Hair cells of the auditory and vestibular systems transform mechanical input from sound and head movement into an electrical current through a process termed mechanoelectrical transduction (MET). At the apical surface of each hair cell is a specialized mechanosensitive organelle called the hair bundle, composed of a collection of actin-filled stereocilia arranged in graded height. The tips of the shorter rows house MET channels (1), nonspecific cation channels hypothesized to be comprised of TMCI/2, TMIE, and a number of other accessory proteins (2–4). MET channels are gated by a tension force imparted by a gating spring, which is part of or connected with the filamentous tip links that span adjacent rows of stereocilia (5–9). Positive stimuli directed along the sensitive axis of the hair bundle (10) result in displacement toward the tallest row and increase tension in the gating spring, increasing the open probability of the MET channel.

Mechanisms that regulate the sensitivity of the MET channel are hypothesized to contribute to the wide dynamic range and frequency selectivity of the auditory system, with MET adaptation being the most widely studied (11–14). Based on the gating spring theory, changes in gating spring stiffness alter the setpoint and the sensitivity of the MET channel (5, 6). The second messenger cyclic adenosine monophosphate (cAMP) has been proposed to modulate the sensitivity of the MET channel in turtle and bullfrog hair cells. In turtle auditory hair cells, cAMP shifts the operating point of the MET channel after a period of minutes, extending the dynamic range of the channel (15). In bullfrog hair cells, pharmacological up-regulation of cAMP reduced the frequency of spontaneous hair bundle oscillations (16). Furthermore, in humans, a loss of function mutation in adenyl cyclase 1 (ADCY1, an enzyme that catalyzes cAMP production) is associated with hearing loss (17) and zebrafish carrying an equivalent mutation (adcy1b morphants) exhibited significantly reduced FM1-43 dye uptake in lateral line hair cells, indicating dysfunctional MET. These studies indicate a potential role for cAMP in modulating MET channel sensitivity in mammals.

In this study, we determine the mechanism of cAMP modulation of the MET channel in a mammalian cochlear hair cell model. We first characterized the cAMP effect in rat outer hair cells (OHCs). Second, we describe a long depolarization manipulation (LDM), which we found mirrored the effects of cAMP up-regulation. Third, by measuring the mechanical changes of the hair bundle, we confirmed that cAMP and long depolarization-induced effects on channel sensitivity are due to a reduction in gating spring stiffness. Finally, we find that cAMP targets the exchange protein directly activated by cAMP (EPAC) rather than protein kinase A (PKA) for modulating MET sensitivity. This cAMP regulation of gating spring stiffness provides a cell signaling means for controlling the sensitivity of MET channels.
**Results**

**cAMP Regulation of Mammalian MET.** To characterize the cAMP effect in rat OHCs, we recorded MET currents and hair bundle displacements in response to a family of fluid-jet (FJ) force steps and generated activation curves before and after bath application (10 min) of hydrolysis-resistant cAMP analogs (0.1 mM 8-Bromoadenosine 3',5'-cyclic monophosphate, 8-BrcAMP, or dibutyryl cyclic adenosine monophosphate, dbcAMP) (Fig. 1A and B). In rat OHCs, 8-BrcAMP produced both a rightward shift in the half activation (from 25.1 ± 6.3 nm to 38.6 ± 5.4 nm, n = 6, P = 0.0033, paired t test) (Fig. 1C) and increased width (from 53.6 ± 13.3 nm to 76.2 ± 20.8 nm, P = 0.0025, paired t test) (Fig. 1D) of the activation curve. To further support a cAMP pathway-specific effect, we repeated these experiments using 0.1 mM dbcAMP. Consistent with the results from 8-BrcAMP, 0.1 mM dbcAMP produced a rightward shift in the half-activation (from 27.3 ± 4.8 nm to 56.6 ± 18.7 nm, n = 9, P = 0.0011, paired t test) (Fig. 1C) and increased width (from 66.5 ± 13.9 nm to 97.6 ± 20.1 nm, P = 0.0057, paired t test) (Fig. 1D) of the activation curve.

Consistent with a right-shifted activation curve, resting open probability measurements from cells treated with 8-BrcAMP and dbcAMP were significantly decreased (8-BrcAMP: from 5.3 ± 1.1% to 3.7 ± 0.9%, P = 0.0025, paired t test; dbcAMP: from 5.1 ± 0.9% to 2.6 ± 1.2%, P = 0.00025, paired t test) (Fig. 1E).

It is important to note that we also observed that in our control condition (Norm Ext), the resting open probability showed a statistically significant decrease (Norm Ext: from 5.0 ± 1.0% to 4.0 ± 0.9%, n = 8, P = 0.043, paired t test) (Fig. 1E). However, one-way ANOVA analysis with Tukey–Kramer post hoc test for multiple comparisons revealed that the change in resting open probability between the two time points was significantly different for control and dbcAMP, but not between control and 8-BrcAMP (P = 0.014 for control vs. dbcAMP, P = 0.42 for control vs. 8-BrcAMP, P = 0.28 for 8-BrcAMP vs. dbcAMP) (SI Appendix, Fig. S1D), suggesting a true effect of dbcAMP on resting open probability beyond the decrease we observed in the control condition. Furthermore, we performed one-way ANOVA analyses for the other summary data presented in Fig. 1. All one-way ANOVA with post hoc comparisons were consistent with a statistically significant effect of dbcAMP vs. the control (SI Appendix, Fig. S1).

Adaptation of the MET process is hypothesized to extend the dynamic range and increase the frequency selectivity of the hair cell (11–13). Experimentally, adaptation manifests as a decay of the MET current in response to a constant stimulus and a rightward shift of the activation curve. We analyzed the amount of MET current decay in response to cAMP analogs before and after applying either 8-BrcAMP or dbcAMP. We observed a nonstatistically significant trend toward increased adaptation with 0.1 mM 8-BrcAMP (from 21.4 ± 8.9% to 29.1 ± 13.2%,

![Fig. 1](https://doi.org/10.1073/pnas.2107567119)
Long Depolarization and cAMP Have Similar Effects on MET. Previously, we reported that depolarizing a hair cell to $+76$ mV produces a transient increase in MET channel resting open probability that subsequently decays over a period of several seconds (23, 24). In this previous work, we attributed the increase in resting open probability, which has a time course exceeding 100 ms, to a mechanism involving the lipid membrane, but we did not investigate the decay in resting open probability. Here, we describe the effect of the decay in resting open probability at depolarized potentials that occurs during a depolarization of $\sim 10$ s or longer, which we term “long depolarization modulation” or LDM.

We subjected OHCs to LDM, and using a stiff probe, we elicited MET current families to generate activation curves at various time points during the depolarization (SI Appendix, Fig. S2). We observed that during the prolonged depolarization, the activation curve shifted rightward and became less sensitive, which remained upon repolarization. To compare the effect before and after LDM, we performed experiments using the Fj that were similar to our experiments with cAMP analogs (Fig. 2 A and B, black and gray traces). The activation curve as measured with the Fj (Fig. 2B) exhibited both a significant rightward shift (Fig. 2C) (Half-Act, from $32.8 \pm 14.7$ nm to $59.8 \pm 17.7$ nm, $P = 2.0E-5$, paired t test) and increased width after depolarization (Fig. 2C) (Width$_{10-90}$, from $70.8 \pm 19.7$ nm to $95.1 \pm 26.2$ nm, $P = 0.039$, paired t test). Resting open probability was similarly decreased (Fig. 2E) (Resting $P_{\text{open}}$, from $4.7 \pm 1.9\%$ to $1.6 \pm 1.0\%$, $P = 1.3E-5$, paired t test), while peak current was unchanged (Fig. 2D) (from $0.75 \pm 0.08$ nA to $0.73 \pm 0.11$ nA, $P = 0.37$, paired t test). To determine the effect of LDM on MET adaptation, we first examined the effect on fast adaptation using stiff probe stimuli before and after LDM and found no effect on fast adaptation (SI Appendix, Fig. S3).

Next, we investigated the effect of LDM on slow adaptation. Using 50-ms step-like Fj stimuli (Fig. 2A), we found that following LDM the adaptation magnitude was significantly increased (Fig. 2E) (Adaptation %, from $32.8 \pm 21.4\%$ to $70.9 \pm 9.0\%$, $n = 15$, $P = 5.5E-7$, paired t test) and adaptation time constants were significantly decreased (Fig. 2E) ($\tau_A$, from $20.1 \pm 9.1$ ms to $9.7 \pm 2.6$ ms, $P = 4.0E-4$, paired t test). All of the LDM effects mirrored the cAMP up-regulation effects, raising the possibility that these two manipulations activate a similar mechanism.

**cAMP and LDM Are Not Additive.** We hypothesized that cAMP and depolarization operate through or engage a common mechanism regulating the MET channel in hair cells. Therefore, we predicted that activation of the cAMP pathway prior to LDM would prevent any further change to MET. Indeed, we found that in OHCs exposed to 0.1 mM dbcAMP, activation curve width, resting open probability, slow adaptation magnitude, and slow adaptation time constant were not significantly different after LDM (dbcAMP, Width$_{10-90}$, $P = 0.97$; Resting $P_{\text{open}}$, $P = 0.23$; % Adaptation, $P = 0.58$; $\tau_A$, $P = 0.23$; $n = 12$, paired t tests) (Fig. 2C–E). In dbcAMP-treated cells, activation curve half activation was slightly right-shifted after depolarization (Fig. 2C) (from $46.7 \pm 14.8$ nm to $55.7 \pm 12.3$ nm, $P = 0.038$, paired t test), but the magnitude of the shift was significantly smaller from that in control cells ($P = 0.0044$, unpaired t test). With multiple methods to affect the resting open probability and magnitude of adaptation, we observed a negative linear correlation between the resting open probability and the magnitude of slow adaptation that a cell exhibited ($r = -0.62$, $P = 2.89E-16$) (Fig. 2H). These data suggest that in rat OHCs, the resting open probability can predict the amount of slow adaptation the cell will exhibit when stimulated with 50-ms duration Fj step stimuli. Together, these data demonstrate that cAMP and LDM are not additive, supporting the hypothesis that LDM and cAMP modulate the same mechanism.
spring stiffness would result in changes in the activation curve since changes to \(k_{GS}\) do not modify gating (Fig. 3Q (5, 6, 25).

To further support the hypothesis that the gating spring stiffness is decreased with cAMP up-regulation and LDM, we measured the relative change in total hair bundle stiffness with FJ hair bundle stimulation and high-speed imaging at 5 min after entering whole-cell mode (control) followed by 10 min of 0.2 mM dbcAMP application (dbcAMP) (Fig. 3D). In these experiments, extreme care was used to ensure that the position of the FJ in relation to the hair bundle (in X, Y, and Z) was consistent between the 5-min and 10-min postdrug conditions so that the force from the FJ on the hair bundle remained consistent between the two time points. This allowed us to use the driving voltage command to the FJ piezo as a proxy for the relative force on the hair bundle. These values only apply to a given cell and cannot be compared across different cells due to the different hair bundle morphologies and characteristics and positioning of the FJ in each given hair cell; therefore, we normalized the stiffness values against the control condition for each cell. We plotted driver voltage against the hair bundle displacement at the end of the 50-ms FJ step, where the displacement is more consistent between the 5-min and 10-min postdrug conditions so that the force from the FJ on the hair bundle remained consistent.

We also assayed the effect of LDM on total hair bundle stiffness using the same method as in Fig. 3D–H. We found that LDM significantly decreased the total stiffness of the hair bundle in control cells (\(-22 \pm 15\%\), \(n = 15\)) but had no effect in dbcAMP-treated cells (\(2 \pm 19\%\), \(n = 12\)) (Fig. 3F), which further supports that the two manipulations are not additive. With these changes in total hair bundle stiffness, it was surprising not to see any consistent effects on the kinetics of the hair bundle movement (SI Appendix, Fig. S4). It could be that the changes in hair bundle mechanics were not large enough to elicit a sufficient change in the movement kinetics. In summary, these data demonstrate that after cAMP up-regulation or LDM, hair bundle stiffness decreased, consistent with a decrease in \(k_{GS}\) or \(k_{SP}\).

To delineate between an effect on \(k_{GS}\) or \(k_{SP}\), we tested whether the changes in hair bundle stiffness were correlated with changes in the activation curve. The inverse of the width is linearly related to \(k_{GS}\) (SI Appendix, Materials and Methods). We indeed found that \(k_{CS}\) and 1/width are correlated (Fig. 3F) \((r = 0.57, P = 4.6E-5)\). These data suggest that gating spring stiffness is decreased upon cAMP up-regulation or LDM.

**LDM Results in Decreased Gating Spring Stiffness.** The shorter timescale of the LDM process allows continuous monitoring of
the mechanical changes of the hair bundle over a period of seconds. Delivering cAMP drugs by a perfusion system over the course of minutes precludes accurate resting position measurements because the movement noise is too high on that timescale. With the shorter activation time of LDM over the time course of ~10 s, we designed an experiment to monitor the changes in the stiffness of the hair bundle, the resting position of the hair bundle, and the change in the activation curve simultaneously (Fig. 3A). Using our high-speed imaging technique and FJ stimulation (19), we subjected rat OHCs to intermittent sine wave stimuli of 4 Hz, and the change in the activation curve simultaneously (Fig. 3C). Concurrently, the hair bundle underwent mechanical changes that developed over a time course similar to the decline in resting open probability during the depolarization (Fig. 4B). In response to sine wave stimulations of identical driver voltage, the resting position of the hair bundle moved forward (Fig. 4D, E, and H) (\(\Delta x_{\text{rest}}\) ~84 mV, \(\pm 27.3\ pm 30.4\) nm), and the normalized displacement of the hair bundle increased (Fig. 4D, E, and H) (\(x_{\text{end-pk}}/x_{\text{start-pk}}\) ~1.20 ± 0.16), indicating that total hair bundle stiffness decreased, which is consistent with our previous observations (Fig. 3H). We observed a small increase in hair bundle stiffness immediately upon depolarization (first +76 mV, 3.6 ± 7.2%), which differed from a previous report (27). Similar observations on resting position and total hair bundle stiffness were made when 10 mM intracellular BAPTA was used (SI Appendix, Fig. S5A–D). The observation that the resting position increases with a decrease in total hair bundle stiffness is only consistent with the hypothesis that LDM decreases \(k_{\text{GS}}\) (Fig. 3A and B).
To increase our confidence in these data, we performed several control experiments to ensure that the mechanical changes observed were not an artifact. First, we performed the same mechanical protocol but did not depolarize the cell (Fig. 4E–I, brown circles (“−84 mV Control”). Next, the effects of depolarization on resting open probability and hair bundle mechanics only occur for hair cells that have not been recently depolarized, since after inducing LDM, the cell requires time (on the order of minutes) to return from the effects of LDM with the rate depending on the intracellular BAPTA concentration (SI Appendix, Fig. S5E–O). Therefore, subsequent depolarizations delivered immediately following the initial depolarization have little to no effect (Fig. 4A, B, and D, gray (“2nd +76 mV”)). Third, cells that were treated with either 0.1 mM dbcAMP or forskolin did not exhibit the increase in resting open probability, change in resting open probability during the depolarization, or changes in hair bundle mechanics (Fig. 4D, red).
cAMP and LDM Decrease the Contribution of the Gating Springs to Total Hair Bundle Stiffness. A third method to confirm a change in $k_{\text{GS}}$ was to measure the contribution of $k_{\text{GS}}$ to $k_{\text{HB}}$ by disrupting tip links. We performed experiments where hair cells were either depolarized or treated with 0.2 mM dbcAMP, and we measured hair bundle displacements before and after severing tip links with iontophoresis of the calcium-chelator EDTA (26, 28). Assuming the stiffness of the hair bundle dbcAMP, 0.42 ± 0.13, $P = 0.011$, unequal variance $t$ test). Together, both depolarization in IHCs, and dbcAMP treatment in OHCs, reduced the contribution of the gating springs to the total stiffness of the hair bundle, indicating that both manipulations decreased the stiffness of the gating springs.

Fig. 5. Depolarization or cAMP reduce the contribution of $k_{\text{GS}}$ to $k_{\text{HB}}$. (A) Image of the hair bundle with an iontophoretic pipette for an IHC (Upper) and OHC (Lower). MET currents were recorded in all experiments to measure tip-link disruption. (B) Hair bundle displacement and MET current data from the IHC shown in A before and after iontophoretic application of EDTA. Notice the change in displacement size and absence of MET current after EDTA. (C) EDTA application produced near-complete tip-link breakage ($> 75 \text{ pA}$ remaining) as measured by the peak MET current in response to a family of force steps (not shown) before and after EDTA treatment ($n = 18$, $P = 4.7E-16$, paired $t$ test). (D) IHC bundle displacement size in response to Fj sine wave stimulation increased more after EDTA application in control than after LDM (displacement ratio, control: $2.30 \pm 0.37$, $n = 7$, dbcAMP: $1.81 \pm 0.41$, $n = 11$, $P = 0.022$, unequal variance $t$ test). (E) Similar to depolarization, dbcAMP decreases $k_{\text{GS}}$ in OHCs (from $0.56 \pm 0.06$ to $0.42 \pm 0.13$, $P = 0.011$, unequal variance $t$ test). Error bars indicate the mean ± SD. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.}

"1st Presentation w/cAMP"), and appeared like a cell that was already subjected to LDM (Fig. 4B, F–l, comparing gray and red) (Max $P_{\text{open}}$ after EDTA; $P = 0.65$; $\Delta P_{\text{open}}$, $P = 0.27$; $\Delta x_{\text{rest}}$, $P = 0.95$; $x_{\text{pk-pk}}$, $P = 0.40$, Tukey–Kramer post hoc test). This observation also supports that the activation of the cAMP pathway and LDM function through the same mechanism.

Finally, we repeated these experiments after calcium-chelator treatment (EDTA) via iontophoresis to sever tip links and, therefore, the gating springs (18, 26, 28). Hair bundles that lack tip links only have $k_{\text{GS}}$ contributing to the hair bundle stiffness and should not exhibit any of the mechanical changes in response to LDM that modulates gating springs. EDTA-treated hair bundles exhibited neither the positive change in resting position nor the increase in displacement size after depolarization (Fig. 4H–I, comparing black to blue) ($\Delta x_{\text{rest}}$, $P = 0.0055$; $x_{\text{pk-pk}}$, $P = 0.0017$, Tukey–Kramer post hoc test). This experiment also indicates that in the absence of tip links, LDM does not change hair bundle stiffness, suggesting that LDM does not affect $k_{\text{GS}}$. One-way ANOVA analysis revealed statistical significance for all measured parameters (Fig. 4E–I), indicating that the experimental condition of LDM was different from the control conditions. Together, these control experiments provide evidence that the mechanical changes we observed are real, thus indicating that LDM causes a decrease in gating spring stiffness.

In all experiments performed in Fig. 4, we observed a negative correlation between the maximum resting open probability that a cell exhibited during the depolarization (typically occurring in the first second (Fig. 4D, black) and the amount of adaptation that the cell exhibited before being depolarized. In other words, OHCs that exhibited smaller magnitudes of slow adaptation before being depolarized typically displayed higher maximum resting open probability values upon depolarization (Fig. 4I) ($r = -0.66$, $P = 5.62E-9$). Higher maximum resting open probability suggested that the cell was further from the LDM state, consistent with a lower adaptation magnitude. This finding supports that cells may exist in a continuum of states where LDM moves the cell toward exhibiting a larger slow adaptation magnitude and lower maximum resting open probability.
cAMP Modulates MET through EPAC, Not PKA. The classic downstream target of cAMP is PKA, also known as the cAMP-dependent protein kinase. cAMP promotes the dissociation of the regulatory and catalytic subunits of PKA to allow phosphorylation of proteins via the catalytic subunit (29). To determine if the effects we observed with cAMP pharmacology were a result of downstream activation of PKA, we inhibited PKA activity while simultaneously activating the cAMP pathway. Using two different PKA inhibitors, H89 and PKI, we tested if PKA inhibition could prevent the changes in the activation curve observed with cAMP activation. H89 inhibits PKA by competing for the ATP binding site on the catalytic subunit (29). We used H89 at 10 μM, over 200 times its $K_i$ (30) and 10 times greater than the concentration used in IHCs to inhibit PKA modulation of potassium currents (31). At this concentration, data were similar to that using an activator alone (Fig. 6C–G, blue) (Half-Act: from 29.1 ± 9.7 nm to 55.0 ± 34.4 nm, $P = 0.013$; Width$_{10,90}$: from 65.1 ± 15.0 nm to 96.8 ± 50.0 nm, $P = 0.038$; Resting $P_{\text{open}}$: from 4.9 ± 0.8% to 3.3 ± 1.4%, $P = 0.0018$; Adaptation: from 22.6 ± 7.8% to 44.7 ± 23.5%, $P = 0.0021$; $\tau_c$: from 28.9 ± 47.5 ms to 12.9 ± 5.9 ms, $P = 0.23$; paired $t$ test). PKI is also an inhibitor of the catalytic subunit of PKA by binding to the substrate-binding pocket while unable to be phosphorylated (29). PKI has a $K_i$ of 1.7 nM according to the manufacturer, and we used PKI intracellularly at 1 μM and 5 μM and did not observe any difference. Data with PKI corroborated the data with H89 and were also similar to data with activator alone (Fig. 6C–G, purple) (Half-Act: from 29.1 ± 9.0 nm to 60.1 ± 19.8 nm, $P = 0.0013$; Width$_{10,90}$: from 73.7 ± 10.1 nm to 90.6 ± 22.7 nm, $P = 0.048$; Resting $P_{\text{open}}$: from 5.0 ± 1.1% to 3.0 ± 1.3%, $P = 0.0076$; Adaptation: from 24.6 ± 16.7% to 54.1 ± 28.6%, $P = 0.0080$; $\tau_c$: from 31.6 ± 50.6 ms to 15.8 ± 12.6 ms, $P = 0.41$; paired $t$ test). Unexpectedly, these data indicate that PKA is not responsible for the rightward shift and increased width of the activation curve observed with cAMP activation.

Another potential cAMP signaling pathway is through the EPAC (32, 33). To test whether EPAC activation can recapitulate the cAMP effect, we used the EPAC-specific agonist 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-CPT). 8-CPT is a potent and selective superagonist of EPAC over PKA (34). 8-CPT can be used up to about 10 μM, where there is about 20% activation of PKA and near 100% activation of EPAC (34). When 8-CPT was included in the intracellular solution at 1 μM and 5 μM, hair cells behaved similarly to when the cAMP pathway is activated with dbcAMP or forskolin with a rightward shift of the activation curve and increase in adaptation magnitude over time (Fig. 6, green) (Half-Act: from 38.9 ± 1.9 nm to 56.8 ± 23.8 nm, $P = 0.0087$; Width$_{10,90}$: from 87.8 ± 34.1 nm to 102.1 ± 33.2 nm, $P = 0.046$; Resting $P_{\text{open}}$: from 4.7 ± 1.1% to 3.4 ± 1.5%, $P = 0.013$; Adaptation: from 25.8 ± 10.2% to 48.7 ± 23.9%, $P = 0.0035$; $\tau_c$: from 22.6 ± 15.3 ms to 20.2 ± 12.1 ms, $P = 0.71$; paired $t$ test). The results from all three pharmacological manipulations (H89, PKI, and 8-CPT) are consistent with the cAMP effect being mediated by EPAC. We also tested whether there was a baseline level of cAMP activity by using the cAMP inhibitor Rp-cAMPS at 0.1 mM, near the IC$_{50}$ for EPAC1 (35). We found that there was no effect with Rp-cAMPS on the hair cell activation curve (SI Appendix, Fig. 5E). Taken together, these data suggest that cAMP functions through an EPAC mediated pathway, not PKA, to decrease gating spring stiffness.

Discussion

Regulation of hair cell MET is critical to our auditory system's high precision and wide dynamic range (11–13). Growing evidence indicates the existence of other mechanisms that modulate the activity of the MET channel apart from the classic adaptation processes (24, 36). The present study expands on early work from turtle and bullfrog hair cells (15, 16) and demonstrates that up-regulation of the second messenger cAMP, as well as LDM, modulates the MET channel by decreasing the gating spring stiffness. Data are also consistent with the cAMP effect occurring through activation of EPAC and not the classic PKA mediated pathways.

In this study, we first demonstrated that in rat OHCs, pharmacological agents that increased intracellular cAMP signaling reduced the sensitivity of the MET channel (Fig. 1). We subsequently characterized another manipulation, LDM. Previously, we characterized the changes that occur during depolarization, where there is initially an increase in the resting open probability followed by a subsequent decrease in open probability (24). The increase in resting open probability involved the lipid bilayer and is a separate mechanism from the LDM described here. LDM results in a decline in resting open probability and alters MET properties similarly to cAMP up-regulation (SI Appendix, Figs. S2 and S3). We also hypothesize that hair cells exist in a continuum of states where the MET channel resting open probability and the magnitude of slow adaptation are inversely related. Further experiments demonstrated that cAMP up-regulation combined with LDM did not affect MET or hair bundle mechanics in an additive manner, suggesting that the two manipulations act at a common end effector site (i.e., gating spring) to modulate MET (Fig. 2), potentially through different signaling cascades. Both manipulations resulted in decreased MET channel sensitivity, consistent with a decrease in gating spring stiffness (6).

To test the hypothesis of reduced gating spring stiffness, we performed experiments that allowed us to quantify mechanical changes in the hair bundle. In a simplistic view, the stiffness of the hair bundle consists of the contributions of the stiffnesses of the gating springs ($k_{\text{sp}}$) and stereocilia pivots ($k_{\text{SP}}$), which encompasses contributions by other interstereociliary links as well. The gating springs can contribute ~50% of the total hair bundle stiffness (18, 26). At rest, the hair bundle position is determined by the tension in the tip link pulling the hair bundle in the negative direction and the resistive force of the stereocilia pivots. Alterations in $k_{\text{sp}}$, but not $k_{\text{SP}}$, are associated with changes in the activation curve (Fig. 3A–Q). We first found that cAMP up-regulation and LDM were associated with a decrease in hair bundle stiffness, which was correlated with a widening of the activation curve (Fig. 3J), all being consistent with an effect on $k_{\text{sp}}$. Next, we found that in response to LDM, the hair bundle exhibited a positive change in the resting position (toward the tallest row of stereocilia) and an increase in the displacement size (Fig. 4), which is consistent with a gating spring stiffness decrease, not a decrease in stereocilia pivot stiffness (Fig. 3A and B). Additionally, LDM in the absence of tip links did not change the stiffness of the hair bundle, indicating no effect on $k_{\text{SP}}$.

Finally, using an iontophoresis technique to sever tip links, we could estimate the change in the stiffness of the gating springs in response to either cAMP analogs or depolarization by measuring the displacement of the hair bundle in response to a PJ stimulus of the same fluid drag force before and after severing the tip-links. We found that both cAMP and depolarization decreased the contribution of the gating springs to the total hair bundle stiffness (Fig. 5). The gating spring theory posits that changes in gating spring stiffness will result in a change in the MET channel sensitivity (6). With cAMP up-regulation using dbcAMP, we observed a 47% average increase in the width of
Fig. 6. cAMP modulates MET through EPAC, not PKA. (A) Example traces of hair bundle displacement and MET currents from a rat OHC elicited by FJ force steps with 5 μM 8-CPT in the intracellular solution. Early traces were obtained ~5 min after entering whole-cell mode. The late data were collected ~15 min after entering whole-cell mode. (B) MET current versus hair bundle displacement (activation curve) for the data presented in A. (C and D) Summary plots for the half activation (Half-Act) and 10–90 width (Width10–90) of the activation curve. H89 was used at 0.01 mM, and PKI was used at 1 and 5 μM with either forskolin or dbcAMP as the activator. Data were taken in the presence of the inhibitor and after 10 min of activator application. 8-CPT was used in the intracellular solution at 1 and 5 μM and traces were taken early and late after entering whole-cell mode. Number of cells: H89 and activator n = 15, PKI and activator, and 8-CPT produced a significant increase in adaptation magnitude (P = 0.0021, P = 0.0080, P = 0.0035, paired t tests). Error bars indicate the mean ± SD. *P ≤ 0.05, **P ≤ 0.01.

the activation curve (Fig. 1D). According to the gating spring theory, this would be the result of a 32% decrease in gating spring stiffness. When combining the measured changes in $k_{1B}$ (Fig. 3H) and $k_{G}/k_{1B}$ (Fig. 5H), we estimate a 54% decrease in $k_{G}$ on average upon cAMP pathway activation. These two values, which are calculated based on different experimental measures, are consistent with each other, suggesting that $k_{G}$ changes can account for most of the stiffness changes we observed. These data indicate that cAMP and LDM modulate hair cell MET by decreasing gating spring stiffness.

Knowing that the gating spring is the target of cAMP activation, we sought to determine the signaling pathway. PKA is the classic cAMP target; however, two specific PKA inhibitors were unable to block the CAMP effect (Fig. 6C–G, blue and purple). Instead, we found that cAMP functions through EPAC to mediate the decrease in gating spring stiffness by using an EPAC-specific activator (Fig. 6, green). Failure of PKA-specific inhibitors to block the CAMP effect along with the specific EPAC activator (8-CPT) mimicking the CAMP effect strongly suggests the EPAC pathway mediating the decreased gating spring stiffness. Ideally, inhibition of the EPAC pathway would help to further support the pathway, however, current inhibitors of the EPAC pathway, like ESI-09 and HJC0197, have general protein denaturing capabilities and may not be competitive inhibitors of EPAC (37). Further experiments are still required to identify an appropriate inhibitor of the pathway, but current data are supportive of an EPAC pathway regulating hair cell MET.
Implications for Auditory Function. cAMP signaling is a ubiquitous pathway in many cell types and serves a variety of different cellular functions. Although the effect of cAMP on MET was documented over two decades ago, the mechanism of action and signaling pathway has not been elucidated until now. These findings provide a potential mechanism for G protein-coupled receptors (GPCRs) to modulate gating spring stiffness and the sound transduction process.

A potential function of the cAMP pathway in hair cells is to provide some level of protection during levels of high-intensity stimulation. cAMP could be up-regulated to decrease MET sensitivity to protect against damage. Another potential pathway could operate through GPCRs and downstream activation of G\(\alpha\) to stimulate adenyl cyclase activity to modulate MET activity, but the participation of GPCRs in hair cell MET regulation is yet to be explored.

A resting level of cAMP could allow for bidirectional control of gating spring stiffness, hence hair cell sensitivity. This has been demonstrated to modulate spontaneous oscillation frequency in bullfrog saccular hair cells (16). However, in mammalian hair cells, there does not appear to be a resting level of cAMP activity since Rp-cAMPS, which inhibits both PKA and EPAC (to a lesser extent) activation (35, 38), did not cause a leftward shift in the activation curve (SI Appendix, Fig. S6). This result argues against cAMP levels being the determinant of where a given hair cell sits on the continuum of states shown by the resting open probability and baseline level of slow adaptation (Figs. 2F and 4F); however, this hypothesis cannot be fully ruled out since further experiments using higher concentrations of Rp-cAMPS and different EPAC inhibitors are required.

Potential Molecular Mechanisms of Modulating Gating Spring Stiffness. The molecular and structural composition of the gating springs is still debated (36). The tip link was originally thought to be the gating spring (6), however, electron microscopy and molecular dynamics simulations later suggest that the tip link itself may not be the gating spring due to its rigidity (39, 40). Even more recent data have revived the suggestion that the tip link could contribute to the gating spring (41, 42). Even if the tip link contributes to the gating spring stiffness through the structure of its extracellular domains, it is unlikely to be the target of the cAMP effect since the EPAC signaling pathway is likely restricted to intracellular targets. In addition to the tip link, gating spring stiffness may arise from any component in series with the tip link. Potential elements could be the myosin motors (like myosin VIIa) or their adapter proteins at the upper tip-link insertion point (9, 43, 44), as well as other components at the lower tip-link insertion important for MET, such as CIB2, TMIE, LHFPL5, or TMC1 (2, 3, 45–48). The gating spring stiffness may also potentially arise from its interaction with the lipid bilayer of the stereocilia membrane, where the upper and lower ends of the tip link insert before being anchored to the F-actin core (49). Any of these components could be the target of the EPAC-mediated regulation.

EPAC acts as a guanine exchange factor for the Rap1 and Rap2 small GTPases. Rap has many potential downstream effectors, pathways, which could lead to modulating gating spring stiffness (33). A notable pathway is one that down-regulates the RhoA signaling pathway to increase smooth muscle relaxation (50) and could target the actin cytoskeleton in hair bundles. More detailed studies are required to elucidate the complete signaling pathway. A recent study has localized both EPAC1 and EPAC2 to hair cells in both auditory and vestibular hair cells (51). Only one other study has investigated EPAC in hair cells, where EPAC1 was up-regulated in hair cells following noise exposure and promoted the loss of hair cells (52). Our observations on decreasing gating spring stiffness appear opposite to the effect observed in this study, since decreasing the sensitivity would likely be a protective mechanism to help prevent excitotoxicity of the hair cell. Reconciling these findings may lie in the timescale of effects since our study looked at the effect on the minute timescale, whereas the other study looked at effects on the timescale of days. Nonetheless, these published studies confirm the presence of EPAC in hair cells.

A recent study demonstrated that the diffusivity of the lipid membrane is correlated with the resting \(P_{\text{open}}\) of the MET channel; manipulations that decrease membrane diffusivity increase the resting \(P_{\text{open}}\) of the channel (53). Importantly, during a long depolarization (i.e., LDM), the membrane diffusivity increased, suggesting that in the LDM state, membrane diffusivity is increased. It is reasonable to hypothesize that lipid diffusivity may contribute to gating spring stiffness by altering how rigidly the tip link is held in place within the membrane (at either the upper or lower tip-link insertion) or the compliance of the membrane surrounding the channel if MET channels are gated via the lipid membrane itself. Further work is necessary to determine if cAMP/EPAC signaling also increases membrane diffusivity.

In summary, our findings demonstrate that cAMP and long depolarization both modulate the activity of auditory MET by decreasing the stiffness of the gating springs that gate the MET channel. These results demonstrate cAMP regulation of the MET process in mammalian hair cells and introduce a potential mechanism for GPCR regulation of hair cell function.

Methods Summary

Animals were killed using methods approved by the University of Colorado Institutional Animal Care and Use Committee. Patch-clamp electrophysiology, mechanical stimulation, and high-speed imaging of hair bundles are similar to previously described methods on rat cochlear hair cells (18, 19, 24). Iontophoresis was performed using an MVCS 02 (NPI Electronic). Hair bundle stiffness estimates were performed similarly to the methods previously described (26). Data were analyzed using jClamp (SciSoft Company), MATLAB (MathWorks), and Excel (Microsoft). Data are presented as mean ± SD unless otherwise noted. Detailed materials and methods are provided in SI Appendix, Materials and Methods.

Data Availability. All data are available upon request by contacting the corresponding author.

ACKNOWLEDGMENTS. We thank Jong-hoon Nam and Gregory Frolenkov for valuable input regarding the work in this manuscript; and Mark Dell’Acqua for steering us toward investigating EPAC-mediated pathways. Initial characterization of LDM was done at Stanford University under support of K99 DC013299 and R01 DC003896. This work was also supported by National Institute on Deafness and Other Communication Disorders Grants R00 DC013299 and R01 DC016868 (to A.W.P.), F31 DC018457 (to A.A.M.), and R21 DC019701 (to G.A.C.).

1. M. Beurg, R. Fettiplace, J. H. Nam, A. J. Ricci, Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. Nat. Neurosci. 12, 553–558 (2009).
2. B. Pan et al., TMC1 forms the pore of mechanosensitive transduction channels in vertebrate inner ear hair cells. Neuron 99, 736–753.e6 (2018).
3. C. L. Cunningham et al., TMIE defines pore and gating properties of the mechanotransduction channel of mammalian cochlear hair cells. Neuron 107, 126–143.e8 (2020).
4. M. Beurg, W. Xiong, B. Zhao, U. Müller, R. Fettiplace, Subunit determination of the conductance of hair-cell mechanotransducer channels. Proc. Natl. Acad. Sci. U.S.A. 112, 1589–1594 (2015).
