Chloride secretion by semicircular canal duct epithelium is stimulated via $\beta_2$-adrenergic receptors

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The lumen of the vestibular labyrinth is filled with endolymph, a fluid with a high concentration of K$^+$ (149 mM) and a low concentration of Na$^+$ (9 mM) (36). This composition is necessary to support the transduction of acceleration by the vestibular sensory cells into nerve signals to the brain. The epithelium forming the boundary of the endolymphatic compartment is composed of many epithelial cell types, including the neuroepithelial sensory hair cells. Vestibular dark cells are known to be responsible for K$^+$ secretion (19) under adrenergic control (31, 34), and transitional cells are known to be responsible for cation reabsorption (15). Net cation movements cannot occur in isolation and must be balanced by transport of anions to maintain bulk electroneutrality. The transcellular and/or paracellular routes of Cl$^-$ movements in the inner ear have not previously been determined. It was of interest to determine whether the canal ducts provide this function, because a polarized primary culture of epithelial cells of the semicircular canal duct from neonatal rats was recently developed that produced an apical-negative transepithelial voltage ($V_T$) and associated apical-to-basal short-circuit current ($I_{sc}$) (21). This $I_{sc}$ could be due to anion secretion and/or cation absorption.

The goals of the present study were to determine whether the semicircular canal duct epithelium engages in anion secretion and/or cation absorption, whether it is under adrenergic control, and whether the primary culture has a phenotype that represents the native tissue. Dysfunction of transport and its regulation by this epithelium may be one basis of pathological states such as Meniere’s disease.

METHODS

Temporal bones were removed after decapitation from neonatal Wistar rats (3–5 days after birth) and adult gerbils (4- to 5-wk-old females), and the semicircular canal ducts were dissected from the vestibular labyrinth. Gerbils were anesthetized before euthanasia by injection of pentobarbital sodium (50 mg/kg, ip). All procedures conformed to protocols approved by the Institutional Animal Care and Use Committees. Canals were dissected and prepared for primary culture, transferred to a perfusion chamber on the stage of an inverted microscope (Nikon TE-300) for measurement of $I_{sc}$ density, or used for measurement of cAMP accumulation.

Epithelial cultures. Cells from neonatal rat semicircular canal epithelium, exclusive of the common crus, were dispersed and seeded on permeable culture dish inserts and cultured as described previously (21). Cells were seeded at a density of 10,000 cells/cm$^2$.
density of 5–18 canals/cm² on inserts with 0.4-μm pores in 15-μm-thick polyester membrane (1.6 × 10⁶ pores/cm²). The inserts were either 6.5 (Transwell; Costar, Cambridge, MA) or 12 mm in diameter (Snapwell; Costar).

Confluent monolayers of primary cultures were mounted in an Ussing chamber (catalog no. AH 66-0001; Harvard Apparatus, Holliston, MA) maintained at 37°C. For most experiments, both sides of the epithelium were bathed in bicarbonate-buffered physiological saline, which was stirred by bubbling with a mixture of 95% O₂ and 5% CO₂. The composition of the solution was (in mM) 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, and 5 glucose, pH 7.4. A HEPES-buffered solution bubbled with air was used for the Ba²⁺ experimental series to avoid potential problems with Ba²⁺ precipitation; its composition was (in mM) 150 NaCl, 10 Na-HEPES, 3.6 KCl, 1 MgCl₂, 0.7 CaCl₂, and 5 glucose, pH 7.4. The HEPES-buffered solution did not alter the response of Iₛₑ to forskolin.

Experimental agents were added to the bath at 1,000X concentrations. Histamine (catalog no. H-7375, Sigma, St. Louis, MO), vasopressin (catalog no. V-8975, Sigma), (-)-isoproterenol (catalog no. I-6504, Sigma), (-)-norepinephrine (catalog no. A-9512, Sigma), CGP-20712A (catalog no. C-231, Sigma), and ICI-118551 (catalog no. I-127, Sigma) were dissolved in H₂O, whereas forskolin (catalog no. F-6886, Sigma), ouabain (catalog no. O-3125, Sigma), glibenclamide (catalog no. G-0639, Sigma), 4,4’-disothiocyanostilbene-2,2’-disulfonic acid (DIDS; catalog no. D-3514, Sigma), and 5’-(N-ethylcarboxamido)adenosine (NECA; catalog no. E-2387, Sigma) were dissolved in dimethyl sulfoxide (DMSO). DMSO never exceeded 0.6% final concentration.

Fluxes of Cl⁻, Na⁺, and Rb⁺ (K⁺). The following isotopes were used for measuring transepithelial ion fluxes from cultured neonatal rat semicircular canal epithelium: ³²Na for sodium was used at 84 Bq/μL, ⁸⁶Cl for chloride was used at 38 Bq/μL, and ⁸⁶⁶Rb for potassium was used at 260 Bq/μL. We assumed that the tracers moved in the same ways as nonradioactive Na⁺, Cl⁻, and K⁺. Cultures grown on 12-mm inserts were used for flux measurements. Electrodes connecting the voltage-current clamp to the Ussing chamber consisted of Ag-AgCl connected to the bath solutions via a bridge of 1 M KCl and 2% agarose (catalog no. Fluka 05066, Sigma).

The experimental protocol consisted of a 20-min initial period during which Vₜ reached a steady state. The radioisotope was added to the apical or basal compartment, and the epithelium was voltage clamped to zero and allowed to reach a steady state for >50 min. Three samples of 100 μl were collected from each compartment at this time and again 20 min later. Isoproterenol (10 μM) was added to the basal compartment, a steady-state current was reached after 5–10 min, and three samples of 100 μl were again collected from each compartment at this time and 20 min later. After each withdrawal, fresh buffer was added to maintain a constant volume. Samplings were accompanied by measurement of current and open-circuit voltage.

³²Cl and ⁸⁶⁶Rb were counted in 2 ml of liquid scintillation fluid (Aquasafe 500 plus, Zinsser Analytic, Frankfurt, Germany) for 5 min per sample. ²²Na was counted in a gamma counter for 10 min per sample, up to eight times because of high background.

Unidirectional flux = (C × [B])/[(S × T × [R])

where C, expressed in counts per minute (cpm), is the quantity of isotope arriving into the cold (unlabeled) compartment. C is corrected for background and dilution due to samplings and refillings. [B] (in μmol/ml) is the total concentration of ion under study. S (in cm²) is the surface area of the epithelium. T (in min) is the duration of the flux measurement. [R] (in cpm/ml) is the concentration of radioactivity in the hot compartment.

Net fluxes were obtained by subtraction of the mean apical-to-basal flux from the mean basal-to-apical flux.

Electrophysiological recordings. Vₜ, Iₛₑ, and resistance (Rₛₑ) were measured from cultured neonatal rat canal with an epithelial voltage-current clamp amplifier (model VCC600, Physiologic Instruments, San Diego, CA; or model DVC 1000p, World Precision Instruments, Sarasota, FL). Vₜ and Rₛₑ were measured during current clamp, and the equivalent Iₛₑ was calculated from Iₛₑ = Vₛₑ/Rₛₑ. During flux measurements, the epithelium was voltage-clamped to zero and Iₛₑ was measured directly.

Table 1. Ion flux responses to isoproterenol of primary cultures of semicircular canal duct epithelium

<table>
<thead>
<tr>
<th>condition</th>
<th>Rₛₑ (n)</th>
<th>Iₛₑ (n)</th>
<th>Jₛₑ, 10⁻³ (n)</th>
<th>Jₛₑ, 10⁻³ (df)</th>
<th>Jₛₑ, 10⁻³ (df)</th>
</tr>
</thead>
<tbody>
<tr>
<td>³²Cl⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.70 ± 1.40(14)</td>
<td>0.51 ± 0.16(14)</td>
<td>1.35 ± 0.16(7)</td>
<td>2.35 ± 0.29(7)</td>
<td>1.0 ± 0.12(12)</td>
</tr>
<tr>
<td>Iso</td>
<td>5.20 ± 0.95(19)</td>
<td>2.33 ± 0.53(19)</td>
<td>3.86 ± 0.47(10)</td>
<td>5.19 ± 0.42(9)</td>
<td>1.52 ± 0.61(17)</td>
</tr>
<tr>
<td>⁸⁶⁶Rb⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.40 ± 1.60(12)</td>
<td>0.71 ± 0.18(12)</td>
<td>0.07 ± 0.009(6)</td>
<td>0.12 ± 0.017(6)</td>
<td>0.05 ± 0.02(10)</td>
</tr>
<tr>
<td>Iso</td>
<td>5.70 ± 1.18(12)</td>
<td>1.44 ± 0.26(12)</td>
<td>0.09 ± 0.003(6)</td>
<td>0.16 ± 0.017(6)</td>
<td>0.07 ± 0.04(10)</td>
</tr>
<tr>
<td>³²⁷Na⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.00 ± 0.76(12)</td>
<td>0.48 ± 0.14(12)</td>
<td>2.96 ± 0.44(6)</td>
<td>4.81 ± 0.75(6)</td>
<td>1.82 ± 0.95(10)</td>
</tr>
<tr>
<td>Iso</td>
<td>4.90 ± 0.55(12)</td>
<td>3.31 ± 0.73(12)</td>
<td>3.21 ± 0.18(6)</td>
<td>3.32 ± 0.77(6)</td>
<td>0.13 ± 0.87(10)</td>
</tr>
</tbody>
</table>

Transepithelial resistance (Rₛₑ), short-circuit current (Iₛₑ), apical-to-basolateral flux (Jₛₑ,ab), basolateral-to-apical flux (Jₛₑ,ba), net flux (Jₛₑ,net = Jₛₑ,ab - Jₛₑ,ba), and the difference between Iₛₑ and Jₛₑ,net expressed as current (Jₛₑ,net, μA/cm²) are listed for confluent epithelial cultures grown on Snapwell (12-mm diameter) permeable supports; epithelial cultures were selected for high resistance (>5 KΩcm²). Iso, isoproterenol (10 μM). Fluxes are expressed as nmol·min⁻¹·cm⁻². Values are means ± SE; n, no. of confluent epithelia; df, degrees of freedom. *P < 0.05; †P < 0.01; ns, not significant.
cAMP was measured in the supernatant with a colorimetric immunoassay according to the manufacturer’s protocol (RPN 225, Amersham, Piscataway, NJ). The sensitivity of the assay ranged from 12.5 to 3,200 fmol cAMP per well. Results were normalized to the cAMP production induced by 1 μM isoproterenol.

Voltage-sensitive vibrating probe. The vibrating probe technique was identical to that previously described (15). Briefly, the current density (proportional to the $I_{sc}$) was monitored from neonatal rat or adult gerbil semicircular canal ducts by vibrating (200–400 Hz) a Pt-Ir wire microelectrode with a Pt-black tip positioned 20–30 μm from the apical surface of the epithelium with computer-controlled, stepper-motor manipulators (Applicable Electronics, Forest Dale, MA) and probe software (ASET version 1.05, Science Wares, East Falmouth, MA). The bath references were 26-gauge Pt-black electrodes. The signals from the phase-sensitive detectors were digitized (0.5 Hz, 16 bit), and the output was expressed as current density at the electrode. In this series of experiments, the HEPES-buffered solution was used. The solution in the chamber was exchanged 0.6 times per second and maintained at 37°C.

Pharmacology. EC$_{50}$ and $K_{DB}$ values were calculated as described previously (26, 33, 34). The agonist concentration that caused a half-maximal effect (EC$_{50}$) was obtained by fitting data to the Hill equation: $E = E_{\text{max}} \times C^n / (EC_{50}^n + C^n)$, where $E_{\text{max}}$ is the maximal effect, $C$ is the concentration of the agonist, and $n$ defines the slope. The affinity of the antagonists to the receptor ($K_{DB}$) was obtained from cumulative dose-response curves in the absence and presence of antagonist. $K_{DB}$ was obtained from the Schild equation: $p(K_{DB}) = \log(y) - \log(DR - 1)$, where $y$ is the concentration of the antagonist and DR is the dose ratio. The DR was obtained according to DR = EC$_{50}$ antagonist/EC$_{50}$ agonist, where “EC$_{50}$ antagonist” is the EC$_{50}$ of isoproterenol in the presence of antagonist and “EC$_{50}$ agonist” is the EC$_{50}$ in the absence of the antagonist. All nonlinear curve fits were obtained by a least-squares algorithm using a programmable spreadsheet and plotting software (Origin 6.1, OriginLab, Northampton, MA). The $\beta_1$, $\beta_2$, and $\beta_3$-adrenergic receptor subtypes can be distinguished by the relative affinity of the antagonists ICI-118551 and CGP-20712A (27, 33, 34).

Statistical analysis. The Student’s $t$-test was used to determine statistical significance of paired samples. Variance homogeneity was verified with Fisher’s or Bartlett’s test before computing unpaired Student’s $t$-test or ANOVA, respectively, for ion flux data (30). A logarithmic transformation of data or the Aspin Welch test (a modified Student’s unpaired $t$-test) was used when the variances were significantly different (30). Data are expressed as means ± SE (n = no. of tissues). Dose-response curves of agonists were normalized to the response to 10 μM forskolin or 10 μM isoproterenol.

![Fig. 1. Cl⁻ fluxes under stimulation by isoproterenol. Open bars represent unidirectional ³⁶Cl tracer fluxes during stimulation with isoproterenol (10 μM). Hatched bars represent net flux expressed in current and short-circuit current ($I_{sc}$), which are not significantly different; net flux is significantly >0. BA, basolateral-apical flux; AB, apical-basolateral flux. * $P < 0.05$; ns, not significant.](http://ajpcell.physiology.org/)

![Fig. 2. Representative recordings of transepithelial voltage ($V_T$) from primary cultures of semicircular canal on permeable supports. A: response to forskolin (10⁻⁵ M) on the basolateral side. B: response to increasing concentrations of isoproterenol (10⁻¹⁰–10⁻⁶ M) and to forskolin (FSK; 10⁻⁵ M) on the basolateral side. C: absence of response to vasopressin (VP; 10⁻⁸ M) and histamine (Hist; 10⁻⁴ M). Pulses are the responses of $V_T$ to current pulses (1 μA, 0.3-s duration, repeated every 10 s).](http://ajpcell.physiology.org/)
shown). However, several Cl⁻/H11002 propyl amiloride (EIPA) were not significant (data not shown). The synthetic and natural agonists for β-adrenergic receptors, isoproterenol and norepinephrine, increased the magnitude of $I_{sc}$ by stimulation of β-adrenergic receptor activation (2, 6, 16, 23, 24).

**RESULTS**

**Confluent primary cultures of neonatal rat canal epithelium.** The previously-found apical-side negative $V_T$ of primary cultured epithelium from the semicircular canal ducts was hypothesized to be due to Cl⁻ secretion and/or Na⁺ absorption. Preliminary experiments showed that the responses to apical addition of the Na⁺ transport inhibitors amiloride and ethylisopropyl amiloride (EIPA) were not significant (data not shown). However, several Cl⁻-secreting epithelia are known to be stimulated by β-adrenergic receptor activation (2, 6, 16, 23, 24).

**Net fluxes of Cl⁻, Na⁺, and Rb⁺ across cultured neonatal rat canals.** To determine the ionic basis of the electrophysiological transport by this epithelium, we measured unidirectional fluxes of Cl⁻, Na⁺, and Rb⁺ (for K⁺) and calculated the net fluxes. Inserts of high $R_T$ (≥5 kΩ·cm²) were selected to minimize the background of passive paracellular fluxes. Net fluxes were also measured across epithelia stimulated by the β-adrenergic receptor agonist isoproterenol.

In the absence of isoproterenol, a net Cl⁻ secretion was observed, but no net absorption of Na⁺ (Table 1). All of the $I_{sc}$ could be accounted for by the net Cl⁻ flux, because the difference was not significantly different from zero. A small net basolateral-to-apical Rb⁺ (K⁺) flux was seen that amounted to only ~5% of the net Cl⁻ flux. This Rb⁺ flux was not due to the presence of K⁺-secreting dark cells in the cultured epithelium because cells from the common crus were assiduously excluded from the present series of experiments. We functionally tested for the presence of dark cells by addition of DIDS (500 μM) to the apical bath and found no response of $V_T$ (data not shown and Fig. 5). DIDS strongly increases the positive $V_T$ across dark cells (29).

Addition of isoproterenol (10 μM) to the basolateral compartment led to a strong increase in the net Cl⁻ secretory flux but no change in either the Na⁺ or Rb⁺ (K⁺) net fluxes (Table 1). All of the $I_{sc}$ could be accounted for by the net Cl⁻ flux (Fig. 1, Table 1). $I_{sc}$ increased significantly and $R_T$ decreased significantly after addition of isoproterenol.

Increase in $I_{sc}$ by stimulation of β2-adrenergic receptors. The synthetic and natural agonists for β-adrenergic receptors, isoproterenol and norepinephrine, increased the magnitude of $I_{sc}$ of cultured neonatal rat canals with an EC₅₀ of 3 nM (pEC₅₀ = 8.6 ± 0.1, n = 15) and 15 nM (pEC₅₀ = 7.8 ± 0.3, n = 12; $P < 0.05$), respectively, on the basal side (Figs. 2B and 3, Table 2). Isoproterenol had no effect when added to the apical solution (not shown).

β-Adrenergic receptors are usually linked to adenyl cyclase via the heterotrimeric G protein of the Gₛ type. Activation of adenyl cyclase by forskolin (10 μM) caused a substantial increase in $I_{sc}$ from 1.0 ± 0.3 to 2.4 ± 0.3 μA/cm² ($n = 20$), although $R_T$ in this experimental series did not change between control and forskolin conditions (1.8 ± 0.2 vs. 1.8 ± 0.2 kΩ·cm²) (Fig. 2A). Inserts were not selected for high resistance in this series of experiments. At full stimulation with either isoproterenol or norepinephrine, there was no further change in $V_T$ or $I_{sc}$ with addition of forskolin (Fig. 2B).

The β-adrenergic receptor antagonist CGP-20712A (1 μM) had no effect (1.9 ± 1.3%, n = 10) after stimu-

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**Table 2. Electrophysiological response to isoproterenol of primary cultures of semicircular canal duct epithelium**

<table>
<thead>
<tr>
<th></th>
<th>6.5-mm-Diameter Supports</th>
<th>12-mm-Diameter Supports</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated ($n = 15$)</td>
<td>Isoproterenol (10 μM) ($n = 15$)</td>
</tr>
<tr>
<td>$V_T$, mV</td>
<td>-1.26 ± 0.21</td>
<td>-3.02 ± 0.45*</td>
</tr>
<tr>
<td>$R_T$, (kΩ·cm²)</td>
<td>1.56 ± 0.26</td>
<td>1.60 ± 0.23</td>
</tr>
<tr>
<td>$I_{sc}$, μA/cm²</td>
<td>-0.84 ± 0.11</td>
<td>-2.02 ± 0.30*</td>
</tr>
</tbody>
</table>

Transepithelial voltage ($V_T$), $R_T$, $I_{sc}$, and equivalent $I_{sc}$ ($I_{sc,eq} = V_T/R_T$) are listed for cultures grown on Transwell (6.5-mm diameter) and Snapwell (12-mm diameter) permeable supports; the latter were selected for high resistance (≥5 kΩ·cm²). *$P < 0.05$. 

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Fig. 4. Specific inhibition of the current from cultured epithelia stimulated by \( \beta \)-adrenergic receptors. The current stimulated by isoproterenol (0.1 \( \mu \)M) was inhibited by CGP-20712A or ICI-118551, specific antagonists of \( \beta_1 \)- or \( \beta_2 \)-adrenergic receptors, respectively.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Specific inhibition of the current from cultured epithelia stimulated by \( \beta \)-adrenergic receptors.}
\end{figure}

lnation by isoproterenol (100 \( \mu \)M), whereas the antagonist ICI-118551 inhibited \( I_{sc} \) with an \( IC_{50} \) of 6 ± 2 \( \mu \)M \((n = 15)\) and a \( K_{DB} \) of 0.20 ± 0.06 \( \mu \)M, indicating that ion transport by this epithelium was stimulated via \( \beta_2 \)-adrenergic receptors (Fig. 4; see DISCUSSION).

We tested agonists for several other \( G_{s} \)-linked receptors: histamine for histamine receptors, arginine vasopressin for vasopressin receptors, and NECA for adenosine receptors. The agonists were added at high concentration to the basolateral compartment of the epithelium, and \( V_T \) and \( R_T \) were continuously recorded. No significant changes in \( V_T \) or \( R_T \) were observed for histamine (10\(^{-4}\) M) and vasopressin (10\(^{-8}\) M) (34) (Fig. 2C) or for NECA (10\(^{-5}\) M) (22).

**Pharmacological test for apical CFTR.** \( Cl^- \) secretion across the apical membrane in many epithelia is mediated by the CFTR \( Cl^- \) channel (28). Although an unequivocal pharmacological criterion for the presence of functional CFTR has not been developed, it is widely accepted that stimulation of secretory current by apical genistein (30 \( \mu \)M), inhibition by glibenclamide (50–300 \( \mu \)M), and no effect of the broad-spectrum anion transport inhibitor DIDS (500 \( \mu \)M) indicate mediation of the current by CFTR (1, 28).

Genistein (30 \( \mu \)M) significantly increased \( I_{sc} \) in primary cultures of neonatal rat canal epithelium in the absence of forskolin and in the presence of submaximal (1 \( \mu \)M) forskolin (Fig. 5, A and B), consistent with CFTR. However, there was no effect of apical genistein following stimulation of \( I_{sc} \) with a higher concentration of forskolin (10 \( \mu \)M) (Fig. 5C), and, importantly, apical glibenclamide (300 \( \mu \)M) as well as DIDS (500 \( \mu \)M) had no effect on \( I_{sc} \) (Fig. 5, A and C).

**Decrease in \( I_{sc} \) by blockers of \( K^- \) channels and \( Na^- \)-\( K^- \)-ATPase.** Basolateral addition of \( Ba^{2+} \) decreased the magnitude of \( I_{sc} \) of forskolin-stimulated cultured neonatal rat canals with an \( IC_{50} \) of 0.27 mM \((n = 3–6)\) (Fig. 6, A and C). \( I_{sc} \) was reduced 55 ± 4% \((n = 6)\) by 1 mM \( Ba^{2+} \). Basolateral ouabain (1 mM) decreased the magnitude of \( I_{sc} \) by 30 ± 3% \((n = 4)\) within 5 min (Fig. 6, B and D). Preliminary results showed no effect of either \( Ba^{2+} \) (1 mM) or ouabain (1 mM) on \( I_{sc} \) from the apical side. These findings are consistent with the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Stimulation of \( I_{sc} \) by genistein at submaximal forskolin concentrations and lack of effect of glibenclamide and DIDS. A: representative recording of \( V_T \) during addition of apical amiloride (A; 10 \( \mu \)M), apical genistein (G; 30 \( \mu \)M), basolateral forskolin (FSK; 10 \( M \)), apical glibenclamide (Gb; 300 \( \mu \)M), and apical DIDS (D; 500 \( \mu \)M). B: summary of experiments showing stimulation by genistein [after control conditions (C) and block of residual \( Na^- \) absorption by apical amiloride (10 \( \mu \)M)] and further stimulation by forskolin (F10; 10 \( \mu \)M) \((n = 6)\); right: stimulation by genistein (30 \( \mu \)M) after submaximal forskolin (F1G; 1 \( \mu \)M) \((n = 5)\). C: left: no further stimulation by genistein (30 \( \mu \)M) after forskolin (F10; 10 \( \mu \)M) \((n = 6)\); right: no inhibition of stimulated \( I_{sc} \) by either glibenclamide (Gl; 300 \( \mu \)M) or DIDS (500 \( \mu \)M) \((n = 5)\). *P < 0.05.}
\end{figure}
presence of K\(^+\) channels and the Na\(^+\)-K\(^+\)-ATPase in the basolateral membrane of these cells. The basis for the incomplete inhibition of \(I_{sc}\) by ouabain is not clear, but it could be due to submaximal concentration, the presence of other ion pumps, or a slower secondary phase of rundown.

Native tissue. The native tissue was used (1) to determine whether the primary cultures had the same phenotype as the original tissue with respect to the adrenergic receptor and cAMP signal pathway and (2) to demonstrate that the assumed increase in cAMP during exposure to agonists of the receptor or adenylyl cyclase did indeed occur. The results showed that native canals had the same responses as the primary cultured epithelium.

The vibrating probe was used to measure current generated by the native epithelium in neonatal rats and adult gerbils. The probe detected a negative current (toward the epithelium) when the probe tip was positioned near the apical cell surface of neonatal rat canals (Fig. 7A) and a positive current (away from the epithelium) when the probe tip was positioned near the basolateral cell surface of adult gerbil canals (Fig. 7B).

The current from neonatal rat canals \((n = 3)\) was stimulated by isoproterenol (100 nM) and forskolin \(10 \mu M\), and the isoproterenol-stimulated current was inhibited by ICI-118551 \(10 \mu M\) but not CGP-20712A \(10 \mu M\) (Fig. 7A). Similarly, the current from adult gerbil canals \((n = 6)\) was stimulated by isoproterenol \((10 \mu M\) and forskolin \(10 \mu M\) (Fig. 7B).

Isoproterenol caused a dose-dependent stimulation of cAMP production in isolated native neonatal rat canals (Fig. 8). The \(EC_{50}\) for isoproterenol-induced cAMP production was 5 nM \(pEC_{50} = 8.3 \pm 0.4, n = 7\). The presence of 1 \(\mu M\) CGP-20712A had no effect on the \(EC_{50}\) \(3 nM, pEC_{50} = 8.5 \pm 0.8, n = 7\). In the presence of 10 \(\mu M\) ICI-118551, the dose-response curve was shifted to the right and had an \(EC_{50}\) of \(\approx 10 \mu M\). The data provide evidence for the presence of \(\beta_2\)-but not \(\beta_1\)-receptors in semicircular canals of neonatal rats.

**DISCUSSION**

The vestibular labyrinth is comprised of the sensory hair cells and many other epithelial cell types including transitional cells, dark cells, several “wall” cells, and the cells of the semicircular canal ducts. Sensory hair cell function depends on maintenance of endolymph ion composition and volume, which is the function of the other epithelial cells of the vestibular labyrinth. The contributions of vestibular dark cells...
Ion transport by a variety of epithelia is controlled by β-adrenergic receptors (4, 11, 24, 34). The classic view is that stimulation of these receptors leads to an increase of intracellular cAMP through coupling to heterotrimeric G proteins of the Gs type and subsequent activation of adenyl cyclase. Three β-adrenergic receptor subtypes (β1, β2, and β3) have been identified (37), and the subtypes can be distinguished by the affinity of the antagonists ICI-118551 and CGP-20712A (33). The Cl− secretion by semicircular canal duct epithelium is clearly regulated by the β2-adrenergic receptor acting via elevation of cAMP. The Isc and cAMP level of canal epithelium from neonatal rats were stimulated by agonists of β-adrenergic receptors. The affinity for ICI-118551 of the receptor in the canal epithelium is distinctly greater than for CGP-20712A, a constellation fitting only that for the β2-adrenergic receptor and not β1 or β3 (27, 33, 34).

Furthermore, this signal pathway is not restricted to early development because isoproterenol and forskolin stimulated Isc in adult canals. The finding that addition of forskolin after maximal stimulation by isoproterenol had no additional effect on Isc suggests that adenyl cyclase is mainly linked to β-adrenergic receptors rather than to multiple receptors. This signal pathway is likely functional in vivo and may be stimulated by agonists in the serum, because measured concentrations of norepinephrine in human (14) and rat serum are in the nanomolar range (Fig. 3).

Cl− transport by several epithelia has been shown to be under control of a cAMP signal pathway. Transporter proteins whose activities are modified by cAMP include Cl− channels (10, 28), anion exchanger (25), and Na+−K+−2Cl− cotransporter (13). The constellation of transporters in semicircular canal duct epithelium that accounts for the observed Cl− secretion remains to be determined. The decrease in Rf during isoproterenol stimulation in the radioisotope flux series is consistent with the activation of an apical Cl− channel, such as CFTR.

Anion transport. The major anions in fluids of the inner ear are Cl− and HCO3− (36). It is likely that HCO3− secretion by semicircular canal duct epithelium is small because 36Cl− flux accounted for the basal and isoproterenol-stimulated Isc in the presence of HCO3−. Very recent evidence points to the participation of vestibular transitional cells and cochlear outer sulcus cells in the secretion of HCO3− via an apical pendrin transporter (T. Wu, E. White, P. Wangemann, and D.C. Marcus, unpublished observations).

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![Fig. 7. Representative recordings of Isc in native semicircular canals stimulated by increase in cAMP; current density was recorded by vibrating probe (VP; see micrographs in insets). A: Isc in neonatal canals was reversibly stimulated by isoproterenol (ISO; 100 nM) and inhibited by ICI-118551 (ICI; 10 μM) but not by CGP-20712A (CGP; 10 μM); forskolin (10 μM) was also reversibly active. B: Isc in adult gerbil canals was stimulated by cAMP via forskolin (10 μM) and isoproterenol (10 μM). SCC, short-circuit current.](image-url)
Experiments were performed to test for electrophysiological responses to genistein, glibenclamide, and DIDS; these agents are generally accepted as pharmacologically defining the presence of functional CFTR (1, 28). We found that the transepithelial current in the cultured canal epithelium did not fully fit this profile, suggesting that Cl⁻ secretion may be carried by another cAMP-dependent pathway.

Our current understanding of Cl⁻ transport by the semicircular canal duct epithelium is illustrated in Fig. 9. K⁺ is taken up into the cell across the basolateral membrane by the Na⁺-K⁺-ATPase, and the resulting high intracellular K⁺ concentration is expected to develop a negative basolateral membrane voltage via the Ba²⁺-sensitive basolateral K⁺ channels. Because the transepithelial voltage in the vestibular labyrinth is within a few millivolts of zero (17), the apical membrane voltage would also be negative and provide an electrical driving force for the exit of Cl⁻ into the lumen. This secretory pathway in the apical membrane does not fully fit the pharmacological profile of CFTR. Cl⁻ secretion in this epithelium is regulated by cAMP via β₂-adrenergic receptors. The molecular basis of this control is not yet known.

**Cation transport.** Previous investigations of the physiological function of the semicircular canal ducts have focused on transport of monovalent cations, because K⁺ and Na⁺ concentrations are maintained farthest from equilibrium between endolymph and peri-lymph (36). Cellular mechanisms of K⁺ secretion have been well characterized in vestibular dark cells of the utricle and ampulla (18–20). Na⁺ was shown to be absorbed by the frog ampulla, and the absorptive flux was partially inhibited by amiloride (9). More recently, it was shown that mammalian transitional cells of the ampulla are responsible for Na⁺ absorption and that this occurs through amiloride-sensitive nonselective cation channels in the apical cell membrane (7, 15).

We found a small K⁺ secretion by semicircular canal ducts, but there was no evidence for Na⁺ absorption. The relatively small flux of K⁺ under basal conditions and the absence of K⁺ flux in the presence of isoproterenol suggest that K⁺ is of little or no physiological significance. The previous report of a small K⁺ secretion by this epithelium (21) may have been the result of a minor presence of dark cells in the culture from inadvertent inclusion of parts of the common crus. The common crus is the confluence of the anterior and posterior canal ducts that is partially composed of dark cells (12). Cells from the common crus were assiduously excluded from the present series of experiments, and a functional test for dark cells with DIDS confirmed their absence. Furthermore, isoproterenol would have caused an increase in K⁺ secretion (31, 34), contrary to our observations.

**Physiological significance.** The present study demonstrated for the first time that semicircular canal duct epithelium contributes to the homeostasis of vestibular endolymph by secretion of Cl⁻ under adrenergic regulation. K⁺ secretion by vestibular dark cells has recently been shown to be stimulated by β₁-adrenergic receptors and by downstream events in the signal pathway, including activation of adenylyl cyclase and increase of intracellular cAMP levels (31, 34).

The vestibular labyrinth, therefore, has the means to control vestibular endolymph composition not only by cation secretion (dark cells) and absorption (transitional cells) but also by the primary anion, Cl⁻. β-Adrenergic receptor agonists carried to both the dark cells and semicircular canal duct cells would increase secretion of both K⁺ and Cl⁻. These two processes would be physiologically linked, because both cells respond to a similar range of agonist. Pathological dysfunctions of the vestibular labyrinth include vertigo associated with endolymphatic hydrops (Meniere’s disease). The possible involvement of adrenergic receptors in Meniere’s disease has been discussed (5, 33). The current work points to one possible etiology of endolymphatic hydrops in Meniere’s disease and may provide a basis for intervention.

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