Functional Expression of Exogenous Proteins in Mammalian Sensory Hair Cells Infected With Adenoviral Vectors

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1Department of Neurobiology, Harvard Medical School and Massachusetts General Hospital; 2Howard Hughes Medical Institute, Boston, Massachusetts 02114; 3Section of Molecular and Cellular Cardiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and 4Centre for Research in Neuroscience, Montreal General Hospital, Montreal, Quebec H3G 1A4, Canada

Holt, Jeffrey R., David C. Johns, Sam Wang, Zheng-Yi Chen, Robert J. Dunn, Eduardo Marban, and David P. Corey. Functional expression of exogenous proteins in mammalian sensory hair cells infected with adenoviral vectors. J. Neurophysiol. 81: 1881–1888, 1999. To understand the function of specific proteins in sensory hair cells, it is necessary to add or inactivate those proteins in a system where their physiological effects can be studied. Unfortunately, the usefulness of heterologous expression systems for the study of many hair cell proteins is limited by the inherent difficulty of reconstituting the hair cell’s exquisite cytoarchitecture. Expression of exogenous proteins within hair cells themselves may provide an alternative approach. Because recombinant viruses were efficient vectors for gene delivery in other systems, we screened three viral vectors for their ability to express exogenous genes in hair cells of organotypic cultures from mouse auditory and vestibular organs. We observed no expression of the genes for β-galactosidase or green fluorescent protein (GFP) with either herpes simplex virus or adeno-associated virus. On the other hand, we found robust expression of GFP in hair cells exposed to a recombinant, replication-deficient adenovirus that carried the gene for GFP driven by a cytomegalovirus promoter. Titors of 4 × 107 pfu/ml were sufficient for expression in 50% of the ~1,000 hair cells in the utricular epithelium; < 1% of the nonhair cells in the epithelium were GFP positive. Expression of GFP was evident as early as 12 h postinfection, was maximal at 4 days, and continued for at least 10 days. Over the first 36 h there was no evidence of toxicity. We recorded normal voltage-dependent and transduction currents from infected cells identified by GFP fluorescence. At longer times hair bundle integrity was compromised despite a cell body that appeared healthy. To assess the ability of adenovirus-mediated gene transfer to alter hair cell function we introduced the gene for the ion channel Kir2.1. We used an adenovirus vector encoding Kir2.1 fused to GFP under the control of an edecysone promoter. Unlike the diffuse distribution within the cell body we observed with GFP, the ion channel–GFP fusion showed a pattern of fluorescence that was restricted to the cell membrane and a few extranuclear punctate regions. Patch-clamp recordings confirmed the expression of an inward rectifier with a conductance of 43 nS, over an order of magnitude larger than the endogenous inward rectifier. The zero-current potential in infected cells was shifted by ~17 mV. These results demonstrate an efficient method for gene transfer into both vestibular and auditory hair cells in culture, which can be used to study the effects of gene products on hair cell function.

INTRODUCTION

Gene transfer into sensory hair cells presents numerous opportunities for auditory and vestibular neuroscience. Potential applications include localization of proteins by expression of tagged constructs, dominant-negative or antisense knockout of endogenous proteins, rescue of mutant phenotypes to identify disease genes, and perhaps even treatment of auditory or vestibular disorders.

Unfortunately, conventional gene transfer techniques such as cationic liposomes, electroporation, or a gene gun run the risk of substantial damage to the cell membrane and possibly the transduction machinery. Viral vectors, on the other hand, offer an attractive alternative for several reasons (Breakefield et al. 1998). 1) The transfer event is via receptor-mediated endocytosis or membrane fusion and thus poses little threat to cell integrity. 2) Viruses provide a highly efficient means of delivery and expression of exogenous genes in host cells. 3) Virus-mediated gene transfer has the potential for therapeutic use in vivo, whereas most of the nonviral techniques are much less effective.

The goal of this study was to screen several viral vectors for their ability to express exogenous genes in hair cells of cultured mouse auditory and vestibular organs. Although our screen was not exhaustive, we determined adenovirus to be a suitable vector for gene transfer into inner ear hair cells. Adenovirus-mediated gene transfer was selected because it satisfied the following criteria: efficient and specific infection of hair cells, ease of vector construction, ability to produce vector at high titers, and minimal toxic effects.

METHODS

Tissue preparation

Utricles and cochlea were excised from young CD-1 mice (Charles River, Wilmington, MA) between postnatal day 0 and postnatal day 3, as described by Rüsch and Eatock (1996a). The mice were killed by cervical dislocation and decapitation. Removal of the sensory epithelium was performed in MEM (GIBCO, Gaithersburg, MD) to which 10 mM HEPES (pH 7.4) was added. From the medial surface, the bony labyrinth was opened, and the epithelium were exposed. To facilitate removal of the otoconia and otolithic membrane, the tissue was treated with protease XXVII (Sigma, St. Louis, MO) at a concentration of 100 µg/ml in MEM for 20 min. After removal of the otolithic membrane, the utricles were excised, and the surrounding...
tissue and nerve were trimmed away. The sensory epithelia were then mounted on round glass coverslips with Cell Tak (Collaborative Biomedical Products, Bedford, MA) and placed in an incubator at 37°C and 5% CO₂ for ≤10 days. The culture medium was identical to that described previously except that it contained 10% heat-inactivated horse serum (GIBCO). Viruses were added directly to the culture media for 24 h. All times were measured from the beginning of the viral infection.

Viral vectors

HERPES SIMPLEX VIRUS. A recombinant herpes simplex virus (HSV) type 1 vector, hrR3, which contained most of the viral genome (Goldstein and Weller 1988), was used at a titer of 10⁷ transducing units/ml (tu/ml). This vector contains the lacZ gene in place of the deleted ribonucleotide reductase gene and was driven by the endogenous HSV ribonucleotide reductase promoter. As such, the vector is replication deficient in postmitotic cells. An HSV-amplicon vector (Johnston et al. 1997), in which the viral genome was deleted, was packaged with the helper virus-free system described by Frael et al. (1996). In brief, a cytomegalo virus (CMV)-driven enhanced-GFP expressing ampiclon was cotransfected with the helper virus cosm id set into Vero 2.2 cells. Cells were harvested 2.5 days later by scraping followed by three freeze-thaw cycles and sonication. Cellular debris was pelleted at 3,000 g, and the cell lysate containing 10⁷ tu/ml (titered on Vero 2.2 cells) was used for experiments.

AAV. The University of Florida Vector Core provided recombinant adeno-associated virus vectors containing enhanced GFP under three promoters: CMV at 10⁷ infectious particles/ml (ip/ml), α-elongation factor promoter at 10¹¹ ip/ml (Zolotukhin et al. 1996), and neuron-specific promoter at 10¹⁰ ip/ml (Peel et al. 1997).

ADGFP. Replication-deficient (E1a/b deleted) recombinant adenovirus containing the cDNA for enhanced-GFP driven by a CMV promoter (rAdGFP) was obtained from Genzyme (Cambridge, MA) at a titer of 10¹ plaque forming units/ml (pfu/ml).

AD-GFP-KIR2.1. The coding sequence for the human Kir2.1 gene was fused in frame to eGFP in the plasmid vector pEGFP-C3 (Clontech, Palo Alto, CA) creating pGFP-Kir2.1. The adeno virus shuttle vector pAdLox (Hardy et al. 1997) was modified to replace the CMV promoter with the edycysone inducible promoter from pNLD-1 (Invitrogen, San Diego, CA) making the vector pAdEcd. The coding sequences from pGFP-Kir2.1 was cloned into the multiple cloning site of pAdEcd making the vector pAd-GFP-Kir2.1.

ADGVRXRX. The plasmid pAdVgRXR was made by cloning the dual expression cassette from pVgRXR (Invitrogen) into pAdLox. This vector constitutively expresses a modified edycysone receptor and the retinoid X receptor. Reombinant adeno virus vectors were generated by co-transfecting CRE8 cells with 2.1 µg of purified Ψ5 viral DNA and 2.1 µg of purified shuttle vector DNA with Lipofectamine Plus (Life Technologies). Cells were incubated 5–9 days until cytopathic effects (CPEs) were observed and then freeze-thawed. Cellular debris was removed by centrifugation, and 2 ml of the supernatant was added to 90% confluent CRE8 cells and returned to the incubator until CPEs were observed. This procedure was repeated three to four times. Viruses were expanded and purified as previously described (Johns et al. 1995). Titers were determined by plaque assays. Expression was induced by adding 4 µM mursisterone A (Invitrogen). All virus stocks were aliquoted and stored at −80°C.

Microscopy

Live utricle cultures were imaged with an Axioskop FS (Zeiss, Germany) fitted for differential interference contrast (DIC) and fluorescence microscopy. A FITC long-pass filter set (No. 41012, Chroma Technology, Brattleboro, VT) was used to observe GFP fluorescence. Images were acquired with an integrating charge-coupled device (CCD) camera (C2400, Hamamatsu Photonics, Japan) and analyzed with Photoshop 4.0 (Adobe Systems, Mountain View, CA).

Fixed epithelia were imaged with a BioRad MRC 1000 confocal microscope (BioRad Laboratories, Hercules, CA). These cultures were fixed in 4% formaldehyde for 20 min. rinsed in PBS (pH 7.4), and permeabilized with 0.5% Triton X-100 in PBS for 20 min. To visualize the hair bundles, the tissue was stained with 100 nM rhodamine-conjugated phalloidin for 1 h (Molecular Probes, Eugene, OR). Then the tissue was rinsed with PBS and mounted with BioRad mounting medium which contained p-phenylenediamine to prevent photobleaching. To estimate the number of hair cells in the sensory epithelium we counted the number of hair bundles. This method may have resulted in a slight under estimation of the total number of hair cells present as some cells may have lost their bundles during preparation of the tissue.

Recording

We recorded voltage-dependent and transduction currents, as previously described (Holt et al. 1997, 1998). Briefly, cultures were bathed in extracellular solution that contained (in mM) 144 NaCl, 0.7 NaH2PO4, 7 KCl, 1.3 CaCl2, 0.9 MgCl2, 5.6 glucose, and 10 HEPES-NaOH, vitamins, and amino acids as in MEM, pH 7.4. Recording pipettes had resistances between 3 and 5 MΩ and contained (in mM) 130 KCl, 0.1 CaCl2, 5 EGTA-KOH, 3.5 MgCl2, 2.5 MgATP, and 5 HEPES-KOH, pH 7.4. Currents were recorded at room temperature (22–24°C) with an Axopatch 200B (Axon Instruments, Foster City, CA), filtered at 2 kHz with an 8-pole bessel filter (Frequency Devices, Haverhill, MA), digitized at ≥4 kHz with a 12-bit acquisition board and pClamp6.0 (Axon Instruments) and stored on disk for off-line analysis with Origin 5.0 (MicroCal Software, Northampton, MA). Data are presented as means ± SD.

Stimulation

The mechanical stimulus was a fluid jet delivered from a glass micropipette and controlled by a fast pressure-clamp system (Denk and Webb 1992; McBride and Hamill 1995), as described previously (Holt et al. 1997, 1998). Stimulus pipettes were pulled to a tip diameter of ~10 µm, filled with standard extracellular solution, and positioned ~50 µm from the hair bundle. Stimuli were step and sinusoidal bundle deflections controlled by pClamp 6.0 software. Hair bundle deflections were monitored with a C2400 CCD camera (Hamamatsu, Japan) and recorded onto videotape. Deflections were measured off-line directly from the video image.

RESULTS

Screen of viral vectors

We tested three types of viral vectors for their ability to transfer reporter genes into the hair cells of organotypic cultures of the mouse utricle. Cultures exposed to HSV-amplicon vectors at titers up to 5 × 10⁸ tu/ml for 24 h were checked daily for expression of GFP. Between days 1 and 7 postinfection, ~20% of fibroblasts expressed GFP as well as a few glial cells. However, no hair cells appeared fluorescent throughout the duration of the experiment. We did not observe expression of either of the reporter genes, GFP or lacZ, in hair cells with any of the HSV vectors we tested, which included hrR3 and ampiclon vectors with titers that ranged between 10⁶ tu/ml and 5 × 10⁸ tu/ml.

Adeno-associated virus was introduced into the culture medium at titers of ≤10⁷ ip/ml. Three constructs were tested that contained GFP driven by different promoters: CMV, α-elongation factor 1, and neuron-specific enolase. We observed no
GFP expression regardless of promoter, titer, or duration of infection in any cell type.

However, with a recombinant, replication-deficient adenovirus that contained GFP driven by a CMV promoter (rAdGFP), we observed robust expression of GFP in both fibroblasts and hair cells but not supporting cells. The following sections elaborate on the infection of hair cells by rAdGFP.

Properties of rAdGFP infection of hair cells

Figure 1A shows a confocal image of a mouse utricle culture viewed from above. The culture was treated with $10^6$ pfu/ml of rAdGFP for 24 h and fixed and stained 36 h postinfection. The sensory epithelium of the utricle contains 600–1,000 hair cells and perhaps an equal number of supporting cells. The plane of focus appear the brightest. Those farther from the plane of focus appear more dim. E: fluorescence image of mouse cochlear culture exposed to $10^6$ pfu/ml rAdGFP counterstained with rhodamine-conjugated phalloidin; 15 of 71 hair cells visible in this image were GFP positive. F: at the periphery of the cochlear culture fibroblasts were also found to express GFP.
At 10^5 pfu/ml a few hair cells expressing GFP were observed except fibroblasts at the periphery of the culture (Fig. 1). The frequency (data not shown). No other cell type was fluorescent. Inner hair cells were also found to express GFP but at a lower level. Although only outer hair cells are fluorescent in this image, 15 of 71 (21%) of the hair cells were fluorescent. When exposed to 10^6 pfu/ml of rAdGFP. In the image shown in Fig. 1B, all cells could be infected at higher titers, we fit our data for the time course of GFP expression. E: percentage of hair cells infected as a function of titer. The titers we used were not sufficient to infect all hair cells. By assuming that all cells could be infected at higher titers, we fit our data with a sigmoidal curve and calculated a titer for half-maximal expression of 4 × 10^7 pfu/ml.

Viability of infected cells

Although the strong immune response reported for adenoviral vectors used in vivo (Byrnes et al. 1996) presents little concern for our culture system, there was still the possibility that toxic effects could result from the expression of viral genes within the infected hair cells. To address the viability of the cells we used the patch-clamp technique to record both voltage-dependent and transduction currents from rAdGFP-infected hair cells. Individual infected hair cells were identified by GFP fluorescence, and recording electrodes were sealed onto those cells. Figure 3A shows a family of voltage-dependent currents recorded from a fluorescent cell in response to voltage steps that ranged between −124 and +36 mV in 10-mV increments. The large outward currents are similar in both magnitude and kinetics to a delayed rectifier found in normal type II cells of the mouse utricle (Rüsch et al. 1998). The inward currents resembled the fast inward rectifiers normally found in both type I and type II cells (Rüsch et al. 1998). Normal voltage-dependent currents were recorded from hair cells expressing GFP ≤6 days postinfection (n = 6).

Perhaps a more rigorous test of the general health of a hair cell is the presence of transduction currents. A fluid-jet stimulus was used to deflect the sensory hair bundles of rAdGFP infected hair cells. We recorded transduction currents evoked by a 30-Hz sinusoidal stimulus (6-cycle burst) from a type II hair cell at 20 h postinfection (Fig. 3B). The currents were age-dependent and plateaued after 4 days. The line through the data is an exponential fit with a time constant of 35 h and a maximum of 33% of the hair cells in the epithelium. In other cultures, expression was stable for at least 10 days. Longer times were not tested.

To determine the efficacy of adenovirus infection, we tested titers that ranged between 10^4 and 10^8 pfu/ml. The cultures were infected for 24 h, and at 3 days postinfection the proportion of hair cells that expressed GFP was measured (Fig. 2B). At 10^5 pfu/ml a few hair cells expressing GFP were observed (Fig. 1C). At the highest titer we tested, 10^8 pfu/ml, more than one-half the hair cells were infected (Fig. 1D). The titers we used were not sufficient to infect all hair cells. By assuming that all cells could be infected at higher titers, we fit our data with a sigmoidal curve and calculated a titer for half-maximal expression at 4 × 10^7 pfu/ml.

Cultures of the mouse cochlea revealed similar expression when exposed to 10^6 pfu/ml of rAdGFP. In the image shown in Fig. 1E, 15 of 71 (21%) of the hair cells were fluorescent. Although only outer hair cells are fluorescent in this image, inner hair cells were also found to express GFP but at a lower frequency (data not shown). No other cell type was fluorescent except fibroblasts at the periphery of the culture (Fig. 1F).
asymmetric with an amplitude of $\sim$50 pA peak to peak. Step deflection of the hair bundle evoked a current with a rapid onset that decayed back toward its initial value despite a maintained deflection (Fig. 3C). The current decay, or adaptation, was fit with an exponential function that had a 32-ms time constant. Similar transduction and adaptation were reported previously from uninfected type II hair cells of the mouse utricle (Holt et al. 1997).

However, at times longer than 36 h we observed a detrimental effect of rAdGFP on the hair bundles of infected cells; they were tilted by $>45^\circ$ in the negative direction (toward the shortest stereocilia), a condition never observed in uninfected cells. Figure 4A shows a DIC image from a utricle culture 2 days postinfection. Five healthy-looking hair bundles of noninfected cells surround a single tilted hair bundle of an infected cell. Figure 4, B and C, shows DIC and fluorescence images, respectively, of the same field focused at the cell body layer. It is apparent that the hair cell with the tilted bundle expresses GFP. Almost all infected cells had tilted bundles beyond 36 h postinfection. Although fluid jet-evoked bundle deflections resulted in no transduction currents in the five infected hair cells we tested, the cells were otherwise viable. Normal voltage-dependent currents were recorded as late as 6 days postinfection, and the cell bodies of infected cells appeared healthy for $\geq10$ days.

**Infection with rAd-GFP-Kir2.1**

To assess the ability of adenovirus to deliver genes with functional consequences into hair cells we choose to overexpress a K$^+$-selective inward rectifier channel, Kir2.1, for several reasons; Navaratnum et al. (1995) have shown Kir2.1 is expressed in the hair cells of the chick cochlea. The currents predicted to result from overexpression of Kir2.1 should be easily distinguished from the small, endogenous, voltage-dependent currents present at hyperpolarized potentials (Rusch et al. 1998). Finally, the contributions of inward rectifiers to normal hair cell physiology were well documented for frog (Holt and Eatock 1995), turtle (Goodman and Art 1996), goldfish (Sugihara et al. 1996), and chick (Fuchs et al. 1990; Navaratnum et al. 1995) but are less well understood in mammalian hair cell organs.

Mouse utricle cultures were co-infected with two viruses: $5 \times 10^7$ pfu/ml of rAdVgRXR, which contained the gene for the ecdysone receptor under control of a CMV promoter and the gene for the retinoid X receptor under the control of a RSV promoter, and $10^8$ pfu/ml of rAd-GFP-Kir2.1, which contained the Kir2.1 gene fused to the gene for enhanced GFP under control of the inducible ecdysone promoter. Cells infected with rAdVgRXR were expected to express both the ecdysone and retinoid X receptors. To activate the ecdysone receptor complex we added 4 $\mu$M of the ecdysone analogue muristerone A after 24. The activated complex consists of a heterodimer that binds the ecdysone inducible promoter of rAd-GFP-Kir2.1. In other systems muristerone A enhances the expression of genes driven by the ecdysone promoter by $>30$-fold, and protein expression was near half-maximal within 12 h (Johns et al. 1998). Thus cells infected with both rAdVgRXR and rAd-GFP-Kir2.1 and treated with muristerone A were expected to express the GFP-Kir2.1 fusion protein. Because Kir2.1 is a membrane-bound ion channel, we predicted the fusion protein would still be targeted to the cell membrane. Figure 5 shows a ring of fluorescence localized to the cell membrane. Additionally, several punctate regions are visible and may reflect sites of protein synthesis or processing, such as the endoplasmic reticulum or Golgi apparatus.

To assess the effects of rAd-GFP-Kir2.1 on the electrophysiology of the cells, we first recorded from cells that showed no fluorescence and thus were apparently not infected with rAd-GFP-Kir2.1. Figure 6A shows a family of voltage-dependent currents recorded from a hair cell classified as type I based on the criteria of Rüschi et al. (1998). The instantaneous currents present at the beginning of the voltage steps are evidence of the low-voltage activated K$^+$ conductance ($g_{K, L}$) present exclusively in type I cells (Rüschi and Eatock 1996a). The data of Fig. 6B are from an uninfected type II hair cell. The outward currents were likely through delayed rectifier K$^+$ channels, and the small inward...
currents, which were $<100$ pA at $-104$ mV, were likely through the endogenous $K^+$-selective inward rectifier channels (Rüsch et al. 1998). Figure 6C shows data from a cell with a visible ring of fluorescence outlining its cell body; on the basis of the shape thus illuminated, it was classified as a type II hair cell. Unlike normal type II cells, voltage steps to $-104$ mV evoked inward currents of $\sim 2$ nA. Taken together the following evidence suggests that these large inward currents were the result of the viral-mediated transfer and expression of the gene for inward rectifier channel, Kir2.1.

The reversal potential of the peak tail currents after a step to $-124$ mV was $-75$ mV (data not shown). The proximity of the reversal potential to $E_K$ ($-76$ mV) indicated the currents were carried primarily by potassium, consistent with the selectivity of Kir2.1 reported by Navaratnum et al. (1995). The average inward rectifier conductance in infected cells was $43 \pm 20$ nS ($n = 6$) when fully activated, whereas the inward rectifier conductance from normal type II cells was $3.6 \pm 1.7$ nS ($n = 107$) (Rüsch et al. 1998). The outward current at the holding potential ($-64$ mV) and the large instantaneous currents evoked by voltage steps (Fig. 6C) suggest that a substantial fraction of the Kir2.1 conductance was active at the holding potential. In frog and turtle, inward rectifiers are active at potentials up to $-40$ mV positive to the holding potential (Goodman and Art 1996; Holt and Eatock 1995). Thus the input conductance at the holding potential is expected to be larger in a cell that expresses a large inward rectifier conductance. Indeed at $-64$ mV the infected type II cells had a mean input conductance of $31 \pm 6$ nS ($n = 6$), whereas the control type I and type II cells of Fig. 6, A and B, had input conductances of 22 and 0.7 nS, respectively. The physiological consequences of overexpression of Kir2.1 in type II hair cells are consistent with the role of $K^+$-selective inward rectifiers in frog saccular hair cells (Holt and Eatock 1995). These authors showed that hair cells that had the $K^+$-selective inward rectifier conductance, $g_{K1}$, had zero-current potentials that were more negative than those that did not, $-68$ versus $-50$ mV. Figure 6D shows that the zero-current potential (the presumed resting potential) of the infected type II cell was $-72$ mV ($\bullet$), $17$ mV more negative than the uninfected type II cell ($-55$ mV; $\triangle$).

**FIG. 6.** Voltage-dependent currents recorded from 2 control cells and a cell infected with rAd-Kir2.1-GFP. Currents shown in A–C were evoked by stepping the membrane potential to voltages that ranged between $-104$ and $46$ mV in $10$-mV increments. Membrane potentials for select traces are shown to the right. Capacitive transients were removed for clarity. A: currents recorded from an uninfected type I hair cell. B: currents recorded from an uninfected type II hair cell. C: currents recorded from a type II hair cell that based on its fluorescent membrane was infected with rAd-Kir2.1-GFP. Data collected 20 h after introduction of muristerone A to the medium. D: current-voltage relationship for the cells shown in A–C. Steady-state currents were sampled $90$ ms after the onset of the voltage steps and are plotted vs. membrane potential. $\nabla$, type I cell; $\triangle$, type II cell; $\bullet$, infected type II.

**FIG. 5.** Confocal image of hair from a mouse utricle culture. After a 24-h exposure to $5 \times 10^3$ pfu/ml of rAd-RXR, $10^6$ pfu/ml of rAd-Kir2.1-GFP and $4 \mu$M muristerone A induce expression of the Kir2.1-GFP fusion protein. The hair cell is viewed from above focused approximately halfway down the length of the cell body. The protein seems to be localized to the cell membrane and a few punctate regions within the cytoplasm.
expression of exogenous Kir2.1 in hair cells

As we predicted, adenoviral delivery of the gene for Kir2.1 resulted in the expression of large inwardly rectifying currents in hair cells. Overexpression of Kir2.1 channels altered the normal physiology of the type II cells by shifting the zero-current potential to $-72$ mV, close to $E_K$ ($-76$ mV). In addition to $g_{k1}$ contributions to resting potential, Holt and Eatock (1995) showed that a substantial fraction of $g_{k1}$ was on at rest and that it resulted in a larger input conductance, which in turn resulted in smaller but faster voltage responses to input currents. Thus we expect that the larger input conductance of the type II cells infected with rAd-GFP-Kir2.1 would have a similar effect on their receptor potentials. Indeed Johns et al. (1998) infected cultured superior cervical ganglion neurons with identical viral constructs and showed that the large Kir2.1 conductance decreased the voltage response to injected currents.

To quantify the magnitude of the Kir2.1 protein expression, we compared the mean whole cell conductance ($43$ nS) recorded from type II hair cells infected with rAd-GFP-Kir2.1 with the Kir2.1 single-channel conductance ($17$ pS) reported by Navaratnam et al. (1995). By this method, we estimate $\sim 2,500$ functional channels were present $20$–$26$ h after introduction of muristerone A; because the channel is tetrameric, we estimate $\sim 10,000$ protein monomers. Because this electrophysiological assay of protein expression could only measure functional channels inserted into the membrane, the total amount of GFP-Kir2.1 expressed was presumably greater, perhaps significantly. Thus we can achieve protein expression significant enough to alter hair cell physiology at early times with adenoviral vectors.

The inducible promoter used in this system offers several advantages over a constitutively active promoter. Chief among them is the ability to terminate protein expression by removal of the ec dysone analogue; after allowing time for protein degradation the effects of the induced protein should be reversed (Johns et al. 1998).

Use of gene transfer to elucidate protein function in hair cells

A number of proteins expressed in hair cells were cloned; these include motor proteins (myosin-Ib, myosin-VI, myosin-VIIa, my-
osin XV), ion channels (AChR α9, CSLO, VSCC1D, BIR10, and ROMK1), and transcription factors (Bm 3.1). In addition, some of these are defective in inherited disorders of hearing and balance. Generation of transgenic mice in which these genes are altered would be a traditional approach to study their function, but this method can be time consuming and expensive, and proper interpretation may be confounded by developmental effects. The advantages of adenovirus-mediated gene transfer in vitro, which include rapid construction of viral vectors, ease of viral delivery at high titer, identification of living infected cells, the presence of negative controls in the same tissue, and the ability to disrupt function in fully developed cells, all make this an attractive system with which to study the location, function, and identity of proteins within hair cells.

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REFERENCES


