L-α-Difluoromethylornithine (DFMO) is a chemopreventive agent for colon cancer in clinical trials. Yet, the drug produces an across-frequency elevation of the hearing threshold, suggesting that DFMO may affect a common trait along the cochlear spiral. The mechanism for the ototoxic effects of DFMO remains uncertain. The cochlear duct is exclusively endowed with endolymphatic potential (EP). EP is a requisite for normal sound transduction, as it provides the electromotive force that determines the magnitude of the receptor potential of hair cells. EP is generated by the high throughput of K⁺ across cells of the stria vascularis, conferred partly by the activity of Kir4.1 channels. Here, we show that the ototoxicity of DFMO may be mediated by alteration of the inward rectification of Kir4.1 channels, resulting in a marked reduction in EP. These findings are surprising given that the present model for EP generation asserts that Kir4.1 confers the outflow of K⁺ in the stria vascularis. We have proposed an alternative model. These findings should also enable the rational design of new pharmaceuticals devoid of the untoward effect of DFMO.

Functional Consequences of Polyamine Synthesis Inhibition by L-α-Difluoromethylornithine (DFMO)

CELLULAR MECHANISMS FOR DFMO-MEDIATED OTOTOXICITY*

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L-α-Difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (ODC), a key enzyme in the synthesis of polyamines. Inhibition of polyamine synthesis suppresses carcinogen-induced epithelial cancers in animal models (1), which has raised the possibility that DFMO can be used effectively as a chemopreventive agent for colon cancer. However, the therapeutic potential of DFMO is hindered by its unaccounted mechanisms of ototoxicity. DFMO decreases the level of polyamines in the cochlea (2, 3) and causes an across-frequency hearing loss (4), suggesting that the drug may alter a fundamental feature of cochlear function.

The acute sensitivity of the auditory system is conferred partly by the base-line spontaneous activity of hair cells. Moreover, to overcome the intrinsic membrane noise, the sensitivity of hair cells is dependent on the endocochlear potential (EP >80 mV). Thus, EP is indispensable for normal sound transduction (5, 6). EP is generated by the high throughput of outward K⁺ flux across cells of the stria vascularis (StV) into the scala media by K⁺ transporters, pumps, and channels together with Cl⁻ channels (7–12). A congenital deficiency in intermediate cells (ICs), melanocytes forming the middle cellular layer of the StV, results in a reduction in EP and an increase in the hearing threshold (13, 14). For this reason, as well as the fact that ICs express Kir4.1, it has been asserted that the Kir4.1 channel is responsible for the high throughput of outward K⁺ movement across the IC membrane that leads to generation of EP (15–17). Consistent with this assertion is the evidence that Ba²⁺, a nonspecific blocker of Kir channels, decreases the magnitude of EP (15, 18, 19). Indeed, null deletion of the channel resulted in marked reduction in EP and a decreased concentration of endolymphatic K⁺ (17, 20, 21). Despite the circumstantial evidence, it is uncertain how an inward rectifier channel can produce a robust outward K⁺ efflux to generate EP. It is conceivable, albeit unlikely, that the Kir4.1 channels in the StV may be specialized to confer a tissue-specific function. Alternatively, the channel may not be a major player in K⁺ extrusion from ICs into the interstitial space (IS) but may contribute toward the removal of K⁺ from the IS.

The inward rectification of Kir channels is caused by both blockage of the outward current by cytoplasmic Mg²⁺ and by intrinsic polyamines (21–26). We surmised that DFMO-induced ototoxicity is mediated through inhibition of polyamine synthesis, consequently altering inward rectification of inner ear-specific Kir4.1. Thus, we cloned mouse cochlear lateral wall-specific Kir4.1 and analyzed the phenotypic features of the channel both with and without DFMO to determine the effects of the drug on EP. It was reassuring to observe that DFMO reduced the inward rectification of cochlear lateral wall Kir4.1. However, it was startling to determine that the drug produces a marked reduction in EP. These results indicate that although Kir4.1 channels are involved in the generation of EP, the existing model for the generation of EP should be refined. The underlying cellular mechanisms for the contribution of Kir4.1 to EP generation are reevaluated, and the possible mechanism of DFMO-mediated ototoxicity is outlined.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AY374423.

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† The abbreviations used are: DFMO, L-α-difluoromethylornithine; ODC, ornithine decarboxylase; EP, endocochlear potential; StV, stria vascularis; IC, intermediate cell; IS, interstitial space; ABR, auditory brainstem response.

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PCR and cDNA Cloning—Total RNA was purified from 12-week-old mouse inner ear StV using TRIzol reagent following the protocol suggested by the manufacturer (Invitrogen). RNA (~1 μg) was then used as a template to synthesize cDNA using SuperScript II reverse transcriptase. Aliquots of this cDNA were used as a DNA template for PCR. The sequences of the 5’- and 3’-primers for amplification of the Kir4.1 channel were derived from the mouse brain sequence (GenBank accession number AF322631) as follows, 5’-GCCGCCACCATGACGTCGTCGGCGTAAAAG-3’ and 5’-AGGATATACAGCGTGGTCG-3’, respectively. PCR was performed according to the following schedule: predenaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min. The PCR products were recovered after agarose gel separation and subcloned into a pCR-H100-TOPO vector (Invitrogen). Four clones were selected and checked by the digestion of internalBgII and HindIII sites. The cDNA sequences were determined by the chain termination method. A representative clone, pKJ-E3, was analyzed using the Xenopus oocyte expression system.

Expression in Heterologous Systems—For expression in the Xenopus oocyte system, the cDNA fragment containing the complete open reading frame of the Kir4.1 potassium channel was subcloned into the vector, pNLE, which included the 5’- and 3’-untranslated regions of a Xenopus β-globin gene (27). From the resulting expression plasmid, RNAs of Kir4.1 were transcribed in vitro using T7 RNA polymerase and injected into stage V–VI oocytes as described (28).

Electrophysiological Recordings—Two-electrode voltage clamp experiments were carried out with a commercially available amplifier (Waner Instrument Corp., Hamden, CT) with microelectrodes, which were filled with 3 M KCl. Oocytes were bathed in a solution that contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, and 1 mM niflumic acid to block endogenous chloride currents in oocytes (pH adjusted to 7.6 with NaOH). The current was activated in oocytes (pH adjusted to 7.6 with NaOH). The current was activated in oocytes (pH adjusted to 7.6 with NaOH). The current was activated in oocytes (pH adjusted to 7.6 with NaOH).

Materials and Methods

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tones (8, 16, and 32 kHz) were presented to the ear of the animal in 10-dB increments, starting from 0 dB to 100 dB sound pressure level. The ABR sweeps were computer-averaged (time-locked with onset of 128–1024 stimuli, at 20/s) from the continuous electroencephalographic activity. The threshold of hearing was determined as the lowest intensity of sound required to elicit a characteristic waveform.

RESULTS

The Kir4.1 channel has been cloned from the brain (32). However, given the presumed specific role of the channel in the inner ear, we performed reverse transcription-PCR using the primers related to mouse brain Kir4.1 and cDNA from mouse inner ear lateral wall to amplify StV-specific Kir4.1. A single band with the expected size of 1155 bp was obtained. The PCR product was cloned into a pCRII-TOPO vector and sequenced. We identified the Kir4.1 channel cDNA from the StV that contained a complete open reading frame (GenBank™ accession number AY374423) encoding a putative protein with 379 amino acids. The analysis of the amino acid sequences of putative proteins from Kir4.1 cDNAs revealed high similarities between StV-specific and other cloned Kir4.1 channels (15, 32–35) and bore the signature of an intermediate inward rectifier (Fig. 1), which gave the assurance that the channel is likely to be subjected to partial polyamine block and hence to be influenced by ODC (Fig. 1).

The phenotypic features of Kir4.1 expressed in Xenopus oocytes are illustrated in Fig. 2. The current rectifies inwardly, showing stunted growth at depolarized step potentials. By contrast, the current magnitude was robust in response to hyperpolarized step potentials from a holding potential of −40 mV (Fig. 2A). Consistent with a K+-selective channel, the whole-cell current-voltage relations shifted to depolarized potentials (Fig. 2B), and the estimated reversal potentials (E_{rev}) were close to the calculated Nernst potential as the bath K⁺ was elevated systematically (Fig. 2C). The current was blocked by Ba²⁺ with a half-blocking concentration at ~30 ± 10 μM, binding to ~20% of the functional electrical field of the channel (Fig. 2, E and F). These results were in accord with previous reports (32, 34, 36). Moreover, the resting potential of oocytes expressing Kir4.1 was controlled exclusively by the Ba²⁺-sensitive...
current, indicating that the channel serves as a background current that clamps the membrane potential close to the net resting potential of the oocytes (Fig. 2D).

To determine the effects of DFMO on the cloned Kir4.1 channels, *Xenopus* oocytes were injected with RNA and incubated in ND-96 solution containing 500 μM DFMO for 24 h. Experiments were also performed by injecting DFMO directly into oocytes. The ensuing whole-cell currents were compared with currents from control oocytes (Fig. 3, B and C). In accord with previous findings from Kir2.3 and other Kir channels, DFMO and other inhibitors of ODC produced a marked in-}

**current records, cell-attached patches from DFMO-treated oocytes had single-channel openings at depolarized potentials, and the probability of channel openings increased substantially in the excised patch mode (data not shown). The single channel conductance obtained for the Kir4.1 channels (29.1 ± 5.3 pS, n = 7) was in accord with reports from the channel cloned from other systems (39), giving further assurance that the identified Kir4.1 in the StV is not a unique channel.**

To ensure the high throughput of K⁺ across the ICs of the StV, the K⁺ concentration in the IS should be kept at relatively low levels. Inward rectification of Kir4.1 in the ICs may promote removal of K⁺ from the IS. Alternatively, like many other inward rectifier currents, the role of Kir channels in ICs may optimize the activity of an unidentified outward current by setting the membrane potential to a level that generates EP. Thus, we surmised that the modification of the inward rectification of Kir4.1 in vivo should alter EP and hearing threshold. ABRs were analyzed in DFMO-treated (1 mg/g of body weight/ day for 4 weeks, Ref. 4) CBA-mice and compared with their age-matched sham controls to determine the sound pressure levels at which typical ABR waveforms are seen. The control mice exhibited the characteristic ABR waveform beginning at sound pressure levels of −40, 30, 15, and 30 dB using broadband clicks and pure tones of 8-, 16-, and 32-kHz stimuli (Fig. 4). The DFMO-treated mice yielded elevated thresholds for broadband clicks and the three pure pips tested. The generation of across-frequency elevation of hearing threshold by

**Fig. 3. Effects of DFMO on inward rectification of Kir4.1 channel currents.** A, continuous perfusion of a bath solution containing 20 mM K⁺ produced an expected membrane depolarization. The time course of recovery after washout (arrow) with control saline (ND 96) was faster (τ = 2.3 ± 1.8 min, n = 6) in DFMO-treated than in control (τ = 12 ± 4.7 min, n = 6) oocytes. B, Kir4.1 current traces after incubation of *Xenopus* oocytes in 500 μM DFMO. C, the corresponding current-voltage relation shows that inward rectification of control oocytes was substantially reduced (n = 9). Single-channel Kir4.1 currents in *Xenopus* oocytes. D, representative and consecutive single-channel traces recorded in a cell-attached patch using pipette K⁺ = 120 mM. E, closed level. The bath solution contained 120 mM K⁺, and the resting potential of oocytes was −60 mV. The holding potential was −40 mV; the step potentials are indicated. Example of amplitude histograms used to generate the I-V relation as shown in E is depicted as an inset. E, the single-channel conductances of control and DFMO-treated oocytes were 29.1 ± 5.3 pS and 31.2 ± 3.6 (n = 7), respectively.
DFMO-treated reduction in EP and increased hearing threshold is striking. DFMO-treated tial (EP at the basal turn of the cochlea, control and pure tones at 8, 16, and 32 KHz. There were significant increases compared with age-matched saline-injected controls. B, in addition, DFMO-treated mice had a significant reduction in endocochlear potential (EP at the basal turn of the cochlea, control = 87 ± 9 mV and DFMO-treated = 49 ± 14 mV; apical turn, control = 83 ± 5 mV and DFMO-treated = 45 ± 8 mV; p < 0.05). The correlation between the reduction in EP and increased hearing threshold is striking.

DFMO raised the possibility that the drug may affect a common trait along the cochlear spiral. The measurement of EP in DFMO-treated and sham-control mice suggested that the ototoxic effects of the drug are mediated at least partly through reduction of EP (Fig. 4B). The relation between increased hearing threshold and a decline in EP (0.8 dB/1 mV) was consistent with previous reports on several animal models, which demonstrated a correlation between hearing loss and EP (40–43).

**DISCUSSION**

DFMO is potentially an efficient cancer-preventive drug. Yet, its insidious ototoxic side effect hinders its use as a mainstream cancer remedy. We have identified, cloned (GenBank accession number AY374423), and functionally expressed an inner ear lateral wall Kir4.1 channel that may serve as a target for the underlying mechanism for DFMO-induced ototoxicity. We report that the Kir4.1 channel in the lateral wall resembles and has properties that are consistent with an intermediate inward rectifier channel. Furthermore, in contrast to the suggestion that the channel may confer high outward throughput of K+ across ICs to the IS in the StV (17, 44), we propose that Kir4.1 in the ICs (16) may control the membrane potential of the cell and operate to reduce the K+ concentration in the IS. This action would serve to promote the steep K+ gradient necessary to generate EP. An increase in the outward component of Kir4.1 current by DFMO is likely to render the gradient shallow, reducing the flux of K+ and consequently resulting in a drop in EP. Alternatively, as an upshot of DFMO-induced reduction in inward rectification, the channel may clamp the IC membrane closed to the E_{rev} of K+, which would produce a similar outcome. Finally, the findings of this report transcend the outline of mechanisms for DFMO-induced ototoxicity. The data suggest that an unidentified outward K+ channel current that confers extrusion of K+ across the ICs should be sought, thus leading to a new understanding of the mechanisms for EP generation.

The rationale for the inhibition of ODC by DFMO as a cancer chemopreventive strategy is underpinned by the evidence that the enzyme is transactivated by the c-myc oncogene in certain cell/tissue types and cooperates with the ras oncogene in malignant transformation of epithelial tissues (45, 46). Thus, DFMO is a cancer chemopreventive drug that is currently used in clinical trials for the prevention of various types of cancer. Its major toxicity is reversible sensorineural hearing loss that has been found in adult humans at moderate doses (47) and in developing animals at low doses (48). Previous studies have demonstrated that the organ of Corti and the lateral wall of rat inner ear have the highest ODC activity, which is consistent with the distribution of polyamines (49, 50) and the suggestion that ODC may play a developmental role in the cochlea. Although the effects of polyamines in suppressing the outward component of Kir currents in other systems have been shown as a biophysical phenomenon (51), until now the functional and clinical significance has not been apparent. Moreover, the production of across-frequency hearing loss by DFMO (48) raises the possibility that the drug may alter a common trait along the cochlear spiral. It has been demonstrated that DFMO decreases the level of polyamines in the cochlea (2, 3). The fact that the drug alters the inward rectification of StV Kir4.1 was not surprising. However, it was startling to observe that the DFMO-induced suppression of inward rectification reduced EP.

EP is generated across cells in the StV to produce a composite of the main driving force for sensory transduction (5, 6). It has been demonstrated that the high throughput of K+ across ICs membrane yields EP (14, 52, 53). A combination of immunohistochemistry (16, 54), pharmacological (44), and null mutant mice experiments (17) have resulted in the assertion that Kir4.1 channels residing at membranes of intermediate cells in the StV confer the extrusion of K+ to polarize the membrane. Marcus et al. (17) noted the paradox that the present model posesses because of the inward rectification of the channel. However, given the paucity of data, they offer no plausible explanation for the inconsistency in the model.

The Kir channel family is a superfamly of channel proteins that play important roles in maintaining the resting membrane potential and in the secretion as well as absorption of K+ ions across cell membranes (55). They are tetramers with each subunit consisting of cytoplasmic N and C termini, two transmembrane domains, and a pore-forming region. The Kir channel family consists of more than 20 members, which can be classified into seven major subfamilies (56, 57). It has been demonstrated that two negatively charged amino acids (Fig. 1) are crucial for the inward rectification of Kir channels. One is in the second transmembrane segment (21, 23, 24), and the other is in the cytoplasmic C-terminal domain (25, 26). Channels such as Kir2.1–Kir2.4, with two negatively charged amino acids in these positions, confer strong rectification (24, 58), whereas those with neutral or positively charged amino acids produce weak rectification, e.g., Kir1.1 (Fig. 1, and Refs. 26, 33, 59). Kir4.1 has one negatively charged residue (Glu-158) and a neutral residue (Gly-210). As expected, it shows an intermediate rectification (Fig. 2).
It is conceivable that modulation of Kir4.1 in the STV may yield sufficient outward flux of $K^+$ to generate EP. For example, Kir5.1 has been found to form heteromeric channels with Kir4.1 in the renal tubular epithelia, retina, and brainstem (60–64). Compared with the homomeric Kir4.1 channel, the heteromeric Kir4.1–Kir5.1 channel has a large conductance and distinct kinetics. It has been suggested that Kir5.1 may be responsible for physiological modulation of functional Kir channels in the kidney (60–62). Although the modulation of Kir4.1 channels by other channels or second messengers remains a viable option, an alternative to the present model for the function of the channel in the generation of EP is made apparent by the effects of suppression of inward rectification by DFMO.

We propose that the main role of Kir4.1 in the ICs of STV is to contribute toward the maintenance of the membrane potential, decrease the $K^+$ concentration in the IS to improve the function of other outward $K^+$ channels, and confer a steep $K^+$ gradient between ICs and the IS. This model neither challenges nor refutes the prevailing evidence that Kir4.1 is involved in the generation of EP (65). It is implicit in our present replica that null deletion of the Kir4.1 channels will alter the resting membrane potential of ICs to more positive values, which will produce a crippling effect on $K^+$ extrusion resulting in a reduction in EP. A DFMO-induced increase in outward $K^+$ is expected to produce a similar outcome by reducing the $K^+$ gradient between the IS and ICs shallow. These findings suggest that other $K^+$ currents may be responsible for the high throughput of $K^+$ ions across IC membranes. Recently, a membrane protein with the signature sequence of TWIK-1 $K^+$ channels (66–69) has been identified in the STV. The TWIK-1 channel has weak inward rectifying properties when expressed in Xenopus oocytes and may serve as a background channel (70). In addition, although preliminary, the STV-specific $K^+$ channel, MERG1a, has been identified and is poised to contribute to $K^+$ flux (71). Thus, EP may be produced and maintained by a cadre of $K^+$ channels in the membrane of ICs and marginal cells in conjunction with $K^+$ transporters and pumps.

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