J. Membrane Biol. 122, 1–10 (1991)
002226319100075X

The Journal of
Membrane Biology

HCO_3^--Coupled Na^+ Influx is a Major Determinant of Na^+ Turnover and Na^+/K^+ Pump Activity in Rat Hepatocytes

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Summary. Recent studies in hepatocytes indicate that Na^+-coupled HCO_3^- transport contributes importantly to regulation of intracellular pH and membrane HCO_3^- transport. However, the direction of net coupled Na^+ and HCO_3^- movement and the effect of HCO_3^- on Na^+ turnover and Na^+/K^+ pump activity are not known. In these studies, the effect of HCO_3^- on Na^+ influx and turnover were measured in primary rat hepatocyte cultures with $^{22}Na^+$, and $[Na^+]_i$ was measured in single hepatocytes using the Na^+-sensitive fluorochrome SBFI. Na^+/K^+ pump activity was measured in intact perfused rat liver and hepatocyte monolayers as Na^+-dependent or ouabain-suppressible ^{86}Rb uptake, and was measured in single hepatocytes as the effect of transient pump inhibition by removal of extracellular K^+ on membrane potential difference (PD) and $[Na^+]_i$. In hepatocyte monolayers, HCO_3^- increased $^{22}Na^+$ entry and turnover rates by 50–65%, without measurably altering $^{22}Na^+$ pool size or cell volume, and HCO_3^- also increased Na^+/K^+ pump activity by 70%. In single cells, exposure to HCO_3^- produced an abrupt and sustained rise in $[Na^+]_i$ from ≈8 to 12 mM. Na^+/K^+ pump activity assessed in single cells by PD excursions during transient K^+ removal increased ≈2.5-fold in the presence of HCO_3^-, and the rise in $[Na^+]_i$ produced by inhibition of the Na^+/K^+ pump was similarly increased ≈2.5-fold in the presence of HCO_3^-. In intact perfused rat liver, HCO_3^- increased both Na^+/K^+ pump activity and O_2 consumption. These findings indicate that, in hepatocytes, net coupled Na^+ and HCO_3^- movement is inward and represents a major determinant of Na^+ influx and Na^+/K^+ pump activity. About half of hepatic Na^+/K^+ pump activity appears dedicated to recycling Na^+ entering in conjunction with HCO_3^- to maintain $[Na^+]_i$ within the physiologic range.

Key Words Na^+/HCO_3^- cotransport · symport · microelectrodes · membrane potential · SBFI · ion transport

Introduction

Hepatocytes exhibit a mechanism for electrogenic Na^+-coupled transport of HCO_3^- across the basolateral membrane (Fitz, Persico & Scharschmidt, 1989*a*; Gleeson, Smith & Boyer, 1989; Renner et al., 1989*b*). This recently described mechanism appears to contribute importantly to transmembrane HCO_3^- flux as well as to regulation of intracellular pH (pH_i) (Fitz & Scharschmidt, 1989), but little is known regarding its contribution to Na^+ transport or turnover. In cultured hepatocytes, $^{22}Na^+$ influx and efflux are increased fivefold or more in the presence of an imposed HCO_3^- gradient (Renner et al., 1989*b*), and HCO_3^--associated Na^+ movement appears to be a predominant pathway for electrogenic Na^+ transport (Fitz & Scharschmidt, 1989). These observations suggest that movement of Na^+ in association with HCO_3^- contributes importantly to total Na^+ transport and turnover. However, the direction of net HCO_3^--coupled movement and the contribution of this mechanism to Na^+ transport under more physiological conditions are unknown.

In certain other epithelia, Na^+-coupled HCO_3^- transport mediates net HCO_3^- extrusion (Soleimani, Grassl & Aronson, 1987). Because the HCO_3^- : Na^+ stoichiometry appears to be 3 : 1, coupled Na^+ and HCO_3^- transport is associated with movement of negative charge, and the interior negative membrane potential difference (PD) is therefore an effective driving force favoring efflux. Hepatocytes, however, exhibit a comparatively low resting PD, and indirect evidence suggests that the net direction of HCO_3^--coupled Na^+ transport is inward (Fitz et al., 1989*a*). If so, Na^+ influx through this mechanism might significantly affect intracellular Na^+ homeostasis as well as the activity of the Na^+/K^+ pump, which is sensitive to changes in intracellular Na^+ concentration ($[Na^+]_i$) (Van Dyke & Scharschmidt, 1983) and accounts for up to 30% of hepatic oxygen consumption (Van Dyke, Gollan & Scharschmidt, 1983). The purpose of these studies was to determine the direction of HCO_3^--associated Na^+ movement under basal conditions and its effects on $[Na^+]_i$,

Na^+ turnover, Na^+/K^+ pump activity, and hepatic oxygen utilization.

Materials and Methods

Chemicals

Radioisotopes ($^{86}Rb^+$, $^{22}Na^+$, 3-O-[methyl-^{14}C]-glucose) were obtained from New England Nuclear (Boston, MA). Sodium-binding benzofuran isophthalate acetoxy methylester (SBFI-AM) and pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Gramicidin D was obtained from Boerhinger-Mannheim (Indianapolis, IN). Ouabain, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), and all other reagents were purchased from Sigma Chemical (St. Louis, MO).

Solutions

Nominally HCO_3^--free solutions were made fresh daily from concentrated stock solutions and contained (in mM): 135 Na^+, 5 K^+, 1.2 Ca^{2+}, 0.8 Mg^{2+}, 115.8 Cl^-, 0.8 SO_4^{2-}, 0.8 PO_4^{2-}, 5 glucose, and 10 HEPES. pH was adjusted to ~7.4 with NaOH/HCl, and solutions were equilibrated with 100% O_2. HCO_3^--containing solutions included 25 mM HCO_3^- (substituted for Cl^-) and were equilibrated with 95% O_2/5% CO_2. Na^+-free solutions were made by isosmotic substitution of choline for Na^+. All studies were performed at 37°C, and solutions were prewarmed and equilibrated with the appropriate gas prior to the study.

Studies in Hepatocyte Monolayers

$^{22}Na^+$, 3-O-[methyl-^{14}C]-glucose, and $^{86}Rb^+$ uptake were measured in hepatocytes in monolayer culture as previously described (Scharschmidt & Stephens, 1981; Van Dyke & Scharschmidt, 1987). For these studies, hepatocytes were isolated from livers of male Sprague-Dawley rats by collagenase perfusion, separated by centrifugal elutriation, and plated on collagen-coated coverslips for 24–36 hr before use. Measurements were performed in triplicate in each batch of cells, and each experimental maneuver repeated on five or more batches of cells. Results are presented as the means ±SE of the daily averages. There was some variation in the absolute rate of cation transport from different cell batches. However, within each batch, experimental maneuvers tended to produce a consistent deviation from the control value. Therefore, in addition to determining overall mean values (e.g., Tables 1 and 2), the effects of experimental maneuvers were analyzed as previously reported by determining the mean ±SE of experimental values for each day expressed as a percentage of concurrent control values (Van Dyke & Scharschmidt, 1983).

$^{22}Na^+$ Uptake Rate and Turnover

$^{22}Na^+$ uptake was linear for at least 150 sec, and the initial rate of $^{22}Na^+$ uptake (nmol · min^{-1} · mg cell protein^{-1}) was obtained by linear regression from multiple time points (generally four) over this period (Scharschmidt & Stephens, 1981). Previously published and preliminary studies showed that $^{22}Na^+$ reached its equilibrium value within 45 min (Scharschmidt & Stephens, 1981; Van Dyke & Scharschmidt, 1983). Consequently, steady-state $^{22}Na^+$ content or pool size (nmol · mg cell protein^{-1}) was measured after 60 min. Turnover rate for the intracellular Na^+ pool was calculated as $^{22}Na^+$ uptake rate/$^{22}Na^+$ content and expressed as percent · min^{-1}.

Ouabain-Sensitive Rubidium Uptake

$^{86}Rb^+$ uptake (nmol · min^{-1} · mg cell protein^{-1}) with or without ouabain (5 mM) was linear for at least 10 min, and Na^+/K^+ pump activity was determined as previously reported from the difference between $^{86}Rb^+$ uptake in the absence and presence of ouabain when measured at 8 min (Van Dyke & Scharschmidt, 1983, 1987).

Cell Volume

Cell volume was calculated from the steady-state (60 min) accumulation of 2 mM ^{14}C-3-O-methylglucose (3-OMG) as previously described (Scharschmidt & Stephens, 1981; Van Dyke & Scharschmidt, 1983). $[Na^+]_i$ was calculated as $^{22}Na^+$ content/cell volume and expressed as mmol · liter^{-1} cell water (Van Dyke & Scharschmidt, 1983).

Studies in Individual Hepatocytes

Na^+/K^+ pump activity in individual cells was determined from the effects of transient inhibition of the pump on membrane PD (Griff, Shirao & Steinberg, 1985; Scharschmidt, Griff & Steinberg, 1988) and on $[Na^+]_i$. Because electrogenic pump activity contributes to the negative PD and low $[Na^+]_i$ characteristic of hepatocytes, inhibition of pump activity by removal of extracellular K^+ (or exposure to ouabain) results in a decrease in the contribution of electrogenic pumping to PD and an increase in $[Na^+]_i$. Consequently, comparison of the changes in PD and $[Na^+]_i$ in response to inhibition of pump activity in the presence and absence of HCO_3^- provides information regarding changes in Na^+/K^+ pump activity in individual cells over time (*see* Results).

Measurement of Membrane Potential Difference

PD was measured in individual hepatocytes grown on biomatrix (EHS)-coated coverslips using intracellular microelectrodes as previously described (Fitz et al., 1989*a*; Fitz & Scharschmidt, 1989; Fitz, Trouillot & Scharschmidt, 1989*b*). Coverslips were placed in a perfusion chamber mounted on the stage of an Olympus IMT-2 microscope, and cells were viewed at a magnification of 400×. The chamber (volume ~1 ml) was open at the top to allow access of the microelectrodes and was perfused by gravity at 10 ml · min^{-1}. Microelectrode resistance was compensated with a bridge circuit before impalement (Axoprobe-1A, Axon Instruments, San Mateo, CA), and voltage deflections (V) corresponding to 0.1 nA current pulses (I) were used to estimate cell conductance (g_{cell}), where $g_{cell} = I/V$. This allowed for stable intracellular recording for 1 hr or more, permitting repetition of experimental maneuvers in a single cell over time.

Measurement of $[Na^+]_i$

Intracellular sodium concentration ($[Na^+]_i$) was measured in individual hepatocytes using the Na^+-sensitive fluorochrome SBFI (Harootunian et al., 1989; Minta & Tsien, 1989). Hepatocytes plated on EHS-coated glass coverslips were incubated at room temperature for 90 min in standard buffer containing 5 μM SBFI and 0.025% pluronic F-127. Following dye loading, each coverslip was rinsed and placed in a perfusion chamber (volume ~1 ml) mounted on a Nikon Diaphot CM microscope (Nikon, Garden City, NY). Cell monolayers were perfused at 10 ml · min^{-1} at 37°C for a 5- to 10-min stabilization period. Subsequently, individual hepatocytes were optically isolated via a pinhole imaging system. Fluorescence measurements were performed using a SPEX CMII computer-controlled dual-wavelength excitation system (SPEX Industries, Edison, NJ) with excitation wavelengths of 340 and 380 nm, and emission was focused through a 480-nm cut-off filter. Following the completion of each study, *in situ* calibration was performed by equilibrating $[Na^+]_i$ with extracellular sodium concentration ($[Na^-]_e$) using the ionophore gramicidin. The calibration solutions contained 4 μM gramicidin in 100 mM K^+ gluconate, 10 mM HEPES (pH 7.20), and varying amounts of NaCl and KCl to produce a final constant total Na^+-plus-K^+ concentration of 140 mM and $[Na^+]_e$ of 0, 10, or 40 mM. $[Na^+]_i$ was calculated from the ratio of fluorescence with excitation at 340 nm to that with excitation at 380 nm after subtraction of background fluorescence, according to the equation

$$[Na^-]_i = \propto (R - R_0)/(R_m - R) \quad (1)$$

where

$$R_m = \frac{10R_0R_{10} + 30R_{10}R_{40} - 40R_0R_{10}}{40R_{10} - 30R_0 - 10R_{40}} \quad (2)$$

$$\alpha = 10\frac{R_M - R_{10}}{R_{10} - R_0} \quad (3)$$

and R_0, R_{10}, and R_{40} are the observed ratios at the extracellular Na^+ concentrations of 0, 10, and 40 mM used for *in situ* calibration (Harootunian et al., 1989).

Studies in Perfused Rat Liver

For studies in the intact organ, livers were removed from 220- to 260-g male Sprague-Dawley rats under ether anesthesia and perfused via the portal vein with 50 ml of recirculating fluorocarbon emulsion (Oxypherol FC-43, Alpha Therapeutics, Los Angeles, CA) at 22–26 ml · min^{-1} in a specially designed perfusion chamber as previously described (Brissot et al., 1985). After a 30-min stabilization period, single-pass perfusion with HCO_3^--containing media was initiated for an additional 10-min equilibration period.

^{86}Rb uptake was determined by measuring the accumulation of total liver ^{86}Rb with time under standardized conditions. A NaI scintillation crystal (Model TD76S76, Tennelec, Oak Ridge, TN) was suspended 5–10 mm above the liver, and the stepwise increase in liver radioactivity determined for a series of bolus injections of ^{86}Rb (~2 μCi each) into the portal vein catheter. To avoid measuring extracellular radioactivity, the liver was perfused with nonradioactive buffer for at least 30 sec before each measurement. Control studies indicated that uptake rates determined by this method remained linear for at least 20 min with correlation coefficients > 0.99, consistent with minimal efflux of ^{86}Rb from the liver over this period. After establishing the baseline uptake rate from nominally bicarbonate-free buffer, bicarbonate was abruptly introduced and the uptake rate again determined. Results were expressed as percent change in uptake relative to the basal period.

In separate studies, the partial pressure of oxygen in the effluent perfusate was measured continuously using an oxygen-sensitive electrode located at the hepatic vein catheter, and hepatic oxygen consumption was calculated from the difference in perfusate oxygen partial pressure of the portal and hepatic veins, perfusion flow rate, and liver weight (Van Dyke et al., 1983; Weisiger, Fitz & Scharschmidt, 1989).

Data Analysis

Results are reported as mean ±SE. The effects of experimental maneuvers were analyzed using Student's t test (paired or unpaired, as appropriate); $P < 0.05$ was considered to be statistically significant.

Results

Studies in Hepatocyte Monolayers

$^{22}Na^+$ *Uptake, Content, and Turnover*

To determine the effect of HCO_3^- on Na^+ uptake rate and turnover under steady-state conditions, monolayers were preincubated in nominally HCO_3^--free or HCO_3^--containing media for 60 min. The results are summarized in Table 1. The initial rate of $^{22}Na^+$ uptake in the absence of HCO_3^- averaged 3.44 ± 0.86 nmol · min^{-1} · mg cell $protein^{-1}$ and was consistently greater (5.65 ± 1.47) in each batch of cells when measured in the presence of HCO_3^-. This reflected an increase in initial rate of uptake in individual experimental *vs.* control monolayers of 65 ± 7% ($P < 0.03$) under steady-state conditions. To serve as a frame of reference, Na^+ uptake rate was also measured in the presence of alanine (2–5 mM) which is known to enter hepatocytes via a Na^+-coupled uptake mechanism (Van Dyke & Scharschmidt, 1983), and an increase of 105 ± 24% in individual monolayers was observed.

Intracellular $^{22}Na^+$ pool size measured after 60 min increased in the presence (22.1 ± 1.15 nmol · mg $protein^{-1}$) as compared to absence (19.7 ± 0.84 nmol · mg $protein^{-1}$) of HCO_3^-, although this increase did not achieve statistical significance. Cell volume was unchanged and calculated $[Na^+]_i$ also exhibited only a modest increase which was not statistically significant (Table 1). The observation that HCO_3^- causes an increase in the initial rate of $^{22}Na^+$

Table 1. Effect of HCO_3^- and alanine on $^{22}Na^+$ uptake and turnover and ouabain-suppressible ^{86}Rb uptake in hepatocyte monolayers

Conditions			
$[HCO_3^-]$	0	25 mM	0
[Alanine]	0	0	2–5 mM
Absence of Ouabain			
$^{22}Na^+$			
Initial uptake rate ($nmol \cdot min^{-1} \cdot mg^{-1}$)	3.44 ± 0.86	5.65 ± 1.47[a]	6.15 ± 0.97[b]
Pool size ($nmol \cdot mg^{-1}$)	19.7 ± 0.84	22.1 ± 1.15	24.9 ± 1.10[a]
Turnover ($\% \cdot min^{-1}$)	18.3 ± 0.06	26.8 ± 0.09[b]	24.8 ± 0.04[b]
Concentration ($mmol \cdot liter^{-1}$)	12.2 ± 0.58	13.7 ± 0.80	15.5 ± 0.76[a]
^{86}Rb			
Ouabain-sensitive uptake ($nmol \cdot mg^- \cdot min^{-1}$)	1.32 ± 0.20	1.92 ± 0.15[a]	2.50 ± 0.18[a]
Presence of Ouabain			
$^{22}Na^+$			
Pool size	91.0 ± 3.0	147.4 ± 9.2[b]	ND

Hepatocyte monolayers were preincubated in HEPES-buffered solutions in the nominal absence of HCO_3^-, presence of alanine (2–5 mM), or presence of HCO_3^-/CO_2, all at pH 7.4. The initial rates of $^{22}Na^+$ and ^{86}Rb uptake and ^{22}Na pool size were determined as described in the text. Na^+ turnover was calculated from pool size and uptake rate on individual days and apparent Na^+ concentration was calculated from pool size and cell volume determined using ^{14}C-3-O-methyl-D-glucose, which averaged 1.61 ± 0.05 μl/mg and did not differ between HCO_3^--containing and nominally HCO_3^--free incubations. Results represent the mean ± SE of five or more separate studies done in triplicate in each batch of cells, where [a] indicates $P < 0.05$ and [b] indicates $P < 0.01$ when compared with values in HCO_3^--free media. ND means not done.

uptake with a comparatively modest effect on $[Na^+]_i$ and pool size implies increased $^{22}Na^+$ turnover. Indeed, calculated turnover of the Na^+ pool increased from 18.3 ± 0.06% min^{-1} in the absence of HCO_3^- to 26.8 ± 0.09% min^{-1} in the presence of HCO_3^-, representing an increase of 46 ± 3% ($P < 0.01$). Alanine had similar effects, increasing turnover by 39 ± 4% ($P < 0.01$).

To further explore the effect of HCO_3^- on Na^+ transport, additional studies were performed in the presence of ouabain. In the nominal absence of HCO_3^-, exposure of ouabain increased intracellular Na^+ content from 19.7 ± 0.84 to 91.0 ± 3.0 nmol · mg protein^{-1} when measured at 60 min. In the presence of HCO_3^-, the increase in intracellular Na^+ content with ouabain exposure was much greater (22.1 ± 1.15 to 147 ± 9.2 nmol · mg protein^{-1}, $P < 0.01$, Table 1). These findings suggest that HCO_3^--associated Na^+ movement is inward, and that intact Na^+/K^+ pump function is essential to maintain Na^+ homeostasis in the presence of HCO_3^-. The failure to demonstrate a statistically significant increase in calculated $[Na^+]_i$ in the presence of HCO_3^- may reflect the difficulty in detecting small changes in $[Na^+]_i$ in the presence of variability among monolayers and prompted us to study the effects of HCO_3^- on $[Na^+]_i$ in individual hepatocytes (*see below*).

Ouabain-Sensitive ^{86}Rb Uptake

An HCO_3^--dependent increase in $^{22}Na^+$ turnover with only a modest increase in $[Na^+]_i$ implies that exposure to HCO_3^- is also accompanied by increased Na^+ efflux. Additional studies were performed to determine whether this was mediated by an increase in Na^+/K^+ pump rate, assessed as ouabain-sensitive ^{86}Rb uptake. The results are summarized in Table 1. In hepatocyte monolayers, 74 ± 8% of total uptake was ouabain sensitive, and the proportion was not different among the separate study groups. Ouabain-sensitive ^{86}Rb uptake in individual experimental *vs.* control monolayers was 70 ± 27% greater ($P < 0.025$) in the presence, as compared with the absence, of HCO_3^-. Alanine, which also enters via a Na^+-coupled mechanism, increased ouabain-sensitive ^{86}Rb uptake in individual experimental *vs.* control monolayers by 101 ± 38% ($n = 5$, $P < 0.002$) (Van Dyke & Scharschimdt, 1983). In separate studies, SITS (1 mM) had no effect on the increase in ouabain-sensitive ^{86}Rb uptake produced by alanine, but inhibited by 40% ($P < 0.05$; $n = 4$) the increase produced by HCO_3^-.

Studies were also done to assess the possibility that HCO_3^- might affect Na^+/K^+ pump activity indirectly via alteration in pH_i. When the pH of the incubation medium was varied from 7.0 to 7.8 to

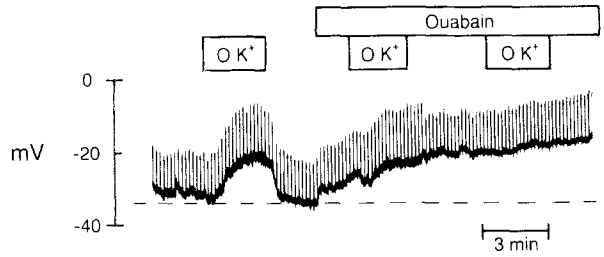

Fig. 1. Effect of Na^+/K^+ pump inhibition on membrane PD. PD was measured from an individual hepatocyte as described in the text. Periodic upward deflections represent the voltage response to 0.1-nA current pulses and were used to estimate membrane conductance. Under basal conditions resting PD was −35 mV (broken line) and acute removal of extracellular K^+ resulted in depolarization of ≈11 mV with prompt recovery to basal levels on return to control perfusate. Inhibition of Na^+/K^+ pump activity by ouabain (2 mM) depolarized the cell to a similar degree with a slower time course. In the presence of ouabain, the effect of removing extracellular K^+ on PD was largely abolished

alter pH_i (Fitz et al., 1989*b*), there was no effect on ouabain-suppressible ^{86}Rb uptake (101 and 103% of control, respectively).

Studies in Individual Hepatocytes

The studies in hepatocyte monolayers described above indicate that Na^+ entry and Na^+/K^+ pump activity are increased in the presence of HCO_3^-. However, they provide limited information about the rapidity with which HCO_3^- affects the Na^+/K^+ pump and do not directly indicate the direction of *net* HCO_3^--coupled Na^+ movement. Although HCO_3^- may stimulate both Na^+ influx and efflux (Renner et al., 1989*b*), only net inward HCO_3^--coupled Na^+ flux would be expected to stimulate Na^+ extrusion mediated via the Na^+/K^+ pump.

Electrophysiologic Measurement of Na^+/K^+ Pump Activity

The resting PD in HCO_3^--containing perfusate averaged −40 ± 2 mV (n = 41) and ranged between −24 and −54 mV. Apparent cell conductance (g_{cell}) measured simultaneously was 14.5 ± 2.1 nS, similar to values previously reported (Fitz et al., 1989*a,b*; Fitz & Scharschmidt, 1989). Removal of extracellular K^+ had biphasic effects on PD in most cells, with transient initial hyperpolarization followed by sustained depolarization of greater magnitude (Figs. 1 and 2). Several observations indicate that these responses are attributable to changes in K^+ equilibrium potential and electrogenic Na^+/K^+ pumping, respectively. Previous studies using this preparation have shown that more negative values for resting potential are associated with higher resting K^+ conductance (g_{K^+}) (Fitz et al., 1989*b*). Consequently, the shift in K^+ equilibrium potential caused by lowering $[K^+]_o$ would be expected to hyperpolarize the membrane. In these studies, the initial hyperpolarization was not evident at resting potentials of −30 mV or less (where resting g_{K^+} is relatively low), but increased in magnitude (range 2–6 mV) with more negative values of resting PD (where resting g_{K^+} is relatively high, r = 0.76, $P < 0.01$). When present, hyperpolarization appeared within ~15 sec, corresponding to the lag time due to the dead space of the perfusion apparatus, and was eliminated in the presence of Ba^{2+} (eight out of eight studies) in concentrations previously shown to inhibit g_{K^+} (1 mM; Fitz et al., 1989*b*).

In contrast to early hyperpolarization seen in some cells, the predominant response to lowering $[K^+]_o$ in all cells was membrane depolarization which developed more slowly. Depolarization was evident within 30 sec and reached near maximal values within 2 min. There was considerable cell-to-cell variation in the magnitude of the response (range 3–20 mV), but the response in a single cell was stable over time (c.v. 9% measured in eight cells at 5-min intervals). The magnitude of the depolarization response was not related to resting potential (r = 0.52, NS) but was inhibited by ouabain, as shown in Fig. 1. Under basal conditions, removal of extracellular K^+ resulted in depolarization with prompt recovery on return to K^+-containing perfusate. Inhibition of pump activity by ouabain depolarized the cell to a similar degree over a longer time course and eliminated the effects of removal of extracellular K^+, consistent with a common effect. In 13 such studies, ouabain decreased the voltage response to removal of extracellular K^+ from 9 ± 1 mV to 2 ± 1 mV ($P <$ 0.001). By contrast, inhibition of membrane K^+ conductance with Ba^{2+} (1 mM) also decreased the resting voltage, but had no significant effect on this response (7 ± 1 mV *vs.* 6 ± 2 mV, n = 7).

The effects of HCO_3^- on the PD response to transient pump inhibition are illustrated in Fig. 2 and summarized in Table 2. In 13 studies, resting PD in the absence of HCO_3^- averaged −24 ± 1 mV, and lowering $[K^+]_o$ caused a depolarization of 5 ± 1 mV (range 3–11 mV) with rapid recovery on return to control conditions. Exposure to HCO_3^- caused rapid hyperpolarization to −46 ± 4 mV, and lowering $[K^+]_o$ produced a greater depolarization in each cell tested, averaging 13 ± 1 mV (range 8–20 mV, $P < 0.001$). The effects of HCO_3^- on this response were apparent within 2 min (it was not possible to evaluate earlier points

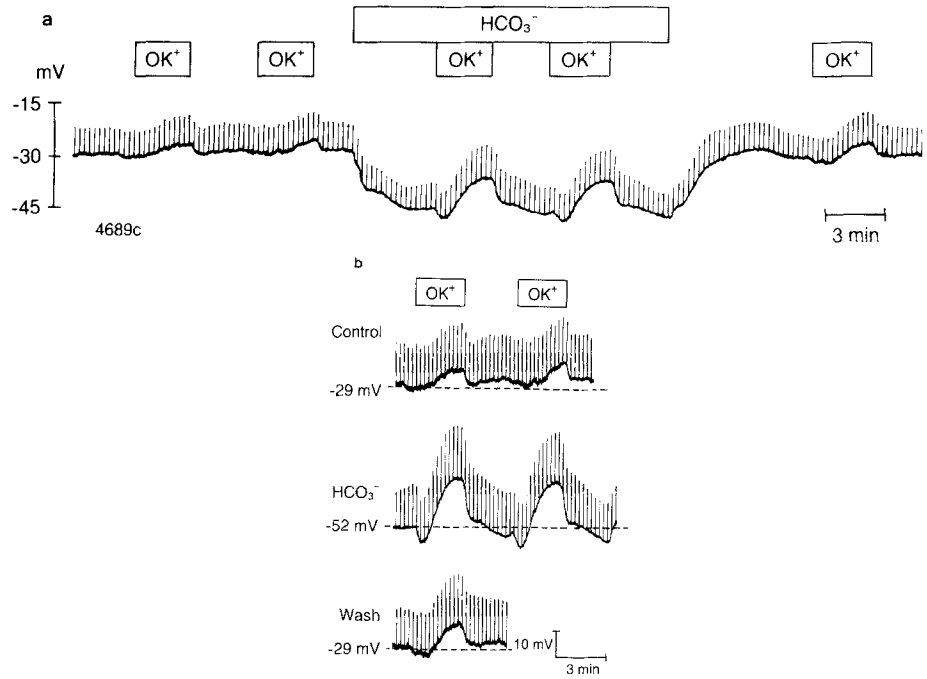

Fig. 2. Effect of HCO_3^- on membrane PD and response to inhibition of the Na^+/K^+ pump by removal of extracellular K^+ in a single hepatocyte. (*A*) In the continuous recording, reversible hyperpolarization of PD upon exposure to HCO_3^- is apparent. (*B*) Sections of the tracing in *A* are depicted at higher amplification, and the effects of HCO_3^- on the response to removal of extracellular K^+ are demonstrated. In the absence of HCO_3^-, removal of extracellular K^+ depolarized the cell by ~6 mV with rapid recovery upon reintroduction of K^+. In the presence of HCO_3^-, K^+ removal resulted in transient initial hyperpolarization that was followed by depolarization of greater magnitude. The lower tracing recorded following the removal of HCO_3^- resembles the control response

Table 2. Effect of HCO_3^- on the change in membrane potential difference (PD) and $[Na^+]_i$ in response to inhibition of Na^+/K^+ pump activity in individual hepatocytes by removal of extracellular K^+

	Potential difference (mV)		$[Na^+]_i$ (mM)	
	Resting	Change with K^+ removal	Resting	Change with K^+ removal
HCO_3^- free	−24 ± 1.0 (13)	5 ± 1 (13)	10.8 ± 1.3 (20)	3.4 ± 0.5 (6)
HCO_3^- containing	−47 ± 4[a] (13)	13 ± 1[a] (13)	12.0 ± 1.0 (20)	9.2 ± 1.3[a] (6)

[a] Potential difference was measured in each cell in the absence and presence of extracellular HCO_3^- under resting conditions as well as following inhibition of Na^+/K^+ pump activity by removal of extracellular K^+, as described in Materials and Methods (*see also* Fig. 2). Using a similar protocol, $[Na^+]_i$ was measured with SBFI fluorescence under resting conditions as well as following removal of extracellular K^+. Results are presented as mean ±SE, and the number of experiments is indicated in parentheses.
[a] $P < 0.001$ as compared with HCO_3^--free incubations.

because of the rapidly changing baseline following exposure to HCO_3^-; Fig. 2), and these effects were rapidly reversible on return to HCO_3^--free perfusate. This corresponds to an increase of 195 ± 34%, suggesting a large increase in the contribution of electrogenic Na^+/K^+ pump activity to resting potential in the presence of HCO_3^-.

Effects of Pump Inhibition on $[Na^+]_i$

Whereas these studies indicate that plasma membrane Na^+ turnover and Na^+/K^+ pump rate are increased in the presence of HCO_3^-, they do not directly indicate the direction of net HCO_3^--associated Na^+ movement. To address this important

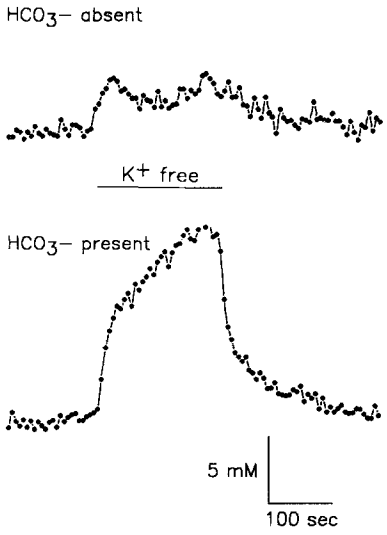

Fig. 3. Effect of HCO_3^- on the change in $[Na^+]_i$ observed in response to inhibition of the Na^+/K^+ by removal of extracellular K^+. $[Na^+]_i$ was measured in individual hepatocytes with SBFI fluorescence in the absence (top) and presence (bottom) of perfusate HCO_3^-. In the presence of HCO_3^-, the increase in $[Na^+]_i$ observed upon removal of extracellular K^+ was greater than in the absence of HCO_3^-. In both recordings, $[Na^+]_i$ promptly recovered to basal levels with reintroduction of extracellular K^+

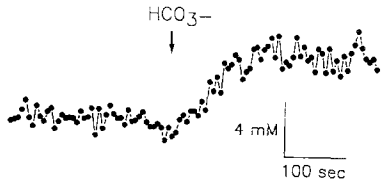

Fig. 4. Effect of acute HCO_3^- exposure on $[Na^+]_i$ in an individual hepatocyte. $[Na^+]_i$ was measured with SBFI fluorescence before and following acute exposure to 25 mM HCO_3^-. The steady-state value for $[Na^+]_i$ after HCO_3^- exposure is greater than that measured under basal conditions in HCO_3^--free media

point, the effects of inhibition of Na^+/K^+ pumping on $[Na^+]_i$ were determined in the presence and absence of HCO_3^-. If the direction of net HCO_3^--associated Na^+ movement is inward, then $[Na^+]_i$ should increase more rapidly in the presence, as compared with the absence, of HCO_3^- when the Na^+/K^+ pump is inhibited.

In individual cells, resting $[Na^+]_i$ as measured with SBFI fluorescence was higher in the presence (12.2 ± 1.0 mM, $n = 20$), as compared to the absence (10.8 ± 1.3 mM, $n = 20$), of HCO_3^-, although this difference did not achieve statistical significance (Table 2). The effects of acute inhibition of Na^+/K^+ pump activity by removal of extracellular K^+ in the absence (top) and presence (bottom) of HCO_3^- are shown in Fig. 3. In the absence of HCO_3^-, inhibition of pump activity was associated with an increase in $[Na^+]_i$ of 3.4 ± 0.5 mM ($n = 6$). By contrast, in the presence of HCO_3^-, the same maneuver caused a rapid increase in $[Na^+]_i$ of 9.2 ± 1.3 mM ($n = 6$), with prompt recovery on restoration of pump activity following return to normal concentrations of extracellular K^+. Notably, $[Na^+]_i$ increased promptly with K^+ removal in all studies, even though the initial PD response to K^+ removal in some cells is hyperpolarization (Fig. 2). Thus, the change in $[Na^+]_i$ predominantly reflects acute inhibition of Na^+/K^+ pump activity rather than PD-driven changes in HCO_3^--coupled Na^+ movement.

Dynamic Effects on HCO_3^- on $[Na^+]_i$

The studies described above demonstrate that under steady-state conditions Na^+/K^+ pump activity is increased in the presence of HCO_3^- and further suggest that the increase is due to enhanced HCO_3^--coupled Na^+ influx. However, the trend toward an increase in $[Na^+]_i$ did not achieve statistical significance. To better detect potentially small HCO_3^--induced changes in $[Na^+]_i$ against the background variability in $[Na^+]_i$ among cells, studies were conducted in which the response to HCO_3^- exposure in individual cells was continuously monitored. A representative study is illustrated in Fig. 4. On exposure to HCO_3^-, $[Na^+]_i$ promptly increased and reached a new steady state higher than its basal value. In four such studies in which each hepatocyte served as its own control, $[Na^+]_i$ was increased by 4.0 ± 0.9 mM in the presence of HCO_3^- ($P < 0.02$), yet mean $[Na^+]_i$ did not significantly differ before and after HCO_3^- exposure (8.2 ± 1.9 *vs.* 12.2 ± 2.4 mM). Thus, HCO_3^- does produce a significant increase in $[Na^+]_i$, although this increase is difficult to detect in populations of cells because of cell-to-cell variation in $[Na^+]_i$.

Studies in Intact Rat Liver

The studies in primary hepatocyte cultures summarized above suggest that intact liver should respond to the presence of HCO_3^- with an increase in Na^+/K^+ pump activity and O_2 consumption. Indeed, HCO_3^- had similar effects on ^{86}Rb uptake by the intact perfused liver. Under basal conditions in the absence of HCO_3^-, total ^{86}Rb uptake was linear with time for at least 20 min (Fig. 5*A*). The slope of this line gives the uptake rate and was used as a control to determine the effect of ion substitution on the relative uptake rate. Exposure to HCO_3^--containing perfusate caused an abrupt increase in the uptake rate to 176 ± 6% of control. Replacement of

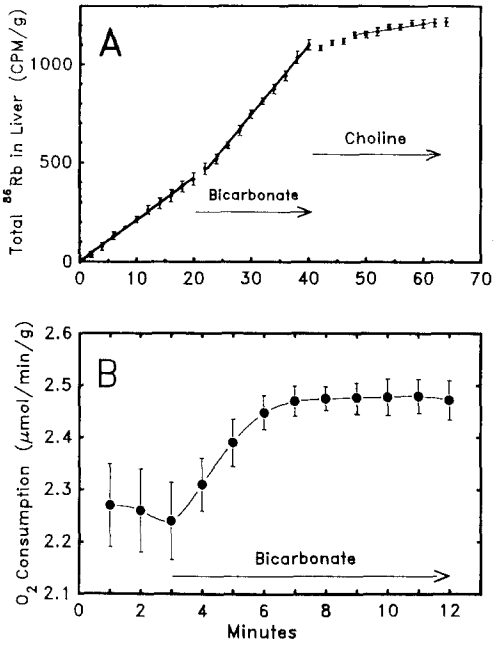

Fig. 5. Effect of HCO_3^- on ^{86}Rb uptake (*A*) and oxygen consumption (*B*) in the perfused liver. ^{86}Rb uptake and oxygen consumption were measured in separate studies as described in the text. In the nominal absence of perfusate HCO_3^-, ^{86}Rb uptake was linear for at least 20 min. Following exposure to HCO_3^-, there was a prompt increase in the rate of ^{86}Rb uptake to 176 ± 6% of control and oxygen consumption to 110 ± 5% of control. Changes were detectable within 1 min and maximal within 3 to 5 min

perfusate Na^+ with choline at the end of the study decreased ^{86}Rb uptake to 13.8 ± 3.5% of control. Thus, as previously demonstrated (Renner et al., 1988), the majority of ^{86}Rb uptake by the intact liver is Na^+ dependent and likely reflects Na^+/K^+ pump activity.

The increase in ^{86}Rb uptake in the presence of HCO_3^- was accompanied by an increase in total hepatic oxygen consumption from 2.26 ± 0.09 to 2.48 ± 0.04 μmol/min/g liver (mean ± SE, $n = 4$, $P < 0.05$), an increase of 10% (Fig. 5*B*). The increase was detectable within 1 min and was maximal within 4–5 min, similar to the time course noted for ^{86}Rb uptake by perfused liver and electrophysiologic measurement of Na^+/K^+ pump activity in individual hepatocytes. These findings are all consistent with rapid modulation of Na^+/K^+ activity by HCO_3^-.

Discussion

The present studies have characterized the effects of HCO_3^- on Na^+ transport and Na^+/K^+ pump activity in hepatocytes. Our major findings are as follows: (*i*) $^{22}Na^+$ entry rate and turnover are increased by ~65 and ~46%, respectively, in the presence as compared to the absence of HCO_3^-; (*ii*) Na^+/K^+ pump rate, measured in single cells, monolayers, and intact liver increased from 70 to 160% depending on the method used; (*iii*) the increase in Na^+/K^+ pump rate is associated with an increase in oxygen consumption by the intact liver; and (*iv*) the net direction of Na^+-coupled HCO_3^- transport is inward. These observations indicate that HCO_3^--coupled Na^+ movement represents a major contributor to total Na^+ influx and turnover in hepatocytes. Rapid modulation of pump rate following exposure to HCO_3^- appears to be an adaptive response to maintain Na^+ homeostasis. The metabolic cost is substantial as this results in a significant increase in pump rate and oxygen consumption by the intact organ. Before discussing these results further, it is appropriate to consider certain aspects of the study in more detail.

We have previously demonstrated that HCO_3^--associated Na^+ uptake is electrogenic, inhibited by stilbene derivatives, and not affected by Cl^- or amiloride, consistent with Na^+/HCO_3^- cotransport (Fitz & Scharschmidt, 1989; Renner et al., 1989*b*). In the present study, $^{22}Na^+$ entry rate and turnover were measured under steady-state conditions following preincubation in the presence or absence of HCO_3^-. The increase in initial rate of $^{22}Na^+$ uptake observed in these studies was less than the 4- to 10-fold stimulation reported previously (Renner et al., 1989*b*), reflecting the fact that earlier studies were performed in the presence of ouabain, a transmembrane HCO_3^- gradient, and 5 mM Na^+ for the purpose of minimizing HCO_3^--independent Na^+ flux and maximizing any effects of HCO_3^-. The present studies indicate that, under more physiologic conditions, one-third or more of total Na^+ flux is HCO_3^- dependent.

Although both HCO_3^- and alanine, which we employed as a positive control and frame of reference, increased the initial rate of $^{22}Na^+$ uptake by 65% or more (Table 1), $[Na^+]_i$ increased only slightly. Indeed, the increase was detected only in studies in which individual hepatocytes served as their own control (Fig. 4) and did not achieve statistical significance when populations were studied (e.g., Table 1). $^{22}Na^+$ turnover, however, increased approximately 50% in the presence of HCO_3^-. This increase in Na^+ influx and turnover implies that increased uptake of Na^+ in the presence of HCO_3^- is associated with increased activity of the Na^+/K^+ pump, and this was directly demonstrated using several approaches.

In hepatocyte monolayers, Na^+/K^+ pump activity measured as ouabain-suppressible ^{86}Rb uptake was increased by approximately 70%. Na^+/K^+

pump activity was also assessed using an electrophysiologic technique previously applied to retinal pigment epithelium (Griff et al., 1985; Scharschmidt et al., 1988), in which the PD response to inhibition of the electrogenic Na^+/K^+ pump by removal of extracellular K^+ is measured with conventional microelectrodes. K^+ removal in both tissues elicits a biphasic response. In retinal pigment epithelium, hyperpolarization due to alteration of the K^+ equilibrium potential is the predominant response and masks depolarization due to Na^+/K^+ pump inhibition. In hepatocytes, depolarization due to Na^+/K^+ pump inhibition is the predominant response. The presumably reflects greater contribution of electrogenic Na^+/K^+ pumping.

Using each cell as its own control, the presence of HCO_3^- more than doubled the effects on PD of removal of K^+ (Fig. 2 and Table 2), consistent with a rapid increase in Na^+/K^+ pump activity. The reversible hyperpolarization of basal PD produced by exposure to HCO_3^- was considerably larger ($\simeq -22$ mV) than the change in PD ($\simeq -8$ mV) attributable to increased pump activity, and likely reflects electrogenic HCO_3^- transport (Fitz et al., 1989*a*; Fitz & Scharschmidt, 1989) as well as the effects of intracellular pH (pH_i) on membrane K^+ conductance (Fitz et al., 1989*b*).

Exposure to HCO_3^- likely has a variety of effects on the metabolic activity of hepatocytes. However, several observations suggest that the large increase in Na^+/K^+ pump rate by HCO_3^- results predominantly from the effects of HCO_3^- on Na^+ influx. *First*, the effects of HCO_3^- on $^{22}Na^+$ uptake and turnover imply an increase in pump rate, and the magnitude of the increase in $^{22}Na^+$ influx and the increase in Na^+/K^+ pump rate measured as ouabain-suppressible ^{86}Rb uptake in monolayers is very similar. Moreover, the increase in Na^+/K^+ pump rate produced by HCO_3^-, but not the increase produced by alanine, was partially inhibited by SITS, which inhibits Na^+-coupled HCO_3^- transport. *Second*, inhibition of Na^+/K^+ pump activity produced a greater increase in $[Na^+]_i$ in the presence, as compared with the absence, of HCO_3^-. This provides direct evidence that the direction of net coupled movement of Na^+ and HCO_3^- is inward under basal conditions, and suggests that an increase in pump activity is necessary to keep $[Na^+]_i$ in a physiologic range. *Third*, the small increase in $[Na^+]_i$ in individual hepatocytes exposed to HCO_3^- would be expected to increase Na^+/K^+ pump activity (Van Dyke & Scharschmidt, 1983). *Finally*, although exposure to HCO_3^- resulted in large changes in PD and presumably pH_i, neither appears to account for the observed changes in pump activity.

Studies in cardiac myocytes suggest that the voltage-sensitive step is not rate limiting (Bahinski, Nakao & Gadsby, 1988), and membrane hyperpolarization should inhibit, not increase, pump turnover (DeWeer, Gadsby & Rakowski, 1988). In the present studies, there was no significant correlation between PD and the magnitude of the depolarization associated with inhibition of the pump in the presence or absence of HCO_3^-. Na^+/K^+ pump rate in certain other epithelia appears to be sensitive to changes in pH_i (Eaton, Hamilton & Johnson, 1984). We therefore examined the effect of pH_i on Na^+/K^+ pump activity by measuring ouabain-suppressible ^{86}Rb uptake in hepatocytes incubated in media of varying pH. We have previously demonstrated that preincubation of hepatocytes in media with pH 7.0 or 7.8 alters pH_i by ~ -0.10 and ~ 0.15 pH units, respectively (Fitz et al., 1989*b*). In the present studies, these maneuvers had no effect on ouabain-suppressible ^{86}Rb uptake in monolayers, suggesting that changes in pH_i are unlikely to account for the observed effects on pump rate.

To establish the relevance of our findings in primary hepatocyte cultures to intact liver, the effects of HCO_3^- on Na^+/K^+ pump activity and O_2 consumption were studied in perfused liver. As expected, introduction of HCO_3^- increased both ^{86}Rb uptake rate and O_2 consumption within 1 min. Moreover, the magnitude of the increase in ^{86}Rb uptake was very similar to that in the monolayers, and the increase in O_2 consumption of about 10% is approximately that predicted for an increase of 70% in the Na^+/K^+ pump, which accounts for about 20–25% of hepatic O_2 consumption (Van Dyke et al., 1983).

The demonstration that basolateral Na^+-coupled HCO_3^- transport is inward under basal conditions implies that it may function differently from similar mechanisms in other epithelia where it mediates net HCO_3^- efflux. In renal tubular cells, for example, Na^+-coupled HCO_3^- transport on the basolateral membrane mediates efflux of HCO_3^-, which is accumulated intracellularly due to the action of apical Na^+/H^+ exchange. In hepatocytes, both Na^+/H^+ exchange and Na^+-coupled HCO_3^- transport are localized to the basolateral membrane. Because Na^+/H^+ appears to be inactive at physiologic pH_i (Renner et al., 1989*a*), Na^+-coupled HCO_3^- transport in hepatocytes may represent the predominant base loader under resting conditions. Recent studies suggest that Na^+-coupled HCO_3^- transport plays an important role in regulation of pH_i following intracellular acidification as well (Fitz & Scharschmidt, 1989; Lidofsky et al., 1990). The role of Na^+-coupled HCO_3^- transport in other hepatocellular functions including formation of HCO_3^--rich bile is unknown. However, the present studies indicate that HCO_3^- coupling represents a major deter-

minant of Na^+ influx and turnover and that the metabolic cost for this mechanism is high. Indeed, up to one-half of Na^+/K^+ pump activity, representing 10% of total hepatic O_2 consumption, appears dedicated to recycling the Na^+ entering in conjunction with HCO_3^- so as to maintain $[Na^+]_i$ in physiologic range.

The authors wish to acknowledge the important support of UCSF Liver Center Core Facilities, including the Cell Culture Facility (Dr. D.M. Bissell, Director), Biomathematics Core Facility (Dr. V. Licko, Director), and Editorial Core Facility (D. Fedorchak and M. Karasik, Editors).

This work was supported in part by NIH grants AM-26270, DK-43278, DK-26743, DK-08311, and DK-01987; an American Liver Foundation Postdoctoral Research Fellowship and Liver Scholar Awards (S.D.L.); and an American Gastroenterological Association/G.D. Searle Research Scholar Award (J.G.F.).

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Received 14 September 1990; revised 4 December 1990