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Growth Factors Mobilize Multiple Pools of K_{Ca} Channels in Developing Parasympathetic Neurons: Role of ADP-Ribosylation Factors and Related Proteins

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Chae, Kwon-Seok, Kwang-Seok Oh, and Stuart E. Dryer. Growth factors mobilize multiple pools of K_{Ca} channels in developing parasympathetic neurons: role of ADP-ribosylation factors and related proteins. *J Neurophysiol* 94: 1597–1605, 2005. First published April 20, 2005; doi:10.1152/jn.00296.2005. In developing ciliary ganglion (CG) neurons, movement of functional large-conductance (BK type) Ca^{2+}-activated K^+ (K_{Ca}) channels to the cell surface is stimulated by the endogenous growth factors TGFβ1 and β-neuregulin-1 (NRG1). Here we show that a brief NRG1 treatment (0.5–1.5 h) mobilizes K_{Ca} channels in a post-Golgi compartment, but longer treatments (>3.5 h) mobilize K_{Ca} channels located in the endoplasmic reticulum or Golgi apparatus. Specifically, the effects of 3.5 h NRG1 treatment were completely blocked by treatments that disrupt Golgi apparatus function. These include inhibition of microtubules, or inhibition of the ADP-ribosylation factor-1 (ARF1) system by brefeldin A, by over-expression of dominant-negative ARF1, or over-expression of an ARF1 GTPase-activating protein that blocks ARF1 cycling between GTP- and GDP-bound states. These treatments had no effect on stimulation of K_{Ca} evoked by 1.5 h treatment with NRG1, indicating that short-term responses to NRG1 do not require an intact Golgi apparatus. By contrast, both the acute and sustained effects of NRG1 were inhibited by treatments that block trafficking processes that occur close to the plasma membrane. Thus mobilization of K_{Ca} was blocked by treatments than inhibit ADP-ribosylation factor-6 (ARF6) signaling, including overexpression of dominant-negative ARF6, dominant-negative ARNO, or dominant-negative phospholipase D1. TGFβ1, the effects of which on K_{Ca} are much slower in onset, is unable to selectively mobilize channels in the post-Golgi pool, and its effects on K_{Ca} are completely blocked by inhibition of microtubules, Golgi function and also by plasma membrane ARF6 and phospholipase D1 signaling.

INTRODUCTION

The intrinsic membrane properties of excitable cells are a highly differentiated trait that emerges over an extended period of time during the development of postmitotic cells. For several years, we have studied the regulation of this process in identified populations of vertebrate neurons, especially in the parasympathetic neurons of the chick ciliary ganglion (Dryer et al. 2003). In chick ciliary neurons, significant functional expression of large-conductance (BK-type) Ca^{2+}-activated K^+ (K_{Ca}) channels occurs relatively late in embryonic development at stages that coincide with the formation of synapses with target tissues (E8-13) (Dourado and Dryer 1992).

The expression of ciliary neuron K_{Ca} channels requires inductive interactions with target tissues in the eye and with midbrain preganglionic neurons (Dourado et al. 1994) that are mediated by TGFβ1 (Cameron et al. 1998) and β-neuregulin-1 (NRG1) (Cameron et al. 2001; Subramony and Dryer 1997), respectively. The effects of TGFβ1 and NRG1 on ciliary neuron K_{Ca} channels persist when protein synthesis is blocked (Cameron et al. 1998; Chae et al. 2005; Lhuillier and Dryer 2000, 2002; Subramony and Dryer 1997; Subramony et al. 1996) and are associated with movement of preexisting K_{Ca} channels into the plasma membrane (Chae et al. 2004; Lhuillier and Dryer 2002). However, the mechanisms of growth factor-evoked K_{Ca} trafficking are poorly understood.

We have observed that NRG1 can evoke a robust increase in functional K_{Ca} in as little as 30 min (Chae et al. 2005), whereas a reliable response to TGFβ1 is not usually observed in <6 h (Subramony et al. 1996). By contrast, both growth factors can activate the PI3K/Akt signaling cascades in a matter of minutes. One possible explanation for the different time courses of the physiological responses to the two factors is that NRG1 and TGFβ1 cause mobilization of different pools of K_{Ca} channels. In the present study, we test that hypothesis.

The trafficking of membrane proteins is orchestrated by a host of small GTPases, including members of the ADP-ribosylation factor (ARF) subfamily (Donaldson 2003; Spang 2002; Takai et al. 2001). Two phylogenetically conserved members of this subfamily, ARF1 and ARF6, have received extensive attention in the context of membrane trafficking. ARF1 and its associated proteins regulate the budding and fusion of vesicles within the Golgi apparatus, in part by recruiting coatomer protein I to Golgi membrane surfaces (Presley et al. 2002; Stamnes 2002). By contrast, ARF6 regulates the movement of vesicles in and out the plasma membrane and mediates changes in cortical actin dynamics and membrane lipid composition that are associated with protein trafficking and cell motility (Donaldson 2003). As with other small GTPases, ARF1 and ARF6 exist in GTP-bound (active) and GDP-bound (inactive) states, and normal function requires that they cycle between these states. Moreover, ARF1 and ARF6 are regulated by a variety of guanine nucleotide exchange factors (GEFs), which mediate ARF activation, and GTPase-activating proteins (GAPs), which stimulate the intrinsic GTPase activity of ARFs and thereby terminate their action.

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In the present study, we show that K_{Ca} channels can be mobilized from multiple intracellular pools. These include a post-Golgi pool that can be rapidly mobilized in response to NRG1 by a process that requires activation of ARF6 but that does not require activation of ARF1, microtubules, or an intact Golgi apparatus. A different pool of K_{Ca} channels is mobilized more slowly and in a more sustained manner in response to TGFβ1, as well as in response to higher concentrations of NRG1 (Chae et al. 2005). Mobilization of these K_{Ca} channels into the plasma membrane requires an intact Golgi apparatus based on the requirement for activation of ARF1 and related proteins and functional microtubular transport.

METHODS

Cell isolation, culture, and transfection

Chick ciliary ganglion neurons were isolated at embryonic day 9 (E9) and cultured in a medium containing 20 ng/ml recombinant rat ciliary neurotrophic factor as described previously (Cameron et al. 1998, 1999, 2001). Recombinant human TGFβ1 and NRG1 were obtained from R&D Systems (Minneapolis, MN). For some experiments, colchicine, nocodazole, and brefeldin-A were obtained from Calbiochem (San Diego, CA). Cells were pretreated with these agents for 30 min before the addition of TGFβ1 and NRG1, grown for various times as indicated in figure legends, and then used for electrophysiology. In other experiments, cells were transfected using biolistic methods (Lhuillier and Dryer 2003) and then used for electrophysiology or confocal microscopy. For transfection, E9 neurons were dissociated, cultured for 12 h, and plasmids were introduced into neurons using a biolistic particle-delivery system (PSD-1000; Bio-Rad, Hercules, CA). Plasmids were precipitated onto 1.0-μm gold beads according to the manufacturer's protocol, and a helium shock wave with a pressure gradient of 650 psi was used to accelerate the coated beads onto cultured CG neurons. In these experiments, plasmids encoding dominant-negative or constitutively active mutants of signaling proteins were co-transfected with *Renilla* green fluorescent protein (GFP) (Stratagene, La Jolla, CA) at a ratio of 1:1. Plasmids encoding ARF6 (T27N), ARF1 (T31N), and ARF6 (Q67L) were provided by Dr. Julie G. Donaldson (NHLBI, National Institutes of Health, Bethesda, MD). A dominant-negative form of ARNO (E156K) was obtained from Dr. James E. Casanova (University of Virginia Health Sciences Center, Charlottesville, VA). Plasmids encoding phospholipase D1 (PLD1 K898R) and wild-type PLD1 were obtained from Dr. Michael Frohman (University Medical Center at Stony Brook, Stony Brook, NY). GFP-ARF-GAP273, in which sequences encoding 273 carboxyl-terminal noncatalytic amino acids of ARF-GAP1 were joined to the carboxyl-terminus of GFP, were obtained from Dr. Michael G. Roth (University of Texas Southwestern Medical Center, Dallas, TX).

Electrophysiology

Whole cell recordings of K_{Ca} and voltage-activated Ca^{2+} currents were made using methods that we have standardized in many previous studies on ciliary neurons (Cameron et al. 1998, 1999, 2001; Chae and Dryer 2005; Chae et al. 2005; Dourado and Dryer 1992; Dryer et al. 1991; Lhuillier and Dryer 2000, 2002, 2003; Subramony and Dryer 1997; 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 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). Ciliary neurons are nearly spherical, and surface areas were calculated from cell diameters measured in two orthogonal axes and used to estimate current density. Similar results were obtained when currents were normalized to cell capacitance estimated as described in Martin-Caraballo and Dryer (2002). 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. This solution buffers the bulk cytosolic levels of Ca^{2+} to low levels and prevents slow changes in intracellular Ca^{2+} associated with longer distance diffusion, such as diffusion that would occur between endosomal stores and plasma membranes but does not effectively buffer rapid Ca^{2+} changes in the immediate vicinity of Ca^{2+} channels. Normal external salines for measurements of K_{Ca} 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 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 from a minimum of two platings of ciliary ganglion neurons (i.e., from multiple cultures).

Golgi staining

The Golgi apparatus was visualized using the fluorescent marker *N*-({4-[4, 4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl]phenoxy}acetyl)sphingosine (BODIPY TR C_5 ceramide) as described by Gangalum et al. (2004). This probe is commercially available as a complex with bovine serum albumin (Molecular Probes, Eugene, OR). Cells were treated with neuregulin in the presence or absence of brefeldin A for 1.5 or 3.5 h, washed in PBS, fixed in 4% paraformaldehyde for 6 min, blocked in PBS containing 1% BSA and 0.1% Triton X-100 for 30 min, and then stained with 15 μM BODIPY TR C5 ceramide for 30 min in the dark. Cells were then rinsed, mounted, and the integrity of the Golgi determined by fluorescence microscopy. The excitation wavelength was 580 nm and emission was monitored at 620 nm using a $\times$60 objective and an Olympus confocal microscope.

RESULTS

Role of the Golgi apparatus, microtubules and ARF1 in NRG1 stimulation of K_{Ca}

Previous studies from our laboratory (Lhuillier and Dryer 2002) showed that the stimulatory effects of TGFβ1 on K_{Ca} expression require an intact Golgi apparatus because the effects of this growth factor were blocked by the Golgi inhibitor brefeldin-A as well as by inhibitors of microtubules (Lhuillier and Dryer 2002; see also Hamm-Alvarez and Sheetz 1998). TGFβ1-evoked mobilization of K_{Ca} is a relatively slow process and is best observed $\geq$5 h after the onset of treatment. Functional mobilization of ciliary K_{Ca} channels by NRG1 is much more rapid and can occur in as little as 30–90 min (Chae et al. 2005). Therefore in the initial experiments of the present study, we examined the role of microtubules and the Golgi apparatus in NRG1-evoked trafficking of K_{Ca} channels. We quantified increases in macroscopic K_{Ca} by means of standard whole cell recordings (Fig. 1*A*) immediately after 1.5- or 3.5-h exposures to 10 nM NRG1. We observed that pretreatment with the microtubule inhibitors nocodazole (10 μM) or colchicine (5 μM) for 30 min prior to the onset of NRG1 exposure blocked the responses observed after 3.5 h but had no significant effect on responses observed at 1.5 h. A similar pattern was observed

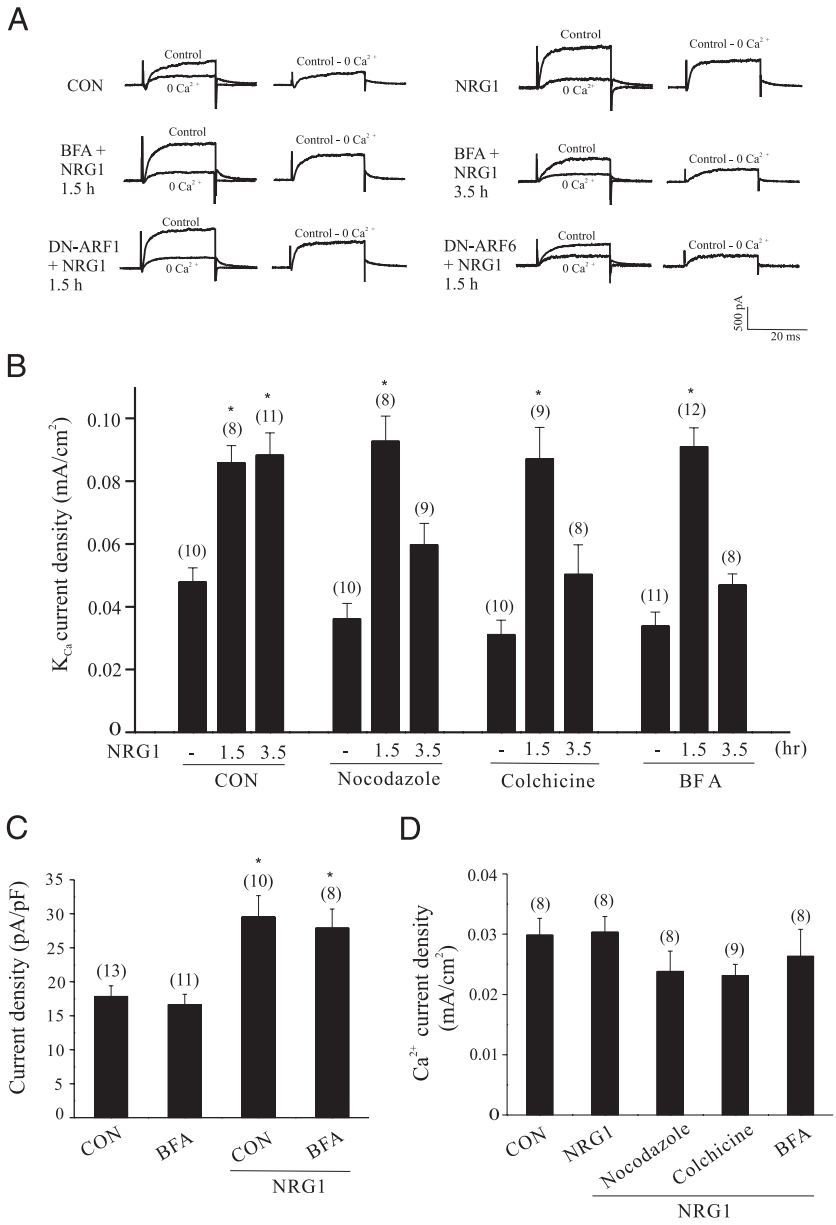

FIG. 1. Inhibition of microtubules or the Golgi apparatus blocks sustained but not acute mobilization of K_{Ca} by NRG1. Embryonic day 9 (E9) ciliary ganglion (CG) neurons were incubated for 1.5 or 3.5 h in the presence or absence of 10 nM β-neuregulin-1 (NRG1), 20 μM nocodazole, 5 μM colchicine, or 1 μg/ml brefeldin A, as indicated. Treatment with the inhibitors agents commenced 30 min prior to the onset of growth factor treatment. K_{Ca} and voltage-activated Ca^{2+} currents were quantified at the end of the treatment by whole cell recordings. *A*: examples of typical whole cell recordings in ciliary neurons treated with growth factors, brefeldin A (BFA), or overexpressing dominant-negative forms of signaling proteins described in subsequent figures. *B*: treatment with nocodazole or colchicines (which block microtubules) or brefeldin-A (which disrupts the Golgi apparatus) had no effect on K_{Ca} density measured 1.5 h after the start of NRG1 but blocked the effect of NRG1 measured 3 h after the start of treatment. *C*: in a different group of cells, brefeldin A produced the same quantitative pattern when currents were normalized for whole cell capacitance. *D*: voltage-activated Ca^{2+} currents measured at 3 h were not affected by these drugs.

in ciliary neurons pretreated with 1 μg/ml brefeldin-A, an agent that disrupts the Golgi apparatus (Lippincott-Schwartz et al. 1989) (Fig. 1*B*). The same quantitative conclusion was obtained when currents were normalized for cell size by means of whole cell capacitance (Fig. 1*C*). Consistent with our previous observations (Lhuillier and Dryer 2002), none of these treatments affected the density of voltage-activated Ca^{2+} currents (Fig. 1*D*). These treatments, and the others used in subsequent experiments in this study, had no effect on kinetics of Ca^{2+} current activation or deactivation (data not shown). The ability of brefeldin A treatment to disrupt the Golgi apparatus in ciliary neurons, as it does in other cell types, was addressed directly by means of the fluorescent Golgi marker BODIPY TR C_5 ceramide (Fig. 2). The Golgi apparatus in ciliary neurons was completely disrupted by brefeldin A treatment. Therefore these data are consistent with a model in which NRG1 initially causes mobilization of K_{Ca} channels located in post-Golgi pools but subsequently recruits channels from the Golgi apparatus.

To further test this model, we examined responses to NRG1 in E9 ciliary neurons over-expressing a form of ARF1 (T31N) that preferentially binds GDP and thereby functions as a dominant-negative (Dascher and Balch 1994). Cells were concurrently transfected with GFP to allow for identification of transfected cells during electrophysiology (Chae and Dryer 2005; Chae et al. 2005; Lhuillier and Dryer 2003). Control neurons were transfected with GFP alone. Transfections were carried out using biolistic methods, and growth factor treatments and whole cell recordings were carried out 24 h after transfection. Our earlier studies have shown that expression of constructs from CMV promoters in chick ciliary neurons is robust 6 h after transfection (Lhuillier and Dryer 2003). We observed that 1.5-h exposure to 10 nM NRG1 continued to cause mobilization of K_{Ca} in ciliary neurons co-expressing

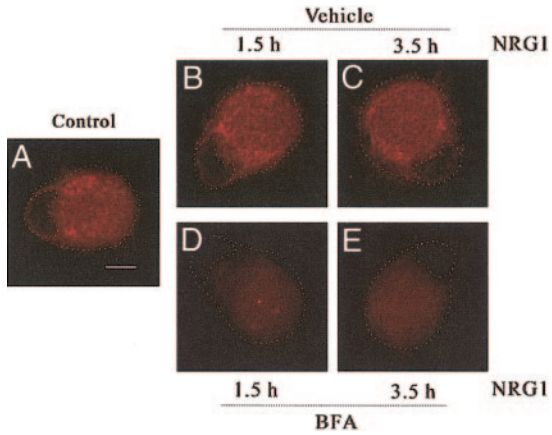

FIG. 2. Brefeldin A treatment causes complete disruption of the Golgi apparatus. Confocal images of Golgi were obtained using the fluorophore marker *N*-({4-[4, 4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl]phenoxy}acetyl)sphingosine (BODIPY TR C_5 ceramide). · · · , outline of cell surface and nucleus. Control shows Golgi apparatus in an untreated ciliary neuron. Other images are from different cells treated with 10 nM NRG1 for 1.5 h (*left*) or 3.5 h (*right*) in the absence (*top*) or presence (*bottom*) of 1 μg/ml brefeldin A.

GFP and dominant-negative ARF1, and the responses were indistinguishable from those obtained in ciliary neurons expressing GFP alone. By contrast, overexpression of dominant-negative ARF1 completely blocked responses to NRG1 monitored 3.5 h after the onset of growth factor treatment (Fig. 3*A*). Dominant-negative ARF1 and GFP overexpression had no effect on voltage-activated Ca^{2+} currents monitored after 3.5 h of NRG1 (Fig. 3*B*). A similar conclusion was obtained using a different method to disrupt ARF1 function. ARF-GAP1 is the founding member of a class of GTPase-activating proteins known to terminate the actions of ARF1 (Cukierman et al. 1995). Overexpression of wild-type ARF-GAP1 causes disruption of the Golgi apparatus, most likely owing to increased GTP hydrolysis on ARF1 (Aoe et al. 1997). In the present experiments, we used an ARF1-GFP fusion protein (GFP-ARF-GAP273) that blocks protein translocation through the Golgi when overexpressed (Yu and Roth 2002). As with dominant-negative ARF1, overexpression of GFP-ARF-GAP273 for 24 h had no effect on the response of NRG1 monitored after a 1.5-h exposure but blocked responses observed after a 3.5-h exposure (Fig. 4*A*). Overexpression of this construct had no significant effect on the expression or properties of Ca^{2+} currents (Fig. 4*B*). Collectively these data point to multiple pools of K_{Ca} channels in different cellular compartments that can be mobilized by NRG1 on different time scales.

ARF6 signaling is required for both the acute and sustained stimulation of K_{Ca}

ARF6 regulates a number of protein trafficking events that occur in and near the plasma membrane (Donaldson 2003). To determine whether ARF6 is involved in mobilization of K_{Ca} channels, E9 ciliary ganglion neurons were transfected with a mutant form of ARF6 (T27N) defective in GTP binding that thereby functions as a dominant-negative (D'Souza-Schorey et al. 1998; Macia et al. 2004). As with ARF1 constructs, transfection was carried out 24 h before application of growth factors. We observed that overexpression of ARF6 (T27N) blocked responses to 10 nM NRG1 monitored both 1.5 and 3.5 h after the onset of treatment (Fig. 5*A*). However, expression of this construct had no effect on voltage-activated Ca^{2+} currents (Fig. 5*B*). The activity of ARF6 is regulated by a guanine nucleotide exchange factor known as ARF nucleotide binding site opener (ARNO), one of a conserved family of Sec7-domain containing proteins that regulate various ARF proteins (Jackson and Casanova 2000). The Sec7 domain is the catalytic center of these proteins, and a conserved glutamate residue within this domain is required for catalytic activity in all members of this family (Beraud-Dufour et al. 1999). Therefore we examined NRG1 mobilization of K_{Ca} in E9 ciliary neurons over-expressing an ARNO mutant (E156K) that lacks this conserved residue and that functions as a dominant nega-

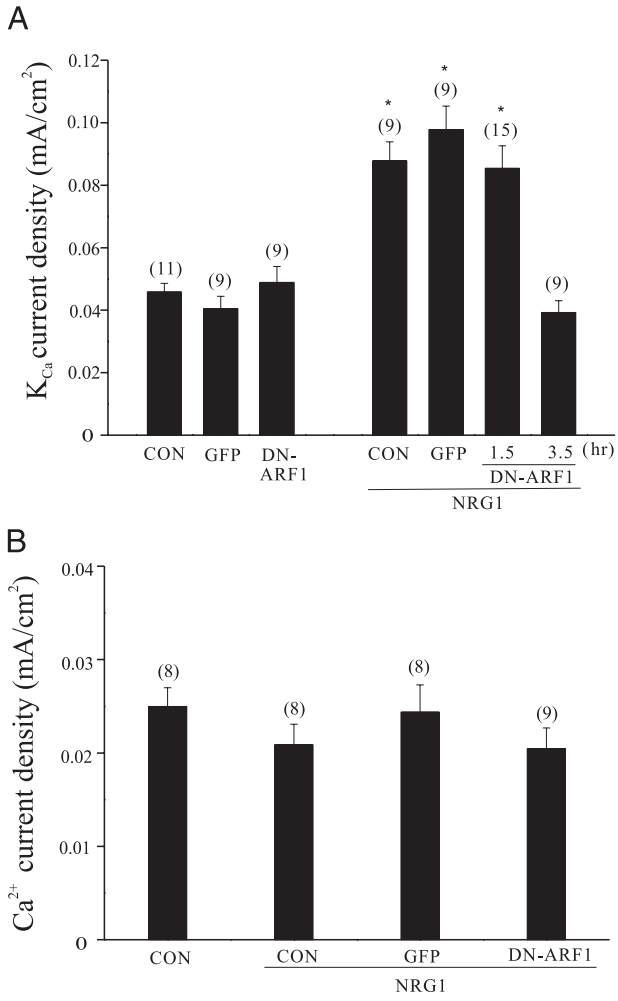

FIG. 3. ADP-ribosylation factor-1 (ARF1) is necessary for the sustained but not for the acute mobilization of K_{Ca} evoked by NRG1. These experiments utilized biolistic overexpression of ARF1 (T31N), a dominant-negative form of ARF1. *A*: macroscopic K_{Ca} density in ciliary neurons transfected with *Renilla* green fluorescent protein (GFP), a combination of GFP and ARF1 (T31N), or from nontransfected cells in the same dish (CON). Note robust stimulation by NRG1 in nontransfected cells, in cells expressing only GFP, and in cells incubated for 1.5 h with NRG1 after overexpressing of GFP and ARF1 (T31N). However, over-expression of ARF1 (T31N) with GFP completely blocked the responses to NRG1 measured 3.5 after the start of treatment. *B*: overexpression of ARF1 (T31N) or GFP had no effect on mean Ca^{2+} current density in any treatment group. *C*: E9 ciliary neurons overexpressing a GFP-tagged form of ARF1 were incubated for 1.5 or 3.5 h in the presence or absence of 10 nM NRG1. NRG1 treatment promoted movement of ARF1 into the Golgi apparatus, especially after 3.5 h.

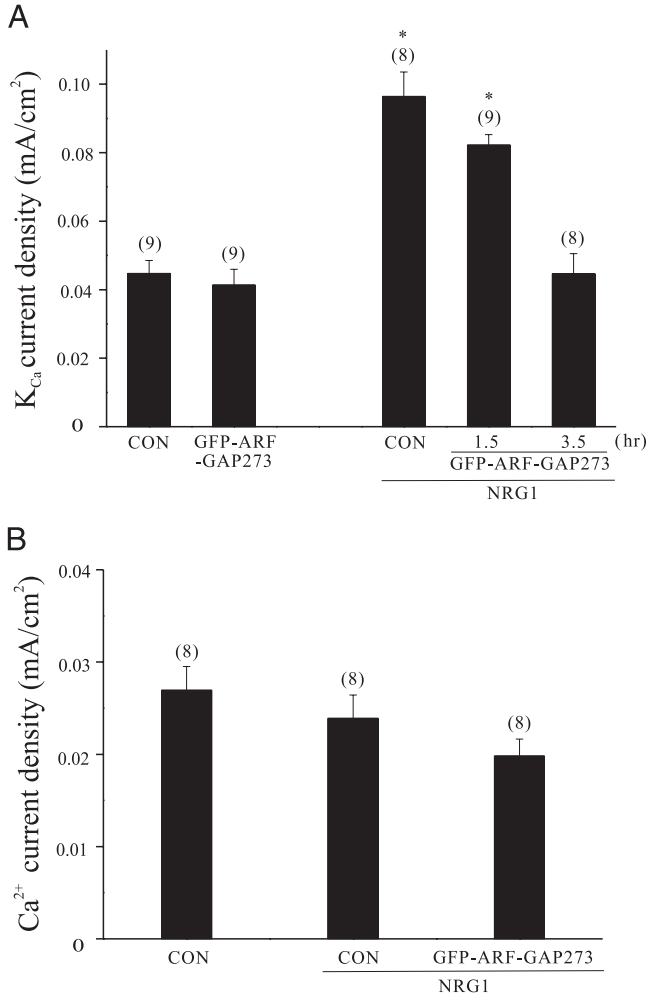

FIG. 4. ARF1 is necessary for the sustained but not for the acute mobilization of K_{Ca} evoked by NRG1. These experiments used overexpression of ARF-GAP273, an agent that stimulates the intrinsic GTPase activity of ARF1, and thereby inhibits its function. *A*: overexpression of ARF-GAP273 has no effect on response to NRG1 measured at 1.5 h but completely blocked responses to NRG1 measured at 3 h. These results are similar to those obtained with dominant-negative ARF1. *B*: overexpression of ARF-GAP273 did not affect voltage-activated Ca^{2+} currents.

tive (Mukherjee et al. 2001). We observed a pattern similar to that obtained with the ARF6 dominant negative; specifically, both the acute (1.5 h) and sustained (3.5 h) responses to 10 nM NRG1 were abolished, but Ca^{2+} currents were fully normal (Fig. 6).

ARF6 affects membrane and cytoskeleton dynamics by regulating multiple effector molecules (Donaldson 2003). One of these is PLD1, which is thought to be necessary to induce transient changes in membrane lipid composition required for vesicle trafficking to the plasma membrane of neurons and neuroendocrine cells (Caumont et al. 1998; Vitale et al. 2001). To explore the role of PLD1 in K_{Ca} trafficking in ciliary neurons, we overexpressed a catalytically dead form of PLD1 (K898R) that functions as dominant-negative (Denmat-Ouisse et al. 2001; Rizzo et al. 1999; Vitale et al. 2002). We observed that over-expression of PLD1 (K898R) in E9 ciliary neurons blocked the increase in macroscopic K_{Ca} evoked by a 1.5-h treatment with NRG1 (Fig. 7*A*) or a 3-h treatment with NRG1 (data not shown). Overexpression of this construct had no effect on expression of voltage-activated Ca^{2+} channels in E9 neurons (Fig. 7*B*). We also examined the role of PLD1 using a different experimental design that entailed use of a constitutively active form of ARF6 (Q67L). This mutant cannot hydrolyze bound GTP and is therefore unable to cycle between GTP- and GDP-bound states. As with other constitutively-active small GTPases, this mutant is able to recapitulate some but not all of the actions of active wild-type ARF6 (Santy 2002). We observed that overexpression of ARF6 (Q67L) had no effect on basal K_{Ca} expression in E9 ciliary neurons (Fig. 7*C*). Similarly, overexpression of wild-type PLD1 had no effect on basal K_{Ca} density. However, overexpression of ARF6 (Q67L) together with wild-type PLD1 caused a modest but statistically significant increase in K_{Ca} (Fig. 7*C*). As an aside, it bears noting that over-expression of ARF6 (Q67L) not only

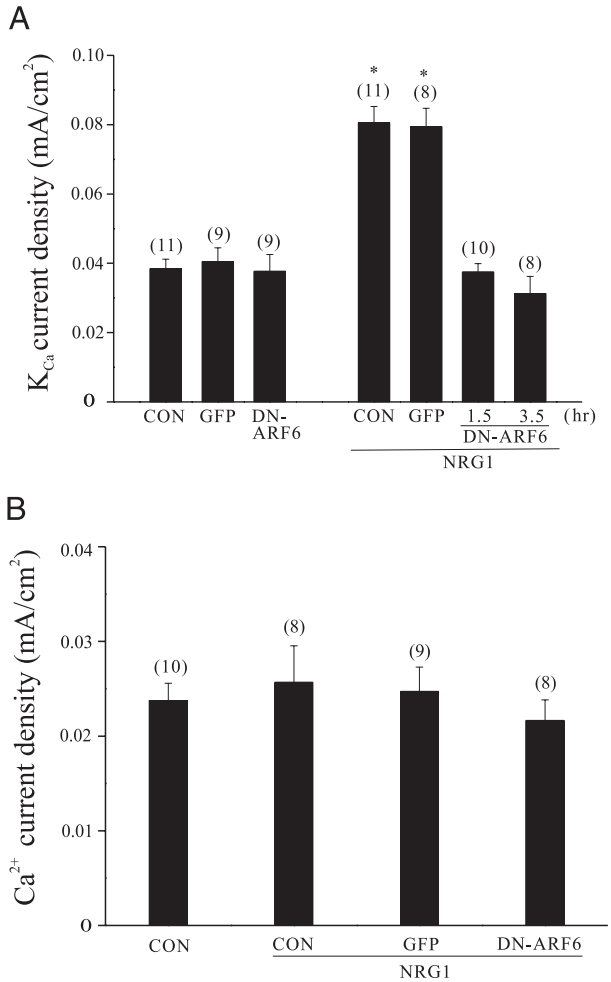

FIG. 5. ARF6 is necessary for both the acute and sustained mobilization of K_{Ca} evoked by NRG1. Macroscopic K_{Ca} and Ca^{2+} currents were recorded from ciliary neurons overexpressing GFP or a combination of GFP and ARF6 (T27N), a dominant-negative form of ARF6. Recordings were also made from nontransfected cells in the same culture dish (CON). *A*: overexpression of ARF6 (T27N) completely blocked the effect of NRG1 on functional expression of K_{Ca} measured at all times after treatment. *B*: voltage-activated Ca^{2+} currents were not affected by overexpression of ARF6 (T27N). *C*: E9 ciliary neurons overexpressing a GFP-tagged form of ARF6 were incubated for 1.5 or 3.5 h in the presence (NRG1) or absence (control) of 10 nM NRG1. NRG1 treatment evoked some movement of tagged ARF6 toward the periphery of the cell at 1.5 h, with some movement back into intracellular compartments apparent at 3 h. The latter phenomenon may reflect accumulation of GDP-bound ARF6 that has completed its cycle.

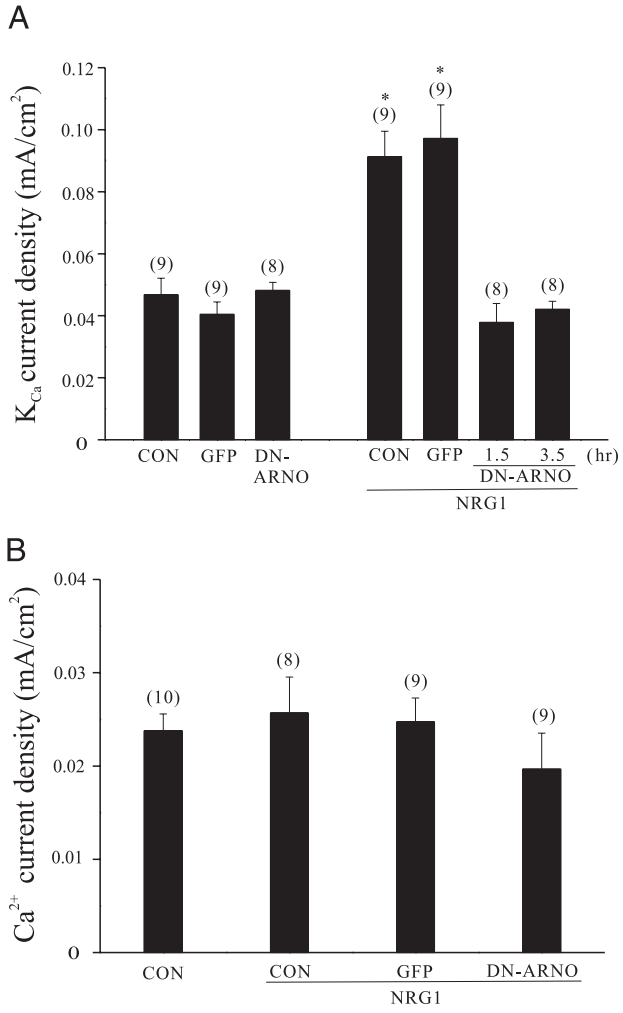

FIG. 6. ARF nucleotide-binding site opener (ARNO) is required for both the acute and sustained mobilization of K_{Ca} channels evoked by NRG1. These experiments employed overexpression of the dominant-negative mutant ARNO (E156K). *A*: overexpression of ARNO (E156K) completely blocked mobilization of K_{Ca} measured 1.5 and 3.5 h after the onset of NRG1 treatment. *B*: voltage-activated Ca^{2+} currents were not affected by overexpression of ARNO (E156K).

failed to increase trafficking of K_{Ca} channels by itself, it also blocked the stimulation of K_{Ca} normally observed following 1.5- or 3.5-h treatments with NRG1 (data not shown). In other words, with respect to mobilization of K_{Ca}, the GTPase-deficient ARF6 mutant also behaved as a dominant-negative, as reported in other cell types (Zhang et al. 1998). Collectively, these data indicate that an ARF6-PLD1 mechanism is necessary for K_{Ca} trafficking to the plasma membrane.

Finally, it bears noting that ARF1, ARF6, ARNO, and PLD1 are also necessary for sustained posttranslational responses to TGFβ1, another growth factor required for normal K_{Ca} expression in developing ciliary neurons (Fig. 8). In those experiments, we examined K_{Ca} expression in transfected E9 ciliary neurons after a 6.5-h exposure to TGFβ1 using the constructs already described. The results suggest that two growth factors

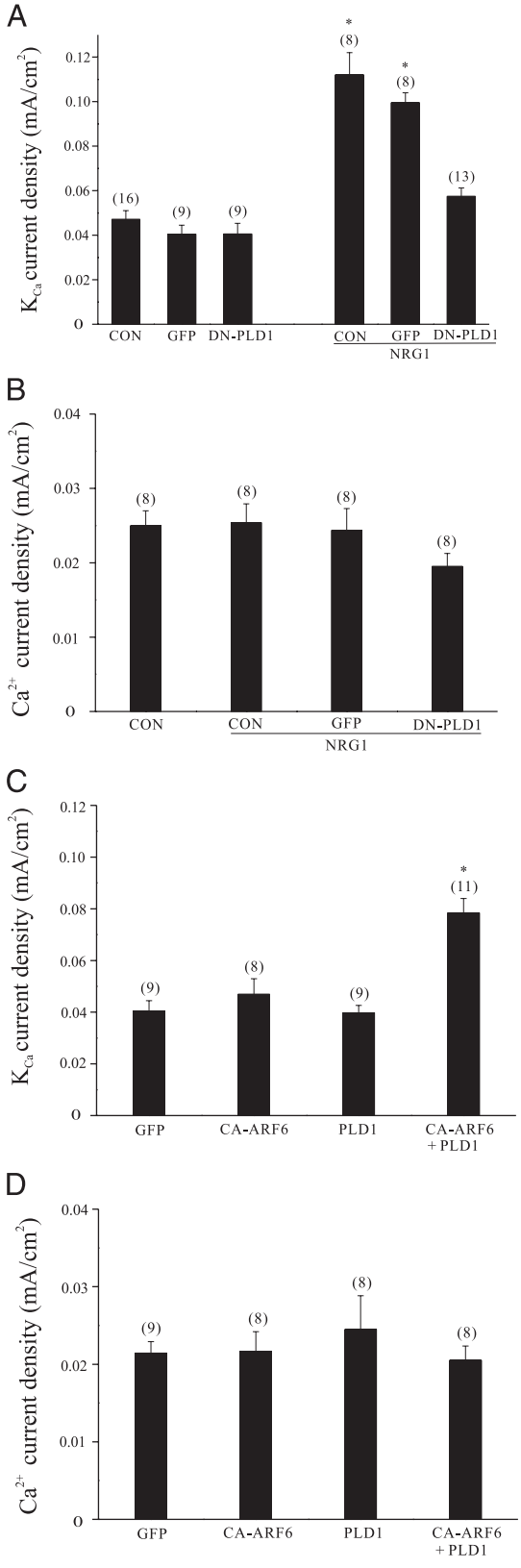

FIG. 7. PLD1 is required for both the acute and sustained mobilization of K_{Ca} evoked by NRG1. *A*: overexpression of dominant-negative phospholipase D1 (PLD1) (K898R) completely blocked the effect of NRG1 on functional expression of K_{Ca} measured 1.5 h after the onset of growth factor treatment. *B*: voltage-activated Ca^{2+} currents were not affected by overexpression of PLD1 (K898R). *C*: overexpression of the constitutively-active mutant ARF6 (Q67L) together with wild-type PLD1 caused a modest but statistically significant increase in K_{Ca}, even in the absence of growth factor treatment. Overexpression of these constructs by themselves was not sufficient to cause mobilization of K_{Ca}. *D*: voltage-activated Ca^{2+} currents were not affected by overexpression of constitutively active ARF6 (Q67L) and/or PLD1.

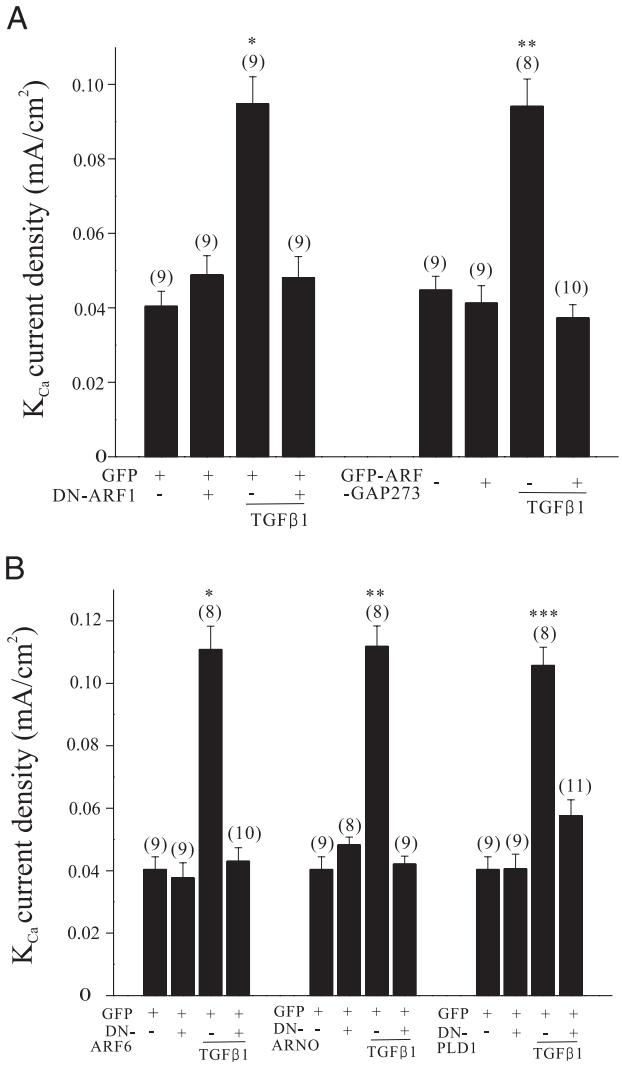

FIG. 8. ARF1, ARF6, ARNO, and PLD1 are required for sustained mobilization of K_{Ca} evoked by TGFβ1. These experiments used over-expression of the constructs described in previous figures. Mobilization of K_{Ca} was quantified in E9 ciliary neurons by whole cell recording after a 6.5-h exposure to 1 nM TGFβ1. Shorter treatments (e.g., 1.5–3 h) do not cause statistically significant stimulation of K_{Ca}. *A*: overexpression of ARF1 (T31N) or ARF-GAP273 completely blocked the effect of TGFβ1 on functional expression of K_{Ca}. *B*: overexpression of ARF6 (T27N), ARNO (E156K) or PLD1 (K898R) completely blocked the effect of TGFβ1 on functional expression of K_{Ca} measured at 6.5 h. Voltage-activated Ca^{2+} currents were not affected by overexpression of these mutants as noted in previous figures.

share at least some of the pathways required for mobilization of K_{Ca} channels in the endoplasmic reticulum and/or Golgi apparatus of ciliary neurons. Responses to TGFβ1 with shorter treatment times are weak and highly variable and are not detectable at all at 1.5 h. Thus TGFβ1 appears to be much less effective than NRG1 at mobilizing the pool of K_{Ca} channels associated with post-Golgi compartments.

DISCUSSION

The data in this study are consistent with a model in which functional BK-type K_{Ca} channels of developing ciliary neurons are mobilized into the plasma membrane from two separate compartments in response to physiologically relevant growth factors. One pool of channels in the ER and/or Golgi is mobilized slowly (>3 h) in response to TGFβ1 or high concentrations of NRG1 (Chae et al. 2005; Lhuillier and Dryer 2002). A second K_{Ca} pool in a post-Golgi compartment moves into the plasma membrane more rapidly after the onset of NRG1 exposure (0.5–1.5 h) but cannot be effectively mobilized by TGFβ1. We have previously shown that stimulation of K_{Ca} evoked by lower concentrations (1 nM) of NRG1 is transient; responses are significant 1–1.5 after the onset of treatment but return to baseline after 3 h, even in the continuous presence of NRG1 (Chae et al. 2005). By contrast, treatment with 10 nM NRG1, the concentration used in the present study, evokes responses that last essentially as long as NRG1 is present. This suggests that the post-Golgi pool appears to be preferentially mobilized by low concentrations of NRG1 (Chae et al. 2005). For reasons as yet unknown, TGFβ1 is unable to rapidly mobilize the K_{Ca} channels in the post-Golgi pool.

The two-pool model of K_{Ca} regulation is supported by several lines of evidence. Thus the sustained physiological responses to NRG1 and TGFβ1 are completely inhibited by treatments that cause disruption of Golgi function, including microtubule inhibitors (Hamm-Alvarez and Sheetz 1998), overexpression of dominant-negative forms of ARF1 (Dascher and Balch 1994), brefeldin-A (Lippincott-Schwartz et al. 1989), and expression of a mutant form of ARF-GAP-1 that blocks ARF1 cycling (Yu and Roth 2002). None of these treatments affected the acute responses to NRG1, defined in this study as increases in macroscopic K_{Ca} observed $\leq$1.5 h after the onset of treatment. Further, none of these treatments affected the expression or properties of voltage-activated Ca^{2+} currents, consistent with our previous studies (Lhuillier and Dryer 2002).

Both the acute and sustained responses to growth factors ultimately entail insertion of K_{Ca} channels into the plasma membrane. Thus NRG1 and TGFβ1 increase the number of immunochemically detectable cell-surface SLO1 proteins (Chae et al. 2005). Moreover, we observed here that inhibition of ARF6 signaling cascades completely blocked the increases in macroscopic K_{Ca} evoked by NRG1 or TGFβ1. This was observed with both short (1.5 h) and long (>3.5 h) growth factor treatments that are differentially sensitive to inhibition of ARF1 cascades, and equivalent blockade was produced by overexpression of dominant-negative forms of ARF6 (D'Souza-Schorey et al. 1998), ARNO (Mukherjee et al. 2001), and PLD1 (Rizzo et al. 1999). Clearly, ARF6 activation is necessary for growth factor mobilization of K_{Ca}. However, it does not appear to be sufficient, as overexpression of a constitutively active form of ARF6 (Dascher and Balch 1994) did not lead to an increase in macroscopic K_{Ca}, except for a modest effect in cells that were concurrently overexpressing wild-type PLD1. Indeed, the constitutively-active form of ARF6, which is limited in its ability to cycle between GTP- and GDP-bound states, not only failed to cause mobilization of K_{Ca}, it actually inhibited the physiological responses to NRG1. A similar pattern has been observed with constitutively-active forms of ARF6 in other systems (Brown et al. 2001; Santy 2002) and with some other small GTPases (e.g., Lin et al. 1997).

It is likely that ARF6 acts on multiple effectors to regulate insertion of functional K_{Ca} complexes into the plasma membrane. In other systems, ARF6 causes activation of PLD1, leading to focal changes in the composition of the plasma

membrane (Donaldson 2003). Consistent with this, our current data indicate that an ARF6-PLD1 pathway is an essential component of the cascades leading to K_{Ca} mobilization in ciliary neurons. This is true when mobilization is evoked by either NRG1 or TGFβ1. However, ARF6 activation also causes changes in the dynamics of a dense layer of filamentous actin adjacent to the plasma membrane of most cell types (Schafer et al. 2000). This so-called cortical actin layer can play a variety of roles in the late stages of membrane protein trafficking (Eitzen 2003). In a recent study, we observed that cortical actin acts as a barrier to prevent the constitutive insertion of K_{Ca} channels into the plasma membrane of ciliary neurons. Specifically, we observed that agents that cause depolymerization of cortical F-actin, such as cytochalasin D, rapidly stimulate insertion of K_{Ca} into the plasma membrane. Conversely, treatments that stabilize F-actin, such as phalloidin, tend to suppress ciliary neuron K_{Ca} mobilization (Chae and Dryer 2005). These data raise the possibility that actin rearrangements mediate some of the effects of ARF6 on K_{Ca} mobilization.

The mechanisms whereby NRG1 and TGFβ1 lead to activation of ARFs in ciliary neurons are not known. However, we have previously shown that PI3 kinase and Akt activation are required for NRG1- or TGFβ1-evoked mobilization of K_{Ca} channels (Chae et al. 2005; Lhuillier and Dryer 2000). Indeed, over-expression of a membrane-targeted (myristoylated) form of Akt is sufficient in itself to cause mobilization of K_{Ca} (Chae et al.2005). Several ARF-associated GEFs of the Sec7 family, such as ARNO and GRP1, contain tandem pleckstrin homology (PH) domains that allow for binding of the lipid products of PI3 kinase, and a consequent association with cell membranes (Klarlund et al. 1998; Venkateswarlu et al. 1998). Akt also contains a PH domain that is necessary for its activation, and it is therefore possible that ARF-related proteins and Akt are brought into close proximity on endomembranes and plasma membranes of ciliary neurons in response to NRG1 or TGFβ1. To date there have been no published reports of direct interactions between ARFs or ARF-associated proteins and Akt. However, a number of Akt substrates, such as p70 S6 kinase-1 (Qian et al. 2004) and p21-activated kinase (Bokoch 2003) are able to modulate cortical F-actin dynamics. Thus there are several potentially interacting mechanisms whereby Akt and ARF6 cascades could converge to coordinately regulate trafficking of K_{Ca} in ciliary neurons. Indeed, we cannot exclude that overexpression of a membrane-targeted form of Akt can induce dynamic changes in trafficking that, under more physiological circumstances, are mediated by ARFs.

It is surprising that two growth factors regulate different pools of K_{Ca} channels in ciliary neurons, and the question arises as to the functional significance of the rapid regulation of macroscopic K_{Ca} evoked by NRG1. TGFβ1 and NRG1 are both required for developmental regulation of K_{Ca} in ciliary neurons developing in vivo (Cameron et al. 1998, 2001). It is possible that NRG1 regulation persists past development and plays a role in fully formed ciliary ganglion, as occurs with growth factor regulation of potassium channels in other systems (Fadool et al. 2000). By analogy, previous work suggests that NRG1 plays a role in the maintenance as well as the formation of nicotinic acetylcholine receptors in the developing mammalian neuromuscular junction (Sandrock et al. 1997).

In summary, we have shown that developing ciliary neurons maintain at least two distinct pools of K_{Ca} channels. These include a rapidly accessible pool located in a post-Golgi compartment and a major reserve pool located in the ER-Golgi. ARF1 and associated proteins are required for movement of K_{Ca} channels through the Golgi apparatus, whereas an ARF6 cascade that also requires PLD1 regulates the late stages of translocation of functional K_{Ca} channels into the plasma membrane. These data also provide plausible mechanisms for growth-factor-evoked regulation of the neuronal cytoskeleton in the context of protein trafficking and the developmental regulation of excitability.

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REFERENCES

Aoe T, Cukierman E, Lee A, Cassel D, Peters PJ, and Hsu VW. The KDEL receptor, ERD2, regulates intracellular traffic by recruiting a GTPase-activating protein for ARF1. *EMBO J* 16: 7305–7316, 1997.

Beraud-Dufour S, Paris S, Chabre M, and Antonny B. Dual interaction of ADP ribosylation factor 1 with Sec7 domain and with lipid membranes during catalysis of guanine nucleotide exchange. *J Biol Chem* 274: 37629–37636, 1999.

Bokoch GM. Biology of the p21-activated kinases. *Annu Rev Biochem* 72: 743–781, 2003.

Brown FD, Rozelle AL, Yin HL, Balla T, and Donaldson JG. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J Cell Biol* 154: 1007–1017, 2001.

Cameron JS, Dryer L, and Dryer SE. Regulation of neuronal K^+ currents by target-derived factors: opposing actions of two different isoforms of TGFβ. *Development* 126: 4157–4164, 1999.

Cameron JS, Dryer L, and Dryer SE. β-Neuregulin-1 is required for the in vivo development of functional Ca^{2+}-activated K^+ channels in parasympathetic neurons. *Proc Natl Acad Sci* 98: 2832–2836, 2001.

Cameron JS, Lhuillier L, Subramony P, and Dryer SE. Developmental regulation of neuronal K^+ channels by target-derived TGFβ in vivo and in vitro. *Neuron* 21: 1045–1053, 1998.

Caumont AS, Galas MC, Vitale N, Aunis D, and Bader MF. Regulated exocytosis in chromaffin cells. Translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. *J Biol Chem* 273: 1373–1379, 1998.

Chae KS and Dryer SE. The p38 MAPK pathway negatively regulates K_{Ca} channel trafficking in developing parasympathetic neurons. *J Neurochem* In press.

Chae KS, Martin-Caraballo M, Anderson M, and Dryer SE. Akt activation is necessary for growth factor-induced trafficking of functional K_{Ca} channels in developing parasympathetic neurons. *J Neurophysiol* 93: 1174–1182, 2005.

Cukierman E, Huber I, Rotman M, and Cassel D. The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization. *Science* 270: 1999–2002, 1995.

Dascher C and Balch WE. Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus. *J Biol Chem* 269: 1437–1448, 1994.

Denmat-Ouisse LA, Phebidias C, Honkavaara P, Robin P, Geny B, Min do S, Bourgoin S, Frohman MA, and Raymond MN. Regulation of constitutive protein transit by phospholipase D in HT29-cl19A cells. *J Biol Chem* 276: 48840–48846, 2001.

Donaldson JG. Multiple roles for ARF6: sorting, structuring, and signaling at the plasma membrane. *J Biol Chem* 278: 41573–41576, 2003.

Dourado MM, Brumwell C, Wisgirda ME, Jacob MH, and Dryer SE. Target tissues and innervation regulate the characteristics of K^+ currents in chick ciliary ganglion neurons developing in situ. *J Neurosci* 14: 3156–3165, 1994.

Dourado MM and Dryer SE. Changes in the electrical properties of chick ciliary ganglion neurones during embryonic development. *J Physiol* 449: 411–428, 1992.

D'Souza-Schorey C, van Donselaar E, Hsu VW, Yang C, Stahl PD, and Peters PJ. ARF6 targets recycling vesicles to the plasma membrane: insights from an ultrastructural investigation. *J Cell Biol* 140: 603–616, 1998.

Dryer SE, Dourado MM, and Wisgirda ME. Characteristics of multiple Ca^{2+}-activated K^+ channels in acutely dissociated chick ciliary-ganglion neurones. *J Physiol* 443: 601–627, 1991.

Dryer SE, Lhuillier L, Cameron JS, and Martin-Caraballo M. Expression of K_{Ca} channels in identified populations of developing vertebrate neurons: role of neurotrophic factors and activity. *J Physiol* 97: 49–58, 2003.

Eitzen G. Actin remodeling to facilitate membrane fusion. *Biochim Biophys Acta* 1641: 175–181, 2003.

Fadool DA, Tucker K, Phillips JJ, and Simmen JA. Brain insulin receptor causes activity-dependent current suppression in the olfactory bulb through multiple phosphorylation of Kv1.3. *J Neurophysiol* 83: 2332–2348, 2000.

Gangalum RK, Schibler MJ, and Bhat SP. Small heatshock protein alpha B chrystallin is part of cell cycle-dependent Golgi reorganization. *J Biol Chem* 279: 43374–43377, 2004.

Gaschet J and Hsu VW. Distribution of ARF6 between membrane and cytosol is regulated by its GTPase cycle. *J Biol Chem* 274: 20040–20045, 1999.

Hamm-Alvarez SF and Sheetz MP. Microtubule-dependent vesicle transport: modulation of channel and transporter activity in liver and kidney. *Physiol Rev* 78: 1109–1129, 1998.

Jackson CL and Casanova JE. Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol* 10: 60–67, 2000.

Klarlund JK, Rameh LE, Cantley LC, Buxton JM, Holik JJ, Sakelis C, Patki V, Corvera S, and Czech MP. Regulation of GRP1-catalyzed ADP ribosylation factor guanine nucleotide exchange by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 273: 1859–1862, 1998.

Lhuillier L and Dryer SE. Developmental regulation of neuronal K_{Ca} channels by TGFβ1: transcriptional and posttranscriptional effects mediated by Erk MAP kinase. *J Neurosci* 20: 5616–5622, 2000.

Lhuillier L and Dryer SE. 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.

Lhuillier L and Dryer SE. Ras is a mediator of TGFβ1 signaling in developing chick ciliary ganglion neurons. *Brain Res* 982: 119–124, 2003.

Lin R, Bagrodia S, Cerione R, and Manor D. A novel Cdc42Hs mutant induces cellular transformation. *Curr Biol* 7: 794–797, 1997.

Lippincott-Schwartz J, Yuan LC, Bonifacino JS, and Klausner RD. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* 56: 801–813, 1989.

Macia E, Luton F, Partisani M, Cherfils J, Chardin P, and Franco M. The GDP-bound form of Arf6 is located at the plasma membrane. *J Cell Sci* 117: 2389–2398, 2004.

Martin-Caraballo M and Dryer SE. Activity- and target-dependent regulation of large-conductance Ca^{2+}-activated K^+ channels in developing chick lumbar motoneurons. *J Neurosci* 22: 73–81, 2002.

Mukherjee S, Casanova JE, and Hunzicker-Dunn M. Desensitization of the luteinizing hormone/choriogonadotropin receptor in ovarian follicular membranes is inhibited by catalytically inactive ARNO(+). *J Biol Chem* 276: 6524–6528, 2001.

Presley JF, Ward TH, Pfeifer AC, Siggia ED, Phair RD, and Lippincott-Schwartz J. Dissection of COPI and ARF1 dynamics in vivo and role in Golgi membrane transport. *Nature* 417: 187–193, 2002.

Qian Y, Corum L, Meng Q, Blenis J, Zheng JZ, Shi X, Flynn DC, and Jiang BH. PI3K induced actin filament remodeling through Akt and p70S6K1: implication of essential role in cell migration. *Am J Physiol Cell Physiol* 286: C153–163, 2004.

Rizzo MA, Shome K, Vasudevan C, Stolz DB, Sung TC, Frohman MA, Watkins SC, and Romero G. Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J Biol Chem* 274: 1131–1139, 1999.

Sandrock AW Jr, Dryer SE, Rosen KM, Gozani SN, Kramer R, Theill LE, and Fischbach GD. Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. *Science* 276: 599–603, 1997.

Santy LC. Characterization of a fast cycling ADP-ribosylation factor 6 mutant. *J Biol Chem* 277: 40185–40188, 2002.

Schafer DA, D'Souza-Schorey C, and Cooper JA. Actin assembly at membranes controlled by ARF6. *Traffic* 1: 896–907, 2000.

Spang A. ARF1 regulatory factors and COPI vesicle formation. *Curr Opin Cell Biol* 14: 423–427, 2002.

Stamnes M. Regulating the actin cytoskeleton during vesicular transport. *Curr Opin Cell Biol* 14: 428–433, 2002.

Subramony P and Dryer SE. Neuregulins stimulate the functional expression of Ca^{2+}-activated K^+ channels in developing chicken parasympathetic neurons. *Proc Natl Acad Sci USA* 94: 5934–5938, 1997.

Subramony P, Raucher S, Dryer L, and Dryer SE. Posttranslational regulation of Ca^{2+}-activated K^+ currents by a target-derived factor in developing parasympathetic neurons. *Neuron* 17: 115–124, 1996.

Takai Y, Sasaki T, and Matozaki T. Small GTP-binding proteins. *Physiol Rev* 81: 153–208, 2001.

Venkateswarlu K, Gunn-Moore F, Oatey PB, Tavare JM, and Cullen PJ. Nerve growth factor- and epidermal growth factor-stimulated translocation of the ADP-ribosylation factor-exchange factor GRP1 to the plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain. *Biochem J* 335: 139–146, 1998.

Vitale N, Caumont AS, Chasserot-Golaz S, Du G, Wu S, Sciorra VA, Morris AJ, Frohman MA, and Bader MF. Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. *EMBO J* 20: 2424–2434, 2001.

Vitale N, Chasserot-Golaz S, and Bader MF. Regulated secretion in chromaffin cells: an essential role for ARF6-regulated phospholipase D in the late stages of exocytosis. *Ann NY Acad Sci* 971: 193–200, 2002.

Yu S and Roth MG. Casein kinase I regulates membrane binding by ARF GAP1. *Mol Biol Cell* 13: 2559–2570, 2002.

Zhang Q, Cox D, Tseng CC, Donaldson JG, and Greenberg S. Requirement for ARF6 in Fcgamma receptor-mediated phagocytosis in macrophages. *J Biol Chem* 273: 19977–19981, 1998.