Extracellular Carbonic Anhydrase in the Dogfish, *Squalus acanthias*: A Role in CO_2 Excretion

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ABSTRACT

In Pacific spiny dogfish (*Squalus acanthias*), plasma CO_2 reactions have access to plasma carbonic anhydrase (CA) and gill membrane–associated CA. The objectives of this study were to characterise the gill membrane–bound CA and investigate whether extracellular CA contributes significantly to CO_2 excretion in dogfish. A subcellular fraction containing membrane-associated CA activity was isolated from dogfish gills and incubated with phosphatidylinositol-specific phospholipase C. This treatment caused significant release of CA activity from its membrane association, a result consistent with identification of the dogfish gill membrane–bound CA as a type IV isozyme. Inhibition constants (K_i) against acetazolamide and benzolamide were 4.2 and 3.5 nmol L^{-1}, respectively. Use of a low dose (1.3 mg kg^{-1} or 13 μmol L^{-1}) of benzolamide to selectively inhibit extracellular CA in vivo caused a significant 30%–60% reduction in the arterial-venous total CO_2 concentration difference, a significant increase in P_{CO_2} and an acidosis, without affecting blood flow or ventilation. No effect of benzolamide on any measure of CO_2 excretion was detected in rainbow trout (*Oncorhynchus mykiss*). These results indicate that extracellular CA contributes substantially to CO_2 excretion in the dogfish, an elasmobranch, and confirm that CA is not available to plasma CO_2 reactions in rainbow trout, a teleost.

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Introduction

The conventional view concerning the distribution of the enzyme carbonic anhydrase (CA) in fish gills has been that it is restricted to the intracellular compartment; that is, it is located in the red blood cells (RBC) and gill epithelial cells but is not available to plasma CO_2-HCO_3^- H^+ reactions (reviewed by Gilmour 1998; Henry and Heming 1998; Tufts and Perry 1998). Hence, CO_2 excretion at the gills requires the movement of HCO_3^- ions, which are carried primarily in the plasma, into the RBC via the band 3 anion exchanger for CA-catalysed dehydration to CO_2. The CO_2 then diffuses into the ventilatory water along its partial pressure gradient (Perry 1986; Tufts and Perry 1998).

Several recent studies on dogfish have challenged this view of CA distribution for elasmobranch fish. CA activity has been found, using two different assays, in separated plasma samples from two species of dogfish (Wood et al. 1994; Perry et al. 1996; Gilmour et al. 1997; Henry et al. 1997). Dogfish also appear to possess gill membrane–bound CA activity that is exposed to the blood. A subcellular fraction of dogfish gill tissue containing external and internal membranes was found to have a CA activity about three times higher than that of an equivalent fraction from trout gills (Henry et al. 1997). Physiological evidence that this gill membrane–bound CA activity is exposed to the blood has been obtained using selective CA inhibitors to examine the effect on acid-base status of inhibiting extracellular CA activity (Swenson et al. 1995, 1996; Gilmour et al. 1997; Wilson et al. 2000).

The availability of CA activity to plasma CO_2 reactions at the gas exchange surface in dogfish resembles the situation in mammals (Crandall and O'Brasky 1978; Effros et al. 1978; Klocke 1978) or reptiles (Stabenau et al. 1996), where pulmonary capillary endothelial CA activity (type IV isozyme; Waheed et al. 1992*a*) is exposed to the blood (see review by Henry and Swenson 2000). In teleost fish, by contrast, plasma CO_2 reactions do not have access to CA activity (e.g., Henry et al. 1988, 1993; Perry et al. 1997; Sender et al. 1999). Despite the convincing physiological and biochemical evidence for a plasma-accessible membrane-associated CA in dogfish gills that is analogous to mammalian pulmonary capillary CA IV, the

dogfish isozyme has not yet been identified. Therefore, one objective of this study was to characterise this CA activity by an examination of its sensitivity to sulphonamide inhibitors and phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves the characteristic phosphatidylinositol-glycan linkage between CA IV and the plasma membrane (Zhu and Sly 1990; Waheed et al. 1992*b*).

In addition, the physiological function of elasmobranch extracellular CA activity remains uncertain. Such CA activity could, in theory, participate in CO_2 excretion by providing an extracellular pathway for rapid HCO_3^- dehydration in addition to the standard route through the RBC. However, it has been argued that pulmonary capillary CA IV in mammals contributes little to CO_2 excretion (<10%) owing to the low nonbicarbonate buffer capacity of the plasma relative to the RBC; that is, HCO_3^- dehydration in the plasma is limited by proton availability (Heming et al. 1986; Heming and Bidani 1992; Henry and Swenson 2000). Thus, an alternative role for the extracellular CA activity has been proposed in which it functions to maintain a rapid chemical equilibrium between CO_2 and H^+ for the purposes of ventilatory control (Heming et al. 1993, 1994).

Similar arguments have been applied to the function of extracellular CA activity in dogfish (Henry et al. 1997), as elasmobranch fish appear to possess a strong CO_2/H^+-sensitive ventilatory drive (Heisler et al. 1988; Graham et al. 1990; Wood et al. 1990; Perry and Gilmour 1996), and indeed, selective inhibition of extracellular CA activity was not found to elicit a significant rise in arterial P_{CO_2} (Swenson and Maren 1987; Gilmour et al. 1997). However, partial inhibition of HCO_3^- flux through the erythrocytes using the anion exchange inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), also did not result in a significant increase in arterial P_{CO_2}, leading to the hypothesis that extracellular CA activity in dogfish might function as a reserve capacity for CO_2 excretion under conditions of increased CO_2 production, such as exercise (Gilmour et al. 1997; Henry et al. 1997). Thus, the second objective of this study was to assess the role of extracellular CA activity in CO_2 excretion in dogfish under resting conditions and under conditions of increased CO_2 loading. For comparative purposes, rainbow trout were also examined because CA activity is not available to plasma CO_2 reactions in teleost fish.

Material and Methods

Experimental Animals

Pacific spiny dogfish (*Squalus acanthias* L.; 565–2,300 g; experimental $N = 33$) were trawled or angled off the coast of Vancouver Island, Canada, in July and August 1997, and held at Bamfield Marine Station (British Columbia) for up to 4 wk in a 75,000-L circular tank provided with flowing seawater at 11°C. The dogfish were maintained under a 12L : 12D photoperiod and were fed herring twice weekly. A second set of experiments was carried out on freshwater rainbow trout (*Oncorhynchus mykiss*) at the University of Ottawa (Ottawa, Ontario) in December 1997. Rainbow trout (323–1,072 g; experimental $N = 11$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and maintained on a 12L : 12D photoperiod in a large fibreglass aquarium supplied with flowing, aerated, and dechlorinated City of Ottawa tap water (in mmol L^{-1}, $[Ca^{2+}] = 0.43$, $[Na^+] = 0.13$, $[K^+] = 0.023$, $[Cl^-] = 0.14$, pH = 6.8, temperature = 15°C). Trout were fed daily to satiation on a diet of commercial trout pellets, and food was withheld for 24 h before experimentation.

Inhibitor Sensitivity of Dogfish Gill Membrane–Bound CA Activity

Gills from dogfish and trout were perfused with heparinised saline to obtain erythrocyte-free tissue, dissected out of the fish, frozen in liquid nitrogen, and stored at −80°C until analysis as described by Henry et al. (1997). Gill tissue was homogenised and fractionated according to the procedures of Henry et al. (1993, 1997). Tissue (8–10 g) was added to 5 volumes of cold buffer (in mmol L^{-1}, 300 mannitol-sucrose, 10 Tris-PO_4, pH 7.40) and homogenised by a minimum of 30 passes in a motor-driven (600 rpm) Teflon/glass homogeniser. The homogenate was then subjected to differential centrifugation (Henry et al. 1993). The final supernatant (cytoplasm, containing gill epithelial intracellular CA activity) and pellet (microsomes; containing membrane-bound CA activity) fractions were saved for analysis of CA activity by means of the electrometric ΔpH method (Henry 1991).

Inhibitor Titrations. To generate inhibitor titration curves, 50 μL of sample was used in the electrometric ΔpH assay to produce an increase in CO_2 hydration of about eight times the uncatalysed rate. The CA activity was then titrated with increasing volumes of either 5 μmol L^{-1} acetazolamide or 5 μmol L^{-1} benzolamide. The resultant data were plotted using the method of Easson and Stedman (1937) and the equation $Io/i = K_i/(1 - i) + Eo$, where Eo is the concentration of free enzyme, K_i is the inhibition constant, and i is the fractional inhibition of CA activity at a given inhibitor concentration.

PI-PLC Sensitivity. To examine the sensitivity of the gill membrane–bound CA activity to PI-PLC, microsomal pellets were resuspended in 1.5 mL of buffer. A 50-μL aliquot was used to measure suspension CA activity. The CA activity that was not integrally associated with the microsomal pellet was assessed by centrifuging a 400-μL aliquot of the suspension to repellet the microsomes and assaying 50 μL of the resultant supernatant for CA activity. This measurement also provided the time = 0 supernatant CA activity, against which were compared the supernatant activities following incubation for 30, 60, or 90 min at room temperature (22°C) of aliquots of the suspension with PI-PLC (1 unit; Sigma) or buffer (control). As a control for the effects of PI-PLC, the above procedure

was also carried out on the cytoplasmic fraction obtained by differential centrifugation; cytoplasmic CA activity should be unaffected by treatment with PI-PLC. This experiment was carried out on four independent microsomal preparations each for dogfish and trout.

Selective CA Inhibition and CO_2 Excretion In Vivo

Surgical Procedures and Extracorporeal Blood Circulation. All fish were anaesthetised using ethyl-m-aminobenzoate (MS-222; 0.1 g L^{-1}; neutralised to pH 7.5–8.0 with $NaHCO_3$ if required), then transferred to an operating table that permitted continuous irrigation of the gills with aerated anaesthetic solution. In dogfish used for extracorporeal experiments (1,115–2,300 g; $N = 25$), two cannulae (Clay-Adams, PE50) were implanted into the coeliac artery (Graham et al. 1990) in the orthograde and retrograde directions to create the extracorporeal blood circulation. Two additional cannulae (PE 50) were placed in the caudal vein and artery for the withdrawal of pre- and postbranchial blood samples for plasma total CO_2 concentration (C_{CO_2}) measurements. The caudal artery and vein samples were found, by comparison with samples withdrawn from the ventral aorta and coeliac artery, to accurately reflected the pre- and postbranchial C_{CO_2} values, except during HCO_3^- infusion. A catheter (PE160) was inserted into a spiracle and sutured in place for the measurement of ventilatory pressures. Finally, cardiac output was monitored by fitting dogfish with a 3S or 4S ultrasonic flow probe (Transonic Systems, Ithaca, N.Y.), as described by Bernier et al. (1999). An additional group of dogfish (565–1,020 g; $N = 8$) was used for HCO_3^- loading experiments because the caudal vein samples did not provide an accurate estimate of prebranchial plasma C_{CO_2} under these conditions. In these dogfish, prebranchial blood was sampled from a cannula (PE 60) implanted into the conus arteriosus according to the method of Olson et al. (1997). Cannulae were also placed in the caudal artery and caudal vein for the withdrawal of postbranchial blood and the infusion of the HCO_3^- load, respectively.

Rainbow trout were fitted with two dorsal aortic cannulae, as well as caudal artery and caudal vein cannulae, such that pre- and postbranchial blood samples could be withdrawn without disturbing the flow of blood through the extracorporeal blood circulation. Ventilation amplitude (VAMP) and ventilation frequency (f_R) were measured by means of small (1 cm^2) brass-plate electrodes sutured to each operculum and connected to an impedance converter. A 3S or 4S ultrasonic flow probe was placed around the bulbus arteriosus for the measurement of cardiac output (see Bernier et al. 1999).

After surgery, fish were transferred to experimental chambers of an appropriate size for a 24-h recovery period. The chambers were supplied with aerated flow-through seawater or freshwater (flow rate > 2.5 L min^{-1}) at the experimental temperature. Cannulae were flushed with heparinised (100 IU mL^{-1} sodium heparin) saline; dogfish saline consisted of 500 mmol L^{-1} NaCl, while Cortland saline was used for trout.

The extracorporeal blood circulation (Thomas 1994) consisted of an external circuit (of approximately 1 mL volume) containing pH, P_{CO_2}, and P_{O_2} electrodes through which blood was pumped at a rate of 0.55 mL min^{-1}. To prevent clotting, the circuit was rinsed with 10 mL of heparinised (540 IU mL^{-1}) saline before initiating the blood flow. A Metrohm combination glass pH electrode (model 6.0204.100) and a Radiometer PHM 73 meter were used to measure arterial pH (pHa), while P_{CO_2} and P_{O_2} electrodes together with the PHM 73 analyser were used to monitor arterial P_{CO_2} (Pa_{CO_2}) and P_{O_2} (Pa_{O_2}). The PHM 73 analyser, in turn, was connected to a data acquisition system (BioPac Systems, Goleta, Calif.) and a computer. All electrodes were housed in thermostatted cuvettes maintained at ambient water temperature.

VAMP and f_R were monitored in dogfish by connecting the spiracular catheter to a pressure transducer (model 1050BP, UFI, Morro Bay, Calif.) to measure pressure changes associated with breathing. In rainbow trout, the frequency and amplitude of opercular displacements were assessed using an impedance converter that measured the changes in impedance between the electrodes sutured to the opercula. The f_R was determined automatically using a rate function feature of the data acquisition system, while the arithmetic difference between inspiratory and expiratory pressures, or impedance values, was used as a measure of VAMP. The flow probe was connected to a blood flowmeter (Model T106, Transonic Systems, Ithaca, N.Y.); cardiac output ($\dot{V}b$) was calculated by correcting blood flow measurements for fish weight.

Experimental Protocol for Spiny Dogfish. Experiments on dogfish were designed to assess the role of extracellular CA activity in CO_2 excretion under conditions of normal and increased CO_2 loading. Indices of CO_2 excretion were evaluated in dogfish treated with a low dose of the CA-inhibitor benzolamide (1.3 mg kg^{-1} or 13 μmol L^{-1}) to inhibit only extracellular CA activity or with acetazolamide (30 mg kg^{-1} or 450 μmol L^{-1}) to inhibit both extracellular and RBC CA activities. The efficacy of these treatments was established previously using the same species (Gilmour et al. 1997). However, RBC CA activity was quantified in this study to monitor the effects of the inhibitors (see below). Increased CO_2 loading was accomplished either by treating dogfish with the RBC anion exchange inhibitor DIDS (final concentration in the blood of 1×10^{-4} mol L^{-1}), a treatment previously found to inhibit HCO_3^- flux through dogfish erythrocytes by approximately 40% (Gilmour et al. 1997) or by the infusion of a HCO_3^- load to augment HCO_3^- clearance from the plasma at the gills.

Following initiation of the extracorporeal circulation and stabilisation of the measured variables (usually within 10–30 min), control pre- and postbranchial blood samples (0.6 mL) were withdrawn simultaneously to measure plasma C_{CO_2} (50 μL plasma, duplicate samples; Corning model 965 CO_2 ana-

lyser). Dogfish were then subjected to one of three treatments to inhibit extracellular CA activity: injection of benzolamide (Bz), injection of DIDS followed by benzolamide, or infusion of a HCO_3^- load, via the caudal vein cannula, followed by injection of benzolamide. Alternatively, dogfish were treated with bovine CA to examine the effect of increasing extracellular CA activity, followed by acetazolamide (Az) to inhibit both extracellular and RBC CA activities. Blood samples for Cco_2 measurements were withdrawn 5, 10, 15, and 30 min after administration of the CA inhibitor. Arterial-venous Cco_2 differences for HCO_3^--infused dogfish before and after benzolamide treatment were determined on the additional dogfish that were fitted only with conus arteriosus and caudal vessel cannulae; no blood gas, acid-base, ventilatory, or cardiovascular variables were measured in these experiments.

Drugs were administered as a bolus injection into the return cannula of the extracorporeal loop. Benzolamide was dissolved in a stock solution of saline with added NaOH (pH approximately 10); the pH was then slowly titrated to approximately 8.5. DIDS was dissolved in dogfish saline containing 20% dimethyl sulphoxide (DMSO) and injected into the dogfish to achieve a final concentration in the blood of 1×10^{-4} mol L^{-1} (0.05% DMSO). Pre- and postbranchial blood samples were withdrawn 15 min after DIDS injection and benzolamide was then injected. Similarly, pre- and postbranchial blood samples were withdrawn 15 min after bovine CA (2 mg kg^{-1}) in dogfish saline was injected, and the fish was then treated with acetazolamide. Acetazolamide was prepared as was benzolamide but injected at a dose of 30 mg kg^{-1}.

Bicarbonate loading was achieved by the protocol of Wood et al. (1995). A 500 mmol L^{-1} $NaHCO_3$ solution was infused into the caudal vein at a rate of 1,600 μmol kg^{-1} h^{-1} for approximately 3 h until stable ventilatory, cardiovascular, and blood gas readings were once again observed. At this time, postbranchial (extracorporeal experiments) or pre- and postbranchial (nonextracorporeal experiments) blood samples were collected, and benzolamide was injected. The $NaHCO_3$ infusion was maintained throughout the experiment. That this infusion procedure resulted in the attainment of a new steady state condition was tested by injecting a group of dogfish ($N = 7$–10) with saline after collection of the high HCO_3^- blood samples and resampling after 15 min; no significant differences between the two samples were observed for any variable measured.

Experimental Protocol for Rainbow Trout. The experiments on rainbow trout were designed to examine the effects of extracellular CA inhibitors on CO_2 excretion in a species that appears to lack branchial extracellular CA activity. In these experiments, blood gases and pH, ventilation, and cardiac output were measured, and CO_2 excretion ($\dot{M}co_2$) was determined directly using flow-through respirometry. $\dot{M}co_2$ was calculated as the difference in water Pco_2 between inflowing and outflowing water from the experimental chamber, taking into account the solubility coefficient, water flow rate, and mass of the fish. The solubility coefficient of CO_2 in freshwater was determined experimentally as the slope of the CO_2 combining curve for dechlorinated City of Ottawa tap water at 15°C (water Cco_2 in mmol L^{-1} vs. Pco_2 in Torr yielded the regression equation $Cwco_2 = 0.49 + 0.13\ Pco_2$, $r = 0.99$, $P < 0.05$).

Following initiation of the extracorporeal circulation and stabilisation of the measured variables (usually within 10–30 min), control pre- and postbranchial blood samples were withdrawn simultaneously to measure plasma Cco_2 (20 μL, triplicate samples; Capni-Con 5 total CO_2 analyser, Cameron Instrument Company). Trout were then injected with benzolamide (1.3 mg kg^{-1}), and blood samples were collected at 5-, 10-, 15-, and 30-min postdrug administration. Subsequently, trout were injected with acetazolamide (30 mg kg^{-1}) and monitored for an additional 30 min.

Validation Experiments. Because the use of low concentrations of benzolamide to selectively inhibit extracellular CA activity relies largely on its slower rate of equilibration across the RBC membrane than that of acetazolamide (Maren 1967), the effects of the inhibitor treatments were monitored by quantifying RBC CA activity using the electrometric ΔpH method. RBC pellets collected at each sampling time following inhibitor injection were frozen in liquid nitrogen and stored at −80°C until analysis. Pellets were thawed, diluted fourfold in 5 mmol L^{-1} EDTA solution, and sonicated; 20-μL samples of the resultant lysate were used in the CA assay. Dogfish plasma CA activity was also assessed using 100-μL samples before and 5 min following the injection of benzolamide.

As an additional test of the extent to which RBC CA activity was inhibited by acetazolamide or benzolamide, a radioisotopic HCO_3^- dehydration assay (Wood and Perry 1991) was used to measure blood HCO_3^- dehydration rates in vitro in the presence of the inhibitors. Measurement of RBC CA activity provides information about the catalytic potential of the enzyme itself, whereas measurement of blood HCO_3^- dehydration rates takes into account the fact that HCO_3^- dehydration may be limited by factors other than CA catalytic activity. Indeed, HCO_3^- entry into the RBC via the Cl^-/HCO_3^- exchanger is thought to be the rate-limiting step to CO_2 excretion in vivo (see Tufts and Perry 1998). Samples (0.8 mL) of rainbow trout blood adjusted to 15% haematocrit were equilibrated with a humidified gas mixture of 0.5% CO_2 in air (GF-3/MP gas mixing flowmeter, Cameron Instrument Company) for 60 min (at 10°C); rainbow trout blood was used in preference to dogfish blood because there is no CA activity in the plasma to complicate the interpretation of inhibitor effects. Acetazolamide or benzolamide was added to the blood at 5% by volume to achieve final concentrations of 450 μmol L^{-1} or 13 μmol L^{-1}, respectively. These concentrations were chosen to mimic the concentrations used in vivo, assuming distribution of the inhibitor throughout the extracellular fluid. The inhibitors were added to the blood sample 5, 10, 15, or 30 min before carrying out the assay to

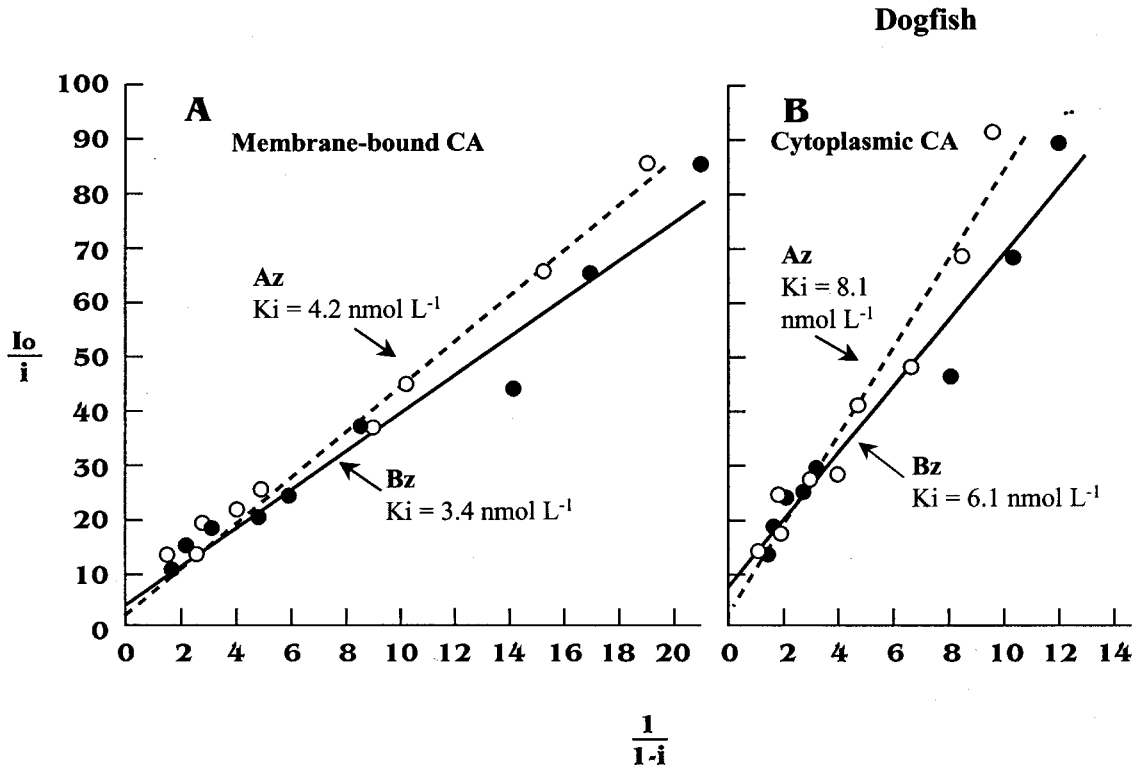

Figure 1. Double reciprocal plots for the titration of *A*, branchial membrane-associated CA activity, and *B*, cytoplasmic CA activity with increasing aliquots of 5 μmol L^{-1} acetazolamide (*Az*; *unfilled symbols*) or benzolamide (*Bz*; *filled symbols*). Values are means for duplicate assays. K_i values are given by the slope of the line; linear regression equations were fitted by the least squares method and are as follows: $y = 4.2x + 4.2$, $r^2 = 1.00$, membrane-associated CA with Az (*A*; *dashed line*). $y = 3.4x + 5.2$, $r^2 = 0.98$, membrane-associated CA with Bz (*A*; *solid line*). $y = 8.1x + 2.8$, $r^2 = 0.97$, cytoplasmic CA with Az (*B*; *dashed line*). $y = 6.1x + 7.21$, $r^2 = 0.98$, cytoplasmic CA with Bz (*B*; *solid line*).

match the sampling times used in vivo; blood was also assayed following 1 h of incubation with acetazolamide to determine the HCO_3^- dehydration rate when RBC CA was fully inhibited. Following the 3 min assay, filter paper and plasma ^{14}C activities were determined by liquid scintillation counting (Packard TR 2500) with automatic quench correction. Filter papers were counted in 10 mL of Bio-Safe NA (Research Products International), while 50 μL of plasma was counted in 10 mL of ACS (Amersham) scintillation cocktail. Plasma Cco_2 was measured on 20-μL duplicate samples (Capni-Con 5 total CO_2 analyser).

The radioisotopic HCO_3^- dehydration assay was also used to monitor CA activity in separated plasma samples obtained from dogfish at different points during the experimental protocol. Because dogfish lack an endogenous plasma CA inhibitor (Henry et al. 1997), RBC lysis induced by the experimental procedures could artificially elevate plasma CA activity, confounding the effects of subsequent selective CA inhibition. To control for this possibility, blood samples were collected from four dogfish immediately before surgery, 24 h postsurgery, and 1 h into experiments with the extracorporeal circulation. Separated plasma from these samples, together with a sample to which 4.5×10^{-4} mol L^{-1} acetazolamide was added in vitro, were then assayed for CA activity using the radioisotopic approach as described above.

Statistical Analyses

Data are presented as means ± 1 standard error of the mean (SEM; *N*). Mean blood gas, acid-base, ventilatory, and cardiovascular data were compiled for 1-min periods corresponding to the blood-sampling periods. Statistical differences between control and treatment values within a treatment group were determined by one-way repeated-measures ANOVA followed by Dunnett's post hoc multiple-comparisons test to compare all data points against the appropriate control or by Student-Newman-Keuls post hoc multiple-comparisons test to make comparisons among all data points. The effects of different treatments were compared using one-way ANOVA followed by Dunnett's post hoc multiple-comparisons test or by unpaired Student's *t*-tests, as appropriate. Microsomal CA activities for

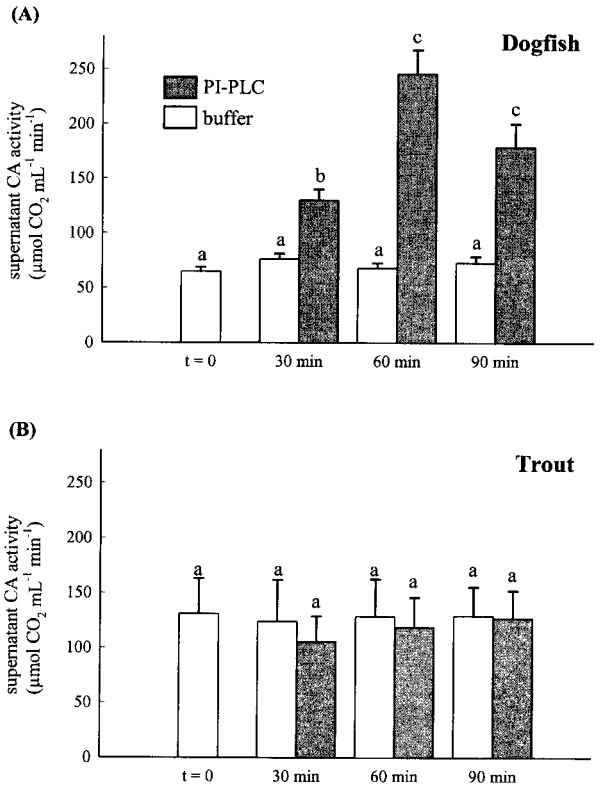

Figure 2. The CA activity (μmol CO_2 mL^{-1} min^{-1}) associated with the supernatant derived from centrifugation of microsomal pellet suspensions for *A*, dogfish, and *B*, trout. Microsomal pellet suspensions were incubated for 30, 60, and 90 min with either mannitol-sucrose Tris buffer (*unfilled bars*) or 1 unit of PI-PLC dissolved in mannitol-sucrose Tris buffer (*filled bars*). The $t = 0$ value represented the initial activity associated with the supernatant before any incubations were carried out. Bars represent mean values ± SEM ($N = 4$). Values sharing the same letter are not significantly different from one another (one-way ANOVA followed by the Student-Newman-Keuls multiple-comparisons test, $P < 0.05$).

dogfish and trout gills were compared using an unpaired Student's *t*-test, while the effects of incubation with PI-PLC, as well as the effects of inhibitor treatments in the $[^{14}C]HCO_3^-$ assay, were analysed using a one-way ANOVA followed by the Student-Newman-Keuls post hoc multiple-comparisons test. Where assumptions of normality or equal variance were violated, data were analysed by equivalent nonparametric tests. The fiducial limit of significance in all cases was 5%.

Results

Characterisation of CA Activity in Dogfish Gills

The CA activity in both the membrane and cytoplasmic fractions of dogfish gill was highly sensitive to acetazolamide and benzolamide, with K_i values for gill membrane–bound CA of 4.2 and 3.4 nmol L^{-1}, respectively (Fig. 1). Corresponding K_i values for cytoplasmic CA activity titrated against acetazolamide and benzolamide were 8.1 and 6.1 nmol L^{-1}, respectively (Fig. 1). Furthermore, the CA activity in the membrane fraction of dogfish gills, but not trout gills, was sensitive to treatment with PI-PLC. When suspensions of microsomal pellets from dogfish gills were incubated with 1 unit of PI-PLC for 30–90 min and then centrifuged to repellet the microsomes, the CA activity associated with the supernatant was increased significantly, by 70%–262%, over that of control samples incubated with buffer; incubation with buffer had no significant effect on the supernatant CA activity (Fig. 2*A*). By contrast, incubation of suspensions of microsomal pellets from trout gills with PI-PLC had no significant effect on supernatant CA activity (Fig. 2*B*), nor was the CA activity associated with the cytoplasmic fraction of either trout or dogfish gills affected by incubation with PI-PLC (data not shown). While the CA activity of the microsomal suspension for dogfish (394 ± 27 μmol CO_2 mL^{-1} min^{-1}, $N = 4$) gills was almost double that for trout (202 ± 14 μmol CO_2 mL^{-1} min^{-1}, $N = 4$), determination of the CA activity associated with the supernatant obtained by repelleting the microsomes revealed that much of the CA activity of the trout microsomal suspension was not integrally associated with the pellet. When the high supernatant CA activity of trout versus dogfish ($t = 0$ samples, Fig. 2*A*) was taken into account, the

Table 1: Effect of acetazolamide (450 μmol L^{-1}) or benzolamide (13 μmol L^{-1}) on the rate of HCO_3^- dehydration in vitro for rainbow trout blood

	HCO_3^- Dehydration Rate (μmol mL^{-1} h^{-1})
Blood	19 ± .7[A]
Plasma	2.7 ± .3[B]
Blood + Az (1 h)	2.1 ± .1[B]
Blood + inhibitor:	
Bz:	
5 min	16.9 ± 1.1[A]
10 min	18 ± 1.3[A]
15 min	16.3 ± .8[A]
30 min	17.1 ± .9[A]
Az:	
5 min	2.8 ± .1[B]
10 min	2.2 ± .3[B]
10 min	2.2 ± .4[B]
30 min	2.0 ± .3[B]

Note. Az, acetazolamide; Bz, benzolamide. Values are means ± 1 SEM; $N = 4$. Values having different letters are significantly different from one another (one-way ANOVA followed by the Student-Newman-Keuls post hoc test for multiple comparisons, $P < 0.05$).

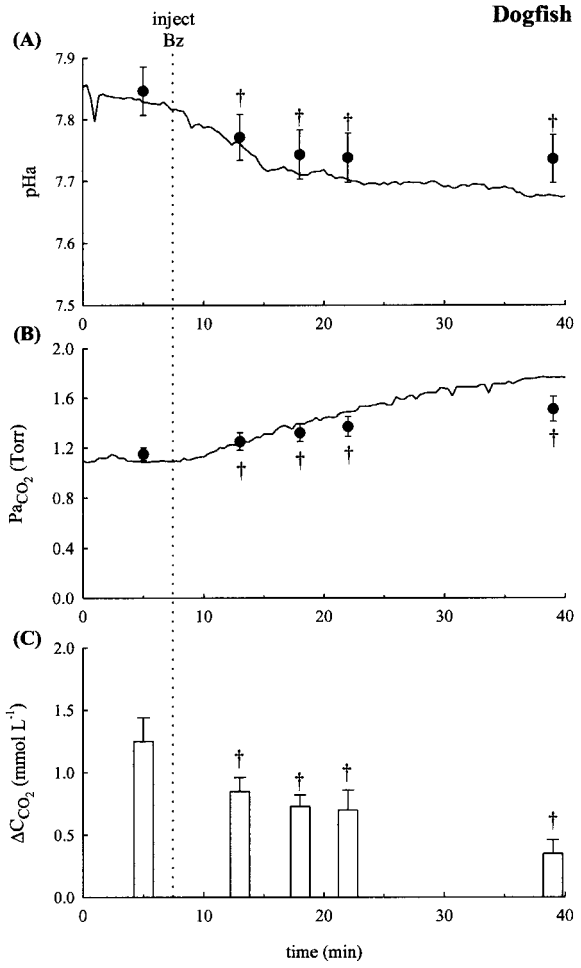

Figure 3. The effects in dogfish (*Squalus acanthias*) of intra-arterial benzolamide (*Bz*; 1.3 mg kg^{-1}) injection on *A*, arterial blood pH (pHa), *B*, arterial blood P_{CO_2} ($Paco_2$), and *C*, the arterial-venous total CO_2 concentration difference (ΔCco_2). The dotted vertical line indicates the time of injection. The symbols represent mean values ± 1 SEM (N = 5–7). Data for pHa and $Paco_2$ were compiled for 1-min periods corresponding to the times at which pre- and postbranchial blood samples were withdrawn for Cco_2 measurements (i.e., before Bz injection [control] and at 5, 10, 15, and 30 min after Bz injection). A dagger indicates a significant difference from the control value (one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test, $P < 0.05$). The lines in panels *A* and *B* present a representative original data recording illustrating the effects of benzolamide treatment on pHa and $Paco_2$.

true membrane-bound CA activity of dogfish gills, at 329 ± 28 μmol CO_2 mL^{-1} min^{-1} (N = 4), was nearly five times that of trout gills, 71 ± 35 μmol CO_2 mL^{-1} min^{-1} (N = 4), a difference that was highly significant (P = 0.001).

Validation Experiments

Haemolysis. Statistical analysis of HCO_3^- dehydration rates for plasma samples obtained from four dogfish immediately before surgery (3.5 ± 0.6 μmol mL^{-1} h^{-1}), 24 h postsurgery (3.9 ± 0.8 μmol mL^{-1} h^{-1}), and 1 h after initiation of the extracorporeal blood circulation (4.1 ± 0.5 μmol mL^{-1} h^{-1}) revealed that no significant differences existed among the different sampling times. Furthermore, the HCO_3^- dehydration rates of all three groups were significantly higher than that for plasma samples incubated with 4.5 × 10^{-4} mol L^{-1} acetazolamide (1.6 ± 0.1 μmol mL^{-1} h^{-1}). These data confirmed that low levels of CA activity are present in dogfish plasma (Gilmour et al. 1997; Henry et al. 1997) and demonstrated that plasma CA activity was elevated by experimentally induced haemolysis to a minimal extent.

Selective Inhibition of Extracellular CA Activity. The efficacy of the inhibitor treatments in achieving selective inhibition of extracellular CA activity (benzolamide) or inhibition of both extracellular and RBC CA activities (acetazolamide) was assessed using the electrometric ΔpH assay to quantify plasma or RBC CA activity for samples withdrawn from dogfish before and following administration of a CA inhibitor. As in previous studies (Gilmour et al. 1997; Henry et al. 1997), low but measurable CA activity (28.1 ± 4.6 μmol CO_2 mL^{-1} min^{-1} [N = 10] where the uncatalysed rate of hydration was 4.40 ± 0.2 μmol CO_2 min^{-1} [N = 65]) was detected in plasma samples from dogfish. However, by 5 min following the injection of benzolamide (1.3 mg kg^{-1} or 13 μmol L^{-1}), the plasma CA activity was not significantly different from the uncatalysed rate. While RBC CA activity also was decreased by the benzolamide treatment, the RBC CA activity at 5 min following benzolamide injection was still 51.2% ± 6.2% (N = 5) of the control value and remained at this level throughout the remainder of the 30-min experimental period. By contrast, RBC CA activity was decreased to 11.6% ± 2.9% (N = 5) of the control value 5 min after the injection of acetazolamide (30 mg kg^{-1} or 450 μmol L^{-1}) and by 15 min of acetazolamide treatment, only 4.6% ± 1.8% (N = 5) of the RBC CA activity remained. It should be noted that sample dilution before and during the electrometric assay results in dissociation of the inhibitor from the enzyme, such that the measured values are likely to be conservative estimates of CA inhibition in vivo. Thus, as it is thought that >98% inhibition of CA activity is required to produce a measurable physiological effect (Maren 1967), benzolamide treatment effectively inhibited only extracellular CA activity, whereas both extracellular and RBC CA activities were probably fully inhibited by 15 min of acetazolamide treatment.

The in vitro assessment of the effects of the inhibitors on RBC CA activity in rainbow trout supported the results obtained for the plasma and RBC CA activities of samples withdrawn from dogfish during in vivo experiments. Because the aim of these in vitro assays was solely to determine the extent

Table 2: Effect of benzolamide (1.3 mg kg^{-1}) on Pao_2, ventilation parameters, and $\dot{V}b$ in dogfish

	Control	5 Min	10 Min	15 Min	30 Min
Pao_2 (Torr)	105.9 ± 7.1	105.3 ± 7.5	102.2 ± 8.3	102.0 ± 9.9	97.9 ± 11.9
f_R (min^{-1})	40 ± 2	40 ± 2	41 ± 1	40 ± 2	39 ± 2
V_{AMP} (cm)	4.1 ± 1.8	4.4 ± 1.6	4.5 ± 1.8	4.1 ± 1.7	4.0 ± 1.7
$\dot{V}b$ (mL kg^{-1} min^{-1})	28.6 ± 5.1	27.8 ± 4.8	27.9 ± 4.9	26.9 ± 4.8	23.0 ± 3.7*

Note. f_R, breathing frequency; V_{AMP}, ventilation amplitude; $\dot{V}b$, blood flow. Values are means ± 1 SEM; $N = 6–7$.

* A significant difference (one-way repeated-measures ANOVA followed by Dunnett's post hoc test for multiple comparisons, $P < 0.05$) between the treatment value and its associated control.

to which RBC CA activity was impacted by the inhibitor treatments, the blood samples used for the $[^{14}C]HCO_3^-$ assays were obtained from rainbow trout, which lack plasma CA activity. Incubation of trout blood with 450 μmol L^{-1} acetazolamide for 1 h significantly decreased the HCO_3^- dehydration rate in comparison to the rate obtained in the absence of any inhibitor (Table 1). Indeed, the HCO_3^- dehydration rate following incubation with acetazolamide for 1 h did not differ significantly from the rate obtained for separated plasma and was assumed to represent the situation where RBC CA was fully inhibited. The HCO_3^- dehydration rate measured for trout blood in vitro was unaffected by incubation with 13 μmol L^{-1} benzolamide, but treatment with 450 μmol L^{-1} acetazolamide for any time decreased the HCO_3^- dehydration rate to a value that was not significantly different from the fully inhibited rate (Table 1). Thus, even though benzolamide enters the RBC and inhibits RBC CA activity, the degree of inhibition of RBC CA activity is not sufficient to significantly impact upon the whole blood rate of CO_2 production in vitro.

The Effects of CA Inhibition in Dogfish

Treatment of dogfish with benzolamide (1.3 mg kg^{-1}) to inhibit extracellular CA activity had a significant impact on measures of CO_2 excretion by 5 min following injection of the inhibitor (Fig. 3). In addition to a significant increase in $Paco_2$ and a corresponding fall in pHa (Fig. 3*A*, 3*B*), the arterial-venous difference in plasma Cco_2 (ΔCco_2) exhibited a significant 40% decrease at 5 min (Fig. 3*C*). It should be noted that it did not prove practical to calculate CO_2 excretion from measurements of ΔCco_2 and $\dot{V}b$ for either dogfish or trout because of difficulties in obtaining consistently both sets of measurements for individual fish. Pao_2, f_R, V_{AMP}, and $\dot{V}b$ were unaffected by benzolamide treatment, with the exception of a small but significant fall in $\dot{V}b$ at 30 min (Table 2).

Similar results were obtained under DIDS treatment or after the infusion of a HCO_3^- load. The injection of DIDS to inhibit HCO_3^- flux through the RBCs caused a small but significant increase in pHa (Fig. 4*A*) but was otherwise without effect. Subsequent treatment with benzolamide decreased ΔCco_2 (Fig. 4*C*) and resulted in a significant increase in $Paco_2$ (Fig. 4*B*) together with a decrease in pHa (Fig. 4*A*). The infusion of a HCO_3^- load was associated with significant increases in pHa, $Paco_2$, and ΔCco_2 (Fig. 5) but did not affect ventilation parameters or $\dot{V}b$ (data not shown). Injection of benzolamide into HCO_3^--loaded dogfish again resulted in a significant increase in $Paco_2$ (Fig. 5*B*), a corresponding fall in pHa (Fig. 5*A*), and a significant decrease in ΔCco_2 (Fig. 5*C*). Indeed, the impact of benzolamide injection on the arterial-venous plasma Cco_2 difference, pHa, and $Paco_2$ did not differ significantly among the three experiments (Fig. 6). Arterial O_2 tension, ventilation parameters and $\dot{V}b$ were unaffected by benzolamide injection in DIDS-treated and HCO_3^--loaded dogfish (data not shown).

Increasing extracellular CA activity by the addition to the circulation of bovine CA had no effect on measures of CO_2 excretion in dogfish (Fig. 7). Inhibition of extracellular and RBC CA activities by the subsequent injection of acetazolamide, on the other hand, had a negative impact on CO_2 excretion, resulting in a significant increase in $Paco_2$ (Fig. 7*B*) and a significant reduction in ΔCco_2 to a value not significantly different from zero (Fig. 7*C*). The initial rise in pHa with acetazolamide, which was a consequence of the high pH of the injected solution (see Gilmour et al. 1994), tended to confound the effect of inhibiting both extracellular and RBC CA, such that the pHa changes observed with acetazolamide treatment were not significant (Fig. 7*A*).

Comparisons of the effects of the two CA inhibitors indicated that the inhibitor-induced depression of ΔCco_2 and elevation of $Paco_2$ were significantly greater in acetazolamide-injected dogfish than with benzolamide treatment by, respectively, 10 and 15 min following administration of the CA inhibitor (Fig. 8*B*, 8*C*). The differences between benzolamide and acetazolamide treatments with respect to pHa were primarily a reflection of the initial rise in pHa with acetazolamide due to the high pH of the injection vehicle (Fig. 8*A*). Ventilation parameters, $\dot{V}b$ and Pao_2, were largely unaffected by CA or acetazolamide treatments (data not shown).

The Effects of CA Inhibition in Rainbow Trout

In contrast to the significant impact of benzolamide injection in dogfish, treatment of rainbow trout with benzolamide (1.3 mg kg^{-1}) had no effect on any measure of CO_2 excretion 5,

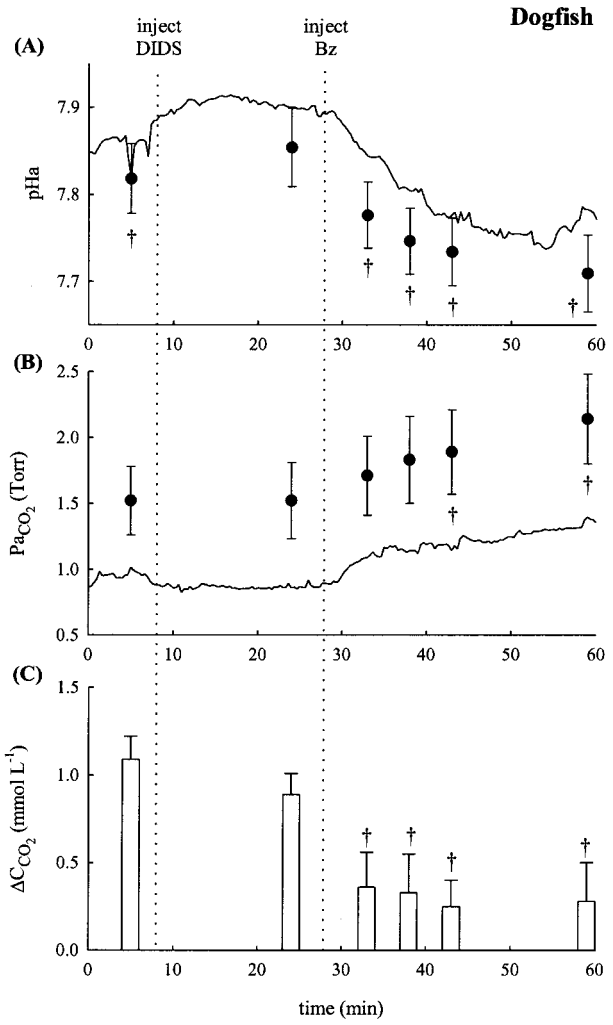

Figure 4. The effects of intra-arterial injection of DIDS (final concentration in the blood of 1×10^{-4} mol L^{-1}) followed by benzolamide (*Bz*; 1.3 mg kg^{-1}) on *A*, arterial blood pH (pHa), *B*, arterial blood Pco_2 ($Paco_2$), and *C*, the arterial-venous total CO_2 concentration difference (ΔCco_2), in dogfish. The dotted vertical lines indicate the time of DIDS and Bz injections. The symbols represent mean values ± 1 SEM ($N = 6$). Data for pHa and $Paco_2$ were compiled for 1-min periods corresponding to the times at which pre- and postbranchial blood samples were withdrawn for Cco_2 measurements (i.e., before any treatment [control]; 15 min after DIDS injection; and at 5, 10, 15, and 30 min after Bz injection). A dagger indicates a significant difference from the DIDS value (one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test, $P < 0.05$). The lines in panels *A* and *B* present a representative original data recording illustrating the effects of benzolamide treatment on pHa and $Paco_2$.

10, 15, or 30 min following the benzolamide injection. Carbon dioxide excretion in trout was significantly impacted by the subsequent injection of acetazolamide (30 mg kg^{-1}; Fig. 9). ΔCco_2 was unaffected by benzolamide injection (Fig. 9*D*), as were pHa, $Paco_2$, and $\dot{M}co_2$ (Fig. 9*A*–9*C*). Acetazolamide treatment, on the other hand, caused a significant elevation of $Paco_2$ (Fig. 9*B*) and significant reductions in pHa and $\dot{M}co_2$ (Fig. 9*A*, 9*C*); ΔCco_2 was not measured after acetazolamide injection. As in dogfish, the initial rise in pHa with acetazolamide was a consequence of the high pH of the injected solution. The remaining variables measured (Pao_2, f_R, V_{AMP}, and $\dot{V}b$) were not affected by either benzolamide or acetazolamide treatment (data not shown).

Discussion

Characterisation of CA Activity in Dogfish Gills

The membrane-associated CA isozyme (CA IV) has been found in mammals in a variety of locations (Sly and Hu 1995). While minor differences have been found among tissues and species, mammalian CA IV characteristically is anchored to the cell membrane by a phosphatidylinositol-glycan linkage and can be released from the cell membrane by treatment with the enzyme PI-PLC (Zhu and Sly 1990; Waheed et al. 1992*b*). Like the cytosolic CA II, CA IV is a high-activity, sulphonamide-sensitive enzyme (Baird et al. 1997).

With respect to lower vertebrates, Stabenau et al. (1996) used a saline-perfused lung preparation to demonstrate the presence of a PI-PLC-sensitive pulmonary CA activity in turtles. In fish, a membrane-associated CA with CA IV-like characteristics was found in the air bladder of bowfin (Gervais and Tufts 1998). The results of this study indicate that the gill membrane–bound CA activity of the dogfish, *Squalus acanthias*, is likely also to be a type IV isozyme. Previous studies provided evidence for a membrane-associated CA located on the basolateral membrane of the gill in dogfish and accessible to plasma reactions (Swenson et al. 1995, 1996; Gilmour et al. 1997; Henry et al. 1997; Wilson et al. 2000). The capacity of PI-PLC to release this CA activity in vitro from the microsomal pellet into the supernatant (Fig. 2*A*) is consistent with identification of the membrane-associated CA activity of dogfish gills as a type IV-like isozyme. Like mammalian CA IV, the CA IV-type activity of dogfish gills exhibits a high sensitivity to sulphonamide inhibitors as evidenced by the low K_i values measured for benzolamide and acetazolamide (Fig. 1). However, the sulphonamide sensitivity of dogfish gill CA IV-like activity is similar to that of the cytoplasmic CA, a situation that is also true of the sulphonamide sensitivity of bowfin air bladder CA IV-like and gill cytoplasmic CA activities (Gervais and Tufts 1998). Mammalian CA IV, on the other hand, is typically seven- to 33-fold less sensitive to sulphonamide inhibition than CA II (Maren et al. 1993; Baird et al. 1997).

Dogfish gill cytoplasmic CA activity was also highly sensitive to inhibition by both sulphonamide inhibitors examined in this study, yielding K_i values (6–8 nmol L^{-1}; Fig. 1) similar to those reported for trout, lamprey, bowfin, and flounder against acetazolamide (K_i = 2–8 nmol L^{-1}; Henry et al. 1993; Gervais and

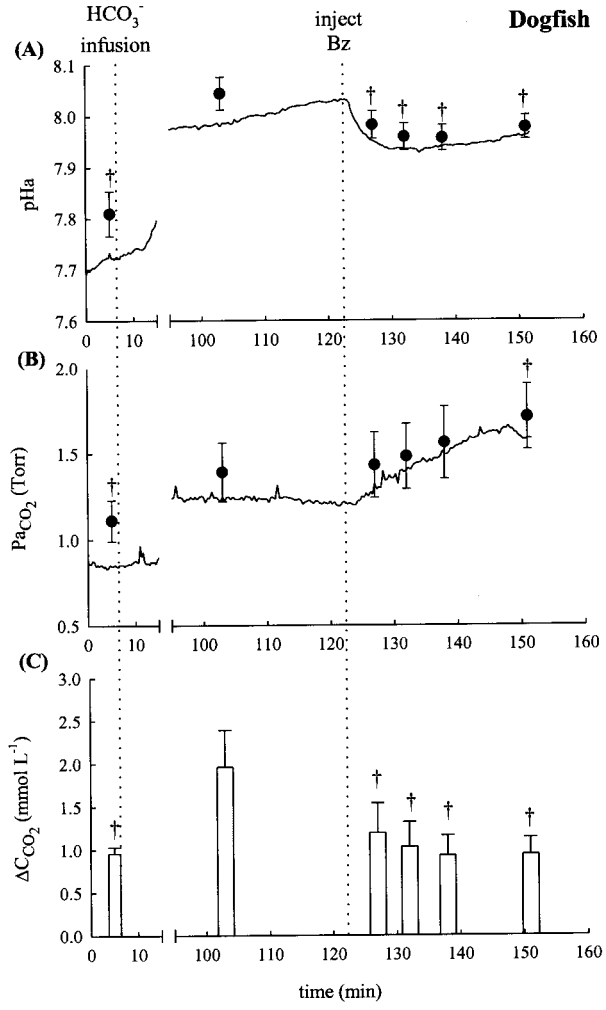

Figure 5. The effects on *A*, arterial blood pH (pHa), *B*, arterial blood P_{CO_2} ($Paco_2$), and *C*, the arterial-venous total CO_2 concentration difference (ΔCco_2) of HCO_3^- infusion (500 mmol L^{-1} $NaHCO_3$ solution infused at a rate of 1,600 μmol kg^{-1} h^{-1}) and benzolamide (*Bz*; 1.3 mg kg^{-1}) injection during HCO_3^--infusion, in dogfish. The dotted vertical lines indicate the time at which HCO_3^- infusion was initiated and Bz was injected. The symbols represent mean values ± 1 SEM (N = 6 for pHa and $Paco_2$, N = 6 for ΔCco_2). Data for pHa and $Paco_2$ were compiled for 1-min periods corresponding to the times at which postbranchial blood samples were withdrawn for Cco_2 measurements (i.e., before any treatment [control], when the measured variables had restabilised during HCO_3^- infusion [high HCO_3^-] and at 5, 10, 15, and 30 min following Bz injection); note that the ΔCco_2 measurements were made on a separate group of dogfish (see "Material and Methods"). A dagger indicates a significant difference from the high HCO_3^- value (one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test, $P < 0.05$). The lines in panels *A* and *B* present a representative original data recording illustrating the effects of HCO_3^--loading and benzolamide treatment on pHa and $Paco_2$.

Tufts 1998; Sender et al. 1999). The similarity of these inhibition constants contrasts with the situation for RBC cytoplasmic CA, where K_i values against acetazolamide differ by two orders of magnitude; trout, flounder, and bowfin 2–8 nmol L^{-1} (Henry et al. 1993; Gervais and Tufts 1999; Sender et al. 1999), lamprey 22 nmol L^{-1} (Henry et al. 1993), and dogfish 200 nmol L^{-1} (Maren et al. 1980; see also Henry and Heming 1998).

Physiological Role of Extracellular CA Activity in Dogfish

The physiological function of pulmonary capillary CA IV in higher vertebrates remains unclear. Given its high efficiency for HCO_3^- dehydration, its availability to plasma reactions, and the carriage of the majority of CO_2 in the blood as HCO_3^- ions in the plasma, pulmonary capillary CA IV would appear to be ideally situated to contribute to CO_2 excretion. Specifically, it could provide an extracellular site for HCO_3^- dehydration at the catalysed rate, in addition to the catalysed dehydration of HCO_3^- via the RBC. However, it has been argued on the basis of mathematical models that HCO_3^- dehydration in the plasma would be limited by proton availability, such that CA IV could account for less than 10% of total CO_2 excretion (Bidani 1991). Furthermore, the presence of endogenous CA inhibitors in the plasma of some species (Hill 1986; Roush and Fierke 1992) would presumably lower the activity of CA IV in vivo, further limiting its ability to contribute to CO_2 excretion (Heming et al. 1993).

In this study, the arterial-venous plasma Cco_2 difference, $Paco_2$, and pHa were monitored during selective inhibition of extracellular CA activity in dogfish to assess the contribution of gill membrane-bound and plasma CA activities to CO_2 excretion in vivo. It was originally intended to calculate CO_2 excretion in vivo from measurements of blood flow and arterial-venous Cco_2 differences, but the difficulties encountered in simultaneously measuring $\dot{V}b$ and collecting pre- and postbranchial blood samples in sufficient numbers of fish precluded such an analysis. However, as both $\dot{V}b$ and ventilation parameters were in general unaffected by the experimental treatments, the arterial-venous plasma total CO_2 concentration difference (i.e., ΔCco_2) provided a useful indicator of CO_2 excretion.

The efficacy of CA inhibitor treatments was tested both in vivo, by assaying samples withdrawn during experiments for plasma and RBC CA activities by the electrometric ΔpH method, and in vitro, using a radioisotopic assay to examine RBC CO_2 production in the absence/presence of CA inhibitors. As in earlier work (e.g., Swenson and Maren 1987; Gilmour et al. 1997), a low concentration (1.3 mg kg^{-1}) of benzolamide, which has a low diffusibility, selectively inhibited extracellular CA activity. Inhibition of both RBC and extracellular CA activities was achieved with a higher dose (30 mg kg^{-1}) of the more highly diffusible acetazolamide. Given its high sensitivity to sulphonamides, gill cytoplasmic CA activity would be expected to be inhibited by both treatments (see also Swenson

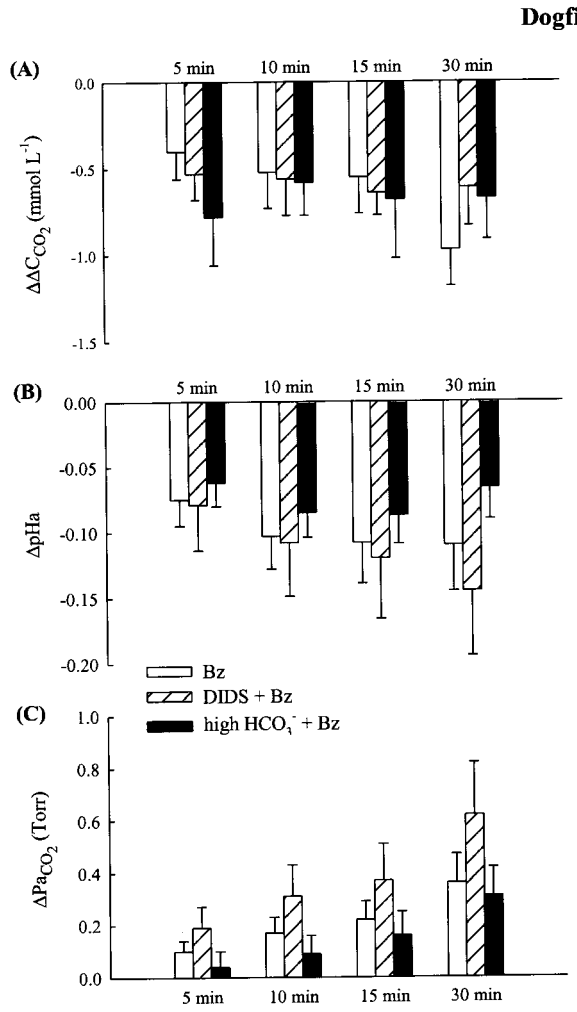

Figure 6. A comparison of the effects of benzolamide (*Bz*; 1.3 mg kg^{-1}) treatment in dogfish under control conditions (*Bz*), following DIDS injection (DIDS + Bz) and during HCO_3^- infusion (high HCO_3^- + Bz). Data are replotted from Figures 2–4 as the difference between the control value and the value at 5, 10, 15, or 30 min following Bz injection for *A*, the arterial-venous total CO_2 concentration difference ($\Delta\Delta Cco_2$), *B*, arterial pH (ΔpHa), and *C*, arterial Pco_2 ($\Delta Paco_2$). Values are means ± 1 SEM (N = 5–7). No significant differences were observed among the three treatments (one-way ANOVA followed by Dunnett's multiple-comparisons test, $P > 0.05$).

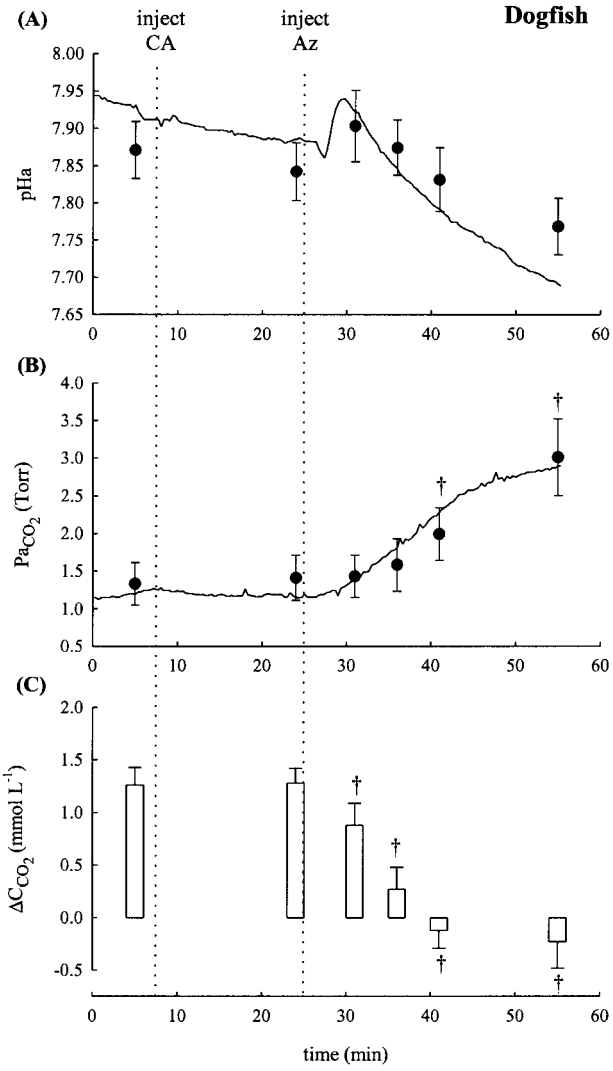

Figure 7. The effects in dogfish on *A*, arterial blood pH (pHa), *B*, arterial blood Pco_2 ($Paco_2$), and *C*, the arterial-venous total CO_2 concentration difference (ΔCco_2) of intra-arterial injection of bovine CA (2 mg kg^{-1}) followed by acetazolamide (*Az*; 30 mg kg^{-1}). The dotted vertical lines indicate the time of CA and Az injections. The symbols represent mean values ± 1 SEM (N = 5–6). Data for pHa and $Paco_2$ were compiled for 1 min periods corresponding to the times at which pre- and postbranchial blood samples were withdrawn for Cco_2 measurements (i.e., before any treatment [control]; 15 min after bovine CA injection; and at 5, 10, 15, and 30 min following Az injection). A dagger indicates a significant difference from the CA value (one-way repeated-measures ANOVA followed by Dunnett's multiple-comparisons test, $P < 0.05$). The lines in panels *A* and *B* present a representative original data recording illustrating the effects CA and Az treatments on pHa and $Paco_2$.

and Maren 1987). However, inhibition of this CA activity should not substantially influence conclusions with respect to CO_2 excretion since the very low permeability of gill cell basal membranes to HCO_3^- ions limits its contribution to CO_2 excretion (Perry et al. 1982, 1984; Tufts and Perry 1998).

The significant 30%–60% reduction in the arterial-venous plasma Cco_2 difference of dogfish in vivo following inhibition of plasma and gill membrane-bound CA by benzolamide (Fig.

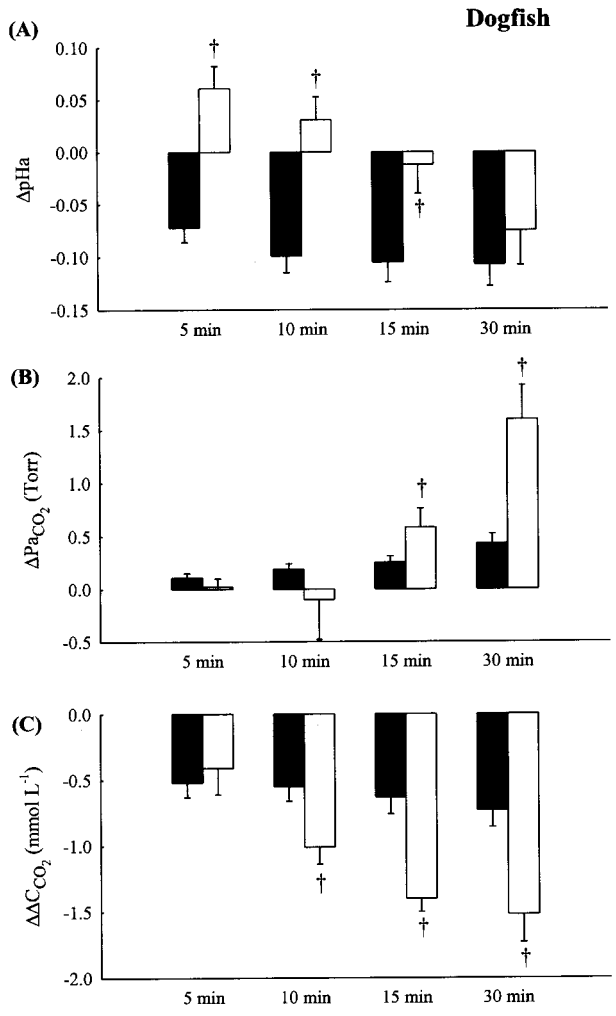

Figure 8. A comparison of the effects of benzolamide (*filled bars*; 1.3 mg kg^{-1}) and acetazolamide (*unfilled bars*; 30 mg kg^{-1}) treatments in dogfish. As no significant differences were detected among the three benzolamide treatments, data from all three benzolamide treatments, as presented in Figure 5, were combined. Data are replotted from Figures 5 and 6 as the difference between the appropriate control value and the value at 5, 10, 15, or 30 min following injection of the CA inhibitor for *A*, arterial pH (ΔpHa), *B*, arterial P_{CO_2} (ΔPa_{CO_2}), and *C*, the arterial-venous total CO_2 concentration difference ($\Delta\Delta C_{CO_2}$). Values are means ± 1 SEM (N = 17–19 for benzolamide, N = 5–7 for acetazolamide). A dagger indicates a significant difference between the benzolamide and acetazolamide treatments (unpaired Student's t-test, $P < 0.05$).

6*A*) argues strongly that extracellular CA activity contributes substantially to CO_2 excretion in this elasmobranch fish. In all three benzolamide treatments, ΔC_{CO_2} was significantly inhibited 5 min after benzolamide injection (Figs. 3*C*, 4*C*, 5*C*), even though RBC CA activity remained well within the range required for normal function. Similarly, a significant 30% reduction in ΔC_{CO_2} was observed 5 min after acetazolamide injection (Fig. 7*C*), that is, at a time when RBC CA was probably not yet fully inhibited. Indeed, the reduction in ΔC_{CO_2} did not differ between acetazolamide and benzolamide at this time (Fig. 8*C*). RBC CA function was, however, likely impacted to a physiologically relevant extent 10–15 min postacetazolamide injection, accounting for the significantly greater inhibition of ΔC_{CO_2} with acetazolamide than benzolamide beyond 5 min (Fig. 8*C*).

The reduction in the arterial-venous ΔC_{CO_2} following benzolamide in dogfish was accompanied in each case by a significant fall in arterial pH (Figs. 3*A*, 4*A*, 5*A*) that was probably the product of two factors; CO_2 retention and a change in the status of the postbranchial blood acid-base disequilibrium (Gilmour et al. 1997). The retention of CO_2 resulting from the decreased clearance of C_{CO_2} from the plasma was reflected in the significant increases in Pa_{CO_2} observed in all three benzolamide treatments. This increase was significant at 5 min in dogfish treated with benzolamide alone (Fig. 3*B*) but did not become significant until at least 15 min in HCO_3^--loaded or DIDS-treated dogfish (Figs. 4*B*, 5*B*). Similarly, Gilmour et al. (1997) reported a nonsignificant increase in Pa_{CO_2} 6 min after benzolamide injection. In view of the small size of the Pa_{CO_2} increases early in benzolamide treatment (approximately 9% at 5 min) and individual variation among dogfish, that the Pa_{CO_2} increases 5-min postbenzolamide injection were not consistently significant was not surprising. In the absence of compensatory adjustments to $\dot{V}b$ or ventilation following the inhibition of extracellular CA (Table 2), the elevation of Pa_{CO_2} acts to reset the P_{CO_2} gradient for CO_2 diffusion across the gills, establishing a new steady state for CO_2 excretion. When both extracellular and RBC intracellular CAs are inhibited with acetazolamide, the Pa_{CO_2} increase must be correspondingly larger (as at 15 and 30 min in Fig. 8*B*).

The significant effects of benzolamide treatment on ΔC_{CO_2}, Pa_{CO_2}, and pHa (Figs. 3–5) clearly indicate that extracellular CA is involved in CO_2 excretion in resting dogfish. Furthermore, analysis of plasma samples obtained from dogfish at different times during the experimental protocol confirmed that treatment effects in this study were due to the inhibition of naturally occurring extracellular CA and not the result of inhibiting plasma CA that had been elevated artificially through experimentally induced haemolysis. In this regard, the very similar ΔC_{CO_2} responses to benzolamide injection of fish that were not subjected to the extracorporeal blood circulation (Fig. 5*C*) and fish used in extracorporeal experiments (Figs. 3*C*, 4*C*), with their attendant higher risk of haemolysis, were noteworthy. Thus, the inhibition of CO_2 excretion reported in this study in response to selective inhibition of extracellular CA activity is a real effect, and not the result of experimental artefact.

The potential involvement of extracellular CA in CO_2 excretion in dogfish has been the subject of some discussion

(reviewed by Henry and Heming 1998) owing to the results of several studies in which selective inhibition of extracellular CA did not appear to substantially affect CO_2 excretion. For example, Swenson and coworkers did not detect a significant increase in arterial P_{CO_2} in resting dogfish treated with the selective inhibitor polyoxyethylene-aminobenzolamide (Swenson et al. 1995) or 2 h after benzolamide administration (1 mg kg^{-1}; Swenson and Maren 1987). However, the equivalent effects of inhibitors with access to different cellular compartments in the clearance of an injected HCO_3^- load led Swenson and coworkers to conclude that membrane-bound CA activity plays an important role in the correction of a metabolic alkalosis in vivo in dogfish (Swenson et al. 1995), a conclusion that is consistent with the results of this study. Interestingly, P_{CO_2} was significantly elevated by benzolamide treatment in active dogfish (Swenson and Maren 1987), suggesting that extracellular CA may contribute to CO_2 excretion under conditions of increased CO_2 loading. Furthermore, no increase in P_{aCO_2} or reduction of ΔC_{CO_2} occurred in dogfish treated with DIDS in vivo to decrease the rate of Cl^-/HCO_3^- exchange (Fig. 4; see also Gilmour et al. 1997). As any reduction in the speed of this rate-limiting process (Perry 1986; Tufts and Perry 1998) might be expected to inhibit CO_2 excretion significantly, the absence of such inhibition in DIDS-treated dogfish also suggests that extracellular CA may contribute to CO_2 excretion.

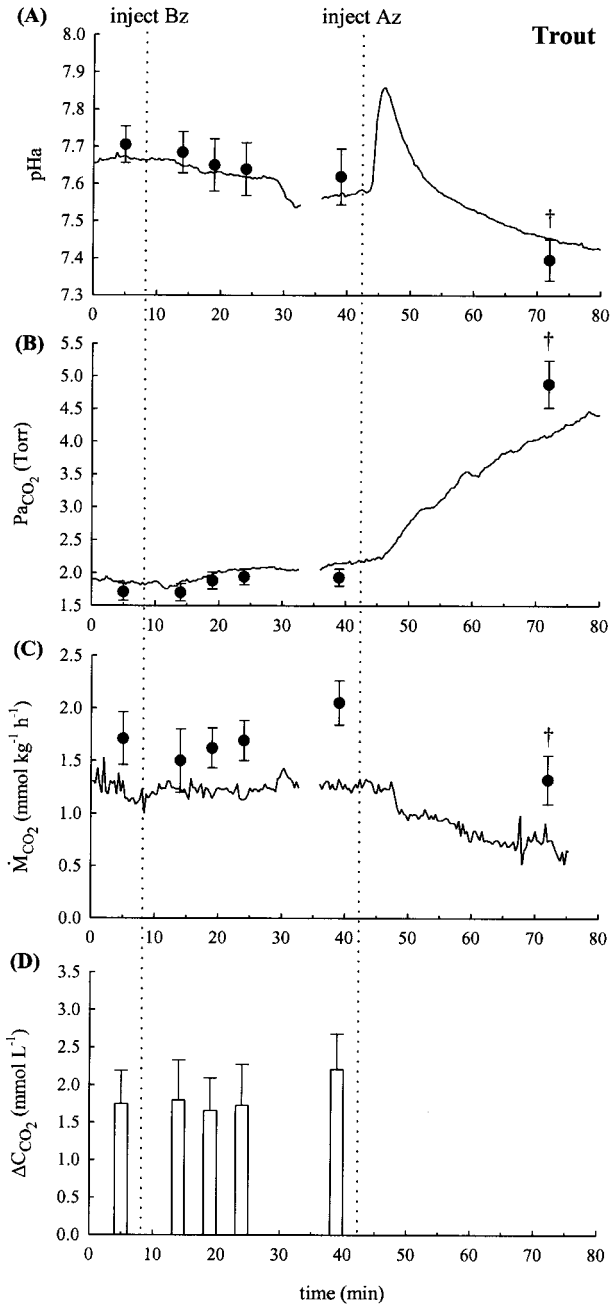

Figure 9. The effects in rainbow trout (*Oncorhynchus mykiss*) of intra-arterial injection of benzolamide (*Bz*, 1.3 mg kg^{-1}) followed by acetazolamide (*Az*, 30 mg kg^{-1}) on *A*, arterial blood pH (pHa), *B*, arterial blood P_{CO_2} (Pa_{CO_2}), *C*, CO_2 excretion determined by respirometry ($\dot{M}_{CO_2}$), and *D*, the arterial-venous total CO_2 concentration difference (ΔC_{CO_2}). The dotted vertical lines indicate the times of injection. The symbols represent mean values ± 1 SEM (N = 5–6). Data for pHa, Pa_{CO_2}, and $\dot{M}_{CO_2}$ were compiled for 1-min periods corresponding to the times at which the pre- and postbranchial blood samples were withdrawn for C_{CO_2} measurements (i.e., before Bz injection [control]; 5, 10, 15, and 30 min after Bz was injected; and 30 min after Az was injected). A dagger indicates a statistically significant difference from the 30-min Bz data point (one-way repeated-measures ANOVA followed by the Student-Newman-Keuls multiple-comparisons test, $P < 0.05$). The lines in panels *A–C* present a representative original data recording illustrating the effects of benzolamide followed by acetazolamide treatment on pHa, Pa_{CO_2}, and $\dot{M}_{CO_2}$.

Such findings led to the hypothesis that extracellular CA in dogfish functions to provide a reserve capacity for CO_2 excretion under conditions of increased CO_2 loading, such as exercise (Gilmour et al. 1997; Henry et al. 1997). However, based on the results of this study, it would be more appropriate to postulate that extracellular CA always contributes to CO_2 excretion, but to a fixed, limited extent. As in a previous study (Gilmour et al. 1997), injection of DIDS elicited a significant increase in pHa without affecting Pa_{CO_2} or ΔC_{CO_2} (Fig. 4), probably owing to changes in the magnitude of the acid-base disequilibrium in the postbranchial blood caused by the partial inhibition of anion exchange (Gilmour et al. 1997). However, neither CO_2 loading via HCO_3^- infusion nor DIDS treatment enhanced the benzolamide effect over the control value (Fig. 6), suggesting that extracellular CA is making a maximal contribution to CO_2 excretion under control conditions, perhaps because plasma HCO_3^- dehydration is constrained by limited proton availability.

Limitations on proton availability owing to the low buffering capacity of plasma relative to that of the RBC are thought to restrict the contribution of pulmonary CA IV to CO_2 excretion

in mammals to <10% (Bidani 1991). The results of this study, however, suggest that extracellular CA could potentially contribute up to 30%–60% of total CO_2 excretion in dogfish. In this regard, it is notable that the plasma in certain elasmobranchs contributes more than the RBCs to whole-blood buffering capacity, in contrast to the situation in teleost fish and higher vertebrates (Tufts and Perry 1998). For example, the plasma buffer values of *S. acanthias* (-6.5 mmol L^{-1} pH $unit^{-1}$; Lenfant and Johansen 1966) and *Raja ocellata* (-6.6 mmol L^{-1} pH $unit^{-1}$; Graham et al. 1990) constitute 72% and 60% of whole-blood buffering, respectively, whereas the plasma buffering capacity in humans, which at -6.5 mmol L^{-1} pH $unit^{-1}$ is similar to that of *S. acanthias* (Lenfant and Johansen 1966), constitutes only 22% of the whole-blood buffer value. Thus, the elevated importance of extracellular CA in CO_2 excretion in dogfish may reflect the greater buffering capacity of the extracellular compartment relative to that of the RBC, a reversal of the mammalian situation.

If the contribution of extracellular CA to CO_2 excretion is maximal under control conditions owing to proton availability constraints, then the lack of effect on ΔC_{CO_2} or P_{aCO_2} of increasing plasma CA activity by administering bovine CA would be expected (Fig. 7*B*, 7*C*). The trend for pHa to decrease following bovine CA injection, while not significant (Fig. 7*A*), probably resulted from the establishment of an equilibrium condition in the postbranchial blood. As discussed by Gilmour et al. (1997), the equilibrium pH for arterial blood in dogfish is lower than the pH measured in vivo in postbranchial blood because the latter is not at equilibrium. Therefore, addition to the circulation of sufficient CA activity to establish equilibrium causes a fall in the pH measured in vivo in the postbranchial blood.

Unlike the situation in the spiny dogfish, CO_2 excretion in rainbow trout was not affected by the administration of benzolamide (Fig. 9). By contrast, treatment of rainbow trout with acetazolamide to inhibit RBC CA activity elicited the classic response (e.g., Hoffert and Fromm 1973) of a profound respiratory acidosis; $\dot{M}_{CO_2}$ was reduced by 36%, while P_{aCO_2} was more than double the preinjection value by 30-min postacetazolamide injection. These results are consistent with those of previous studies on teleost fish, which have demonstrated that no change occurs in the status of the postbranchial blood acid-base disequilibrium of rainbow trout following benzolamide injection (Perry et al. 1997), and that the CA activity associated with the microsomal fraction of teleost fish gills is slight (Henry et al. 1988, 1993, 1997; Sender et al. 1999). Furthermore, significantly less CA activity is integrally associated with the microsomal pellet in trout than in dogfish gills, and no CA activity is released from the microsomal fraction of trout gills by treatment with PI-PLC (Fig. 2*B*), implying that CA IV is not present in trout gills. Thus, the absence of a benzolamide effect on CO_2 excretion in rainbow trout confirms that plasma CO_2-HCO_3^--H^+ reactions in the gills of teleost fish do not have access to CA activity and contrasts with the substantial contribution to CO_2 excretion made by extracellular CA in dogfish.

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