Increased activity of ionised calcium in gall bladder bile in gall stone disease

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Abstract

The actual activity of ionised calcium (Ca^{2+}) in gall bladder bile determined with an ion-selective electrode was significantly higher in patients with gall stone disease ($n=15$) than in patients without gall stones ($n=10$) (0.43 mmol/kg v 0.31 mmol/kg ; $p<0.05$). No change in the Ca^{2+} activity in any of the gall bladder bile samples was observed during equilibration with CO_2 . During titration with HCl/NaOH , however, the Ca^{2+} activity fell with increasing pH in a biphasic manner, with the breaking point occurring at a significantly lower median pH in patients with gall stones than in patients without (pH 7.1 v 8.2 ; $p<0.0001$). The combination of a higher activity of calcium in bile and precipitation of bile salts taking place at a lower pH in patients with gall stone disease than in patients without gall stones suggests a major role for calcium and pH in the pathogenesis of gall stones. Strict anaerobic sampling is not necessary for the measurements of Ca^{2+} in gall bladder bile, because the Ca^{2+} was not significantly affected by the changes in pCO_2 . The metabolic studies suggest, however, that simultaneous measurements of the activity of Ca^{2+} and pH is important in order to interpret data for the calcium activity in gall bladder bile.

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In the Western world cholesterol is the predominant component of stones in the gall bladder.¹ Cholesterol gall bladder stones may be initiated by precipitation of calcium salts to form a nidus, with subsequent laying down of cholesterol from its supersaturated state on this nidus.^{2,3} Bile salts are important buffers for calcium ion, which may act to reduce free calcium ions (Ca^{2+}) in the bile thereby reducing calcium lithogenicity.²

Although several studies have focused on bile calcium, specifically the Ca^{2+} as a major factor involved in gall stone formation, conflicting results have been achieved on the level of Ca^{2+} in bile. Studies in artificial solution of bile salts or in animal bile report increased concentration of Ca^{2+} in gall stone formation.⁴⁻⁸ In man both increased and decreased concentrations of ionised calcium in bile have been reported.⁹⁻¹¹ All studies convert the measured activity to a concentration, although such a conversion may be inaccurate because of the unknown activity coefficient (variation in ionic strength and effects of bile salts anions).

Gall bladder bile collection is traditionally collected anaerobically, although the effect of aerobic/anaerobic – that is, CO_2 loss from the bile sample, sampling techniques on the activity of Ca^{2+} is unknown.

We present results for the actual activity of Ca^{2+} and pH in human gall bladder bile from patients with gall stone disease and in patients without gall stones (controls). We also investigated the relationship between the activity of Ca^{2+} and pH in vitro by equilibration with two different CO_2 tensions and by titration with HCl/NaOH .

Methods

PATIENTS

Fifteen patients, 12 women and three men (age range 31–57 years) with gall bladder stones were included. Patients with previous cholecystitis diagnosed by scintigraphic examination of the gall bladder were excluded. Ten patients, six women and four men (age range 29–67 years) without gall bladder stones, who underwent cholecystectomy and liver resection comprised the control group. Liver resection was performed for hepatic metastases (eight), cystic echinococcus (one), and hepatic haemangioma (one). All patients had normal serum concentrations of liver enzymes and bilirubin and were included consecutively in the study. Informed written consent was obtained before the study. The study was approved by the local ethical committee.

In patients with gall bladder stones gall bladder bile was aspirated after ligation of ductus cysticus during elective cholecystectomy. In controls the liver was examined before resection by ultrasound in order to assess whether liver resection was possible or there were multiple hepatic metastasis. In 10 patients who were found suitable for biliary/hepatic surgery the ductus cysticus was then ligated and gall bladder bile was aspirated. The latter procedure was in agreement with the recommendations from the local ethical committee.

BIOCHEMICAL ANALYSIS

Biochemical analysis and titration studies were done without knowledge of the diagnosis.

Whole bile samples were collected anaerobically and into sterile tubes. Measurements of Ca^{2+} and pH were carried out immediately after the collection.

The Ca^{2+} in bile was measured in duplicate at 37°C with an ICA 1 analyser (Radiometer, Copenhagen, Denmark), which provides simultaneous measurements for the activity of Ca^{2+} and pH. The primary calibration solution had a molality of $\text{Ca}^{2+}=1.25 \text{ mmol/kg}$; $m\text{Ca}^{2+}$; ionic strength, $I=160.0 \text{ mmol/kg}$; molal activity coefficient 0.304^{12} ; activity molality= $m\text{Ca}^{2+}$ (1.25 mmol/l) $\times$ molal activity coefficient (0.304)

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Activity of ionised calcium (Ca^{2+}), pH, the substance concentrations of sodium (Na^+), potassium (K^+), chloride (Cl^-), albumin and bilirubin in human gall bladder bile from patients with gall stone disease and without

	Patients with gall stones (n=15)	Controls (n=10)
Ca^{2+} , active molality (mmol/kg)	0.43 (0.37–0.60)	0.31* (0.24–0.33)
pH	7.50 (6.78–7.80)	7.89* (7.68–8.20)
Na^+ (mmol/l)	159 (131–192)	144 (104–176)
K^+ (mmol/l)	7.5 (4.8–11.5)	6.8 (3.9–12.0)
Cl^- (mmol/l)	54 (42–95)	69 (56–124)
Albumin ($\mu\text{mol/l}$)	200 (138–250)	185 (140–200)
Bilirubin (mmol/l)	1150 (10–4070)	2000 (10–4420)

* $p < 0.05$. Results are given as median and 95% confidence limits.

=0.380 mmol/kg.¹³ The relative standard deviation within series (CVs) and between series (CVd) were 0.9% and 1.2% (n=22), respectively.

IN VITRO Ca^{2+} EQUILIBRATION

The relationship between pH and the activity of Ca^{2+} was studied by equilibrating bile in duplicate for 11 patients with gall stones and nine controls at two different CO_2 tensions (5.3 kPa and 10.6 kPa). CO_2 tension was changed by the use of the BMS2 Mk2 Blood Micro System analyser (Radiometer, Copenhagen, Denmark).

TITRATION STUDIES

Titration studies in gall bladder bile were undertaken with HCl and NaOH in the same patients as studied by CO_2 equilibration. We used 2 ml of each gall bladder bile sample for titration studies. The titration curve was established by stepwise addition of approximately 5 μl 1 molar HCl, or NaOH. Each titration curve comprised

Figure 1: In vitro relation between the active molality of ionised calcium (Ca^{2+}) and pH at two different CO_2 tensions (5.3 kPa and 10.6 kPa) in gall bladder bile from patients (●) and controls (■). The dashed lines denotes not continuous measurements. Median and 95% confidence limits are presented.

approximately 25 measurements of the activity of Ca^{2+} and pH.

Bile sodium (Na^+), potassium (K^+) were measured potentiometric by a multichannel analyser (Technicon SMAC analyser, Tarrytown, USA), which also was used for measurements of albumin and bilirubin concentrations. Chloride (Cl^-) was measured by colourmetric titration (CMT10, Radiometer, Copenhagen).

LIQUID JUNCTION POTENTIAL (E_j)

The liquid junction potential of the junction sodium formate (4.6 mol/kg) and 'test solution' was calculated using the Henderson equation¹⁴ to be -2.1 mV for the calibration solution and in gall bladder bile (Table) (containing 150, 7.0, 60, 30 mmol/kg) for Na^+ , K^+ , Cl^- , HCO_3^-) to be -3.4 mV. The limiting molar conductance for cholic acid conjugated with either glycine or taurine is theoretically negligible considering the molar conductance of the other electrolytes. These results indicate that the residual liquid junction potential (E_j) = E_j (gall bladder bile) - E_j (calibr) is -1.3 mV corresponding to a bias on the measured Ca^{2+} activity of -9.5%. The presented data for the activity of ionised calcium have not been corrected.

STATISTICAL ANALYSIS

The difference between median values were examined by Mann-Whitney test. Linear regression analysis was used to evaluate the relationship between the activity of Ca^{2+} and pH. The relationship between the activity of Ca^{2+} and pH during titration studies included approximately 25 measurements of Ca^{2+} and pH in each bile sample. The best fit between these measurements for each patient was calculated by non-linear regression analysis. The breaking points were defined without knowledge of the diagnosis. The statistical analysis were performed by Statistical Analysis System computer package 4.10 (SAS Institute Inc). A p value less than 0.05 was chosen as statistically significant.

Results

As shown in Table the activity of Ca^{2+} was significantly increased and pH significantly lower in gall stone patients compared with controls ($p < 0.05$), whereas no significant differences were observed as regards other electrolytes, albumin, or bilirubin.

IN VITRO STUDIES

CO_2 equilibration

The slope $\Delta \lg \text{Ca}^{2+} / \Delta \text{pH}$ measured by equilibrating whole gall bladder bile at two different CO_2 tensions (5.3 kPa, 10.6 kPa) from gall stone patients (n=10) and controls (nine) gave a mean value of -0.09 and -0.04, respectively (NS) (Fig 1).

Titration with HCl and NaOH

When the relationship between the Ca^{2+} activity

Figure 2: Active molality of ionised calcium (Ca^{2+}) (on a logarithmic scale) as a function of pH in human bile titrated with HCl and NaOH from patients with gall stone disease and controls. The dotted lines denote the breaking points for gall stones (pH 7.1, range 6.1–7.5) and without (pH 8.2, range 7.9–8.8).

and pH was studied by titrating each collected gall bladder bile with HCl and NaOH (Fig 2), a highly significant negative relationship was found between the activity of Ca^{2+} and pH ($r = -0.98$; $p < 0.001$). In bile from patients with gall bladder stones all curves showed a significant 'breaking point' at pH 7.1, where the slope of the curve changed from -0.09 to -0.21 (median values). In controls the slope changed from -0.07 to -0.27 at pH 8.2. The breaking point was located at a significantly lower pH in patients with gall stones than in controls (pH 7.1 *v* pH 8.2) ($p < 0.0001$).

Discussion

The actual activity of Ca^{2+} in gall bladder bile in patients with gall stone disease was significantly higher combined with a significantly lower pH than in the controls. It is observed that pH in the samples from patients without gall stones was higher than what is normally reported in human gall bladder bile.¹¹ Whether this may be because of some sort of 'contamination' of gall bladder bile with common duct bile (pH in the range of 7.5 to 8.05) during liver examination can not be discounted. Theoretically this influence could have been eliminated by including controls

(patients) who were not scheduled for biliary/hepatic surgery. We did not find it feasible, however, because of potential ethical problems, to collect bile from such a patient group. We have, therefore, chosen a control group, which comprised patients with normal liver function tests and in whom the gall bladder was removed during liver resection. Furthermore, the levels of other biliary electrolytes did not differ among patients with gall stones and controls suggesting that contamination had not occurred.

We observed an inverse relationship between Ca^{2+} and pH whether the change in Ca^{2+} and pH was the result in variations of CO_2 tensions or to added HCl or NaOH. Therefore some of the higher activity of Ca^{2+} observed in gall bladder bile from patients with gall stone disease may be caused by the lower pH. When we recalculated the activity of Ca^{2+} at the same pH (pH 7.7) using the formula for changes in pCO_2 ,¹⁵ however, the difference between the two groups was still apparent, indicating that the difference in pH may have only a minor influence on the observed difference in the Ca^{2+} activity when the pH changes are caused by variations in pCO_2 (recalculating the actual activity in gall bladder bile from patients with gall stone disease and controls to a pH 7.7 gives an activity of 0.407 and 0.315 mmol/kg, respectively) (Fig 1).

Previous authors have attempted to convert the measured ionised calcium activity to the substance concentration of free calcium. From a physicochemical point of view such a conversion seems inappropriate because the activities of the participating ions determine the solubility product for a given salt and hence the possibility for precipitation. Furthermore, calculation of the substance concentration as the activity divided by the activity coefficient is rather inaccurate. The activity coefficient of Ca^{2+} may vary considerably depending upon the ionic strength in gall bladder bile according to the Debye-Hückel equation.¹⁶ The activity coefficient in gall bladder bile may vary from 0.3 to 0.9 as estimated from measurements of the concentrations of sodium and potassium in pure bile salt solutions.² Because of these difficulties artificial bile solutions have been prepared to a total ionic strength of 0.295 mmol/l. From these artificial bile solutions formation constants and solution products for different calcium complexes have been calculated.² If the previous data on substance concentration of free Ca^{2+} in gall bladder bile are converted back to active molality we find values in the same range as our values.⁸

The concentration of electrolytes in bile from patients with stones in the gall bladder did not differ significantly from controls. From our data the interference of the major cations in bile on the calcium electrodes may be considered very small (selective coefficients for Na^+ , K^+ about 10^{-1} , and for Mg^{2+} 10^{-3}).¹⁵ We have previously shown that hydrogen interference is negligible in the pH interval 5.0 to 9.0.¹³

The small negative slope after *in vitro* CO_2 equilibration of gall bladder bile ($\Delta \lg \text{Ca}^{2+} / \Delta \text{pH}$ of -0.09 and -0.04) is much lower than that found in human serum of -0.23 . In serum the changes in Ca^{2+} with the changes in pH are caused predominantly by the albumin concen-

tration,¹⁵ which explains the lower slope of $\Delta \lg \text{Ca}^{2+}/\Delta \text{pH}$ in gall bladder bile (median albumin concentration 200 $\mu\text{mol/l}$ compared with 650 $\mu\text{mol/l}$ in human serum). The theoretical calculated slope in gall bladder bile containing an albumin concentrations of 200 $\mu\text{mol/l}$ gives a slope of -0.05 close to the observed value. Therefore, anaerobic sampling which is a necessity in serum because of the possible CO_2 loss from the sample is not as crucial for the measurements of gall bladder bile because of the small effect on Ca^{2+} activity with a CO_2 loss from the sample where pH changes only minimal. The titration studies, with a pH change of 4 to 10 units, however, showed that it is essential to measure the activity of Ca^{2+} and pH simultaneously because of the strong relationship between the Ca^{2+} activity and the pH of gall bladder bile. The increased activity of Ca^{2+} and the location of the breaking point at a significantly lower pH in patients with gall stone disease than in controls ($\text{pH}=7.1$ v 8.2) may increase the risk for precipitation of bile salts and may, as suggested by Moore, be a principal factor in the initiation of gall stone formation.^{2,17}

In conclusion we find a higher activity of Ca^{2+} in gall bladder bile in patients with gall stone disease than in controls. Our results suggest that the activity of Ca^{2+} and pH may be important factors in the nucleation and ultimately the growth of gall stones. For the measurements of Ca^{2+} in gall bladder bile strictly anaerobic sampling is not crucial, as the activity of Ca^{2+} was not significantly affected by the small changes in pH caused by different pCO_2 tensions. The close relationship between the Ca^{2+} activity and pH as a result of metabolic changes, however, may indicate the need to measure both parameters simultaneously in

order to interpret data for the Ca^{2+} activity in gall bladder bile.

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