Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dried milk before overnight incubation with either a polyclonal antibody specific for Ser-473 phosphorylated-Akt/PKB (P-Akt) or a polyclonal antibody insensitive to the phosphorylation state of Akt/PKB (Akt-P) and an antibody insensitive to the phosphorylation state of Akt/PKB (Akt). For measurements of Akt/PKB phosphorylation, 10 E9 ciliary ganglia were cultured in the presence of 1 nM TGFβ1 for various lengths of time, as indicated. Cells were then lysed and the phosphorylation state of Akt/PKB was assessed by immunoblot analysis using an antibody selective for Ser-473 phosphorylated-Akt/PKB (Akt-P) and an antibody insensitive to the phosphorylation state of Akt/PKB (Akt). Each sample was analyzed with both antibodies. TGFβ1 increases Akt/PKB phosphorylation in developing ciliary ganglion (CG) neurons. Embryonic day 9 (E9) CG neurons were cultured in the presence of 1 nM TGFβ1 for various lengths of time, as indicated. Cells were then lysed and the phosphorylation state of Akt/PKB was assessed by immunoblot analysis using an antibody selective for Ser-473 phosphorylated-Akt/PKB (Akt-P) and an antibody insensitive to the phosphorylation state of Akt/PKB (Akt). Each sample was analyzed with both antibodies. TGFβ1 increases Akt/PKB phosphorylation within the first 30 min of application and this activation lasts for more than 12 h. This experiment was repeated 3 times. Typical immunoblots; bottom: time course of Akt phosphorylation determined by densitometric analysis of the blots.

FIG. 1. TGFβ1 increases Akt/PKB phosphorylation in developing ciliary ganglion (CG) neurons. Embryonic day 9 (E9) CG neurons were cultured in the presence of 1 nM TGFβ1 for various lengths of time, as indicated. Cells were then lysed and the phosphorylation state of Akt/PKB was assessed by immunoblot analysis using an antibody selective for Ser-473 phosphorylated-Akt/PKB (Akt-P) and an antibody insensitive to the phosphorylation state of Akt/PKB (Akt). Each sample was analyzed with both antibodies. TGFβ1 increases Akt/PKB phosphorylation within the first 30 min of application and this activation lasts for more than 12 h. This experiment was repeated 3 times. Top: typical immunoblots; bottom: time course of Akt phosphorylation determined by densitometric analysis of the blots.
**Immunocytochemistry**

Cells were grown on poly-α-lysine-coated coverslips as described earlier. Cultures were fixed in Zamboni's fixative and blocked overnight at room temperature in blocking solution (PBS containing 10% horse serum, 0.5% Triton X-100, and 0.2% NaN₃). Cultures were then incubated overnight with various primary antibodies (described further below) in blocking solution at room temperature. After several washes, cultures were blocked for 30 min in a second solution (PBS containing 10% normal goat serum, 5% BSA, 1% fish gelatin, and 0.1% Triton X-100), followed by a 1-h incubation with the appropriate secondary antibodies. Coverslips were then mounted in Vectashield medium (Vector Labs, Burlingame, CA) and examined using a Zeiss confocal microscope. Anti-SLO antibodies used were kindly provided by Dr. Irwin Levitan (University of Pennsylvania, Philadelphia, PA; antibody G18, abbreviated SLO Ab in the figures; and antibody MP, abbreviated SLO Ab(H11032) in the figures). G18 antibody was generated against aa 972–1,135 of mSLO (73% identity at the amino acid level with chicken SLO). MP antibody was raised against aa 913–926 of mSLO (90% identity with chicken SLO). SLO Ab(H11033)

**Fig. 2.** Inhibitors of phosphatidylinositol 3-OH kinase (PI3K) block the stimulatory effects of TGFβ1 on functional expression of KᵥCa. E9 CG neurons were cultured for 12 h in medium containing 1 nM TGFβ1, LY294002 (10 or 50 μM) or wortmannin (500 nM). Macroscopic KᵥCa and Ca²⁺ currents were measured by whole cell recording at the end of the treatment. Numbers in parentheses indicate the number of cells recorded for each condition. A: LY294002 (a PI3K inhibitor) at 2 different concentrations blocks TGFβ1 stimulation of KᵥCa expression. B: wortmannin, a structurally dissimilar PI3K inhibitor, also blocks TGFβ1 stimulation of KᵥCa expression. C and D: voltage-activated Ca²⁺ currents were not affected by PI3K inhibitors. In this and subsequent figures, *, P < 0.05 compared with control; n.s., no significant difference (P > 0.05).
antibody was raised against aa 499–898 of mSLO (94% identity with chicken SLO), but it bears noting that the antigen sites are not overlapping. All three antibodies are directed against C-terminal domains of mammalian SLO proteins. An anti-KDEL antibody (Stress-Gen, San Diego, CA) was used as a marker for endoplasmic reticulum. Secondary antibodies were Cy3-conjugated anti-rabbit IgG (Vector Labs) and Alexa-Fluor 488 anti-mouse IgG (Molecular Probes, Eugene, OR).

RESULTS

TGFβ1 actions are associated with activation of phosphatidylinositol 3-OH kinase

In initial experiments, we examined the effects of TGFβ1 on phosphorylation of the protein kinase Akt/PKB at Ser-473 in CG neurons. Akt/PKB is activated by PtdIns[3,4]P 2 - or PtdIns[3,4,5]P 3 -dependent phosphorylation at Thr-308 and Ser-473, a process that generally requires activation of PI3K in intact cells (Corvera and Czech 1998; Vanhaesebroeck and Alessi 2000). Akt/PKB therefore represents a convenient biochemical marker for PI3K activation. In dissociated E9 CG neurons, application of 1 nM TGFβ1 evokes a robust increase in the ratio of phospho-Akt/PKB to total Akt/PKB within 30 min after the onset of trophic factor treatment (Fig. 1). Increased Akt/PKB phosphorylation can be sustained for at least 12 h in the continuous presence of TGFβ1 (Fig. 1B). This contrasts with TGFβ1 activation of Erk in CG neurons, which returns to baseline within 1 h even in the continuous presence of TGFβ1 (Lhuillier and Dryer 2000).

Is PI3K activation required for TGFβ1-induced stimulation of K_Ca? To address this question, the functional expression of K_Ca was assayed using whole cell recordings from E9 CG neurons cultured for 12 h in the presence and absence of 1 nM recombinant TGFβ1. Currents were evoked by a depolarizing step to 0 mV from a holding potential of −40 mV in the presence of 250 nM tetrodotoxin to block voltage-activated Na⁺ channels. In each cell, currents were evoked in the presence and absence of external Ca²⁺ ions and the net Ca²⁺-dependent currents were obtained by digital subtraction and normalized for cell size to obtain an estimate of K_Ca current density. As with our previous studies (Cameron et al. 1998, 1999; Lhuillier and Dryer 2000), application of 1 nM TGFβ1 evoked a three- to fivefold increase in the density of macroscopic K_Ca (Fig. 2, A and B). However, the stimulatory effect of TGFβ1 was abolished in CG neurons treated with the PI3K inhibitors LY294002 (at 10 and 50 μM; Fig. 2A) or wortmannin (at 500 nM; Fig. 2B). These drugs had no effect on the basal expression of K_Ca in E9 neurons. Moreover, these structurally dissimilar PI3K inhibitors had no effect on K_Ca channels of acutely isolated E13 CG neurons that were already in the plasma membrane (data not shown; see METHODS) or on voltage-activated Ca²⁺ currents in E9 neurons (Fig. 2, C and D). These experiments indicate that the stimulatory actions of TGFβ require activation of PI3K and that the effect on K_Ca cannot be attributed to changes in Ca²⁺ dynamics.

Akt/PKB and Erk appear to be parallel as opposed to serial consequences of TGFβ1 stimulation of CG neurons.
To ascertain this point, dissociated E9 CG neurons were treated with 1 nM TGFβ1 or control medium, and proteins were extracted from cells 5 min, 30 min, or 3 h after the onset of trophic factor treatment. The phosphorylation state of Erk or Akt/PKB was then determined by immunoblot analysis (Fig. 3). Somewhat surprisingly, a significant basal phospho-Akt/PKB signal (at Ser-473) was detected in the presence of the PI3K inhibitor LY294002 (50 μM). However, this signal was undetectable in cells treated with 1 nM TGFβ1 in the presence of LY294002. Application of LY294002 had no effect on basal or TGFβ1-evoked Erk phosphorylation monitored in the same cells (Fig. 3). Conversely, application of the MEK1 inhibitor PD98059 had no effect on basal or TGFβ1-stimulated Akt/PKB phosphorylation. However, PD98059 inhibited the increase in Erk phosphorylation evoked by TGFβ1 (Fig. 3B) as noted in an earlier study (Lhuillier and Dryer 2000). In other words, Erk and the downstream targets of PI3K represent parallel consequences of TGFβ receptor stimulation in CG neurons, as each cascade can proceed normally in the absence of the other.

**Pharmacological evidence that TGFβ1 actions are associated with translocation of proteins to the plasma membrane**

Some of the downstream targets of PI3K cascades are proteins involved in membrane targeting and insertion (Cantrell 2001; Corvera and Czech 1998; Rameh and Cantley 1999). TGFβ1-evoked stimulation of macroscopic \( K_{Ca} \) in CG neurons persists in the presence of protein synthesis inhibitors, but a complete response is not seen until 3–7 h after the onset of trophic factor treatment (Lhuillier and Dryer 2000; Subramony et al. 1996). This raises the possibility that TGFβ1 actions entail translocation of a preexisting pool of \( K_{Ca} \) channels or associated proteins from an intracellular pool to the plasma membrane.
membrane. We have obtained several additional lines of evidence to support this hypothesis.

Intracellular trafficking of membrane proteins often entails tubulovesicular structures (Klumperman 2000; Prekeris et al. 1999) or other elements of the cytoskeleton (Shoop et al. 2000). We have observed that the stimulatory actions of TGFβ1 on macroscopic KCa channels require intact microtubules. To ascertain this, 1 nM TGFβ1 was applied to CG neurons for 30 min, at which time the cells were treated with the microtubule disrupting agents colchicine (5 μM) or nocodazole (20 μM; Fig. 4). Macroscopic KCa density was monitored by whole cell recording 6 h after the onset of TGFβ1 treatment. Negative control cells did not receive TGFβ1, whereas positive control cells were treated with TGFβ1 but did not receive microtubule-disrupting agents. Our earlier studies showed that Erk activation is maximal 5 min after the onset of TGFβ1 treatment (Lhuillier and Dryer 2000). The present experimental design ensured that disruption of microtubules did not block the initial steps of TGFβ1 signal transduction because the inhibitors were not applied until well after these initial steps were complete. We observed that treatment with either colchicine (Fig. 4A) or nocodazole (Fig. 4B) completely blocked the stimulatory effects of TGFβ1. In separate control experiments, we observed that microtubule disruption did not disrupt plasma membrane KCa channels of acutely isolated E13 CG neurons (data not shown) and did not affect expression of voltage-activated Ca2+ currents in E9 cells (Fig. 4, C and D).

Using a similar experimental design, we observed that profound perturbation of the Golgi apparatus also prevented TGFβ1-induced increases in macroscopic KCa (Fig. 5). In these experiments, E9 CG neurons were treated with brefeldin-A (5 μg/ml), which causes complete disassembly of the Golgi apparatus and thereby prevents processing and translocation of membrane proteins from Golgi or pre-Golgi compartments (Sciacy et al. 1997). Brefeldin-A treatment blocked the stimulatory effects of TGFβ1 but did not alter basal levels of KCa in E9 CG neurons (Fig. 5A). Moreover, this drug did not affect fully established macroscopic KCa (in E13 CG neurons) and blocked TGFβ1 actions under conditions that did not affect expression of voltage-activated Ca2+ channels in E9 neurons (Fig. 5B).

Two different botulinum toxins also blocked the stimulatory effects of TGFβ1 on KCa channels of ciliary neurons (Fig. 6). Botulinum toxin A (BoNT/A) and botulinum toxin E (BoNT/E) are zinc-endopeptidases that disrupt membrane targeting and exocytosis by cleaving different SNARE proteins required for docking and fusion of intracellular vesicles (Jahn et al. 1995; Montecucco and Schiavo 1995). Treatment of E9 CG neurons with either of these neurotoxins prevented the increase in macroscopic KCa evoked by 1 nM TGFβ1 (Fig. 6B) but had no effect on expression of Ca2+ channels (Fig. 6, C and D) or on KCa channels that were already in the plasma membrane (data not shown). These data indicate that TGFβ1 stimulation of KCa requires fusion of intracellular vesicles with docking sites on the plasma membrane.

**Immunohistochemical localization of KCa channels in ciliary ganglion neurons**

The experiments described in the preceding text provided pharmacological evidence that KCa channels or an essential auxiliary protein are retained within an intracellular pool and are translocated to the plasma membrane by a PI3K-dependent process in response to TGFβ1. Therefore it was of interest to attempt to localize the subcellular distribution of these channels directly. To do this, we utilized three different polyclonal antisera directed against nonoverlapping portions of the C-terminus of mammalian SLO alpha subunits (kindly provided by Drs. Irwin Levitan of the University of Pennsylvania and David McCobb of Cornell University). SLO is the protein product of the gene that encodes for large-conductance KCa channels. The C-terminal domains of SLO are well conserved in mammals and birds (Jiang et al. 1997; Navaratnam et al. 1997; Rosenblatt et al. 1997), and immunoblot analyses indicated that all three antisera selectively labeled SLO proteins of...
chick brain and CG (data not shown). Immunostaining of E9 CG neurons (Figs. 7 and 8) indicated that SLO alpha subunits are expressed at this developmental stage, consistent with our earlier studies on slo transcript expression obtained by RT-PCR (Lhuillier and Dryer 2000; Subramony et al. 1996).

Surprisingly, the most intense SLO signal appeared to be nuclear in E9 CG neurons (Figs. 7 and 8). Initially we considered that this signal could arise from the nuclear envelope, but we have never been able to obtain confocal optical sections with a "halo" pattern, suggesting that this signal is originating at least in part from the nuclear matrix. Moreover, we obtain robust signal from immunoblots prepared from isolated nuclei from CG neurons (Fig. 8B). It is not clear why a plasma membrane ion channel protein should appear to be heavily expressed in the nucleus, but it again bears noting that this pattern was observed with all three of the polyclonal antisera available to us, and that these antisera were raised against different C-terminal peptides. Moreover, the apparent nuclear localization of plasma membrane proteins is not without precedent and has been observed with small-conductance (SK) type Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels under some conditions (Miller et al. 2001). It bears noting that we also observed nuclear SLO signal in E13 CG neurons, which express maximal densities of large-conductance KCa channels in the plasma membrane (data not shown).

Outside of the cell nucleus, we observed substantial amounts of punctate intracellular SLO staining as well as lower levels of more diffuse signal that presumably arises from plasma membrane (Figs. 7 and 8). Antibodies against KDEL, an endoplasmic reticulum marker, also co-localize with some of the intracellular punctate SLO staining (Fig. 7). These images provide direct evidence for intracellular stores of SLO of the sort that would be likely to participate in membrane targeting. To date we have been unable to demonstrate a TGF\beta1-evoked change
in the pattern or intensity of the SLO confocal immunofluorescence signal, especially in the diffuse extra-nuclear label most likely to arise from plasma membrane K<sub>Ca</sub> channels (data not shown). There are several possible reasons for this result, which will be outlined in the DISCUSSION.

DISCUSSION

Previous work from our laboratory demonstrated that an avian ortholog of TGF<sub>β1</sub> is the target-derived factor required for normal developmental regulation of large-conductance K<sub>Ca</sub> channels in ciliary neurons of the chick ciliary ganglion (Cameron et al. 1998; Lhuillier and Dryer 1999, 2000). The short-term effects of TGF<sub>β1</sub>, while requiring several hours to manifest, are posttranslational (Cameron et al. 1998; Subramony et al. 1996) and require activation of Erk MAP kinase (Lhuillier and Dryer 2000). Here we have shown that TGF<sub>β1</sub> also causes activation of Akt/PKB, a downstream biochemical readout of the signaling enzyme PI3K, and that PI3K activation is essential for stimulation of K<sub>Ca</sub> by TGF<sub>β1</sub> in developing ciliary neurons. In addition, we show that the acute effects of TGF<sub>β1</sub> are associated with insertion of proteins into the plasma membrane, and we present immunofluorescence data indicating that substantial amounts of SLO protein are present in intracellular compartments of developing CG neurons. Surprisingly, a considerable amount of SLO protein appears to be expressed in the cell nucleus at both E9 and at E13.

An essential role for PI3K in the developmental regulation of K<sub>Ca</sub> channels is indicated by experiments performed with two structurally dissimilar inhibitors, wortmannin and LY294002. Both of these PI3K inhibitors blocked the short-term effects of TGF<sub>β1</sub> on the functional expression of K<sub>Ca</sub> channels but had no direct effect on the K<sub>Ca</sub> channels that were already in the plasma membrane or on voltage-activated Ca<sup>2+</sup> channels. A role for PI3K in K<sub>Ca</sub> regulation is not surprising. This enzyme catalyzes formation of several phosphoinositol-3-phosphate phospholipids from phosphoinositides and becomes active in many different growth factor signaling cascades. The products of PI3K can then cause direct or indirect activation of a wide variety of intracellular signaling enzymes, many of which contain pleckstrin-homology (PH) and FYVE-finger domains that can bind PtdIns[3,4,5]P<sub>3</sub> or PtdIns[3,4]P<sub>2</sub>. Several of these enzymes, including small GTPases, guanine nucleotide exchange factors, and ADP-ribosylation factors, are involved in processing and targeting of membrane proteins (Cantrell 2001; Corvera and Czech 1998; Rameh and Cantley 1999). Although other TGF<sub>β</sub> transduction cascades have been more extensively studied (reviewed in Massague and Chen 2000), there is evidence that TGF<sub>β1</sub> can signal through PI3K.

FIG. 7. Slowpoke (SLO) proteins are retained in an intracellular pool in CG neurons. E9 CG neurons were cultured overnight and immunocytochemistry was carried out as described in METHODS. Top: cells treated only with primary antibody against SLO; middle: cells treated only with primary antibody against KDEL, an ER marker; and bottom: cells treated with both primary antibodies, as indicated on the left of the panel. SLO immunoreactivity (right) was revealed using a Cy3-conjugated secondary antibody and KDEL immunoreactivity (left) was visualized with an Alexa 488-conjugated secondary antibody. Both secondary antibodies were present in all experiments. An overlay of SLO and KDEL immunoreactivities is displayed in the right column. SLO immunoreactivity is robustly expressed in a perinuclear zone, and there is also punctate staining throughout the intracellular areas. A portion of this punctate signal appears to colocalize with the endoplasmic reticulum (ER) marker (arrows), and the remaining signal is light and diffuse. The punctate SLO signal that co-localizes with KDEL appears as yellow, shown on the inset at a higher magnification.

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cascades in nonneuronal cells (Bakin et al. 2000; Chen et al. 1998). The mechanism whereby TGFβ1 causes PI3K activation is not known, but is unlikely to involve Smad signaling. However, TGFβ1 can evoke activation of small GTPases of the Ras or Rho families in nonneuronal cells (Hartsough and Mulder 1996; Mucsi et al. 1996), and these proteins can feed into PI3K and/or Erk signaling pathways.

Consistent with these data, we observed that TGFβ1 causes a robust increase in Akt/PKB phosphorylation in CG neurons, and that this effect could be observed in the presence of inhibitors of the Erk signaling cascade. In these experiments, we used Akt/PKB phosphorylation at Ser-473 as a biochemical index for Akt/PKB activation. Phosphorylation at this site and at Thr-308 is necessary for Akt/PKB to become catalytically active, but the mechanism whereby Akt/PKB becomes phosphorylated at these residues is complex (reviewed in Toker 2000). Phosphoinositide-dependent kinase-1 (PDK-1) phosphorylates Akt/PKB at Thr-308 (Alessi et al. 1997; Stokoe et al. 1997; Toker and Newton 2000). PtdIns[3,4,5]P_3 is required by PDK-1 but must also bind to the PH-domain of Akt/PKB to allow it to be phosphorylated at Thr-308 (Stokoe et al. 1997). There is evidence that Akt/PKB then autophosphorylates at Ser-473, thereby allowing the enzyme to detach from the plasma membrane and phosphorylate other substrates (Toker and Newton 2000). We observed that inhibition of PI3K with LY294002 eliminated Ser-473 phosphorylation in the presence of TGFβ1, which is consistent with this model of Akt/PKB regulation. However, we were surprised that LY294002 produced at most a small inhibition of basal Akt/PKB phosphorylation. This was a consistent observation, we obtained a similar result with wortmannin, and it suggests the existence of PI3K-independent pathways that can lead to Akt/PKB phosphorylation at Ser-473. There is some precedent for the existence of such cascades in other cell types (Filippa et al. 1999). Akt/PKB has been generally associated with various cell survival cascades (reviewed in Yuan and Yankner 2000), but it is also required for insulin-evoked translocation of glucose transporters to the plasma membrane (Hill et al. 1999). A role for Akt/PKB in K_{Ca} regulation in ciliary neurons is therefore quite plausible, but other PtdIns[3,4,5]P_3 or PtdIns[3,4]P_2-sensitive proteins could also be involved in regulation of these channels.

An interesting result is that Erk and PI3K pathways are independently activated. However, when either one of these pathways is blocked, TGFβ1-evoked K_{Ca} stimulation is inhibited. Taken together these results demonstrate that Erk and PI3K signaling cascades must converge at some yet undeter-
regulated step to promote $K_{Ca}$ stimulation. It bears noting that independent PI3K and Erk signaling is also observed in sympathetic neurons (Xue et al., 2000).

Stimulation of plasma membrane $K_{Ca}$ channels by TGFβ1 is first detectable some 3 h after the onset of growth factor treatment but persists in the presence of protein synthesis inhibitors. By contrast, TGFβ1 stimulation of Erk phosphorylation (Lhuillier and Dryer 2000) and Akt/PKB phosphorylation is maximal within 5 and 30 min of treatment, respectively. Three independent lines of pharmacological evidence indicate that the short-term effects of TGFβ1 on whole cell $K_{Ca}$ are associated with insertion of proteins into the plasma membrane. Thus inhibition of microtubules (with colchicine or associated with insertion of proteins into the plasma membrane. Thus inhibition of microtubules (with brefeldin-A), all cause blockade of TGFβ1 effects on $K_{Ca}$. Therefore some essential but unidentified membrane protein is retained in a proximal compartment prior to growth factor treatment. SLO proteins can produce fully functional $K_{Ca}$ channels in the absence of other proteins (reviewed in Vergara et al. 1998), and therefore it is reasonable to hypothesize that it is the SLO protein itself that is translocated to the plasma membrane in response to TGFβ1.

The immunofluorescence data are consistent with this hypothesis. Confocal images indicate that a large component of the SLO signal is found in intracellular compartments, especially in and around the nucleus, but also in other structures. This signal is observed with three different polyclonal SLO antisera and also in immunoblot analyses of isolated CG nuclei. There is also substantial punctate labeling that appears to represent SLO protein in intracellular compartments outside of the nucleus, an observation that strongly supports the membrane insertion hypothesis for TGFβ1 action. However, we have been unable to detect a TGFβ1-evoked increase in the more diffuse nonpunctate signal that presumably reflects plasma membrane SLO proteins in E9 ciliary neurons (data not shown). In retrospect, our failure to obtain direct evidence for SLO translocation is not surprising and do not allow us to exclude the hypothesis. The increase in macroscopic current density evoked by TGFβ1 in ciliary neurons is about three- to fivefold (Cameron et al. 1998), and the unitary current associated with $K_{Ca}$ channels at 0 mV under the ionic conditions used in whole cell recordings is approximately 5 pA. Therefore the average macroscopic $K_{Ca}$ current in a control E9 neuron at 0 mV is caused by activation of around 50–200 channels, and this increases to 250–1000 channels in TGFβ1-treated cells. It is doubtful whether immunoochemical procedures can have the sensitivity and dynamic range needed to observe translocation of anything close to this number of SLO proteins in response to TGFβ1 even if one assumes that only a small percentage of the translocated channels ever become active in macroscopic recordings (Gola and Crest 1993).

In summary, we have shown that TGFβ1 stimulation of functional plasma membrane $K_{Ca}$ channels in developing chick ciliary neurons is associated with activation of PI3K and translocation of proteins to the plasma membrane. It is possible that growth factor-induced mobilization of stored ion channels represents a common mechanism to provide precise regulation of the excitable properties of neuronal membranes in developing and mature cells.

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