

# Calcium-Dependent Regulation of Secretion in Biliary Epithelial Cells: The Role of Apamin-Sensitive SK Channels

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**Background & Aims:** Increases in intracellular  $Ca^{2+}$  are thought to complement cAMP in stimulating  $Cl^{-}$  secretion in cholangiocytes, although the site(s) of action and channels involved are unknown. We have identified a  $Ca^{2+}$ -activated  $K^{+}$  channel (SK2) in biliary epithelium that is inhibited by apamin. The purpose of the present studies was to define the role of SK channels in  $Ca^{2+}$ -dependent cholangiocyte secretion. **Methods:** Studies were performed in human Mz-Cha-1 cells and normal rat cholangiocytes (NRC). Currents were measured by whole-cell patch clamp technique and transepithelial secretion by Ussing chamber. **Results:**  $Ca^{2+}$ -dependent stimuli, including purinergic receptor stimulation, ionomycin, and increases in cell volume, each activated  $K^{+}$ -selective currents with a linear IV relation and time-dependent inactivation. Currents were  $Ca^{2+}$  dependent and were inhibited by apamin and by  $Ba^{2+}$ . In intact liver, immunofluorescence with an antibody to SK2 showed a prominent signal in cholangiocyte plasma membrane. To evaluate the functional significance, NRC monolayers were mounted in a Ussing chamber, and the short-circuit current ( $I_{sc}$ ) was measured. Exposure to ionomycin caused an increase in  $I_{sc}$  2-fold greater than that induced by cAMP. Both the basal and ionomycin-induced  $I_{sc}$  were inhibited by basolateral  $Ba^{2+}$ , and ~58% of the basolateral  $K^{+}$  current was apamin sensitive. **Conclusions:** These studies demonstrate that cholangiocytes exhibit robust  $Ca^{2+}$ -stimulated secretion significantly greater in magnitude than that stimulated by cAMP. SK2 plays an important role in mediating the increase in transepithelial secretion due to increases in intracellular  $Ca^{2+}$ . SK2 channels, therefore, may represent a target for pharmacologic modulation of bile flow.

Intrahepatic bile duct cells, or cholangiocytes, contribute importantly to bile formation through absorption and secretion of fluid and electrolytes. Although they compose only 2%–5% of the nuclear mass of the liver, they form an extensive branching network and may account for 20%–40% of bile flow in humans. Secretion is initiated through cAMP stimulation of apical  $Cl^{-}$  channels, where increases in apical membrane  $Cl^{-}$  per-

meability permit movement of  $Cl^{-}$  out of the cell and into the duct lumen.<sup>1</sup> Cholangiocyte  $Cl^{-}$  secretion is thought to provide the driving force for  $Cl^{-}/HCO_3^{-}$  exchange and water efflux and, therefore, the dilution and alkalization of bile. The observation that the non-selective  $K^{+}$  channel inhibitor  $Ba^{2+}$  blocks secretion suggests that membrane  $K^{+}$  channels are necessary to maintain the electrical driving force for continued  $Cl^{-}$  efflux and, hence, biliary secretion.<sup>2</sup> However, the location and regulation of the channels involved are not known.

Previous studies have emphasized roles for cAMP as an intracellular signal and cystic fibrosis transmembrane conductance regulator (CFTR) as an apical  $Cl^{-}$  channel involved in the secretory response. There is increasing evidence, however, that intracellular  $Ca^{2+}$  may mediate a complementary response. Cholangiocytes possess a dense population of  $Ca^{2+}$ -activated  $Cl^{-}$  channels,<sup>3</sup> and, in isolated cells, the current density of the  $Ca^{2+}$ -activated  $Cl^{-}$  conductance is approximately 2-fold greater than the cAMP-activated  $Cl^{-}$  conductance.<sup>3,4</sup> Interestingly, in cells with the cystic fibrosis (CF) phenotype (absent or nonfunctioning CFTR), increases in intracellular  $Ca^{2+}$  concentration still result in potent secretory responses,<sup>5</sup> suggesting that  $Ca^{2+}$ -dependent secretion complements that of cAMP and may serve as a strategy to bypass the  $Cl^{-}$  secretory defect associated with CF.

Membrane  $K^{+}$  channels are necessary for both cAMP- and  $Ca^{2+}$ -dependent  $Cl^{-}$  secretory responses in other epithelial cell models.<sup>6</sup> In the basolateral domain,  $K^{+}$  channel activation leads to membrane hyperpolarization to maintain the electrical driving force for continued  $Cl^{-}$  secretion.<sup>7</sup> In the apical membrane,  $K^{+}$  efflux may enhance the  $Cl^{-}$  secretory response.<sup>8</sup> Little is known re-

**Abbreviations used in this paper:** CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance;  $I_{sc}$ , short-circuit current; NRC, normal rat cholangiocytes; PKC, protein kinase C.

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garding the potential role of  $K^+$  channels in the regulation of biliary secretion. We have previously identified a member of the  $Ca^{2+}$ -activated  $K^+$  channel family known as SK2 (small conductance  $K^+$  channel) in human biliary epithelia.<sup>9</sup> SK2, an ~63-kilodalton protein with 6 transmembrane domains, is activated by small increases in intracellular  $Ca^{2+}$  (<100 nmol/L) and is sensitive to the bee venom apamin, which inhibits channel function through interaction at the pore domain between transmembrane segments 5 and 6.<sup>10</sup> Given that intracellular  $Ca^{2+}$  appears to complement the role of cAMP in mediating secretion in other epithelial cells, the purpose of the present studies was to determine the cellular distribution of SK2 channels in biliary epithelia and to characterize their potential role in  $Ca^{2+}$ -dependent  $Cl^-$  secretion.

## Materials and Methods

### Reagents

Ionomycin, NPPB, and adenosine triphosphate (ATP) were purchased from Calbiochem (San Diego, CA). Apamin, barium chloride, and other chemicals were obtained from Sigma (St. Louis, MO).

### Cell Models

Studies in isolated cells were performed in Mz-Cha-1 cells, originally isolated from human adenocarcinoma of the gallbladder.<sup>11</sup> Studies in polarized monolayers were performed utilizing normal rat cholangiocytes (NRC) in culture.<sup>12</sup> Each model system expresses phenotypic features of differentiated biliary epithelium, including receptors, signaling pathways, and ion channels similar to those found in primary cells.<sup>13–15</sup> Moreover, increases in Mz-Cha-1 and NRC intracellular  $[Ca^{2+}]$  are followed by opening of membrane  $K^+$  and  $Cl^-$  channels.<sup>16</sup> Mz-Cha-1 cells also express an apamin-sensitive  $K^+$  conductance that can be activated during metabolic stress.<sup>17</sup> Mz-Cha-1 cells were passaged at weekly intervals and maintained in culture at 37°C in a 5%  $CO_2$  incubator in  $HCO_3^-$ -containing CMRL-1066 media (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100  $\mu$ g/mL).<sup>16</sup> NRC monolayers were cultured on rat tail collagen slabs as previously described and passaged onto collagen-coated semipermeable (24 mm diameter, 0.4  $\mu$ m pore) Costar transwell supports (Corning) 7–10 days before all studies.<sup>12,14</sup> This protocol permits the development of a high transepithelial resistance ( $R_t > 1000 \Omega/cm^2$ ) and net apical fluid secretion.

### Immunohistochemistry

Rat liver sections were stained for immunofluorescence, as previously described.<sup>20</sup> Briefly, livers were flushed with phosphate-buffered saline, perfused with 3% paraformal-

dehyde, and cut into blocks. Blocks were then incubated in 3% paraformaldehyde (30 minutes, room temperature), 5% sucrose (1 hour; 4°C), 10% sucrose (1 hour; 4°C), and 25% sucrose (overnight, 4°C); frozen in liquid nitrogen-cooled isopentane; and sectioned (6  $\mu$ m). Sections were rehydrated, blocked (10% serum, 1% albumin; 1 hour), incubated with SK2 antibody (Alomone; 1:100 dilution; 1 hour), washed, incubated with fluorescein isothiocyanate–anti-rabbit IgG antibody (Jackson Laboratories; 1:50; 30 minutes), washed, and mounted in 60% glycerol with 10% DABCO. Sections stained for F-actin did not receive any primary antibody and rhodamine-phalloidin (Molecular Probes; 1:20 dilution) in place of the secondary antibody. Staining was visualized by confocal microscopy (Bio-Rad;  $\times 600$ ).

### Measurement of $K^+$ Currents

Whole-cell currents were measured using patch clamp recording techniques.<sup>18</sup> Studies were performed at room temperature (22°C–25°C) 24–48 hours after plating of cells on 35-mm collagen-covered plates. The standard extracellular solution contained (in mmol/L): 140 NaCl, 4 KCl, 1  $KH_2PO_4$ , 2  $MgCl_2$ , 1  $CaCl_2$ , 5 glucose, and 10 HEPES/NaOH (pH 7.3). The standard pipette (intracellular) solution contained (in mmol/L): 130 KCl, 10 NaCl, 2  $MgCl_2$ , 10 HEPES/KOH (pH 7.3); calculated free  $[Ca^{2+}] \sim 100$  nmol/L. In other studies, the concentration of  $Cl^-$  was decreased by partial replacement with glutamate to minimize the contribution of volume-sensitive  $Cl^-$  currents to the observed response. The low  $Cl^-$  extracellular solution contained (in mmol/L): 144 NaCl, 8 NaCl, 4 KCl, 1  $KH_2PO_4$ , 2  $MgCl_2$ , 2  $CaCl_2$ , 10 D-glucose, and 10 HEPES/NaOH; and the low  $Cl^-$  pipette (intracellular) solution contained (in mmol/L): 130 K glutamate, 20 NaCl, 2  $MgCl_2$ , 10 HEPES/KOH, 1 EGTA, and 0.5  $CaCl_2$  (calculated free  $Ca^{2+} \sim 100$  nmol/L). With these solutions, the  $K^+$  equilibrium potential is  $-82$  mV, and outward currents at a test potential of 0 mV are carried by  $K^+$  ions ( $I_K$ ).<sup>9,16</sup> Cells were viewed through an inverted phase contrast microscope using Hoffman optics at a magnification of  $\times 600$  (Olympus IMT-2). Patch pipettes were pulled from Corning 7052 glass and had resistances of 3–6 M $\Omega$ . Recordings were made with an Axopatch IC amplifier (Axon Instruments, Foster City, CA), and signals filtered at 2-kHz bandwidth using a 4-pole low-pass Butterworth filter. Currents were analyzed using pClamp software (version 6.0, Axon Instruments, Foster City, CA). Three voltage protocols were utilized: (1) holding potential  $-40$  mV, with 200 ms steps to 0 mV and  $-80$  mV at 10-second intervals (for real-time tracings); (2) holding potential  $-40$  mV, with 400 ms steps from  $-100$  mV to  $+100$  mV in 20 mV increments; and (3) holding potential  $-40$  mV and voltage ramp from  $-100$  mV to  $+100$  mV over 200 ms. Current-voltage (I-V) relations were generated from the “step” or “ramp” protocols as indicated. Pipette voltages ( $V_p$ ) are referred to the bath. In the whole-cell configuration,  $V_p$  corresponds to the membrane potential, and upward deflections of the current trace indicate outward membrane current. Results are compared with control studies measured on

the same day to minimize any effects of day-to-day variability and reported as current density (pA/pF) to normalize for differences in cell size.<sup>19</sup>

### Transepithelial Transport Measurements

NRC cells were used to study vectorial  $K^+$  movement and transepithelial secretion across monolayers. Cells were grown to confluency on collagen-treated polycarbonate filters with a pore size of  $0.4 \mu\text{m}$  (Costar, Cambridge, MA) until a resistance of  $>1000 \Omega \text{ cm}^2$  was achieved as measured by an epithelial tissue voltmeter (EVOHM; World Precision Instruments [WPI], Sarasota, FL). Cells were mounted in a Trans-24 miniperfusion system for tissue culture cups (Jim's Instrument Manufacturing Inc., Iowa City, IA). All experiments were carried out at  $37^\circ\text{C}$ , and basolateral and apical (luminal) sides were perfused continuously and independently in a closed system with the standard extracellular buffer solution (as described above) by bubbling  $\text{O}_2$  through air-lift circulators. Transepithelial voltage ( $V_t$ ) was clamped to 0 mV, and short-circuit current ( $I_{sc}$ ) was recorded through agar bridges (3% agar in 3 mol/L KCl) connected to Ag-AgCl electrodes (cartridge electrodes; WPI). The  $I_{sc}$  is the net sum of electrogenic ion movement from the basolateral to the apical chamber and, hence, a reflection of transepithelial secretion.<sup>15</sup> Experimental results were compared with control studies (basal and ionomycin-induced  $I_{sc}$ ) performed on the same day to minimize any potential effects of day-to-day variability in current amplitude.

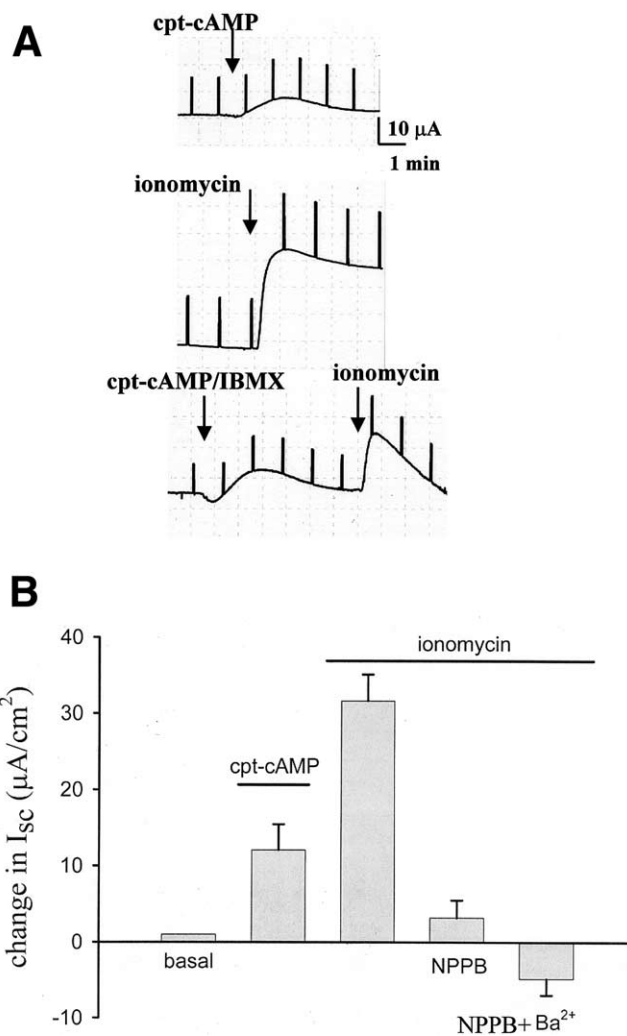
### Statistics

Results are presented as the mean  $\pm$  standard error, with "n" representing the number of cells for patch clamp studies and the number of culture plates or repetitions for other assays. Student paired or unpaired  $t$  test was used to assess statistical significance as indicated, and  $P$  values  $<0.05$  were considered to be statistically significant.

## Results

### $\text{Ca}^{2+}$ -Stimulated Transepithelial Secretion in Polarized Biliary Monolayers

Stimuli that increase intracellular cAMP levels have previously been shown to result in an increase in  $I_{sc}$  in NRC monolayers. This cAMP-stimulated increase in  $I_{sc}$  is thought to be mediated in large part by apical  $\text{Cl}^-$  efflux via CFTR. The present studies were designed to evaluate the relative contribution of  $\text{Ca}^{2+}$ -stimulated secretion in biliary epithelium. Accordingly, inserts of NRC monolayers were mounted in the recording chamber and allowed to equilibrate with the standard extracellular buffer, and basal  $I_{sc}$  was recorded ( $14.47 \pm 2.33 \mu\text{A}/\text{cm}^2$ ,  $n = 12$ ). Addition of cpt-cAMP (500  $\mu\text{mol}/\text{L}$ ), a cell permeable form of cAMP to the apical bath, resulted in an increase of  $12.09 \pm 3.34 \mu\text{A}/\text{cm}^2$  from the basal  $I_{sc}$  ( $n = 8$ ,  $P < .01$ , Figure 1A, top tracing). In



**Figure 1.**  $\text{Ca}^{2+}$ -stimulated transepithelial secretion in polarized biliary monolayers. Short-circuit current ( $I_{sc}$ ), a reflection of transepithelial secretion, across NRC monolayers was measured under voltage-clamp conditions in a Ussing chamber. (A) In these representative recordings, agonists are added to the apical chamber. Although addition of cpt-cAMP (500  $\mu\text{mol}/\text{L}$ ) increases  $I_{sc}$  (top tracing), addition of ionomycin (5  $\mu\text{mol}/\text{L}$ ), to increase  $[\text{Ca}^{2+}]_i$ , results in a significantly greater increase in  $I_{sc}$  (middle tracing). The peak  $I_{sc}$  response also occurred rapidly and tended to be more sustained than the cpt-cAMP-induced response. In separate experiments, cpt-cAMP (500  $\mu\text{mol}/\text{L}$ ) and 3-isobutyl-1-methylxanthine (IBMX, 450  $\mu\text{mol}/\text{L}$ ) were added together to the apical chamber (to stimulate maximally CFTR), followed by the subsequent addition of ionomycin (5  $\mu\text{mol}/\text{L}$ ). Although addition of cpt-cAMP and IBMX increased  $I_{sc}$ , subsequent addition of ionomycin resulted in a further increase in the  $I_{sc}$  (bottom tracing). (B) The average change in  $I_{sc}$  in response to either addition of cpt-cAMP (500  $\mu\text{mol}/\text{L}$ ) or ionomycin (5  $\mu\text{mol}/\text{L}$ ). The y axis values are reported as  $\Delta I_{sc}$  (maximum  $I_{sc}$  - basal  $I_{sc}$ ). Individual inhibitors of  $\text{Cl}^-$  and  $\text{K}^+$  channels were added in separate experiments. Addition of the  $\text{Cl}^-$  channel blocker NPPB (100  $\mu\text{mol}/\text{L}$ ), to the apical chamber significantly inhibited the ionomycin-induced increase in  $I_{sc}$ . Although a residual NPPB-insensitive  $I_{sc}$  remained, subsequent addition of the nonselective  $\text{K}^+$  channel blocker,  $\text{Ba}^{2+}$  (5 mmol/L), to the basolateral chamber abolished this residual  $I_{sc}$ , as well as decreased the basal  $I_{sc}$ .

separate experiments, addition of cpt-cAMP to the basolateral bath resulted in a similar increase in  $I_{sc}$  ( $10.31 \pm 4.12 \mu\text{A}/\text{cm}^2$ ,  $n = 4$ ). The responses occurred rapidly and were of short duration, returning to basal levels within 3–5 minutes.

In separate experiments, addition of ionomycin ( $5 \mu\text{mol}/\text{L}$ ) to the apical chamber to increase intracellular  $[\text{Ca}^{2+}]_i$ , resulted in an increase of  $31.61 \pm 3.46 \mu\text{A}/\text{cm}^2$  from the basal  $I_{sc}$  ( $n = 12$ ,  $P < .01$ , Figure 1A, middle tracing). This  $\text{Ca}^{2+}$ -stimulated increase in  $I_{sc}$  was  $\sim 2$ -fold greater than that induced by cAMP ( $P < .01$ , Figure 1A and 1B). The peak  $I_{sc}$  response also occurred rapidly, but tended to be more sustained than the cAMP-induced response, returning to basal values over 10–15 minutes. Thapsigargin ( $1 \mu\text{mol}/\text{L}$ ) had similar effects, increasing the  $I_{sc}$  by  $+19.37 \pm 4.06 \mu\text{A}/\text{cm}^2$  ( $n = 8$ ,  $P < .01$ , data not shown). To determine whether the effects of cAMP and ionomycin are additive, cpt-cAMP ( $500 \mu\text{mol}/\text{L}$ ) and 3-isobutyl-1-methylxanthine (IBMX;  $450 \mu\text{mol}/\text{L}$ ) were added to the apical chamber (to stimulate maximally CFTR), followed by the subsequent addition of ionomycin ( $5 \mu\text{mol}/\text{L}$ ). The addition of cpt-cAMP and IBMX increased  $I_{sc}$  from  $14.69 \pm 2.06$  to  $23.11 \pm 4.87 \mu\text{A}/\text{cm}^2$  ( $n = 8$ ), and subsequent addition of ionomycin resulted in a further increase in the  $I_{sc}$  to a maximum of  $59.23 \pm 5.22 \mu\text{A}/\text{cm}^2$  ( $n = 8$ ,  $P < .01$ , Figure 1A, bottom tracing).

To determine the conductances involved in the ionomycin-induced secretory response, individual inhibitors of  $\text{Cl}^-$  and  $\text{K}^+$  channels were added in separate experiments. Addition of the nonselective  $\text{Cl}^-$  channel blocker NPPB ( $100 \mu\text{mol}/\text{L}$ ) to the apical chamber significantly inhibited the ionomycin-induced increase in  $I_{sc}$  ( $\Delta I_{sc}$   $3.21 \pm 2.31 \mu\text{A}/\text{cm}^2$ ,  $n = 8$ ,  $P < .01$ , Figure 1B), suggesting that a component of the  $\text{Ca}^{2+}$ -activated  $I_{sc}$  is due to opening of  $\text{Cl}^-$  channels in the apical membrane. However, in all experiments, a residual NPPB-insensitive  $I_{sc}$  remained. Subsequent addition of high concentrations of the nonselective  $\text{K}^+$  channel blocker  $\text{Ba}^{2+}$  ( $5 \text{mmol}/\text{L}$ ) to the basolateral chamber abolished this residual  $I_{sc}$  ( $\Delta I_{sc}$   $-4.84 \pm 2.17 \mu\text{A}/\text{cm}^2$ ,  $n = 8$ ,  $P < .01$ , Figure 1B), suggesting a complementary role of basolateral  $\text{K}^+$  channels in  $\text{Ca}^{2+}$ -activated transepithelial secretion. Additionally, exposure resulted in a decrease in  $I_{sc}$  to values below basal levels, suggesting that basolateral  $\text{K}^+$  channels also contribute to maintaining the basal  $I_{sc}$  in NRC monolayers.

Taken together, these studies suggest that, in polarized biliary monolayers, increases in intracellular  $[\text{Ca}^{2+}]_i$  levels increase transepithelial secretion to values over and above that induced by cAMP. In fact, the magnitude of the  $\text{Ca}^{2+}$ -stimulated response was reliably 2-fold or more

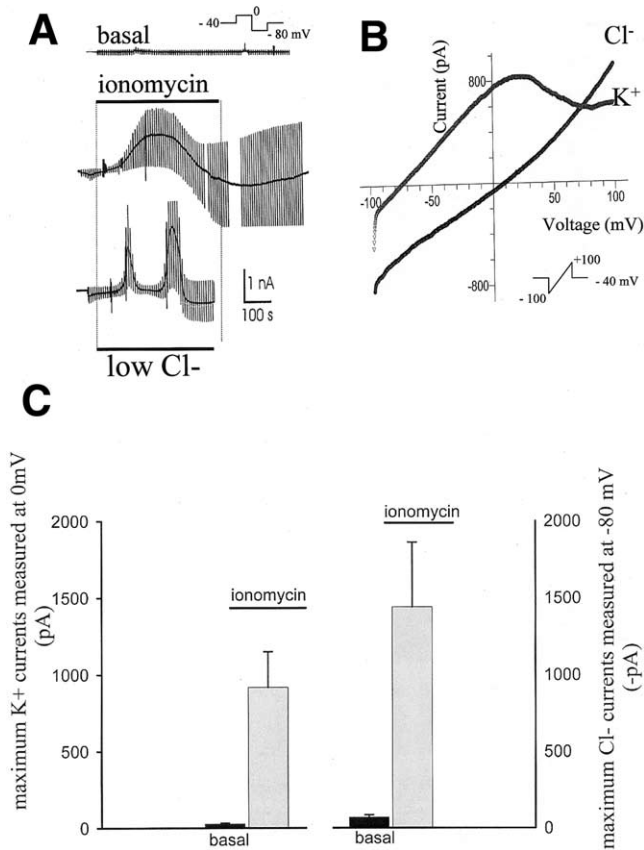
than the cAMP response over multiple study days. Additionally,  $\text{Ca}^{2+}$ -stimulated secretion depends on simultaneous activation of both  $\text{Cl}^-$  and  $\text{K}^+$  conductances. Further studies were undertaken to characterize the  $\text{Ca}^{2+}$ -induced secretory response and the role of the  $\text{K}^+$  conductance with emphasis on its functional location and overall contribution to secretion in biliary cell models.

### Ionomycin Stimulates Both $\text{Cl}^-$ and $\text{K}^+$ Conductances in Isolated Biliary Cells

To evaluate in isolated cells the conductances activated by increases in intracellular  $[\text{Ca}^{2+}]_i$ , whole-cell patch clamp studies were performed in Mz-Cha-1 cells. Utilizing standard extracellular and intracellular solutions (Materials and Methods), basal currents were small (Figure 2A, top tracing). Exposure to ionomycin ( $2 \mu\text{mol}/\text{L}$ ), to increase  $[\text{Ca}^{2+}]_i$ , resulted in a transient outward current because of  $\text{K}^+$  efflux (shown by an upward deflection of the current trace), followed by a sustained  $\text{Cl}^-$  current (downward deflection of the current trace) in 9 of 11 cells (Figure 2A, middle tracing). Ionomycin increased  $\text{Cl}^-$  currents from  $68.66 \pm 6.02 \text{pA}$  to  $1439.8 \pm 421 \text{pA}$  at  $-80 \text{mV}$  ( $n = 9$ ,  $P < 0.001$ , Figure 2C). The  $\text{Cl}^-$  currents demonstrated mild outward rectification and reversal at  $0 \text{mV}$ , consistent with the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance that we have previously described in biliary epithelial cells (Figure 2B).<sup>3</sup> When the standard extracellular buffer solution was replaced with a low  $\text{Cl}^-$  solution (Materials and Methods section), ionomycin ( $2 \mu\text{mol}/\text{L}$ ) increased macroscopic  $\text{K}^+$  currents from  $25.7 \pm 18.5 \text{pA}$  to  $917.5 \pm 31.7 \text{pA}$  at  $0 \text{mV}$  ( $n = 11$ ,  $P < 0.001$ , Figure 2C) without subsequent activation of  $\text{Cl}^-$  currents (Figure 2A, bottom tracing). In the majority of studies,  $\text{K}^+$  currents revealed an oscillatory pattern. The  $\text{K}^+$  currents demonstrated a reversal potential of  $-79.6 \pm 8.2 \text{mV}$  and partial inactivation at depolarizing potentials above  $+30 \text{mV}$ , as expected for a primary  $\text{K}^+$  conductance (Figure 2B). These studies in single cells demonstrate that ionomycin activates distinct  $\text{Cl}^-$  and  $\text{K}^+$  conductances in biliary cells.

### Stimuli That Increase Intracellular $[\text{Ca}^{2+}]_i$ Activate Apamin-Sensitive $\text{K}^+$ Channels

To further characterize the  $\text{Ca}^{2+}$ -stimulated  $\text{K}^+$  conductance, whole-cell patch clamp studies were performed in Mz-Cha-1 cells using low concentrations of  $\text{Cl}^-$  in the pipette and bath solutions (Materials and Methods section). A representative IV plot is shown in Figure 3A, and the cumulative data are shown in Figure 3B and reported as current density to correct for differences in individual cell size. Under basal conditions, cells

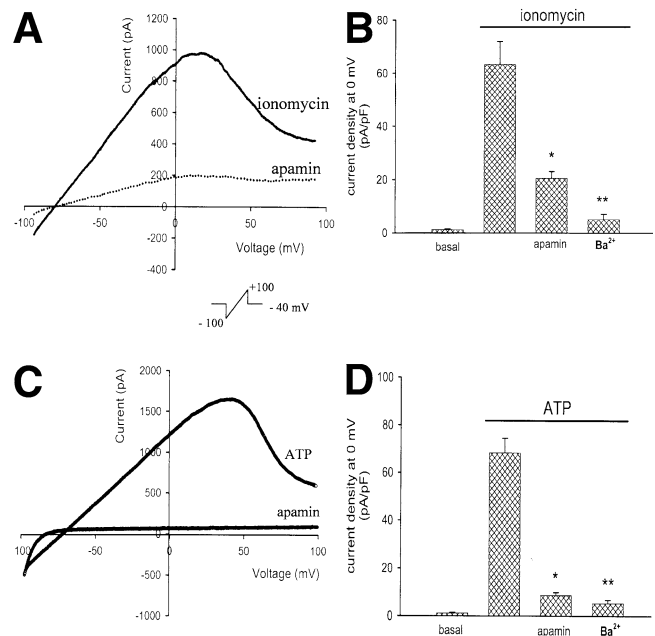


**Figure 2.** Ionomycin stimulates both Cl<sup>-</sup> and K<sup>+</sup> conductances in isolated biliary cells. Whole-cell currents were measured in Mz-Cha-1 cells under basal conditions and during exposure to ionomycin. (A) Representative whole-cell recording. Currents at -80 mV (downward deflection of the current tracing) correspond to I<sub>Cl<sup>-</sup></sub> and currents at 0 mV (upward deflection of the current tracing) represent I<sub>K<sup>+</sup></sub>. Under basal conditions, no currents are observed (top tracing). Exposure to ionomycin (2 μmol/L) results in initial outward currents because of K<sup>+</sup> followed by sustained Cl<sup>-</sup> currents (middle tracing). In low Cl<sup>-</sup> solution, ionomycin increased macroscopic K<sup>+</sup> currents without subsequent activation of Cl<sup>-</sup> currents (bottom tracing). (B) Current-voltage relation of whole-cell currents measured during exposure to ionomycin, utilizing the “ramp” protocol (Materials and Methods section). Cl<sup>-</sup> currents were characterized by a near linear current-voltage relation and reversal near 0 mV. Replacement of Cl<sup>-</sup> in the extracellular buffer with gluconate resulted in activation of primary K<sup>+</sup> currents, with a reversal potential of  $-79.6 \pm 8.2$  mV and partial inactivation at depolarizing potentials above +30 mV. (C) The average maximum currents (pA) in response to ionomycin. K<sup>+</sup> currents were measured at 0 mV and Cl<sup>-</sup> currents at -80 mV.

had a low current density of  $1.21 \pm 0.30$  pA/pF ( $n = 12$ ), which increased to  $86.4 \pm 8.4$  pA/pF ( $n = 11$ ,  $P < 0.001$ ) with the addition of ionomycin (2 μmol/L, Figure 3A and 3B). Ionomycin-stimulated currents demonstrated properties as described above and were completely inhibited by the nonselective K<sup>+</sup> channel blocker Ba<sup>2+</sup> (5 mmol/L,  $8.87 \pm 1.79$  pA/pF,  $n = 6$ ,  $P < 0.001$ ) and partially inhibited in the presence of apamin (50 nmol/L,  $33.61 \pm 6.86$  pA/pF,  $n = 5$ ,  $P < 0.005$ ). The biophysical and pharmacologic properties are consistent

with small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (SK) previously described.<sup>9</sup>

In addition to ionomycin, other stimuli known to increase intracellular Ca<sup>2+</sup> were utilized to further characterize the Ca<sup>2+</sup>-activated K<sup>+</sup> conductance, including activation of purinergic receptors by extracellular ATP, and hypotonic exposure. Once again, utilizing low Cl<sup>-</sup> buffer solutions, exposure to ATP (20 μmol/L) increased K<sup>+</sup> conductance from  $1.31 \pm 0.34$  pA/pF to  $68.31 \pm 6.13$  pA/pF at 0 mV ( $n = 10$ ,  $P < .001$ , Figure 3C and 3D). Currents demonstrated a reversal of  $-78.1 \pm 6.5$  mV and inactivation at membrane voltages >30 mV, again consistent with SK channels. Currents were completely inhibited by either Ba<sup>2+</sup> (5 mmol/L,  $5.27 \pm 1.43$  pA/pF,  $n = 6$ ,  $P < .001$ ) or by apamin (50 nmol/L,  $8.53 \pm 1.36$  pA/pF,  $n = 7$ ,  $P < .001$ , Figure 3C and 3D). In separate experiments, exposure of Mz-Cha-1 cells to hypotonicity (20% decrease in bath NaGluconate, ~230 mOsm) resulted in activation of a K<sup>+</sup> conductance



**Figure 3.** Stimuli that increase intracellular [Ca<sup>2+</sup>] activate apamin-sensitive K<sup>+</sup> channels. Mz-Cha-1 cells were exposed to stimuli to increase Ca<sup>2+</sup> under whole-cell patch clamp conditions with low Cl<sup>-</sup> solutions. IV plots were generated utilizing the “ramp” protocol (Materials and Methods section). (A) Representative IV plot. Exposure to ionomycin (2 μmol/L) results in characteristic K<sup>+</sup> channel activation, which is inhibited by apamin (50 nmol/L). (B) Average current density (pA/pF) at 0 mV was measured under basal conditions and during exposure to ionomycin. When compared with control cells, both apamin (50 nmol/L) and Ba<sup>2+</sup> (5 mmol/L) significantly inhibited ionomycin-stimulated currents. (C) Representative IV plot. Exposure to ATP (20 μmol/L) results in characteristic K<sup>+</sup> channel activation, which is completely inhibited by apamin. (D) Average current density (pA/pF) at 0 mV was measured under basal conditions and during exposure to ATP. When compared with control cells, both apamin and Ba<sup>2+</sup> significantly inhibited ATP-stimulated currents.

( $48.35 \pm 4.51$  pA/pF,  $n = 11$ ,  $P < .001$ ), which was completely inhibited by either exposure to  $Ba^{2+}$  (5 mmol/L,  $4.58 \pm 2.09$ ,  $n = 5$ ,  $P < .001$ ) or apamin ( $9.96 \pm 1.71$  pA/pF,  $n = 6$ ,  $P < .001$ ), consistent with previous observations (data not shown).<sup>9</sup> Taken together, these studies demonstrate that multiple stimuli that increase intracellular  $Ca^{2+}$  concentration result in activation of an apamin-sensitive  $K^+$  conductance. These observations extend the previous observations of the importance of SK2 in cell volume regulation and demonstrate a role in purinergic regulation of membrane  $K^+$  permeability.

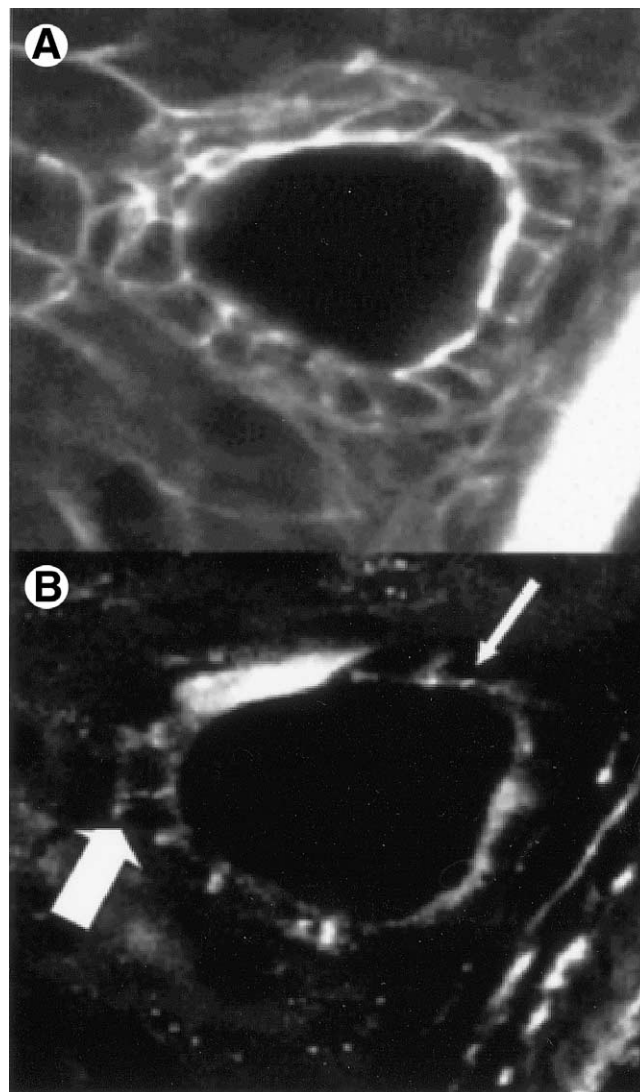
#### Localization of SK2 Channels in Intrahepatic Bile Duct Epithelial Cells

To evaluate the cellular localization of SK2 channels, immunofluorescence with a specific antibody to SK2 (Alomone Labs) was performed in intact rat liver. Staining intensity was greater in cholangiocytes than hepatocytes and was observed primarily in the plasma membrane. To highlight the cell boundaries, sections were dual labeled for F-actin. In sequential confocal images, bile duct staining with F-actin reveals individual cholangiocytes (Figure 4A). Dual staining with SK2 antibody (Figure 4B) reveals signal in both apical and basolateral plasma membranes (Figure 4B, arrows). These studies, utilizing a specific antibody, demonstrate SK2 staining in cholangiocytes with a predominant plasma membrane distribution.

#### Transepithelial $K^+$ Transport

To determine the functional significance of SK channels polarized NRC monolayers were mounted in a Ussing chamber, the basal  $I_{sc}$  was measured, and the effects of apical vs. basolateral delivery of  $K^+$  channel inhibitors (apamin and  $Ba^{2+}$ ) were assessed. Because the  $I_{sc}$  is a reflection of the net movement of ions across the membrane, with net negative charge causing an upward deflection of the current trace, inhibition of  $K^+$  permeability on the apical membrane would be expected to cause an increase in the  $I_{sc}$ ; conversely, inhibition of  $K^+$  permeability on the basolateral membrane would be expected to decrease the  $I_{sc}$  response.

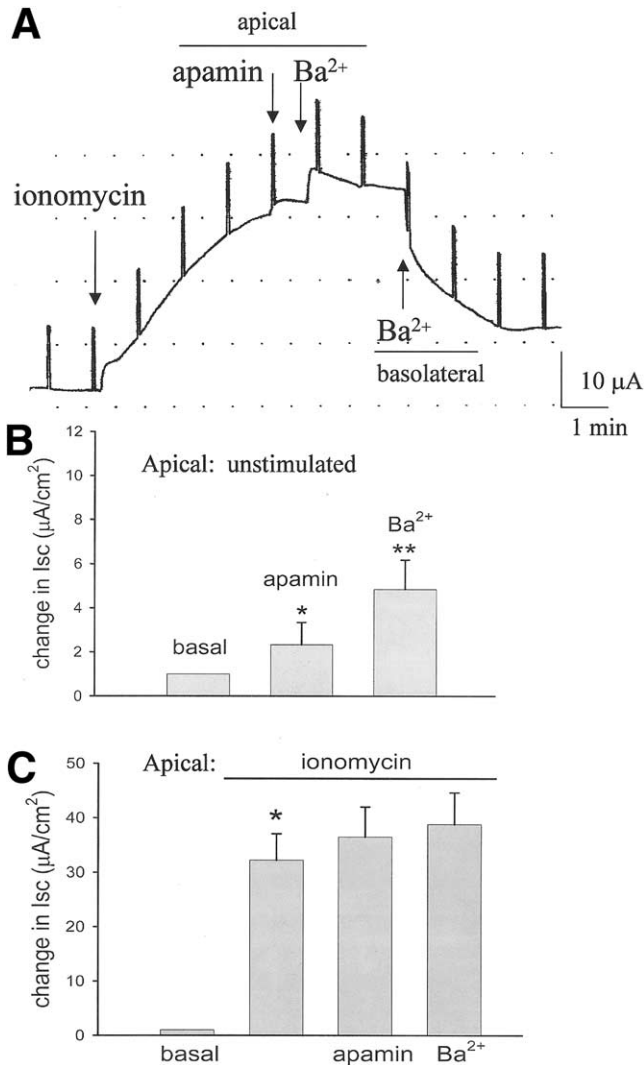
First, the effects of  $K^+$  channel inhibitors on the basal  $I_{sc}$  was assessed. From basal  $I_{sc}$  ( $14.5 \pm 3.7$   $\mu A/cm^2$ ,  $n = 7$ ) and resistance ( $462.7 \pm 33.1$   $\Omega cm^2$ ,  $n = 7$ ) values, apical addition of apamin (50 nmol/L) resulted in a small increase in  $I_{sc}$  consistent with inhibition of apical SK2 channels ( $\Delta I_{sc} +2.33 \pm 1.01$   $\mu A/cm^2$ , Figure 5B). Subsequent addition of  $Ba^{2+}$  (5 mmol/L) resulted in a further small increase in the  $I_{sc}$  ( $\Delta I_{sc} +4.85 \pm 1.33$   $\mu A/cm^2$ , Figure 5B). In contrast, addition of apamin to the baso-



**Figure 4.** Localization of SK2 channels in intrahepatic bile duct epithelial cells. Immunofluorescence with a specific antibody to SK2 in intact rat liver. Sections were dual labeled for F-actin. (A) In sequential confocal images, bile ducts were stained for membrane F-actin, highlighting individual cholangiocytes. (B) Thin arrow points out staining of SK2 on apical membrane, and thick arrow points out staining of SK2 on basolateral membrane.

lateral chamber had much greater effects and decreased the basal  $I_{sc}$  significantly ( $\Delta I_{sc} -7.22 \pm 1.12$   $\mu A/cm^2$ ,  $n = 9$ ,  $P < .01$ ); once again, subsequent addition of  $Ba^{2+}$  further decreased the  $I_{sc}$  ( $\Delta I_{sc} -11.66 \pm 1.19$   $\mu A/cm^2$ ,  $n = 9$ ,  $P < .01$ , Figure 6B). Overall, 61.9% of the basal  $Ba^{2+}$ -inhibitable  $I_{sc}$  was apamin sensitive. These findings suggest that apamin-sensitive  $K^+$  channels play an important role in the maintenance of the basal  $I_{sc}$  in NRC monolayers.

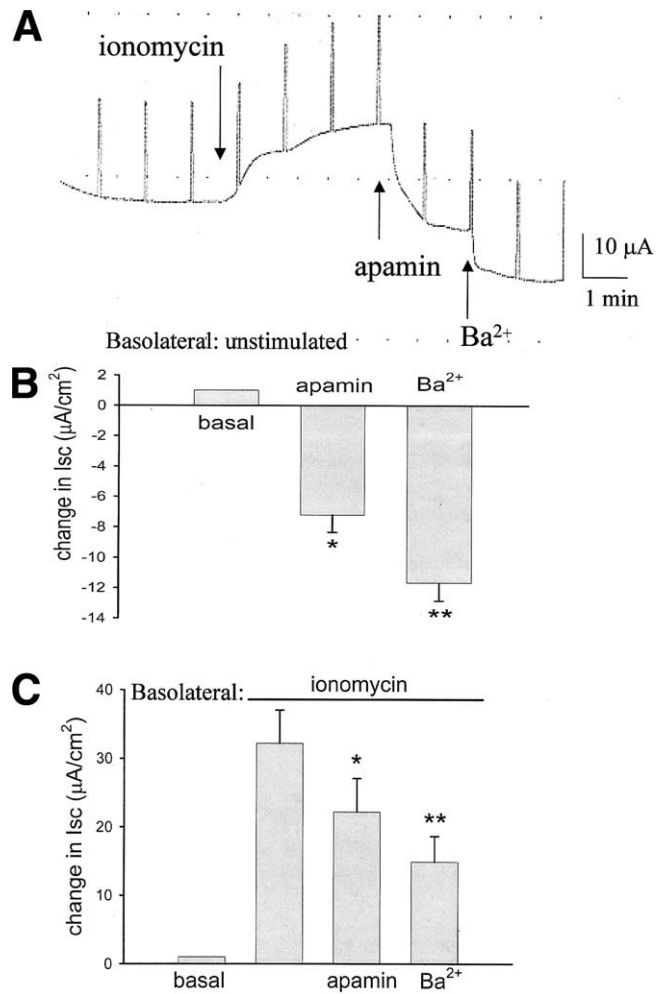
Next, the effects of  $K^+$  channel inhibitors on the  $Ca^{2+}$ -stimulated  $I_{sc}$  were ascertained. In control monolayers, exposure to ionomycin (5  $\mu mol/L$ ) once again increased the basal  $I_{sc}$  ( $\Delta I_{sc} +32.22 \pm 4.83$   $\mu A/cm^2$ ,  $n =$



**Figure 5.** Transepithelial secretion with  $K^+$  channel inhibitors added to apical membrane. Short-circuit current ( $I_{sc}$ ) across NRC monolayers was measured under voltage-clamp conditions in a Ussing chamber. (A) In this representative recording, ionomycin (5  $\mu\text{mol/L}$ ) increases  $I_{sc}$ . Addition of apamin (50  $\text{nmol/L}$ ) or  $Ba^{2+}$  (5  $\text{mmol/L}$ ) to the apical membrane results in small additional increases in  $I_{sc}$ . Addition of  $Ba^{2+}$  (5  $\text{mmol/L}$ ) to the basolateral membrane significantly decreases the ionomycin-stimulated  $I_{sc}$ . (B) Average change in basal  $I_{sc}$  when apamin or  $Ba^{2+}$  added to apical membrane of unstimulated NRC monolayers. Compared with control monolayers, apamin or  $Ba^{2+}$  result in small increases in the basal  $I_{sc}$ . (C) Average change in ionomycin-stimulated  $I_{sc}$  with apical addition of apamin or  $Ba^{2+}$ . There is little change observed in the ionomycin-stimulated  $I_{sc}$  with addition of apamin or  $Ba^{2+}$  to the apical membrane.

4). Subsequent *apical* addition of apamin ( $\Delta I_{sc} + 4.25 \pm 1.5 \mu\text{A/cm}^2$ ,  $n = 4$ ,  $P = \text{ns}$ ) or barium ( $\Delta I_{sc} + 6.54 \pm 1.83 \mu\text{A/cm}^2$ ,  $n = 4$ ,  $P = \text{ns}$ ) caused small additional increases in  $I_{sc}$  (Figure 5A and 5C), suggesting that increases in intracellular  $[Ca^{2+}]_i$  stimulate a small increase in apical  $K^+$  secretion. In separate experiments, *basolateral* addition of apamin decreased the ionomycin-induced  $I_{sc}$  significantly ( $\Delta I_{sc} - 10.01 \pm 4.92 \mu\text{A/cm}^2$ ,  $n = 8$ ,  $P < .001$ , Figure 6A and 6C), and subsequent addition of  $Ba^{2+}$  to the basolateral bath further decreased the magnitude of the  $I_{sc}$  ( $\Delta I_{sc} - 17.33 \pm 3.75 \mu\text{A/cm}^2$ ,  $n = 8$ ,  $P < .001$ , Figure 6A and 6C). In fact, the cumulative data demonstrate that  $\sim 58.8\%$  of the basolateral  $Ba^{2+}$ -sensitive  $Ca^{2+}$ -stimulated  $K^+$  current was apamin sensitive. Taken together, these results demonstrate that SK channels are important in maintaining the basal  $I_{sc}$  as well as mediating the large increase in secretion in response to increases in  $[Ca^{2+}]_i$ . The effects of the  $K^+$  channel inhibitors were more pronounced when

apamin (50  $\text{nmol/L}$ ) or  $Ba^{2+}$  (5  $\text{mmol/L}$ ) to the basolateral membrane significantly decreases  $I_{sc}$ . Subsequent addition to  $Ba^{2+}$  (5  $\text{mmol/L}$ ) to the basolateral membrane results in a further significant decrease in  $I_{sc}$ . (B) Average change in basal  $I_{sc}$  when apamin or  $Ba^{2+}$  is added to basolateral membrane of unstimulated NRC monolayers. (C) Average change in ionomycin-stimulated  $I_{sc}$  with basolateral addition of apamin and subsequent addition of  $Ba^{2+}$ . Compared with control monolayers, apamin and  $Ba^{2+}$  significantly decrease ionomycin-stimulated  $I_{sc}$ .



**Figure 6.** Transepithelial secretion with  $K^+$  channel inhibitors added to basolateral membrane. Short-circuit current ( $I_{sc}$ ) across NRC monolayers was measured under voltage-clamp conditions in a Ussing chamber. (A) In this representative recording, ionomycin (5  $\mu\text{mol/L}$ ) increases  $I_{sc}$ . Addition of apamin (50  $\text{nmol/L}$ ) to the basolateral membrane significantly decreases  $I_{sc}$ . Subsequent addition to  $Ba^{2+}$  (5  $\text{mmol/L}$ ) to the basolateral membrane results in a further significant decrease in  $I_{sc}$ . (B) Average change in basal  $I_{sc}$  when apamin or  $Ba^{2+}$  is added to basolateral membrane of unstimulated NRC monolayers. (C) Average change in ionomycin-stimulated  $I_{sc}$  with basolateral addition of apamin and subsequent addition of  $Ba^{2+}$ . Compared with control monolayers, apamin and  $Ba^{2+}$  significantly decrease ionomycin-stimulated  $I_{sc}$ .

added to the basolateral chamber (during both basal and ionomycin-stimulated conditions), but there is a smaller apical  $K^+$  secretion as well.

## Discussion

The principal findings of these studies in biliary epithelium are that (1) increases in cytosolic  $[Ca^{2+}]$  elicit a robust  $Cl^-$  secretory response, (2) the response depends in large part on parallel activation of apical  $Cl^-$  and basolateral  $K^+$  conductances, and (3) this large basolateral  $K^+$  conductance ( $\sim -10 \mu A/cm^2$ ), as well as a smaller apical  $K^+$  secretory response ( $\sim 4 \mu A/cm^2$ ), are mediated in large part by apamin-sensitive SK2 channels. Accordingly, the approaches to modulate cytosolic  $Ca^{2+}$  levels, the SK2 channel activity, and the  $Ca^{2+}$ -activated  $Cl^-$  channels identified previously, represent attractive targets for regulation of the volume and composition of bile through effects on cholangiocyte secretion.

The formation of bile by the liver depends on complementary contributions from both hepatocytes and cholangiocytes. At the level of cholangiocytes, the role of cAMP in activation of secretory responses is unquestioned and involves stimulation of apical exocytosis,<sup>20</sup> activation of CFTR,<sup>21</sup> and regulation of other transport proteins in apical signaling complexes.<sup>22</sup> Activation of secretin receptors, which are present in cholangiocytes but not hepatocytes, stimulates cAMP-dependent  $Cl^-$  efflux through CFTR and increases bile flow in humans from 0.67 to 1.54 mL/min.<sup>23</sup> Although CFTR is expressed in both airway and biliary epithelia, it is notable that mutations in CFTR result in near universal expression of pulmonary disease, but significant biliary disease is detected in only 10%–25% of patients.<sup>24–27</sup> By analogy with observations in the *cfr*<sup>-/-</sup> mouse,<sup>5</sup> these observations suggest that the cAMP-CFTR axis is not the only pathway involved in cholangiocyte secretion.

Notably, in isolated cholangiocytes, increases in intracellular calcium stimulate an increase in membrane  $Cl^-$  permeability,<sup>4</sup> and cholangiocytes also possess receptors for ATP, acetylcholine, and other agonists known to increase intracellular  $[Ca^{2+}]$ .<sup>28</sup> In these studies of isolated human cholangiocarcinoma cells and polarized normal rat cholangiocytes in monolayer culture, increases in intracellular  $Ca^{2+}$  increased membrane  $Cl^-$  and  $K^+$  permeability 2-fold or more above levels produced by cAMP. Because the increase in  $I_{sc}$  represents the electrophysiologic signature of epithelial ion secretion across biliary cell monolayers, this represents, to our knowledge, the first demonstration of  $Ca^{2+}$ -stimulated secretion across biliary cell monolayers and the first demon-

stration of the complementary role of  $K^+$  channels in expression of the  $Cl^-$  secretory response. The findings also raise the possibility that the underlying mechanisms involved may account in part for the relative resistance to defects in biliary transport in the presence of CFTR mutations.

$Ca^{2+}$ -activated  $K^+$  channels are categorized into classes based on their unitary conductance, with SK channels demonstrating a single channel conductance of  $<50$  pS ("small" conductance) and  $Ca^{2+}$  sensitivity in the range of 10 to 100 nmol/L. Although 3 SK isoforms (SK1, SK2, SK3) have been identified, only SK2 contains the specific apamin-sensitive pore domain and is distinctly apamin sensitive. Accordingly, inhibition by apamin represents a reliable marker for  $K^+$  efflux through SK2 channels. By way of caution, other SK members have been shown to exhibit variable degrees of apamin sensitivity depending on the model studied.<sup>29</sup> However, only SK2 RNA was detectable in biliary cells.<sup>9</sup>

Although, in excitable cells, activation of SK channels results in  $K^+$  efflux and membrane hyperpolarization, which inhibits cell firing, thus mediating the slow after hyperpolarization ( $I_{AHP}$ ) phase,<sup>30,31</sup> the function in epithelial cells has not been as clearly defined. Nonetheless, SK channels have been described in several epithelial cell types, including intestine and liver. In colon, SK contributes to secretion into the intestinal lumen in response to increases in  $Ca^{2+}$ ,<sup>32</sup> and inhibition of intestinal basolateral SK channels blocks luminal  $Cl^-$  secretion and, hence, may be a therapeutic target in the treatment of secretory diarrheas.<sup>33,34</sup> In liver cells, SK plays an important role in cell volume recovery and the adaptive response to metabolic stress.<sup>16,35</sup> The present studies extend these observations and demonstrate for the first time that SK channels play a fundamental role in biliary epithelial cell secretion.

Although 3 complementary methods were used to assess the effects of  $Ca^{2+}$  mobilization, including exposure to ionomycin or thapsigargin, increases in cell volume (hypotonic exposure), and purinergic receptor stimulation, it is acknowledged that significant differences may exist in the signaling pathways and subsequent SK2 channel activation among these approaches. Hypotonic exposure activated SK channels, similar to previous findings, at which cell swelling activates an adaptive response consisting of  $K^+$ ,  $Cl^-$ , and water efflux.<sup>16</sup> It is unclear whether the effects of hypotonicity are mediated through primary modulation of intracellular  $Ca^{2+}$  concentration or through other signaling pathways. We have previously shown that increases in cholangiocyte cell volume result in ATP release, P2 receptor activation, and  $K^+$  and  $Cl^-$  channel activation.<sup>16,36</sup> However, both cur-

rent activation and volume regulation depend on  $[Ca^{2+}]_i$ . Therefore, SK channel activation in response to cell swelling may be a primary event through direct channel gating, or secondary to P2 receptor activation,  $[Ca^{2+}]_i$  modulation, or other signaling pathways. In Mz-Cha-1 cells, extracellular ATP applied to the membrane results in large increases in an apamin-sensitive  $K^+$  conductance. This is presumably through ATP binding to P2 receptors on the membrane and second-messenger-mediated release of  $Ca^{2+}$  from internal stores and/or through ATP-dependent  $Ca^{2+}$  influx from external sources. In other cell types, ATP activates  $Ca^{2+}$ -dependent  $K^+$  channels through activation of P2X receptors and direct  $Ca^{2+}$  influx.<sup>37</sup> The types of receptors and pathways coupling receptor binding to SK channel activation are unknown.

Because the ability to study electrophysiologically primary human cholangiocytes in monolayer culture does not exist at the present time, 2 different models of biliary epithelia were utilized, including human cholangiocarcinoma cells and NRC monolayers. Demonstrating the role of SK2 channels in  $Ca^{2+}$ -stimulated secretion in both epithelial models (rat and human) provides stronger evidence for the role of this channel in  $Ca^{2+}$ -stimulated biliary secretion. However, if these studies in biliary epithelial cell models are relevant to *in vivo* conditions, then several observations merit further investigation.

First, the present studies suggest that the current paradigm concerning the mechanisms of biliary secretion, in which cAMP-dependent activation of CFTR is felt to provide the driving force for secretion, needs to be modified. In these studies, utilizing polarized biliary monolayers,  $Ca^{2+}$ -stimulated secretion was 2-fold greater than that mediated by cAMP. Therefore,  $Ca^{2+}$ -stimulated secretion in fact may be the predominant pathway mediating secretion in biliary epithelium. These observations may provide a mechanistic explanation for the fact that ~80% of patients with CF, and hence no functional CFTR protein in biliary epithelium, do not develop clinical evidence of liver disease. Clearly, further studies are indicated to define the role of  $Ca^{2+}$ -stimulated secretion in both normal and disease states.

Second, the molecular identity of  $Ca^{2+}$ -activated  $Cl^-$  channels remains unresolved. A  $Cl^-$  channel activated by calcium/calmodulin protein kinase II (CaMKII) was recently cloned from a cDNA library derived from bovine trachea and termed *CaCC* (or CLCA-1).<sup>38,39</sup> The cloned protein acts as a CaMKII-modulated  $Cl^-$  channel when reconstituted in a lipid bilayer.<sup>40</sup> However, because reverse-transcription polymerase chain reaction failed to detect transcripts for CaCC in other epithelia, it appears unlikely that this gene product is related to all  $Ca^{2+}$ -

activated  $Cl^-$  channels. In fact, we have been unable to detect transcripts using specific CaCC primers in liver or biliary epithelia, despite a robust  $Ca^{2+}$ -activated  $Cl^-$  current in these cells (unpublished observations).

Third, the observation that the nonselective  $K^+$  channel blocker  $Ba^{2+}$  caused a greater degree of inhibition than apamin indicates that other apamin-insensitive  $K^+$  channels may be involved in the  $Ca^{2+}$ -dependent response, such as BK, IK, or other SK isoforms (SK1, SK3). The biophysical properties of the calcium-activated  $K^+$  channel described here are not consistent with those of BK, which expresses a much larger conductance (>100 pS) and has a different range of sensitivity to  $Ca^{2+}$  (1–10  $\mu\text{mol/L}$ ) IK channels, however, demonstrate a  $[Ca^{2+}]_i$  sensitivity (0.1–10  $\mu\text{mol/L}$ ) and conductance (50–100 pS) between that of SK and BK with substantial overlap in the response of these channels to  $K^+$  channel inhibitors. Therefore, the present studies do not exclude the possibility of some contribution of IK to the  $Ca^{2+}$ -activated secretory response. Additionally, these studies do not exclude the possibility of other SK isoforms in the  $Ca^{2+}$ -dependent response. However, the response here was, in large part, sensitive to apamin, which the other isoforms are not. Although SK3 has been found in hepatocytes, its functional significance is unknown.<sup>29</sup> SK1 has not been found in liver or biliary epithelium.<sup>9</sup> The possible role of other  $Ca^{2+}$ -activated  $K^+$  channel family members in biliary epithelium requires further study.

Fourth, although specific staining of liver sections revealed SK2 protein on both apical and basolateral cholangiocyte membranes, the functional studies in polarized preparations demonstrated a significantly greater basolateral  $Ca^{2+}$ -stimulated  $K^+$  conductance. Because single channel patch clamp studies of separate membrane fractions (apical vs. basolateral) or impedance analysis of monolayers is technically difficult and beyond the scope of the present work, it is impossible to define differences in the number of channels per unit membrane between domains (based on electrophysiology). However, because  $K^+$  ions are recycled actively by the  $Na^+/K^+/ATPase$  pump located on the basolateral membrane, it is conceivable that these channels are also located in close proximity to the pump to provide coordinated responses to changing ion concentration.  $K^+$  efflux, therefore, results in an increase in the local concentration of  $K^+$  ions and, hence, an available pool for reuptake through the pump to maintain intracellular stores. The relative contribution of apical vs. basolateral SK channels requires further study.

Last, although SK2 is dependent on modulations of intracellular  $Ca^{2+}$  concentrations, the other regulatory

pathways have not been elucidated. In these studies, the standard intracellular buffer contained  $\sim 100$  nmol/L  $[Ca^{2+}]$ , and the ionophore ionomycin was utilized to increase acutely intracellular  $[Ca^{2+}]$ . However, regulation of SK2 does not appear to involve a direct interaction between  $Ca^{2+}$  and the channel protein. Instead, regulation depends on an association of calmodulin with the proximal portion of the intracellular C-terminus.<sup>41,42</sup> The roles of calmodulin and CAMKII in the regulation of  $Ca^{2+}$ -stimulated biliary secretion require further study. Additionally, other kinases and regulatory proteins may be involved; in fact, we have previously shown that activation of SK in liver in response to metabolic stress depends on translocation of the  $\alpha$ -isoform of protein kinase C (PKC) to the plasma membrane.<sup>17</sup> The SK2 protein contains multiple consensus PKC phosphorylation motifs, suggesting that PKC may directly affect channel function; however, the role of PKC, as well as other kinases, in  $Ca^{2+}$ -stimulated secretion requires further study.

In conclusion, these are the first studies in polarized biliary epithelial cells to demonstrate that  $Ca^{2+}$ -stimulated secretion is functionally of greater magnitude than cAMP-dependent secretion. Additionally, these studies demonstrate that SK2 channels are fundamental to the regulation of  $Ca^{2+}$ -stimulated cholangiocyte secretion. Although cAMP works through stimulation of CFTR, it is important to note that  $Ca^{2+}$ -dependent secretion appears to take place through non-CFTR pathways. Therefore, elucidation of these pathways may serve to suggest possible therapeutic strategies to bypass the  $Cl^-$  secretory defect associated with CF. Regulation of SK channels, therefore, may represent an important target to modulate biliary secretion in CF as well as other conditions associated with poor bile flow.

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