

Intracellular Signals That Control Cell Proliferation in Mammalian Balance Epithelia: Key Roles for Phosphatidylinositol-3 Kinase, Mammalian Target of Rapamycin, and S6 Kinases in Preference to Calcium, Protein Kinase C, and Mitogen-Activated Protein Kinase

Mireille Montcouquiol and Jeffrey T. Corwin

Departments of Otolaryngology and Neuroscience, University of Virginia School of Medicine, Charlottesville, Virginia 22908

In fish, amphibians, and birds, the loss of hair cells can evoke S-phase entry in supporting cells and the production of new cells that differentiate as replacement hair cells and supporting cells. Recent investigations have shown that supporting cells from mammalian vestibular epithelia will proliferate in limited numbers after hair cells have been killed. Exogenous growth factors such as glial growth factor 2 enhance this proliferation most potently when tested on vestibular epithelia from neonates. In this study, the intracellular signaling pathways that underlie the S-phase entry were surveyed by culturing epithelia

in the presence of pharmacological inhibitors and activators. The results demonstrate that phosphatidylinositol 3-kinase is a key element in the signaling cascades that lead to the proliferation of cells in mammalian balance epithelia *in vitro*. Protein kinase C, mammalian target of rapamycin, mitogen-activated protein kinase, and calcium were also identified as elements in the signaling pathways that trigger supporting cell proliferation.

Key words: cell proliferation; regeneration; supporting cells; mammals; inner ear; vestibule; phosphatidylinositol 3-kinase; hearing; rodents; MAPK; p70/p85 S6 kinase

The hearing, balance, and lateral line epithelia of fish, amphibians, and birds have the capacity to recover from sensory deficits by regenerating hair cells (for review, see Corwin and Oberholtzer, 1997). At sites of hair cell loss, supporting cells divide and give rise to progeny that can differentiate as replacement hair cells. In mammals, hearing and balance deficits also arise from hair cell loss, but those deficits are typically permanent (Nadol, 1993). Mammals produce hair cells late in embryonic development. Cell divisions in their otic sensory epithelia decline precipitously and were believed to cease around the time of birth (Ruben, 1967). Recent evidence, however, supports a revised view of the potential for cell proliferation and self-repair in the balance epithelia of mammals.

Balance epithelia in juvenile rodents can heal damage caused by sublethal doses of ototoxic antibiotics administered *in vivo* (Forge et al., 1993). *In vitro* treatments with higher levels of antibiotics can kill the majority of the hair cells in vestibular epithelia from adult rodents and humans; when this occurs, limited numbers of supporting cells enter S-phase (Warchol et al., 1993). Subsequent investigations have shown that transforming growth factor- α , epidermal growth factor (EGF), insulin, and insulin-like growth factors can all increase proliferation in cultured mammalian balance epithelia (Lambert, 1994; Yamashita

and Oesterle, 1995; Zheng et al., 1997; Kuntz and Oesterle, 1998), as can recombinant human glial growth factor 2 (rhGGF2). RhGGF2 is a secreted member of a family of factors encoded by alternatively spliced transcripts from the neuregulin gene (Marchionni et al., 1993; Riese and Stern, 1998). When vestibular epithelia from newborn rats are cultured in rhGGF2 for 72 hr, >40% of the cells enter S-phase (R. Gu, M. Montcouquiol, M. Marchionni, and J. T. Corwin, unpublished observations).

Binding of growth factors to receptor tyrosine kinases leads to activation of intracellular cascades composed of enzymes and signaling intermediates that could be potential targets for therapeutic control over the regeneration of hair cells; therefore, these targets need to be identified (Corwin and Oberholtzer, 1997). High concentrations of forskolin stimulate supporting cell proliferation in chicken cochleas *in vitro*, and inhibitors of protein kinase A reduce that effect (Navaratnam et al., 1996), but no signaling cascades that mediate proliferation in mammalian hair cell epithelia have been identified. The potent mitogenic effect of rhGGF2 provided an opportunity to survey intracellular signaling cascades for roles in triggering proliferation in mammalian balance epithelia by culturing epithelia with inhibitors and activators of signaling intermediates in the different pathways that can participate in the control of proliferation in other cell types. Activation of phosphatidylinositol-3 kinase (PI-3K), mammalian target of rapamycin (mTOR), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK), as well as increased intracellular calcium were found to contribute to triggering different levels of S-phase entry and proliferation of cells in balance epithelia from rats.

MATERIALS AND METHODS

Preparation of epithelial cell cultures. Experiments were conducted in accordance with an approved animal use protocol that adhered to practices outlined in the *NIH Guide for the Care and Use of Laboratory*

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Correspondence should be addressed to Mireille Montcouquiol, Departments of Otolaryngology and Neuroscience, University of Virginia School of Medicine, Charlottesville, VA 22908. E-mail: mm7vy@virginia.edu.

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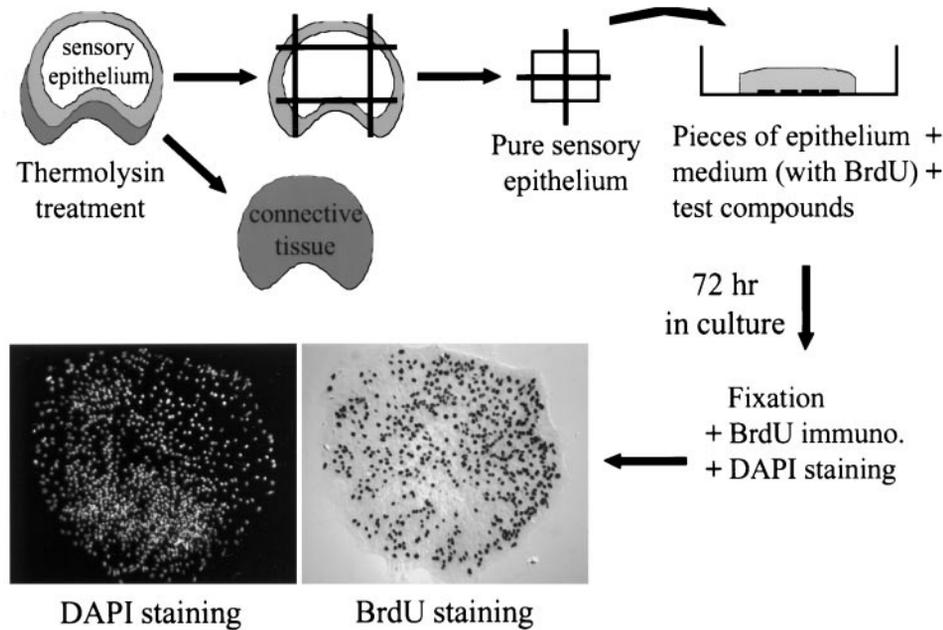


Figure 1. Methods used to analyze S-phase entry in vestibular sensory epithelia. Utricles were dissected from 2-d-old rats and incubated with the enzyme thermolysin to separate the sensory epithelium from the underlying connective tissue. The nonsensory epithelium (light gray) was trimmed away, and the remaining pure sensory epithelium was cut into four equal pieces. Pieces of epithelium were transferred to a glass-bottom culture dish in a medium containing BrdU and either vehicle or one of the test compounds. After 72 hr, the cultures were fixed and processed for immunocytochemistry and DAPI staining; next, the fraction of the cells that had entered S-phase and incorporated BrdU during replication of their DNA was calculated from counts of all of the labeled and unlabeled cells.

Animals under the supervision of the University of Virginia Animal Care Advisory Committee. This report is based on sensory epithelia cultured from 89 2-d-old (postnatal day 2) Sprague Dawley rats that were killed with carbon dioxide and decapitated. Each head was skinned and disinfected in ice-cold 70% ethanol for 10 min. Next, the vestibular organs from both ears were aseptically transferred to ice-cold DMEM/F-12 medium (Life Technologies, Gaithersburg, MD), and the otoconia and otolithic membranes were removed from the utricles. To separate the sensory epithelium from the underlying tissue, each utricle was incubated in thermolysin at 0.5 mg/ml (Sigma, St Louis, MO) in DMEM/F-12 for 45 min at 37°C in a 5% CO₂ atmosphere (Saffer et al., 1996). Whole utricles were then transferred to ice-cold DMEM/F-12 containing 5% fetal bovine serum (FBS) (HyClone, Logan, UT) to stop the digestion, and the epithelium was removed with fine forceps. The surrounding nonsensory epithelium and the outer edges of the sensory macula were trimmed away with a diamond microscalpel and discarded. The remaining pure sensory epithelium was cut into four approximately equal pieces (Fig. 1). The pieces of epithelium were transferred to a glass-bottom culture dish (MatTek, Ashland, MA) that was precoated with poly-L-lysine (5 μg/ml for 1 hr at 37°C; Sigma) and fibronectin (100 μg/ml overnight at 37°C; Sigma). The epithelia were allowed to adhere for 1 hr at 37°C in a 5% CO₂ atmosphere in medium containing 5% FBS. Next, the adherent epithelia were cultured for 72 hr in DMEM/F-12 containing 3 μg/ml 5-bromo-2-deoxyuridine (BrdU) (Sigma), 2.5% FBS, and either DMSO or one of the test compounds.

Test compounds and media. The intracellular signaling mechanisms that are responsible for the entry of supporting cells into S-phase were assessed by incubating sheets of epithelium with the pharmacological activators and inhibitors listed in Table 1. Inhibitor concentrations were chosen on the basis of studies that demonstrated inhibition of the target enzyme and/or significant inhibition of the incorporation of BrdU or tritiated thymidine in cultures (references are listed in Table 1). We did not test pathways related to neurotransmitter receptors or ion channels.

The standard (or control) medium was DMEM/F-12 supplemented with 2.5% FBS and 3 μg/ml BrdU. When test compounds were solubilized in DMSO, control medium also contained DMSO as a vehicle control. Test compounds were added to the standard medium at the concentrations indicated in Results. Each inhibitor was added to the epithelial cultures for 1 hr before the addition of rhGGF2 (Cambridge Neuroscience, Cambridge, MA). RhGGF2 was used at 50 ng/ml throughout the study. The medium was then replaced by the standard medium containing the inhibitor and rhGGF2 for the 72 hr culture period. Wortmannin was added to the medium every 8 hr because of its instability at 37°C (Yao and Cooper, 1996; Parrizas et al., 1997).

Activators were added to the epithelial cultures 15 min before the addition of either standard medium or medium containing rhGGF2, as described in Results. The activators were then removed by replacing the

medium with the standard medium, with or without rhGGF2. Anisomycin was added at the same time as standard medium that contained rhGGF2, and remained throughout the 72 hr culture period.

To downregulate PKC, 20 pieces of epithelium were cultured for 16 hr in 5 μM PMA in the standard medium. The medium was then replaced with standard medium that contained rhGGF2 for the remaining 56 hr of the culture.

Bromodeoxyuridine labeling. After culture, epithelia were fixed in 4% paraformaldehyde for 30 min, then rinsed three times in PBS and immersed in 1 N HCl for 15 min to denature nucleic acids. Immunocytochemical identification of nuclei that had incorporated BrdU was performed at room temperature. The cultures were preincubated for 1 hr in TPBS (PBS with 0.2% Triton X-100) with 10% normal horse serum (NHS) and incubated for 2 hr in a mouse monoclonal antibody against BrdU (Becton Dickinson, San Jose, CA) that was diluted 1:50 in TPBS with 2% NHS. After three PBS rinses, the specimens were incubated for 30 min in a secondary antibody solution containing biotinylated rat-adsorbed anti-mouse IgG (Vector Laboratories, Burlingame, CA) that was diluted 1:100 in TPBS with 2% NHS. Next, they were processed with avidin, horseradish peroxidase conjugated to biotin, and diaminobenzidine using an Elite avidin–biotin complex (ABC) kit with nickel intensification (Vector Laboratories). The specimens were then incubated in 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) at 10 μg/ml in PBS with 0.1% Triton X-100 for 30 min to stain DNA. After three rinses with PBS, the specimens were examined by epifluorescence microscopy.

MetaMorph software (Universal Imaging, Media, PA) was used to acquire images from a cooled CCD camera (Princeton Instruments, Trenton, NJ) interfaced to a Zeiss Axiovert 135 (Zeiss, Thornwood, NY). All of the nuclei that were stained by DAPI and all of the nuclei that were labeled by BrdU were counted in each piece of epithelium. The labeling index was calculated for each piece of sensory epithelium by dividing the number of BrdU-labeled nuclei by the total number of nuclei in the piece of epithelium (Fig. 1). For each test condition, 24–44 pieces of utricular sensory epithelium were analyzed. Statistical significance was determined using the two-tailed Student's *t* test.

MAPK immunocytochemistry. Thirty two pieces of sensory epithelium were isolated and trimmed as described above and cultured for 48 hr in DMEM/F-12 with 5% FBS. Next, they were incubated overnight in DMEM/F-12 with 2.5% FBS to reduce the endogenous level of MAPK activation. The medium was then replaced in one set of eight cultures by DMEM/F-12 with 2.5% FBS in the presence of 20 μM U0126, and for eight others by 100 μM PD98059. After 1 hr, eight cultures were treated with DMEM/F-12 with 2.5% FBS (control), eight were treated with DMEM/F-12 with 2.5% FBS in the presence of 20 μM U0126 and 50 ng/ml rhGGF2, eight were treated with DMEM/F-12 with 2.5% FBS in the presence of 100 μM PD98059 and

Table 1. List of all the activators and inhibitors used in this study

Compound name	Target(s)	References
Inhibitors		
Tyrphostin AG 825 (1)	ErbB2	Osherov et al., 1993
Wortmannin (2)	PI-3K	Okada et al., 1994a,b Vlahos et al., 1994
LY294002 (1)	PI-3K	Sanchez-Margalet et al., 1994 Chung et al., 1992
Rapamycin (1)	mTOR	Brown et al., 1994
FK506 (3)	FKBP12	Lin et al., 1995 Tamaoki et al., 1990
Calphostin C (1)	cPKCs, nPKCs	Seynaeve et al., 1994 Toullec et al., 1991
BIM (1)	PKCs	Martiny-Baron et al., 1993
1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) (1)	MEK1 and MEK2	Favata et al., 1998 Alessi et al., 1995
PD98059 (4)	MEK1	Dudley et al., 1995
Apigenin (1)	ERK/MAPK	Kuo and Yang, 1995
SB203580 (1)	p38-MAPK	Cuenda et al., 1995
Activators		
Anisomycin (5)	JNKs	Cano et al., 1994
DAG (6)	cPKCs, nPKCs	
PMA (2)	cPKCs, nPKCs	Toker et al., 1994 Franke et al., 1997
PI-3,4-P2 (1)	PKCs, Akt/PKB	Klippel et al., 1997
A23187 (2)	Calcium	
Ionomycin (2)	Calcium	

Drug sources (in parentheses): 1, Calbiochem, La Jolla, CA; 2, Sigma, St. Louis, MO; 3, Fujisawa, Deerfield, IL; 4, New England Biolabs, Beverly, MA; 5, Biomol, Plymouth Meeting, PA; 6, Avanti Polar Lipids, Alabaster, AL.

50 ng/ml rhGGF2, and eight were treated with DMEM/F-12 with 2.5% FBS in the presence 50 ng/ml rhGGF2. After 1 hr, all of the cultures were rinsed with PBS and fixed in 4% paraformaldehyde in PBS at room temperature for 30 min followed by a methanol permeabilization at -20°C for 10 min. The cultures were rinsed with PBS, blocked in TPBS with 5% NHS, and incubated overnight with a polyclonal antibody to phosphorylated extracellular regulated kinase-1 (ERK-1) and ERK-2 (diluted 1:200 in TPBS with 2% NHS at 4°C) (Cell Signaling, Beverly, MA). After three rinses in PBS, cultures were incubated with biotinylated rat-adsorbed anti-mouse IgG (diluted 1:100 in TPBS with 2% NHS) and processed using an Elite ABC kit with nickel intensification (Vector Laboratories) for 11 min.

MAPK SDS-PAGE and immunoblot analysis. One hundred and ninety-eight pieces of utricular sensory epithelium were cultured in DMEM/F-12 with 5% FBS. After 48 hr, the cultures were incubated overnight at 4°C in DMEM/F-12 with 2.5% FBS and then treated with U0126, PD98059, and rhGGF2 as described above for the MAPK immunocytochemistry. The cultures were then briefly rinsed with cold PBS containing 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ pepstatin, 10 $\mu\text{g/ml}$ aprotinin, 1 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 50 mM NaF, and 5 mM EDTA. The pieces of sensory epithelium were harvested in that buffer by scraping. For each of the four conditions, the cells from 48–52 pieces were pooled in an Eppendorf tube and centrifuged at $16,000 \times g$ for 10 min at 4°C ; the pellets were stored at -80°C and subsequently resuspended in lysis buffer containing 2% SDS, 10% glycerol, and 62.5 mM Tris-HCl, pH 6.8, heated at 95°C for 5 min, and sonicated for 10 sec. Protein was measured using the bicinchoninic acid method according to the manufacturer's instructions (Pierce, Rockford, IL).

Proteins were separated by SDS-PAGE (20 $\mu\text{g/lane}$ in 4–20% gradient gels) (Novex, San Diego, CA) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were incubated in 5% (w/v) nonfat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline containing 0.2% Tween 20 (TTBS) overnight at 4°C . Membranes were washed and incubated with a 1:1500 dilution of the phosphospecific antibody to ERK-1 and ERK-2 in TTBS for 2 hr, washed again, and incubated for 1 hr with a peroxidase-conjugated anti-rabbit antibody (1:10,000 in TTBS). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham, Princeton, NJ). The membranes were stripped in 2% SDS, 62.5 mM Tris-HCl, and 100 mM β -mercaptoethanol at pH 6.8, with shaking for 45 min at 60°C . Next, they were reprocessed with a mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:1000 in TTBS) (Chemicon, Temecula, CA). Films were scanned and analyzed using a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer and ImageQuant software.

RESULTS

RhGGF2 induces proliferation in an ErbB2 receptor-dependent manner

The fraction of cell nuclei that entered S-phase and were labeled with BrdU was >17 times greater in utricular sensory epithelia that were cultured for 72 hr in medium that contained 50 ng/ml rhGGF2 (373 ± 38.6 BrdU-labeled cells; mean \pm SEM; $n = 39$ pieces of epithelium) than in parallel cultures in the standard medium (5.6 ± 1.9 labeled cells; $n = 25$) (Fig. 2). The rhGGF2-stimulated proliferation was reduced by 60% when epithelia were cultured in medium that also contained the ErbB2 receptor kinase inhibitor AG825 ($p < 0.05$) (Fig. 2).

The role of PI-3K and mTOR

Wortmannin and LY294002 were used to assess the role of PI-3K in proliferation stimulated by rhGGF2. Treatment of epithelia with the PI-3K inhibitor LY294002 caused a dose-dependent inhibition of the rhGGF2-mediated response, with inhibition of nearly all S-phase entry at 30 μM ($p < 0.05$) (Fig. 3*B*, bottom). LY294002 has been reported to inhibit mTOR at 30 μM (Brunn et al., 1996); therefore, it is possible that the inhibition observed in 30 μM LY294002 resulted from the inhibition of both PI-3K and mTOR. Wortmannin at 10 nM, a concentration that has been reported to inhibit PI-3K specifically (Okada et al., 1994a), also caused a significant reduction in the level of S-phase entry induced by rhGGF2 (Fig. 3, bottom).

Incubation of sensory epithelia with rhGGF2 and rapamycin resulted in a dose-dependent inhibition of S-phase entry compared with the level induced in rhGGF2 alone. At 20 nM, rapamycin reduced the level of S-phase entry by $\sim 60\%$ ($p < 0.05$) (Fig. 4*A*). The specificity of the inhibition achieved with rapamycin in this system was confirmed by culturing epithelia in medium containing rhGGF2 together with rapamycin and an excess of FK506, a structurally related drug that competes with rapamycin for the same binding site on FKBP12 (Abraham et al., 1996). The presence of FK506 blocked the inhibition observed with rapamycin (Fig. 4*B*).

The role of PKCs

To determine whether PKCs are involved in the rhGGF2-mediated cell proliferation in utricular epithelia, we treated cultures with rhGGF2 in the presence of calphostin C, a specific inhibitor that binds to the regulatory domain of diacylglycerol (DAG)-dependent PKCs (IC_{50} , 50 nM). Calphostin C at 100 nM, a concentration that has been reported to inhibit classical PKCs (cPKCs), did not inhibit rhGGF2-induced proliferation (Fig. 5). At 500 nM, a concentration reported to inhibit 50% of the activity of two novel PKC (nPKC) isoforms (δ and ϵ), the inhibitor

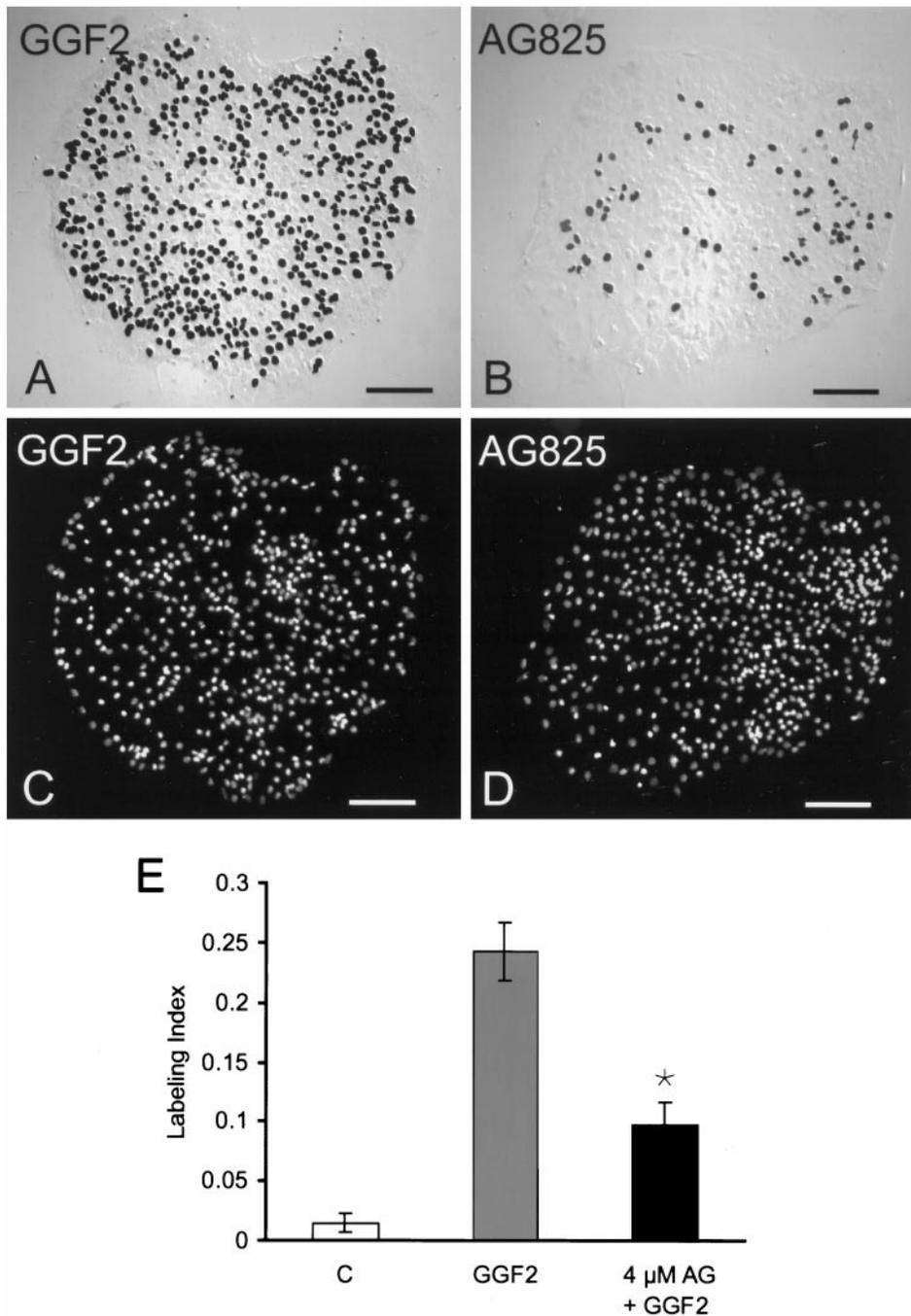


Figure 2. Selective inhibition of ErbB2 decreases the incidence of S-phase entry induced by rhGGF2. *A, C*, Pieces of utricular sensory epithelium fixed after 72 hr in culture in the standard medium with 50 ng/ml rhGGF2. *A*, Nuclei of cells that entered S-phase and incorporated BrdU were stained black after immunocytochemistry and were visualized by differential interference contrast microscopy. *C*, Fluorescent DAPI staining revealed the nuclei that did not enter S-phase in the same piece of epithelium. *B, D*, A piece of epithelium that was preincubated with the ErbB2 inhibitor AG825 at 4 μ M for 60 min and then cultured for 72 hr in the standard medium containing rhGGF2 and 4 μ M AG825. All other inhibitors were tested in the same manner unless otherwise noted. The incidence of cells that entered S-phase was qualitatively different in epithelia cultured in the presence of AG825 as shown by the smaller number of darkly stained nuclei in *B, E*. The fraction of cells that had entered S-phase in the standard control medium (*C*), in that medium supplemented with 50 ng/ml rhGGF2 (*GGF2*), and in medium containing both rhGGF2 and 4 μ M AG825 (*4 μM AG + GGF2*). Each bar represents data from 26 to 37 pieces of epithelium. An asterisk denotes significance compared with cultures treated with rhGGF2 alone unless otherwise noted ($p < 0.05$). Scale bars, 100 μ m.

induced a small but not significant decrease in the mean level of S-phase entry. At 1 μ M, calphostin C reduced the level of S-phase entry by 72% from the level in cultures supplemented with rhGGF2 alone ($p < 0.05$).

Bisindolylmaleimide I (BIM), an inhibitor that competitively binds to the ATP-binding site of PKCs (IC_{50} , 10 nM), was used to provide an independent assessment of the role of PKCs in pathways that trigger rhGGF2-induced proliferation. At 100 nM, BIM did not inhibit rhGGF2-induced proliferation, but treatments with BIM at 1 and 2 μ M resulted in mean levels of S-phase entry that decreased in a dose-dependent manner (Fig. 5). At 2 μ M, BIM reduced the level of rhGGF2-induced S-phase entry by ~66% ($p < 0.05$).

The role of MAP kinase

To assess the role of the ERK–MAPK pathway in the cell proliferation induced by rhGGF2, we used U0126, an inhibitor of MAPK–ERK kinase 1 (MEK1) and MEK2 (IC_{50} values of 72 and 58 nM, respectively). At 20 μ M, U0126 reduced the level of rhGGF2-induced S-phase entry by 34% ($p < 0.05$) (Fig. 6*A*); lower concentrations were less effective.

Other pieces of epithelium were incubated with the MEK1 inhibitor PD98059 (IC_{50} , 5–10 μ M) to provide an independent assessment of the role of the ERK–MAPK pathway in the rhGGF2 response. PD98059 reduced the rhGGF2-induced S-phase entry by 39% at 50 μ M and by 53% at 100 μ M ($p < 0.05$) (Fig. 6*A*). Treatment with 13 μ M apigenin, another inhibitor of the

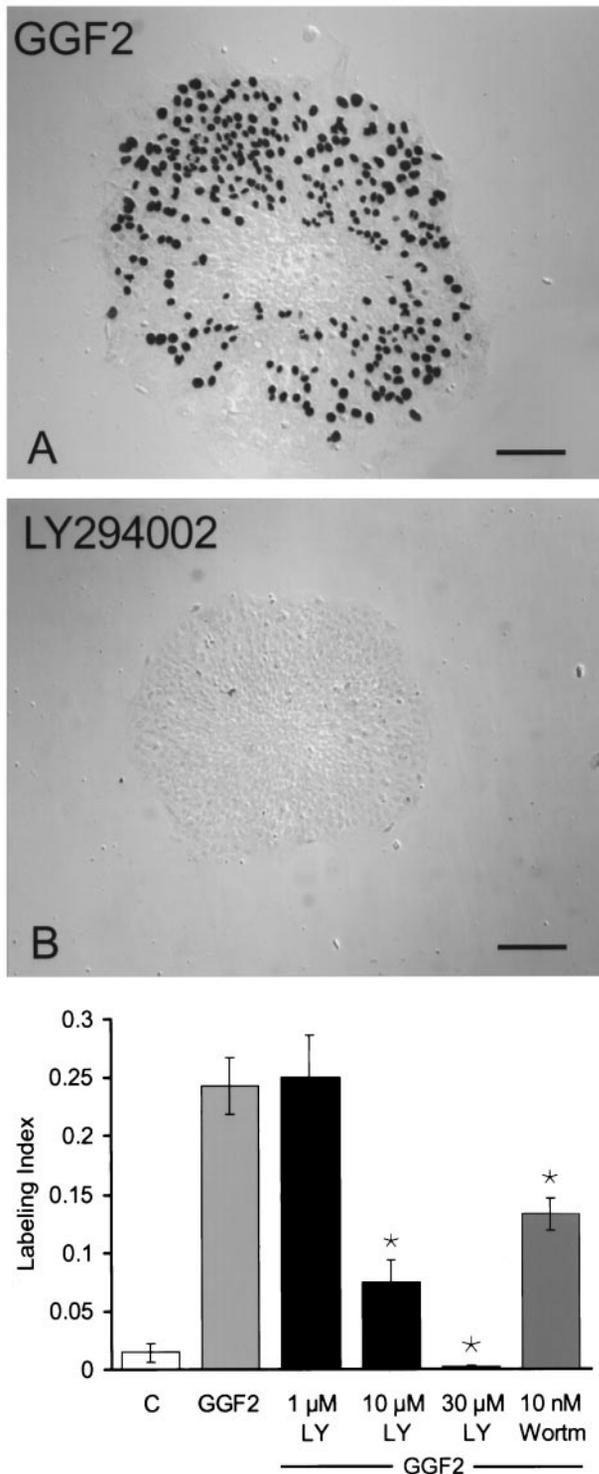


Figure 3. Inhibition of the enzymatic activity of PI-3K effectively blocked S-phase entry. *A*, A differential interference contrast micrograph showing many black BrdU-labeled nuclei that entered S-phase in a piece of utricular epithelium during 72 hr in culture with rhGGF2. *B*, Only one black BrdU-labeled nucleus was visible in this piece of epithelium that was treated with the PI-3K inhibitor LY294002 at 30 μ M in the presence of rhGGF2. The treatment with LY294002 at 30 μ M resulted in a pronounced qualitative reduction in the amount of S-phase entry that occurred in the presence of rhGGF2, reducing S-phase entry to levels below those observed in control cultures. *C*, Quantitative measures of BrdU labeling that resulted from the inhibition of rhGGF2-induced S-phase entry by LY294002 (LY) at 1, 10, and 30 μ M and by 10 nM wortmannin (Wortm). Each bar represents data from 21–37 pieces of epithelium. Scale bars, 100 μ m.

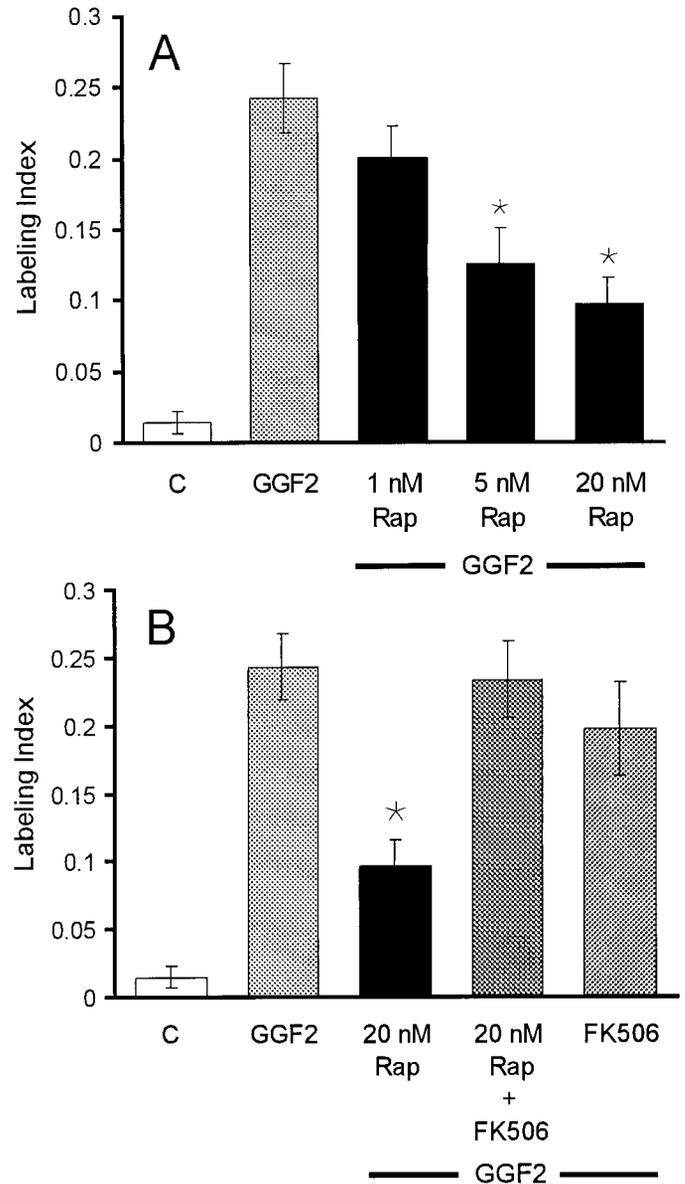


Figure 4. Specific inhibition of mTOR reduced the incidence of S-phase entry induced by rhGGF2. *A*, A histogram of the level of BrdU labeling in epithelia that were treated with rapamycin (Rap) at 1, 5, and 20 nM in the presence of rhGGF2. *B*, The inhibitory effect of 20 nM rapamycin on S-phase entry was reversed by FK506, which competes with rapamycin for the same binding site of the immunophilin FKBP12. FK506 was added 1 hr before rapamycin, rhGGF2, and BrdU. When tested together with rhGGF2, FK506 did not change the level of S-phase entry significantly from the level induced by rhGGF2 alone. Each bar represents data from 20 to 44 pieces of epithelium.

ERK–MAPK pathway, resulted in approximately the same level of inhibition observed with PD98059 at 100 μ M (56%; $p < 0.05$).

To determine whether U0126 and PD98059 were effectively inhibiting the activation of the ERK–MAPK pathway in this culture system, we used a phosphospecific antibody to localize the activated (phosphorylated) forms of ERK-1 and ERK-2. Figure 6*B* shows immunostaining in control cultures and in cultures maintained in media containing rhGGF2 alone or rhGGF2 in combination with the U0126 or PD98059. The eight cultures that were maintained with rhGGF2 alone exhibited strong staining for the phosphorylated forms of ERK-1 and ERK-2 in both the

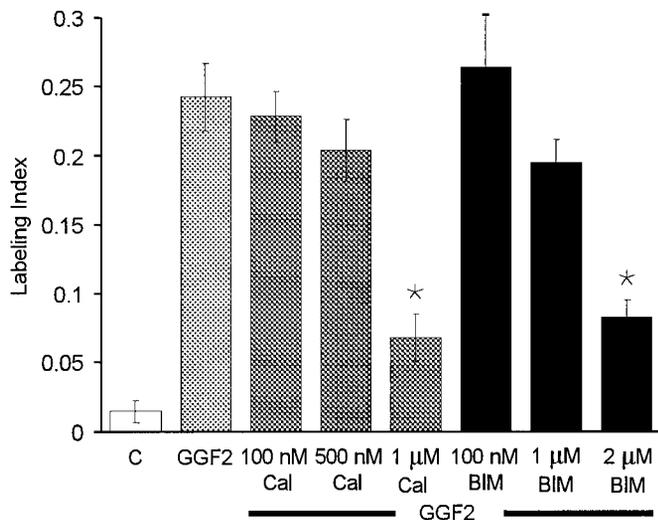


Figure 5. Inhibition of the enzymatic activity of PKCs reduced the incidence of S-phase entry induced by rhGGF2. The PKC inhibitor calphostin C (*Cal*) at 0.1, 0.5, and 1 μ M in the presence of rhGGF2 reduced S-phase entry by 70% compared with the level induced in the presence of rhGGF2 alone. The PKC inhibitor BIM at 0.1, 1, and 2 μ M in the presence of rhGGF2 also produced dose-dependent reductions in S-phase entry compared with the level induced in rhGGF2 alone. Each bar in Figures 5–8 represents data from 20 to 37 pieces of epithelium.

cytoplasmic and nuclear regions of many cells. In contrast, there was virtually no staining for phosphorylated ERK-1 and ERK-2 in the eight cultures that were maintained in the same level of rhGGF2, but with 20 μ M U0126, in the eight cultures that were maintained in rhGGF2 with 100 μ M PD98059, or in the eight cultures that were maintained in the control medium.

To further assess the possibility for incomplete inhibition of the ERK–MAPK pathway, cultures were maintained with either rhGGF2 alone, rhGGF2 together with U0126, rhGGF2 together with PD98059, or in control medium; next, the cells were harvested for SDS-PAGE and immunoblot analysis for the level of ERK-1 and ERK-2 activation. Samples from 48–52 cultured pieces of epithelium per condition yielded two replicate immunoblots that showed that 96% of the phosphorylation of ERK-1 and 95% of the phosphorylation of ERK-2 was inhibited by 20 μ M U0126 and that 96% and 92% of the phosphorylation, respectively, was inhibited by 100 μ M PD98059 when cultures were maintained in those inhibitors together with rhGGF2 (Fig. 6C).

Another family of MAP kinases includes p38-MAPK and the c-Jun N-terminal kinases (JNKs), which are involved in stress-activated responses. Treatment with 10 μ M SB203580, a specific inhibitor of p38-MAPK (IC₅₀, 600 nM), reduced the mean level of rhGGF2-induced S-phase entry, but the effect was not statistically significant in the 20 pieces of epithelium tested (Fig. 7). JNKs can be activated by the antibiotic anisomycin. Treatment with 10 ng/ml anisomycin for 72 hr reduced S-phase entry in the presence of rhGGF2 by 67% (Fig. 7; $p < 0.05$).

Direct pharmacological stimulation of S-phase entry

Epithelia were treated with the physiological PKC activator DAG and the phorbol ester PMA to determine whether activation of PKCs would stimulate S-phase entry directly in the absence of rhGGF2. When epithelia were treated with 10 μ M DAG for just 15 min and subsequently maintained in the standard medium for 72 hr, S-phase entry was more than five times the level observed in control epithelia cultured in the same medium without pre-

treatment ($p < 0.05$) (Fig. 8A). A 15 min treatment with 5 μ M PMA also resulted in a fivefold increase in S-phase entry ($p < 0.05$) (Fig. 8A). When the epithelia were preincubated with the MEK1 inhibitor PD98059 at 50 μ M for 1 hr before treatment with 5 μ M PMA, the PMA-induced S-phase entry was reduced significantly (Fig. 8A). Epithelia treated for 15 min with PMA and then with rhGGF2 responded with a mean level of S-phase entry that was nearly the same as that observed with rhGGF2 alone (Fig. 8). Downregulation of PKCs by a 16 hr treatment with 5 μ M PMA caused a partial inhibition of the rhGGF2-induced S-phase entry, consistent with a role for PKCs in the induction of that proliferation (Fig. 8A).

PKCs and Akt [also known as protein kinase B (PKB)] are important downstream targets of PI-3K. The major lipid phosphorylation products of PI-3K are phosphatidylinositol-3,4-bisphosphate (PI-3,4-P₂) and phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P₃), which activate PKCs and Akt–PKB. Epithelia were treated with synthetic PI-3,4-P₂ to assess signaling downstream from PI-3K after they were permeabilized with saponin at 10 ng/ml for 1 min to allow the phospholipid to cross the cell membrane. The saponin treatment increased the mean level of S-phase entry by ~2.5-fold compared with control cultures, but that difference was not significant ($p = 0.07$), whereas permeabilization followed by a 15 min treatment with PI-3,4-P₂ at 2 μ M resulted in an eightfold increase in S-phase entry during the subsequent 72 hr period of culture in the standard medium compared with control ($p < 0.05$) (Fig. 8B). The increase was approximately threefold when compared with the level of S-phase entry in the saponin-treated controls ($p < 0.05$).

Incubation of the epithelia with the calcium ionophore A23187 at 100 nM for 15 min resulted in 2.4 times the level of S-phase entry that occurred in the controls ($p < 0.05$, Fig. 8C). A 15 min treatment with the calcium ionophore ionomycin at 100 nM raised S-phase entry to approximately three times the control level ($p < 0.05$). When epithelia were treated with either A23187 or ionomycin at 100 nM for 15 min and the medium was replaced by the standard medium containing rhGGF2, S-phase entry was increased significantly over that observed after rhGGF2 alone. Treatment with three 15 min pulses of ionomycin at 100 nM (one pulse per hour) followed by rhGGF2 did not significantly increase S-phase entry beyond that induced by a single 15 min ionomycin pulse that was followed by rhGGF2 (Fig. 8C).

DISCUSSION

PI-3K

The results show that inhibitors of PI-3K most effectively block signaling that is required for rhGGF2 induction of S-phase entry and cell proliferation in vestibular epithelia cultured from the ears of neonatal rats. PI-3K is a heterodimeric enzyme that phosphorylates phosphoinositides at the D3 position of the inositol ring to produce phosphatidylinositol-3-phosphate, PI-3,4-P₂, and PI-3,4,5-P₃ (for review, see Carpenter and Cantley, 1996). The strong inhibition of the rhGGF2-mediated S-phase entry that occurred in epithelia treated with a low concentration of wortmannin and different concentrations of LY294002 suggests that PI-3K cascades play pivotal roles in triggering cell proliferation in utricular sensory epithelia. LY294002 is a synthetic compound that is much more specific for PI-3K inhibition than the fungal metabolite wortmannin. The reduction in S-phase entry in the presence of 30 μ M LY294002 together with rhGGF2 was particularly pronounced and lower than in control cultures.

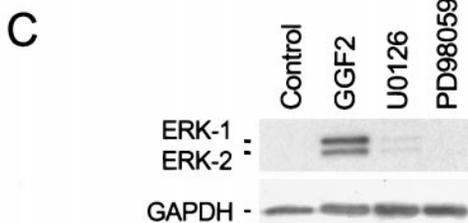
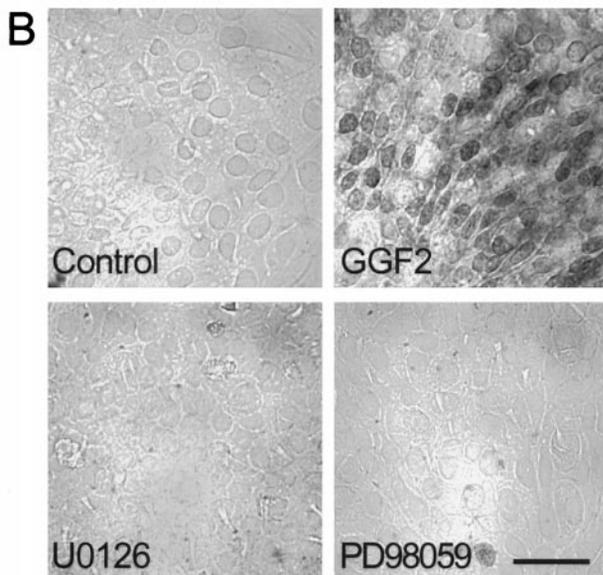
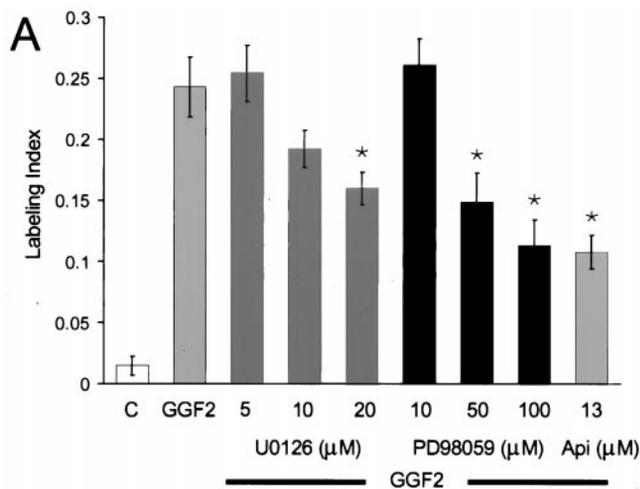


Figure 6. Inhibitors of the ERK-MAPK cascade resulted in moderate reductions in the level of S-phase entry induced by rhGGF2. *A*, U0126 at 5 and 10 μM did not significantly reduce the level of S-phase entry induced by rhGGF2, but 20 μM reduced the response by 34% ($p < 0.05$). The MEK1 inhibitor PD98059 at 10, 50, and 100 μM produced a dose-dependent reduction of rhGGF2-induced S-phase entry. The ERK-MAPK inhibitor apigenin (*Api*) at 13 μM produced approximately the same level of reduction observed for PD98059 at 100 μM when tested in the presence of rhGGF2. *B*, Immunocytochemical analysis with an antibody recognizing activated ERK-1 and ERK-2 showed inhibition of phosphorylated forms of ERK-1 and ERK-2 in the presence of U0126 and PD98059. When pieces of utricular epithelium were treated with 20 μM U0126 or 100 μM PD98059 in the presence of rhGGF2 and then processed for immunocytochemistry, levels of phosphorylated ERK-1 or ERK-2 were virtually undetectable, similar to the control cultures. By comparison, pieces treated for 1 hr with rhGGF2 exhibited strong cytoplasmic and nuclear staining for the phosphorylated forms of ERK-1 and ERK-2. *C*, Immunoblot analysis of pieces of sensory epithelium that were treated with U0126 or PD98059. Phosphorylated forms of ERK-1 and ERK-2 were readily detected in samples treated with rhGGF2 (*lane 2*) but were nearly undetectable in samples treated with rhGGF2 in the presence of 20 μM U0126 (*lane 3*) or 100 μM PD98059 (*lane 4*) or in control cultures (*lane 1*). Scale bar, 50 μm .

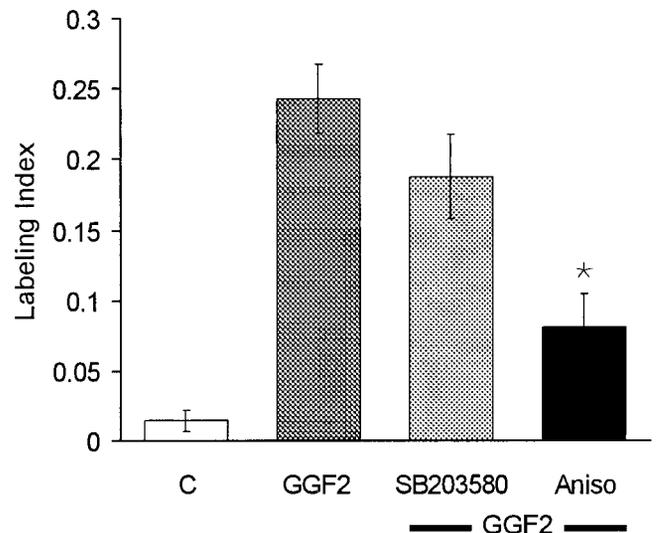


Figure 7. Inhibition of p38-MAPK and activation of JNKs were tested for effects on the level of S-phase entry that could be induced by rhGGF2. In the presence of rhGGF2, treatments with the p38-MAPK inhibitor SB203580 at 10 μM resulted in a reduced average incidence of S-phase labeling, but this effect was not significant when compared with the level of entry obtained with rhGGF2 alone. In contrast, treatments with 10 ng/ml anisomycin (*Aniso*), an activator of JNKs, significantly reduced the level of S-phase entry in the presence of rhGGF2.

mTOR and p70/p85 S6Ks

Inhibition of the kinase activities of mTOR by rapamycin also led to a significant reduction in the incidence of rhGGF2-mediated S-phase entry. Rapamycin inhibition of mTOR blocks downstream activation of the p70/p85 isoforms of S6 kinase (p70/p85 S6Ks) (Brown et al., 1995; Abraham and Wiederrecht, 1996). Activated S6K induces an increase in protein synthesis that is required for cells to make the transition from G_0 to G_1 (for review, see Pullen and Thomas, 1997). The p70/p85 S6K isoforms are generated from alternative translation of the same transcript and differ in an N-terminal extension, which constitutively targets p85 S6K to the nucleus (Reinhard et al., 1992). The p85 isoform of S6K is much less abundant than the p70 isoform, and its accumulation in the nucleus has been shown to trigger G_1 progression (Reinhard et al., 1994). In our experiments, rapamycin produced a dose-dependent inhibition of rhGGF2-induced S-phase entry, which suggests that mTOR participates in the mitogenic signaling pathway in supporting cells. In established pathways, mTOR and p70/p85 S6Ks are downstream from PI-3K (Weng et al., 1995; Brunn et al., 1996; McIlroy et al., 1997). One hypothesis is that the activation of PI-3K could lead, via PKC and/or Akt-PKB, to the activation of mTOR and p70/p85 S6Ks to trigger the proliferation of supporting cells.

ErbB receptors

Inhibition of the kinase activity of the ErbB2 receptor led to a significant reduction in the incidence of S-phase entry induced by rhGGF2. The EGF receptor (EGFR)/ErbB family includes the receptor tyrosine kinases EGFR (also known as ErbB1), ErbB2, ErbB3, and ErbB4. Signaling requires the formation of homodimeric or heterodimeric complexes of these receptors (Alroy and Yarden, 1997). ErbB2 does not have ligand-binding capacity, but it is the preferred dimerization partner for the other members (Graus-Porta et al., 1997). When epithelia were treated with rhGGF2 and the tyrphostin AG825, a specific ErbB2 kinase inhibitor, the level of S-phase entry was reduced by 60%. We hypothesize that the proliferation induced by rhGGF2 is initiated in part through signals that arise when ErbB2 dimerizes with either ErbB3

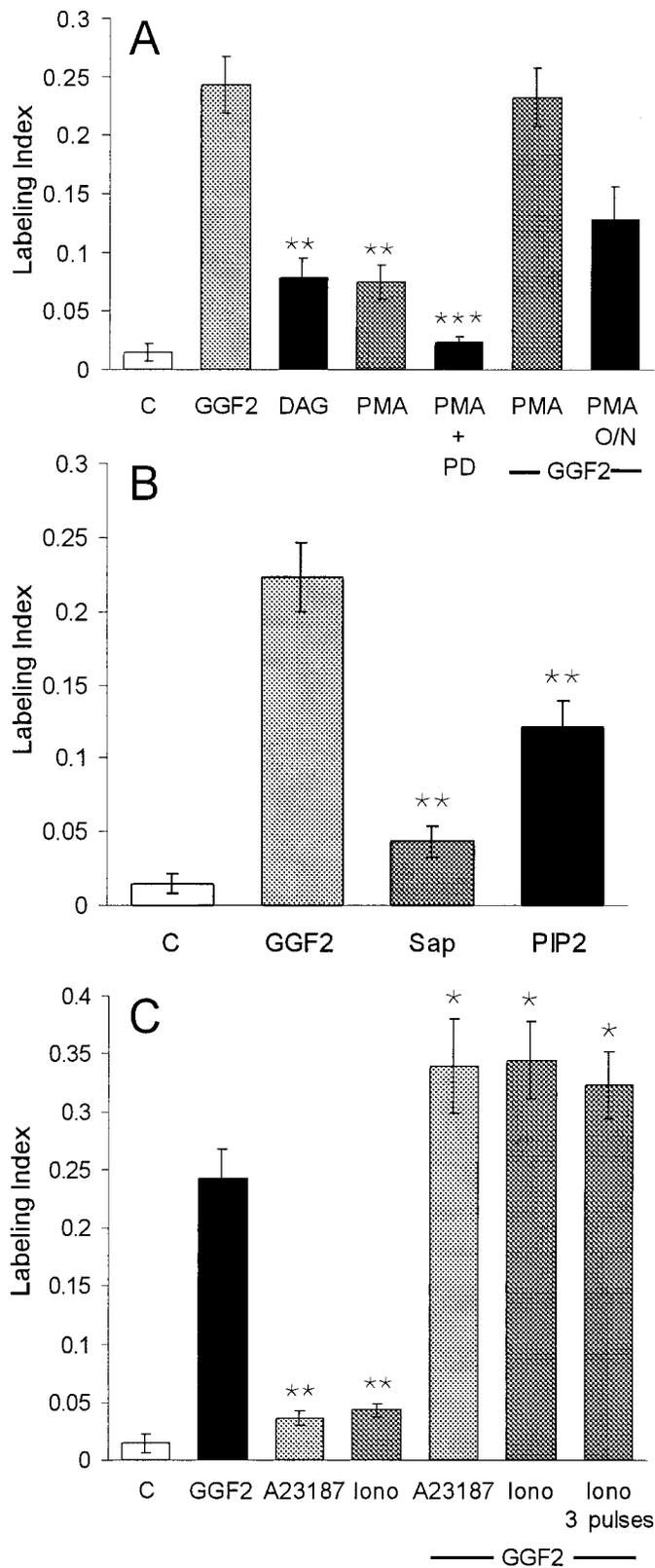


Figure 8. Brief treatments with activators for PKCs, PKB–Akt, and increased intracellular calcium induced S-phase entry in utricular epithelia. *A*, When pieces of sensory epithelium were treated for 15 min with either 10 μ M DAG or 5 μ M PMA and then cultured in the standard medium for 72 hr, S-phase entry increased to approximately five times the level seen in control cultures. To inhibit signaling via the ERK–MAPK pathway, other pieces of epithelium were pretreated for 60 min with 50 μ M PD98059 (PD) before the 15 min treatment with PMA. The medium

or ErbB4. The ErbB3 receptor can associate with the p85 regulatory subunit of PI-3K (Fedi et al., 1994; Kita et al., 1994; Prigent and Gullick, 1994). RT-PCR and immunohistochemistry have shown that ErbB2, ErbB3, and ErbB4 are all expressed in sensory epithelia from the utricles of neonatal rats (J. Corwin, Q. Shi, and T. Karaoli, unpublished observations).

PKC isoforms

PKCs are a large family of serine–threonine kinases composed of at least 11 isotypes that are classified into three groups (Nishizuka, 1995). cPKCs are activated by calcium and DAG, nPKCs are activated by DAG, and atypical PKCs (aPKCs) are not activated by calcium, DAG, or phorbol esters. BIM produced a modest but not significant mean reduction in the rhGGF2 response at 1 μ M, a concentration that inhibits only cPKCs (Martiny-Baron et al., 1993). However 2 μ M BIM, a concentration that blocks both cPKCs and nPKCs, significantly attenuated rhGGF2-induced S-phase entry. The involvement of nPKCs is also indicated by the strong inhibition in 1 μ M calphostin C, a concentration reported to inhibit cPKCs and nPKCs (Seynaeve et al., 1994). Calphostin C used at concentrations reported to block cPKCs specifically did not significantly inhibit the rhGGF2-induced S-phase entry (Tamaoki et al., 1990; Seynaeve et al., 1994).

Treatments with DAG or PMA in the absence of rhGGF2 resulted in a fivefold increase in S-phase entry compared with controls, providing additional evidence for the involvement of cPKCs or nPKCs. The MEK1 inhibitor PD98059 blocked the increase in S-phase entry that resulted from the direct activation of PKC by PMA (Fig. 8). This result suggests that signals downstream from PMA-activated PKCs are transmitted through the ERK–MAPK cascade, as has been reported in several other cell types (Cai et al., 1997; Schonwasser et al., 1998).

Prolonged treatment with the phorbol ester PMA can result in

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 was then changed to one containing 50 μ M PD98059 and BrdU for the remainder of the 72 hr culture period. Inhibition of the ERK–MAPK pathway significantly reduced the level of S-phase entry induced by the PMA treatment. Other pieces of epithelium were treated with 5 μ M PMA for 15 min and subsequently changed to a medium that contained rhGGF2 for 72 hr. The sequential treatment with PMA and rhGGF2 did not increase the level of S-phase entry over that induced by rhGGF2 alone. To downregulate PKC activity, other cultures were incubated with 5 μ M PMA for 16 hr [PMA overnight (O/N)] before rhGGF2 was added for the remaining 56 hr. This resulted in a 32% decrease in the level of labeling compared with that expected for 56 hr in rhGGF2 alone. *B*, Pieces of sensory epithelium were permeabilized by a 1 min treatment with 10 ng/ml saponin (Sap). Next, half of the pieces were changed to standard medium and cultured for 72 hr. The other half were changed to 2 μ M PI-3,4-P2 (PIP2) for 15 min and then changed into standard medium and cultured for 72 hr. Compared with controls, the brief treatment with PI-3,4-P2 resulted in a ninefold increase in S-phase entry measured over the subsequent 72 hr culture period in the presence of BrdU. This was ~50% of the level that was induced during 72 hr in the continuous presence of rhGGF2. *C*, When pieces of sensory epithelium were treated with either A23187 or ionomycin (Iono) at 100 nM for just 15 min and then cultured for 72 hr in the standard medium, a level of S-phase entry was induced that was 2.4 and 2.9 times the level that was measured in controls. When other pieces of epithelium were treated with A23187 for 15 min and then cultured for 72 hr with rhGGF2 and BrdU, a level of S-phase entry was observed that was 139% of the level induced by rhGGF2 (142% in the case of ionomycin). Treatments with three 15 min pulses of ionomycin (1 pulse per hour) followed by 72 hr in culture in the presence of rhGGF2 and BrdU did not produce an increase over the level measured for a single 15 min pulse. **Significance compared with control cultures ($p < 0.05$); ***Significance compared with cultures treated for 15 min with PMA alone ($p < 0.05$).

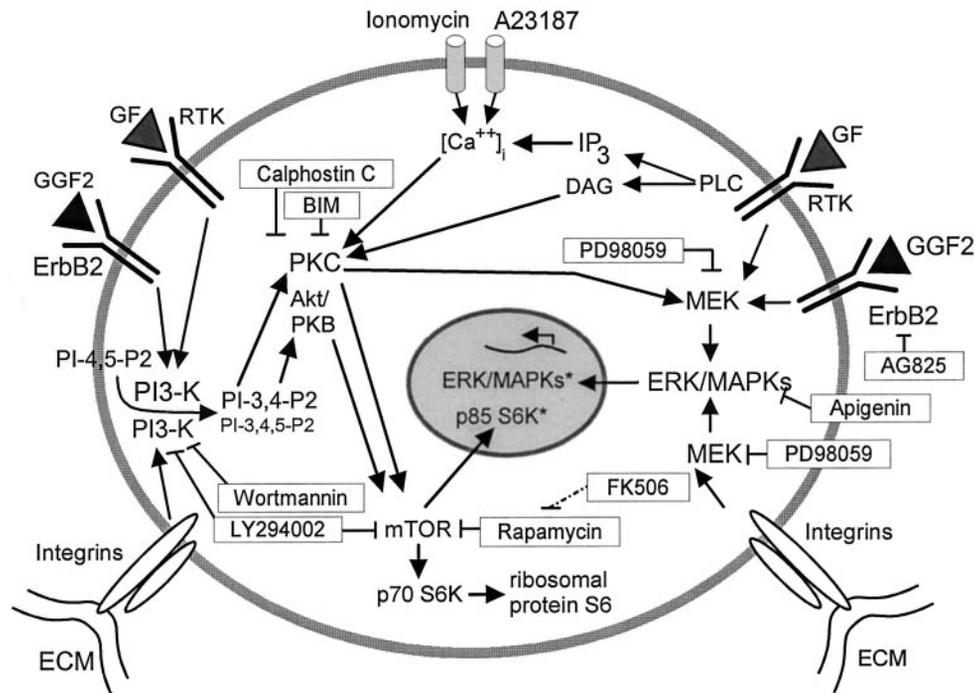


Figure 9. A schematic model of intracellular signal components that control S-phase entry in vestibular epithelia from neonatal mammals. Inhibitors and activators that were used to identify the elements in the signaling cascades are shown in boxes. Binding of GGF2 and other growth factors (GF) to receptor tyrosine kinases (RTK, ErbB2) leads to activation in both the PI-3K cascade and the MAPK cascade. Activated ERK–MAPKs can translocate to the nucleus, resulting in changes in gene expression. Active PI-3K leads to the production of PI-3,4-P2 and PI-3,4,5-P3, which can activate PKCs and Akt–PKB. Certain activated isoforms of PKC and Akt–PKB phosphorylate and activate mTOR, which leads to the activation of p70/85 S6Ks. When p70 S6K is active, it phosphorylates and activates the S6 ribosomal protein, which is required for the synthesis of proteins involved in S-phase entry. Activated p85 S6K is translocated to the nucleus and mediates cell-cycle progression. Phospholipase C (PLC) induces the production of DAG and IP₃. DAG can activate certain PKCs, which can activate the MAPK pathway. The production of IP₃ increases the release of intracellular calcium ([Ca²⁺]_i), which can also lead to the activation of PKCs. The anchorage-dependent proliferative response depends on the level of signals from growth factor receptors as well as anchorage of the cell to the extracellular matrix (ECM) via integrins. Bindings of integrins to specific components of the ECM can lead to the activation of the PI-3K cascade and the MAPK cascade. GGF2, Glial growth factor; ErbB2, receptor tyrosine kinase of the ErbB family; MEK, mitogen-activated protein kinase kinase; p70 S6K, the 70 kDa S6 protein kinase; p85 S6K, the 85 kDa S6 protein kinase; Akt–PKB, protein kinase B.

the degradation of PKCs (Ueda et al., 1996; Cai et al., 1997; Soltoff, 1998). Treatment of sensory epithelia for 16 hr with PMA resulted in partial inhibition of rhGGF2-induced S-phase entry (Fig. 8), as expected for a signaling pathway that depends in part on activation of PKCs. Combined stimulation with PMA and rhGGF2 did not produce an additive effect on S-phase entry, which suggests that DAG-responsive cPKCs or nPKCs may already have been activated in response to rhGGF2.

The observation that a 15 min treatment with PI-3,4-P2 significantly increases S-phase entry in the absence of rhGGF2 provides further evidence for a role for PKCs in the pathways that lead to proliferation of supporting cells. PI-3,4-P2 and PI-3,4,5-P3 are products of PI-3K that can activate certain nPKCs and aPKCs (for review, see Toker, 1998; Rameh and Cantley, 1999). PI-3,4-P2 can also activate Akt–PKB (Franke et al., 1997; Klippel et al., 1997), which has been reported to induce the activation of p70 S6K (Kohn et al., 1998).

The MAP kinase cascades

The MAPK family includes ERK-1 and ERK-2 (also known as p42/44 MAPK), JNKs, and p38-MAPK. The level of rhGGF2-induced S-phase entry was reduced by 34% by 20 μ M U0126, a MEK1 and MEK2 inhibitor, whereas 50 and 100 μ M of the MEK1 inhibitor PD98059 produced 39% and 53% reductions. These levels of S-phase inhibition are noticeably less than have been reported from studies of rat hepatocytes and mouse endothelial cells, in which 50 μ M PD98059 resulted in ~80% inhibition

of EGF-induced and serum-induced proliferation (Talarmin et al., 1999; Vinals et al., 1999). Both immunocytochemistry with a phosphospecific antibody and immunoblotting confirmed that 20 μ M U0126 and 100 μ M PD98059 inhibited 92–96% of the rhGGF2-induced phosphorylation of ERK-1 and ERK-2 in our cultures. A similar level of S-phase reduction occurred with apigenin, which has been reported to inhibit ERK-2 specifically. Although >92% of the phosphorylation of ERK-1 and ERK-2 was inhibited, 47% of cells continued to enter S-phase in the presence of rhGGF2, indicating that activation of the ERK–MAPK pathway is not critical for the proliferative responses of supporting cells. In other cell types, the effectiveness of the ERK–MAPK cascade can depend on activation of PI-3K (Duckworth and Cantley, 1997), and in some cells proliferative signaling depends on parallel activation of a PI-3K cascade and a ERK–MAPK cascade (Wennstrom and Downward, 1999).

Activation of the JNKs by anisomycin also resulted in a strong and significant inhibition of the rhGGF2 effect. A balance between the JNK and the ERK–MAPK pathways may play a role in determining whether cells in the vestibular sensory epithelium proliferate or die by apoptosis, as has been shown in other systems (Xia et al., 1995), but potential contributions of cell death have not been assessed here.

Calcium

Increased levels of intracellular Ca²⁺ have been reported to induce phosphorylation of the EGF receptor (Rosen and Green-

berg, 1996), and treatments with calcium ionophores have been shown to activate PKCs and the ERK–MAPK pathway (Lev et al., 1995; Daulhac et al., 1997; Romanelli and van de Werve, 1997). Other studies have shown that an ionomycin-induced increase in intracellular calcium levels is sufficient for full activation of p70 S6K (Graves et al., 1997; Conus et al., 1998). In our experiments, 15 min exposures to different calcium ionophores increased S-phase entry significantly in the absence of rhGGF2, and combined treatments with rhGGF2 and each ionophore produced additive effects. The results are consistent with the possibility that the signaling pathways activated by intracellular calcium intersect with those that are also activated in response to rhGGF2, and may also function in parallel.

In summary, this investigation has shown that intracellular signals that influence the triggering of S-phase entry in vestibular epithelia from neonatal rodents are components of two established cascades (Fig. 9). One cascade appears to involve activation of PI-3K, PKCs or Akt–PKB, mTOR, and presumably S6Ks. This cascade is critical for S-phase entry of mammalian supporting cells and showed the greatest sensitivity to inhibitors. The other pathway involves PKCs and the ERK–MAPK cascade. That pathway appears to have a supporting or permissive role that is less critical for the proliferation of cells in mammalian vestibular sensory epithelia. Future investigations should lead to the identification of the specific isoforms of the kinases that function in the proliferation cascades in these cells. The identification of those isoforms ultimately holds the potential to define drug targets suitable for modulation of progenitor cell proliferation and the capacity for regenerative replacement of sensory hair cells in mammalian ears.

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