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Physiology

Regulation of activity and apical targeting of the Cl^-/HCO_3^- exchanger in rat hepatocytes

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ABSTRACT **To test the hypothesis that rat hepatocyte canalicular Cl^-/HCO_3^- exchange activity might be regulated by HCO_3^-- or protein kinase-induced changes in the apical targeting of vesicles, isolated rat hepatocytes were cultured in the presence or absence of HCO_3^-/CO_2. Cl^-/HCO_3^- exchange activity increased in cells cultured in the presence of HCO_3^-/CO_2 or when stimulated by dibutyryl cAMP. Both of these effects were blocked by either colchicine or the protein kinase C agonist phorbol 12,13-dibutyrate. Fluorescence and confocal microscopy, respectively, revealed increased pericanalicular–apical membrane localization of two canalicular markers, peanut agglutinin and a 110-kDa canalicular ecto-ATPase, when hepatocyte couplets were preincubated in HCO_3^-/CO_2-containing medium, an effect that was again blocked by colchicine. Dibutyryl cAMP also stimulated canalicular localization of the 110-kDa protein. These findings suggest that hepatocyte Cl^-/HCO_3^- exchange activity is regulated by HCO_3^-/CO_2 and by protein kinase A and protein kinase C agonists through microtubule-dependent targeting of vesicles containing this exchanger to the canalicular domain.**

The location of individual cell surface proteins to specific membrane domains is an essential property of epithelial cells, including hepatocytes, that establishes their structural and functional polarity. The mechanisms by which these plasma membrane proteins are localized to different cell membrane domains and the signals used to guide this "targeting" are largely unknown.

In hepatocytes, the canalicular membrane represents only 13% of the plasma membrane surface and is demarcated from the basolateral cell surface by the tight junctions. This apical domain expresses specific transport systems for excretion of bile acids and other organic anions and cations (1–4).

A Na^+-independent Cl^-/HCO_3^- exchanger has been localized to the canalicular domain (5). This ion exchanger is the mechanism by which the hepatocyte loads acid in defense of increases in intracellular pH (pH_i) above its normal set point and is activated by an alkaline load (6).

We originally observed that basal Cl^--dependent HCO_3^- fluxes were higher in rat hepatocytes cultured in the presence as opposed to the absence of HCO_3^-/CO_2-containing medium. Apical insertion and removal of ion transporters have been suggested as regulatory mechanisms in cells from the renal collecting tubule that secrete H^+ or HCO_3^- in response to changes in pH of their environment (7). Therefore, we hypothesized that the increased activity of the apical Cl^-/HCO_3^- exchanger might also involve targeting of the exchanger to the canalicular domain when stimulated by changes in pH_i and HCO_3^-.

To test this hypothesis, we studied the effects of (*i*) HCO_3^-/CO_2, (*ii*) inhibitors of microtubule and microfilaments, and (*iii*) activators of protein kinases A and C on Cl^-/HCO_3^- exchange activity in rat hepatocyte monolayers. Morphologic studies were also performed in isolated rat hepatocyte couplets to examine the distribution of two canalicular membrane proteins: peanut agglutinin receptors (PNA) (7) and a 110-kDa ecto-ATPase, a putative bile acid transporter (8, 9).

Our results suggest that the activity of this Cl^-/HCO_3^- exchanger is highly regulated by stimuli that can modulate microtubule-dependent targeting of apical proteins to the canalicular domain.

METHODS

Hepatocyte Isolation and Culture. Isolated hepatocytes (viability, 85–92%) were prepared as described (10, 11) and cultured on Matrigel-precoated coverslips at 37°C for 2–6 hr, forming a subconfluent monolayer. Depending on the experimental protocol, cells were cultured in the following media: Liebovitz's 15 phosphate-buffered medium (L-15) or minimal essential medium containing 25 mM $NaHCO_3$ and 5% CO_2 (MEM + HCO_3^-/CO_2) or containing 25 mM Hepes (MEM + Hepes). All media were supplemented with 10% (vol/vol) fetal calf serum (GIBCO) and 100,000 units of penicillin and streptomycin per 100 mg per liter (Sigma).

pH_i Measurement and Assessment of Cl^-/HCO_3^- Exchange Activity. Hepatocyte monolayers were loaded with the acetoxymethyl ester (AM) derivative of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) (10 μM), and pH_i was measured in a Perkin–Elmer LS-5B spectrofluorimeter as described (6). Cover slips with attached cells were placed into a thermostatically controlled cuvette (37°C) of a Perkin–Elmer LS-5B spectrofluorometer. BCECF-AM in Krebs–Ringer bicarbonate buffer (KRB) was loaded for 10 min and washed for 10 min, and baseline pH was measured for 5 min. Cells were alternately excited at 450 and 500 nm, whereas the emitted fluorescence was recorded at 530 nm. The 500/450-nm fluorescence ratio was calibrated in terms of pH units by using nigericin in high K^+ buffer (12–14). After 5-min baseline readings, the perfusate solution was changed to a Cl^--free solution for 15 min. Cl^- was then readmitted, and pH_i was monitored for an additional 10 min. Similar data were obtained with hepatocytes cultured in L-15 or MEM + Hepes medium (see Fig. 1 *a* and *b*).

Cl^-/HCO_3^- exchange activity was assessed by (*i*) determining the maximum rate of pH_i alkalinization or recovery over a 2-min period immediately after acute Cl^--removal and

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Abbreviations: pH_i, intracellular pH; PNA, peanut agglutinin; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; DBcAMP, dibutyryl cAMP; $[HCO_3^-]_i$ and $[cAMP]_i$, intracellular HCO_3^- and cAMP concentrations; KRB, Krebs–Ringer bicarbonate buffer; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester; IBMX, isobutylmethylxanthine; PDB, phorbol 12,13-dibutyrate; clpm F°/CF° ratio, ratio of peak fluorescence on the canalicular liver plasma membrane/average cytosolic fluorescence.

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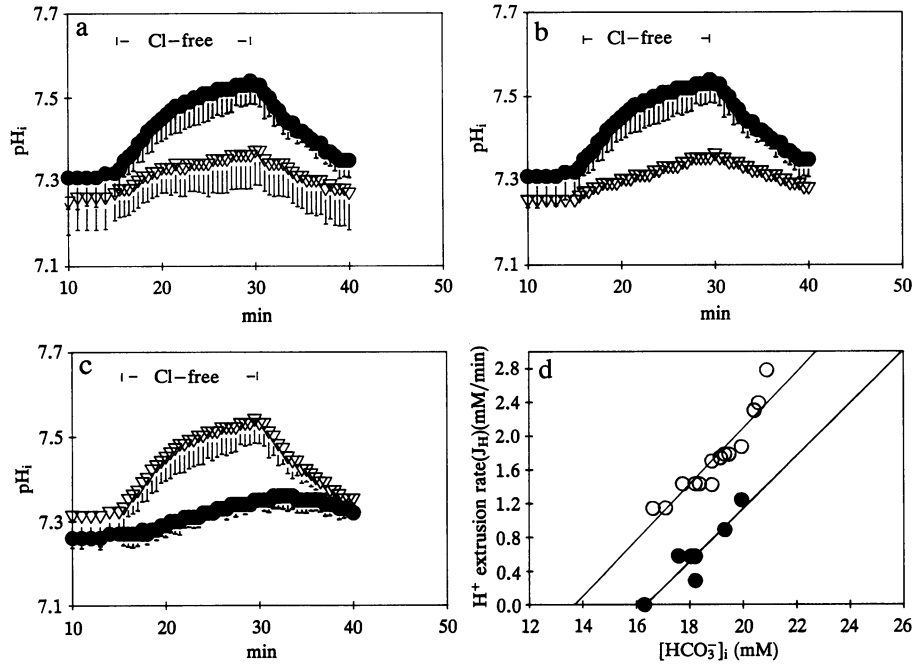

FIG. 1. (*a*) Effects of Cl^- removal in cells cultured in MEM + HCO_3^-/CO_2 medium (n = 8) (●) vs. cells cultured in L-15 medium (n = 6) (▽). (*b*) Effects of Cl^- removal in cells cultured in MEM + HCO_3^-/CO_2 (n = 8) (●) vs. cells cultured in MEM + Hepes (n = 5) (▽). (*c*) Effect of DIDS on cells cultured in the presence of HCO_3^-/CO_2. In four experiments, hepatocytes were preincubated with (●) or without (▽; control) 0.5 mM DIDS for 40–50 min (●). The same protocol was used as in *a*. (*d*) Correlation between H^+ fluxes (J_H) and $[HCO_3^-]_i$ in the two culture media. The intercept (H^+ flux) is significantly higher ($P < 0.01$) for hepatocytes cultured in the presence of HCO_3^-/CO_2 (●) vs. cells cultured in HCO_3^-/CO_2-free medium (○): $y = -0.45 \pm 0.054 + 0.033 \pm 0.0028x$ vs. $y' = -0.61 \pm 0.087 + 0.031 \pm 0.0048x'$.

readmission (this parameter is defined as $(\delta pH_i/\delta t)_{max}$ min^{-1}; the pH_i at which this rate was measured was obtained after 1 min of alkalinization or recovery) and (*ii*) converting these rates to maximum acid influx (J_H) or efflux rates (J_{OH}) by multiplying $(\delta pH_i/\delta t)_{max}$ and the calculated hepatocyte total buffering capacity {β_{tot} = intrinsic buffering capacity (β_i) + 2.303 × intracellular HCO_3^- concentration ($[HCO_3^-]_i$)} according to the following formula:

$$J_{H \text{ or } OH} = \beta_{tot} \times (\delta pH_i/\delta t)_{max}.$$

All experiments were performed in CO_2/HCO_3^- solutions. The standard HCO_3^- solution contained 140 mM Na^+, 5.9 mM K^+, 1.25 mM Ca^{2+}, 1.0 mM Mg^{2+}, 120.9 mM Cl^-, 25 mM HCO_3^-, 1.0 mM SO_4^{2-}, 1.2 mM phosphate, and 5 mM glucose and was equilibrated with 5% CO_2/95% O_2 at pH 7.4. In Cl^--free solutions, gluconate replaced Cl^-.

PNA Binding Studies. Immediately after isolation, hepatocytes were washed with 10 mM Hepes (pH 7.4) at 4°C. Cell suspensions (4 × 10^6 per ml) were incubated with PNA (50 μg/ml) for 45 min at 4°C, washed with 10 mM Hepes, and observed with a Nikon Microphot FX epifluorescence microscope equipped with Nomarski optics. Control experiments were performed in the absence of PNA or by preincubating hepatocytes with PNA and 200 mM D-galactose for 1 hr at room temperature to inhibit the lectin binding. Hepatocyte couplets labeled with PNA were plated on Matrigel-coated coverslips and incubated in L-15 medium at 37°C for 5–10 min before microscopic examination. Other PNA-labeled hepatocyte couplets were cultured for up to 4–6 hr either in MEM + HCO_3^-/CO_2 or in L-15. PNA linear fluorescent staining on the canalicular membrane was then determined in samples from six preparations.

Quantitative Immunofluorescence Study of the 110-kDa ecto-ATPase. A specific antibody against the 110-kDa putative bile acid transporter (8, 9) was used to localize this protein on hepatocyte couplets by immunofluorescence. Three culture conditions were used (L-15, MEM + Hepes, and MEM + HCO_3^-/CO_2). Hepatocyte couplets were fixed with 4% paraformaldehyde and 2% Triton X-100 and were labeled with the anti-100 kDa canalicular bile acid transporter antibody and rhodamine-conjugated anti-rabbit IgG (Tago). Preimmune rabbit serum was used as a control. Couplets with expanded canalicular spaces were selected randomly by bright-field light microscopy without knowledge of the culture conditions. The specimens were examined in a Bio-Rad MRC-600 confocal image system with an argon or krypton/argon laser. A line-scanning technique was used to assess changes in domain fluorescence.

Four sets of experiments were performed, each using 60–72 couplets from five or six isolations: (*i*) MEM + Hepes vs. MEM + HCO_3^-/CO_2 (five isolations), (*ii*) L-15 vs. MEM + HCO_3^-/CO_2 (six isolations), (*iii*) L-15 ± 0.1 mM dibutyryl cAMP (DBcAMP) + 0.5 mM isobutylmethylxanthine (IBMX) (five isolations), and (*iv*) MEM + HCO_3^-/CO_2 ± 10 μM colchicine (six isolations) or 10 μM β-lumicolchicine (two isolations).

cAMP Determination. Intracellular cAMP concentrations ($[cAMP]_i$) in hepatocytes cultured in MEM medium with or without HCO_3^-/CO_2 were determined by RIA (15). Eight preparations were cultured (8 × 10^5 cells per dish) for 4 hr in MEM + Hepes or in MEM + HCO_3^-/CO_2 containing 50 μM IBMX. The cells were washed rapidly three times with ice-cold phosphate-buffered saline, frozen in dry ice and methanol, and stored at −70°C until assayed for cAMP. The frozen cells were then thawed and extracted with 300 μl of 90% (vol/vol) isopropanol for 1 hr at 4°C. The extracts from six dishes were combined, dried under a stream of air, and reconstituted in assay buffer for cAMP measurement.

Statistical Analysis. Data are presented as means ± SDs. Statistical evaluations were conducted by using the paired or

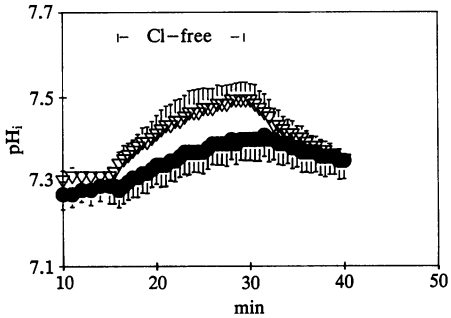

FIG. 2. Effects of pretreatment for 4–5 hr with 10 μM colchicine (n = 10) (●) vs. 10 μM β-lumicolchicine (n = 7) (▽) in hepatocytes cultured in MEM + HCO_3^-/CO_2 medium.

Table 1. Effects of DBcAMP on Cl^-/HCO_3^- exchange activity

Measurement	Experiment A: L-15 culture (absence of HCO_3^-/CO_2)			Experiment B: MEM + HCO_3^-/CO_2 culture	
	Control	+ DBcAMP	+ DBcAMP/+ DIDS	Control	+ DBcAMP
Basal pH_i	7.28 ± 0.011	7.24 ± 0.023	7.23 ± 0.014	7.32 ± 0.055	7.28 ± 0.010
Alkalinization rate, δpH per min	0.008 ± 0.003	0.029 ± 0.010	0.005 ± 0.005	0.023 ± 0.008	0.028 ± 0.009
H^+ extrusion rate (J_H), mM/min	0.45 ± 0.15	1.69 ± 0.55*	0.23 ± 0.29	1.42 ± 0.46	1.65 ± 0.51
Recovery rate, δpH per min	0.014 ± 0.008	0.031 ± 0.009	0.006 ± 0.002	0.038 ± 0.018	0.035 ± 0.008
OH^- extrusion rate (J_{OH}), mM/min	0.95 ± 0.59	2.11 ± 0.64†	0.19 ± 0.17	2.62 ± 1.26	2.56 ± 0.67

Experiment A: DBcAMP (0.1 mM) was added to L-15 medium 10 min before measurement of pH_i and Cl^-/HCO_3^- activity (n = 10) and compared with controls (n = 7) and with experiments in which cells were pretreated with 0.5 mM DIDS for 40–50 min prior to addition of DBcAMP (n = 4). Experiment B: In separate experiments, Cl^-/HCO_3^- activity was also measured when DBcAMP was added to MEM + HCO_3^-/CO_2 medium (n = 6) and compared with controls (n = 8). *, $P < 0.001$ vs. control; †, $P < 0.01$ vs. control.

unpaired Student t test as appropriate; $P < 0.05$ was considered statistically significant.

RESULTS

Effects of HCO_3^- on Cl^-/HCO_3^- Exchange Activity. To evaluate the effects of preincubation of hepatocytes in HCO_3^-/CO_2 on Cl^-/HCO_3^- exchange activity, cells were cultured in the presence of 25 mM $NaHCO_3$ equilibrated with 5% CO_2/95% O_2 (MEM + HCO_3^-/CO_2 medium) or in the nominal absence of HCO_3^- (MEM + Hepes or L-15 media), for 2–6 hr. All preparations were then equilibrated for 25 min in HCO_3^-/CO_2-containing KRB before Cl^- withdrawal.

In cells cultured in HCO_3^-/CO_2-containing medium, Cl^--dependent HCO_3^- fluxes after Cl^- removal and readmission were significantly higher than in cells from the same isolation that had been incubated in a nominally HCO_3^-/CO_2-free medium (MEM + Hepes or L-15) at the same extracellular pH [J_H = 1.42 ± 0.46 mM/min in MEM + HCO_3^-/CO_2 vs. 0.69 ± 0.16 mM/min in MEM + Hepes ($P < 0.01$) and vs. 0.53 ± 0.53 mM/min in L-15 ($P < 0.01$)] (Fig. 1 *a* and *b*). pH_i changes induced by Cl^- removal and readmission were strongly inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (Fig. 1*C*), consistent with carrier-mediated molecular coupling between Cl^- and HCO_3^- fluxes (6). Although baseline pH_i was slightly higher in hepatocytes cultured in the presence of HCO_3^- (7.31 ± 0.05 vs. 7.25 ± 0.01 in MEM + Hepes and 7.26 ± 0.06 in L-15 medium), the increased Cl^-/HCO_3^- exchange activity was not due to the known pH_i dependency of the anion exchanger. In fact, when alkalinization rates (J_H) during Cl^--removal were plotted against baseline pH_i (not shown) or against the calculated $[HCO_3^-]_i$ (Fig. 1*D*), the intercept of the curve was higher for hepatocytes cultured in the presence of HCO_3^-/CO_2. This indicates that, for any given concentration of intracellular HCO_3^-, Cl^-/HCO_3^- exchange activity was higher when cells were cultured in HCO_3^-/CO_2. In addition, the absence of changes in slope between the two curves suggests that the prior exposure to HCO_3^-/CO_2 most likely results in an increase in transporter number rather than a change in its affinity for HCO_3^-.

Differences in protein synthesis between the two culture conditions cannot explain these results because 50 μM cycloheximide did not significantly decrease Cl^-/HCO_3^- exchange activity in HCO_3^-/CO_2-cultured hepatocytes after 4–6 hours of preincubation. [J_H = 1.29 ± 0.23 mM/min in control hepatocytes vs. 1.15 ± 0.32 mM/min in the presence of cycloheximide (n = 6)].

Effects of Microfilament and Microtubule Inhibitors. Pretreatment of hepatocytes cultured in HCO_3^-/CO_2 with cytochalasin D (5 μg/ml; a microfilament inhibitor) for 2 hr had no effect on J_H [J_H = 1.65 ± 0.26 vs. 1.43 ± 0.37 mM/min in control hepatocytes (P = not significant)]. However, inhibition of microtubule polymerization with 10 μM colchicine for 4–5 hr reduced J_H to 0.38 ± 0.24 mM/min as compared with 1.70 ± 0.53 mM/min ($p < 0.001$) in hepatocytes pretreated with the inactive colchicine analog β-lumicolchicine (Fig. 2). In contrast, colchicine had no significant effect on Cl^--dependent ion fluxes in hepatocytes cultured in the absence of HCO_3^- [J_H = 0.80 ± 0.13 vs. 0.67 ± 0.43 mM/min (P = not significant)].

Effects of cAMP Analogs. Administration of DBcAMP to hepatocytes cultured in L-15 medium resulted in a DIDS-inhibitable increase in J_H when compared with controls (Table 1). This DBcAMP-stimulated Cl^-/HCO_3^- exchange activity was also inhibited by pretreatment with colchicine (Table 2). In addition, an analysis of the pH_i vs. activity plots showed significant differences in the intercept between DBcAMP-treated hepatocytes (−0.47 ± 0.058) and controls (−0.68 ± 0.038) ($P < 0.01$), similar to what had been observed previously in HCO_3^-/CO_2 cultured cells. Although not significant, their slopes were also different (0.040 ± 0.0052 vs. 0.031 ± 0.0046). DBcAMP did not stimulate Cl^-/HCO_3^- exchange activity further when cells were cultured in HCO_3^-/CO_2.

Effects of Phorbol 12,13-Dibutyrate (PDB). The C kinase agonist PDB, which inhibits DBcAMP-stimulated vesicular transport of horseradish peroxidase and bile flow in the isolated perfused rat liver (16, 17), also inhibited both HCO_3^- and DBcAMP-stimulated Cl^-/HCO_3^- exchange activity (Table 3), findings consistent with a counter-regulatory effect of protein kinase C.

Domain Localization of the Lectin PNA. In the rabbit renal cortical collecting duct, rhodamine-conjugated PNA binds to glycoproteins present in cell domains expressing an apical Cl^-/HCO_3^- exchanger (7). In isolated hepatocytes, rhodamine–PNA fluorescently labeled only remnants of the canalicular (apical) membrane domain immediately after isolation. Within 10 min, PNA fluorescence could be detected beneath the surface membrane of hepatocytes couplets as apical remnants underwent endocytosis. After 4–6 hr in

Table 2. Effects of colchicine on DBcAMP stimulation of Cl^-/HCO_3^- exchange activity

Measurement	DBcAMP + β-lumicolchicine	DBcAMP + colchicine
Basal pH_i	7.23 ± 0.023	7.24 ± 0.018
Alkalinization rate, δpH/min	0.023 ± 0.009	0.010 ± 0.006
H^+ extrusion rate (J_H), mM/min	1.37 ± 0.50	0.57 ± 0.35*
Recovery rate, δpH/min	0.030 ± 0.014	0.009 ± 0.004
OH^- extrusion rate (J_{OH}), mM/min	1.91 ± 0.89	0.58 ± 0.27*

Effect of pretreatment with 10 μM colchicine (n = 5) or β-lumicolchicine (n = 4) for 4–5 hr + 0.1 mM DBcAMP (same protocol as in Table 1) on Cl^-/HCO_3^- exchange activity in hepatocytes cultured in the absence of HCO_3^-/CO_2 (L-15 medium). *, $P < 0.02$.

Table 3. Effects of phorbol esters

Measurement	Experiment A: MEM + HCO_3^-/CO_2		Experiment B: L-15 (no HCO_3^-/CO_2)	
	PDD	PDB	DBcAMP	DBcAMP + PDB
Basal pH_i	7.29 ± 0.018	7.31 ± 0.038	7.23 ± 0.023	7.29 ± 0.016
Alkalinization rate, δpH/min	0.025 ± 0.004	0.002 ± 0.004	0.023 ± 0.009	0.005 ± 0.005
H^+ extrusion rate (J_H), mM/min	1.48 ± 0.25	0.13 ± 0.28*	1.37 ± 0.50	0.30 ± 0.34†
Recovery rate, δpH/min	0.021 ± 0.002	0.003 ± 0.004	0.030 ± 0.014	0.006 ± 0.002
OH^- extrusion rate (J_{OH}), mM/min	1.50 ± 0.23	0.21 ± 0.32*	1.91 ± 0.89	0.40 ± 0.15†

Experiment A: Effects of 30-min preincubation with PDB on Cl^-/HCO_3^- exchange activity in cells cultured in MEM + HCO_3^-/CO_2. These experiments were performed either in the presence of the active phorbol ester PDB (0.1 μM) (n = 5) or the inactive phorbol ester 4α-phorbol 12,13-didecanoate (PDD) (0.1 μM) in controls (n = 4). Experiment B: Effects of 0.1 mM DBcAMP and 0.1 μM PDB on Cl^-/HCO_3^- exchange activity in cells cultured in the absence of HCO_3^-/CO_2 (L-15 medium). Hepatocytes were pretreated for 30 min with PDB and for 10 min with DBcAMP. PDB and DBcAMP were then present throughout the experiment (n = 4). Control experiments (n = 4) were performed in the absence of DBcAMP. *, $P < 0.001$; †, $P < 0.02$.

culture, PNA fluorescence localized exclusively to the pericanalicular cytoplasm and the canalicular membrane.

PNA fluorescence staining was preferentially localized to the canalicular membrane area in 40 ± 13% of hepatocyte couplets cultured in the presence of HCO_3^-/CO_2 compared with 9 ± 7% of cells incubated in the absence of HCO_3^-/CO_2 (L-15 medium) ($P < 0.001$).

Domain Localization of the 110-kDa ecto-ATPase. Like PNA, the 110-kDa ecto-ATPase was also primarily localized to the apical region of hepatocyte couplets after 4 hr of culture irrespective of the medium (Fig. 3 *b* and *c*). However, when analyzed quantitatively, the clpm F°/CF° intensity ratios (see Fig. 3 legend) increased by an average of 29% in hepatocytes cultured in MEM + HCO_3^-/CO_2 compared with those cultured in L-15 medium and by an average of 30% in hepatocyte couplets cultured in MEM + HCO_3^-/CO_2 compared with those cultured in MEM + Hepes (Fig. 4). With 0.1 mM DBcAMP and 0.5 mM IBMX in L-15 medium, the clpm F°/CF° ratio increased by 46%. In contrast, the clpm F°/CF° ratios in hepatocytes declined with colchicine pretreatment in MEM + HCO_3^-/CO_2 medium, compared with control hepatocytes or hepatocytes cultured with β-lumicolchicine (Fig. 4).

$[cAMP]_i$ Levels. No differences were observed in $[cAMP]_i$ determined from eight isolations incubated in MEM + Hepes (3.4 ± 1.3 pmol per 10^6 cells) vs. MEM + HCO_3^-/CO_2 (3.8 ± 1.4 pmol per 10^6 cells).

DISCUSSION

Several significant findings concerning regulation of the activity of the apical Cl^-/HCO_3^- exchanger emerge from this study. (*i*) Cl^-/HCO_3^- exchange activity in isolated rat hepatocytes is stimulated by preincubation with HCO_3^-/CO_2 in the culture medium. (*ii*) Protein kinase A agonists up-regulate the exchanger in the absence of HCO_3^-/CO_2. (*iii*) Protein kinase C agonists counteract these increases in exchange activity. (*iv*) The up-regulation of Cl^-/HCO_3^- exchange activity appears to involve microtubule-dependent targeting of the exchanger either to or immediately adjacent to the apical membrane. In the present study, the changes in apical localization of PNA and the 110-kDa ecto-ATPase paralleled the changes observed in the activity of the apical Cl^-/HCO_3^- exchanger, suggesting that these phenomena are related.

Increased Cl^-/HCO_3^- exchange activity cannot be explained by an acute effect of HCO_3^- addition at a modifier site of the exchanger (5, 6). Rather, for any given concentration of intracellular HCO_3^-, the activity of the anion exchanger was higher if the cells had been chronically exposed to HCO_3^-/CO_2 in the culture medium. Since the Cl^-/HCO_3^- exchanger K_m for Cl^- is 5.1 mM (6), well below both intracellular and extracellular Cl^--concentrations, a change in Cl^- affinity would not have major influences on Cl^-/HCO_3^- exchange activity. Therefore, the data suggest that there must be more exchanger units available for transport under these conditions.

Microtubules are involved in vesicle-mediated secretion in a number of epithelia, including the liver (29). Since colchicine blocked HCO_3^-/CO_2-induced Cl^-/HCO_3^- exchange activity, we conclude that HCO_3^-/CO_2 increased targeting of the anion exchanger to the apical membrane. In freshly isolated hepatocytes placed in culture, retargeting of cana-

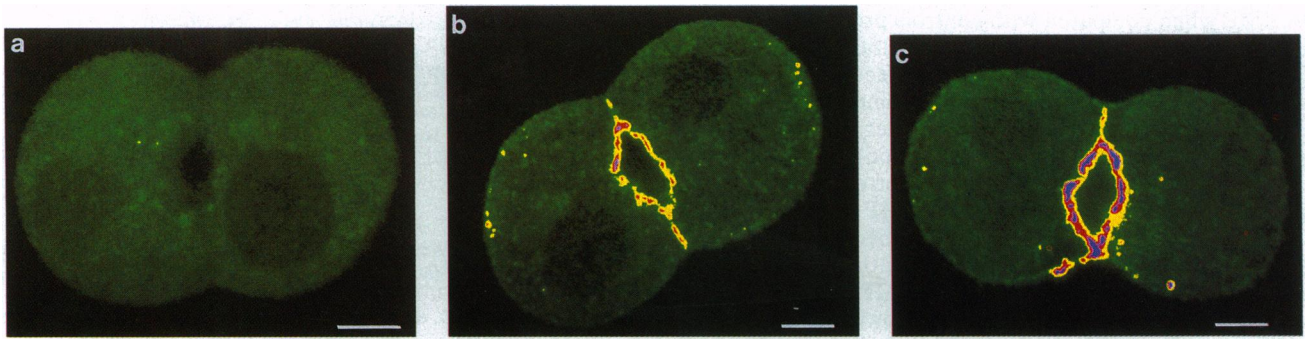

FIG. 3. Pseudo-color confocal images of hepatocyte couplets labeled with preimmune serum (*a*) and of hepatocyte couplets cultured in MEM + Hepes (*b*) or MEM + HCO_3^-/CO_2 (*c*) and labeled with anti-110-kDa ecto-ATPase antibody. The transporter was preferentially localized to the canalicular domain of the hepatocyte couplets (*b* and *c*). Green represents cytosolic fluorescence; yellow, red, and blue represent 3×, 3.8×, and 4.5× higher fluorescence than the average cytosolic fluorescence, respectively. (Bar = 10 μm.) Images of hepatocyte couplets were collected at the full-power laser beam, which was attenuated with a density filter (10% transmission). The aperture, black, and gain level were set at 4, 4.71, and 10 for all image acquisitions. The fluorescence intensity of each of these hepatocyte couplets was then quantitated by GBS analytical image-processing software (Infrascan, Richmond, BC, Canada). Three lines were randomly overlayed on the hepatocyte couplet transecting the bile canaliculus. The software generated a graphic display of pixel intensity along these lines. From the line profile, the peak fluorescence (F°) on the canalicular liver plasma membrane (clpm F°) and the average cytosolic fluorescence of the cell (CF°) could be obtained. The six clpm F°/CF° ratios from each couplet were expressed as a mean value.

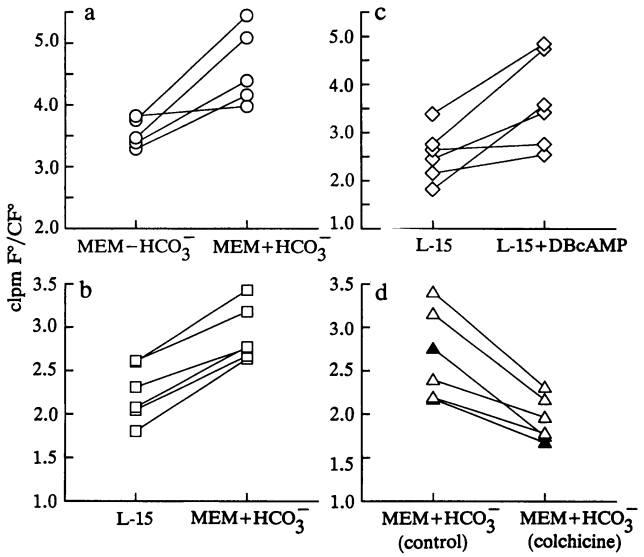

FIG. 4. The clpm F°/CF° ratios obtained from hepatocyte couplets cultured in L-15, L-15 containing DBcAMP and IBMX, MEM + Hepes, or MEM + HCO_3^-/CO_2 as in Fig. 3. (*a* and *b*) Hepatocyte couplets cultured in MEM + HCO_3^-/CO_2 increased clpm F° by 29–30% compared with hepatocytes cultured in L-15 medium ($P < 0.01$) (*b*) or MEM + Hepes ($P < 0.02$) (*a*). (*c*) When hepatocyte couplets were cultured in L-15 medium containing DBcAMP and IBMX, clpm F° increased 46% ($P < 0.05$). (*d*) Colchicine-treated hepatocyte couplets displayed a 27% decrease in clpm F° ($P < 0.01$) (▲; β-lumicolchicine vs. colchicine). Each data point equals the mean of 12 determinations obtained from six couplets from each of five or six separate isolations. A krypton/argon and an argon laser were used in the experiments in *a* and *c* and in *b* and *d* respectively.

licular membrane markers such as Mg^{2+}-ATPase (3, 10) is probably due to their lateral intramembranous dislocation, since redistribution of this enzyme requires intact microfilaments but not microtubules (10). Thus, the increase in Cl^-/HCO_3^- exchange activity induced by preincubation with HCO_3^-/CO_2 is unlikely to be explained by a general redistribution of apical membrane. Therefore, these data are most consistent with localization of the Cl^-/HCO_3^- exchanger in an intracellular compartment where the apical targeting is stimulated by HCO_3^- and is colchicine sensitive.

In the isolated perfused rat liver, cAMP stimulates microtubule-dependent vesicular transport and the biliary excretion of fluid-phase markers (18). In the present study, DBcAMP when compared with controls produced a DIDS-inhibitable increase in J_H only in cells cultured in a HCO_3^-/CO_2-free medium. Inhibition of this effect by colchicine, also suggests that DBcAMP acts by stimulating microtubule-dependent insertion of the transporter at the excretory domain. Activation (as well as inhibition) of Cl^-/HCO_3^- exchanger by DBcAMP has been reported in other cell types (19, 20) by modulating a cAMP-activated Cl^--channel. A Cl^--channel has been described in the canalicular membrane of hepatocytes (21), and although its cAMP-sensitivity is not known, part of the cAMP effect could be secondary to Cl^--channel activation. However, the inhibition by colchicine strongly supports the existence of a pool of Cl^-/HCO_3^- exchanger units that can be targeted to the apical membrane by stimuli acting through the cAMP cascade.

In our experiments, pH_i was slightly but consistently higher in hepatocytes cultured in HCO_3^-/CO_2-containing medium, probably because of the preponderance of HCO_3^--dependent acid extrusion mechanisms. A number of observations suggest that pH_i and $[cAMP]_i$ are interrelated in the liver (22–25). In the range of physiological pH_i, the activity of adenylate cyclase, is pH-dependent, increasing steeply for small changes in pH_i (26). The net effect would be an increase in $[cAMP]_i$ as pH_i increases. In livers from alkalotic and acidotic rats, $[cAMP]_i$ has been shown to be increased and decreased, respectively (24), thus suggesting a possible link between HCO_3^-/CO_2 and cAMP stimulation of the Cl^-/HCO_3^- exchanger. However, direct measurement of $[cAMP]_i$ in hepatocytes cultured with and without HCO_3^-/CO_2 failed to demonstrate significant differences.

The results of this study are also relevant for understanding biliary physiology in the intact liver, since bile acid-independent bile flow and biliary HCO_3^--excretion (27) can be increased by cAMP and by hormones acting through the cAMP cascade—phenomena that are also inhibited by colchicine (18, 27).

Recent studies in isolated perfused rat livers indicate that an acute intracellular alkalinization, produces a colchicine- and DIDS-inhibitable increase in bile flow and fluid phase marker (horseradish peroxidase) excretion and an increase in biliary HCO_3^- secretion (28). These findings in the intact liver also suggest that the canalicular Cl^-/HCO_3^- exchanger is regulated by rapid insertion of vesicles into the apical domain.

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1. Boyer, J. L., Graf, J. & Meier, P. J. (1992) *Annu. Rev. Physiol.* **54**, 415–438.
2. Meier, P. J. (1988) *Semin. Liver Dis.* **8**, 293–307.
3. Boyer, J. L., Gautam, A. & Graf, J. (1988) *Semin. Liver Dis.* **8**, 308–316.
4. Kamimoto, Y., Gatmaitan, Z., Hsu, J. & Arias, I. M. (1989) *J. Biol. Chem.* **264**, 11693–11698.
5. Meier, P. J., Knickelbein, R., Moseley, R. H., Dobbins, J. H. & Boyer, J. L. (1985) *J. Clin. Invest.* **75**, 1256–1263.
6. Benedetti, A., Strazzabosco, M., Corasanti, J. G., Haddad, P., Graf, J. & Boyer, J. L. (1991) *Am. J. Physiol.* **261**, G512–G522.
7. Satlin, L. M. & Schwartz, G. J. (1989) *J. Cell Biol.* **109**, 1279–1288.
8. Sippel, C. J., Ananthanarayanan, M. & Suchy, F. J. (1990) *Am. J. Physiol.* **258**, G728–G737.
9. Sippel, C. J., Suchy, F. J., Ananthanarayanan, M. & Perlmutter, D. H. (1993) *J. Biol. Chem.* **268**, 2083–2091.
10. Gautam, A., Ng, O. C. & Boyer, J. L. (1987) *Hepatology* **7**, 216–223.
11. Graf, J., Gautam, A. & Boyer, J. L. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6516–6520.
12. Thomas, J. A., Bucksbaum, R. N., Zimniak, A. & Racker, E. (1979) *Biochemistry* **81**, 2210–2218.
13. Rink, T. J., Tsien, R. Y. & Pozzan, T. (1982) *J. Cell Biol.* **95**, 189–196.
14. Ganz, M. B., Boyarsky, G., Boron, W. F. & Sterzel, R. B. (1988) *Am. J. Physiol.* **254**, F787–F794.
15. Goldberg, M. L. (1977) *Clin. Chem.* **23**, 576–580.
16. Hayakawa, T., Corasanti, J. G., Philips, K. A. & Boyer, J. L. (1989) *Hepatology* **10**, 598 (abstr.).
17. Corasanti, J. G., Smith, N. D., Gordon, E. R. & Boyer, J. L. (1989) *Hepatology* **10**, 8–13.
18. Hayakawa, T., Bruck, R., Ng, O. C. & Boyer, J. L. (1990) *Am. J. Physiol.* **259**, G727–G736.
19. Dunk, C. R., Brown, C. D. & Turnberg, L. A. (1989) *Pflügers Arch.* **414**, 701–705.
20. Reuss, L. (1987) *J. Gen. Physiol.* **90**, 173–196.
21. Sellinger, M., Weinman, S. A., Henderson, R. M., Zweifach, A., Boyer, J. L. & Graf, J. (1992) *Am. J. Physiol.* **262**, G1027–G1032.
22. Friedman, N. (1972) *Biochim. Biophys. Acta* **274**, 214–225.
23. Reynolds, R. C. & Haugaard, N. (1967) *J. Pharmacol. Exp. Ther.* **156**, 417–425.
24. Yajima, M. & Ui, M. (1975) *Am. J. Physiol.* **228**, 1053–1059.
25. Busa, W. B. & Nuccitelli, R. (1984) *Am. J. Physiol.* **246**, R409–R438.
26. Roos, A. & Boron, W. F. (1981) *Physiol. Rev.* **61**, 296–433.
27. Lenzen, R., Hruby, V. J. & Tavoloni, N. (1990) *Am. J. Physiol.* **259**, G736–G745.
28. Bruck, R., Benedetti, A., Strazzabosco, M. & Boyer, J. L. (1993) *Am. J. Physiol.* **265**, G347–G353.
29. Sakisaka, S., Ng, O. C. & Boyer, J. L. (1988) *Gastroenterology* **95**, 793–804.