Cholestatic effects of the K^+ channel blockers Ba^{2+} and TEA occur through different pathways in the rat liver

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Hill, Ceredwyn Elizabeth, and Jody Elisabeth Jacques. Cholestatic effects of the K^+ channel blockers Ba^{2+} and TEA occur through different pathways in the rat liver. *Am. J. Physiol.* 276 (*Gastrointest. Liver Physiol.* 39): G43–G48, 1999.—The role of K^+ channels in bile acid-independent bile flow (BAIF) was studied in the isolated and bile duct-cannulated perfused rat liver by changing the driving force on K^+ and by using a variety of K^+ channel blockers. Bile flow rate, effluent perfusate K^+ content, and portal pressure were measured. Increase in perfusate K^+ from 5.9 to 80 mM caused inhibition of bile flow that could be fitted to a Boltzmann distribution, indicating partial dependence of bile formation on the K^+ equilibrium potential and hence K^+ channel activity. To investigate this further, the effects of compounds established as K^+ channel blockers in liver or other tissues were surveyed. Ba^{2+} (1–5 mM) inhibited mean bile flow by 20%. Tetraethylammonium (TEA) inhibition of basal bile flow was biphasic with saturable (IC_{50} ~0.7 mM) and linear components. In contrast, infusion of the K^+ channel blockers 4-aminopyridine (5 mM), cesium (2.5 mM), quinidine (0.1 mM), iberiotoxin (90 nM), or paxilline (100 nM) did not affect bile flow. As expected for a K^+ channel blocker, Ba^{2+} caused a net K^+ uptake. Conversely, TEA did not affect basal K^+ fluxes, although TEA-induced cholestasis was accompanied by a 26% decrease in biliary glutathione excretion. These results suggest that the partial cholestasis induced by the K^+ channel blockers Ba^{2+} and TEA occurs by significantly different mechanisms. Whereas the Ba^{2+} response implicates K^+ channel activity as a significant driving force in BAIF, TEA-sensitive K^+ channels are not present or are not involved in bile formation.

hepatocyte; bile acid independent; basolateral; tetraethylammonium

BILE, THE AQUEOUS SECRETORY product of the liver, is vital to absorption of fat-soluble nutrients and elimination of metabolic by-products and xenobiotics. Bile is generated by the hepatocytes and elaborated by the bile ductular epithelium in transit through the liver. The major driving force on fluid movement across the hepatocyte epithelium is the accumulation of bile salts in the bile canaliculus. However, bile acid-independent bile flow (BAIF), determined in the presence of low or essentially absent bile acid secretion, can account for between 30 and 60% of total bile flow (7). The forces underlying bile acid-independent bile generation are not understood, although they are generally thought to be osmotic (26). For example, the concentrative accumulation of nonbile acid organic anions, including cyclic guanosine monophosphate (25, 27), or glutathione or its conjugates (1) may provide some of the osmotic driving force for BAIF formation. Glutathione accumulation in bile under conditions reflecting the resting physiological state accounts for about 25% of BAIF (1). Although other as yet unidentified organic molecules may also contribute, it has been suggested that inorganic electrolytes may provide some of the driving force for BAIF (7), either as counterions for organic anion or cation transit through the hepatocyte and into the canaliculus or as independent osmolytes.

K^+ channels are the major contributing elements to resting plasma membrane potential in hepatocytes as in other cell types. Experimental manipulations that cause increased passive K^+ efflux such as hypotonicity or intracellular alkalinization are associated with increased bile flow (5, 15). In contrast, some cholestatic compounds such as progesterone depolarize hepatocyte membranes by inhibiting K^+ conductance (30), further implicating a role for K^+ channels in bile secretion. Lastly, perfusion of the rat liver with the nonspecific K^+ channel blocker Ba^{2+} for 2 min inhibited both K^+ release and bile flow (14) and caused membrane depolarization in isolated hepatocytes (30). There is a dearth of information regarding the involvement and identification of K^+ channel type(s) in resting bile flow, and such data would help to further define the physiological process of BAIF.

We herein report that a significant fraction of BAIF in the bile acid-deprived perfused rat liver is membrane-potential dependent and inhibited by the K^+ channel blockers tetraethylammonium (TEA) and Ba^{2+}. Whereas Ba^{2+} depressed effluent K^+ concentration as expected for K^+ channel blockade, the cholestasis induced by the organic cation TEA was independent of changes in K^+ flux and could be explained by decreased glutathione excretion. The results suggest that Ba^{2+}-sensitive K^+ channels are involved in BAIF, perhaps as a driving force source and/or counterion for canalicular anion excretion. Conversely, organic cation accumulation appears to cause a glutathione-dependent cholestasis.

MATERIALS AND METHODS

Animals and materials. Male Sprague-Dawley rats weighing 200–225 g (Charles River Laboratories, Montreal, PQ) were maintained on a 12:12-h light-dark cycle with access to rat chow and water ad libitum according to the regulations of the Animal Care Committee of Canada. Channel modulators were obtained from Research Biochemicals (Natick, MA) and Sigma Chemical (St. Louis, MO); all other chemicals were from British Drug Houses (Toronto, ON).

Liver perfusions. Livers were perfused via the portal vein with Krebs-Henseleit bicarbonate-buffered (KH) saline using a nonrecirculated, flow-constant perfusion system as de-

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scribed previously (21). $MgCl_2$ was used throughout rather than $MgSO_4$ to cancel the vascular effects of $BaSO_4$ precipitates. Under these conditions we never observed any increase in portal pressure. KH saline was warmed to 37°C, saturated with 95% O_2-5% CO_2 (vol/vol), respectively, and perfused at 3–4 $ml \cdot min^{-1} \cdot g\ liver^{-1}$. Portal pressure was monitored throughout the perfusions. Bile samples were collected over consecutive 5-min intervals from a cannula placed in the common bile duct. Potential modulators were infused in line just upstream from the liver, from 150- to 300-fold concentrated stock solutions. TEA chloride or KCl solutions of 10 mM or greater were prepared in KH saline with NaCl reduced mole-for-mole TEA or KCl and perfused directly. Livers were perfused for at least 30 min before introduction of test substances to the KH saline. In some experiments K^+ balance was measured with a K^+-selective electrode set in the effluent perfusion line (13).

Glutathione assay. Total glutathione [oxidized glutathione (GSSG) plus glutathione (GSH)] was determined in freshly generated bile samples by measuring the initial rate of 5,5′-dithio-bis(2-nitrobenzoate) reduction in the presence of glutathione reductase and NADPH (12).

Statistical analysis. Data are presented as means ± SE of at least three perfusions for each condition. Student's *t*-test was used to compare different experimental regimens. $P < 0.05$ was considered significant.

RESULTS

K^+ diffusion potential contributes to bile flow. Basal bile flow from the isolated rat liver perfused in the absence of bile acids declined over the first 25–30 min to a constant rate that was maintained for the duration of the experiments (Fig. 1*A*, open squares). Between 35 and 55 min the perfusate K^+ concentration was increased from 5.9 to 26, 50, or 80 mM. Results for 50 mM K^+ are illustrated (Fig. 1*A*, solid squares) and are qualitatively representative of results obtained at 26 or 80 mM K^+. Mean bile flow rate showed a transient depression during perfusion with 50 mM K^+ from 1.69 ± 0.03 (5.9 mM K^+, $n = 5$) to 1.53 ± 0.01 ($n = 3$). Return to 5.9 mM K^+ resulted in a transient increase in bile flow whose amplitude was linearly correlated with the test K^+ concentration (data not shown). For experiments in which K^+ was increased to 50 or 80 mM, 30 μM nifedipine was infused throughout the entire perfusion to reduce the vasoconstriction in response to K^+ depolarization and to allow attainment of a new steady-state rate of bile flow due to the choleretic activity of nifedipine before changing the K^+ concentration. In the presence of nifedipine, a transient rather than more sustained increase in portal pressure, as reported earlier (21), occurred at the onset of 50 mM K^+ (peak at 1.5 min of 0.4 cmH_2O, 4 min duration). With 80 mM K^+, the peak, at 0.5 min, was larger in amplitude (4.2 ± 0.6 cmH_2O) and was followed by a new steady state of 0.7 ± 0.1 cmH_2O above basal that fully recovered after return to 5.9 mM K^+ (data not shown). Peak inhibition of bile flow at each K^+ concentration was best fitted by a Boltzmann distribution (Fig. 1*B*, solid line), having an *e*-fold% decrease in bile flow per 15 ± 3 mM increase in perfusate K^+ and an apparent IC_{50} of 90 ± 3 mM K^+.

K^+ channel inhibitors and bile flow. When livers were perfused with Krebs saline containing either 2 mM

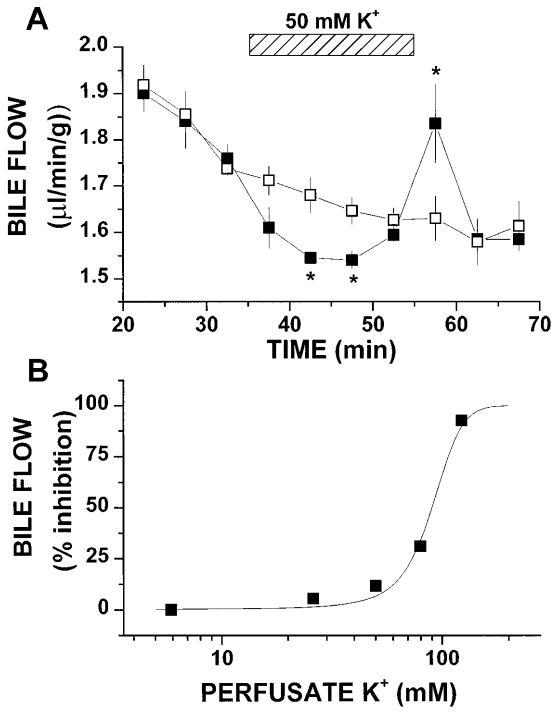

Fig. 1. Effects of increased perfusate K^+ concentration on bile flow. *A*: mean bile flow rates were measured in the presence of 5.9 mM (□, $n = 5$) or 50 mM K^+ (■, $n = 3$) between 35 and 55 min. *B*: peak inhibition of bile flow plotted as average percent decrease between livers perfused in the presence of 26–122.7 mM K^+ ($n = 3$, means ± SE at each concentration) relative to control flow rates with 5.9 mM ($n = 5$). Data were fitted to a Boltzmann relationship as described in text (solid line). * Significantly different from control ($P < 0.05$).

$BaCl_2$ (Ba^{2+}) or 10 mM TEA chloride (TEA) between 35 and 45 min, bile flow was significantly inhibited compared with control rates (Con, Fig. 2). Mean bile flow between 35 and 45 min was 1.06 ± 0.04 ($n = 5$) and 0.86 ± 0.04 ($n = 8$) or 0.85 ± 0.04 ($n = 3$) $\mu l \cdot min^{-1} \cdot g$

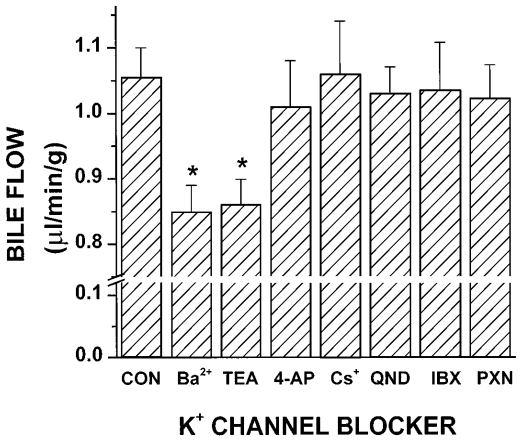

Fig. 2. Effects of various K^+ channel modifiers on bile flow rate. Livers were perfused under control conditions for 35 min and then in the absence (Con) or presence of Ba^{2+} (2 mM), tetraethylammonium (TEA, 10 mM), 4-aminopyridine (4-AP, 5 mM), cesium (Cs^+, 2.25 mM), quinidine (QND, 0.1 mM), iberiotoxin (IBX, 90 nM), or paxilline (PXN, 100 nM) from 35 to 45 min. Bile flow rates were calculated within each perfusion between 35 and 45 min, and means of 3 perfusions under each condition are plotted. * Significantly different from control ($P < 0.05$).

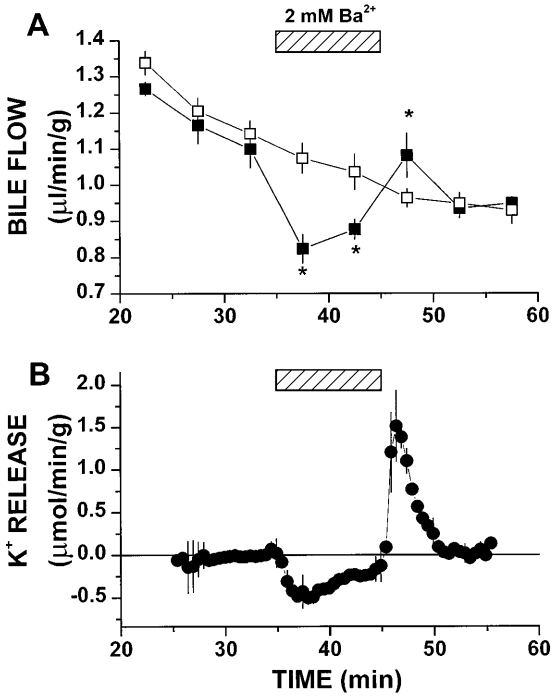

Fig. 3. Effect of Ba^{2+} on bile flow and K^+ balance. *A*: livers were perfused in the absence (□, $n = 3$) or presence (■, $n = 3$) of 2 mM Ba^{2+} between 35 and 45 min, and mean ± SE bile flow rates were plotted. *Significantly different from control ($P < 0.05$). *B*: net K^+ release (positive) or uptake (negative) in same livers plotted as function of perfusion time ($n = 3$, means ± SE).

liver^{-1} in the absence and presence of TEA or Ba^{2+}, respectively. The depression in bile flow with 1 or 5 mM Ba^{2+} was not significantly different from that seen with 2 mM Ba^{2+}, indicating that the Ba^{2+}-sensitive component of bile flow was fully blocked at 1 mM (not shown). Additional K^+ channel blockers were tested for their ability to inhibit bile flow to obtain a pharmacological profile of the type of K^+ channel involved in bile generation. Both relatively nonselective and subtype-specific antagonists were used, the latter focusing on the large conductance Ca^{2+}- and voltage-dependent (BK) channel since there is, respectively, either substantial or no evidence that SK and K_{ATP} channels are expressed in rat hepatocytes (6, 28). Whereas Ba^{2+} or TEA significantly inhibited mean bile flow by 20 or 18.5%, respectively, 10-min infusions of 4-aminopyridine (4-AP, 5 mM), cesium (Cs^+, 2.25 mM), quinidine (0.1 mM), or the specific maxi-K^+ channel antagonists iberiotoxin (90 nM) or paxilline (100 nM) did not significantly depress bile flow (Fig. 2).

Ba^{2+}-induced cholestasis is associated with decreased K^+ release. Figure 3 shows the time dependence of the effects of 2 mM Ba^{2+} on bile flow (Fig. 3*A*) and in the same perfusions net K^+ flux (Fig. 3*B*). As observed with increased perfusate K^+, Ba^{2+} stimulated a transient reduction in bile flow that was followed by a brief increase above control immediately on perfusion with Ba^{2+}-free Krebs. Perfusate K^+ followed a similar pattern, with Ba^{2+} causing a rapid decrease in K^+ release that decayed slowly over the 10-min infusion period. Removal of Ba^{2+} transiently increased the rate of K^+ release above control conditions to about 1.5 μmol·min^{-1}·g^{-1}. Integrating the K^+ trace during and after Ba^{2+} exposure showed that the 3.9 μmol/g released following washout of Ba^{2+} fully compensated for the amount retained during Ba^{2+} perfusion (3.2 μmol/g). At no time during or following Ba^{2+} exposure were changes in portal pressure observed.

TEA-induced cholestasis is associated with glutathione excretion but not K^+ release. In contrast to Ba^{2+}, TEA-induced cholestasis followed a different pattern that was not associated with changes in K^+ flux but was accompanied by decreased efflux of glutathione (GSSG + GSH) into the bile. Figure 4*A* shows that a 15-min exposure to 10 mM TEA inhibited mean bile flow by 22.7 ± 3.5% ($n = 3$). Glutathione excretion was reversibly inhibited by 26% from 1.51 ± 0.05 nmol·min^{-1}·g^{-1} in control perfusions to 1.10 ± 0.05 nmol·min^{-1}·g^{-1} during the final 10 min of TEA perfusion (Fig. 4*B*). In the same experiments K^+ flux into the perfusate was not different from control levels (not shown). Glutathione release in the presence of 2 mM Ba^{2+}, rather than TEA, was not significantly different from basal (not shown).

Lastly, the concentration dependence of TEA-induced cholestasis was measured with the aim of determining whether the pharmacological properties would inform us as to the mechanism of TEA action. Figure 5 shows the percentage decrease in bile flow, calculated from the averaged rate between livers perfused in the absence ($n = 5$) or presence of TEA (2–40 mM, $n \geq 3$ at each

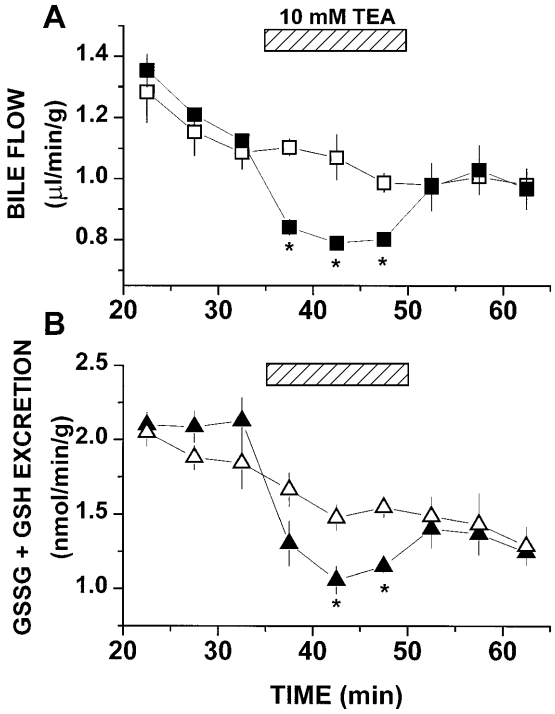

Fig. 4. Effect of TEA on bile flow and glutathione excretion. *A*: livers were perfused in the absence (□ and △, $n = 3$) or presence (■ and ▲, $n = 3$) of 10 mM TEA between 35 and 45 min and mean ± SE bile flow rates were plotted. *B*: rate of total glutathione [oxidized glutathione (GSSG) plus glutathione (GSH)] appearance in bile of same livers plotted as function of perfusion time ($n = 3$, means ± SE). *Significantly different from control ($P < 0.05$).

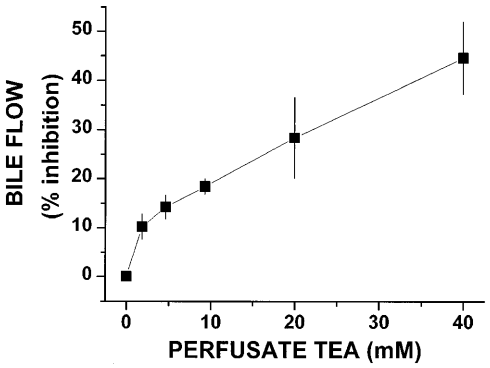

Fig. 5. Concentration dependence of TEA inhibition of bile flow. Mean bile flow rates were measured in the absence (n = 5) or presence of 2–40 mM TEA between 35 and 55 min, and percent inhibition from control rates was plotted (n = 3 at each concentration, means ± SE).

concentration) plotted as a function of the perfusate TEA concentration. The plot shows a biphasic relationship between TEA and inhibition of bile flow, suggesting high (apparent IC_{50} of near 0.7 mM TEA) and low affinity or linear components.

DISCUSSION

The results presented in this report demonstrate that a significant fraction of basal bile flow in the bile acid-deprived perfused rat liver is correlated with the K^+ equilibrium potential and therefore is related to K^+ channel activity. This was confirmed by the observation that the inorganic K^+ channel blocker Ba^{2+} reversibly inhibited K^+ release in parallel with the depression in bile flow rate. Conversely TEA, a small organic cation and K^+ channel blocker in other tissues, was cholestatic but did not affect passive K^+ flux. TEA decreased biliary glutathione concentration, suggesting that TEA-induced cholestasis occurs through perturbation of an organic anion excretory pathway. The contradiction between the two cholestatic and channel blocking agents is discussed in terms of the different fates of these compounds in the liver. It is proposed that a basolateral Ba^{2+}-sensitive K^+ channel provides some of the driving force for anion and hence water accumulation in the canalicular space, whereas TEA-sensitive K^+ channels are not likely to be involved in bile formation.

We used two independent approaches to assess the contribution of K^+ channels and membrane potential to BAIF. First, we showed that the bile flow rate vs. perfusate K^+ relationship followed a Boltzmann distribution (Fig. 1), implying a dependence of a component of bile flow on membrane potential. Membrane depolarization in response to increased perfusate K^+ in isolated hepatocytes or hepatocyte couplets qualitatively reflects the change in the K^+ equilibrium potential (E_K), so that at 20 mM or higher external E_K is more positive than the recorded membrane potential (3, 8, 11). Therefore, although K^+ channels may remain open, and in fact hepatocyte K^+ conductance increases under these conditions (11), current flow is inward. This would decrease the driving force on canalicular anion efflux and, according to our model, decrease BAIF, as was observed.

The second approach specifically addressed the hypothesis that passive K^+ flux provides the underlying mechanism for potential-dependent BAIF. It was predicted that pharmacological block of outward K^+ flux would inhibit anion and water flow into the biliary compartment. From a variety of specific and nonspecific K^+ channel blockers (Fig. 2), Ba^{2+} was identified as the sole cholestatic compound also capable of inhibiting K^+ release (Fig. 3), confirming and extending earlier reports that a short exposure (2 min) to Ba^{2+} inhibits bile flow and K^+ release (13, 14). Ba^{2+} has been reported to depolarize the membrane of isolated hepatocytes likely as a consequence of Ba^{2+}-induced increase in membrane resistance and decrease in K^+ transference number (3, 8). Therefore, Ba^{2+} acts mainly as a K^+ channel blocker in the liver. Although we did not directly measure membrane potential in the intact perfused liver, the earlier reports all suggest that membrane depolarization would occur in response to either Ba^{2+} infusion or increased perfusate K^+ concentration (3, 8). In addition the present experiments do not exclude the possibility that other hyperpolarizing forces may contribute to BAIF. Alternatively, novel K^+ channels insensitive to the blockers used in the present study may be expressed in the liver and mediate some of the driving force behind BAIF. Both of these possibilities must be considered because pharmacological intervention with Ba^{2+} decreased BAIF by 20%, whereas 122.7 mM perfusate K^+ caused 92.5% inhibition, leaving a Ba^{2+}-insensitive but potential-dependent fraction of total BAIF of 72.5%.

In both the increased perfusate K^+ and the TEA experiments presented here the cations replaced Na^+ on a mole-for-mole basis, raising the possibility that decreased perfusate Na^+ was limiting BAIF generation. However earlier studies report that complete replacement of Na^+ with Li^+ resulted in either a 17% decrease or a 20% increase in bile flow (10, 30) compared with a >80% block when K^+ was the substituting ion (Ref. 10 and Fig. 1*B*). Replacement of Na^+ with cations that are less permeant to the paracellular pathway, such as choline or Tris, also inhibited bile flow by up to 90% (10, 29). Whereas the organic cations were thought to decrease osmotic water flow as a result of their decreased paracellular permeability, or reflection coefficient, relative to Na^+ (10) this is not likely to be the situation for K^+, which is similar in ionic radius to Na^+ and Li^+.

The mechanism by which TEA, and perhaps other small organic cations such as choline, is cholestatic is as yet undefined, but the primary event may involve membrane depolarization. This prediction is supported by reports that both TEA and choline are transported into the hepatocyte through potential-sensitive conductive pathways (22–24), and cloned TEA transporters generate inward currents under voltage clamp (9). Such electrogenic uptake of TEA would result in membrane depolarization and, as reported here, inhibition of BAIF. Depolarization could depress BAIF by inhibit-

ing one or more of the canalicular organic anion transport processes. Because oxidized glutathione is the major choleretic organic anion in the rat liver under resting conditions (1) and excretion is at least partially dependent on membrane potential (2), we predicted that TEA exposure would decrease the rate of glutathione excretion. Our results demonstrated a correlation between the degree of cholestasis and the rate of glutathione appearance in the bile (Fig. 4). Lastly, TEA inhibited bile flow with similar kinetic parameters (IC_{50} of ~0.7 mM, Fig. 5) as transport [Michaelis constant 1.1 ± 0.5 mM (23)], indirectly suggesting that transport into the hepatocyte is required for the cholestatic effect.

Earlier studies have established that exposure of the perfused rat liver to 4-AP and Cs^+ caused K^+ channel blocker-associated changes in liver hemodynamics after 1 or 20 min, respectively, and it was concluded that the difference in response times was related to the permeability of the channel blockers (17, 18). Therefore Cs^+, which blocks most K^+ channels, may have been ineffective in the present experiments because it may not have accumulated within the hepatocyte over the time course of the experiments (20 min, Fig. 2). Conversely, it is likely that 4-AP and quinidine would have easily permeated the hepatocyte membrane. Because they were ineffective, we surmise that 4-AP and quinidine-sensitive K^+ channels are not involved in bile formation. The lack of effect on bile flow of two different large-conductance, Ca^{2+}-activated K^+ channel blockers, iberiotoxin and paxilline, indicate that BK-type channels, although identified in avian hepatocytes (20), are not involved in BAIF in the rat liver. The blocker profile suggests that inward rectifying K^+ channels would contribute to BAIF, and these channels have been demonstrated in rat and embryonic chick hepatocytes (4, 16, 19). However, because Ba^{2+}-sensitive K^+ channels have not yet been identified in the rat hepatocytes at the single channel level we cannot conclude what specific channels may be involved in BAIF.

In conclusion, our results identify a significant role for membrane potential in BAIF and further show that this is mediated by passive K^+ flux. Partial cholestasis appears to be a consequence of disruption of the electrogenic outward flow of cations that is proposed to be involved in maintaining anion sequestration in the canaliculus. We suggest that the basolateral localization of K^+ channels would provide charge balance and possibly a driving force for Cl^- and/or organic anion release into the bile canaliculus. Accumulation of the latter would serve as an osmotic stimulus for water flow into the secretory compartment.

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