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Bicarbonate Permeability of the Outwardly Rectifying Anion Channel

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Summary. Single anion-selective channels have been studied in cultured human epithelial cells using the patch-clamp technique. Three cell types were used as models for different anion transport systems: (*i*) PANC-1, a cell line derived from the pancreatic duct, (*ii*) T_{84}, a Cl-secreting colonic cell line, and (*iii*) primary cultures of sweat duct epithelium. Outwardly rectifying anion-selective channels were observed in all three preparations and were indistinguishable with respect to conductance, selectivity and gating. Striking similarities between HCO_3- and Cl-secreting epithelia, and the high density of outward rectifiers in pancreatic cells prompted us to study HCO_3 permeation through this channel. HCO_3 permeability was significant when channels were bathed in symmetrical 150 mM HCO_3 solutions, Cl-HCO_3 mixtures, and under bi-ionic conditions with outwardly and inwardly directed HCO_3 gradients. Permeability ratios (P_{HCO_3}/P_{Cl}) estimated from bi-ionic reversal potentials ranged from 0.50 to 0.64, although conductance ratios greater than 1.2 were observed with high extracellular pH. Chloride did not inhibit HCO_3 permeation noticeably but rather had a small stimulatory effect when present on the opposite side of the membrane. The prevalence of outward rectifiers in PANC-1 and their permeability to bicarbonate suggests the channel may have a dual role in HCO_3 secretion; to allow Cl recycling at the apical membrane and to mediate some of the HCO_3 flux. Defective modulation of this channel in cystic fibrosis might provide a common basis for dysfunction in epithelia having very different anion transport properties (e.g., HCO_3 secretion, Cl secretion and Cl absorption).

Key Words bicarbonate secretion · chloride channel · epithelia · cystic fibrosis

Introduction

Electrogenic anion transport provides the driving force for fluid secretion across many epithelia [23]. Chloride enters epithelial cells of the airway and colon by a sodium-coupled mechanism at the basolateral membrane and exits through a conductance in the apical membrane [16, 38, 55, 57, 66]. Apical permeability determines the transepithelial Cl flux rate and is a site of regulation by second messengers such as cAMP (3′-5′ cyclic adenosine monophosphate). Cl conductance has been studied at the single-channel level and correlates with the presence of an outwardly rectifying anion channel in many epithelial preparations [2, 5, 15, 20, 22, 24, 31, 34, 65]. The outward rectifier is thought to mediate Cl secretion because its open-state probability is increased by secretagogues and because it can be activated in inside-out patches by exposure to a solution containing Mg, ATP and the catalytic subunit of cAMP-dependent protein kinase [4, 41, 56].

In epithelia of the duodenum and pancreatic duct, most fluid secretion is driven by transport of bicarbonate rather than chloride and high luminal HCO_3 concentrations (>140 mM) can be achieved by some tissues. Bicarbonate secretion resembles Cl secretion because it is electrogenic and stimulated by secretagogues that elevate cAMP [17, 19, 51, 52, 58, *see also* 59], however, the pathways mediating apical HCO_3 exit are not well established and there is evidence for electroneutral and electrogenic mechanisms. A model for HCO_3 secretion across turtle urinary bladder has been proposed in which HCO_3 exits the cells in exchange for luminal Cl and most Cl ions entering on the exchanger leak back to the lumen (along with some HCO_3) through a cAMP-activated conductance [60]. Recently, this scheme has been applied to other HCO_3-secreting tissues, particularly the interlobular duct of the rat pancreas [25, 46]. However, data from rat pancreatic duct are more consistent with HCO_3 exiting by Cl/HCO_3 exchange than through a conductive pathway [46] and the analogy with Cl secretion is further weakened by the recent description of a low-conductance Cl channel in rat pancreatic duct that is clearly different from the one described in Cl-secreting tissues. It has been proposed that this novel low-conductance chloride channel mediates Cl recycling at the apical membrane of pancreatic ductal cells [26].

The purpose of this study was to determine if the outwardly rectifying anion channel described in

Cl transporting epithelia is also present in human pancreatic ductal cells, and also to measure its HCO_3 permeability under various conditions. Our results indicate that the outward rectifier is expressed at unusually high density in PANC-1, a pancreatic cell line that maintains many differentiated properties of ductal epithelium (43). Outward rectifiers from PANC-1 were indistinguishable from those in T_{84}, a Cl-secretory cell line, and in primary cultures of the reabsorptive sweat duct, a tissue with high passive Cl conductance [*see* 47]. HCO_3 current flowing through the outwardly rectifying channel was studied when the membrane was bathed with symmetrical HCO_3 solutions, under bi-ionic conditions, and with mixtures of HCO_3 and Cl. Bicarbonate permeability was significant under all conditions examined and was 50–64% that of chloride according to most protocols. Although intracellular [Cl] is several times higher than [HCO_3] and the channel prefers Cl, currents carried by each anion will be proportional to their net electrochemical gradients and ionic conductances, not their intracellular concentrations. If the outward electrochemical gradient for Cl declines during secretagogue activation as a result of Cl efflux and membrane depolarization [46], the fraction of anion current carried by HCO_3 during steady-state stimulation may be significant.

Materials and Methods

Cells

PANC-1 cells and T_{84} cells were obtained from American Type Culture Collection (Rockville, MD) and studied during the first 100 passages. Cells were plated on glass coverslips at a density of 400,000/cm^2. PANC-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). T_{84} cells were cultured in a 50 : 50 mixture of DMEM and F-12 media supplemented with 5% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Sweat ducts were isolated from skin biopsies from young adult donors and cultured as described previously [14]. Briefly, skin samples were digested for 18 hr with 1 mg/ml Type I collagenase dissolved in alpha MEM medium containing 1% fetal bovine serum. After excising and uncoiling individual sweat glands from the remaining epidermis, the ductal portion was transferred along with a few μl of solution to the surface of a glass coverslip coated with Cell-Tak (BioPolymers, Farmington, CT) and left overnight at 37°C in a humidified 5% CO_2 incubator. Medium was added on the second day and cell outgrowth followed for approximately two weeks prior to electrophysiological studies. All regulations of the Medical Research Council (Canada) and the U.S. Department of Health and Human Services concerning procurement and use of human tissues were followed.

Solutions

Cells were transferred to a recording chamber containing a simple NaCl solution (meq/liter): 150 Na, 154 Cl, 2 Ca, 10 HEPES, 1 ethylene glycol-*bis*(beta-aminoethyl ether) N,N,N′,N′-tetra-acetic acid (EGTA), pH 7.4; or "normal bathing saline" (NBS): 154 Cl, 140 Na, 4 K, 2 Mg, 4 Ca, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 5 glucose, pH 7.4.

We notice during preliminary experiments that 10 mM HEPES reduced channel conductance ~30%, but it was used regardless because the effect on conductance was very consistent and because flickery block was often observed using other pH buffers such as MES (2[N-Morpholino] ethanesulfonate; pK_a = 6.1), MOPS (3[N-Morpholino] propanesulfonate; pK_a = 7.2) and TRIS (tri-imino sulfonate, pK_a = 7.8). The effect of HEPES on this channel is different from its actions on neuronal Cl channels [67] and will be detailed in a later paper. Buffer artifacts were minimized throughout the present study by comparing HCO_3 and Cl currents under identical conditions of [HEPES] and extra- and intracellular pH. The weak buffering provided by HEPES in strongly alkaline and acidic solutions was sufficient to maintain pH constant within 0.02 units. Bicarbonate solutions were equilibrated with air or 5% CO_2 and then titrated to the appropriate pH. Control current-voltage relationships in symmetrical Cl were not affected by exposure of patches to CO_2-containing HCO_3 solutions (*see* Results). The final Cl activity of all solutions was checked using an ion-selective electrode. Experiments were carried out at 20 ± 1°C.

Electrical Recording

Currents were recorded using the inside-out patches [32]. Pipettes were pulled in two stages (PP-83, Narishige Scientific Instrument Lab., Tokyo) and had resistances of 4–6 MΩ when filled with 150 mM NaCl solution. The pipette contained a chlorided Ag-AgCl wire. The bath was grounded through an agar bridge having the same ionic composition as the pipette solution. Original traces in the Results section show the applied membrane potential, but the current-voltage (I/V) relationships and all calculations were corrected for liquid junction potentials as described previously [33]. Liquid junction potentials estimated experimentally by this method (7.3 mV in pure 150 mM solutions) were nearly identical to those calculated from the Nernst-Planck equation and published values for Cl and HCO_3 limiting equivalent conductivities [3, 64].

Currents were amplified using an Axopatch 1B (Axon Instruments, Foster City, CA) or Yale Mk V patch clamp and recorded on video cassette tape by a pulse coded modulation-type recording adapter (DR384, NeuroData Instruments, New York, NY). During playback, single-channel records were low-pass filtered using an 8-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA) set at 600 Hz (final cutoff frequency 514 Hz) and sampled at 0.5- or 1.0-msec intervals by the computer's A/D converter.

Analysis

Data were analyzed and fitted using a laboratory microcomputer (Indec Systems, Sunnyvale, CA). Current-voltage relationships were calculated by a semi-automated procedure that involved

computing amplitude histograms for short segments of record and displaying them on a split screen next to the raw data so that current transitions and peaks could be verified using cursors. At least ten open events were measured at each steady-state potential, averaged, and entered into an I/V curve that was displayed at the end of the run to avoid bias. Slope conductance was determined by linear regression over the voltage ranges specified in the Results section. The zero current potential (E_{rev}) was obtained by interpolation after fitting the entire I/V curve with a fourth-degree polynomial, except when currents were recorded with 30 mM salt solutions, in which case curves had to be fitted with third-degree polynomials and extrapolated to E_{rev}. Relative HCO_3 permeability was calculated from the shift in E_{rev} [36] after substituting HCO_3 for Cl. For example, when HCO_3 was substituted on the intracellular side,

$$P_{HCO_3}/P_{Cl} = \frac{(Cl)_i}{(HCO_3)_i} \exp[(E_{HCO_3} - E_{Cl})F/RT]$$

where P_{HCO_3}/P_{Cl} is the permeability ratio at zero net current, $(Cl)_i$ and $(HCO_3)_i$ are Cl and HCO_3 activities bathing the intracellular surface of the patch, respectively; E_{HCO_3} and E_{Cl} are the reversal potentials measured with HCO_3 and Cl in the bath, respectively, and F, R, and T have their usual meanings. This method was adequate because the channel is highly anion-selective (cation/anion permeability ratio 0.06–0.02; *see* Fig. 2, and [31]. Chloride and bicarbonate have similar activity coefficients (within 3% error, [7]), therefore, the same values (0.73 and 0.84 in 150 and 30 mM solutions, respectively) were used for both anions. Activities were calculated using the modified Debye-Hückel formula [49]. Significant differences were determined at the 95% confidence level using paired or unpaired Student's t tests.

Results

Identification of the Outwardly Rectifying Cl Channel

There was little spontaneous channel activity in cell-attached patches under the conditions used here. However in PANC-1 cells, Cl channels were observed in ~75% of patches that had been excised and held at +60 mV for several minutes, and those patches that contained channels usually had at least two. The density in T_{84} and sweat duct cells was lower, with approximately 30% of patches containing Cl channels, and there was usually only one channel per patch. As shown below, Cl channels were distinctive and easily identified under different conditions by their outward rectification and voltage dependence.

Figure 1*a–c* shows single-channel records obtained from PANC-1, T_{84} and sweat duct cells when inside-out patches were bathed symmetrically with 150 mM NaCl. Traces were obtained during the first 20 sec after clamping the membrane potential. Figure 1*d* shows the I/V relationships computed as described in Materials and Methods. The slope conductance at the reversal potential (calculated between −20 and +20 mV) was 30.3 ± 0.7 pS (n = 12 patches). Rectification was very pronounced; the slope conductance for negative currents (between −20 and −30 mV) was 22.9 ± 1.1 pS and for large positive currents (between +60 and +80 mV) was 73.4 ± 2.0 pS.

Rectification in NaCl solutions can be accounted for using a simple rate theory model in which Cl flux is determined by a single predominant free energy barrier (Fig. 2*a*). The estimated mean height of the barrier under these conditions was 5.3 ± 0.07 kcal/mol. The electrical distance of the barrier was estimated to be 38 ± 0.6% of the way through the membrane field from the intracellular side and was not affected by replacing Cl with HCO_3 (*see below*). This simple model accounts very well for the I/V data under these conditions, however, parameter estimates will change in HEPES-free solution and a more elaborate model will be required to explain channel saturation and the effect of chloride-thiocyanate mixtures on conductance [29, 30]. The important result is that outwardly rectifying channels from pancreatic duct, T_{84} and sweat duct cells are indistinguishable with respect to conductance, and they closely resemble Cl channels in airway cells that function abnormally in cystic fibrosis.

Figure 2*b* shows the effect on the I/V relationship of isosmotically replacing 50 mM bath NaCl with sucrose. The reversal potential shifted by −25.8 mV, indicating a cation/anion permeability ratio at zero net current of 0.033. This ratio was difficult to measure accurately but was similar for channels from all cell types and averaged 0.065. Gating was voltage dependent; that is, the initial open state probability (P_o) after stepping the membrane potential from 0 mV to different test potentials increased as the test potential was made more positive. This corresponds to the increase in P_o with depolarization, which has been reported by others (*see* Fig. 1*a* and *b* and [21, 31, 34]). However, unlike previous studies we also observed voltage-dependent inactivation superimposed on this process: inactivation became progressively faster as the membrane potential was clamped to increasingly positive voltages (Fig. 3) and was not affected noticeably by salt concentration or by pH. In summary, the outward rectifier was present in three cells having very different anion transport systems and was easily recognized under different experimental conditions. Cl channels from the different cell types were indistin-

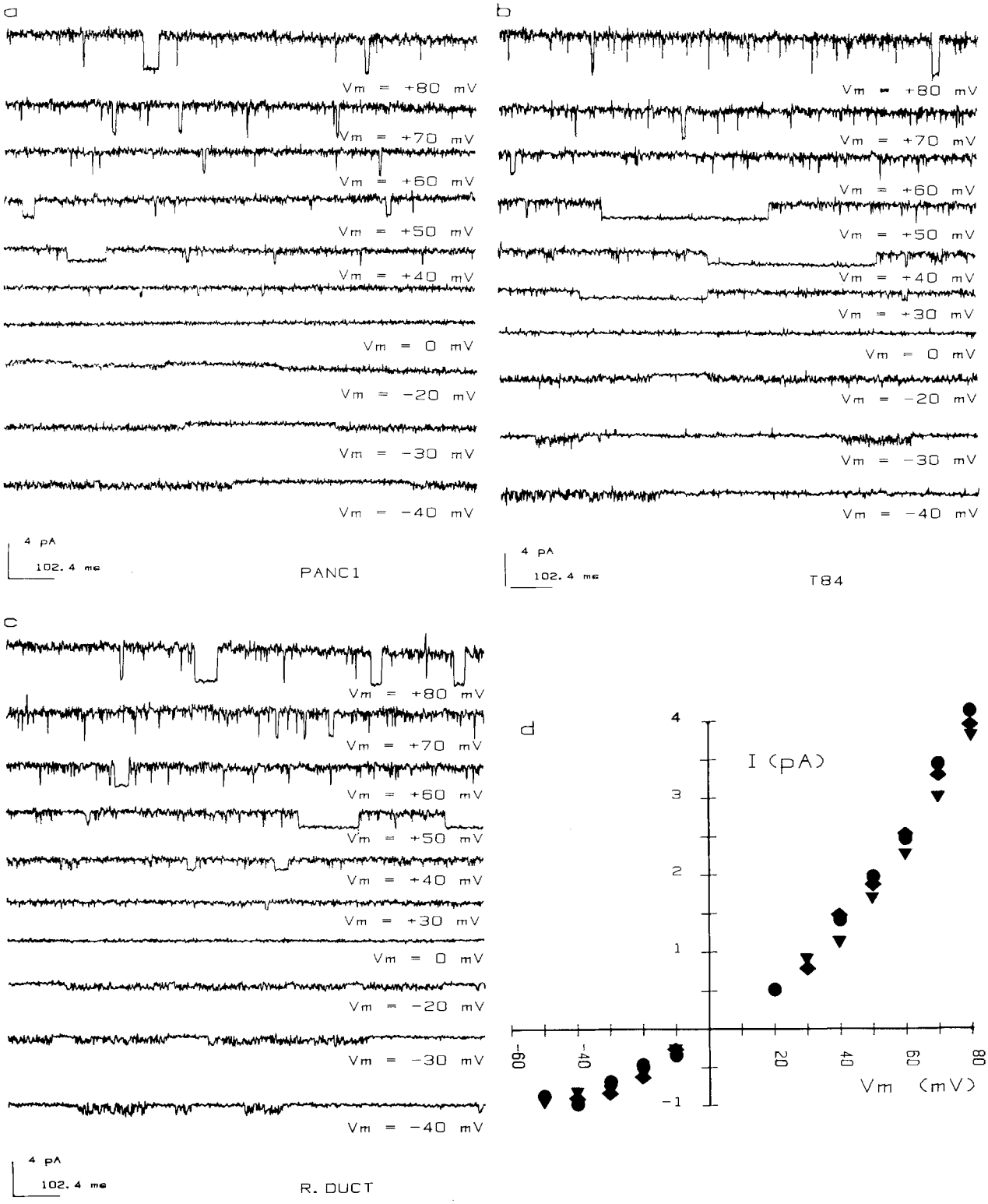

Fig. 1. Identification of the outward rectifier: single channels recorded from (*a*) PANC1, (*b*) T_{84}, and (*c*) reabsorptive sweat duct cells using symmetrical solutions containing 150 mM NaCl, 10 mM HEPES (pH 7.4). Membrane voltage is shown with respect to the extracellular (pipette) solution and positive (outward) membrane current is upward. Currents are larger at positive than at negative potentials, and initial open-state probability at positive voltages is much higher. (*d*) Currents were indistinguishable whether channels were obtained from cells derived from (▼) PANC-1, (◆) T_{84} or (●) reabsorptive sweat duct

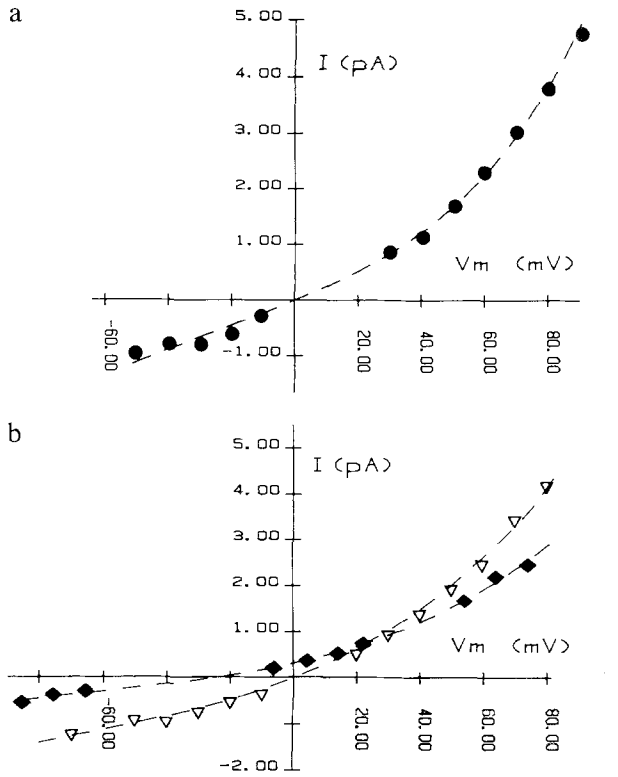

Fig. 2. (*a*) Current-voltage relationship of a channel from PANC-1 bathed symmetrically with 150 mM NaCl solution. The dashed line shows the best-fit single barrier model (height; 5.34 kcal mol^{-1}: electrical distance from inside; 0.397). (*b*) Effect of reducing bath [NaCl] from (▽) 150 mM to (◆) 100 mM by replacing NaCl isosmotically with sucrose (pipette: 150 mM NaCl). Currents are shown after correcting for liquid junction potentials. The dashed lines indicate polynomial best-fits used when estimating reversal potentials. Channel was obtained from a PANC-1 cell

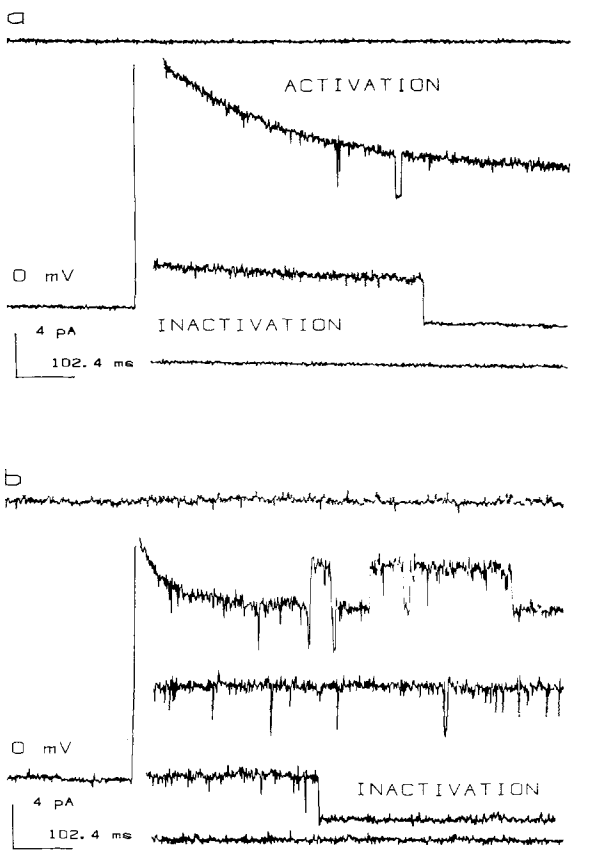

Fig. 3. Voltage activation and inactivation of outwardly rectifying anion channels from (*a*) PANC-1 and (*b*) T_{84} cells. Patches containing inactive channels (at +80 mV) were clamped to 0 mV for 2 sec and then back to +80 mV. The open probability was initially high after returning to +80 mV but anion channels inactivated within 2 sec at this potential. The patch in (*b*) contained two channels

guishable with respect to conductance, selectivity and voltage-dependent gating. The surprisingly high density of Cl channels in the PANC-1 line suggested that this channel might mediate some HCO_3 efflux. Bicarbonate permeability is examined in the following sections.

Conductance in Symmetrical Bicarbonate Solutions

Figure 4*a* shows current traces obtained when both sides of the membrane were bathed with a solution containing nominally Cl-free, 150 mM HCO_3 (5% CO_2, pH 8.23). Replacing Cl on both sides with HCO_3 did not alter the kinetics or voltage-dependent inactivation noticeably; although, currents were smaller at all potentials when carried by HCO_3 (Fig. 4*b*). The slope conductance between +60 and +80 mV was 37.0 ± 4.5 pS in symmetrical 150 mM HCO_3 solutions (mean ± SE, five patches), about half that measured in 150 mM Cl solution (73.4 pS; *see above*). Negative currents were also proportionately smaller, yielding a bicarbonate conductance between −20 and −30 mV of 10.8 ± 1.8 pS. At zero net current, the slope conductance was 10.4 ± 1.3 pS in symmetrical HCO_3 solutions as compared to 29.3 pS with Cl. As shown in Fig. 4*b*, *I/V* curves determined in symmetrical HCO_3 solutions are still well-described by a simple model in which a single barrier is located 38 ± 0.6% of the way through the membrane electric field from the intracellular side, the same electrical distance as calculated for Cl ($P > 0.2$). However the barrier to HCO_3 permeation is significantly higher; 5.88 ± 0.07 kcal/mol ($P <$

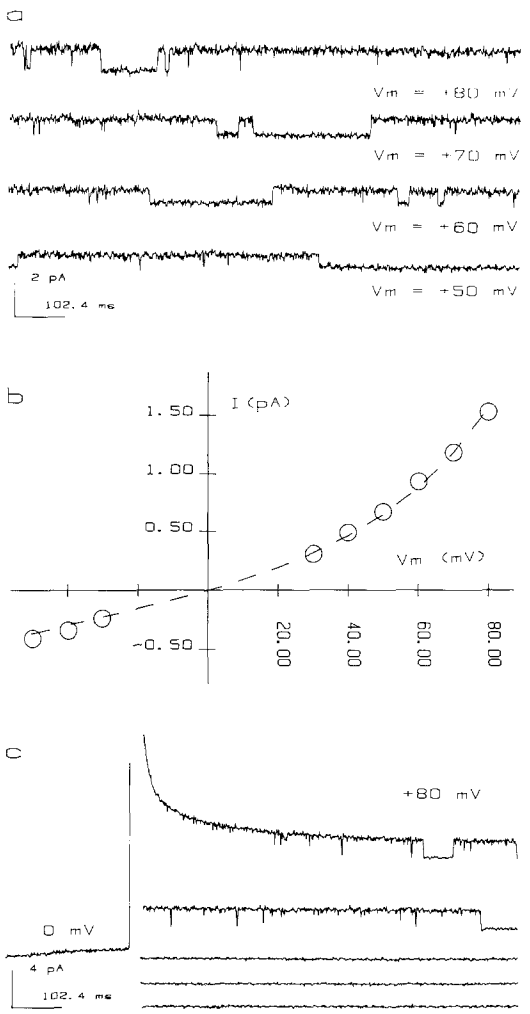

Fig. 4. HCO_3 permeability in symmetrical HCO_3 solutions. (*a*) Recordings obtained with nominally Cl-free, 150 mM $NaHCO_3$ solutions bathing both sides of the membrane. (*b*) *I/V* curve obtained with symmetrical 150 HCO_3. Dashed line indicates the best-fit single barrier model (height; 5.88 kcal mol^{-1}: electrical distance from inside; 0.380). (*c*) Inactivation of the HCO_3 current at +80 mV. Channel was obtained from a T_{84} cell

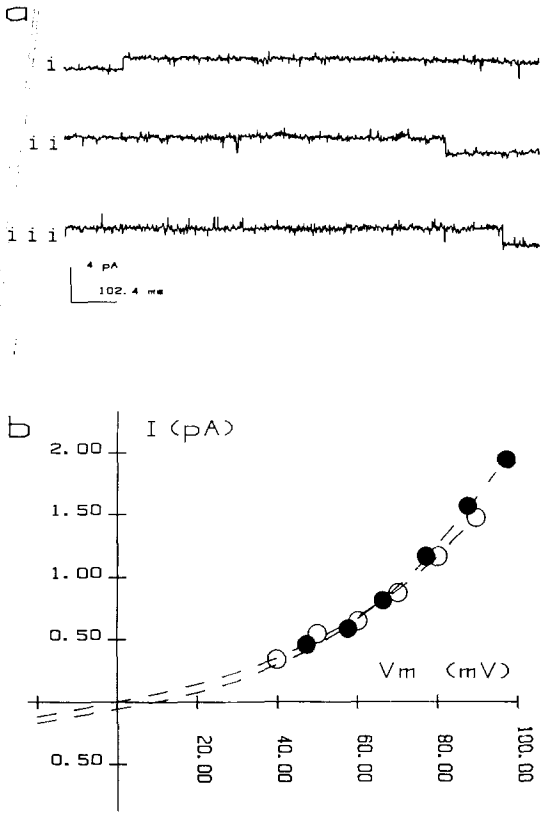

Fig. 5. Inward HCO_3 flow with a large pH gradient and small transmembrane pCO_2 gradient. (*a*) Single-channel currents recorded at +90 mV (*i*) with symmetrical 30 mM NaCl solutions (extracellular pH 9.7, 100% N_2), and (*ii*, *iii*) with 30 mM HCO_3 + CO_3 solution in the pipette (also pH 9.7, air equilibrated). Channels shown in traces *i* and *ii* were obtained from T_{84} cells; trace *iii* shows a channel from sweat duct. (*b*) Comparison of the *I/V* relationship obtained (○) with symmetrical 30 mM NaCl solutions or (●) with 30 mM HCO_3 + CO_3 solution in the pipette. Currents carried by the inward flow of HCO_3 + CO_3 (from the pipette) were larger than those carried by Cl, although extrapolated reversal potentials suggest Cl is more permeant at zero net current. Channels were obtained from reabsorptive sweat duct cells

0.01). Inactivation of the HCO_3 current by large positive voltage steps was identical to that observed when Cl was the charge carrier (*compare* Figs. 4*c* with 3).

HCO_3 Influx Under Bi-ionic Conditions

Measured with a Large pH Gradient and Small pCO_2 Gradient

The concentration of NaCl and $NaHCO_3$ solutions were reduced to 30 mM so that HCO_3 flux from extra- to intracellular solution could be studied under bi-ionic conditions without imposing a large transmembrane pCO_2 gradient. Positive current, which was carried by an inward flow of anions from pipette to bath, was measured with the pipette containing 30 mM Cl solution equilibrated with N_2 at pH 9.7 (the most alkaline pH at which stable patches could be maintained), or 30 mM HCO_3 + carbonate solution equilibrated with air at pH 9.7.

Figure 5*a* shows currents recorded at +90 mV with 30 mM Cl on both sides (trace *i*) and with extracellular solution containing HCO_3 and carbonate (CO_3) at the same pH (traces *ii* and *iii*). Despite channel-to-channel variation, the conductance was consistently larger with external bicarbonate when

compared with extracellular Cl, particularly at large positive voltages (Fig. 5*b*). The slope conductance calculated between +87.4 and +97.4 mV measured with external HCO_3 + CO_3 solution was 35.5 ± 1.16 pS (mean ± SE; five patches), significantly higher than with extracellular Cl (31.1 ± 0.87 pS; $P < 0.025$). However, the mean (extrapolated) reversal potential obtained with HCO_3 + CO_3 in the pipette was 11.1 ± 0.9 mV, indicating a permeability ratio $P_{HCO_3}/P_{Cl} = 0.64 \pm 0.03$ (mean ± SE, six patches). In other words, conductance ratios suggest HCO_3 permeates as well or slightly better than chloride in the presence of a large pH gradient but reversal potentials indicate bicarbonate permeates only 64% as well. In the next section, we show that this discrepancy between conductance and permeability ratios disappears when conductance is measured with less alkaline solutions. Regardless, it is clear that the Cl channel is significantly permeable to bicarbonate and/or carbonate ions, and mediates their influx under these conditions.

Measured with a Large pCO_2 Gradient and Small pH Gradient

Inward HCO_3 permeability was reexamined using a small transmembrane pH gradient (<1 unit) by elevating pCO_2 within the pipette. The pipette was filled with a solution containing 150 mM $NaHCO_3$ (equilibrated with 5% CO_2 at pH = 8.23). Single-channel currents were measured with symmetrical 150 mM $NaHCO_3$, and then again after switching the bath from $NaHCO_3$ to NaCl. A stream of humidified 5% CO_2 was directed onto the bath surface whenever it contained bicarbonate.

Figure 6 shows *I/V* curves obtained before and after replacing bath HCO_3 with Cl. Chloride substitution caused the reversal potential to shift from 0 mV to 17.3 ± 1.1 mV (mean ± SE; six patches), suggesting $P_{HCO_3}/P_{Cl} = 0.50 \pm 0.03$. This ratio is slightly higher than the HCO_3 : Cl conductance ratio obtained by comparing the conductance in symmetrical Cl or HCO_3 solutions. As explained below, this may be attributed to stimulation of conductance by Cl on the opposite side of the membrane.

HCO_3 Efflux Under Bi-ionic Conditions

Outward bicarbonate flow (i.e., from the intra- to extracellular side of the membrane) was studied under bi-ionic conditions with 150 mM HCO_3 in the bath. This protocol was used because rectification made it difficult to measure negative currents with air-equilibrated, 30 mM salt solutions, and because it permitted minimal carbonate ion formation. Ex-

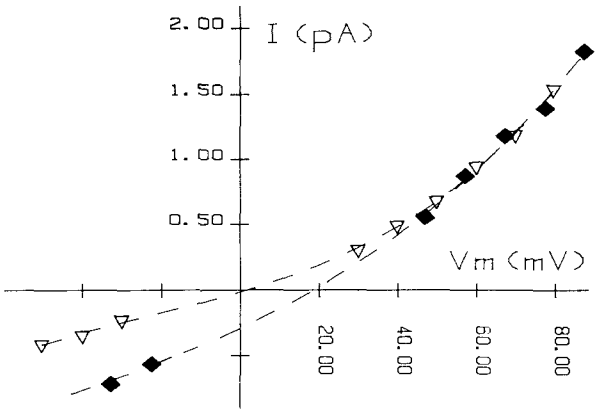

Fig. 6. Inward HCO_3 flow with a large pCO_2 gradient and small pH gradient. *I/V* relationships obtained (▽) with symmetrical 150 mM $NaHCO_3$ (equilibrated with 5% CO_2, pH 8.23) and (◆) after replacing bath HCO_3 solution with Cl (pH 8.23, equilibrated with air). Channel was obtained from a T_{84} cell

cised patches were bathed initially by 150 mM NaCl solution (bath: pH 8.27, pipette: pH 7.3) and currents were recorded at 10–12 potentials. The bath was then flushed with 150 mM $NaHCO_3$ solution (5% CO_2, pH 8.27) and currents were recorded again while a stream of humidified 5% CO_2 was directed onto the bath surface. Measurements with HCO_3 solutions were bracketed by control NaCl solutions (air equilibrated, pH 8.27).

Figure 7*a* and *b* shows currents recorded with symmetrical 150 mM Cl and after replacing NaCl in the bath with $NaHCO_3$ (pH 8.23). The figure shows data obtained from a T_{84} channel but identical results were obtained when PANC-1 channels were used. Substituting HCO_3 for Cl in the bath shifted the reversal potential by −15.4 ± 0.01 mV (mean ± SE; five patches), indicating a HCO_3 : Cl permeability ratio of 0.54 ± 0.01 (Fig. 7*c*). Negative currents were small but were easily resolved. The HCO_3 : Cl conductance ratio was 0.55 ± 0.14 between −30 and −50 mV; i.e., potentials at which current would be carried by anions flowing from bath to pipette.

To determine if [HCO_3] in the pipette tip increased significantly during exposure of patches to CO_2, *I/V* curves obtained after approximately 45-sec exposure to CO_2 were compared with those obtained after 25–30 min exposure. We reasoned that if [HCO_3] increased significantly in the tip during this time period it should cause E_{rev} to shift in the negative direction because HCO_3 can permeate from the extracellular side (*vide supra*). However, Fig. 7*d* shows early and late *I/V* curves were superimposable. Although this time interval brackets the normal duration of CO_2 exposure by wide margins and used the earliest and latest times at which *I/V*

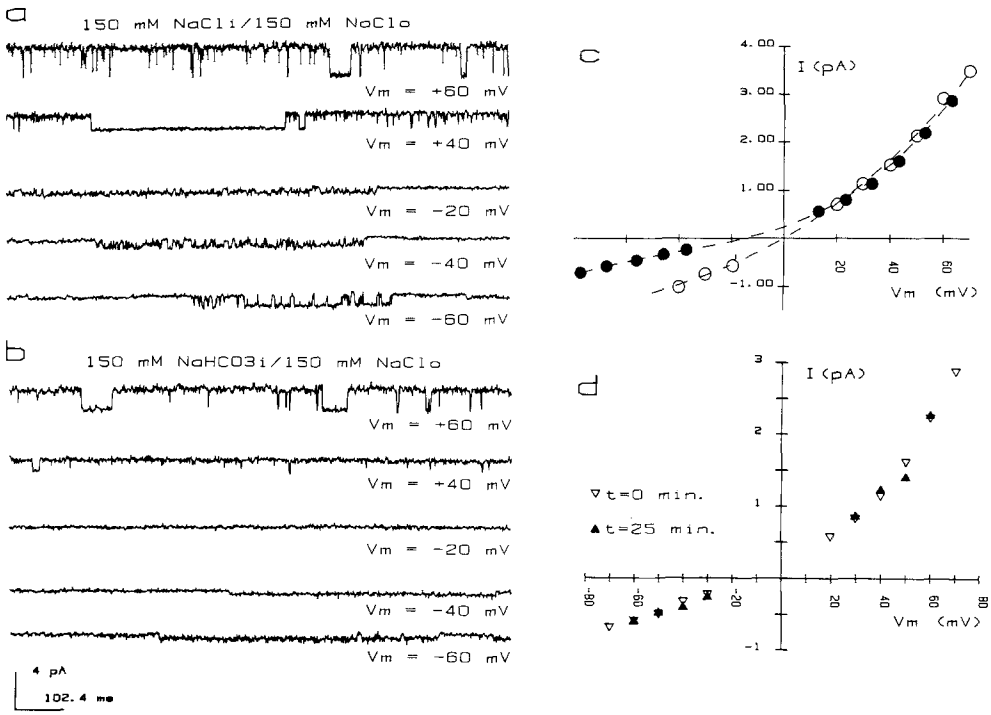

Fig. 7. Outward HCO_3 flow under bi-ionic conditions. Single channel recorded (*a*) with symmetrical 150 mM NaCl (pH 8.23, equilibrated with air), and (*b*) after replacement of bath Cl solution with HCO_3 (pH 8.23, equilibrated with 5% CO_2). (*c*) *I/V* relationships obtained (○) with symmetrical 150 mM NaCl and (●) after replacing the bath NaCl solution with 150 mM $NaHCO_3$. (*d*) *I/V* curve with symmetrical 150 mM NaCl solutions obtained immediately before (▽) and after (◆) exposure of the patch to 150 mM HCO_3, 5% CO_2 in the bath. Channels were obtained from T_{84} cells

curves could be practically obtained, we cannot be certain HCO_3 accumulation would be detectable within this interval. With this caveat, the results suggest the rate of CO_2 diffusion into the pipette under these conditions is not sufficient to cause noticeable HCO_3 accumulation next to the membrane.

Bicarbonate Permeability in HCO_3-Cl Mixtures

Permeability ratios determined in pure solutions may not be physiologically applicable, as illustrated by voltage-dependent calcium channels, which are permeable to Na and K under Ca-free conditions but are highly Ca-selective in physiological salines [1, 35]. To determine if the ratio P_{HCO_3}/P_{Cl} is similar in the presence of Cl, outward anion flow was studied with Cl-HCO_3 mixtures using two protocols: first, *I/V* curves obtained with symmetrical 150 mM HCO_3 solutions were compared with those following replacement of 125 mM bath HCO_3 with Cl. Second, *I/V* curves were compared under bi-ionic conditions (pipette: 150 mM NaCl, bath: 150 mM $NaHCO_3$) and after adding 10 mM Cl to the bicarbonate solution.

Figure 8*a* shows traces obtained with 150 $NaHCO_3$ solution in the pipette and 125 mM NaCl + 25 $NaHCO_3$ in the bath. Changing the bath from 150 mM $NaHCO_3$ to this mixture shifted E_{rev} from 0 mV to +15.0 ± 2.47 mV, consistent with a HCO_3 : Cl permeability ratio of 0.50 ± 0.06 (Fig. 8*b*; mean ± SE, four patches). The slope conductance between −20 and −30 mV increased by 30%, which was expected since the current would be carried by anion flow from the bath to the pipette and Cl is more permeant. More interestingly, the slope conductance at positive voltages also increased when HCO_3 was partially replaced by Cl; i.e., the presence of Cl in the bath enhanced HCO_3 permeation from the opposite (extracellular) side. Figure 8*c* shows the effect of adding a small amount of Cl to the bath solution when it already contained 150 mM HCO_3. When 10 mM NaCl was added to the 150 mM HCO_3 bath solution the reversal potential shifted from −15.4 mV to −6.8 ± 1.4 mV. This corresponds to a HCO_3 : Cl permeability ratio of 0.70 ± 0.04 at zero net current, higher than the ratio obtained under bi-ionic conditions with nominally Cl-free solution in the bath (0.54 ± 0.01; $P \ll 0.01$, *see above*). Again the slope conductance at large de-

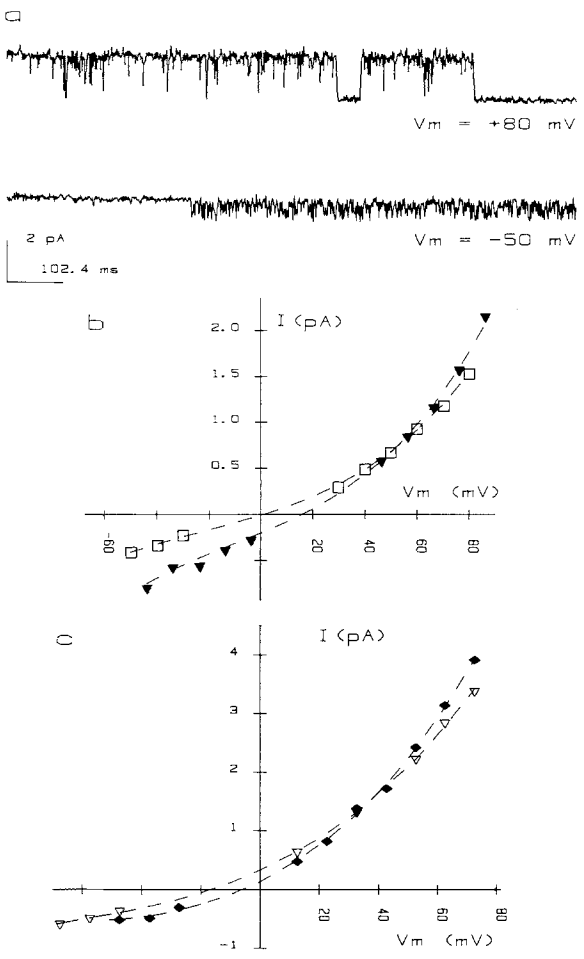

Fig. 8. Bicarbonate-Cl mixtures. (*a*) Recordings obtained with 150 mM Cl in the pipette and 125 mM Cl + 25 mM HCO_3 in the bath (equilibrated with 5% CO_2). (*b*) *I/V* relationships obtained (□) with symmetrical 150 mM $NaHCO_3$ solutions and (▼) after replacing 150 mM $NaHCO_3$ in the bath with 125 mM NaCl + 25 mM $NaHCO_3$. (*c*) *I/V* relationship with 150 mM NaCl in the pipette and (▽) 150 mM $NaHCO_3$ or (◆) 150 mM $NaHCO_3$ + 10 mM NaCl in the bath. Addition of low [Cl] to the bath solution enhanced anion flow from pipette to bath (i.e., in the "*trans*" direction) and shifted the reversal potential towards 0 mV. Channels were obtained from T_{84} cells

polarizing potentials (i.e., +60–+70 mV) was enhanced by adding Cl to the "*trans*" side, increasing from 63.1 ± 1.4 pS when the bath contained 150 mM HCO_3, to 75.0 ± 7.62 pS after Cl addition (mean ± SE; $P < 0.02$).

In summary, bicarbonate currents were not inhibited by the presence of Cl on either side of the membrane but were enhanced when the intracellular solution contained some Cl. This "trans-stimulation" effect may also occur in the opposite direction: the slope conductance for outward HCO_3 flow (at −30 mV) was 22.0 ± 1.8 pS with Cl in the pipette but only 10.8 ± 1.7 pS when the pipette solution contained 150 mM HCO_3 (Figs. 7 and 4).

Discussion

Our results indicate the anion channel believed to mediate Cl transport across many epithelia is also abundant in a pancreatic cell line, PANC-1. Direct microscopic evidence that PANC-1 cells are ductal in origin was obtained by Lieber et al. using sections of the carcinoma [42]. The original tumor contained ducts lined with markedly dysplastic and malignant-type cells. More recently PANC-1 has been shown to express many differentiated properties of pancreatic ductal epithelium in vivo including: (*i*) epithelial intermediate filaments, (*ii*) basolateral localization of Na/K ATPase, (*iii*) formation of complete tight junctions, (*iv*) cuboidal ultrastructure when cells are grown on filters, (*v*) high levels of carbonic anhydrase, (*vi*) expression of gamma-glutamyltranspeptidase, and (*vii*) synthesis of sulfated proteins resembling those secreted by native pancreatic ducts [43].

In this study, HCO_3 permeation through the outward rectifier was studied using several protocols. Inward bicarbonate flow was measured when the channel was bathed with HCO_3 solution on both sides and under bi-ionic conditions in the presence of a large transmembrane pH or pCO_2 gradient. Outward bicarbonate flow was determined with symmetrical HCO_3 solutions, under bi-ionic conditions in the presence of a pCO_2 gradient, and in solutions containing mixtures of HCO_3 and Cl. Bicarbonate permeability was significant under all conditions examined.

Properties of the Anion Channel

Anion channels from PANC-1, T_{84} and sweat duct cells had identical *I/V* curves and resembled the outward rectifier described previously (~150 mM Cl on both sides; [5, 22, 31, 34, 65]). The slope conductances estimated at −30, 0 and +70 mV (23, 30 and 73 pS, respectively) are similar to those reported for T_{84} cells (25, 41 and 71 pS, respectively; [31]), cultured airway cells (29, 30 and 62 pS; [65]: 21, 50 and 71 pS; [22]), and cultured sweat ducts (25, 38 and 80 pS; [5]). The data are also consistent with preliminary reports of outwardly rectifying anion channels in rat epididymal cells [2], *Necturus* small intestine [24] and placental trophoblast [15]. Outward rectification has been observed in another colonic cell line, HT_{29} [34], in which *I/V* relationships at 37°C were well represented by two straight lines having

slopes of ~32 and ~50 pS at negative and positive potentials, respectively.

Channels were not usually active when patches were attached to the cell or immediately after excision, but they could be activated by large positive voltages pulses [41, 56]. Initial open-state probability at each potential increased with voltage as described previously [31, 34]. However, inactivation was also observed when the membrane potential was clamped to potentials greater than +60 mV for more than a few seconds, and the closing rate increased with positive voltage. Inactivation was removed most effectively by reversing the membrane potential. Such voltage dependence has not been reported previously in patch-clamp studies of this channel although there are preliminary reports that whole cell currents in single sweat gland cells inactivate in a similar manner [10, 44, 53]. Anion channels from rat colon incorporated into bilayers inactivate when the *cis* side of the bilayer is clamped at −50 mV. This would correspond to a membrane potential of +50 mV if the channel is outwardly rectified [48]. We did not characterize voltage inactivation in detail because at present it has no obvious physiological function.

The present estimate of $P_{Cl}/P_{Na} > 18$ is in fair agreement with earlier reports in airway (6.5; [65]: > 10; [21]), rat colon (16; [48]) and T_{84} cells (50; [31]). Bi-ionic HCO_3 : Cl permeability ratios ranged from 0.64 to 0.50 for HCO_3 influx when determined at zero net current with large pH or pCO_2 gradients, respectively. The permeability ratio for HCO_3 efflux was 0.54 in the presence of a small pCO_2 gradient, and 0.50 in mixed solutions containing both Cl and HCO_3. HCO_3 : Cl conductance ratios varied from 0.42 for influx in the presence of a large pCO_2 gradient, to 1.44 when the influx was measured with high extracellular pH. The reason for elevated conductance at high extracellular pH is not known, but it is probably a pH effect rather than carbonate ($CO_3^=$) conductance because sulfate, which has a smaller hydrated radius than carbonate (0.184 *vs.* 0.212 nm), was found to be only slightly permeant ($G_{SO_4} : G_{Cl} = 0.19$). Carbonate permeability was not studied directly because patches became unstable at pH > 9.7. Relative permeability of the channel for bicarbonate and other anions closely resembles the transepithelial sequence measured for cat main pancreatic duct (1.9 I > 1.28 Br > 1 Cl > 0.56 HCO_3 > 0.41 F [27]). This sequence was been interpreted with reference to the paracellular pathway, but a cellular component was not ruled out.

Solutions containing 150 mM $NaHCO_3$ were equilibrated with 5% CO_2, thus a CO_2 gradient favoring diffusion through the membrane was unavoidable when measuring selectivity under bi-ionic conditions. Membrane permeability to CO_2 is relatively high, and CO_2 diffusion through the lipid bilayer is not rate limiting when there is little carbonic anhydrase present [28]. Ignoring the membrane, we estimated the CO_2 flux through the tip under these conditions to be $<1.6 \times 10^{-16}$ mol sec^{-1} using the relationship $J = a \cdot pCO_2 \cdot D \cdot \pi \cdot \tan\theta \cdot r_1$, where a is the solubility of CO_2 in water (0.038 mol ml^{-1} atm^{-1}), D is the diffusion coefficient for CO_2 (2×10^{-5} cm^2 sec^{-1}), θ is the semi-angle at the pipette tip (12°), and r is the radius of the tip opening (~0.5 μm). This calculation assumes that (*i*) the membrane is not a significant barrier to net CO_2 flux, (*ii*) the CO_2 flux reaches a steady state, (*iii*) the inside of the pipette tip is conical, and (*iv*) the pCO_2 of the bulk solution in the pipette shank remains constant [*see* 40]. The diffusion coefficient for CO_2 in cell membranes (5×10^{-8} cm^2 sec^{-1}, estimated from methanol diffusion) is lower than for HCO_3 diffusion in water (~10^{-5} cm^2 sec^{-1}), and the uncatalysed rate of CO_2 hydration is relatively slow (14 sec^{-1}). Consequently, CO_2 entering the pipette solution due to the pCO_2 gradient would diffuse an average of 6 μm as CO_2 before conversion to HCO_3. These factors, combined with diffusion of HCO_3 away from the membrane, may explain why the reversal potential was unaffected by exposure to CO_2.

The bicarbonate : Cl permeability ratio of the outward rectifier has not been measured previously using the patch-clamp technique, however, a similar value (0.4) has been reported for colonic anion channels incorporated into planar bilayers [48]. The agreement using different techniques provides further evidence that the reconstituted anion channel originates from apical vesicles and is the same one characterized in patch-clamp studies. The present results also extend the selectivity data to include conductance ratios, equilibrium permeability ratios for HCO_3 flux in both directions, and relative HCO_3 permeability in mixed solutions. HCO_3 permeability is not unique to epithelial Cl channels: patch-clamp studies of neuronal GABA-activated channels yielded a $P_{HCO_3} : P_{Cl}$ ratio of 0.18 [7], although recent microelectrode experiments using crayfish skeletal muscle indicate ratios of 0.46–0.53 [37].

Implications for HCO_3 Secretion and Intracellular pH

Epithelia exhibiting apical HCO_3 conductance include duodenum, choroid plexus, turtle urinary bladder, and pancreatic duct. In frog duodenum, HCO_3 transport occurs through an electroneutral pathway and a larger electrogenic pathway that is

activated by cAMP, prostaglandins, vasoactive intestinal peptide, cholinergic agonists and numerous other secretagogues [18]. Frog choroid plexus secretes HCO_3 into the cerebrospinal fluid in response to beta-adrenergic agonists, cholera toxin, and other maneuvers that elevate intracellular cAMP [51]. Turtle urinary bladder secretes HCO_3 by an electrogenic process that is Na- and Cl-independent [8], stimulated by theophylline, cAMP and IBMX [8, 52], and inhibited by the anion channel blocker anthracene-9-carboxylate [60].

A cellular model has been proposed for electrogenic HCO_3 secretion by turtle bladder that features apical Cl/HCO_3 exchange and anion conductance [60]. According to this scheme, apical anion conductance and Cl/HCO_3 exchange are low in unstimulated cells. cAMP stimulates apical conductance, and the resulting fall in $[Cl]_i$ increases the inward Cl gradient driving anion exchange. Electrodiffusive Cl exit also serves to resupply the exchanger with luminal Cl. Chloride and bicarbonate were both postulated to exit through the anion channel [24].

This model also accounts for many properties of HCO_3 secretion across the pancreatic duct. Anion exchange is consistent with micropuncture measurements of luminal ion levels [e.g., 13, 62], the partial dependence of HCO_3 transport on Cl [11, 50], the effects of luminal SITS on isolated, perfused ducts [46], and optical measurements of intracellular pH during Cl replacement [61]. Moreover, microelectrode data suggest the apical membrane of rat pancreatic duct cells contains anion conductance [46], and patch-clamp studies have revealed a low-conductance (4 pS) anion channel in this membrane [26]. The open probability of the 4-pS channel is increased by secretin when recorded in cell-attached patches. This channel is also present in primary cultures of human pancreatic duct, but a defect in its modulation in cystic fibrosis has not yet been demonstrated. With regard to cystic fibrosis (CF), it is tempting to speculate that outward rectifiers may also participate in pancreatic HCO_3 secretion since a defect in cAMP regulation of this channel could provide a common biochemical basis for abnormal HCO_3 and Cl transport in the pancreas (*see* [39]), and Cl transport in the airways and sweat gland where defective modulation of this channel in CF has already been demonstrated.

Several lines of evidence suggest pancreatic HCO_3 secretion is mediated by multiple pathways. Although whole gland studies must be interpreted with caution, substantial HCO_3 secretion can be maintained when Cl is replaced by impermeant anions [11], indicating there is a Cl-independent component. It is not clear that Cl/HCO_3 antiport could increase luminal $[HCO_3]$ to the levels measured *in situ* when luminal [Cl] varies inversely with $[HCO_3]$. If luminal [Cl] declines from 120 mM to 20–40 mM as luminal $[HCO_3]$ increases, the inward [Cl] gradient would become partially dissipated before luminal $[HCO_3]$ reached its theoretical maximum value. This question of the energetics of HCO_3 transport is most acute for species having high luminal HCO_3 concentrations (e.g., human, >100 mM; cat, >140 mM; and pig, 160 mM). Moreover, transepithelial HCO_3 and Cl gradients are partially dissipated in the main pancreatic duct of the cat and this may be mediated by Cl/HCO_3 exchange [13, 62].

Would the channel carry significant bicarbonate flux under physiological conditions? Intracellular HCO_3 activity is typically 10–20 mM when intracellular pCO_2 approximates that of the extracellular solution and cell pH is 7.0–7.4. If applicable to the pancreatic duct, this range is four- to 10-fold higher than electrochemical equilibrium for HCO_3 (2.5 mM) when the apical membrane potential is −61 mV (perfused ducts; [46]), and two- to four-fold higher than the equilibrium value in stimulated, unperfused ducts (−18 mV; [26]). Channel activation would clearly lead to some HCO_3 efflux, although most current would initially be carried by chloride because it is also above electrochemical equilibrium and its concentration and conductivity in the channel are higher. However, during steady-state stimulation, the relative contribution of bicarbonate to anion current flowing through the apical membrane may increase because the net electrochemical gradient favoring Cl exit would decline as $[Cl]_i$ falls and the apical membrane depolarizes [46]. It is the net electrochemical gradients (and ion conductivities) that will determine the relative magnitudes of Cl and HCO_3 currents, not their intracellular concentrations *per se*. Assuming reasonable values for intracellular and luminal [Cl] and $[HCO_3]$, we estimate that HCO_3 could contribute only about 18% of the apical anion conductance early in the ductal system where luminal [Cl] is high, but this value could approach 50% in the larger ducts due to the inverse relationship between luminal [Cl] and $[HCO_3]$. The rate of channel-mediated HCO_3 efflux from particular cells *in situ* may depend on additional factors such as luminal flow rate and location along the ductal system.

Many anions partially substitute for bicarbonate in supporting fluid secretion. For example, secretion is maintained at approximately 60% of the control level in rabbit and 65% in rat pancreas when HCO_3 is replaced by acetate [54, 63]. The apical exit step for this diverse array of anions may be an exchanger with wide substrate specificity, a collection of exchangers, or a poorly selective anion channel.

It may be relevant that the outward rectifier is permeable to many organic anions that can partially support secretion.

Activation of the anion channel would be expected to cause cell acidification if the channel mediates HCO_3 efflux. In rabbit mandibular gland acinar cells, intracellular pH (pH_i) falls approximately 0.2 units during acetylcholine stimulation [9]. The decline in pH_i is not sensitive to DIDS but is inhibited by the Cl channel blocker diphenylamine 2-carboxylate (DPC), suggesting HCO_3 efflux occurs through a weakly selective anion channel [6]. Further evidence for channel-mediated HCO_3 efflux comes from recent studies of rat parotid glands in which carbachol stimulation caused intracellular pH to fall 0.4 units when Na/H exchange was blocked by amiloride [45]. This effect was reduced by methoxolamide, a carbonic anhydrase inhibitor (e.g. [12]), and by removing exogenous HCO_3, but was accentuated in Cl-free medium. Carbachol-induced acidification was insensitive to the anion exchange inhibitor SITS but was inhibited by DPC. Taken together these results suggest Cl and HCO_3 may exit via the same pathway, perhaps a calcium-activated anion channel [45]. Intracellular acidification was not observed in a recent study of pancreatic acinar cells; however, this may have been masked by Na/H exchange [6].

Relative contributions of conductive *vs.* nonconductive HCO_3 efflux remain to be established, and will probably vary among tissues and between different species. To the extent that results from cultured cells can be extrapolated to natural epithelia, we anticipate channel-mediated HCO_3 secretion will be most significant in branches of the ductal tree where luminal [Cl] has decreased and HCO_3 efflux occurs against a large opposing concentration gradient. Electrogenic HCO_3 secretion may be most pronounced in species that achieve high (>140 mM) luminal HCO_3 concentrations. Establishing the relative importance of apical pathways will require measurements of net HCO_3 flux and net electrochemical gradients for both anions.

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