

Regeneration in avian hair cell epithelia: identification of intracellular signals required for S-phase entry

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Abstract

Balance epithelia in birds closely resemble their mammalian counterparts, but their cells turnover rapidly and they quickly regenerate hair cells, leading to functional recovery from damage that would be permanent for a mammal. We isolated and cultured sheets of the chicken's utricular epithelium in bromo-deoxyuridine and specific inhibitors of different intracellular signalling pathways to identify signals that influence turnover and regeneration. Synthesis (S-phase) entry was effectively blocked by inhibition of PI3-K, TOR or MAPK, and significantly decreased by inhibitors of PKC. Comparisons indicate that activated PI3-K and TOR are required for S-phase entry in both avian and mammalian balance epithelia, but activation of the MAPK pathway appears to have a more significant role in avian utricles than in mammals. The dissimilarities in the requirements for these signalling pathways do not appear sufficient to explain the marked difference in regenerative capacity between the ears of birds and mammals.

Introduction

Hair cells reside in eight neuroepithelia in the inner ears of birds and seven in mammals. Hair cells in the cochlea provide hearing, while those in the three semicircular canals, the saccule, the utricle, elagena, and the macula neglecta comprise the vestibular portion of the ear and provide sensitivity to rotational and linear acceleration of the head (Lewis *et al.*, 1985). In mammals, all cochlear hair cells and virtually all vestibular hair cells are produced before birth (Ruben, 1967), and clinical experience supports the view that those hair cells are not replaced if lost. Permanent deficits in hearing commonly referred to as 'nerve deafness' are most often caused by loss of hair cells, which may result from infection, acoustic trauma, aminoglycoside antibiotic exposure and ageing (Nadol, 1993). Deficits in balance sensitivity can arise from hair cell loss in the vestibular epithelia, and contribute to falls and hip fractures (Kennedy & Clemis, 1990).

Mammals are uniquely vulnerable to permanent deficits caused by hair cell losses. Most other vertebrates are able to produce hair cells postembryonically during the continuous growth of their hair cell epithelia or through regenerative replacement that depends on the proliferation of neighbouring supporting cells and the differentiation of some of their progeny as hair cells (for review, see Corwin & Oberholtzer, 1997). Birds regenerate hair cells in the cochlea when pre-existing hair cells have died and that regeneration leads to functional recovery from hearing deficits that would be permanent for a mammal. Avian vestibular epithelia also regenerate hair cells after

damage and they recover balance sensitivity. Those processes occur via upregulation of ongoing proliferation in their supporting cells (Jørgensen & Mathiesen, 1988; Jones & Nelson, 1992; Roberson *et al.*, 1992; Weisleder & Rubel, 1992, 1993; Kil *et al.*, 1997).

Enzymes of signal transduction pathways are often targets for small molecules that have therapeutic usefulness (Brunton & Workman, 1993; Powis, 1994; Umezawa, 1995; Hamby & Showalter, 1999), so there is considerable interest in determining what signal transduction pathways underlie the initial proliferation that leads to regeneration of hair cells in fish, amphibians and birds. Signal transduction pathways for cell proliferation in the avian ear are largely unknown, but Navaratnam *et al.* (1996) found that treatments that raised intracellular cyclic adenosine monophosphate (cAMP) levels increased proliferation in the avian cochlea. To survey other pathways that may contribute to proliferation in avian ears, we cultured pieces of the utricular sensory epithelium in the presence of pharmacological inhibitors of specific signal transduction enzymes selected because of their participation in mitogenic responses in other cell types. The results indicate that phosphatidylinositol 3-kinase (PI3-K), the target of rapamycin (TOR), the mitogen-activated protein kinase pathway (MAPK), and some protein kinase Cs (PKCs) participate in the induction of synthesis (S-phase) entry in cultured vestibular epithelia from chickens, largely paralleling results in rodents (Montcouquiol & Corwin, 2001).

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Materials and methods

Epithelial cell cultures

Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals using a protocol approved and supervised by the University of Virginia Animal Care Advisory

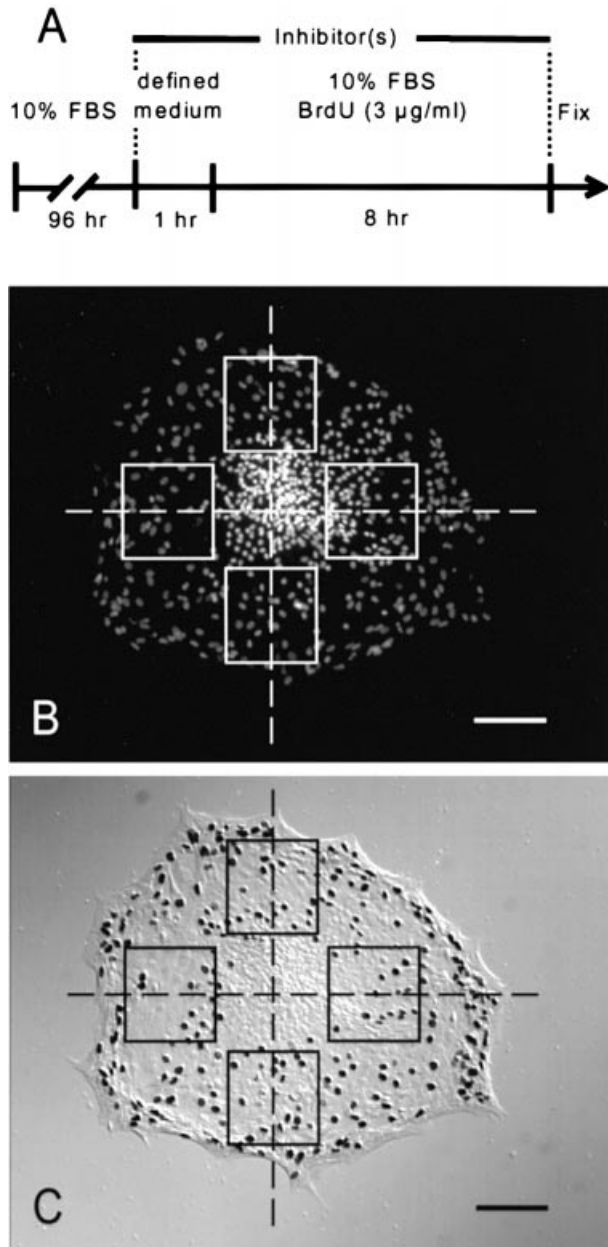


FIG. 1. Epifluorescence and differential interference contrast (DIC) micrographs illustrating the labelling and data collection methods. (A) Time-line used in this study. (B) An epifluorescence image of a sheet of utricular sensory epithelium that was cultured in medium 199 with 10% FBS for 4 days prior to fixation. Staining with DAPI labelled all the cell nuclei that did not take up BrdU. Note the dense packing of the nonproliferative nuclei in the centre of the sheet of epithelium. (C) DIC image of the same sheet of epithelium. BrdU-labelled nuclei are black. Counts were made by randomly orientating the epithelial sheet under the microscope then capturing the images. Four $135 \mu\text{m}^2$ counting regions (shown as boxes) were loaded onto the images and placed along 0° and 90° axes (dashed lines) passing through the centre of the epithelial sheet. The regions were then moved along the axes until their edges just contacted the central nonproliferative zone, then counts were made. Scale bars, $100 \mu\text{m}$.

Committee. For this investigation, 72 white leghorn chickens (*Gallus domesticus*) that were 1–7 days old were anaesthetized with CO_2 and decapitated. The skin and the mandibles were removed and the heads were immersed in chilled 70% ethanol for 15 min. Then the lateral squamous portion of the temporal bone was removed with a sterile

razor blade. The utricles were dissected from the membranous labyrinth and transferred to a chilled solution of medium 199 with Hanks' salts (M199-H), and the utricle's roof and otolithic membrane were removed. Then the utricles were incubated in thermolysin (protease type X, Sigma, St Louis, MO, USA) at 0.5 mg/mL in Hanks' balanced salt solution containing 25 mM HEPES at pH 7.4 in a 5% CO_2 incubator at 37 °C for 45 min (Saffer *et al.*, 1996). The utricles were then transferred to M199-H, which contained 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA) added to retard further enzymatic digestion. The sensory epithelium was carefully dissected free from the underlying connective tissue using fine forceps. The epithelial edges were trimmed away with a diamond microscalpel and discarded to remove all traces of the surrounding nonsensory epithelium. The remaining sheet of pure sensory epithelium was cut into pieces that measured $\approx 0.25 \text{ mm}^2$ each. Eight to 12 pieces were transferred in M199 with Earle's salts (M199-E) and 10% FBS to the glass-bottom well of a Mat-Tek culture dish that had been coated by overnight exposure to 0.1 mg/mL fibronectin (Sigma). A needle was used to orientate the pieces so that their hair bundles were up, then they were allowed to attach and grow undisturbed for 96 h in a 5% CO_2 atmosphere at 37 °C.

After the 96-h growth period the standard tests began with a 1-h preincubation with the inhibitor in M199-E supplemented with 50 $\mu\text{g/mL}$ holo-transferrin, 5 ng/mL sodium selenite and 1 ng/mL bovine insulin (Sigma). Then the medium was replaced with M199-E containing 10% FBS, the inhibitor and 5-bromo-2-deoxyuridine (BrdU) at 3 $\mu\text{g/mL}$ to label DNA during the synthesis (S-phase) of the cell cycle. After 8 h of incubation in the BrdU-containing medium the cultures were fixed with 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) at pH 7.4. The protocol allowed inhibitors to act on their target enzymes in a relatively nutrient-depleted, defined medium for 1 h prior to the test of the inhibitor's effect in a medium that contained 10% FBS to again prompt cell proliferation.

This experimental time-line was modified when FK506 was used to test the specificity of the inhibition by rapamycin. In that experiment the cultures were preincubated with FK506 in the defined medium for 1 h prior to the addition of rapamycin. This step was followed by an additional 1-h preincubation in defined medium containing both FK506 and rapamycin. Then the cultures were incubated for 8 h in medium containing FK506 and rapamycin with 3 $\mu\text{g/mL}$ BrdU and 10% FBS.

One further modification of the experimental time-line was employed when a three-inhibitor combination (PD 98059, rapamycin and LY294002) was used in a prolonged preincubation protocol that preceded the addition of BrdU. In this experiment an additional 12-h preincubation period was added prior to the standard 1-h preincubation in defined medium. In order to avoid deleterious effects caused by abrupt and prolonged withdrawal of the nutrient growth medium, the medium used for the 12-h preincubation contained 10% FBS. The remainder of the procedure followed the standard experimental time-line.

The inhibitors and the concentrations used were LY294002 at 30 μM , wortmannin at 25 nM, rapamycin at 20 nM, FK506 at 12 μM , calphostin C at 0.5 μM , bisindolylmaleimide I (BIM) at 1 μM AND 2 μM , Gö6983 at 100 nM, PD98059 at 50 μM AND 100 μM , U0126 at 10 μM AND 20 μM , and apigenin at 13 μM AND 39 μM . The concentrations chosen for the inhibitors used were based on previous studies and in particular a study of utricle epithelia from rats that used a similar culture technique (Montcouquiol & Corwin, 2001). The FK506 was from Fujisawa (Deerfield, IL, USA). The PD98059 was

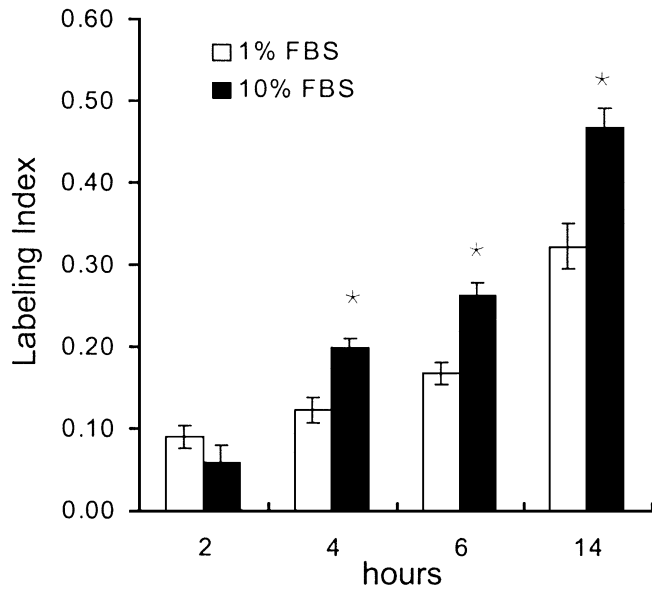


FIG. 2. The concentration of serum in the culture medium had a small but consistent effect on the level of S-phase entry in the chicken utricular epithelial cultures over time. Cultures were grown for 96 h in medium containing 10% FBS, then they were cultured in media that contained BrdU for the indicated times prior to fixation and counting. After 4 h in BrdU-containing medium, the labelling indices for cultures in 10% FBS were significantly higher than for those in 1% FBS. The differences were consistent and significant after 4, 6 and 14 h ($P < 0.05$).

from New England Biolabs (Beverly, MA, USA). All other inhibitors were purchased from Calbiochem (La Jolla, CA, USA).

During each experiment, 10–15 control cultures were run along with the experiments. The control data from all the experiments were pooled and are presented in several of the histograms from the individual experiments.

Bromodeoxyuridine labelling

Immunohistochemical labelling was performed at room temperature. The fixed epithelia were rinsed twice with PBS and incubated in 1 N HCl for 15 min to denature the DNA. After three PBS rinses, they were incubated for 30 min in a blocking solution containing 10% normal horse serum (NHS) and 0.2% Triton X-100 in PBS. Then they were incubated for 1 h with a mouse monoclonal antibody to BrdU (Becton-Dickinson, San Jose, CA, USA), that was diluted 1 : 50 in PBS containing 1% NHS and 0.2% Triton X-100. After three rinses in PBS, they were incubated in a biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 100 in PBS containing 0.2% Triton X-100. Then they were rinsed twice in PBS and incubated for 30 min in avidin-biotin solution (Vectastain ABC, Vector Laboratories) in PBS with 0.1% Triton X-100. The DAB reaction product was developed with nickel intensification, and the nuclei were counterstained in 10 $\mu\text{g}/\text{mL}$ DAPI (Molecular Probes, Eugene, OR, USA) in PBS and 0.1% Triton X-100 for 30 min.

Data analysis

Differential interference contrast microscopy (DIC) was used to image BrdU-labelled nuclei and epifluorescence microscopy was used to image DAPI-stained nuclei that had not incorporated BrdU. For a given field of view, aligned DIC and epifluorescent microscope images were captured sequentially via a cooled CCD camera

(Princeton Instruments, Monmouth Junction, NJ, USA) that was controlled by MetaMorph software (Universal Imaging Inc., Media, PA, USA). The software was used to count all the BrdU-labelled nuclei and all the DAPI-labelled nuclei in four 135 μm^2 regions in each piece of epithelium. The first two regions were positioned along a line passing through the centre of the piece of epithelium at a randomly chosen angle defined as the 0° axis. The two other regions were on a line that ran 90° to that axis. The 0° and 90° lines both passed through the centre of the piece of epithelium. The exact locations of the four regions on those lines were adjusted so that they did not overlap the nonproliferative central zone in each piece of epithelium (Fig. 1). The software automatically determined the centroid of each nucleus, and only those nuclei with centroids within the designated region were counted. For each inhibitor and condition investigated, 20–32 pieces of epithelia were analysed by counting the labelled and unlabelled nuclei in four regions per piece, as described earlier. In total, 458 pieces of utricular sensory epithelium were cultured and analysed.

The proliferative index was calculated for each region by dividing the number of BrdU-labelled nuclei by the total number of nuclei in that region. Data are reported as the average proliferative index, plus and minus the standard error of the mean (SEM). Significance was determined via Students' two-tailed *t*-test.

Results

The pieces of sensory epithelia grew in culture as rounded sheets of contiguous epithelial cells that spread from columnar to squamous polygonal shapes. The cultures appeared healthy after culturing in the presence of all the tested inhibitors over the range of concentrations reported here. Cells in the centre of the pieces did not spread but instead retained a columnar shape. As a result, the central regions were noticeably thicker than the surrounding regions, and their DAPI-labelled nuclei had smaller profiles in the XY plane of view than the nuclei of cells in the periphery. The incidence of BrdU-labelling was markedly lower in the central regions than in the peripheral regions. For that reason, the data collection protocol was designed to exclude the central region of each piece, as described earlier and illustrated in Fig. 1.

S-phase induction by serum

When the sheets of epithelium were cultured in M199-E and 10% FBS for 96 h, then in the same medium with BrdU for 8 h, 34.2 \pm 1.1% of the cell nuclei incorporated BrdU. Cultures labelled this way in the absence of inhibitor were designated as 'controls'. An analysis of 10 pieces of epithelia cultured under these conditions showed that only 2.1% of the BrdU-labelled nuclei were in mitosis at the time of fixation (data not shown).

A pilot study measured the increase in the labelling index that occurred with progressively longer times of incubation with BrdU, in the presence of 1% and 10% FBS (Fig. 2). At the shortest time (2 h), the differences between the samples in 1% and 10% FBS were not significant, but at longer times, the incidence of BrdU labelling was 8–15% lower in the pieces cultured in 1% FBS than in those cultured in 10% FBS. The labelling index increased progressively from 2 to 14 h in BrdU. Signalling inhibitors were tested in 10% FBS during 8 h in the presence of BrdU.

Inhibition of PI3-K

Pieces of epithelia ($n = 21$) were incubated for 96 h in M-199-E containing 10% FBS and then treated for 1 h in defined medium

TABLE 1. Comparison of inhibition of chick and mammalian inner ear epithelial cultures

Target	Labelling index (%)	Labelling index (%) minus 13%	Inhibition (%) compared with control in chicks	Inhibition (%) compared with control in mammals
Control	34.2	21.2		
LY294002 (30 μ M)	16.7	3.7	82.50	98
Wortmannin	19.5	6.5	(25 nM) 69.30	(10 nM) 45.8
Rapamycin (20 nM)	18.5	5.5	74	60
Calphostin (0.5 μ M)	15.4	2.4	88.60	50
BIM (1 μ M)	30.9	17.9	15.50	21
BIM (2 μ M)	23.9	10.9	48.50	60
Go6983 (100 nM)	25.4	12.4	41.50	
PD98059 (50 μ M)	21.7	8.7	58.90	39
PD98059 (100 μ M)	17.0	4.0	81.10	53
U0126 (10 μ M)	24.8	11.8	44.30	21
U0126 (20 μ M)	20.8	7.8	63.20	34
Apigenin (13 μ M)	23.2	10.2	51.80	56
Apigenin (39 μ M)	17.0	4.0	81.10	
PD98059 (50 μ M) + rapamycin (20 nM)	15.1	2.1	90.10	
PD98059 (50 μ M) + rapamycin (20 nM) + LY204002 (30 μ M)	13.1	0.1	99.50	

Under the standard experimental protocol, the maximum degree of inhibition that could be obtained resulted in a 13.1% labelling index. Further modification of the protocol (the addition of 12 h to the standard preincubation) resulted in a 0.1% labelling index for the maximum degree of inhibition, a situation similar to that found in the mammalian model. To facilitate interspecies comparison, 13% was subtracted from the raw data obtained under the standard protocol. To generate the percentage inhibition vs. control, these adjusted labelling indices were then divided by the adjusted control labelling index (21.2%). The last two columns of the table compare values obtained in this experiment with that obtained under identical experimental conditions using rat epithelium as detailed in Montcouquiol & Corwin (2001). Note similarly pronounced inhibitory effects in both species with high dose PI3-K and TOR inhibition; the effect is less pronounced for most inhibitors of the MAPK cascade. In the chick study, 25 nM wortmannin was used, and in the mammalian study, 10 nM.

containing the PI3-K inhibitor LY294002 (Sanchez-Margalet *et al.*, 1994; Vlahos *et al.*, 1994). At this point, the medium was replaced by medium containing LY294002, 10% FBS and BrdU, and incubation continued for 8 h before fixation. Treatment with other inhibitors, unless otherwise specified, followed this same experimental time-line (Fig. 1). LY294002 was used at 30 μ M, a dose that has been shown to inhibit 98% of S-phase entry in epithelia from mammalian utricles (Montcouquiol & Corwin, 2001; Table 1). The treatment with LY294002 reduced the level of BrdU labelling by \approx 51% in the chick epithelium. Only $16.7 \pm 1\%$ of the cells incorporated BrdU (Fig. 3).

Wortmannin was tested at 25 nM, a concentration that specifically inhibits PI3-K (Stephens *et al.*, 1993; Okada *et al.*, 1994). Pieces of epithelia ($n = 29$) were incubated in defined medium containing 25 nM wortmannin and BrdU. Labelling was reduced by 43% relative to control. Only $19.5 \pm 0.9\%$ of the nuclei were labelled with BrdU in the presence of wortmannin. The decreases in BrdU-labelling that were observed after LY294002 and wortmannin inhibition were significant ($P < 0.05$).

LY294002 has been reported to inhibit mammalian TOR (mTOR) at 30 μ M, but wortmannin inhibits PI3-K at concentrations that are 1/100th of those required to inhibit mTOR (Brunn *et al.*, 1996). In our assay, wortmannin was an effective inhibitor at 1/10th of the estimated IC_{50} for its inhibition of mTOR, suggesting that the reduction in S-phase observed with a 25-nM treatment with wortmannin was largely the result of PI3-K inhibition. The treatments with LY294002 resulted in slightly more inhibition than the treatment with wortmannin, but the differences were not statistically significant. Such a difference might be explained by the possibility that LY294002 could have inhibited both PI3-K and TOR. In addition, wortmannin is unstable at 37 $^{\circ}$ C (Kimura *et al.*, 1994) and can lose potency in culture after 4 h (Parrizas *et al.*, 1997).

Inhibition of TOR

Following the standard protocol described earlier, 23 pieces of utricular epithelium were exposed to rapamycin at 20 nM to inhibit the kinase activity of TOR (Chung *et al.*, 1992; Brown *et al.*, 1994). This resulted in the labelling of $18.5 \pm 1\%$ of the cells, a 46% decrease from the labelling in the control medium ($P < 0.05$, Fig. 4). To assess the specificity of inhibition, rapamycin was added together with FK506, a compound that competes with rapamycin for the same binding site (Abraham & Wiederrecht, 1996). In this experiment, 20 pieces of epithelia were incubated for 1 h with 12 μ M FK506, followed by another 1 h incubation in both 12 μ M FK506 and 20 nM rapamycin. Then they were cultured for 8 h in medium containing BrdU, together with both FK506 and rapamycin at the above concentrations. This resulted in BrdU-labelling of $26.5 \pm 1.2\%$ of the epithelial cells, a partial but significant ($P < 0.05$) reversal of the antiproliferative effect of rapamycin.

Inhibition of protein kinase C (PKC)

Calphostin C inhibits classical and novel PKCs by acting on the diacylglycerol (DAG) binding site (Gordge & Ryves, 1994; Seynaeve *et al.*, 1994). Under the standard protocol, pieces of epithelium ($n = 28$) were exposed to 0.5 μ M calphostin C resulting in a 55% decrease relative to control. Only $15.4 \pm 0.9\%$ incorporated BrdU (Fig. 5). In early experiments, it was found that doubling the concentration of calphostin C to 1 μ M was toxic. The sheets of epithelium ($n = 14$) in those cultures fragmented and completely detached from the substrate. Additional tests using 0.75 μ M calphostin C resulted in patchy loss of cells within the epithelial pieces, with shrinkage and substrate detachment along the edges of the sheets (data not shown). The toxicity of calphostin C has been observed in other systems (Ikemoto *et al.*, 1995; Savickiene *et al.*, 1999).

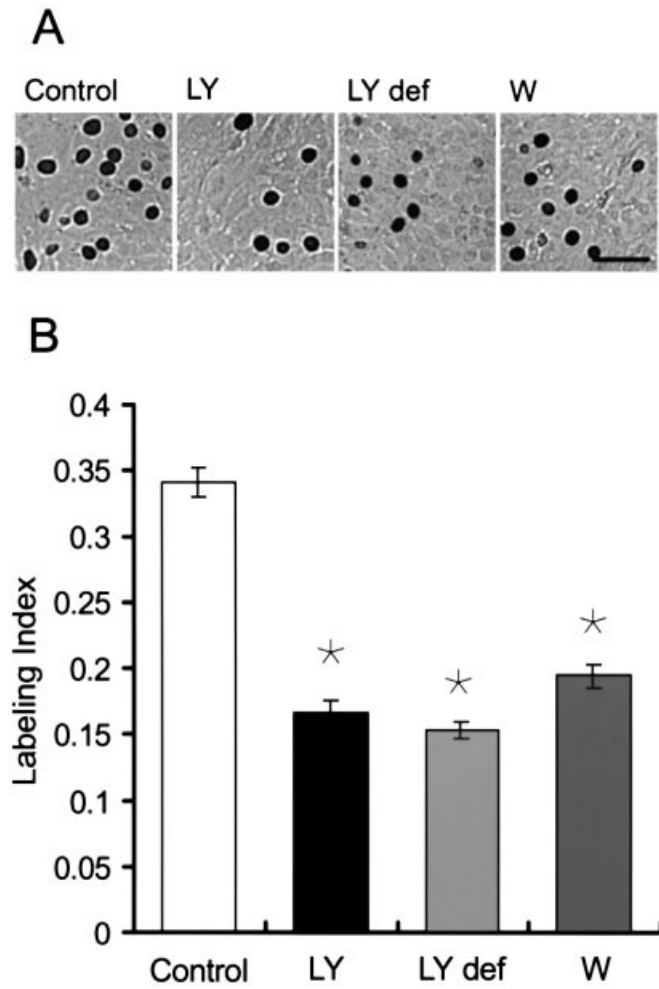


FIG. 3. Inhibitors of PI3-K reduced the level of S-phase entry significantly. (A) Representative $135 \mu\text{m}^2$ counting regions of chick utricular epithelium exposed to 10% FBS (control), $30 \mu\text{M}$ LY294002 together with 10% FBS (LY), $30 \mu\text{M}$ LY294002 in serum-free, defined medium (LY def) and 25 nM wortmannin (W) together with 10% FBS. (B) Histograms showing the proliferative index for control epithelium maintained in 10% FBS and in parallel cultures that were continuously exposed to the PI3-K inhibitors LY294002 and wortmannin. Note the marked reduction in the proliferative index with exposure to $30 \mu\text{M}$ LY294002. Incubation with LY294002 in serum-free medium causes a similar fall in the proliferative index, demonstrating that lack of 10% FBS in the medium has little additive effect on inhibition. Incubation with 25 nM wortmannin, another PI3-K inhibitor, causes a similar degree of inhibition of BrdU labelling. The asterisk in this and subsequent figures denotes significance compared with control ($P < 0.05$). Scale bars, $50 \mu\text{m}$.

Other tests for the effects of PKC inhibition were performed using BIM and Gö6983, which act at the ATP-binding site of PKC (Martiny-Baron *et al.*, 1993; Gordge & Ryves, 1994; Stempka *et al.*, 1997). Pieces of epithelium ($n = 23$) were tested in medium containing $1 \mu\text{M}$ BIM, resulting in a modest decrease in the average level of BrdU labelling. Incubation of 20 pieces in the medium containing $2 \mu\text{M}$ BIM resulted in $23.9 \pm 1.69\%$ BrdU labelling, a moderate but significant reduction from the control level ($P < 0.05$). Testing of sensory epithelium ($n = 24$ pieces) with 100 nM Gö6983 resulted in $25.4 \pm 0.1\%$ labelling, a 26% decrease from control levels ($P < 0.05$).

Inhibitors of MAPK

In epithelia from mammalian utricles $100 \mu\text{M}$ PD98059 and $20 \mu\text{M}$ U0126 each inhibit over 90% of the phosphorylation of p44 and p42

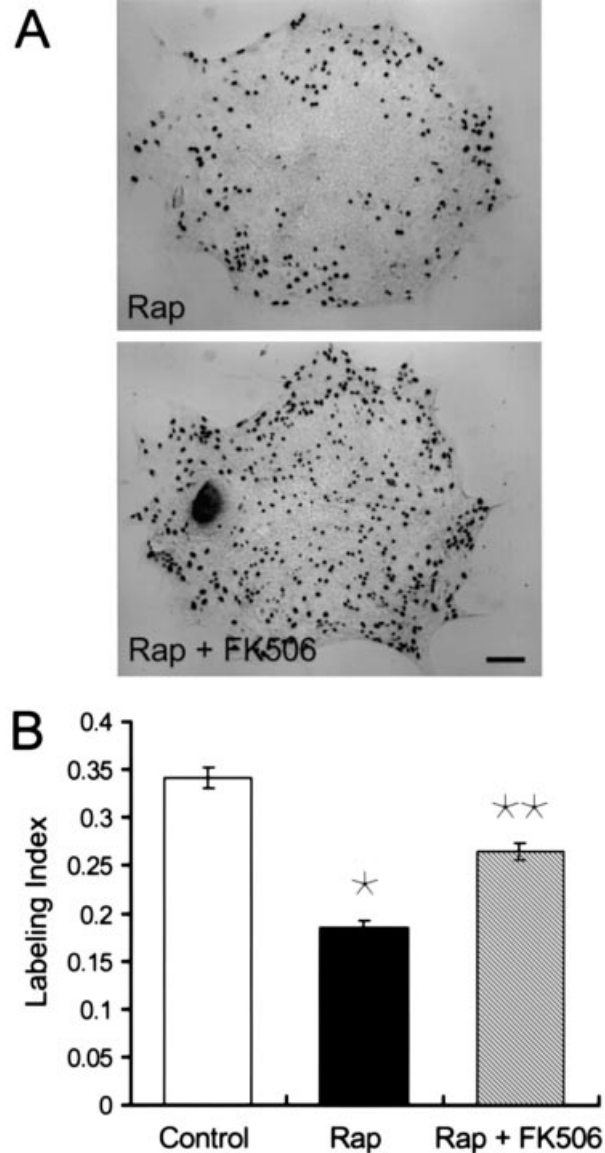


FIG. 4. The TOR inhibitor rapamycin markedly reduced the level of S-phase entry and this effect was reduced by coinubation with a specific competitive inhibitor, FK506. (A) Representative pieces of chicken utricular epithelium that were treated with 20 nM rapamycin (Rap) and 20 nM rapamycin plus $12 \mu\text{M}$ FK506 (Rap + FK506). (B) A marked reduction in the proliferative index was measured after incubation with rapamycin (19% vs. 34% in control cultures). The antiproliferative effect of rapamycin was attenuated partially by simultaneous incubation with FK506, following a 1-h preincubation with FK506 alone. * $P < 0.05$, compared with control. ** $P < 0.05$, compared with Rap. Scale bars, $100 \mu\text{m}$.

MAPK (ERK1 and ERK2) (Montcouquiol & Corwin, 2001). In the chick's epithelium treatment with the MEK1 inhibitor PD98059 (Alessi *et al.*, 1995; Dudley *et al.*, 1995) reduced the incidence of S-phase entry in a dose-dependent manner (Fig. 6). When pieces of epithelium ($n = 22$) were incubated with $50 \mu\text{M}$ PD98059, $21.7 \pm 0.1\%$ of the cells were labelled by BrdU. In 21 pieces cultured with $100 \mu\text{M}$ PD98059, $17.0 \pm 0.8\%$ were labelled, a 50% decrease from the level in control cultures. Both decreases were significant ($P < 0.05$).

Another inhibitor of the MAPK pathway, U0126, interacts with MEK1 and MEK2 with nearly equal affinity (Favata *et al.*, 1998;

Kanterewicz *et al.*, 2000). When pieces of epithelium ($n = 21$) were cultured with $10 \mu\text{M}$ U0126, $24.8 \pm 0.1\%$ of the cells were labelled, a moderate but significant reduction ($P < 0.05$; Fig. 6). In pieces of epithelium ($n = 24$) cultured with U0126 at $20 \mu\text{M}$, $20.8 \pm 0.1\%$ of the cells were labelled with BrdU, which was also a significant decrease from control levels ($P < 0.05$).

Apigenin is a plant flavonoid that has been shown to inhibit the MAPK cascade by dephosphorylation of ERK1 (Kuo & Yang, 1995; Grewal *et al.*, 1999). When pieces of epithelium ($n = 21$) were incubated with $13 \mu\text{M}$ apigenin, $23.2 \pm 1.3\%$ of the cells were labelled. Culturing of pieces of epithelium ($n = 23$) with $39 \mu\text{M}$ apigenin resulted in $17.0 \pm 0.7\%$ labelling with BrdU. Both results were significantly different from the control cultures ($P < 0.05$).

Effects of combinations of inhibitors

Pieces of sensory epithelium ($n = 21$) were exposed to medium that contained a combination of the inhibitors PD98059 ($50 \mu\text{M}$), LY294002 ($30 \mu\text{M}$), and rapamycin (20 nM), following the standard protocol described earlier (Fig. 7). This combination of inhibitors resulted in the labelling of $13.1 \pm 0.8\%$ of the cell nuclei (Fig. 8). To

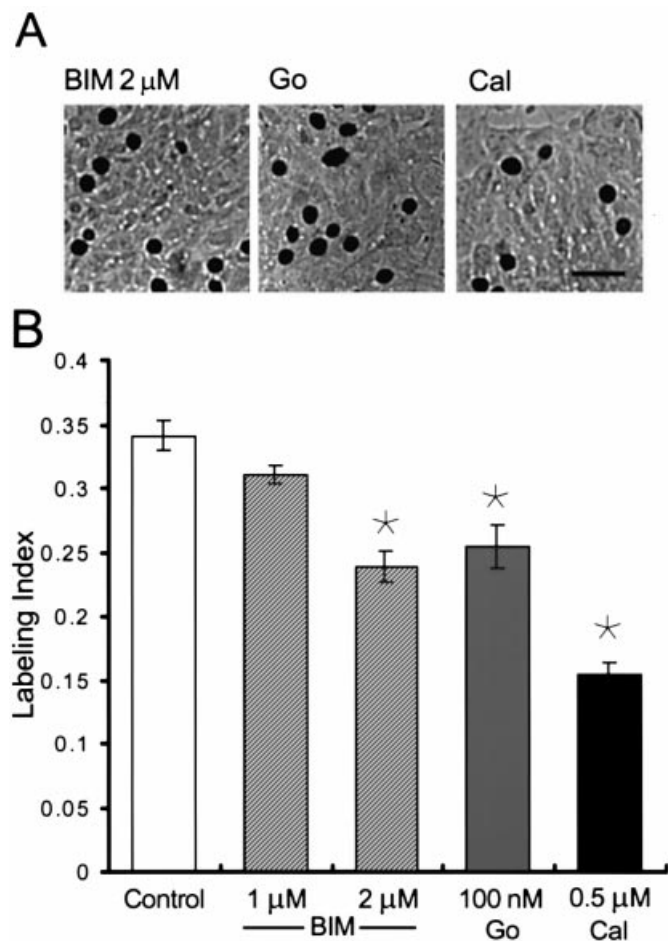


FIG. 5. PKC inhibition also significantly reduces S-phase labelling in the sensory epithelium. Histograms of the proliferative index in controls and in cultures exposed to the PKC inhibitors bisindolylmaleimide I (BIM), Gö6983 (Go), and calphostin C (Cal). Note the stepwise reduction in the proliferative indices that were measured after increasing concentrations of BIM. There was a marked reduction in BrdU labelling following incubation with $0.5 \mu\text{M}$ calphostin C, and a significant reduction with exposure to Gö6983.

assess the possible dependence of the effect of LY294002 on concurrent downstream inhibition of TOR and/or MEK, an identical

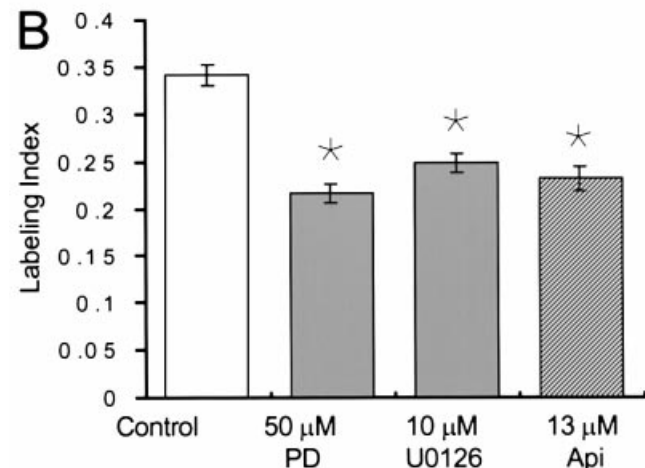
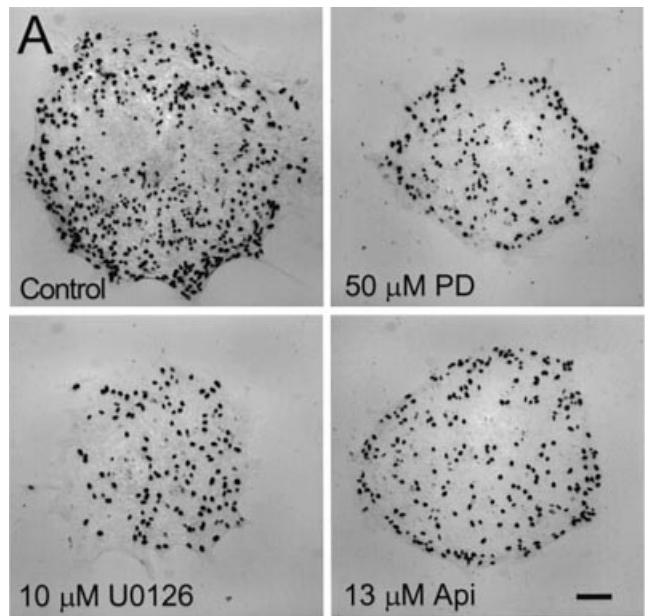


Table 6C: Summary of Inhibitory Effects

Treatment	n	mean	±SEM
Control	32	0.341	0.011
PD98059 50 μM	22	0.217	0.010
PD98059 100 μM	21	0.169	0.008
U0126 10 μM	21	0.248	0.010
U0126 20 μM	24	0.208	0.010
Apigenin 13 μM	21	0.232	0.013
Apigenin 39 μM	23	0.170	0.007

FIG. 6. The effects of three inhibitors of intermediates in the MAP kinase pathway. (A) Pieces of chick utricular epithelium exposed to the standard medium (control), $50 \mu\text{M}$ PD98059 (PD), $10 \mu\text{M}$ U0126 (U0126) and $13 \mu\text{M}$ apigenin (Api). (B) Both PD98059 and U0126, which inhibit at the level of MEK, caused significant dose-dependent inhibition of S-phase entry. Apigenin, an ERK inhibitor, also caused dose-dependent reductions, with a particularly marked reduction at $39 \mu\text{M}$. (C) Doubling the concentration of PD98059, U0126 and apigenin increased the inhibitory effects. Scale bars, $100 \mu\text{m}$.

experiment was performed using only PD98059 and rapamycin. When 25 pieces of epithelia were incubated with 50 μM PD98059 and 20 nM rapamycin, $15.1 \pm 0.7\%$ of the cell nuclei were labelled with BrdU (Fig. 7). The incidence of labelling decreased by 62% in the three-inhibitor experiment and by 56% in the two-inhibitor experiment, as compared with control cultures; the difference between the three-inhibitor and the two-inhibitor results was significant ($P < 0.05$).

We suspected that some of the cells that incorporated BrdU in the presence of the combined inhibitors may have progressed past the G_1

restriction point of the cell cycle before the preincubation in the inhibitors, but had not completed S-phase prior to the onset of exposure to BrdU 60 min later. We tested that hypothesis by increasing the duration of the preincubation in the three-inhibitor medium. Pieces of utricular epithelium ($n = 10$) were preincubated for 12 h with the three-inhibitor combination described earlier, followed by the standard 1-h preincubation, and the 8-h BrdU labelling period. This protocol resulted in a nearly complete block of S-phase entry, with only $0.03 \pm 0.1\%$ of the cells labelled. This is a 99.5% decrease from the incidence of labelling in the control cultures (Figs 7 and 8).

Cytotoxicity assessment

Because 0.75 and 1 μM calphostin C had such a dramatic impact on cell viability, it was deemed important to assess whether the other inhibitors had toxic effects on the cells in our system.

Cytotoxicity can manifest with features of cell necrosis and/or apoptosis (Berke, 1991; Krähenbühl & Tschopp, 1991). Cells

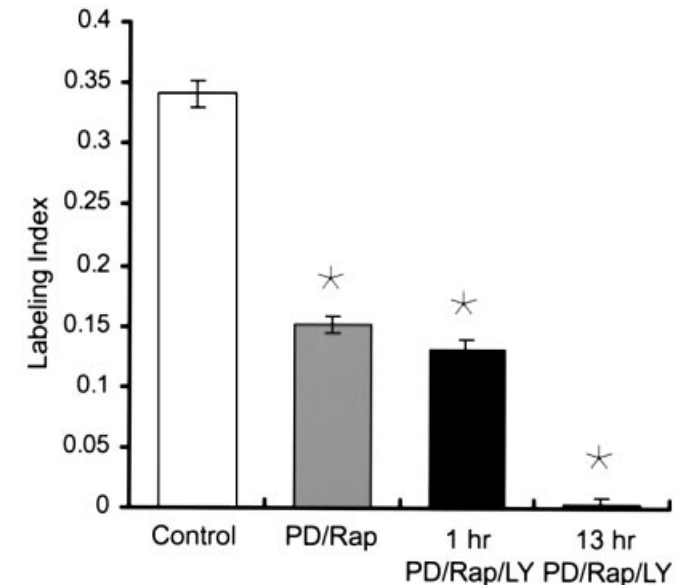
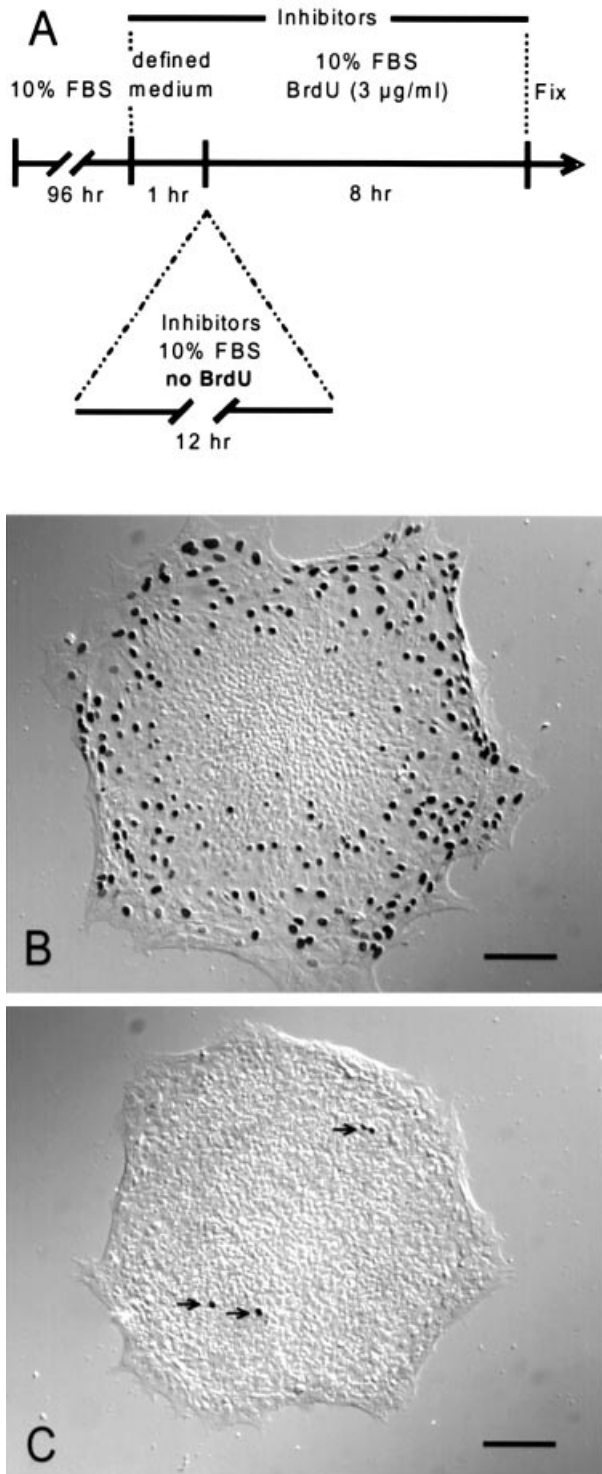


FIG. 8. The use of combinations of inhibitors resulted in marked suppression of S-phase entry and proliferation, with complete inhibition occurring after a 13 h incubation with inhibitors prior to the addition of BrdU. There was a small but significant difference following incubation with the downstream inhibitors of ERK and TOR (PD98059 and rapamycin; PD/Rap) as compared with these inhibitors, plus a PI3-K inhibitor (LY294002; PD/Rap/LY). A 13-h incubation with LY294002, rapamycin and PD98059 prior to the addition of BrdU reduced the incidence of S-phase entry by $< 99\%$.

FIG. 7. Sensory epithelium cultures exposed for 13 h to the combination of three inhibitors prior to the addition of medium containing BrdU. (A) Timeline for the three inhibitors experiments. (B) Photomicrograph of a representative control epithelial culture, grown for 96 h in medium with 10% FBS, followed by an 8 h incubation in medium containing 10% FBS plus 3 $\mu\text{g}/\text{mL}$ BrdU. BrdU-labelled nuclei are black. (C) An epithelial culture that was incubated with PD98059, rapamycin and LY294002 for 13 h prior to commencement of 8-h period in medium containing the inhibitors, 10% FBS and 3 $\mu\text{g}/\text{mL}$ BrdU. Almost no nuclei were labelled with BrdU (arrows) in the epithelia after the 13 h treatment with the three inhibitors. This demonstrated that the 13.1% labelling index measure after a 1-h incubation with the three inhibitors represented BrdU label uptake in cells that had entered S-phase prior to the onset of incubation. Scale bars, 100 μm .

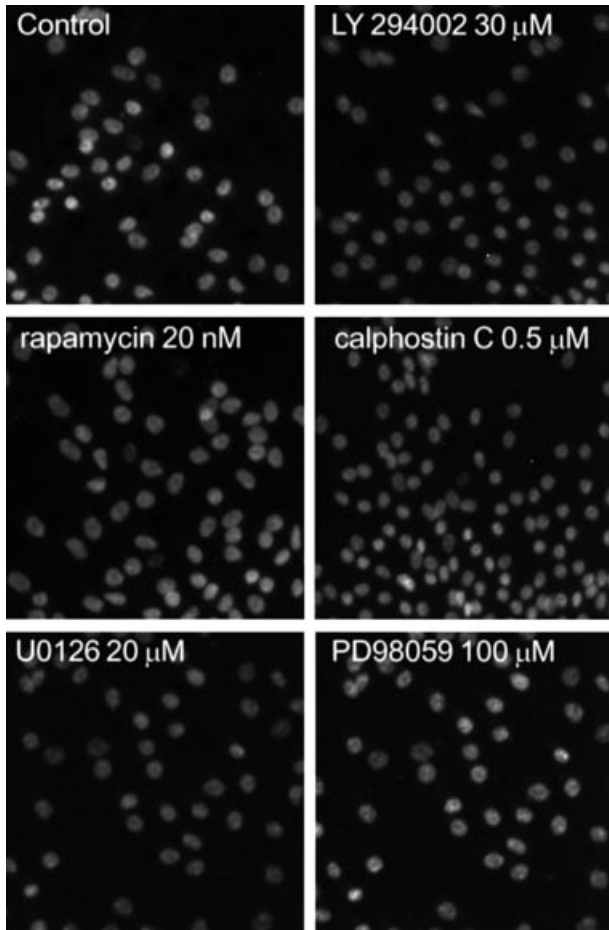


FIG. 9. DAPI staining of pieces of utricle treated with different inhibitors. Representative regions of chick utricular epithelium exposed to 10% FBS (control) or 30 μM LY294002, 20 nM rapamycin, 0.5 μM calphostin C, and 100 μM PD98059 together with 10% FBS. There is no obvious feature of apoptosis in the assayed epithelia. Scale bars, 50 μm .

undergoing apoptosis show characteristic morphological features including chromatin aggregation, nuclear and cytoplasmic condensation, and partition of cytoplasm and nucleus into membrane bound-vesicles. All of these features can readily be observed by staining the cells with the DNA binding dye DAPI (Darzynkiewicz *et al.*, 1997). For all cultures tested, no features of apoptosis were ever observed in the assayed epithelia. Figure 9 shows representative samples for each inhibitor at the highest concentration used in this study.

Discussion

The results from our control cultures confirmed a number of observations that were originally reported by Warchol (1995). Sheets of sensory epithelium isolated from the utricles of chickens retained epithelial characteristics in culture. The central zone of each piece of epithelium retained its columnar morphology and had only a moderate rate of growth when assayed for ≤ 14 h. In contrast the peripheral regions changed from columnar to simple squamous morphology and cells in those regions proliferated at a high rate. Analysis of the peripheral labelling that occurred during BrdU exposures ranging from 2 to 14 h indicated that in cultures grown in the presence of 10% FBS, approximately half the cells in the peripheral regions entered S-phase every 16 h (Fig. 2).

There is a relatively high incidence of S-phase entry in chicken utricular sensory epithelium *in vivo*, as measured by a pulse of BrdU

given 2 h before fixation (Kil *et al.*, 1997). The cell population in avian vestibular hair cell epithelia turns over throughout life (Jørgensen & Mathiesen, 1988; Roberson *et al.*, 1992), and *in vivo* labelling rates suggest that the average lifespan for hair cells in chicken utricles is less than 1 month (Kil *et al.*, 1997). The high rate of turnover in these epithelia can lead to rapid recovery after cell loss through replacement of hair cells that results from upregulation of the ongoing proliferation of the supporting cells (Weisleder & Rubel, 1992). When sheets of sensory epithelia are isolated and placed in culture, it is probable that some hair cells will be damaged. Such damage would appear likely to stimulate supporting cell proliferation in both the centres and the peripheral regions of the cultures. Yet, there is much more proliferation in the peripheral regions than in the centres of the sheets. Changes associated with spreading are believed to contribute to the high rate of S-phase entry based on cultures where cell density and cell proliferation have been investigated quantitatively (Warchol, 2001), and the evidence from our cultures supports that conclusion.

The high rate of proliferation facilitated the pharmacological survey of intracellular signal transduction pathways by allowing measurement of reductions in S-phase entry that could be achieved in treatments with different kinase inhibitors. The marked reductions of BrdU labelling that resulted from treatments with LY294002 and wortmannin suggest that PI3-K activation is critical for triggering S-phase entry in supporting cells from the utricles of chickens.

A kinase known as TOR has been identified as an important target of protein kinase B (PKB/Akt) in a pathway downstream from PI3-K (Brun *et al.*, 1996; McIlroy *et al.*, 1997). TOR in turn is an upstream activator of p70 S6K and p85 S6K in the pathways that trigger S-phase entry in Swiss 3T3 cells and rat embryo fibroblasts REF-52 (Chung *et al.*, 1992; Reinhard *et al.*, 1994). Treatment with rapamycin resulted in decreases in S-phase entry that were intermediate between those resulting from the treatments with LY294002 and wortmannin. The reduction of S-phase entry observed with rapamycin is consistent with the conclusion that a PI3-K/TOR cascade is involved in the S-phase entry of chick-supporting cells.

Pronounced inhibition of S-phase entry was observed after treatments with 500 nM calphostin C, which can block the DAG-binding site of PKC. That result suggests that PKC family members may have roles in the signalling cascades that trigger proliferation in chicken utricles, but higher concentrations of calphostin C appear to be toxic to these cells, raising the possibility for other interpretations. The very similar level of reduction in S-phase entry that resulted from treatments with two other PKC inhibitors, BIM and Gö6983 (48.5% and 41.5% reduction, respectively), both of which act on the ATP-binding site of PKC, lends support to the conclusion that PKC family members are involved in triggering S-phase entry in these cells. PKC isoforms could be part of the PI3-K/TOR cascade, as it has been shown that PKCs could be downstream targets of PI3-K (Toker *et al.*, 1994). PKC isoforms also could contribute to activation of the MAPK pathway via phosphorylation of Raf (Ueda *et al.*, 1996; Cai *et al.*, 1997; Schonwasser *et al.*, 1998).

Treatments with 50 μM AND 100 μM PD98059 inhibited MEK1 (Alessi *et al.*, 1995; Dudley *et al.*, 1995) and reduced the incidence of S-phase entry in a dose-dependent manner. Treatments with U0126, an inhibitor of MEK1 and MEK2 (Favata *et al.*, 1998), resulted in less pronounced, but significant dose-dependent reductions in S-phase entry, as did treatments with 13 μM AND 39 μM apigenin, another inhibitor of the MAPK pathway (Kuo & Yang, 1995). The marked reductions in S-phase entry by these three inhibitors leave little doubt that activation of the MAPK cascade plays a role in supporting cell proliferation in the sensory epithelium of the avian utricle. These results are consistent with results from cultures of whole hearing

organs from chickens, where the MAPK cascade was identified as an important mediator of proliferation (Bell *et al.*, 2000).

The similar reductions in labelling that resulted from the concurrent inhibition of MEK + TOR and the inhibition of PI3-K + MEK + TOR suggest that the PI3-K effects on cell proliferation are mediated primarily through a signalling pathway that impinges on TOR and/or MAPK. Whereas 50 μ M PD98059 alone led to \approx 59% inhibition, and 20 nM rapamycin led to 74% inhibition, the combination of those inhibitors resulted in 90% inhibition of S-phase entry (see Table 1). When 30 μ M LY294002 was combined with PD98059 and rapamycin, S-phase entry was inhibited by 99.5%. PI3-K is upstream of TOR in several systems (for review, see Schmelzle & Hall, 2000), and other studies have shown that PI3-K can act upstream of MEK as well as TOR (Uehara *et al.*, 1995; Urish *et al.*, 1995; King *et al.*, 1997; Penuel & Martin, 1999). A reasonable hypothesis is that PI3-K is an upstream component of one or both pathways that regulate the cell cycle in the cells of the chicken sensory epithelium. In fact, the MAPK and the PI3-K/TOR cascades may be closely interconnected, so that each requires some basal activity of the other for functionally effective triggering of proliferation. PI3-K activity has been shown to be required for integrin-induced MAPK activation in Cos 7 cells (King *et al.*, 1997), and may play a role in the adhesion dependence of cell proliferation in this epithelium.

The data suggest that the relative importance of signalling elements for the triggering of S-phase entry in the sensory epithelium of the chicken utricle is PI3-K \geq TOR \geq MAPK. PKC family members also participate, but the difference in effect between calphostin C and the PKC inhibitors BIM and Gö6983, as well as the cytotoxicity of calphostin C at higher concentrations, make it difficult to judge the relative contribution of PKC activation. A survey of intracellular signalling pathways in samples of sensory epithelium cultured from neonatal rats has shown that PI3-K, TOR, PKC and, to a lesser extent the MAPK pathway, play roles in triggering S-phase entry in mammalian utricles (Montcouquiol & Corwin, 2001; Table 1). The results in the sensory epithelium from chickens differed from those in the mammalian epithelium in the amount of BrdU labelling that was observed after similar treatments with inhibitors. The PI3-K inhibitor LY294002 blocked BrdU labelling almost completely in the sensory epithelia from rats, even in the presence of the potent mitogen glial growth factor. LY294002 was effective in suppressing BrdU labelling in the sensory epithelium from chickens, but 15% of the cells were still labelled in its presence when the preincubation with the inhibitor was for 60 min. In fact, when pieces of the chicken epithelium were cultured in medium that contained a combination of MEK, TOR and PI3-K inhibitors (PD98059, rapamycin and LY294002), the incidence of BrdU labelling was only reduced to 13%. We hypothesized that many of the cells that labelled in this experiment had passed the G₁ restriction point prior to exposure to the combined inhibitors, but did not complete and exit S-phase before the change to the BrdU-containing medium 60 min later. The duration of S-phase ranges from 3 to 25.5 h in other cells (Altman & Dittmer, 1972) and it is less than 8 h in supporting cells of the developing chicken cochlea (Katayama & Corwin, 1993). We tested that hypothesis by increasing the duration of the preincubation from 1 h to 13 h to allow cells that had already passed the G₁ restriction point to exit S-phase prior to their exposure to BrdU. Consistent with the hypothesis, the longer preincubation in medium containing the combination of the three inhibitors reduced the labelling by BrdU by over 99%. Detailed microscopic examination of the epithelial sheets exposed to these conditions revealed normal cellular morphology and small numbers of healthy appearing cells that were unequivocally labelled by BrdU,

indicating that cell cycle and DNA synthesis pathways were intact, although nearly 100% inhibited (see Fig. 7C, arrows).

Results from the long preincubation experiment indicate that the maximum level of inhibition that can be achieved following a protocol that uses a 60-min preincubation with an inhibitor will allow 13% BrdU labelling, even if 99% of the S-phase entry is inhibited from the time of exposure to the inhibitor. Therefore, we subtracted this value from the growth fractions under all experimental conditions. These calculations (Table 1) indicate that certain inhibitors for PI3-K, MAPK downstream components and PKC are individually capable of producing greater than 80% inhibition of S-phase entry under the conditions tested.

Overall, the experimental results support the conclusion that S-phase entry in the vestibular epithelia of chickens requires the activation of PI3-K, TOR and the MAPK pathway. The signalling pathways involved in control of cell proliferation in the chick utricle are similar to those observed in the rat balance epithelia, but seem to be more closely interconnected than the pathways that control proliferation in their mammalian counterparts. The relatively close correspondence between birds and mammals suggests that the markedly different proliferative capacities of their hair cell epithelia reside at another level of cellular regulation.

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Abbreviations

BIM, bisindolylmaleimide I; BrdU, 5-bromo-2-deoxyuridine; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; DIC, differential interference contrast microscopy; ERK, extracellular regulated kinase; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase pathway; MEK, MAPK kinase; mTOR, mammalian TOR; NHS, normal horse serum; PBS, phosphate-buffered saline; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TOR, chick target of rapamycin.

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