The Journal of Experimental Biology 209, 4701-4716
Published by The Company of Biologists 2006
doi:10.1242/jeb.02564

A critical analysis of carbonic anhydrase function, respiratory gas exchange, and the acid–base control of secretion in the rectal gland of *Squalus acanthias*

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Accepted 27 September 2006

Summary

We compared *in vivo* responses of rectal gland secretion to carbonic anhydrase (CA) inhibition (10^{-4} mol l^{-1} acetazolamide) in volume-loaded dogfish with *in vitro* responses in an isolated-perfused gland stimulated with 5×10^{-6} mol l^{-1} forskolin and removed from systemic influences. We also measured respiratory gas exchange in the perfused gland, described the acid–base status of the secreted fluid, and determined the relative importance of various extracellular and intracellular acid–base parameters in controlling rectal gland secretion *in vitro*. *In vivo*, acetazolamide inhibited Cl^- secretion and decreased pHi in the rectal gland, but interpretation was confounded by an accompanying systemic respiratory acidosis, which would also have contributed to the inhibition. In the perfused gland, $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ increased in linear relation to increases in Cl^- secretion rate. CA inhibition (10^{-4} mol l^{-1} acetazolamide) had no effect on Cl^- secretion rate or pHi in the perfused gland, in contrast to *in vivo*, but caused a transitory 30% inhibition of $\dot{M}_{CO_2}$ (relative to stable $\dot{M}_{O_2}$) and elevation in secretion P_{CO_2} effects, which peaked at 2 h and attenuated by 3.5–4 h. Secretion was inhibited by acidosis and stimulated by alkalosis; the relationship between relative Cl^- secretion rate and pHe was almost identical to that seen *in vivo*. Experimental manipulations of perfusate pH, P_{CO_2} and HCO_3^- concentration, together with measurements of pHi, demonstrated that these responses were most strongly correlated with changes in pHe, and were not related to changes in P_{CO_2}, extracellular HCO_3^-, or intracellular HCO_3^- levels, though changes in pHi may also have played a role. The acid–base status of the secreted fluid varied with that of the perfusate, secretion pH remaining about 0.3–0.5 units lower, and changing in concert with pHe rather than pHi; secretion HCO_3^- concentrations remained low, even in the face of greatly elevated perfusate HCO_3^- concentrations. We conclude that pH effects on rectal gland secretion rate are adaptive, that CA functions to catalyze the hydration of CO_2, thereby maintaining a gradient for diffusive efflux of CO_2 from the working cells, and that differences in response to CA inhibition likely reflect the higher perfusion-to-secretion ratio *in vitro* than *in vivo*.

Key words: chloride secretion, O_2 consumption, CO_2 excretion, gas exchange ratio, pHi, pHe, acidosis, alkalosis, shark, acetazolamide.

Introduction

The rectal gland is a small finger-shaped organ in the posterior intestine of elasmobranchs. Its ionoregulatory and volume-regulatory functions were first characterized by Burger and Hess (Burger and Hess, 1960; Burger, 1962), who demonstrated that the rectal gland of the intact dogfish shark (*Squalus acanthias*) secreted an almost pure NaCl solution at a concentration of approximately 500 mmol l^{-1}, almost twice the NaCl level in blood plasma, but close to isosmotic with seawater and the urea-rich plasma. Subsequently, the gland has been used at many levels as a powerful *in vitro* model for understanding the mechanism and control of NaCl secretion. These analyses have included the use of *in situ* preparations in pithed, artificially ventilated dogfish, isolated-perfused whole gland preparations, perfused rectal gland tubules, cultured tubular epithelial cells, and membrane vesicles of the tubules (reviewed by Epstein et al., 1983; Shuttleworth, 1988; Riordan et al., 1994; Silva et al., 1990; Silva et al., 1996; Silva et al., 1997; Olson, 1999; Hazon et al., 2003). The isolated-perfused rectal gland preparation, first developed by Hayslett et al. (Hayslett et al., 1974) and subsequently improved by many others, has been a particularly useful approach, in

which secretion can be directly stimulated using forskolin (Moran and Valentich, 1991) to activate the adenyl cyclase pathway (e.g. Forrest, Jr et al., 1997; Walsh et al., 2006).

The following model has emerged. The oral intake of seawater or salty food during feeding (Mackenzie et al., 2002) is assumed to cause volume expansion *in vivo* (Solomon et al., 1984a; Solomon et al., 1984b; Solomon et al., 1985). The latter is known to mobilize C-type natriuretic peptide (CNP) from the heart (Silva et al., 1987), which stimulates increased rectal gland blood flow and secretion, by both direct and indirect mechanisms. The direct action seems to involve activation of both protein kinase C and guanyl cyclase signalling pathways (Silva et al., 1999). However, at least in *Squalus acanthias*, there is also a very potent indirect action, by which CNP causes the release of vasoactive intestinal polypeptide (VIP) from nerve endings in the rectal gland, and VIP in turn activates the adenyl cyclase pathway (Stoff et al., 1979; Stoff et al., 1988; Silva et al., 1987). A cascade of events (reviewed by Silva et al., 1997) is initiated by the various intracellular signals, in which apical CFTR-like channels are activated for Cl^- extrusion, together with basolateral Na^+,K^+,$2Cl^-$ co-transporters (NKCC), Na^+,K^+-ATPase and K^+ channels. The Na^+ electrochemical gradient powers the NKCC-mediated entry of both Cl^- and K^+. These ions exit *via* their respective apical and basolateral channels, while Na^+ is secreted in equimolar amounts to Cl^- through a paracellular pathway, driven by the negative potential created by apical Cl^- extrusion. The net NaCl transport into the tubule entrains an isosmotic flux of water.

The rectal gland contains high carbonic anhydrase (CA) activity (Maren, 1967; Lacy, 1983) but its function has never been satisfactorily integrated into this model. Several *in vitro* studies with isolated-perfused glands have reported a complete lack of effect of CA inhibition on secretory performance (Siegel et al., 1975; Silva et al., 1977; Swenson and Maren, 1984). However, using an *in situ* preparation in a pithed, artificially ventilated whole dogfish, Swenson and Maren (Swenson and Maren, 1984) reported that CA inhibition reduced rectal gland secretion. They speculated that the normal role of CA is to facilitate the diffusive excretion of CO_2 from the metabolically active gland cells, in accord with the pioneering work of Gros in muscle tissue (Gros et al., 1976; Gros, 1991), but their results may have been confounded by the loss of neural control and depressed circulation in this preparation, as well as systemic effects of CA inhibition.

Recently we developed a method for assessing rectal gland performance in intact, unanaesthetized dogfish subjected to volume loading (Wood et al., 2006). Therefore, our first objective was to use this approach to further investigate the role of CA in the gland by examining the effect of CA blockade with acetazolamide in the intact animal on secretory performance, secretion acid–base status, and intracellular pH in the rectal gland. We followed this up with comparable studies on the effects of CA inhibition using the isolated-perfused rectal gland preparation to separate gland-specific effects from systemic effects of the CA blockade.

One advantage of the isolated-perfused preparation is that it allows the direct collection of not only the secreted fluid itself, but also the outflow through the rectal gland vein. Respiratory gas exchange by the secreting cells can therefore be directly measured, though to our knowledge, this has only been done for O_2 uptake (e.g. Silva et al., 1980), and not for CO_2 excretion. Our second objective was therefore to measure both O_2 and CO_2 exchange of the perfused gland, as well as the acid–base status of the secreted fluid, at rest, after activation with forskolin, under various acid–base manipulations, and during blockade of CA function with acetazolamide, to assess whether CA is involved in facilitating the diffusive excretion of CO_2, as suggested by Swenson and Maren (Swenson and Maren, 1984).

Several early studies reported that acidic perfusates inhibited secretion in the isolated-perfused gland *in vitro* (Siegel et al., 1975; Silva et al., 1992), and Swenson and Maren demonstrated that severe metabolic or respiratory acidosis inhibited secretion of their *in situ* gland preparation (Swenson and Maren, 1984). Recently, we have shown that the NaCl secretion rate of the rectal gland in intact unanaesthetized dogfish is very responsive to blood acid–base status *in vivo*, being stimulated by metabolic alkalosis, and inhibited by both respiratory acidosis and metabolic acidosis in volume-loaded animals (Wood et al., 2006). The nature of the acid–base stimulus (i.e. extracellular or intracellular pH, HCO_3^- level or P_{CO_2}) was not determined in any of these investigations. Our final objective was therefore to use the forskolin-stimulated isolated-perfused preparation together with independent manipulations of perfusate P_{CO_2}, HCO_3^- level and pH to analyze the precise nature of the extracellular and/or intracellular acid–base stimuli controlling secretion rate in the rectal gland.

Materials and methods

Experimental animals

Pacific spiny dogfish (*Squalus acanthias* L., 0.8–5.0 kg) were collected over the course of three summers from Barkley Sound, British Columbia, Canada. The fish were caught as bycatch in the trawls of commercial shrimp fishing. At Bamfield Marine Sciences Centre, they were held for a period of at least 1 week and up to 4 weeks prior to experimentation. Food (herring) was offered, but the fish would not feed in captivity. The outdoor holding tank (3000 l) was served with running seawater at the experimental temperature (11±1°C), salinity (29±2‰) and pH (7.9±0.15).

In vivo preparations

In order to economize on radioisotopes, the smallest animals (0.3–1.0 kg) were selected for intracellular pH measurements, while larger dogfish (1.0–5.0 kg) proved more suitable for cannulation of the secretory duct of the rectal gland. Dogfish were anaesthetized with MS-222 (0.1–0.2 g l^{-1}; Syndel Labs., Vancouver, BC, Canada), weighed, and irrigated on an operating table. Dogfish intended for intracellular pH measurements (N=20) were fitted with only an indwelling

mesenteric artery catheter (PE50 polyethylene tubing; Clay-Adams, Parsipanny, NJ, USA) by the method of Graham et al. (Graham et al., 1990), whereas those intended for secretion collections (*N*=13) were also fitted with a rectal gland duct catheter (PE50 or PE90 depending on animal size) by the method described (Wood et al., 2006). The arterial catheter was filled with dogfish saline [recipe as in Pärt et al. (Pärt et al., 1998), but with the omission of PVP-40 and albumin] containing 50 i.u. ml^{-1} sodium heparin (Sigma-Aldrich, St Louis, MO, USA), and the rectal gland catheter with 500 mmol l^{-1} NaCl to duplicate normal secretion composition. The animal was then revived and placed in an individual Plexiglas^{TM} chamber served with aeration and a constant flow (>0.5 l min^{-1}) of fresh seawater. Dogfish were allowed to recover for at least 36 h before experiments commenced.

In vivo protocols

Secretion measurements

Rectal gland secretion flow proved to be negligible in these unfed resting dogfish. Therefore the animals were infused *via* the arterial catheter with 500 mmol l^{-1} NaCl at a rate of 15 ml kg^{-1} h^{-1} using a Minipuls peristaltic pump (Gilson, Middleton, WI, USA), France). This created a brisk, stable secretory flow from the gland, reaching a stable plateau from 1.5 to 6 h (cf. Wood et al., 2006), against which the effects of carbonic anhydrase inhibition could be assessed. The 1.5–3.0 h period served as the pre-treatment control, and the experiment was terminated at 6 h. Control animals (*N*=7) simply received the infusion alone for 6 h, whereas the experimental animals (*N*=6) received 20 mg kg^{-1} of acetazolamide (as the HCl salt, Wyeth-Lederle, Madison, NJ, USA) in 0.5 ml kg^{-1} dogfish saline *via* the arterial catheter at 3 h, and the experiment continued with 500 mmol l^{-1} NaCl infusion up to 6 h. This acetazolomide dose was calculated to produce an *in vivo* circulating concentration of about 10^{-4} mol l^{-1}, representing the level generally recognized as the maximum safe dose needed to achieve significant CA inhibition while avoiding non-specific effects (Maren, 1977). This concentration has been commonly used to produce effective CA inhibition in previous shark studies (Gilmour et al., 1997; Perry et al., 1999; Wilson et al., 2000; Gilmour and Perry, 2004). Rectal gland secretion flow was collected over successive 0.5 h periods, quantified gravimetrically, analysed immediately for acid–base status (pH and total CO_2) and frozen at –20°C for later ionic analysis. Blood samples (0.6 ml) were drawn from the arterial catheter at 1 h intervals, and analysed immediately for arterial blood gases and pH, while plasma was separated (2 min at 9000 ***g***) and frozen (–20°C) for later ion measurements. The red cell pellet was resuspended in dogfish saline, combined with blood recovered from the electrodes, and the original volume re-infused, so the impact of blood sampling on hematocrit was minimal.

Intracellular pH and fluid volume measurements

Intracellular pH was measured in the rectal gland as well as in white muscle (as a point of reference), together with extracellular acid–base status and fluid volume distribution in these tissues, in three treatments. These were unstimulated control (*N*=8), stimulated control (*N*=6), and stimulated plus acetazolamide-treated (*N*=6). In the first treatment, no infusion was given. The stimulated control (infusion with 500 mmol l^{-1} NaCl for 6 h) and stimulated acetazolamide treatments (infusion with 500 mmol l^{-1} NaCl for 6 h with administration of 20 mg kg^{-1} of acetazolamide at 3 h) were identical to the comparable treatments in the secretion measurement experiments.

The DMO (5,5-dimethyloxazolidine-2,4-dione) method (Waddell and Butler, 1959) was employed, using mannitol as the extracellular fluid volume marker. The two radiolabelled compounds – [^{3}H]mannitol (28 μCi kg^{-1}, specific activity 27.4 mCi $mmol^{-1}$) and [^{14}C]DMO (7 μCi kg^{-1}, specific activity 50.0 mCi $mmol^{-1}$), both from Dupont-NEN, Boston, MA, USA – were administered *via* the arterial catheter in 1 ml kg^{-1} of 500 mmol l^{-1} NaCl approximately 12 h prior to sampling to allow adequate equilibration time (Munger et al., 1991). Immediately prior to sacrifice, an arterial blood sample (1.0 ml) was drawn for blood gas and pH measurements, with a portion of plasma immediately separated by centrifugation (2 min at 9000 ***g***) and divided into aliquots for radioactivity measurements. The dogfish was then killed by a sharp cephalic blow and spinal section. The rectal gland and a section of epaxial muscle were immediately removed, blotted, weighed and processed for radioactivity measurements as described below. The capsule was removed from the rectal gland prior to processing.

In vitro perfused rectal gland preparation

Isolated-perfused rectal gland preparations (*N*=75) were made from dogfish in the 1.5–3.5 kg range. Each dogfish was anaesthetized with MS-222 (0.2 g l^{-1}), injected with 5000 i.u kg^{-1} of sodium heparin (Sigma-Aldrich, St Louis, MO, USA) *via* the caudal haemal arch, then killed by pithing with a steel wire inserted *via* the rostrum through the brain and spinal cord. The rectal gland artery, vein and duct were cannulated *in situ* as described (Shuttleworth, 1983) using PE50 polyethylene tubing (Clay-Adams, Parsipanny, NJ, USA). The arterial and venous catheters were filled with standard dogfish saline, with the following composition: 257 mmol l^{-1} NaCl, 7 mmol l^{-1} Na_2SO_4, 6 mmol l^{-1} $NaHCO_3$, 4 mmol l^{-1} KCl, 3 mmol l^{-1} $MgSO_4.7H_2O$, 2 mmol l^{-1} $CaCl_2.2H_2O$, 0.1 mmol l^{-1} Na_2HPO_4, 30 mmol l^{-1} glucose, 20 mmol l^{-1} trimethylamine oxide, 400 mmol l^{-1} urea. The saline was passed through a 0.45 μm filter and equilibrated with a precision gas mixture (0.256% CO_2, 99.744% O_2) so as to achieve control acid–base conditions (see below). The rectal gland duct catheter was filled with 500 mmol l^{-1} NaCl. The preparation was then dissected free and placed on a thermostatted platform.

Perfusion with control saline was initiated using the peristaltic pump/overflow system (Shuttleworth, 1983) to maintain an inflow pressure of ~20 mmHg. A windkessel reduced pressure pulsatility to ~3 mmHg and served as a

bubble trap. The perfusion reservoirs were fitted with gassing ports and a combination pH electrode (GK2401C, Radiometer-Copenhagen, Copenhagen, Denmark) for monitoring of gas equilibration. The reservoirs and the perfusion platform were jacketed with flowing seawater and the perfusion lines were immersed so as to maintain the experimental temperature at 11±1°C. Perfusion pressure was monitored by a P23 dB pressure transducer (Statham, Hato Rey, Puerto Rico) connected *via* a sidearm to the perfusion line immediately proximal to the gland. This T-junction also served as an arterial sampling point. A second T-junction just distal to the gland served as the venous sampling point. The venous outflow and the secretion outflow from the rectal gland duct were set to 0 mmHg relative to the gland, and drained through infrared drop-sensors connected to custom-built microprocessor-controlled digital flowmeters, so as to provide continuous flow records. The venous outflow (which is always less than the arterial inflow) represents the actual perfusion flow through the secretory parenchyma of the gland, whereas various non-secretory shunt pathways drain diffusely (Kent and Olsen, 1982). Therefore determinations of O_2 consumption ($\dot{M}_{O_2}$) and CO_2 production ($\dot{M}_{CO_2}$) rates by the perfused gland employed the Fick principle using the measured venous outflow rate and simultaneous arterial and venous measurements of total O_2 or CO_2 concentrations (see below).

In vitro protocols

Secretion measurements

After a series of pilot experiments, the following general protocol was established. The preparation was first perfused at ~20 mmHg (1 mmHg=133.3 Pa) with standard saline equilibrated with the 0.256% CO_2, 99.744% O_2 gas mixture so as to establish control acid–base conditions of P_{CO_2} ~1.9 mmHg, pH ~7.8, HCO_3^- ~5.3 mmol l^{-1} and a P_{O_2} >400 mmHg. After 0.75 h, samples of the rectal gland secretion, venous perfusate and arterial perfusate were collected for gas and acid–base analyses, and the secretion was frozen (–20°C) for the later analysis of ions. These sampling procedures took 15 min, and then at 1 h, 5×10^{-6} mol l^{-1} forskolin (Sigma-Aldrich) was added to the perfusion reservoir to stimulate secretion (Moran and Valentich, 1991; Forrest, Jr et al., 1997; Walsh et al., 2006). After a further 0.75–1 h, the sampling and analyses were repeated, and then the perfusion pressure was lowered to ~12 mmHg, a procedure chosen to decrease the perfusion flow through the gland without altering secretion flow so as to increase the precision of measurement of arterial-venous differences (see Results). A third set of sampling and analyses was performed after a further 0.75–1 h; these represented the pre-treatment control measurements. The experimental treatment was then implemented, while maintaining stimulation with 5×10^{-6} mol l^{-1} forskolin. In later preparations, only the pre-treatment control measurements were performed so the first two sets of analyses were omitted, but the gland was put through the same protocol so as to maintain consistency with earlier preparations.

N=6 was the minimum employed for each experimental treatment. The experimental treatments involved either the addition of 10^{-4} mol l^{-1} acetazolamide HCl (Wyeth-Lederle, Madison, NJ, USA) to the inflowing perfusate or alterations in its pH–P_{CO_2}–HCO_3^- status. The latter were accomplished by altering either its gassing or the concentration of $NaHCO_3$ or both. The objective was to achieve desired manipulations of extracellular and intracellular acid–base status (see Results). Manipulations were guided by the Henderson–Hasselbach equation:

$$pH = pK_{app} + \log[HCO_3^-] / \alpha_{CO_2} \times P_{CO_2} ,$$

using the solubility of carbon dioxide (α_{CO_2}) and the apparent pK (pK_{app}) for dogfish at the experimental temperature (Boutilier et al., 1984). When $NaHCO_3$ was experimentally elevated, NaCl was reduced on an equimolar basis, and *vice versa* so as to maintain osmolality. Alterations in P_{CO_2} were achieved by gassing the perfusate with the output from a 301-af gas mixing pump (Wosthof, Bochum, Germany), in which the mixing pistons were fed from various certified mixtures of CO_2 in O_2.

Experimental treatments lasted routinely 2 h, except for acetazolamide where the experimental period was 4 h. Sampling and analysis of rectal gland secretion, venous perfusate, and arterial perfusate were performed at 1 h intervals, except in the acetazolamide treatment where they were performed at 0.5 h intervals. In two series (control acid–base conditions, ~12 mmHg, 5×10^{-6} mol l^{-1} forskolin; 10^{-4} mol l^{-1} acetazolamide, ~12 mmHg, 5×10^{-6} mol l^{-1} forskolin), at the end of the experiment, a portion of the gland tissue was immediately freeze-clamped in liquid N_2 and stored at –80°C for later determination of tissue buffer capacity. In addition, in three experiments (glands from three different series), samples of the secreted fluid and venous effluent were immediately fixed in two volumes of ice-cold 8% $HClO_3$ for the assay of lactate, and the glands were freeze-clamped and stored as above. This tissue was later homogenized in ten volumes of ice-cold 8% $HClO_3$ for lactate analysis.

Intracellular pH and fluid volume measurements

The DMO method (Waddell and Butler, 1959), with mannitol as the extracellular fluid volume marker, was again employed, and measurements were made in each of the acid–base series, and in the acetazolamide treatment. Additional series (N=5–6) were run to measure fluid volume distribution and intracellular pH in the two treatments at the start of the perfusion protocol prior to lowering of the perfusion pressure: (i) non-stimulated preparations (control acid–base conditions, ~20 mmHg, no forskolin); and (ii) stimulated preparations (control acid–base conditions, ~20 mmHg, 5×10^{-6} mol l^{-1} forskolin).

[^{14}C]DMO (12.5 μCi l^{-1}) and [^{3}H]mannitol (50 μCi l^{-1}), both from NEN-Dupont, Boston, MA, USA, were added to the perfusion reservoirs 2 h before terminal sampling – i.e. at the start of most experimental treatments or after 2 h of acetazolamide treatment. Pilot experiments demonstrated that a time period of 2 h was sufficient to achieve full equilibration.

At termination, immediately after the final set of perfusion analyses, the rectal gland was blotted and weighed, and the capsule was removed prior to processing for radioactivity and water content measurements.

Analyses for intracellular pH and fluid volume calculations

The same methods were used for both *in vivo* and *in vitro* samples. Notably, the response time of this [^{14}C]DMO technique is less than 15 min, even in poorly perfused trout trunk muscle (Milligan and Wood, 1985), whereas a minimum of 2 h *in vitro* and 3 h *in vivo* was allowed for label redistribution in the present study on the much better perfused rectal gland. Triplicate samples (50–150 mg each) of the tissue were used for radioactivity measurements, and the remaining tissue was dried to a constant mass at 70°C in order to determine its total water content. Plasma water content was determined by refractometry using a Goldberg refractometer (American Optical TS meter, Buffalo, NY, USA) recalibrated for dogfish plasma. For radioactivity measurements, samples were added to 2 ml NCS (Amersham, Piscataway, NJ, USA) digest medium and incubated at 35–40°C for 12 h in sealed glass scintillation vials. The clear digests were then neutralized with glacial acetic acid, diluted with 10 ml OCS fluor (Amersham), and stored in the dark overnight to reduce chemiluminescence prior to scintillation counting for [^{14}C]DMO and [^{3}H]mannitol d.p.m. on an LKB Rackbeta 1217 (LKB-Wallac, Turku, Finland). Triplicate plasma samples from *in vivo* experiments, or inflow and outflow perfusate samples from *in vitro* experiments (100 μl each) were similarly processed and counted. Separation of ^{3}H and ^{14}C d.p.m. was achieved using on-board dual label quench-correction programs in the Rackbeta 1217, calibrated by the external standard ratio method. The coordinates of the programs were experimentally generated using a range of quenched samples from perfusate, plasma, rectal gland, and muscle tissue in the NCS/OCS system. Recovery of d.p.m. was regularly checked by spiking with known amounts of [^{3}H]mannitol and [^{14}C]DMO (internal standardization).

Tissue intracellular pHi, $[HCO_3^-]_i$, extracellular fluid volume (ECFV) and intracellular fluid volume (ICFV) were calculated from measurements of extracellular acid–base status, water contents of tissue, plasma or perfusate, and [^{14}C]DMO and [^{3}H]mannitol radioactivities in tissue, and plasma or perfusate, using equations given by Wright et al. (Wright et al., 1988). For *in vivo* experiments, arterial measurements of all parameters were used. For *in vitro* experiments, extracellular pH and P_{CO_2} were taken as the average of arterial and venous values, and inflow and outflow [^{14}C]DMO and [^{3}H]mannitol d.p.m. were similarly averaged for these calculations. The pK of DMO at the experimental temperature was interpolated from the measurements of Heisler et al. (Heisler et al., 1976) at comparable ionic strength.

Buffer capacity measurements

In two series, rectal gland tissue buffer capacity was measured by an acid titration of tissue homogenate using the protocol described (Wood et al., 1990). The slope of the curve relating mmol HCl added *versus* pH was linear over the pH range 7.0–7.8 (the operative pHi range *in vivo*) and taken as the buffer capacity of the tissue in mmol pH unit^{-1} kg^{-1}. This was then converted to mmol pH unit^{-1} l^{-1} intracellular water (slykes), taking into account the measured intracellular fluid volume. This technique measures total physico-chemical buffer capacity (i.e. non-HCO_3^- plus HCO_3^- buffering). However, as the original HCO_3^- content of the tissues is low, and the P_{CO_2} is close to zero during titration, the HCO_3^- component is negligible, and the results in essence yield an estimate of the non-HCO_3^- buffer capacity.

Analytical techniques

For *in vivo* analyses, blood samples were withdrawn *via* the arterial catheter into ice-cold, gas-tight Hamilton syringes (Reno, NV, USA). Arterial blood pH (pHa) and oxygen tension (Pa_{O_2}) were measured using Radiometer micro-electrodes (Radiometer-Copenhagen, Copenhagen, Denmark) kept at the experimental temperature with water jackets. Electrode outputs were displayed on Radiometer pHM 71 and pHM 72 acid–base analysers. True plasma CO_2 was measured on plasma obtained from blood samples centrifuged in sealed tubes. In some experiments, the measurements were made using a Corning 965 total CO_2 analyzer (Loughborough, UK) and in others the method of Cameron (Cameron, 1971); the two yielded identical results, though the former was more rapid. The same analytical methods (pH, total CO_2) were used for rectal gland secretions. Because of the low buffer capacity of the secretions, it was necessary to flush the pH electrode capillary 3 times with the sample over a 2 min period followed by a further 2 min of equilibration to achieve stable, reproducible values. Carbon dioxide tensions (P_{CO_2}) and bicarbonate concentrations ($[HCO_3^-]$) were calculated using the solubility of carbon dioxide (α_{CO_2}), the apparent pK (pK_{app}) for dogfish at the experimental temperature, and rearrangements of the Henderson–Hasselbalch equation (Boutilier et al., 1984).

For *in vitro* analyses, venous followed by arterial perfusate samples (typically 300 μl) were obtained anaerobically from the outflow and inflow ports of the perfusion set-up, by gentle withdrawal into ice-cold, gas-tight Hamilton syringes. The rectal gland secretion was diverted from the drop counter into the needle of a Hamilton syringe; the secretion was allowed to overflow directly through the open syringe barrel for 10 min, which was then sealed to yield a sample with negligible air exposure. Arterial (pHa), venous (pHv), and secretion pH, arterial (Pa_{O_2}) and venous oxygen tensions (Pv_{O_2}), and arterial (Ca_{CO_2}) and venous (Cv_{CO_2}) total CO_2 concentrations, were measured using the same equipment as for the *in vivo* samples. Again, because of the low buffer capacity of the perfusate and secretion fluid, it was necessary to pre-condition the pH capillary electrode when reading these samples, as outlined above. Pa_{O_2} and Pv_{O_2} values were converted to total O_2 concentrations (Ca_{O_2}, Cv_{O_2}) using tabulated solubility coefficients (Boutilier et al., 1984).

Na^+ and Cl^- in blood plasma and rectal gland secretions were

measured by atomic absorption spectrophotometry (Varian 1275 AA, Mulgrave, Victoria, Australia) and coulometric titration (Radiometer CMT-10, Radiometer-Copenhagen, Copenhagen, Denmark), respectively. For lactate analyses of fluid and gland samples, the deproteinized $HClO_3$ extracts were neutralized and assayed enzymatically by the L-lactate dehydrogenase method (Bergmeyer, 1983) using reagents from Sigma-Aldrich.

Statistics

Data are reported as means ± 1 s.e.m. (*N*). When a paired experimental design was used, single paired *t*-tests, or Dunnett's paired multiple comparison test, as appropriate, were used to compare treatment values back to pre-treatment measurements in the same glands or the same animals, or measurements of two different parameters in the same preparation. Specific differences between treatments were evaluated using Student's unpaired two-tailed *t*-test, with the Bonferroni correction applied when multiple comparisons were made. For comparisons across a range of treatments, data were first tested for normality and homogeneity of variances, and then analyzed by one-way ANOVA, followed by Tukey's honestly significant difference test to detect specific differences. Regression lines were fitted by linear and exponential models, and the significance of the correlation coefficient assessed. A significance level of 0.05 was used throughout.

Results

Effects of acetazolamide in vivo

Relative to a very stable rectal gland secretion rate in control infused dogfish, injection of 20 mg kg^{-1} of acetazolamide at 3 h caused a progressive decrease in secretion output of the rectal gland, which became significant by 4.5–5 h (Fig. 1A). However, there was also an accompanying fall in pHa (Fig. 1B) and rise in Pa_{CO_2} (Fig. 1C) and plasma HCO_3^- levels (Fig. 1D), all of which became significant by 4 h. By 6 h, pHa had decreased by ~0.35 units to ~7.4 due to a tripling of Pa_{CO_2} to ~4 mmHg, and rectal gland secretion rate had fallen by 53%. Na^+ and Cl^- concentrations in the secretion (both ~520 mmol l^{-1}) were not altered, but its pH was significantly depressed (6.85±0.16 at 5.5–6 h *versus* 7.30±0.07 in the control fish) and its P_{CO_2} elevated (4.38±2.01 *versus* 0.90±0.10 mmHg) by a comparable amount to the elevation in Pa_{CO_2}. Secretion HCO_3^- concentration was only marginally increased (0.99±0.19 *versus* 0.77±0.18 mmol l^{-1} in the control treatment). These changes in secretion P_{CO_2} and HCO_3^- concentration accompanying acetazolamide treatment were significant relative to the pre-treatment period in the same animals, but not relative to the control group. Plasma Na^+ and Cl^- levels (both ~320 mmol l^{-1}) and Pa_{O_2} (~90 mmHg) were unaffected (data not shown).

Intracellular pHi and fluid volume distribution were measured in three experimental treatments *in vivo*: unstimulated (non-infused) control, stimulated control (infused), and stimulated (infused) plus acetazolamide-treated

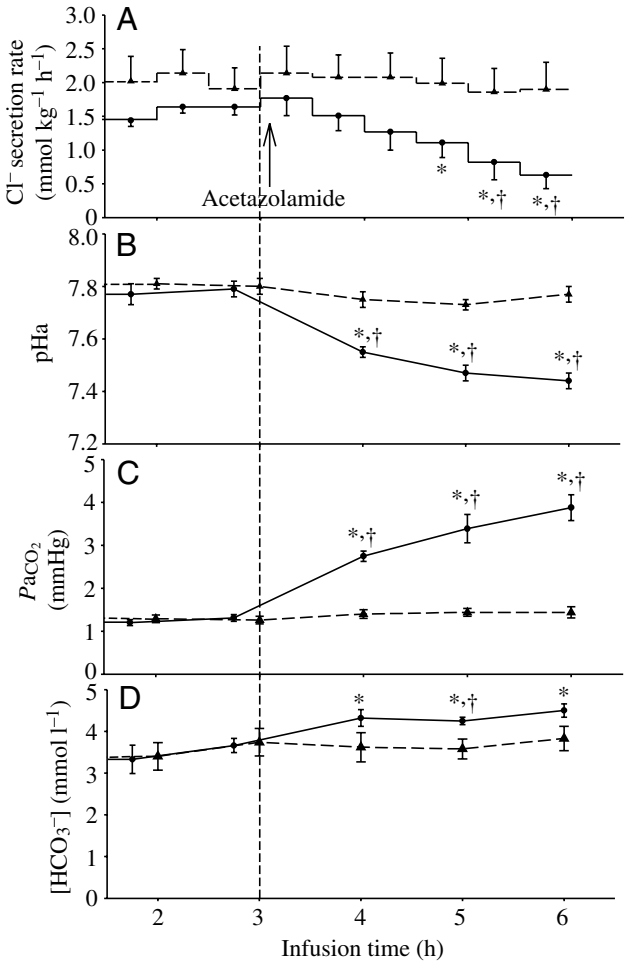

Fig. 1. The influence of acetazolamide (20 mg kg^{-1}) administered at 3 h on: (A) rectal gland Cl^- secretion rate: (B) arterial pH; (C) arterial CO_2 tension; and (D) arterial plasma bicarbonate concentration. Values are means ± 1 s.e.m. Control data are from fish (*N*=7) identically infused with 500 mmol l^{-1} NaCl but not treated with acetazolamide. *Values significantly different (P<0.05) from the simultaneous value in the control treatment; †values significantly different (P<0.05) from the pre-treatment reference values in the same treatment group.

(Table 1). In these smaller dogfish, infusion alone caused a small decrease in pHa, probably associated with dilution of plasma HCO_3^- (i.e. slight metabolic acidosis). Acetazolamide treatment caused the expected respiratory acidosis in the extracellular fluid. Intracellular pHi in the rectal gland and white muscle tended to fall with infusion, though the difference was only significant for the latter (Table 1). However the combination of infusion plus acetazolamide caused a significant intracellular acidosis in the gland tissue. Intracellular HCO_3^- concentration in the rectal gland rose significantly with acetazolamide treatment of volume-loaded fish, whereas there were no differences relative to non-infused fish. Similar trends were seen with intracellular HCO_3^- concentrations in the white muscle. There was also an increase in total rectal gland water content and ECFV in both treatments,

Table 1. *Intracellular pH, intracellular [HCO_3^-], and fractional fluid volume distribution in the rectal gland and white muscle of dogfish which were either not infused, or infused with 500 mmol l^{-1} NaCl for 6 h (15 ml kg^{-1} h^{-1}) in the presence or absence of acetazolamide injection (20 mg kg^{-1}) at 3 h and blood acid–base status in these same animals at 6 h*

	Non-infused (N=8)	Infused (N=6)	Infused + Acetazolamide (N=6)
pHa	7.88±0.02	7.81±0.01*	7.68±0.03*,†
Pa_{CO_2} (mmHg)	1.41±0.11	1.36±0.11	2.43±0.20*,†
[HCO_3^-]a (mmol l^{-1})	4.90±0.24	4.00±0.24*	5.94±0.15*,†
pHi			
Rectal gland	7.55±0.02	7.45±0.05	7.34±0.04*
Muscle	7.39±0.04	7.21±0.04*	7.27±0.03
[HCO_3^-]i (mmol l^{-1} ICF)			
Rectal gland	2.85±0.34	2.08±0.21	3.15±0.24†
Muscle	1.89±0.25	1.09±0.11*	2.60±0.25†
Rectal gland			
Water	0.7407±0.0036	0.7584±0.0043*	0.7623±0.0032*
ECFV	0.2201±0.0030	0.2633±0.0129*	0.2864±0.0120*
ICFV	0.5207±0.0026	0.4951±0.0167	0.4759±0.0141*
Muscle			
Water	0.7324±0.0093	0.7223±0.0068	0.7708±0.0013*,†
ECFV	0.0941±0.0068	0.0826±0.0150	0.0901±0.0034
ICFV	0.6383±0.0128	0.6288±0.0078	0.6798±0.0042*,†

Values are means ± 1 s.e.m.
*Significantly different (P<0.05) from respective value in the non-infused treatment.
†Significantly different (P<0.05) from respective value in the infused treatment.
ECFV, fractional extracellular fluid volume; ICFV, fractional intracellular fluid volume; [HCO_3^-]a/i, arterial/intracellular [HCO_3^-].

as well as an increase in rectal gland ICFV in the infusion + acetazolamide treatment. Muscle water and ICFV increased in the infusion + acetazolamide treatment.

Characteristics of the perfused gland in vitro

In the unstimulated rectal gland preparation perfused initially at an inflow pressure of 20 mmHg, there was no detectable secretion flow (<2 μl g^{-1} gland min^{-1}). After stimulation with 5×10^{-6} mol l^{-1} forskolin, perfusion flow increased modestly (by 18%), and secretion flow was activated at a brisk rate (25–30 μl g^{-1} gland min^{-1}). This was accompanied by increases in the gradients of pH, P_{CO_2}, total O_2 and total CO_2 between arterial inflow and venous outflow, achieved almost entirely by changes in the venous outflow values (Table 2). The activation of secretion was therefore accompanied by 3–4-fold increases in $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ (Table 2). Notably $\dot{M}_{CO_2}$ was approximately twice as large as $\dot{M}_{O_2}$ in both treatment conditions, but the measurements of $\dot{M}_{CO_2}$ were of lower precision, because of the difficulty of resolving small arterial–venous differences against the background total CO_2 concentrations in the perfusate.

To increase the precision of measurement of arterial–venous differences in the stimulated gland, the perfusion pressure was decreased to approximately 12 mmHg, which reduced the perfusion flow rate by 34% (Table 2). There was no significant decrease in rectal gland secretion rate, but the arterial–venous gradients in pH, P_{CO_2}, total O_2, and total CO_2 all increased. Again, these were achieved largely by changes in the venous outflow values, though there were also some small differences in arterial inflow values. $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ did not change significantly, but the discrepancy between them was reduced (Table 2). Notably, while the pH of the secretion was well below the venous outflow level, its P_{CO_2} remained fairly close to arterial rather than venous values. Secretion total CO_2 concentrations were only about 20% of those in the perfusate. In all cases, HCO_3^- concentrations (data not shown) remained similar to but slightly smaller than total CO_2 concentrations in perfusate and secretion samples. Pilot experiments demonstrated that all parameters remained approximately stable for up to 6 h of perfusion under these conditions.

Intracellular pHi (terminal measurements) and [HCO_3^-]i, together with fractional fluid volume distributions, were determined in separate groups of glands under the three perfusion conditions (Table 3). Total O_2 and CO_2 concentrations, as well as acid–base characteristics of both inflowing and outflowing perfusate (data not shown), did not differ significantly from the values for the respective treatments in Table 2. Intracellular pHi was about 0.3 units below mean extracellular pHe at rest, and did not change significantly when the glands were stimulated with 5×10^{-6} mol l^{-1} forskolin, or when inflow pressure was reduced in the continued presence of forskolin. However, all these treatments exhibited a significantly greater [HCO_3^-]i relative to the unstimulated control. The stability of pHi was seen despite the observed decreases in pHe (Table 3) under these conditions due to the falls in pHv (cf. Table 2). Fractional water content remained

Table 2. *Characteristics of perfused dogfish rectal gland preparations*

	Unstimulated (N=14)	Stimulated (N=14)	Stimulated – reduced pressure (N=52)
Perfusion flow (ml g^{-1} gland min^{-1})	2.19±0.23	2.58±0.37*	1.70±0.17*,†
pHa	7.84±0.01	7.84±0.01	7.79±0.01*,†
pHv	7.81±0.01	7.69±0.03*	7.43±0.02*,†
Pa_{CO_2} (mmHg)	1.59±0.06	1.67±0.06	2.02±0.04*,†
Pv_{CO_2} (mmHg)	1.76±0.06	2.63±0.28*	5.37±0.03*,†
Ca_{CO_2} (mmol l^{-1})	5.27±0.05	5.44±0.05	5.62±0.06*
Cv_{CO_2} (mmol l^{-1})	5.36±0.04	5.73±0.07*	6.08±0.06*,†
Ca_{O_2} (mmol l^{-1})	0.81±0.01	0.83±0.01	0.82±0.01
Cv_{O_2} (mmol l^{-1})	0.75±0.02	0.67±0.03*	0.50±0.02*,†
Secretion flow (μl g $gland^{-1}$ min^{-1})	ND	29.3±3.4*	25.3±1.3*
Secretion pH	–	7.32±0.04	7.18±0.02†
Secretion P_{CO_2} (mmHg)	–	1.04±0.22	2.13±0.16†
Secretion CO_2 (mmol l^{-1})	–	0.89±0.14	1.35±0.08
$\dot{M}_{O_2}$ (μmol g^{-1} gland min^{-1})	0.11±0.01	0.38±0.07*	0.43±0.03*
$\dot{M}_{CO_2}$ (μmol g^{-1} gland min^{-1})	0.20±0.05	0.78±0.16*	0.67±0.07*

Values are means ± 1 s.e.m.

*Significantly different (P<0.05; paired test) from respective unstimulated value.

†Significantly different (P<0.05; unpaired test) from respective stimulated value.

The same 14 preparations were examined in the unstimulated and stimulated states (5×10^{-6} mol l^{-1} forskolin) at an inflow perfusion pressure of approximately 20 mmHg. A largely different set of 52 stimulated preparations (5×10^{-6} mol l^{-1} forskolin), including 9 from the original 14, were examined after the inflow perfusion pressure was decreased to approximately 12 mmHg.

unchanged but was clearly re-distributed, with ECFV increasing, and ICFV decreasing reciprocally after stimulation with forskolin, a situation which persisted after reduction of the inflow pressure.

There was a very strong linear relationship (r=0.99, N=127, P<0.0001) between secretion flow rate and Cl^- secretion rate in the perfused, forskolin-stimulated rectal glands, over a wide secretory range (Fig. 2A). The slope of the relationship indicated a mean Cl^- concentration of 520 mmol l^{-1} in the secretion, which may be compared with 270 mmol l^{-1} in the perfusate. While data were initially analysed according to whether the gland was at high pressure (~20 mmHg), reduced pressure (~12 mmHg), or subjected to a particular experimental treatment, it became clear that a single linear relationship fitted all the data. In those samples (N=32) where Na^+ concentration was monitored simultaneously, it was indistinguishable from Cl^- concentration (both ~520 mmol l^{-1}). Because of this consistency, Cl^- secretion rate was used as the indicator of secretory performance in all further analyses, as *in vivo*.

Again using all the data points, including those obtained in the absence of forskolin, there was a strong linear relationship (r=0.73, N=139, P<0.0001) between Cl^- secretion rate and the

Table 3. *Intracellular pH (pHi), bicarbonate ($[HCO_3^-]i$) and fractional fluid volume distributions of perfused rectal gland preparations*

	Unstimulated (N=5)	Stimulated (N=6)	Stimulated – reduced pressure	
			Control (N=6)	Acetazolamide (N=7)
pHe	7.86±0.01	7.73±0.05*	7.58±0.05*	7.56±0.01*
pHi	7.50±0.01	7.57±0.03	7.50±0.05	7.47±0.02
$[HCO_3^-]i$ (mmol l^{-1} ICF)	2.39±0.07	4.60±0.44*	6.18±0.65*	6.07±0.39*
Water	0.7605±0.0017	0.7586±0.0019	0.7581±0.0039	0.7597±0.0036
ECFV	0.2059±0.0046	0.2473±0.0072*	0.2819±0.0156*	0.2560±0.0086*
ICFV	0.5546±0.0049	0.5112±0.0075*	0.4762±0.0164*	0.5037±0.0074*

Values are means ± 1 s.e.m.

*Significantly different (P<0.05) from respective stimulated value.

There were no other significant differences amongst treatments.

Separate groups were examined in the unstimulated and stimulated states (5×10^{-6} mol l^{-1} forskolin) at an inflow perfusion pressure of approximately 20 mmHg, in the stimulated state after reduction in inflow pressure to approximately 12 mmHg (‘control’), and in the stimulated state at reduced pressure 4.5 h after addition of 10^{-4} mol l^{-1} acetazolamide to the perfusate.

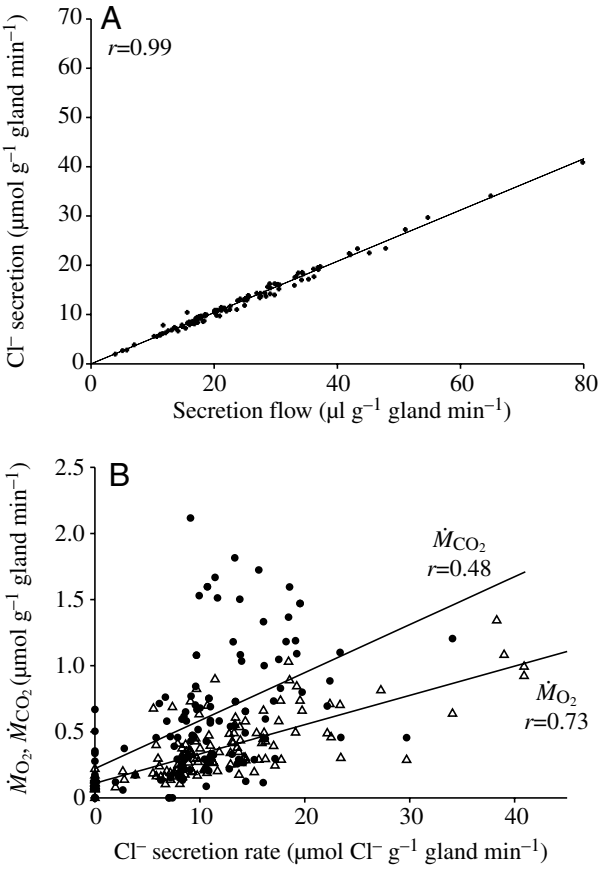

Fig. 2. (A) The relationship between secretion flow rate (*x* axis) and Cl^- secretion rate (*y* axis) in perfused rectal gland preparations stimulated with 5×10^{-6} mol l^{-1} forskolin. $y=0.52x-0.01$ ($r=0.99$, $N=127$, $P<0.0001$). The slope (0.52) of the regression line indicates that the average concentration of Cl^- in the secretion was 520 mmol l^{-1}. Simultaneous measurements obtained at high perfusion pressure (~20 mmHg), reduced perfusion pressure (~12 mmHg), and after all experimental treatments at reduced perfusion pressure (~12 mmHg), are plotted: $N=127$ data points from 71 preparations. (B). Open triangles: the relationship between Cl^- secretion rate (*x*) and oxygen consumption rate ($\dot{M}_{O_2}$; *y*) in all perfused rectal gland preparations. $y=0.022x+0.11$ ($r=0.73$, $N=139$, $P<0.0001$). Simultaneous measurements obtained at high perfusion pressure (~20 mmHg) in the absence of stimulation (no Cl^- secretion), and in the presence of stimulation with 5.5×10^{-6} mol l^{-1} forskolin at high perfusion pressure (~20 mmHg), at reduced perfusion pressure (~12 mmHg), and after all experimental treatments at reduced perfusion pressure (~12 mmHg), are plotted: $N=139$ data points from 71 preparations. Closed circles: the relationship between Cl^- secretion rate (*x*) and carbon dioxide excretion rate ($\dot{M}_{CO_2}$; *y*) in perfused rectal gland preparations. $y=0.036x+0.22$ ($r=0.48$, $N=117$, $P<0.0001$). The same set of simultaneous measurements as used for $\dot{M}_{O_2}$ are plotted, with the exception of points obtained at high perfusate HCO_3^- concentration (where it was impossible to resolve small arterial–venous differences in total CO_2 concentrations) and after treatment with acetazolamide (which transiently inhibited CO_2 excretion). $N=117$ data points from 68 preparations.

simultaneous rate of oxygen consumption ($\dot{M}_{O_2}$) of the perfused glands (Fig. 2B). The relationship was not altered or improved by considering any of the perfusion conditions or experimental treatments separately. The intercept at zero Cl^- secretion was positive, indicating that unstimulated glands consume O_2 at a rate of about 0.11 μmol g^{-1} gland min^{-1}, in accord with the mean measured value (0.11 μmol g^{-1} gland min^{-1}) of Table 2.

The rate of carbon dioxide excretion ($\dot{M}_{CO_2}$) by the perfused glands was more difficult to measure, and essentially undetectable when high HCO_3^- salines were used because it was impossible to resolve small arterial–venous differences in total CO_2 concentrations. The analysis of $\dot{M}_{CO_2}$ (*versus* Cl^- secretion rate) therefore uses the same set of data points as for the analysis of $\dot{M}_{O_2}$, but without those obtained with high HCO_3^- saline, or under acetazolamide treatment (which could potentially inhibit CO_2 excretion). The relationship between Cl^- secretion rate and $\dot{M}_{CO_2}$ was much more variable than for $\dot{M}_{O_2}$, but still highly significant ($r=0.48$, $N=117$, $P<0.0001$; Fig. 2B). The intercept at zero Cl^- secretion was 0.22 μmol g^{-1} gland min^{-1}, comparable to the mean measured value (0.20 μmol g^{-1} gland min^{-1}) for unstimulated glands in Table 2. However, the two regression lines were not statistically different, and there was no significant difference when all simultaneous measurements of $\dot{M}_{CO_2}$ *versus* $\dot{M}_{O_2}$ were compared ($N=117$; paired *t*-test).

Lactate concentrations measured in three perfused glands were 7.15±1.19 mmol kg^{-1}; lactate concentrations in the secreted fluid and venous effluent were below the resolution of the assay (<0.06 mmol l^{-1}).

Responses to acetazolamide in the perfused gland in vitro

Addition of 10^{-4} mol l^{-1} acetazolamide to the perfusate had no significant effect on Cl^- secretion rate (Fig. 3A), $\dot{M}_{O_2}$ (Fig. 3B), or perfusion rate (data not shown) in the stimulated rectal gland. However $\dot{M}_{CO_2}$ exhibited a transient decline of about 30%, which became significant at 2 h, 2.5 h and 3 h, but recovered by 4 h (Fig. 3C). The gas exchange ratio ($R=\dot{M}_{CO_2}/\dot{M}_{O_2}$) declined significantly from 1.41±0.06 under control conditions to 1.04±0.17 at 2 h and 1.01±0.22 at 2.5 h, and then recovered to 1.30±0.08 by 4 h. Pa_{CO_2} (controlled experimentally) and Pv_{CO_2} did not change significantly, but the P_{CO_2} of the secretion was significantly elevated by about 1.5 mmHg at most times from 1.0 to 3.5 h of acetazolamide treatment (Fig. 4). There were no significant changes in pHa, pHv, $[HCO_3^-]a$, or secretion pH, whereas $[HCO_3^-]v$ rose in mirror image to the transient decline in $\dot{M}_{CO_2}$, and secretion HCO_3^- levels increased slightly in accord with increases in secretion P_{CO_2} (data not shown).

Intracellular pH (pHi) and $[HCO_3^-]i$ of the rectal gland, measured at the end of the 4 h acetazolamide treatment, remained unchanged relative to the control value, and fractional water content, ECFV and ICFV were also unaffected (Table 3).

The non-HCO_3^- buffer capacity of the forskolin-activated gland perfused under control conditions (normal perfusate P_{CO_2}

and HCO_3^-, 12 mmHg) was 35.28±2.10 (6) slykes per unit intracellular fluid [16.83±0.79 (6) mmol pH unit^{-1} kg^{-1} on a whole tissue basis] and this value remained unchanged at 33.97±1.03 (6) slykes [16.87±0.32 (6) mmol pH unit^{-1} kg^{-1}] after treatment with 10^{-4} mol l^{-1} acetazolamide for 4 h.

Responses of the perfused gland to acid–base manipulations in vitro

P_{CO_2} and $[HCO_3^-]$ in the inflowing perfusate were experimentally manipulated in order to discern the relative importance of extracellular and intracellular pH, $[HCO_3^-]$, and/or P_{CO_2}, in controlling the rate of secretion in the stimulated rectal gland. The measured acid–base status of the inflowing and outflowing perfusate and estimated $[HCO_3^-]$i for all treatments are detailed in Table 4, and the secretory response of the rectal gland relative to pHe or pHi is illustrated in Fig. 5. The control glands (control P_{CO_2}, control $[HCO_3^-]$) were simply put through the same manipulations but without change in the acid–base status of the inflowing perfusate, and exhibited a 14% decline in secretion rate. There were no significant changes in fractional water content, ECFV or ICFV of the glands in any of the treatments (data not shown), mean values staying close to the control data of Table 3.

Approximately equal depressions of both pHe (by ~0.3 units) and pHi (by about 0.15 units) were accomplished in one group by raising P_{CO_2} at control $[HCO_3^-]$, and in another by lowering $[HCO_3^-]$ at control P_{CO_2}. Both treatments resulted in comparable marked inhibitions (by ~50%) of rectal gland secretion, despite very different changes in intracellular and extracellular $[HCO_3^-]$ and P_{CO_2}, suggesting that pH rather than P_{CO_2} or $[HCO_3^-]$ was the important factor (Fig. 5, Table 4). This conclusion was reinforced by a treatment where both P_{CO_2} and $[HCO_3^-]$ were lowered so as to maintain pHe and pHi unaltered. Rectal gland secretion rate remained unchanged relative to the control treatment despite the changes in intracellular and extracellular $[HCO_3^-]$ and P_{CO_2}. Further support for this conclusion and some distinction between the importance of pHi and pHe were obtained by a treatment where both P_{CO_2} and $[HCO_3^-]$ were raised. Rectal gland secretion rate again remained unchanged despite a marked rise in $[HCO_3^-]$i and a marked fall in pHi, the latter equal to that seen in the two treatments that had caused about a 50% inhibition of rectal gland secretion rate (Fig. 5, Table 4). This suggests that pHe, rather than pHi, may be the key controlling factor. Notably, however, pHe was significantly elevated in this treatment. When pHe was further raised to about 1.0 unit above control

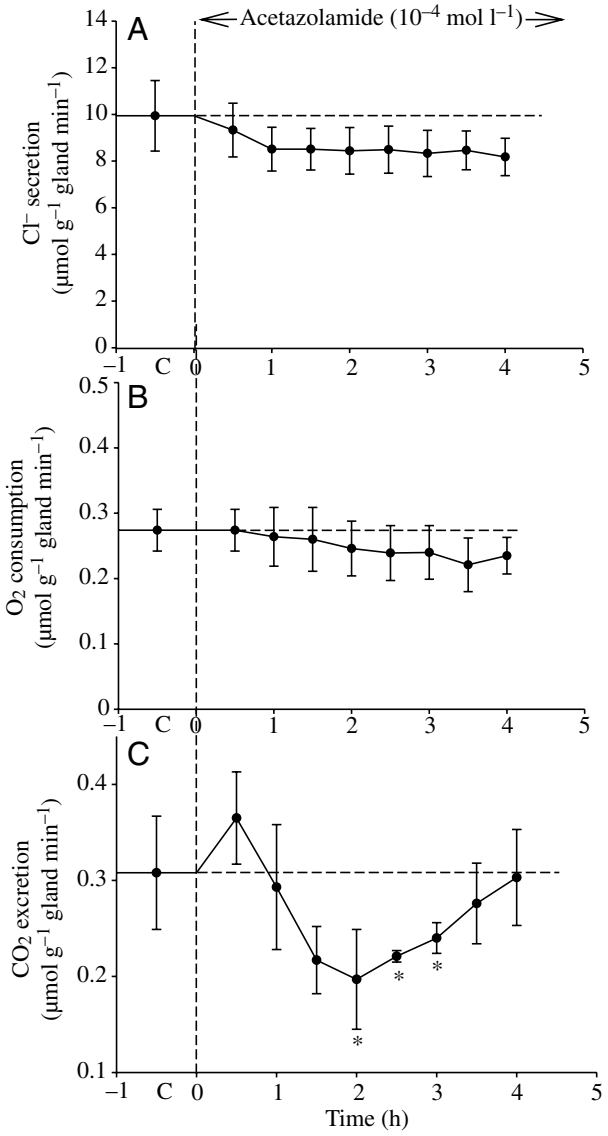

Fig. 3. The influence of 10^{-4} mol l^{-1} acetazolamide added to the perfusion saline at 0 h on (A) Cl^- secretion rate, (B) oxygen consumption rate ($\dot{M}_{O_2}$) and (C) carbon dioxide excretion rate ($\dot{M}_{CO_2}$) in the perfused rectal gland. Asterisks indicate means significantly different (P<0.05) from the control value at 'C' before acetazolamide addition. Values are means ± 1 s.e.m. (N=6).

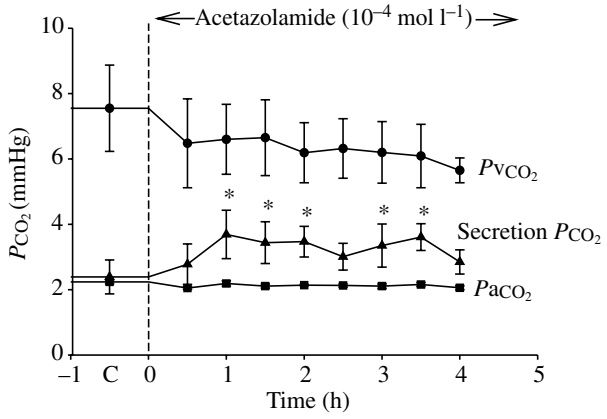

Fig. 4. The influence of 10^{-4} mol l^{-1} acetazolamide added to the perfusion saline at 0 h on P_{CO_2} levels in arterial perfusate inflow, venous perfusate outflow, and secretion fluid. Asterisks indicate means significantly different (P<0.05) from the respective control value at 'C' before acetazolamide addition. Values are means ± 1 s.e.m. (N=6).

Table 4. *Acid–base characteristics of arterial inflow, venous outflow and extracellular and intracellular pH (pHi) and bicarbonate ($[HCO_3^-]i$) in rectal gland preparations perfused at an inflow pressure of approximately 12 mmHg, stimulated with 5×10^{-6} mol l^{-1} forskolin, and subjected to various experimental manipulations of perfusate P_{CO_2} and $[HCO_3^-]$*

	Control P_{CO_2} Control $[HCO_3^-]$ (N=6)	High P_{CO_2} Control $[HCO_3^-]$ (N=6)	Control P_{CO_2} Low $[HCO_3^-]$ (N=7)	Low P_{CO_2} Low $[HCO_3^-]$ (N=6)	High P_{CO_2} High $[HCO_3^-]$ (N=6)	LowP_{CO_2} Control $[HCO_3^-]$ (N=6)	Control P_{CO_2} High $[HCO_3^-]$ (N=6)
pHa	7.76±0.02[a]	7.33±0.03[b]	7.33±0.05[b]	7.72±0.04[a]	7.88±0.02[c]	8.44±0.02[d]	8.65±0.03[e]
pHv	7.40±0.09[a]	7.17±0.06[b]	7.16±0.05[b]	7.38±0.05[a]	7.82±0.01[c]	7.93±0.04[c]	8.52±0.03[d]
pHe	7.58±0.05[a]	7.28±0.03[b]	7.24±0.05[b]	7.55±0.04[a]	7.85±0.01[c]	8.19±0.03[d]	8.58±0.03[e]
pHi	7.50±0.05[a]	7.32±0.02[b]	7.35±0.04[b]	7.49±0.05[a]	7.29±0.07[b]	7.67±0.02[d]	7.68±0.06[a,d]
Pa_{CO_2} (mmHg)	1.98±0.10[a]	6.76±0.38[b]	1.88±0.13[a]	0.70±0.07[d]	11.37±0.86[c]	0.37±0.01[e]	1.60±0.13[a]
Pv_{CO_2} (mmHg)	5.82±1.30[a]	10.41±1.43[b]	3.22±0.38[a]	1.89±0.15[c]	13.27±0.59[b]	1.48±0.15[c]	2.27±0.18[a,c]
$[HCO_3^-]a$ (mmol l^{-1})	5.15±0.10[a]	5.98±0.18[a]	1.72±0.22[b]	1.70±0.18[b]	40.03±0.97[c]	5.43±0.09[a]	38.93±1.23[c]
$[HCO_3^-]v$ (mmol l^{-1})	5.49±0.09[a]	6.02±0.22[a]	1.88±0.21[b]	1.99±0.23[b]	40.63±1.04[c]	5.73±0.12[a]	39.87±1.24[c]
$[HCO_3^-]i$ (mmol l^{-1} ICF)	6.18±0.65[a]	7.85±0.45[a]	2.76±0.24[b]	2.09±0.39[b]	12.20±1.65[c]	1.97±0.13[b]	5.56±1.08[a]

Values are means ± 1 s.e.m. Means sharing the same letter are not significantly different (P>0.05).
Separate groups were examined in each treatment.
ICF, intracellular fluid.

pHe, by increasing $[HCO_3^-]$ at control P_{CO_2}, a treatment that did not significantly alter pHi or $[HCO_3^-]i$, there was a dramatic stimulation (2.3-fold) of rectal gland secretion rate (Fig. 5, Table 4). A smaller increase in pHe (~0.6 units) achieved by reduction of P_{CO_2} at control $[HCO_3^-]$ resulted in a very similar pHi, but a reduced $[HCO_3^-]i$, and a secretion rate which was slightly elevated but not-significantly different from the control. Overall, these results suggest that pHe, rather than P_{CO_2}, or intracellular or extracellular $[HCO_3^-]$, may be the dominant influence on rectal gland secretion rate, but do not eliminate a role for pHi (see Discussion).

As expected from the relationship in Fig. 2B, changes in rectal gland $\dot{M}_{O_2}$ varied in parallel to changes in rectal gland secretion rate in the various experimental treatments (data not shown). However, on a relative basis they tended to be smaller – e.g. ~24% *versus* ~50% inhibitions in the two extracellular

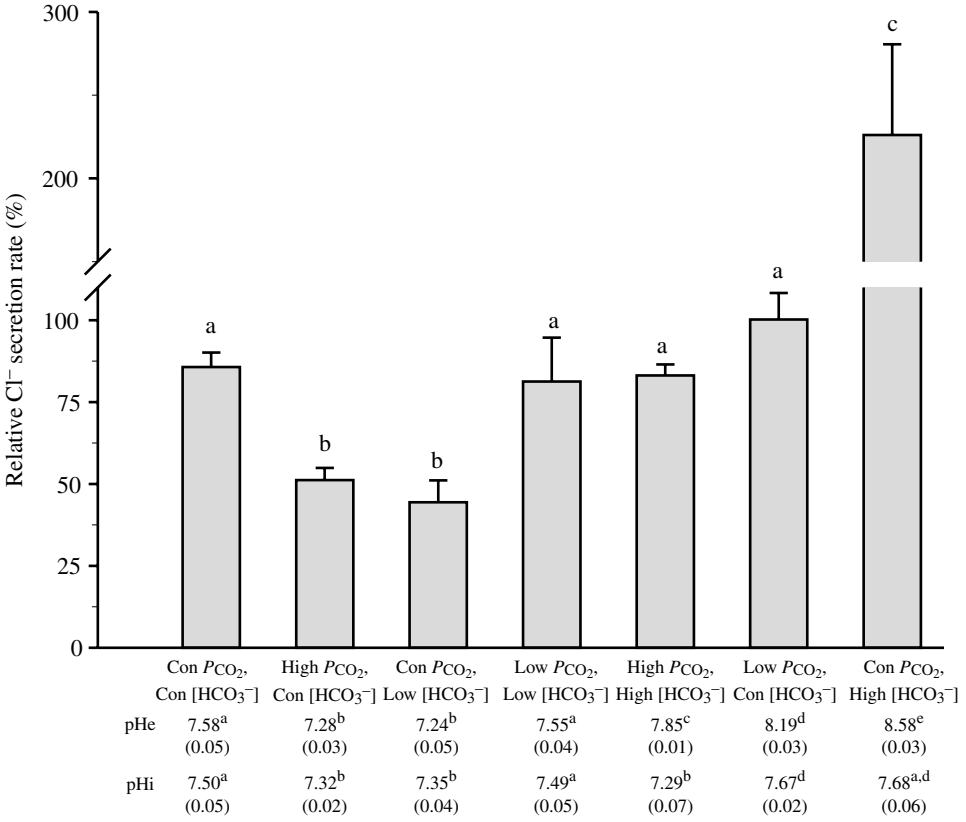

Fig. 5. The influence of experimental manipulations of acid–base status on the relative rate of Cl^- secretion rate in the perfused rectal gland. Secretion rate after 2 h of experimental treatment is expressed as a percentage of the pre-treatment control (Con) measurement. Measured values of extracellular pHe and intracellular pHi are given; for other acid–base parameters, see Table 4. Means sharing the same letter are not significantly different (P>0.05). Values are means (+1 s.e.m.) (N=6–7).

Table 5. *Acid–base characteristics of rectal gland secretion in preparations perfused at an inflow pressure of approximately 12 mmHg, stimulated with 5×10^{-6} mol l^{-1} forskolin, and subjected to various experimental manipulations of perfusate P_{CO_2} and [HCO_3^-]*

	Control P_{CO_2} Control [HCO_3^-] (N=6)	High P_{CO_2} Control [HCO_3^-] (N=6)	Control P_{CO_2} Low [HCO_3^-] (N=7)	Low P_{CO_2} Low [HCO_3^-] (N=6)	High P_{CO_2} High [HCO_3^-] (N=6)	Low P_{CO_2} Control [HCO_3^-] (N=6)	Control P_{CO_2} High [HCO_3^-] (N=6)
pH	7.32±0.09^{a}	7.05±0.05^{a,b}	7.02±0.03^{b}	7.24±0.09^{a,b}	7.28±0.08^{a,b}	7.85±0.09^{c}	8.28±0.04^{d}
P_{CO_2} (mmHg)	2.06±0.63^{a,b}	3.08±0.25^{b}	1.84±0.16^{a,b,c}	0.70±0.36^{a,c,e}	7.73±0.94^{d}	0.17±0.06^{e}	0.32±0.04^{e}
[HCO_3^-] (mmol l^{-1})	1.29±0.28^{a,b}	1.44±0.08^{a}	0.75±0.10^{b}	0.36±0.09^{b}	5.97±1.01^{c}	0.63±0.20^{b}	3.26±0.47^{c}

Values are means ± 1 s.e.m. Means sharing the same letter are not significantly different (P>0.05).
Separate groups were examined in each treatment. See Table 4 for acid–base characteristics of the perfusates.

acidosis treatments, 1.9-fold *versus* 2.3-fold stimulation in the extracellular alkalosis treatment, and only the latter change in $\dot{M}_{O_2}$ was statistically significant. This reflected the fact that a portion of rectal gland $\dot{M}_{O_2}$ is devoted to routine metabolism and occurs even in the absence of secretion, as well as the greater variability in $\dot{M}_{O_2}$ measurements (Table 2, Fig. 2B).

The acid–base characteristics of the secretions from the rectal glands subjected to the various acid–base manipulations are summarized in Table 5. In general, secretion pH was about 0.3–0.5 units below pHa and a more variable amount (0.1–0.5 units) below pHv. Secretion pH declined in those treatments where the extracellular fluid was acidotic, and increased in those treatments where it was alkalotic, though only some of these changes were statistically significant. Thus secretion pH correlated with changes in pHe rather than pHi: note for example, the unchanged secretion pH associated with the high P_{CO_2}, high [HCO_3^-] treatment (where pHi was depressed), and the greatly elevated secretion pH associated with the control P_{CO_2}, high [HCO_3^-] treatment where pHi was unchanged. Changes in secretion P_{CO_2} reflected changes in the Pa_{CO_2} of inflowing perfusate, but were generally smaller than those in Pa_{CO_2}. In all cases (Table 5), secretion P_{CO_2} remained well below Pv_{CO_2} (Table 4). Secretion HCO_3^- concentrations reflected changes in perfusate HCO_3^- concentrations, but remained only a small fraction (8–42%) of the latter.

Discussion

Effects of acetazolamide in vivo

Systemic treatment with 20 mg kg^{-1} acetazolamide (calculated to produce an *in vivo* concentration of about 10^{-4} mol l^{-1}) caused a profound inhibition of rectal gland secretion (by 53±6% after 3 h) in the intact, unanaesthetized dogfish (Fig. 1A). In general, this result is similar to that of Swenson and Maren (Swenson and Maren, 1984) on the *in situ* gland preparation in pithed dogfish subjected to a similar volume-loading protocol, where the degree of inhibition by another CA blocker (methazolamide) was about 50%. However, key points differ. In the present study, there was also a significant fall in rectal gland pHi (Table 1) accompanying CA inhibition, a point to which we return later in our discussion of role of carbonic anhydrase in rectal gland function. Swenson and Maren (Swenson and Maren, 1984) did not measure pHi, but reported a paradoxical 40% decrease in rectal gland CO_2 content after CA inhibition (from 18 to 11 mmol kg^{-1}), for which the mechanism remained unexplained. In the present study, total gland CO_2 content was not measured directly, but [HCO_3^-]i in the rectal gland increased significantly (from 2.08 to 3.15 mmol l^{-1} ICF; Table 1), as might be expected during respiratory acidosis. The gross discrepancy in absolute values and opposite direction of the changes between the two studies suggests that the *in situ* preparation (Swenson and Maren, 1984) may have been severely perfusion-limited due to a depressed circulation resulting in CO_2 retention, such that methazolamide treatment of the whole animal put the gland into metabolic depression or metabolic acidosis. Swenson and Maren attempted a more specific inhibition of gland CA alone using a low dose of benzolamide (Swenson and Maren, 1984), but a systemic P_{CO_2} rise still occurred; they reported a small fall in secretion rate and a large rise in secretion P_{CO_2}. While the present results on the intact animal appear more coherent, in neither study can we attribute the decreases in secretion rate specifically to an effect on rectal gland CA. This is because the accompanying systemic respiratory acidosis, which has been demonstrated in previous studies on CA inhibition in *Squalus acanthias* (e.g. Swenson and Maren, 1984; Swenson and Maren, 1987; Gilmour et al., 1997; Gilmour et al., 2001; Perry et al., 1999; Gilmour and Perry, 2004), could have contributed a major portion of the effect. Indeed Swenson and Maren (Swenson and Maren, 1984) demonstrated inhibitory effects on secretion of severe respiratory and metabolic acidoses alone (without CA inhibition), and fully recognized this complication. Recently, we have established a relationship between pHa and secretion rate in intact *Squalus acanthias* subjected to an identical volume-loading protocol, and in which acid–base status was experimentally manipulated without using acetazolamide (Wood et al., 2006). Based on this regression, the fall in pHa (to 7.455±0.034; Fig. 1B) alone caused by acetazolamide would have caused a 31% inhibition of secretion, whereas the actual inhibition by acetazolamide in the intact animal was 53±6%, right at the 95% confidence limit of the relationship. We therefore turned to the *in vitro* perfused gland preparation to more clearly dissect these influences.

Acid–base control of rectal gland secretion

The present results confirm that secretion in the isolated-perfused rectal gland is very sensitive to acid–base status, in agreement with several early studies on the perfused gland *in vitro* (Siegel et al., 1975; Silva et al., 1992), on the *in situ* gland preparation in the pithed, artificially ventilated dogfish (Swenson and Maren, 1984), and our recent investigation on the intact, unanaesthetized dogfish (Wood et al., 2006). However our data extend these studies by dissecting out the influences of various extracellular and intracellular acid–base parameters. As noted in Results, the results overall point to pHe as the dominant influence, but pHi may also be involved. Thus the inhibitory action associated with low pHe occurred to the same extent regardless of whether pHe was reduced by raising P_{CO_2} at control $[HCO_3^-]$e and $[HCO_3^-]$i (respiratory acidosis) or by lowering $[HCO_3^-]$e and $[HCO_3^-]$i at control P_{CO_2} (metabolic acidosis) (Fig. 5). Furthermore, lowering both P_{CO_2} and $[HCO_3^-]$e (and $[HCO_3^-]$i) so as to leave pHe unchanged had no effect, as did lowering only intracellular pHi (and not pHe) by raising both P_{CO_2} and $[HCO_3^-]$e (and $[HCO_3^-]$i). Raising pHe (and not pHi or $[HCO_3^-]$i) by high $[HCO_3^-]$e treatment at normal P_{CO_2} (metabolic alkalosis) markedly stimulated secretion, whereas simultaneous treatment with both high $[HCO_3^-]$e (and $[HCO_3^-]$i) and high P_{CO_2} had no effect.

To further test this conclusion, we regressed the extent of secretion inhibition against each of the acid–base treatment means separately using both linear and exponential models. The relationships were uniformly non-significant for P_{CO_2}, $[HCO_3^-]$i, and $[HCO_3^-]$e, but significant for pHi (r=0.73, P<0.05, linear model only) and strongest for pHe (r=0.91, P<0.002 by the linear model; r=0.94, P<0.001 by the exponential model). Thus while pHe appears to be the dominant influence, we cannot eliminate a role for pHi as well. Indeed, in the intact animal, it is likely that pHe and pHi may often covary. An additional note of caution is that the [^{14}C]-DMO technique measures mean tissue pHi. While the non-secretory capsule was removed, it remains possible that differences in pHi may have occurred between secreting and non-secreting cells, which would not have been detected.

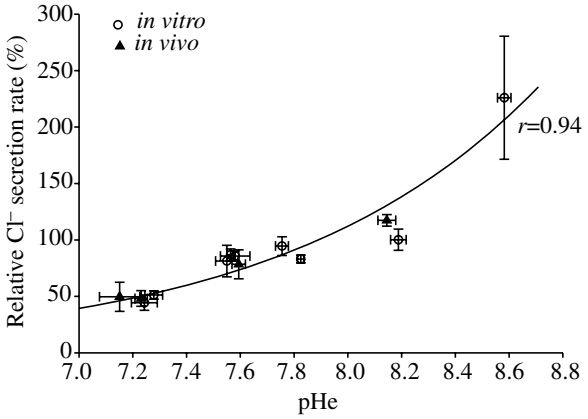

Fig. 6. The relationship between extracellular pHe (x) and relative Cl^- secretion rate (y) of the isolated-perfused rectal gland activated by 5×10^{-6} mol l^{-1} forskolin (closed symbols, data from Fig. 5 of the present study) and the rectal gland of intact, unanaesthetized dogfish activated by systemic volume loading [open symbols, data from Wood et al. (Wood et al., 2006)]. Data from every experimental treatment except acetazolamide are shown. Values are means ± 1 s.e.m. (N=5–7 for each treatment). The *in vitro* and *in vivo* data sets appear to follow a single relationship. The equation of the exponential regression line is: $y=0.0441e^{0.9784x}$ (r=0.94, P<0.0001).

Fig. 6 integrates the *in vitro* data of the present study with the responses of the rectal gland *in vivo* from Wood et al. (Wood et al., 2006) where only extracellular acid–base parameters were measured. Overall, there was good agreement with the *in vivo* data reinforcing the present *in vitro* data, such that the relationship between pHe and relative secretion rate did not change, but became more significant due to the greater number of treatment means (r=0.94, P<0.0001; exponential model). Again there was no significant relationship with $[HCO_3^-]$e. These responses of rectal gland secretion to pHe are probably adaptive in the context of the intact animal because feeding will cause both a metabolic alkalosis (‘alkaline tide’; Wood et al., 2005) and volume loading due to the ingestion of seawater and/or salty food (Mackenzie et al., 2002), necessitating activation of rectal gland secretion (Walsh et al., 2006). The highest pHe of about 8.6 from the *in vitro* experiments is probably outside the physiological range and may explain the non-linearity of response in this range (Fig. 6). However significant stimulation did occur *in vivo* at a pHe of 8.15, which is within the physiological range (Wood et al., 2005). Conversely inhibition of rectal gland output by acidosis would be useful, because metabolic acidosis normally occurs after severe exercise, a time when volume is contracted due to a shift of extracellular fluid into white muscle because of the high intracellular lactate load (Holeton and Heisler, 1983; Richards et al., 2003).

As the effects *in vivo* are similar to those in the forskolin-stimulated gland completely removed from the dogfish (Fig. 6), these data place the site of acid–base action at the level of the gland itself, rather than at the volume detection, neuroendocrine communication, or surface receptor activation level. One possible site of action might be basolateral K^+ channels, which are known to be activated as part of the secretory mechanism (Valentich and Forrest, Jr, 1991; Greiger et al., 1999), and which are known to be very sensitive to pHi in the appropriate fashion (Kerst et al., 2001). Other possibilities might include the apical Cl^- channels, the basolateral Na^+,K^+,2Cl^- co-transporter (NKCC) or the basolateral Na^+,K^+-ATPase; work at the cellular level will be needed to separate these possibilities.

Gas exchange of the rectal gland and acid–base status of the secretion

The present measurements of $\dot{M}_{O_2}$ in relation to secretion rate are in reasonable agreement with the only other detailed study on this topic in the isolated-perfused rectal gland. Using very similar methodology but a higher perfusion pressure (40 mmHg), Silva et al. (Silva et al., 1980) reported that the

$\dot{M}_{O_2}$ of the unstimulated gland was 0.30 μmol g^{-1} gland min^{-1}, but that extrapolation of the regression of $\dot{M}_{O_2}$ on Cl^- secretion rate back to zero Cl^- secretion gave a much lower value, 0.14 μmol g^{-1} gland min^{-1}. Our values were 0.11 μmol g^{-1} gland min^{-1} by both techniques (Table 2, Fig. 2B). From the slope of their regression, Silva et al. calculated that the cost of transporting 1 μmol Cl^- was about 0.030 μmol O_2 (Silva et al., 1980), whereas our value indicated slightly greater efficiency, 0.022 μmol O_2 per μmol Cl^- (Fig. 2B). $\dot{M}_{CO_2}$ is a more difficult measurement, and we are aware of no previous data in the isolated-perfused rectal gland. Our determinations of $\dot{M}_{CO_2}$ were generally higher than simultaneous measurements of $\dot{M}_{O_2}$. For example, resting $\dot{M}_{CO_2}$ was 0.20–0.22 μmol g^{-1} gland min^{-1} by direct measurement or extrapolation of the regression relationship (Table 2, Fig. 2B) and the cost of Cl^- transport from the slope was 0.036 μmol O_2 per μmol Cl^-. However, these differences were not statistically significant. While lactate concentrations in the secreted fluid and venous effluent were below detection [as also reported by Silva et al. (Silva et al., 1980)], lactate levels in the gland tissue itself were about 7 mmol l^{-1}, comparable to those in the white muscle of intact *Squalus acanthias* or an isolated-perfused muscle preparation from this species (Richards et al., 2003). Thus a glycolytic component to the metabolism of the perfused rectal gland cannot be excluded; indeed, high lactate dehydrogenase activities have recently been identified in the rectal gland, which increase after feeding (Walsh et al., 2006).

Measurements of the acid–base status of the secreted fluid from the isolated-perfused gland (Tables 2, 5) were in excellent agreement with those from intact dogfish (Wood et al., 2006). Thus in both data sets, secretion pH was about 0.3–0.5 units below pHa, while secretion P_{CO_2} was very close to Pa_{CO_2}, and secretion HCO_3^- concentration was well below that in the arterial samples. Also in both data sets, changes in secretion acid–base status tended to track those in extracellular acid–base status during experimental disturbances, but were less pronounced. There is substantial disagreement of our values with the sparse earlier measurements (Burger and Hess, 1960; Siegel et al., 1975; Swenson and Maren, 1984). Possibly, earlier workers may not have realized the need to pre-condition the electrodes to the poorly buffered secretion fluid, which would have caused an underestimation of its pH and overestimation of its P_{CO_2}. The present data demonstrate that the secretion barrier has low permeability to HCO_3^- but allows approximate equilibration of Pa_{CO_2} with the secreted fluid. The barrier function was particularly apparent in the high HCO_3^- treatments, where secretion HCO_3^- levels rose to only 3–6 mmol l^{-1} (Table 5), in the face of 40 mmol l^{-1} levels in the perfusate (Table 4). In agreement with studies on the *in situ* gland preparation (Swenson and Maren, 1984) and the intact dogfish (Wood et al., 2006), the present results with the isolated-perfused gland provide no evidence that this organ serves as a significant site of systemic acid–base regulation, but do show that it is effective in minimizing passive HCO_3^- losses. Similarly, the gland is effective in minimizing passive urea losses (Zeidel et al., 2005).

The role of carbonic anhydrase in rectal gland function

The present results clarify the formerly uncertain role of carbonic anhydrase (CA) in the rectal gland by showing that blockade of the enzyme with acetazolamide (10^{-4} mol l^{-1}) affects the dynamics of CO_2 excretion in the isolated-perfused rectal gland. Thus $\dot{M}_{CO_2}$ was depressed by about 30% for several hours in the actively secreting gland (Fig. 3C) without significant change in $\dot{M}_{O_2}$ (Fig. 3B) or Cl^- secretion rate (Fig. 3A). P_{CO_2} in the secreted fluid was also significantly elevated over a similar time course (Fig. 4), as also seen *in vivo* (present study) and in the *in situ* preparation (Swenson and Maren, 1984). These data suggest that CA is not involved in the secretory mechanism itself, but rather serves to facilitate the diffusive efflux of CO_2 into the bloodstream (or perfusate) by ensuring that it is converted into HCO_3^- at the extracellular boundary, thereby maintaining an outwardly directed P_{CO_2} gradient. This might include the recycling of metabolic protons and imported HCO_3^- back into the bloodstream. Indeed the original localization of CA by Lacy (Lacy, 1983) fits well with this interpretation. Lacy concluded that CA appears to lie in the 'intercellular spaces' (i.e. in the extracellular compartment) along the basolateral membranes of the gland cells, close to Na^+,K^+-ATPase and the underlying mitochondria which are the actual sites of CO_2 generation in the working cells (Lacy, 1983). Lacy reported a second site of concentrated CA activity, along the epithelium of the central canal draining the secreted fluid (Lacy, 1983). Blockade at this site would therefore explain the increase in P_{CO_2} of the fluid (Fig. 4).

Our interpretation is therefore similar to the explanation offered by Swenson and Maren based on their experiments with an *in situ* gland preparation in the pithed, artificially ventilated dogfish (Swenson and Maren, 1984). However, there is one important difference: Swenson and Maren interpreted CA as acting in the intracellular compartment as a true facilitator of CO_2 diffusion [i.e. *via* the co-diffusion of HCO_3^- and buffered H^+, which is the model of Gros and coworkers (Gros et al., 1976; Gros, 1991)], whereas we propose that CA serves to catalyze the CO_2 hydration reaction in the extracellular compartment, so as to maintain the P_{CO_2} gradient from cell to blood/perfusate. This interpretation is in accord with Lacy's original localization of CA (Lacy, 1983). As such, its role would be identical to that of extracellular CA in trout muscle where there is a need to accelerate CO_2 excretion after exercise (Henry et al., 1997). An added benefit of this role is that it would ensure that acid–base reactions in the extracellular compartment next to the working cells would be at equilibrium. Therefore any change in systemic acid–base status important in controlling rectal gland function would be instantly transmitted to the secreting cells. However, one note of caution must be raised. Given the concentration of acetazolamide used (10^{-4} mol l^{-1}), a very potent inhibition of extracellular carbonic anhydrase would be expected to occur rapidly, whereas the reduction in CO_2 excretion of the perfused gland did not occur until 1.5–2 h of treatment. This is more in line with slow permeation of acetazolamide into the intracellular compartment.

Regardless of the site of acetazolamide action, if the CO_2 excretion function of CA is important, then a pertinent question is why CA inhibition did not inhibit Cl^- secretion in the isolated-perfused gland in this (Fig. 3A) or earlier studies (Siegel et al., 1975; Silva et al., 1977; Swenson and Maren, 1984). This question was first raised by Swenson and Maren (Swenson and Maren, 1984), and the explanation they offered now seems very reasonable in light of the current data. Swenson and Maren speculated that *in vivo* and *in situ* perfusion flows relative to secretory rates are much lower than in maximally vasodilated *in vitro* preparations (Swenson and Maren, 1984). In the present study, the ratio of perfusion flow to secretion flow in the isolated-perfused gland was about 67 (Table 1), whereas Swenson and Maren estimated that the ratio might be tenfold lower *in vivo*, making perfusive limitations on CO_2 excretion much more severe in the intact animal (Swenson and Maren, 1984). Thus CA inhibition decreased secretory output *in vivo* (Fig. 1A) and *in situ* (Swenson and Maren, 1984), though as earlier noted, interpretation in both studies is confounded by the accompanying systemic acidosis.

Further support for these ideas is provided by the pHi data. Absolute pHi values in the rectal gland were very similar *in vivo* and *in vitro* (both about 7.5), but pHi in the secreting cells was not reduced by CA inhibition in the isolated-perfused gland (Table 3), whereas it was *in vivo* (Table 1). We were initially concerned that this apparent lack of effect was an artifact since acetazolamide itself could act as a buffer if it accumulated to high concentration in the tissue, but in fact measurements demonstrated there was no change in the buffer capacity of the gland. Notably, activation of secretion did not lower pHi in the perfused gland (Table 3), whereas it did *in vivo* (Table 1), again suggesting that CO_2 excretion is a much more serious problem *in vivo.* Acid–base regulatory mechanisms are critical in maintaining a suitable intracellular pHi for secretion in analogous NaCl-secreting systems such as the bird salt gland (Shuttleworth and Wood, 1992; Shuttleworth and Hildebrandt, 1999). Similar Na^+-dependent and HCO_3^--dependent pHi-regulatory mechanisms have been identified in shark rectal gland cells, but only at rest in perfused tubules (Bleich et al., 1998). Clearly there is a need to understand how effective these are in the working rectal gland *in vivo*.

List of abbreviations

CA	carbonic anhydrase
CNP	C-type natriuretic peptide
ECFV	extracellular fluid volume
ICF	intracellular fluid
ICFV	intracellular fluid volume
NKCC	Na^+,K^+,2Cl^- co-transporter
VIP	vasoactive intestinal polypeptide

Supported by an NSERC Discovery Grant to C.M.W. C.M.W. is supported by the Canada Research Chair Program. We thank the director and staff of Bamfield Marine Sciences Centre for their help. Animals were cared for in accord with the principles of the Canadian Council for Animal Care, and protocols were approved by institutional animal care committees.

References

Bergmeyer, H. (1983). *Methods of Enzymatic Analysis*. New York: Academic Press.

Bleich, M., Warth, R., Thiele, I. and Greger, R. (1998). pH-regulatory mechanisms in *in vitro* perfused rectal gland tubules of *Squalus acanthias*. *Eur. J. Physiol.* **436**, 248-254.

Boutilier, R. G., Heming, T. A. and Iwama, G. K. (1984). Appendix: physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology*. Vol. 10A (ed. W. S. Hoar and D. J. Randall), pp. 403-430. Orlando: Academic Press.

Burger, J. W. (1962). Further studies on the function of the rectal gland in the spiny dogfish. *Physiol. Zool.* **35**, 205-217.

Burger, J. W. and Hess, W. N. (1960). Function of the rectal gland in the spiny dogfish. *Science* **131**, 670-671.

Cameron, J. N. (1971). Rapid method for determination of total carbon dioxide in small blood samples. *J. Appl. Physiol.* **31**, 632-634.

Epstein, F. H., Stoff, J. S. and Silva, P. (1983). Mechanism and control of hyperosmotic NaCl-rich secretion by the rectal gland of *Squalus acanthias. J. Exp. Biol.* **106**, 25-41.

Forrest, J. N., Jr, Aller, S. G., Wood, S. J., Ratner, M. A., Forrest, J. K. and Kelley, G. G. (1997). Cadmium disrupts the signal transduction pathway of both inhibitory and stimulatory receptors regulating chloride secretion in the shark rectal gland. *J. Exp. Zool.* **279**, 530-536.

Gilmour, K. M. and Perry, S. F. (2004). Branchial membrane-associated carbonic anhydrase activity maintains CO_2 excretion in severely anemic dogfish. *Am. J. Physiol.* **286**, R1138-R1148.

Gilmour, K. M., Henry, R. P., Wood, C. M. and Perry, S. F. (1997). Extracellular carbonic anhydrase and an acid–base disequilibrium in the blood of the dogfish *Squalus acanthias*. *J. Exp. Biol.* **200**, 173-183.

Gilmour, K. M., Perry, S. F., Bernier, N. J., Henry, R. P. and Wood, C. M. (2001). Extracellular carbonic anhydrase in dogfish, *Squalus acanthias*: a role in CO_2 excretion. *Physiol. Biochem. Zool.* **74**, 477-492.

Graham, M. S., Turner, J. D. and Wood, C. M. (1990). Control of ventilation in the hypercapnic skate *Raja ocellata*. I. Blood and extradural fluid. *Respir. Physiol.* **80**, 259-277.

Greiger, R., Bleich, M., Warth, R., Thiele, I. and Forrest, J. N. (1999). The cellular mechanisms of Cl^- secretion induced by C-type natriuretic peptide (CNP). Experiments on isolated *in vitro* perfused rectal gland tubules of *Squalus acanthias*. *Eur. J. Physiol.* **438**, 15-22.

Gros, G. (1991). The role of carbonic anhydrase within the tissues, with a special reference to mammalian striated muscle. In *Physiological Strategies for Gas Exchange and Metabolism* (ed. A. J. Woakes, M. K. Grieshaber and C. R. Bridges), pp. 35-54. Cambridge: Cambridge University Press.

Gros, G., Moll, W., Hoppe, H. and Gros, H. (1976). Proton transport by phosphate diffusion – a mechanism of facilitated CO_2 transfer. *J. Gen. Physiol.* **67**, 773-790.

Hayslett, J. P., Schon, A. D., Epstein, M. and Hogben, A. M. (1974). *In vitro* perfusion of the dogfish rectal gland. *Am. J. Physiol.* **226**, 1188-1192.

Hazon, N., Wells, A., Pillans, R. D., Good, J. P., Anderson, W. G. and Franklin, C. E. (2003). Urea-based osmoregulation and endocrine control in elasmobranch fish with special reference to euryhalinity. *Comp. Biochem. Physiol.* **136B**, 685-700.

Heisler, N., Weitz, H. and Weitz, A. (1976). Extracellular and intracellular pH with changes of temperature in the dogfish *Scyllorhinus stellaris. Respir. Physiol.* **26**, 249-263.

Henry, R. P., Wang, Y. and Wood, C. M. (1997). Carbonic anhydrase facilitates CO_2 and NH_3 transport across the sarcolemma of trout white muscle. *Am. J. Physiol.* **272**, R1754-R1761.

Holeton, G. F. and Heisler, N. (1983). Contribution of net ion transfer mechanisms to acid–base regulation after exhausting activity in the larger spotted dogfish (*Scyliorhinus stellaris*). *J. Exp. Biol.* **103**, 31-46.

Kent, B. and Olson, K. R. (1982). Blood flow in the rectal gland of *Squalus acanthias*. *Am. J. Physiol.* **243**, R296-R303.

Kerst, G., Beschorner, U., Unsöld, B., von Hahn, T., Schreiber, R., Greiger, R., Gerlach, R. G., Lang, H. J., Kunzelmann, K. and Bleich, M. (2001). Properties and function of KCNQ1 K^+ channels isolated from the rectal gland of *Squalus acanthias*. *Eur. J. Physiol.* **443**, 146-154.

Lacy, E. R. (1983). Carbonic anhydrase localization in the elasmobranch rectal gland. *J. Exp. Zool.* **226**, 163-169.

Mackenzie, S., Cutler, C. P., Hazon, N. and Cramb, G. (2002). The effects of dietary sodium loading on the activity and expression of Na, K-ATPase in the rectal gland of the European dogfish (*Scyliorhinus canicula*). *Comp. Biochem. Physiol.* **131B**, 185-200.

Maren, T. H. (1967). Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol. Rev.* **47**, 598-671.

Maren, T. H. (1977). Use of inhibitors in physiological studiesof carbonic anhydrase. *Am. J. Physiol.* **232**, F291-F297.

Milligan, C. L. and Wood, C. M. (1985). Intracellular pH transients in rainbow trout tissues measured by diamethadione distribution. *Am. J. Physiol.* **248**, R668-R673.

Moran, W. M. and Valentich, J. D. (1991). Cl^- secretion by cultured shark rectal gland cells. II. Effects of forskolin on cellular electrophysiology. *Am. J. Physiol.* **260**, C824-C831.

Munger, R. S., Reid, S. D. and Wood, C. M. (1991). Extracellular fluid measurements in rainbow trout and their effects on intracellular pH and ion calculations. *Fish Physiol. Biochem.* **9**, 313-323.

Olson, K. R. (1999). Rectal gland and volume homeostasis. In *Sharks, Skates, and Rays* (ed. W. C. Hamlett), pp. 329-352. Baltimore: Johns Hopkins University Press.

Pärt, P., Wright, P. A. and Wood, C. M. (1998). Urea and water permeability in dogfish gills (*Squalus acanthias*). *Comp. Biochem. Physiol.* **119A**, 117-123.

Perry, S. F., Gilmour, K. M., Bernier, N. J. and Wood, C. M. (1999). Does gill boundary layer carbonic anhydrase contribute to carbon dioxide excretion: a comparison between dogfish (*Squalus acanthias*) and rainbow trout (*Oncorhynchus mykiss*)? *J. Exp. Biol.* **202**, 749-756.

Richards, J. G., Heigenhauser, G. J. F. and Wood, C. M. (2003). Exercise and recovery metabolism in the Pacific spiny dogfish (*Squalus acanthias*). *J. Comp. Physiol. B* **173**, 463-474.

Riordan, J. R., Forbush, B., III and Hanrahan, J. W. (1994). The molecular basis of chloride transport in shark rectal gland. *J. Exp. Biol.* **196**, 405-418.

Shuttleworth, T. J. (1983). Haemodynamic effects of secretory agents on the isolated elasmobranch rectal gland. *J. Exp. Biol.* **103**, 193-204.

Shuttleworth, T. J. (1988). Salt and water balance – extrarenal mechanisms. In *Physiology of Elasmobranch Fishes* (ed. T. J. Shuttleworth), pp. 171-199. Berlin: Springer-Verlag.

Shuttleworth, T. J. and Hildebrandt, J. P. (1999). Vertebrate salt glands: short- and long-term regulation of function. *J. Exp. Biol.* **283**, 689-701.

Shuttleworth, T. J. and Wood, C. M. (1992). Changes in pH_i associated with activation of ion secretion in avian nasal salt gland cells. *Am. J. Physiol.* **262**, C221-C228.

Siegel, N. J., Silva, P., Epstein, F. H., Maren, T. H. and Hayslett, J. P. (1975). Functional correlates of the dogfish rectal gland during *in vitro* perfusion. *Comp. Biochem. Physiol.* **51A**, 593-597.

Silva, P., Stoff, J., Field, M., Fine, L., Forrest, J. N. and Epstein, F. H. (1977). Mechanism of active chloride secretion by shark rectal gland: role of Na-K-ATPase in chloride transport. *Am. J. Physiol.* **233**, F298-F306.

Silva, P., Stoff, J., Solomon, R. J., Rosa, R., Stevens, A. and Epstein, F. H. (1980). Oxygen cost of chloride transport in perfused rectal gland of *Squalus acanthias*. *J. Membr. Biol.* **53**, 215-221.

Silva, P., Stoff, J. S., Solomon, R. J., Lear, S., Kniaz, D., Greger, R. and Epstein, F. H. (1987). Atrial natriuretic peptide stimulates salt secretion by shark rectal gland by releasing VIP. *Am. J. Physiol.* **252**, F99-F103.

Silva, P., Solomon, R. J. and Epstein, F. H. (1990). Shark rectal gland. *Methods Enzymol.* **192**, 754-766.

Silva, P., Epstein, F. H. and Solomon, R. J. (1992). The effect of mercury on chloride secretion in the shark (*Squalus acanthias*) rectal gland. *Comp. Biochem. Physiol.* **103C**, 569-575.

Silva, P., Solomon, R. J. and Epstein, F. H. (1996). The rectal gland of *Squalus acanthias*: a model for the transport of chloride. *Kidney Int.* **49**, 1552-1556.

Silva, P., Solomon, R. J. and Epstein, F. H. (1997). Transport mechanisms that mediate the secretion of chloride by the rectal gland of *Squalus acanthias. J. Exp. Zool.* **279**, 504-508.

Silva, P., Solomon, R. J. and Epstein, F. H. (1999). Mode of activation of salt secretion by C-type naturiuretic peptide in the shark rectal gland. *Am. J. Physiol.* **277**, R1725-R1732.

Solomon, R. J., Taylor, M., Stoff, J. S., Silva, P. and Epstein, F. H. (1984a). *In vivo* effect of volume expansion on rectal gland function. I. Humoral factors. *Am. J. Physiol.* **246**, R63-R66.

Solomon, R. J., Taylor, M., Rosa, R., Silva, P. and Epstein, F. H. (1984b). *In vivo* effect of volume expansion on rectal gland function. II. Hemodynamic factors. *Am. J. Physiol.* **246**, R638-R640.

Solomon, R. J., Taylor, M., Sheth, S., Silva, P. and Epstein, F. H. (1985). Primary role of volume expansion in stimulation of rectal gland function. *Am. J. Physiol.* **246**, R67-R71.

Stoff, J. S., Rosa, R., Hallac, R., Silva, P. and Epstein, F. H. (1979). Hormonal regulation of active chloride transport in the dogfish rectal gland. *Am. J. Physiol.* **237**, F138-F144.

Stoff, J. S., Silva, P., Lechan, R., Solomon, R. J. and Epstein, F. H. (1988). Neural control of shark rectal gland. *Am. J. Physiol.* **255**, R212-R216.

Swenson, E. R. and Maren, T. H. (1984). Effects of acidosis and carbonic anhydrase inhibition in the elasmobranch rectal gland. *Am. J. Physiol.* **247**, F86-F92.

Swenson, E. R. and Maren, T. H. (1987). Roles of gill and red cell carbonic anhydrase in elasmobranch HCO_3^- and CO_2 excretion. *Am. J. Physiol*. **253**, R450-R458.

Valentich, J. D. and Forrest, J. N., Jr (1991). Cl^- secretion by cultured shark rectal gland cells. I. Transepithelial transport. *Am. J. Physiol.* **260**, C813-C823.

Waddell, W. J. and Butler, T. C. (1959). Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidine dione (DMO): application to skeletal muscle of the dog. *J. Clin. Invest*. **38**, 720-729.

Walsh, P. J., Kajimura, M., Mommsen, T. P. and Wood, C. M. (2006). Metabolic organization and effects of feeding on enzyme activities of the dogfish shark (*Squalus acanthias*) rectal gland. *J. Exp. Biol.* **209**, 2929-2938.

Wilson, J. M., Randall, D. J., Vogl, A. W., Harris, J., Sly, W. S. and Iwama, G. K. (2000). Branchial carbonic anhydrase is present in the dogfish, *Squalus acanthias*. *Fish Physiol. Biochem.* **22**, 329-336.

Wood, C. M., Turner, J. D., Munger, R. S. and Graham, M. S. (1990). Control of ventilation in the hypercapnic skate *Raja ocellata*. II. Cerebrospinal fluid chemistry, and intracellular pH in the brain and other tissues. *Respir. Physiol.* **80**, 279-298.

Wood, C. M., Kajimura, M., Mommsen, T. P. and Walsh, P. J. (2005). Alkaline tide and nitrogen conservation after feeding in the elasmobranch *Squalus acanthius. J. Exp. Biol.* **208**, 2693-2705.

Wood, C. M., Munger, R. S., Thompson, J. and Shuttleworth, T. J. (2006). Control of rectal gland secretion by blood acid–base status in the intact dogfish shark (*Squalus acanthias*). *Respir. Physiol. Neurobiol.* doi: 10.1016/j.resp.2006.09.003.

Wright, P. A., Randall, D. J. and Wood, C. M. (1988). The distribution of ammonia and H^+ ions between tissue compartments in lemon sole (*Parophrys vetulus*) at rest, during hypercapnia, and following exercise. *J. Exp. Biol.* **136**, 149-175.

Zeidel, J. D., Mathai, J. C., Campbell, J. D., Ruiz, W. G., Apodaca, G. L., Riordan, J. and Ziedel, M. L. (2005). Selective permeability barrier to urea in shark rectal gland. *Am. J. Physiol.* **289**, F83-F89.