Evidence of hydrogen ion secretion from the human gall bladder in vitro

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Abstract
Gall bladder bile is more acid that hepatic bile and this has been attributed to bicarbonate absorption by the gall bladder epithelium. The aim of this study was to investigate in vitro the acid base changes that occur across the human gall bladder mucosa. Fresh gall bladder tissue was obtained at cholecystectomy and placed in an Ussing Chamber and perfused with Ringer-Krebs glucose bicarbonate solution. The viability of the gall bladder was assessed by measuring the potential differences across the epithelium and by the morphology of the epithelial cells at the end of the experiments. Aliquots from the solutions were taken at two, 45 and 70 minutes and pCO₂, hydrogen ion and bicarbonate concentrations were measured. In the mucosal side of the chamber a consistent and significant decrease was observed from two minutes to 70 minutes in bicarbonate concentration while pCO₂ and hydrogen ion concentrations significantly increased. The degree of inflammation correlated well with the ability for acidification, the more inflamed the tissue the less it ability to acidify. When the gall bladder was exposed to amiloride or sodium free solution acidification was abolished in the mucosal side. When tissue metabolism was irreversibly inhibited by exposure to formaldehyde, hydrogen ion concentration and pCO₂ were significantly decreased in the mucosal side of the chamber compared with the viable gall bladder. The human gall bladder is capable of secreting acid and this may be an important mechanism for preventing calcium precipitation and gall stone formation.

Gall stone disease is a common cause of morbidity and mortality and cholecystectomy represents a significant percentage of major upper abdominal operations in western society. Although considerable research has been concentrated on the pathogenesis of gall stone formation, this has often focused on the biochemical changes that occur in the bile. It is only within the last 10 years that the importance of the events in the gall bladder and in particular the possible contributions of the gall bladder mucosa to lithogenesis has been recognised. The gall bladder mucosa has one of the highest rates of water absorption in the body and an 80 to 90% decrease in the initial volume of bile is achieved within the gall bladder. This is achieved by coupling of active sodium transport and passive water absorption resulting in isotonic fluid absorption.

Methods
Studies were done using fresh human gall bladder mucosa obtained at the time of elective cholecystectomy.
cholecystectomy usually for gall stone disease. Patient’s sex, age, and type of gall stones, if any, were recorded. The gall bladder tissue was retrieved within 15 minutes of the ligation of the cystic artery. A circular piece of the gall bladder wall (d=12 mm) was removed using a tissue punch and placed in Ringer-Krebs bicarbonate solution (0.9% NaCl (0.154 M), 1.15% KCl (0.154 M), 1.22% CaCl2 (0.11 M), 2.11% KH2PO4 (0.154 M), 3.8% MgSO4, 7H2O (0.154 M), 1.3% NaHCO3, 5.4% glucose (0.3 M)) at 4 °C and preoxygenated with 95% O2, 5% CO2. To minimise tissue hypoxia, this was done as soon as the gall bladder was removed.

The gall bladder tissue was transferred to the laboratory and within five minutes the mucosa was stripped by blunt dissection from the muscular part of the wall, rinsed with Ringer-Krebs bicarbonate (RKGB) solution to remove debris and bile and mounted in an Ussing Chamber. The Ussing Chamber consisted of two 2 ml volume compartments each of which communicated with a glass tube above through two plastic tubes (inlet and outlet) (Fig 1). The gall bladder tissue separated the two compartments creating a mucosal compartment at the mucosal site and a serosal compartment on the opposite side. The term ‘serosa’ is used throughout to indicate the non-luminal surface of the mucous membrane. Both compartments were filled with 10 ml RKGB through the glass tubes (2 ml) in each compartment of the chamber and 8 ml at each glass tube. The mucosal compartment was sealed while 95% O2/5% CO2 (21/min) was bubbled through the serosal compartment only. Preliminary experiments had shown that pO2 between mucosal and serosal compartments did not differ significantly if 95% O2/5% CO2 was bubbled into the serosal compartment only and this was preferred in order to avoid disruption of the ‘unstirred water layer’ in the mucosal side.20-21 The whole system was covered by a thermostatic water jacket to maintain a constant temperature of 37°C throughout the experiment. A pair of silver/silver chloride matrix 1 mm electrodes (Clark Electromedical Instruments, Reading, Berks, UK) monitored the potential difference across the two sites of the tissue and used as an index of viability of the tissue. Each study lasted for 70 minutes. One millilitre of the solution was removed through a micropipette from each compartment of the Ussing Chamber at two minutes, 45 minutes and 70 minutes and was immediately analysed in an 1302 pH/blood gas analyser (Instrumentation Laboratory System, Lexington, MA, USA) for pO2, pCO2, hydrogen ion ([H+] plus bicarbonate ([HCO3-]) concentrations. Gall bladders which were macroscopically grossly distorted or damaged were excluded from further study.

EXPERIMENTS

Forty gall bladders were studied. The experiments were divided into three groups.

First group
Five gall bladders were studied. The effect of oxygen deprivation and immersion in formaldehyde 4% on the resting potential difference was observed. 95% O2/5% CO2 was bubbled at a steady flow rate of 21/min from time 0 to 20 minutes, then stopped for 10 minutes from time 20 to 30 minutes and subsequently continued at the same flow rate until the end of the experiment. Thereafter the gall bladder mucosa was exposed to 4% formaldehyde for two minutes, rinsed with RKGB solution and remounted to the Ussing Chamber and studied for further 10 minutes. The potential differences were monitored continuously throughout the experiment.

Second group
Twenty five gall bladders were studied. Twenty one were processed according to the standard protocol to maintain viability and four were immersed in formaldehyde 4% for two minutes before being mounted on the Ussing Chamber. The hydrogen ion concentration, bicarbonate concentration pCO2 and pO2 were measured at the beginning (two minutes) at 45 minutes and the end of the experiments (70 minutes). The resting transepithelial potential difference was monitored throughout. The aim was to study the acidification capacity of the viable gall bladder compared with non-viable tissue.

Third group
Ten gall bladders were studied. In the first set of experiments involving six gall bladders the mucosal bathing solution was replaced by sodium free Krebs-Ringer solution (NaCl and NaHCO3 removed) and the analytes mentioned above were measured. In a second set of experiments involving four gall bladders 2 mM amiloride (a specific Na+/H+ inhibitor) was introduced into the mucosal compartment and its effect on acidification was observed after 45 minutes.

Figure 1: Potential difference across the human gall bladder mucosa; (A) represents a viable gall bladder with an initial potential difference of 7 mV. Oxygen deprivation or immersion in formaldehyde resulted in a drop in the potential difference across the epithelium but recovered when oxygen was re-introduced. (B) represents a gall bladder with an initial potential difference of less than 2 mV. Although potential difference recovered after 15 minutes, oxygen deprivation resulted in a non-reversible fall in the potential difference. Gall bladders with initial potential difference <2 mV were excluded from further study.
PATHOLOGY

A small sample of the tissue under study was fixed in 10% buffered formalin before each Ussing Chamber experiment. The rest of the tissue was also fixed in 10% buffered formalin at the end of the experiment. An experienced histopathologist examined each gall bladder blindly on two occasions and reported on the degree of cholecystitis and the viability of the tissue according to cell morphology at the beginning and end of the experiments. The degree of cholecystitis was graded from 1 (mild) to 3 (severe) according to the appearance of the mucosa, muscle layer thickness, presence of Rokitansky-Aschoff sinuses and degree of inflammatory process. The morphology changes were recorded as 0 (healthy looking cells), 1 (mild morphological changes such as cell oedema, presence of granules, vacuolation), 2 (moderate but without evidence of cell necrosis).

The gall bladders used in the above experiments were selected according to the following criteria: (a) satisfactory macroscopic appearance with no obvious ulceration or fibrosis at the beginning of the study and no obvious damage during the study; (b) expression of a resting potential difference more than 2 mV of serosa positive; (c) data from mucosa with severe changes in cell morphology were excluded from further analysis.

STATISTICAL ANALYSIS

Data are expressed as mean standard error of mean (SEM). The data were not normally distributed and were analysed using non-parametric tests; Mann-Whitney test was used for unpaired data and Wilcoxon's test for paired data. A p value of 0.05 was taken as significant.

Results

In the first set of experiments the gall bladders (five) documented a mean potential difference of 7.5±3.3 mV serosa positive after five minutes in the chamber. This potential difference remained quite stable throughout the ensuing 15 minutes. There was a significant drop in the potential difference when 95% oxygen 5% CO2 was discontinued, but when reapplied the potential difference recovered to levels similar to those before oxygen deprivation indicating that hypoxia had a direct effect on reducing the resting potential difference. When those gall bladders were immersed in formaldehyde 4% and remounted on the Ussing chamber, however, there was a dramatic and irreversible drop in the potential difference indicating non-viability of the tissue (Fig 1). The PO2 was measured in both compartments at two minutes, 45 minutes, and 70 minutes. Although 95% O2/5% CO2 was bubbled in the serosal compartment only, there was no difference in the PO2 between the mucosal and serosal compartment (25·3±2·1 pKa v 23·5±2·2 pKa respectively) which shows that the diffusion of oxygen through the gall bladder mucosa was adequate. It was thought therefore unnecessary to oxygenate the mucosal compartment directly, as this would disrupt the "unstirred water layer". All gall bladders showed evidence of chronic cholecystitis (three grade 1, two grade 2) without any significant changes in cell morphology at the beginning and end of the experiments.

In the second set of experiments 25 gall bladders were studied (18 women, seven men; 13 contained cholesterol stones, nine pigment stones, three biliary sludge). Four of those were immersed in formaldehyde and used as controls and from the remaining 21, six showed evidence of grade 3 chronic cholecystitis and were not included in the analysis outlined below. There was a significant increase in the hydrogen ion concentration from two minutes to 45 minutes.
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and to 70 minutes observed in the mucosal compartment (p<0.01) while in the serosal compartment there was a significant decrease in hydrogen concentration (p<0.05). By contrast in the non-viable gall bladder (those immersed in formaldehyde) there was a significant drop in hydrogen concentration on the mucosal side without any significant change of hydrogen concentration in the serosal side (Fig 2a). In the viable gall bladder bicarbonate concentration significantly decreased in the mucosal compartment from two minutes to 45 and 70 minutes (p<0.02) while on the serosal side there was a significant increase of bicarbonate concentration from two minutes to 70 minutes. In the non-viable gall bladder an increase of bicarbonate concentration was seen in both compartments (Fig 2b). In the viable gall bladder there was a significant increase in pCO2 between two and 70 minutes on the mucosal site (p<0.03) while in the serosal compartment a significant decrease in pCO2 was observed (p<0.05). In the non-viable gall bladder there was a drop in pCO2 in both compartments being significant in the mucosal side (p<0.01) (Fig 2c).

In the viable gall bladder the mean potential difference did not significantly change throughout the experiments. There was, however, a significant difference in the potential difference between the viable and the non-viable gall bladders studied (6.3 (2.6) to 1.3 (0.9), p<0.01) at five minutes. The potential difference of the non-viable gall bladders rapidly declined to 0. When the acidification ability of the 21 gall bladders was plotted against the degree of chronic cholecystitis there was a progressive and statistically significant decrease of hydrogen ion secretion from grade 1 (mild) to grade 3 (severe) cholecystitis (Fig 3).

The same was true when hydrogen ion secretion was plotted against the degree of cell morphology changes graded from 0 to 2 indicating that gall bladder epithelia with impaired viability had a tendency to secrete less hydrogen ions (Fig 4).

There were no differences in the acidification capacity of the gall bladders with sex or type of gall stones, although epithelia from gall bladders with pigment stones had a higher ability to absorb bicarbonate (Δ[HCO3]=−1.68 (0.49) mmol/l (pigment) v 3.8 (0.74) mmol/l (cholesterol), p<0.01).

In the third set of experiments (10) RKGB solution was replaced with sodium free isotonic solution in the mucosal side. No significant differences in the [H+], [HCO3−] and pCO2 were observed in any site of the Ussing chamber, between two and 70 minutes of the experiment. Similarly, the use of amiloride (2 mM) in the mucosal side abolished acidification (Fig 5).

Discussion

The Ussing chamber method has been adapted and used by many investigators since its introduction by Ussing and Zerhan in 1951. It is a valid method of keeping a biological preparation viable during the period of investigation and has been used to study electrophysiological, secretory, and absorptive properties of tissues in vitro. Tissues previously used include frog skin, intestine, bladder, and animal and human gall bladder.

For years it has been known that gall bladder bile is more acid compared with hepatic and the difference in the pH has been attributed to bicarbonate absorption. In this study we have shown that fresh viable human gall bladder mucosa is capable of acidifying physiological solutions in vitro. This acidification is a function of viable tissue; it was reduced in more inflamed gall bladders or when the epithelial cells became ‘sick’ during the study and was lost when the mucosa was non-viable. Acidification was abolished when the gall bladder epithelium was exposed to sodium free solution or in the presence of high concentration of amiloride in the mucosal compartment. In our study where human gall bladders were used to investigate acid secretion in vitro, the gall bladder epithelium appeared capable of increasing the hydrogen concentration in mucosal side with simultaneous decrease of hydrogen concentration in the serosal side which suggests that hydrogen ions were transferred from the serosal to mucosal side of the tissue. The concomitant decrease of bicarbonate concentration with increased pCO2 in the mucosal side indicates that this acidification is not simply the result of bicarbonate reabsorption. It appears that hydrogen ions produced by the mucosal cells react with bicarbonate to form CO2 and water with a decrease in bicarbonate ions. The observed differences in pCO2 between the viable and non-viable gall bladders could be explained on the basis that a non-viable gall bladder mucosa loses its ability to secrete acid therefore less hydrogen ions are available to react with bicarbonate and also that the production of CO2 from the mucosal cell metabolism is depressed.

Studies on gall bladders of other animal...
species such as rabbit, guinea pig, necturus, and dog have shown that these tissues are capable of acidifying the mucosal solutions in vitro and there is evidence that a sodium/hydrogen antiport is present at the apical site of the epithelial cell. In our experiments the use of sodium free solutions and amiloride, which is a specific sodium/hydrogen inhibitor, abolished acidification which suggests that hydrogen secretion in the human gall bladder depends upon a sodium/hydrogen antiport.

A potential problem, with Ussing chamber studies is related to the viability of the tissue received at cholecystectomy. The ligation of cystic artery which is part of the operative procedure usually takes place between five to 20 minutes before the removal of the gall bladder. The effect of hypoxia during this period has been studied and about 70% of the gall bladders examined immediately after the operation showed evidence of mitochondrial change which was attributed to anoxia and mechanical damage. The human gall bladder, as well as all normal gall bladders, however, is a durable organ which can rapidly recover from hypoxia and can be preserved viable for in vitro experiments for up to four hours. The gall bladder has been used for many years as a model to study ion transport across the epithelia. In this study all the gall bladders showed a transmural potential difference of more than 2.3 mV (range 2.3–11.9 mV) serosa positive. This potential difference remained stable throughout the period of experiment and we, as well as other workers, were able to show that anoxia resulted in a drop at the transmural potential difference reflecting a reducing viability of the tissue which was reversible if the anoxic period was less than 10 minutes. Similar results were produced after immersion in formaldehyde with a permanent drop of the potential difference indicating loss of viability. The continuous transpotential difference monitoring therefore has been used as a reliable method of monitoring the viability of the tissue and allow further study of the mucosal function. The transmural potential difference of the gall bladders under study was in accordance with the literature. Ross and others carried out studies on the electrical properties of the human gall bladder and on 46 gall bladders the mean transpotential difference was 7.6 mV while other investigators later showed that inflamed gall bladders have a lower resting transpotential difference which is dependent upon the degree of inflammation, the variation in time between the ligation of cystic artery, and the transfer to the laboratory and the methodology used for studying the electrical properties of the tissue. For instance, tissues which are clamped at the edges as in the Ussing chamber technique suffer from edge damage which reduces the transpotential difference.

Another potential problem with physiological studies using tissues from routine cholecystectomy is that most are not histologically normal. Although it is not appropriate to extrapolate to normal gall bladders in respect of hydrogen ion secretion, the fact that we could show changes in the acidification capacity with histology, the more inflamed gall bladders being less capable to secrete acid, implies that hydrogen ion secretion is a function of the normal human gall bladder mucosa. The acidification capacity was impaired when the cell morphology was abnormal and was abolished in the non-viable gall bladder; these suggest that hydrogen ion secretion is a function of a viable tissue. There is recent supportive evidence from animal and human studies that the normal gall bladder secretes acid. Moore et al showed that the canine gall bladder is capable of secreting hydrogen ions. Preliminary in vivo studies from the same group have shown that the diseased human gall bladder is associated with decreased acid output. Their conclusions, however, were not based on studies in human gall bladder mucosa directly, but were inferred from biochemical analysis of gall bladder bile from laparotomy.

Our data would support the hypothesis by Moore and colleagues that reduced gall bladder hydrogen ion secretion is associated with gall stone formation. It is postulated that the more electronegative end of the experimental model diseased gall bladders represent only a fraction of the capacity which the normal human gall bladder epithelium might possess to secrete acid. More studies on fresh normal tissue are required to test this suggestion.

It is now accepted that the sequence of events in the process of gall stone formation is supersaturation of bile, nucleation, precipitation, and subsequent growth to stone sizes aggregates. Supersaturation of bile with cholesterol is present in the vast majority of patients with gall stone disease; but 40 to 80% of normal individuals are supersaturated bile without ever forming gall stones. Therefore the process of nucleation is important and this depends upon the presence of certain nucleating agents or the absence of the naturally occuring inhibitors of crystal formation. Calcium bilirubinate or mucous glycoproteins could serve as nucleating factors while a bile protein which is a cholesterol crystal formation inhibitor has been proposed by Holzbach as a gall stone formation protective protein. Calcium has long been implicated in the pathogenesis of cholesterol and pigment gall stones. Pigmented gall stones are predominantly composed of the calcium salts of carbonate, bilirubinate, phosphate, and long chain fatty acids and to a lesser extent carbonate. Calcium carbonate also precipitates on to the surface of the cholesterol gall stones and is present in most cholesterol gall stones. The regulation of calcium concentration in the gall bladder bile is therefore of critical importance. It is postulated that acid secretion may be biologically important because a reduction in the pH of the gall bladder bile effectively lowers the bicarbonate and may reduce the risk of insoluble carbonate formation. As a result, the concentration of ionised calcium is increased in bile. The gall bladder epithelium, however, has the ability to absorb calcium and can reduce its concentration in bile by more than 50%; bile acids also buffer the remaining ionised calcium. Consequently less calcium is available to form insoluble salts. The mechanism of acid secretion also merits...
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Further investigation. In this study we have shown that it is sodium dependent, therefore it is closely linked with the concentrating ability of the gall bladder because it is known that water is passively absorbed consequent upon the absorption of sodium from the epithelial cell. It is, however, necessary to identify other factors which may influence acid secretion and further studies are under way to investigate this.

In conclusion we have shown in this study that the viable human gall bladder is capable of secreting acid. This acid secretion probably occurs through an apical Na+/H+ exchange at the mucosal site of the gall bladder epithelial cell. This may represent a protective mechanism against calcium precipitation. Further studies are needed to investigate more fully the process and identify those factors which regulate acid secretion.

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