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Measurement of intracellular chloride activity in mouse liver slices with microelectrodes

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Steady-state membrane potential (V_m) and intracellular Cl^- activity (a^i_{Cl}) were measured with double-barreled Cl^--selective microelectrodes in mouse liver slices. In bathing solutions (33.8°C) containing pyruvate, glutamate, fumarate, and glucose, V_m and a^i_{Cl} were -27.6 ± 1.0 mV and 32.6 ± 1.5 mM, respectively. This apparent value of a^i_{Cl} exceeded the level required for passive distribution of this ion ($a^{eq}_{Cl} = 26.4 \pm 1.3$ mM) by 6.2 ± 1.0 mM. This difference was essentially unchanged in experiments where (i) Na^+ was replaced by choline, (ii) HCO_3^- was removed, and (iii) Cl^- was replaced by gluconate. These data argue against the presence of Na^+- or HCO_3^--coupled Cl^- transport mechanisms in the plasma membrane of mouse liver cells. This implies that a^i_{Cl} is in fact at equilibrium and interference with the response of Cl^--selective microelectrodes by intracellular anions is responsible for the apparent difference between a^i_{Cl} and a^{eq}_{Cl}. We found that Cl^--selective microelectrodes containing Corning 477315 ligand are sensitive to taurocholate, a representative bile salt. Their selectivity to taurocholate is about 60-times their selectivity towards Cl^-. This suggests that interference of bile acids at concentrations normally present in hepatocytes with determinations of a^i_{Cl} can account for the apparent difference $a^i_{Cl} - a^{eq}_{Cl}$.

Introduction

In many epithelia the steady state intracellular chloride activity (a^i_{Cl}) significantly exceeds the "equilibrium" level (a^{eq}_{Cl}) predicted by the Nernst equation [1]. This implies the presence of active Cl^- transport mechanisms in the cell membrane that utilize either metabolic energy or the electrochemical gradient of some other ionic species. Two such mechanisms have been well characterized in epithelia: an electrically neutral Na^+-Cl^- cotransporter and an electrically silent Cl^-/HCO_3^- exchanger [2]. Each of these transporters generates an inward flux of Cl^-. Attempts to directly demonstrate Na^+-Cl^- cotransport in liver, which is classified as a leaky epithelium [3], have yielded inconsistent results. If Na^+-Cl^- cotransport were present, removal of Na^+ from the medium should result in a decline in intracellular chloride activity towards its equilibrium value. Similarly, removal of Cl^- should decrease Na^+ influx. Scharschmidt et al. [4] showed that $^{22}Na^+$ entry into cultured hepatocytes was reduced if Cl^- was replaced by benzenesulfonate, cyclamate, or sulfate plus mannitol. However, replacement of Cl^- by NO_3^-, gluconate, or thiocyanate caused no reduction in sodium entry. Replacement of Na^+ with choline resulted in a 17% decrease in $^{36}Cl^-$ entry, but replacement with Li^+ caused no significant change. In a recent review, Scharschmidt and Van Dyke [5] summarized current evidence in

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favor of Cl^--HCO_3^- exchange in hepatocytes but questioned its physiological role, particularly with respect to Cl^--transport across the cell membrane.

The question whether or not the intracellular chloride activity is above equilibrium has not been adequately resolved. Three groups of workers have measured the chemical Cl^- content of liver and have compared this value to that calculated from the Nernst equation using companion microelectrode measurements of membrane potential. Williams et al. [6,7] found intracellular Cl^- concentration in intact tissue to be slightly lower than its equilibrium value; Claret and Mazet [8] found values in perfused liver consistent with passive distribution; and Heller and Van Der Kloot [9] reported guinea pig liver chloride concentrations to be far in excess of the calculated values for solely passive distribution. Other studies have utilized $^{36}Cl^-$ to measure exchangeable Cl^- concentration and have compared this quantity to equilibrium values calculated using membrane potentials from the literature. Using cultured hepatocytes Smock et al. [10] and Scharschmidt et al. [4] found Cl^- to be near equilibrium. Direct measurements of intracellular ionic activities with Cl^--selective microelectrodes indicated that in mouse liver slices [11] and in intact rat liver cells [12] the steady-state chloride activity is about 1.2 to 1.3 times the value that corresponds to passive distribution of this ion across the cell membrane. However, preliminary data from this laboratory [13] indicate that this ratio may be due to interference by intracellular anions with the response to Cl^- ions of these microelectrodes. These studies are reported in extenso in the present paper.

Materials and Methods

Male mice (ICR, Harlan) weighing 12–15 g were fasted overnight and killed by a blow on the head. The abdominal cavity was opened and a portion of liver was excised and washed free of blood with the appropriate bathing solution (see below). A thin slice (about 1 mm in thickness) was cut with a razor blade and was placed in an experimental chamber with the undamaged, capsular surface facing upwards. Tissue slices were held stationary by the weight of a small steel washer.

Six different bathing solutions were used during the course of these studies, permitting measurements of membrane potential and intracellular chloride activity in the presence and in the absence of Na^+, HCO_3^-, and Cl^-. The compositions of these solutions are given in Table I. Solutions A, B, and D were adjusted to pH 7.4 with 0.1 M HCl and were bubbled with 100% O_2. Solutions C, E, and F were bubbled with 95% O_2/5% CO_2 and were found to have pH values of 7.4 ± 0.02. In solutions E and F (see Table I) 28.9 mM $NaHCO_3$ was added rather than 25 mM in order to neutralize the excess acidity of the organic acids. Bathing solutions were warmed by gravity flow through Graham condensers in which temperature-regulated water was circulated by a heater pump (FE-2, Haake). Solutions were switched by operation of individual valves located between the condensers and the experimental chamber.

The experimental chamber consisted of a plexiglass block ($9.5 \times 5.2 \times 1.8$ cm) in which three wells were machined. An attached glass plate constituted the bottom of these wells. Bathing solutions first entered a conical well to permit release of gas bubbles. Flow into and out of the central well (diameter 2 cm), which contained the tissue slice, occurred below the surface of the solution, minimizing surface tension related variations in flow. Fluid was removed from the third well by continuous suction. Temperature-regulated water from the heater pump was circulated beneath the glass plate of the chamber to reduce the dependence of the chamber temperature on flow rate. The temperature of the central well was recorded with a thermister probe which touched the bottom of the well. The measured temperature at this point was maintained at 36.8 ± 0.5° C (mean ± S.D., $N = 31$) by adjustments of the temperature of the heater pump and the flow rate of the bathing solutions (typically 5 ml/min). Due to evaporative cooling at the solution surface and heating from below there was a temperature gradient in the central well. Measurement of local temperatures at various positions in the chamber indicated a difference of about 3C° between the bottom of the well and the top surface of the slices. We, therefore, estimate that the impaled cells on the upper surface of the liver slices had a mean temperature of 33.8° C. Slices were perfused

TABLE I

COMPOSITION OF BATHING SOLUTIONS

Salt	Concentration (mM) in solution					
	A	B	C	D	E	F
NaCl	112.42	–	100.00	100.00	113.57	–
KCl	–	–	4.70	4.70	–	–
$CaCl_2$	–	–	2.56	2.56	–	–
$MgCl_2$	–	–	1.30	1.30	–	–
NaH_2PO_4	–	–	1.15	1.15	–	–
Sodium pyruvate	–	–	4.90	4.90	–	–
Sodium glutamate	–	–	4.90	4.90	–	–
Sodium fumarate	–	–	2.70	2.70	–	–
$NaHCO_3$	–	–	25.00	–	25.00 [a]	25.00 [a]
Glucose	2.80	2.80	2.80	2.80	2.80	2.80
Imidazole	5.00	5.00	–	5.00	–	–
Sodium gluconate	20.00	–	–	20.00	–	113.57
KOH	3.55	3.55	–	–	3.55	3.55
$Ca(OH)_2$	2.56	2.56	–	–	2.56	2.56
$Mg(OH)_2$	1.30	1.30	–	–	1.30	1.30
KH_2PO_4	1.15	1.15	–	–	–	–
Pyruvic acid	4.90	4.90	–	–	4.90	4.90
Glutamic acid	4.90	4.90	–	–	4.90	4.90
Fumaric acid	2.70	2.70	–	–	2.70	2.70
Choline chloride	–	112.42	–	–	–	–
Mannitol	–	40.00	–	–	–	–

[a] These solutions contained 25 mM $NaHCO_3$. An additional 3.93 mM $NaHCO_3$ was added to neutralize the acidity of the organic acids.

with a control medium (Solution A, C or E) for a minimum of 20 min before impalements were performed.

Microelectrodes were advanced perpendicularly to the tissue using a hydraulic micromanipulator (MO-10, Narishige). One some occasions a 2–4 μm advance or withdrawal of the microelectrode was necessary following impalement to obtain a stable intracellular potential. Microelectrodes were repositioned horizontally after each impalement to ensure that no two measurements were from the same cell. The criteria for valid cell impalements with microelectrodes were as described elsewhere [14]. These were as follows: (i) A sharp deflection of the microelectrode potential on penetration of a cell, (ii) attainment and maintenance of a steady-state intracellular potential value, (iii) constancy (or virtual constancy) of the microelectrode resistance following impalement, and (iv) a prompt return of the microelectrode tip potential to its initial value following withdrawal from a cell to the bathing solution.

Cl^--Selective microelectrodes

Borosilicate glass capillary tubing (GCF-(2)-150-4; A-M Systems, Inc.) was soaked in 50% HNO_3 overnight, rinsed, dried at 200 °C, and stored over silica gel. Double-barreled microelectrodes were drawn in a vertical puller (Model 700C, D. Kopf instruments) adjusted to produce tip diameters of about 1 μm. The tip of one barrel was silanized by back filling with a droplet of 6.7% (v/v) tri-*n*-butylchlorosilane in 1-chloronaphthalene (Pfaltz and Bauer) and by baking at 240 °C for 45 min. A droplet of liquid ion-exchanger (Corning 477315) was drawn into the tip of a filling pipette by syringe suction, the filling pipette was advanced inside the silanized barrel to near the tip, and the ion-exchanger was expelled slowly, filling the tip of the ion-selective barrel. The remainder of this barrel was then filled with 0.5M KCl. Using a similar technique the open tip barrel was filled with 1 M sodium formate.

Electrodes were calibrated at room temperature in solutions containing 100, 50, 20, 10, 5, 1, and

nominally 0 mM KCl plus constant amounts of interfering anions. Cl^- activities in the calibrating solutions were estimated from Guggenheim's parametric form of the extended Debye-Huckel equation (see Ref. 17, Eqn. 9.13) using constants appropriate for NaCl for 25 °C ($A = 0.512$, $b = 0.16$) and assuming that the activity coefficient of Cl^- was equal to the mean activity coefficient of the solution. Two different sets of calibrating solutions were used with interfering anions in the same concentrations as in the bathing solutions. These contained: (I) 5 mM imidazole, 4.9 mM sodium pyruvate, 4.9 mM sodium glutamate, and 2.7 mM sodium fumarate, and (II) 25 mM $NaHCO_3$, 4.9 mM sodium pyruvate, 4.9 mM sodium glutamate, and 2.7 mM sodium fumarate in addition to the varying amounts of KCl. Data obtained during perfusion with bathing solutions A, B and D were evaluated using the curve from calibrating solutions set I. Data obtained with bathing solutions C, E and F were evaluated using the curve from calibrating solutions set II.

Calibration curves obtained in the presence of interfering anions were fitted with a form of the Nicolsky-Eisenman equation [15]

$$E = E^0 + (RT/z_i F) \ln\left(a_i + \sum_j K_{ij}^{\mathrm{Pot}} a_j^{(z_i/z_j)}\right) \quad (1)$$

where E is the potential of the ion-selective electrode, E^0 is a reference potential, a_j is the activity of ions of type j and K_{ij}^{Pot} is a selectivity coefficient of the electrode for type j ions relative to type i. Other variables have their usual meanings. Making the substitutions

$$S = (RT/z_i F) \ln(10)$$

$$\mathrm{DL} = \sum_j K_{ij}^{\mathrm{Pot}} a_j^{(z_i/z_j)}$$

and

$$B = (E^0 - E^{\mathrm{B}})$$

where E^{B} is the potential measured in the appropriate bathing solution, we obtain

$$(E - E^{\mathrm{B}}) = B + S \log(a_{\mathrm{Cl}} + \mathrm{DL}) \quad (2)$$

In our procedure we used B, S, and DL as adjustable parameters and obtained a least-squares fit of this equation to $(E - E^{\mathrm{B}})$ versus a_{Cl} calibration data by a Newton-Raphson algorithm. The best fit values of DL and S will be referred to as the detection limit [16] and slope, respectively, of the electrode. The value of DL obtained in this way estimates the total interference in the calibrating solutions. If, as we assume, the activities of interfering anions within the cell are less than in the bathing solution the true value of $a_{\mathrm{Cl}}^{\mathrm{i}}$ lies between a_{Cl} and $a_{\mathrm{Cl}} + \mathrm{DL}$, where a_{Cl} is the value read from the calibration curve. Thus, the intracellular chloride activities calculated from our calibrations should be slight underestimates of the true $a_{\mathrm{Cl}}^{\mathrm{i}}$.

To compare the magnitude of interference due to different anions, a few electrodes were also calibrated in KCl solutions containing either 25 mM HCO_3^- but no organic anions, 200 μM sodium taurocholate, or 1 mM sodium taurocholate. In addition, some electrodes were calibrated in solutions of 1, 5, 10, 20, 50 and 100 mM KCl alone and three electrodes were calibrated in solutions of 0.01, 0.1, 1.0 and 10 mM sodium taurocholate. Calibration curves in KCl or sodium taurocholate alone were fitted with the Nernst equation. For sodium taurocholate solutions the activity coefficient was assumed to be unity.

Since calibrations were done at room temperature and experiments were performed near 37 °C it was necessary to compensate for temperature dependence of the electrode response. Errors due to temperature dependence of S and DL were estimated to be less than 2 mM and were ignored. However, changes in E^0 are a potential source of error since it includes factors such as junction potentials which show a less predictable temperature dependence. We compensated for these changes by referencing measured potentials of the ion-selective electrode to the corresponding potential in the bathing solution at that temperature. In other words, changes in E^0 with temperature were assumed to parallel changes in E^{B}, therefore, $B = (E^0 - E^{\mathrm{B}})$ was assumed to be the same at the two temperatures.

The potential of the Cl^- electrode was corrected for the membrane potential by subtracting the membrane potential, V_{m} from the $(E - E^{\mathrm{B}})$ value observed. $a_{\mathrm{Cl}}^{\mathrm{eq}}$ was calculated from the Nernst

equation using V_m and the measured Cl^- activity in the bathing solution. The value of Cl^- activity in each bathing solution was obtained experimentally as the x-intercept of the calibration curve referenced to that solution, that is, the value of a_{Cl} for which $(E - E^B) = 0$. This value varied slightly with each experiment and had a mean of 75.5 ± 0.8 mM ($N = 61$).

In some experiments single-barreled open-tip microelectrodes were used to measure the membrane potential. These were drawn from 'Kwik-Fil' borosilicate glass capillary tubing (1.2 mm o.d.; 0.8 mm i.d.; W.P. Instruments, New Haven, CT) in the same vertical puller used for double-barreled microelectrodes and were back filled with 0.5 M KCl. These electrodes had resistances of about 40 MΩ when their tips were immersed in bathing media.

Electrical circuitry

Micropipette solutions were connected by Ag | AgCl electrodes and guarded coaxial cables to high input impedance ($> 10^{15}\Omega$) FET-input electrometers with unit gain (Analog Devices 515L). Potentials were recorded with respect to a reference electrode consisting of a calomel half-cell connected to the tissue bath by a 3 M KCl/agar bridge. The resistance of the open tip barrel was monitored continuously throughout the experiments by passing a current pulse (0.5 nA, 700 ms duration, 6.7 s period). Voltages were displayed on digital meters and recorded on a two channel strip-chart recorder (Gould Mark 2200).

Measured changes in potential following alterations in the composition of the bathing solution were corrected for liquid junction potentials arising at the tip of the agar bridge. Junction potentials were measured according to the method of Garcia-Diaz et al. [18]. Complete substitution of chloride by gluconate (Solution E to Solution F) generated junction potentials of about -6.0 mV and substitution of bicarbonate by gluconate plus imidazole (Solution C to Solution D) generated junction potentials of -0.3 mV. Repeated measurements of these junction potentials gave values that were constant within ± 0.2 mV.

Chemical estimation of chloride in liver slices

Chemical chloride concentrations were measured as follows. Liver slices weighing between 100 and 200 mg were superfused with either a Cl^--containing medium (Solution E) for 30 min or a Cl^--free medium (Solution F) for 75 min. The tissues were then gently blotted on filter paper and weighed. Total tissue water was calculated by subtracting the weight of the tissue after drying at 105 °C for 24 h. The total chloride content of the samples was determined by conductometric titration following alkaline digestion of the dried residue, deproteinization, and acidification as described by Cotlove [19]. For calculation of intracellular chloride concentration, c^i_{Cl}, the extracellular space was assumed to be 18% [20].

The results are expressed as the mean value ± S.E. Students's 't'-test was applied to test the significance of differences between mean values.

Results

Calibration curves of a representative electrode in various calibrating solutions are shown in Fig. 1. In KCl solutions without interfering anions (top curve) the electrode gave a virtually linear response between 1 and 100 mM with a slope of -52.7 mV/decade change in Cl^- activity. Nine electrodes calibrated under similar conditions had an average slope of -58.3 ± 0.5 mV. Significant deviations from linearity occurred when KCl solutions contained imidazole and organic anions (middle curve of Fig. 1, calibrating solutions set I) or HCO_3^- and organic anions (bottom curve of Fig. 1, calibrating solutions set II). Fifty six electrodes calibrated in calibrating solutions set I had an average slope (S) of -59.2 ± 1.1 mV and a detection limit (DL, Eqn. 2) of 7.3 ± 0.7 mM Cl^-. Fifteen electrodes calibrated with calibrating solutions set II had a mean S of -61.6 ± 0.9 mV and a DL of 9.0 ± 0.4 mM Cl^-. Eleven electrodes calibrated in KCl solutions containing 25 mM HCO_3^- but no organic anions (data not shown in Fig. 1) had a mean S of -61.6 ± 1.5 mV and a DL of 3.8 ± 0.6 mM Cl^-. This DL was significantly ($P < 0.05$) less than that obtained in the presence of HCO_3^- plus pyruvate, glutamate and fumarate.

Results from 20 animals (104 impalements) in which simultaneous measurements of V_m and a^i_{Cl} were made with double-barreled Cl^--selective mi-

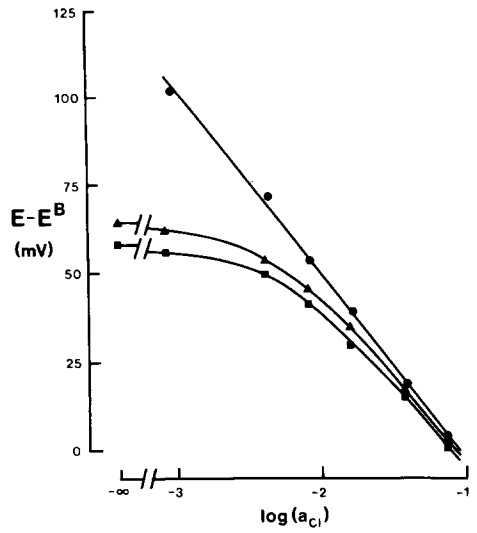

Fig. 1. Calibration of a double-barreled Cl^--selective microelectrode in KCl solutions with and without interfering anions. Top curve: calibration in 1, 5, 10, 20, 50 and 100 mM KCl. Middle curve: calibration in KCl solutions containing 5 mM imidazole, 4.9 mM sodium pyruvate, 4.9 mM sodium glutamate and 2.7 mM sodium fumarate (calibrating solutions set I). Bottom curve: calibration in KCl solutions containing 25 mM $NaHCO_3$, 4.9 mM sodium pyruvate, 4.9 mM sodium glutamate and 2.7 mM sodium fumarate (calibrating solutions set II). The points plotted at '$-\infty$' in the latter two curves represent the potentials recorded by the electrode in nominally Cl^--free solutions containing the other anions listed above.

croelectrodes in liver slices bathed in Solution C (Table I) were -27.6 ± 1.0 mV and 32.6 ± 1.5 mM, respectively. Paired individual comparisons of a^i_{Cl} and a^{eq}_{Cl} (mean value 26.4 ± 1.0 mM) yielded an average difference of 6.2 ± 1.0 mM which is significantly greater than zero ($P < 0.001$). The observation that a^i_{Cl} was significantly greater than a^{eq}_{Cl} suggested the possible presence of active accumulative mechanisms in the plasma membrane of liver cells. These mechanisms might include Na^+-Cl^- symport or Cl^-/HCO_3^- antiport systems similar to those demonstrated in other epithelia [2]. On the other hand, the relatively small magnitude of $(a^i_{Cl} - a^{eq}_{Cl})$ suggested that this apparent difference might be due to uncompensated interference with Cl^--selective electrodes by intracellular anions. To distinguish between these alternative interpretations, experiments were performed as follows to test each hypothesis individually: (i) Na^+ was removed from the external medium to eliminate the contribution of Na^+-Cl^- flux coupling to intracellular chloride activity; (ii) HCO_3^- was removed to evaluate the contribution of Cl^-/HCO_3^- antiport to $(a^i_{Cl} - a^{eq}_{Cl})$; and, (iii) the interference hypothesis was tested by measurement of a^i_{Cl} under conditions of Cl^- depletion, verified by parallel chemical determinations of Cl^- concentrations.

To investigate the involvement of a Na^+-Cl^--coupled transport system in liver, a^i_{Cl} was measured in the presence and complete absence of Na^+ (with choline$^+$ replacing Na^+). During stable impalements in Solution A (Table I), the bathing medium was switched to Solution B. The results from these experiments are summarized in Table II. Complete removal of Na^+ from the external medium resulted in small changes in both V_m and steady state a^i_{Cl}. However, the average difference between paired a^i_{Cl} and a^{eq}_{Cl} values (11.0 ± 1.7 mM) remained significantly greater than zero ($P < 0.01$, $N = 8$) and was not significantly different ($P > 0.05$) from its value in the presence of Na^+ (7.6 ± 2.1 mM).

To test for the presence of a Cl^-/HCO_3^- antiport mechanism in liver cells, a^i_{Cl} was measured in the presence and in the absence of bicarbonate. One such experiment is depicted in Fig. 2. Upon impalement in Solution C (Table I) the open-tip barrel registered a V_m of -25.1 mV (lower trace) and the ion-selective barrel (upper trace) gave a deflection (V_{Cl}) of -5.7 mV. These values remained stable for several min following which the bathing medium was switched to Solution D (Table I). V_m depolarized to -17.6 mV ($\Delta V_m = 7.5$ mV) within a few minutes. During this time there

TABLE II

EFFECT OF Na^+ REMOVAL ON a^i_{Cl} IN MOUSE LIVER SLICES

Values are means ± S.E. with respect to N (number of animals); n = total number of impalements. $N = 8$; $n = 18$. n.s., not significant.

Bathing Solution (Table I)	V_m (−mV)	a^i_{Cl} (mM)	a^{eq}_{Cl} (mM)	$(a^i_{Cl} - a^{eq}_{Cl})$ (mM)
A	21.3±1.7	41.3±4.0	33.6±2.6	7.6±2.1
B	19.1±1.3	45.7±2.8	34.6±2.8	11.0±1.7
Δ	2.2±0.9	4.4±1.8	1.0±1.4	3.4±2.4
P (paired)	< 0.05	< 0.05	n.s.	n.s.

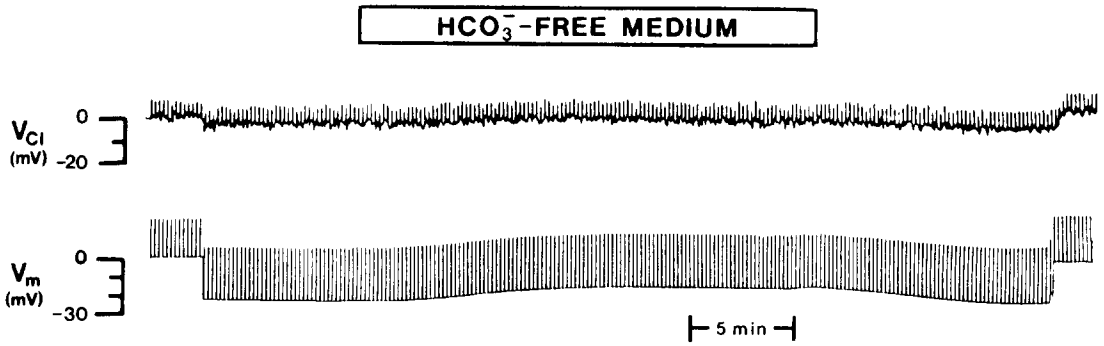

Fig. 2. Record of an impalement with a double-barreled Cl^--selective microelectrode in a liver slice perfused in the presence and in the absence of external HCO_3^-. See text for details.

was a small change in V_{Cl} to -6.4 mV at maximum depolarization. This represents a change in $(V_{Cl} - V_m)$ from 19.4 mV in HCO_3^- medium to 11.2 mV in HCO_3^--free medium, corresponding to a change in a^i_{Cl} from 36.9 to 50.8 mM. On reintroducing HCO_3^- (at the end of the bar) both V_m and a^i_{Cl} returned to values (-26.4 mV and 41.1 mM, respectively) that were close to their steady-state values in the presence of HCO_3^-. Data from several such impalements are tabulated in Table III. These results show that removal of HCO_3^- caused a significant depolarization of V_m($\Delta V_m = 8.0 \pm 0.8$ mV, $P < 0.001$, $N = 5$) with a simultaneous increase in a^i_{Cl}($\Delta a^i_{Cl} = 12.8 \pm 1.8$, $P < 0.01$, $N = 5$). However, the difference between a^i_{Cl} and a^{eq}_{Cl} was essentially unchanged in the presence and absence of HCO_3^-.

To test for uncompensated microelectrode interference due to intracellular anions, the apparent a^i_{Cl} was measured in the absence of Cl^-. Fig. 3 shows the voltage record of an impalement in which Cl^- was replaced by gluconate. Upon impalement in Solution E (Table I), V_m stabilized (upper trace) at -33.6 mV and the difference between the two barrels recorded by a differential amplifier ($V_{Cl} - V_m = -27.5$ mV) corresponded to an a^i_{Cl} of 20.5 mM. Within 8 min after switching to Solution F (at the arrow in Fig. 3), V_m depolarized to -18.1 mV ($\Delta V_m = 15.5$ mV). Thereafter, a slow tendency toward repolarization was observed. Simultaneous with the depolarization, $(V_{Cl} - V_m)$ increased to 44.0 mV, which corresponded to an a^i_{Cl} of 6.1 mM. Fig. 3 was obtained directly from the strip-chart recorder, without correction for the junction potential at the 3 M KCl/agar bridge. Thus V_m appears more

TABLE III

EFFECT OF HCO_3^- REMOVAL ON a^i_{Cl} IN MOUSE LIVER SLICES

Values are means ± S.E. with respect to N (number of animals); $N = 5$, $n = 11$. n.s., not significant.

Bathing Solution (Table I)	V_m (−mV)	a^i_{Cl} (mM)	a^{eq}_{Cl} (mM)	$(a^i_{Cl} - a^{eq}_{Cl})$ (mM)
C	24.3 ± 2.3	39.3 ± 2.4	33.0 ± 2.5	6.3 ± 1.8
D	16.3 ± 1.5	52.1 ± 4.1	43.4 ± 3.5	8.7 ± 2.6
Δ	8.0 ± 0.8	12.8 ± 1.8	10.4 ± 1.3	1.6 ± 1.9
P (paired)	< 0.001	< 0.01	< 0.005	n.s

TABLE IV

EFFECT OF Cl^- REMOVAL ON a^i_{Cl} IN MOUSE LIVER SLICES

Chloride was removed from the bathing solution by switching from Solution E to Solution F (Table I). Values are means ± S.E. with respect to N (number of animals); n = total number of impalements. $N = 3$; $n = 3$. V_m values are corrected for junction potentials.

Time after chloride removal (min)	V_m (−mV)	a^i_{Cl} (mM)
0	28.6 ± 4.0	31.0 ± 5.3
15	11.6 ± 2.4 [a]	10.9 ± 1.2 [a]
60	12.0 ± 2.7 [a]	8.7 ± 1.7 [a]
75	14.2 ± 1.7 [a]	7.8 ± 1.7 [a]

[a] $P < 0.01$ (paired) with respect to value at time zero.

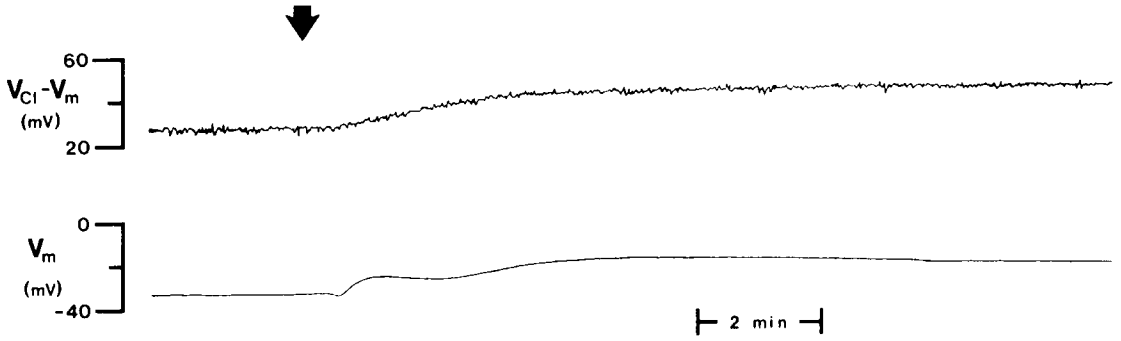

Fig. 3. Record of an impalement with a double-barreled Cl^--selective microelectrode in a liver slice perfused in the presence and in the absence of external chloride. At the arrow Solution E was switched to Solution F. See text for details.

negative than its actual value during Cl^- removal. Following correction, V_m at maximum depolarization in Solution F (Table 1) was -12.2 mV ($\Delta V_m = 21.4$ mV). Data from three such impalements in which changes in V_m and a^i_{Cl} were followed for 75 min are summarized in Table IV. In one other experiment in which the tissue was kept in Cl^--free solution for 180 min, the mean value of the residual apparent a^i_{Cl} was 6.9 ± 0.4 mM (data not included in Table IV).

In a parallel set of experiments, the Cl^- content of liver slices was estimated by conductometric titration. In four slices bathed in Solution E (Table I) for 30 min the intracellular chloride concentration (c^i_{Cl}) was found to be 76.2 ± 5.1 mmol/l of intracellular water. In six slices bathed in Solution F (Table I) for 75 min, c^i_{Cl} was 2.2 ± 1.0 mmol/l.

Because bile salts are concentrated in liver cells [21] and organic anions are known to interfere with Corning 477315 liquid ion-exchanger electrode measurements of Cl^- [22,23], we tested the sensitivity of Cl^--selective microelectrodes to sodium taurocholate (Sigma), a representative bile salt. In Fig. 4, the calibration curve of a representative Cl^--selective microelectrode in sodium taurocholate solutions is shown. This electrode gave a virtually linear response to sodium taurocholate concentrations between 0.01 and 10 mM (slope = -55.2 mV/decade change in sodium taurocholate concentration). Fig. 5 shows the calibration curves of the same electrode in varying concentrations of KCl without sodium taurocholate (top curve), in KCl solutions containing 200 μM sodium taurocholate (middle curve), and in KCl solutions containing 1 mM sodium taurocholate (lower curve). The selectivity coefficient of sodium taurocholate over Cl^- for these electrodes (estimated from the Nicolsky-Eisenman equation) was 60 ± 17 ($N = 3$).

Discussion

Cl^--selective microelectrodes have been used successfully in a variety of cells for the direct measurement of a^i_{Cl} [1]. However, it has recently been shown that several organic and inorganic

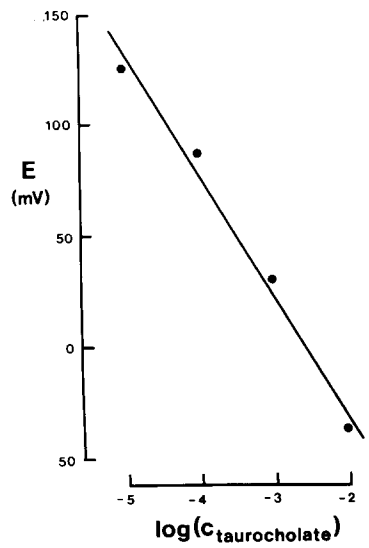

Fig. 4. Calibration of a double-barreled Cl^--selective microelectrode, in 0.01, 0.1, 1.0 and 10 mM sodium taurocholate concentrations.

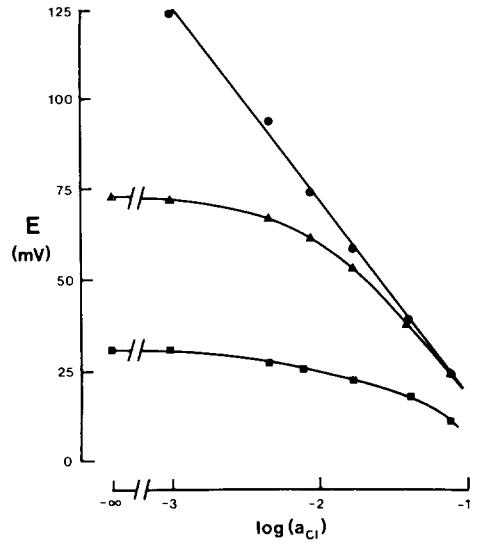

Fig. 5. Calibration of a double-barreled Cl^--selective microelectrode in KCl solutions plus 0 (top curve), 200 μM (middle curve), and 1 mM (bottom curve) sodium taurocholate. Points plotted at '$-\infty$' represent electrode potentials in nominally Cl^--free solutions. See text for for details.

anions often used in physiological experiments interfere with liquid ion-exchanger Cl^--selective electrodes [15,22,23]. Some of these anions have high apparent selectivity factors relative to Cl^-. It is important, therefore, to assess the effect of all other anions on Cl^--selective electrodes and to make corrections through an appropriate calibration protocol. Our results indicate that in addition to the known interfering effects of HCO_3^- on Cl^--selective microelectrodes of the type used herein [15] metabolic substrates (pyruvate, glutamate and fumarate) and possibly imidazole significantly attenuate the response of Corning 477315 liquid ion-exchanger Cl^--selective microelectrodes in KCl solutions. The effects of interfering ions are automatically compensated for if calibration of the electrode is performed in solutions containing appropriate, fixed amounts of the interfering ions. The calibration curve obtained is nonlinear but is well represented by the Nicolsky-Eisenman equation. We chose to calibrate these electrodes in KCl solutions containing the major interfering anions in the same concentrations as in the bathing solutions. Since the intracellular concentrations of these anions are probably less, it follows that the actual a^i_{Cl} values in the mouse liver slices may be slightly higher than those reported in this study.

Impalements with double-barreled microelectrodes of liver slices bathed in a HCO_3^--containing solution (Solution C, Table I) yielded a mean steady state V_m of -27 ± 1.0 mV. This value is somewhat less than those that have been measured by other workers under similar, but not identical, conditions. One difference is that the open-tip barrel of the double-barreled microelectrode was filled with 1 M sodium formate, rather than KCl. To test whether or not this factor was causing depolarization of liver cells V_m was also measured with single-barreled open-tip microelectrodes filled with 0.5M KCl. In a group of 5 animals (16 impalements) V_m was found to be -29.4 ± 1.5 mV, a value not significantly different from the corresponding value obtained with double-barreled microelectrodes. The correct V_m is a subject of some debate but most studies have reported values in the range -30 to -40 mV [24–27]. Slightly lower values have been reported in isolated hepatocytes [28,29] but more recently potentials as high as -78 ± 9 mV have also been reported [30]. Another difference is that most reported data were recorded at temperatures varying between 36 to 38°C, slightly higher than the estimated temperature of our tissues. Interestingly, Wondergem and Castillo [27] found that in mouse liver cells V_m was strongly dependent upon temperature. In the range of 37 to 27°C, the membrane potential varied approximately linearly with temperature and showed a depolarization of about 1.6 mV for each degree below 37°C. Our measurements of V_m were made at an estimated temperature of 33.8°C (see Materials and Methods). If the V_m values reported by Wondergem and Castillo [27] for single barrel microelectrodes filled with 0.5 M KCl are corrected to this temperature a value of -30.8 mV is obtained. Our results do not differ significantly from this value. We, therefore, believe that the slightly lower membrane potentials reported herein are due to a difference in temperature and do not result from any defect in the liver cells themselves or from artifacts of the recording technique.

The average a^i_{Cl} measured with double-barreled Cl^--selective microelectrodes (32.6 ± 1.5 mM) is similar to the chloride concentrations (36.4

$\pm$ 3.9 mmol/l of cell water) reported for liver cells by Claret and Mazet [8] but is higher than that observed by Williams et al. [6,7] in intact rat liver (15.9 $\pm$ 1.1 mM). Our value is also consistent with the apparent steady-state exchangeable intracellular chloride concentration (31.0 mM in 37 control incubations) reported in hepatocytes by Scharschmidt et al. [4]. Rigorous comparisons of these values are not warranted because total and exchangeable chloride concentrations are potentially inaccurate reflections of a^i_{Cl} [31].

Our data also indicate that a^i_{Cl} was significantly greater than the corresponding a^{eq}_{Cl} value calculated from the membrane potential. This difference could result from the presence of ion transfer mechanisms in the liver cell membrane that maintain a^i_{Cl} above its equilibrium value. Removal of Na^+ or HCO_3^- from the bathing solutions was carried out to test for the presence of Na^+-Cl^--coupled or Cl^-/HCO_3^- coupled transport, respectively.

In other epithelia in which the intracellular chloride activity exceeds the value predicted for passive distribution, the most compelling evidence for the presence of Na^+-Cl^--coupled transport has been a decrease in a^i_{Cl} to its equilibrium value upon complete removal of Na^+ from the bathing medium [32]. There is evidence that a variety of anions may substitute for Cl^- but in epithelia, the requirement for Na^+ in Na^+-Cl^--coupled transport appears to be quite specific. Data summarized in Table II show that in tissues initially bathed in imidazole-buffered media, complete removal of Na^+ caused a small but significant ($P < 0.05$) depolarization of V_m($\Delta V_m = 2.2 \pm 0.9$ mV). This was accompanied by a significant increase in a^i_{Cl}($\Delta a^i_{Cl} = 4.4 \pm 1.8$ mM). The depolarization may be a direct effect of choline on the plasma membrane. Similar depolarizations of membrane potentials by choline have been observed in *Necturus* gallbladder [33], rabbit gallbladder [31], flounder intestine [34], and tubular kidney cells [35]. Since the paired difference ($a^i_{Cl} - a^{eq}_{Cl}$) was not significantly different from its value in control Na^+-containing media ($P > 0.05$), the observed increase in a^i_{Cl} can be interpreted as a passive response to the change in V_m. These results do not support the hypothesis of Na^+-Cl^- flux coupling in the liver cell membrane. Scharschmidt et al. [4] also failed to demonstrate Na^+-Cl^- flux coupling in rat liver cells. In their experiments, the steady-state content of intracellular exchangeable chloride was unaltered by replacement of Na^+ by choline.

Another mechanism which is operative in some epithelia in maintaining a^i_{Cl} above equilibrium is Cl^-/HCO_3^- exchange [2]. In this system outward HCO_3^- movement down its electrochemical gradient provides the driving force for intracellular Cl^- accumulation. Removal of bicarbonate might transiently elevate a^i_{Cl} but eventually there should result a decline of a^i_{Cl} towards its equilibrium value. Upon switching to HCO_3^--free medium (Table III) V_m depolarized rapidly ($\Delta V_m = 8.0 \pm 0.8$ mV; $P < 0.001$) with a simultaneous increase in a^i_{Cl}($\Delta a^i_{Cl} = 12.8 \pm 1.8$ mM; $P < 0.01$) and remained stable for 15 min in HCO_3^--free medium. The increase in a^i_{Cl} did not differ significantly from the calculated increase in a^{eq}_{Cl}, that is, ($a^i_{Cl} - a^{eq}_{Cl}$) did not change significantly ($P > 0.05$). Thus, the change in a^i_{Cl} appears to be secondary to a change in V_m and we did not find evidence for Cl^-/HCO_3^- coupled exchange. The depolarization of V_m in the absence of HCO_3^- may reflect a membrane conductance to HCO_3^- or may result indirectly from a change in intracellular pH.

The relatively constant difference between a^i_{Cl} and a^{eq}_{Cl} under control, Na^+-free and HCO_3^--free conditions is consistent with the hypothesis of electrode interference. However, these data do not exclude the possibility of active Cl^- uptake by some hitherto undetermined mechanism. A sufficient proof of electrode interference would be to demonstrate that, under experimental conditions in which the chemical content of chloride inside the cell is essentially zero, the apparent a^i_{Cl} recorded by the ion-selective electrode still gives a significant non-zero value. In our experiments, removal of Cl^- resulted in a rapid depolarization of V_m followed by a slow tendency of the membrane potential to repolarize. No hyperpolarization was observed at any time during the perfusion of Cl^--free media. These observations are consistent with the results of Heller and Van der Kloot [9] in guinea pig and rat liver, but differ from those of Claret et al. [24] and Graf and Petersen [26] who reported a transient depolarization followed by a marked hyperpolarization in

rat and mouse liver cells, respectively. The decrease in a^i_{Cl} concurrent with depolarization (shown in Fig. 3 and Table IV) is suggestive of a high Cl^--conductance in the plasma membrane of these cells. A similar decrease in the intracellular chloride concentration was seen by Claret and Mazet [8] in rat liver on lowering the external chloride concentration.

In Cl^--free media a^i_{Cl} measured by the electrode did not fall below 7.8 ± 1.7 mM. In parallel studies c^i_{Cl}, estimated by conductometric titration, decreased from 76.2 ± 5.1 mmol/l of intracellular water to 2.2 ± 1.0 mmol/l in Cl^--free media. Using control values, the activity coefficient for chloride inside the cell, a^i_{Cl}/c^i_{Cl}, is estimated to be $35.3/76.2 = 0.46$. This is considerably less than the activity coefficient for Cl^- in control HCO_3^--containing media ($75.5/112.4 = 0.67$). Qualitatively similar data have been obtained in bullfrog small intestine by Armstrong et al. [36], and were interpreted by them as suggestive of intracellular binding or compartmentalization of Cl^-. In any event, the activity of Cl^- within the liver cell under Cl^--free conditions should be numerically less than the concentration measured by chemical means. c^i_{Cl} under Cl^--free conditions (2.2 ± 1.0 mmol/l, $N = 6$) was not significantly ($P > 0.05$) different from zero, but was significantly ($P < 0.05$) less than the numerical value of a^i_{Cl} (7.8 ± 1.7 mM, $N = 3$) under these conditions. It follows that a^i_{Cl} measured by Cl^--selective microelectrodes is 6 to 8 mM greater than the true a^i_{Cl} within the liver cell. This difference is probably due to interference by some intracellular anion(s). When a correction of 6–8 mM is applied, the difference between a^i_{Cl} and a^{eq}_{Cl} disappears under all experimental conditions. We conclude that a^i_{Cl} is in fact at equilibrium across the plasma membrane of liver cells. This is consistent with the absence of a demonstrable dependence of a^i_{Cl} on Na^+ or HCO_3^- gradients across the cell membrane. These data and conclusions are in agreement with the earlier work done by Claret and Mazet [8]; Williams et al. [6,7]; Williams and Woodbury [20] and Scharschmidt et al. [4], and with the more recent observations of Fitz and Scharschmidt [12].

One possible source of interference with Cl^--electrodes within hepatocytes is bile salts. Data in Fig. 4 demonstrate the exquisite sensitivity of Cl^--selective microelectrodes to taurocholate, a representative bile salt. A selectivity coefficient ($K^{Pot}_{Cl,taurocholate}$) of approx. 60 can be calculated from data presented in Fig. 5. A free bile salt concentration of about 100 μM could, therefore, produce interference equivalent to 6–8 mM Cl^-. Okishio and Nair [21] found total bile acid concentrations in rat liver to be in the range of 100–300 μM. A free bile salt concentration of 100 μM is plausible in spite of the known tendency of bile salts to bind to intracellular proteins [37,38]. This argument does not eliminate the possibility of other, unrecognized interfering anions within the cell. However, a similar study in *Necturus* gallbladder [39] found a much lower residual interference (1 ± 0.3 mM), suggesting that our higher value may be specific to the liver.

In summary, data obtained from mouse liver slices using double-barreled Cl^--selective microelectrodes contain an artifact, apparently due to uncompensated electrode interference by intracellular anions. When a suitable correction is made, these data indicate that the hepatocyte membrane possesses a high conductance to Cl^- that allows intracellular Cl^- to equilibrate with that of the external medium according to the Nernst equation. Ion substitution experiments failed to reveal coupling of Cl^- transport to that of Na^+ or HCO_3^-. We, therefore, conclude that systems of Na^+-Cl^- symport or Cl^-/HCO_3^- antiport similar to those present in other leaky epithelia either are not present in the mouse liver or, at least, do not contribute significantly to the steady state activity of chloride within the liver cells.

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