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GTP-Binding Proteins Regulate High Conductance Anion Channels in Rat Bile Duct Epithelial Cells

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Summary. Epithelial cells from the intrahepatic bile duct contribute to bile formation, but little is known of the cellular mechanisms responsible. In these studies, we have characterized the endogenous GTP-binding proteins (G proteins) present in these cells and evaluated their role in regulation of high conductance anion channels. G proteins were identified in purified plasma membranes of isolated bile duct epithelial cells using specific antisera on Western blots, and ion channel activity was measured in excised inside-out membrane patches using patch-clamp recording techniques. In patches without spontaneous channel activity, addition of cholera toxin to the cytoplasmic surface had no effect ($n = 10$). Addition of pertussis toxin caused an activation of channels in 13/34 (38%) attempts, as detected by an increase in channel open probability. Activated channels were anion selective (gluconate/Cl^- permeability ratio of 0.17 ± 0.04) and had a unitary conductance of ~380 pS. Channel open probability was also increased by the nonhydrolyzable GDP analogue guanosine 5′-0-(2-thiodiphosphate) in 8/14 (57%) attempts. In contrast, channel open probability was rapidly and reversibly decreased by the nonhydrolyzable analogue of GTP 5′guanylylimidodiphosphate in 7/9 (78%) attempts. Western blotting with specific antisera revealed that both $G_i\alpha$-2 and $G_i\alpha$-3 were present in significant amounts, whereas $G_i\alpha$-1 and $G_o\alpha$ were not detected. These studies indicate that in bile duct epithelial cells, high conductance anion channels are inhibited, in a membrane-delimited manner, by PTX-sensitive G proteins.

Key Words patch clamp · epithelial transport · bile secretion

Introduction

Epithelial cells lining the intrahepatic portion of bile ducts have morphologic characteristics of secretory cells with tight junctions and abundant apical microvilli (Sirica & Cihla, 1984; Ishii, Vroman & LaRusso, 1989; Sirica et al., 1990; Slott, Liu & Tavoloni, 1990). Although their physiologic role is not established, there is increasing evidence that bile duct epithelial (BDE) cells contribute directly to the volume and composition of bile through primary secretion of fluid and electrolytes (Alpini et al., 1988; Alpini et al., 1989; Nathanson & Boyer, 1991). These and other functions are regulated through receptors for secretin (Farouk et al., 1992) and presumably other hormones, but little is known regarding the signal transduction pathways and effector mechanisms involved.

Recently, high conductance anion (HCA) channels have been identified in the plasma membrane of BDE cells in short-term culture (McGill, Basavappa & Fitz, 1992). These channels have a slope conductance of ~380 pS, are anion selective, and have significant permeability to both HCO_3^- and Cl^- with a permeability ratio of ~0.5 (McGill, et al., 1992). HCA channels are generally closed under basal conditions in intact cells consistent with reports of similar channels in epithelial (Schneider et al., 1985; Light et al., 1990) and nonepithelial (Schlichter et al., 1990) preparations.

Heterotrimeric GTP-binding proteins (G proteins) play a central role in coupling membrane receptors to effector mechanisms within the cell. Two classes of G proteins termed G_s and G_i were originally classified based on their stimulatory or inhibitory effects on adenylyl cyclase (Ross & Gilman, 1977; Johnson, Kaslow & Bourne, 1978). More recently, distinct $G_i\alpha$ subunits, which differ in primary amino acid sequences, have been identified (Sunyer et al., 1989). Additionally, G proteins are recognized to regulate a wider variety of effector mechanisms, including membrane ion channels (Yatani et al., 1987; Cerbai, Klöckner & Isenberg, 1988; Cohen-Armon, Garty & Sokolovsky, 1988; Kirsch et al., 1988; Logothetis et al.,

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1988; Yatani et al., 1988; Light, Ausiello & Stanton, 1989; Fargon, McNaughton & Sepúlveda, 1990; Inoue & Isenberg, 1990; Inoue & Kuriyama, 1991). Most studies have focused on regulation of cation-selective (K^+, Ca^{2+}, Na^+) channels in excitable cells (Brown, 1990; Brown & Birnbaumer, 1988; Birnbaumer et al., 1989). By contrast, little is known regarding effects of G proteins on anion permeable channels.

Epithelial anion channels are thought to be involved in maintenance of cell volume and pH, secretion and absorption of fluid and electrolytes, and other physiologic processes. Recently, nonhydrolyzable analogues of GTP, which can activate G proteins in the absence of receptor stimulation (Breitwieser, 1991), have been shown to increase the open probability (P_o) of ~305 pS anion channels in renal epithelia (Schwiebert et al., 1990) and ~20 pS anion channels in HT29 cells from human colon carcinoma (Tilly et al., 1991). This appears to be a direct G-protein effect on channel activity rather than one requiring intermediate signaling pathways. In renal epithelium, the effect on channel P_o is sensitive to pertussis toxin (PTX) and is reproduced by exposure to $G_i\alpha$-3 purified from human erythrocyte membranes. The G proteins endogenous to these cells which may participate in channel regulation are not established.

In the present studies on isolated BDE cells, we demonstrate a distinctive pattern for regulation of HCA channels where PTX-sensitive G proteins mediate inhibition rather than activation of channels. We also demonstrate that $G_i\alpha$-2 and $G_i\alpha$-3 are the principal PTX-sensitive substrates, suggesting that one or both may contribute to regulation of membrane Cl^- and HCO_3^- transport through effects on channel P_o.

Materials and Methods

Cell Isolation and Culture

As previously described (McGill et al., 1992), BDE cells were isolated from the intrahepatic bile duct of male Sprague-Dawley rats (200–250 g) two to six weeks after ligation of the common bile duct, using methods of Sirica et al. (Sirica & Cihla, 1984; Sirica et al., 1990; Sirica, Sattler & Cihla, 1985; Mathis, Walls & Sirica, 1988). This resulted in a relatively pure (>90%) population of cells, 7–10 μm in size, which retain staining for bile duct markers γ-glutamyl transpeptidase (GGT) and cytokeratin-19 (CK-19). BDE cells are readily distinguished from hepatocytes by size and morphology (Mathis, et al., 1988; Ishii, et al., 1989). Isolated cells were plated on 35 mm dishes, previously coated with rat tail collagen, and maintained in HCO_3^- containing CMRL-1066 medium (GIBCO BRL, Grand Island, NY), in 5% CO_2 at 37°C, and studied after ~20 hr.

Purification and Solubilization of Membranes and Immunological Detection of G Proteins

Using the method of Moore and Moore (1989), plasma membranes of freshly isolated BDE cells were purified. They were subsequently solubilized on ice for 1 hr in (mM) 20 Tris, 1 EDTA, 1 dithiothreitol (DTT), 100 NaCl, and 0.9% Na cholate (pH 8.0). The suspension was then centrifuged at 13,000 × g for 10 min and protein in the supernatant was collected. Solubilized membrane protein (200 μg) was loaded onto the stacking gel in one lane, which spanned 90% of the total width. Solubilized plasma membranes were resolved by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide, 0.051% DATD), and electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA) PVDF membranes (Uhing, Polakis & Snyderman, 1987; Casey & Gilman, 1988). Following the procedure of Mumby et al. (1988), PVDF membranes were blocked with 5% dried skim milk in buffer (pH 8.0) containing (in mM) 50 Tris, 2 $CaCl_2$, 80 NaCl, 0.02% Na azide, and 0.2% nonidet P-40 for 1 hr at room temperature with gentle shaking. After blocking, the PVDF membrane was transferred into a Milliblot-MP (Millipore, Bedford, MA) multiple-manifold device which divided the blot into 24 separate lanes. The primary antisera were diluted as specified in low-detergent blotto and added in replicates of 4 lanes for each specific antibody type. The blot was incubated for 2 hr at room temperature with gentle rocking. After four, 10 min washes in low-detergent blotto, ^{125}I-labeled goat anti-rabbit IgG (1 × 10^6 cpm/ml) was incubated with the membranes for 1.5 hr at room temperature. The membranes were subsequently washed three times with low-detergent blotto and twice in Tris-buffered saline without skim milk, blotted dry, and exposed to diagnostic Kodak XAR film with intensifying screens.

Antisera for Western Blots

Antisera were raised against the C-terminal decapeptides of $G_i\alpha$-3 and $G_s\alpha$ (Lynch et al., 1989) and against an internal sequence of $G_i\alpha$-1 (Goldsmith et al., 1988). Antiserum which recognized both $G_i\alpha$-1 and $G_i\alpha$-2 were raised against the C-terminal decapeptide which is shared by both α-subunits of these G proteins (Lynch et al., 1989). The peptides were conjugated to keyhole limpet hemocyanin (KLH) and rabbits were immunized with each conjugate according to the method of Green et al. (1982). The antisera were characterized with respect to titre, specificity and cross reactivity using lysates from bacteria trasfected with the genes for each of the G proteins (Linder & Gilman, 1991). Antisera were used at final dilutions of: $G_s\alpha$, 1 : 16,000; $G_i\alpha$-1, 1 : 1000; $G_a\alpha(1,2)$, 1 : 4000; $G_i\alpha$-3, 1 : 8,000. Antisera specific for $G_o\alpha$ was obtained from Dr. Janet Robishaw (Weis Center for Research, Geisinger Clinic, Danville, PA). Antisera specific for $G_i\alpha$-2 (J-883) was obtained from Dr. Susie Mumby (U. of Texas, Southwestern Medical Center, Dallas, TX).

Patch-Clamp Recording

Single channel currents were measured using patch-clamp recording techniques (Hamill et al., 1981) in excised inside-out membrane patches from cells ~20–30 hr following isolation. Immedi-

ately before study, culture medium was replaced with NaCl-rich electrolyte buffer (*see below*) at room temperature (22–25°C) and isolated cells attached to the dish were selected for study. Patch pipettes were pulled from Corning 7052 glass and had resistances of 3–10 MΩ when filled with the same buffer.

Recordings were made with an Axopatch 1C or 1D amplifier (Axon Instruments, Burlingame, CA), and signals were filtered at 0.5 to 1 kHz bandwidth using a four-pole low-pass Butterworth filter. Currents were digitized (5 kHz) for storage on a Compaq 386/20e computer and analyzed using pCLAMP software (Axon Instruments). Movement of positive charge from pipette to bath is shown as an upward deflection in the current traces; and pipette voltages (V_p) are referred to the bath unless otherwise indicated. $-V_p$ corresponds to the "membrane potential" in excised inside-out patches. All current-voltage plots are shown with outward membrane current and membrane depolarization as positive.

After formation of a high resistance seal (typically 10–60 GΩ), the membrane patch was excised. The pipette potential was held at +15 mV (membrane potential of −15 mV), except for brief (400 msec) test pulses to more polarized potentials to avoid voltage-dependent changes in channel activity (McGill et al., 1992). Currents were recorded under basal conditions and after addition of experimental reagents including: 5′guanylylimidodiphosphate (Gpp(NH)p, 1 μM), guanosine 5′-O-(3-thiotriphosphate) (GTPγS, 1 μM), PTX (1 μg/ml, List Biological Labs., Campbell, CA), cholera toxin (CTX, 5 μg/ml, List Biological Labs.), and guanosine 5′-O-(2-thiodiphosphate) (GDPβS, 100 μM). Unless otherwise indicated, reagents were from Sigma (St. Louis, MO). Channels were identified as HCA channels by a high slope conductance ≥300 pS, steep voltage-dependent inactivation, and reversal at the equilibrium potential for Cl^-. Channels were considered open when the current was ≥50% of the fully open level, and P_o was determined from one or more 10 sec recordings. Current-voltage plots were fitted with first-order least-squares linear regression when bath and pipette contained equal Cl^- concentrations, or second-order regression when bath and pipette contained unequal Cl^- concentrations as previously described (McGill et al., 1992). Values are given as mean ±SE where n denotes the number of patches. Statistical comparisons were made using the paired t-test and $P < 0.05$ was considered significant.

SOLUTIONS

The standard NaCl-rich bath solution contained (in mM) 140 NaCl, 4 KCl, 1 KH_2PO_4, 2 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES/NaOH (pH ~ 7.30) with a total Cl^- of 150 mM. In studies with PTX, 0.01 mM NAD and 0.1 mM ATP were included in the bath solution. In preliminary studies, these agents alone had no effect on channel activity. The standard KCl-rich pipette solution contained (in mM) 130 KCl, 10 NaCl, 2 $MgCl_2$, 0.1 ATP, free Ca^{2+} adjusted to ~100 nM (0.5 $CaCl_2$, 1 EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid), and 10 HEPES/KOH (pH ~ 7.20) with a total Cl^- of 145 mM. Low Cl^- solutions were made by equimolar replacement with gluconate. Although Cl^- and gluconate differ in their affinity for Ca^{2+}, the channel under investigation shows no Ca^{2+} dependence (McGill et al., 1992).

Results

SUMMARY OF CHANNEL CHARACTERISTICS

In previous studies, HCA channels were observed in unstimulated BDE cells in only 11% of cell-attached patches, and in these, P_o remained <0.05 (McGill et al., 1992). Channels showed rapid flickering kinetics with multiple subconductance states, and rarely reached the fully open level. Brief openings were interspersed with long periods of closure lasting minutes and maneuvers that increased intracellular Ca^{2+} or cAMP failed to increase channel activity (McGill et al., 1992). Following excision, channels activated spontaneously in 27% (28/102) of patches. Once activated, channels had a high P_o (>0.8), between −20 and +20 mV, but exhibited voltage-dependent closure outside of this range. These properties appear similar to HCA channels in renal (Schwiebert et al., 1990) and other epithelia (Nelson, Tang & Palmer, 1984; Schneider et al., 1985).

EFFECT OF G PROTEINS ON CHANNEL ACTIVITY

Currents were measured in excised inside-out patches to allow control of membrane potential and access of test reagents to the cytoplasmic surface of the patch, and the results are summarized in Table 1. In patches with no spontaneous channel activity, addition of CTX (5 μg/ml) to the solution bathing the cytoplasmic membrane failed to activate channels in 10 attempts. By contrast, exposure to PTX (1 μg/ml) activated HCA channels in 13/34 (38%) patches with an increase in channel P_o from 0.01 ± 0.01 to 0.90 ± 0.03 ($P < 0.05$). In nonresponding patches, channel activity could not subsequently be induced by either strong depolarization or exposure to air, suggesting that no channels were present. A representative example is shown in Fig. 1. Under basal conditions when pipette voltage was stepped from 0 to −80 mV for 400 msec, no channel currents were detected. After addition of PTX, there was a large increase in the initial current following the voltage step. Sequential closure of channels revealed six current levels, consistent with activation of multiple channels. Channels activated after an average delay of 95 ± 101 sec. In patches where channels were spontaneously active, addition of PTX had no effect (n = 3). Activated channels showed a linear current-voltage relation with a conductance of 380 ± 64 pS and reversed at 1.5 ± 1.1 mV in standard Cl^--containing solutions. In four patches, when pipette Cl^- was partially replaced with gluconate (126 mM), there was a 31 ± 3 mV shift in reversal in the direction anticipated for a Cl^--selective channel. This

Table 1. Channel open probability (P_o) measured in excised, inside-out membrane patches under basal conditions and after exposure to test reagents[a]

Reagent	n	P_o, basal	P_o, experimental	Latency(s)
Control	16	0.82 ± 0.09		
CTX	10	0.03 ± 0.02	0.01 ± 0.04	
PTX	13	0.01 ± 0.01	0.90 ± 0.03[b]	95 ± 101
PTX	3	0.75 ± 0.14	0.83 ± 0.10	
GDPβS	8	0	0.85 ± 0.94[b,c]	17 ± 9
Gpp(NH)p	5	0.92 ± 0.07	0.22 ± 0.17[b]	96 ± 39

[a] Pipette potential was held at +15 mV where voltage-dependent channel closure is not present (McGill, Basavappa & Fitz, 1992).
[b] Indicates $P < 0.05$ as compared to basal values by paired t-test, and n represents the number of patches. There were no statistically significant differences in P_o between spontaneous, PTX, or GDPβS activated channels ($P > 0.9$).
[c] Activation of channels was observed in 8 of 14 attempts. In the majority of these, multiple channels were activated which confounds calculation of P_o. Hence, analysis of P_o was limited to three patches, each having only one channel.

corresponds to a gluconate: Cl^- permeability ratio of 0.17 ± 0.04, similar to values observed in spontaneously active channels (McGill et al., 1992).

PTX slows the rate of receptor-dependent activation of G_i and G_0 (Light et al., 1989), and, in cyc- S49 cells, also slows the spontaneous rate of transition of the inactive GTP-G-protein complex to the active complex in the absence of receptor agonists (Sunyer et al., 1989). These effects occur rapidly in the presence of NAD and ATP (Katada & Ui, 1982). Consequently, activation of channels by PTX suggests that opening of HCA channels may be inhibited by a PTX-sensitive G protein. This was evaluated further by exposure to nonhydrolyzable analogues of GDP and GTP which inhibit and activate G proteins, respectively, in the absence of receptor stimulation (Breitwieser, 1991). Exposure to GDPβS had effects similar to PTX, resulting in activation of HCA channels in 8/14 (57%) attempts with an increase in P_o from 0.0 to 0.85 ± 0.04 ($P < 0.05$). In the six nonresponding patches, channels could not be activated by PTX or other maneuvers. By contrast, exposure to Gpp(NH)p failed to activate channels, insignificantly increasing P_o in only 2/17 attempts (12%). However, when Gpp(NH)p was added to patches with active channels, there was a rapid decrease in P_o in 7/9 (78%) attempts. Similar inhibitory effects were also observed in three patches using GTPγS. In selected studies, channel inhibition by Gpp(NH)p could be reversed by higher concentrations of GDPβS. In the example shown in Fig. 2, a single channel activated spontaneously after excision and had a $P_o > 0.7$ measured over 4 min. Addition of Gpp(NH)p was followed after ~45 sec by a decrease in P_o, with openings occurring as brief bursts. Subsequent addition of higher concentrations of GDPβS rapidly reactivated this channel with a marked decrease in apparent flickering.

Effect of G-Proteins on Voltage-Dependent Closure

P_o of HCA channels in BDE and other cells is greatest between −20 and +20 mV but decreases at more polarized potentials as a result of voltage-dependent channel closure (McGill et al., 1992). This suggests the presence of at least two voltage-sensitive sites which mediate channel closure at depolarized or hyperpolarized potentials (Schlichter et al., 1990). To determine if channel activation by PTX or GDPβS results from a change in voltage sensitivity, the kinetics of channel closure were determined in PTX or GDPβS activated channels, as illustrated in Fig. 3. For these studies, pipette potential was held at 0 mV where channels are generally open, and then stepped repetitively to test potentials between −80 and +80 mV for 400 msec at 2 sec intervals. Summed currents were constructed from 15 to 30 repetitions at a single potential. The time course for inactivation of the summed current could generally be described by a single exponential which decreased as the magnitude of the test voltage increased. Results were not different at positive and negative potentials of equal magnitude and were combined for statistical analysis. In three patches containing a single channel, time constants for channel closure were 198 ± 18 msec at ±40 mV; 94 ± 16 msec at ±60 mV; and 31 ± 6 msec at ±80 mV. As shown in Table 2, these values are not different from values in spontaneously active channels from BDE cells, suggesting that the

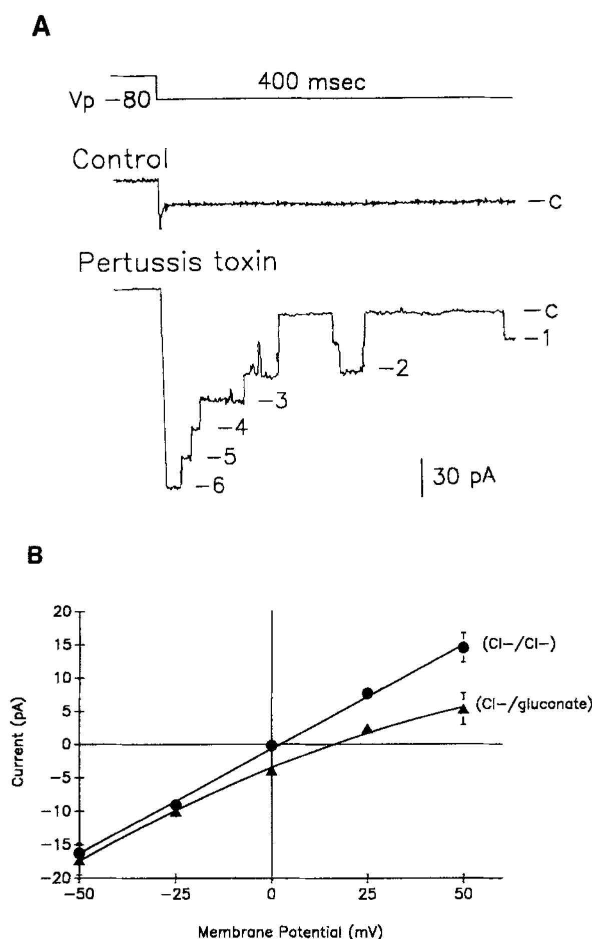

Fig. 1. Effect of pertussis toxin on channel activity. (*A*) In this inside-out membrane patch, pipette potential was stepped from 0 to −80 mV for 400 msec. Under control conditions, no channels were detected. Following pertussis toxin, there was activation of multiple channels within 2 min. In this example, this is seen as a large increment in currents at the onset of the voltage step, and sequential closure of channels over 400 msec. At least six discrete levels, representing individual channels, are present. (*B*) An average current-voltage plot of G-protein-activated channels is shown when the bath contains the NaCl-rich solution with 150 mM Cl^-. When the pipette solution contained 145 mM Cl^- [Cl^-/Cl^-, (n = 5)], slope conductance was 312 pS and currents reversed at ~1 mV. Lowering pipette Cl^- to 24 mM, by substitution with gluconate [Cl^-/gluconate, (n = 5)], caused a shift in reversal potential in the direction anticipated for an anion-selective channel.

effects of G proteins are not likely to result from changes in voltage sensitivity.

Identification of Endogenous G Proteins

PTX-sensitive G proteins present in BDE cell membranes were characterized by Western blotting with specific antisera (Fig. 4). The failure of $G_i\alpha$-1-specific antisera to detect $G_i\alpha$-1 in the first four lanes, coupled with the strong signal in lanes 5–8, using an antibody which recognizes both $G_i\alpha$-1 and $G_i\alpha$-2, suggests that $G_i\alpha$-2 is present in significant amounts. This was also addressed with $G_i\alpha$-2-specific antisera J-883 (Mumby & Gilman, 1991) in separate blots which confirmed that $G_i\alpha$-2 was present (*data not shown*). The results from Fig. 4 also indicate the presence of significant amounts of $G_i\alpha$-3 and $G_s\alpha$. In other separate blots, $G_o\alpha$ was not detected (*data not shown*).

Discussion

These studies in intrahepatic BDE cells demonstrate that the activity of HCA channels in excised patches is regulated by PTX-sensitive G proteins. Exposure to PTX and GDPβS results in channel activation, and the effects are rapidly reversed by Gpp(NH)p. As $G_i\alpha$-2 and $G_i\alpha$-3 are the principal PTX-sensitive substrates present, these findings suggest that endogenous receptors coupled through $G_i\alpha$-2 and/or $G_i\alpha$-3 inhibit channel opening and contribute to regulation of membrane Cl^- and HCO_3^- permeability.

The physiologic role(s) for epithelial HCA channels are not established. In BDE cells, spontaneous or GDPβS-activated channels are observed in ~75% of excised patches, often with multiple channels per patch. Thus, selective regulation of such an abundant high conductance channel would seem to be an efficient site for regulation of membrane anion permeability.

While G proteins have an established role in regulation of K^+, Ca^{2+}, and Na^+ channels in electrically excitable cells (Brown & Birnbaumer, 1988; Kirsch et al., 1988; Birnbaumer et al., 1989), there is limited information regarding regulation of epithelial anion channels in which membrane Cl^- transport plays an important role in a variety of cellular functions. In excised membrane patches, exposure to nonhydrolyzable analogues of GTP activated an inwardly rectified 20 pS anion channel in HT29 cells from colon carcinoma (Tilly et al., 1991) and a ~305 pS anion channel in renal epithelia (Schwiebert et al., 1990). Since GTP analogues are able to activate G proteins in the absence of receptor stimulation (Breitwieser, 1991), these findings are consistent with membrane-delimited channel regulation. In renal epithelia, channel activation was sensitive to PTX and was reproduced by addition of purified $G_i\alpha$-3 subunits from human erythrocytes. While the endogenous receptors and G proteins which regulate this channel have not been identified, this provides support for a role of endogenous $G_i\alpha$-3 in this cell type.

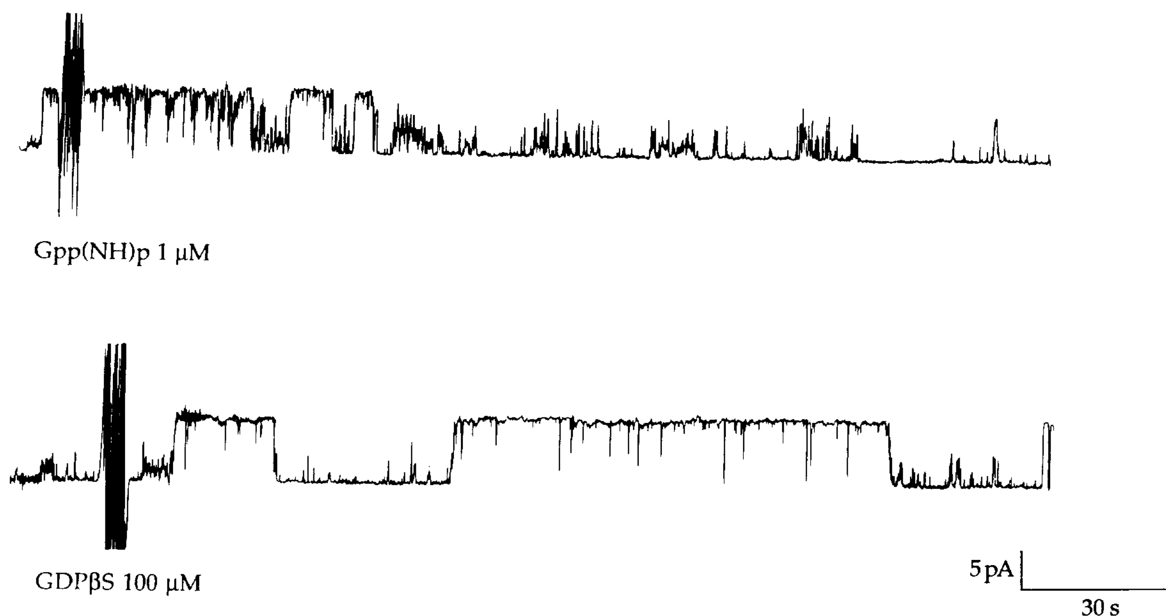

Fig. 2. Effect of GTP and GDP analogues on channel activity. Following excision, a HCA channel activated spontaneously with an open probability (P_o) >0.7, measured over 4 min. Following addition of Gpp(NH)p to the bath (cytoplasmic surface), at the time indicated by the artifact, there was a marked reduction in channel opening with a decrease in P_o, on average, to ~0.2. Addition of higher concentrations of GDPβS was followed by reactivation, as shown in this continuous recording from a single patch.

Despite the close similarities in conductance and permeability of HCA channels in renal and biliary epithelia, biliary HCA channels appear to be under inhibitory rather than stimulatory regulation by G proteins. In the absence of receptor-agonists, PTX ADP ribosylates selected G proteins and reduces the rate of transition from inactive to active G-protein complexes (Sunyer et al., 1989). In these studies, PTX activated HCA channels in 38% of patches. In nonresponding patches, channels could not subsequently be identified, suggesting that no channels were present. The effects of PTX occurred rapidly (95 ± 101 sec) and were mimicked by GDPβS. Moreover, in selected studies, the inhibitory effects of Gpp(NH)p could be overcome by higher concentrations of GDPβS (Fig. 2). Taken together, these observations indicate that an endogenous PTX-sensitive G protein inhibits opening of biliary HCA channels.

These effects of PTX and GDPβS are detectable in cell-free membrane patches. Consequently, regulation appears to be membrane delimited, consistent with direct interactions between G proteins and a regulatory site on or near the channel. The G-protein effect appears distinct from voltage-dependent regulation, since at any given voltage, the time constants for channel closure were not different for spontaneously active *vs.* PTX/GDPβS activated channels. This suggests that G proteins effect gating at a separate site. Once the channel is opened through inactivation of the G protein, voltage-dependent effects on channel activity become apparent. This suggests that channel P_o is regulated cooperatively by both G proteins and membrane voltage.

The identity of the endogenous G protein(s) which regulate this channel is not known. Using specific antisera, $G_i\alpha$-2 and $G_i\alpha$-3 were identified as the primary PTX-sensitive substrates present. While this preparation is highly enriched in BDE cells (~90%), a contribution of G protein from nonbiliary cell types cannot be eliminated. This pattern of expression appears similar to erythrocytes but is different from brain where $G_0\alpha$ and $G_i\alpha$-1 are abundant in the absence of $G_i\alpha$-2 and $G_i\alpha$-3 (Goldsmith et al., 1988; Mumby et al., 1988). We are not aware of previous identification of G-protein subunits in secretory epithelia. $G_i\alpha$-3 from human erythrocytes has been shown to regulate various cation channels, including Na^+ channels in renal medullary collecting duct cells (Light et al., 1989), K^+ channels in atrial myocytes (Brown & Birnbaumer, 1988; Kirsch et al., 1988), and Ca^{2+} channels in cardiac myocytes (Yatani et al., 1987). However, purified $G_i\alpha$-1,2 and 3 are each capable of regulating K^+ channels in guinea pig atrial myocytes (Yatani et al., 1988), demonstrating that multiple signaling pathways may regulate similar channels through different G proteins. Consequently, additional studies are required to determine whether $G_i\alpha$-2, $G_i\alpha$-3 or both are involved in inhibition of these channels.

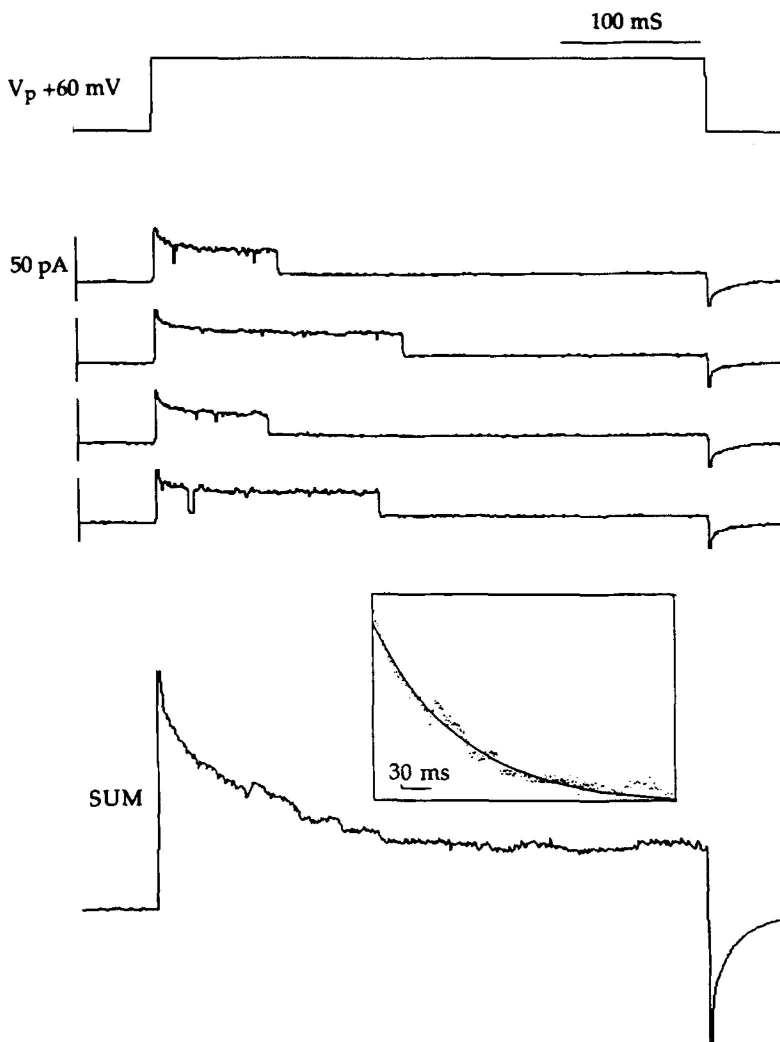

Fig. 3. Voltage-dependent channel closure. In this inside-out patch, channels were activated by exposure to pertussis toxin. Subsequently, the membrane was held at 0 mV, where P_o is high, and repetitively stepped to a test pipette potential of +60 mV. The summed current from 15 repetitions is shown (bottom). The rate of decline of the summed current could be described by a single exponential (*seen in the inset*) representing the time constant for channel closure which averaged 94 ± 16 msec at +60 mV in channels activated by either GDPβS or PTX.

Table 2. Comparison of the time constants for channel closure between spontaneously active and PTX or GDPβS activated HCA channels[a]

Voltage	Excision activated[b]	PTX/GDPβS activated	n	t-test
±80 mV	46 ± 23 msec	31 ± 6 msec	6	$P = 0.75$
±60 mV	97 ± 34 msec	94 ± 16 msec	6	$P = 0.96$
±40 mV	178 ± 54 msec	198 ± 18 msec	6	$P = 0.83$

[a] Since results were not different at positive and negative potentials of equal magnitude, they were combined for statistical analysis. At each voltage, there was no difference in the values from patches with spontaneously active channels and PTX or GDPβS activated channels.
[b] Data from reference (McGill, Basavappa & Fitz, 1992).

Specific antisera also demonstrate the presence of large and small forms of $G_s\alpha$ and the $\beta\gamma$ subunit. This provides a basis for future evaluation of receptor-mediated regulation of secretion and other cellular functions in BDE cells. Receptors for secretin are present (Farouk et al., 1992) and secretin increases adenylyl cyclase in biliary cell membranes (Kato, Gores & LaRusso, 1992). Stimulation of adenylyl cyclase by secretin receptors is presumably coupled through $G_s\alpha$, but the tissue-specific role of G pro-

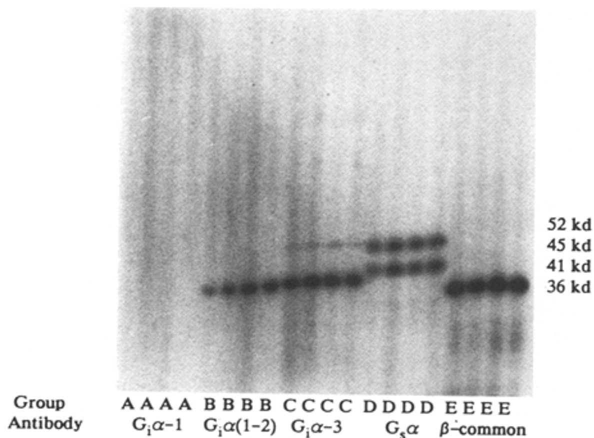

Fig. 4. Identification of endogenous G proteins. Purified plasma membranes were probed with antisera specific for $G_i\alpha$-1 (Group *A*), antisera which recognize both $G_i\alpha$-1 and $G_i\alpha$-2 (Group *B*), antisera specific for $G_i\alpha$-3 (Group *C*), antisera which recognize the large and small forms of $G_s\alpha$ (Group *D*), and antisera which recognize the 35 and 36 kd forms of the β-subunit (Group *E*). $G_i\alpha$-2 and $G_i\alpha$-3 are the PTX-sensitive substrates present, large and small forms of $G_s\alpha$ and β-subunits are also present.

teins in these and other signaling pathways remains to be established.

These studies provide further support for an important role for G proteins in regulation of epithelial anion channels, and emphasize the importance of cell-specific regulatory interactions. PTX-sensitive G proteins appear to inhibit HCA channel activity in biliary cells but activate similar channels in renal epithelia (Schwiebert et al., 1990). These differences may reflect distinct populations of endogenous receptors, G proteins, or channel regulatory sites, and result in differential effects of G proteins on membrane Cl^- and HCO_3^- permeability.

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