Inhibition of Gill Carbonic Anhydrase Blocks CO_2 Excretion in the Isolated Perfused Head of the Dogfish "Pup" Squalus acanthias.

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Introduction: Controversy has existed as to the role of red cell and gill carbonic anhydrase in normal CO_2 exchange of fish. Haswell et al (Am. J. Physiol. 238: R240, 1980) showed that CO_2 exchange occurs across the in situ isolated saline-perfused head of rainbow trout and is reduced by carbonic anhydrase (CA) inhibition. This led them to propose that gill enzyme functions in CO_2 exchange. However, Perry et al (J. Exp. Biol. 101:47, 1982) reached the opposite conclusion, based on experiments in the isolated perfused trout holobranch preparation, which was unable to excrete CO_2 or HCO_3^- when perfused with saline, but did so with blood. Experiments in the intact shark by Swenson et al (Bull. MDIBL 22:72, 1982 and this volume) support the idea that in elasmobranchs gill carbonic anhydrase does contribute to CO_2 exchange (both HCO_3^- excretion in metabolic alkalosis and CO_2 excretion during exercise). To directly examine the role of gill CA in the whole animal requires selective inhibition of red cell enzyme. Since no known CA inhibitors are this specific, we turned to the isolated perfused head of the dogfish pup where saline perfusion allows the study of gill enzyme in the absence of red cell carbonic anhydrase.

Methods: Near-term dogfish "pups" (40g) were removed from females and maintained in running seawater until used. The isolated head preparation utilized was that described by Evans and Claiborne (J. Exp. Biol. 105:363, 1983). Differences in methodology included the use of (0.1 µM) epinephrine in the perfusion fluid and suturing the spiracles. The perfusion rate was 700 µl/min, with a measured afferent pressure of ~ 30 mmHg. The perfusate was dogfish Ringers (Forster et al Comp. Biochem. Physiol. 42A:3, 1972) with a $[NaHCO_3]$ of 8 mM, equilibrated with a gas mixture of 0.4% CO_2, 8% O_2, 91.6% N_2 to achieve a pH of 7.60. Following a 30 minute period of stabilization, three samples of ventral artery perfusate and dorsal artery outflow were taken anaerobically for measurement of total CO_2. Then benzolamide (10 µM) was added to the perfusate and 3-5 samples of perfusate and outflow were collected at 10 minute intervals after a period of 30-60 minutes. Total CO_2 was measured conductometrically in duplicate (Capni-Con II, Cameron Instruments Inc., Port Aransas, Texas).

Results: Eight fish were studied. During the control period ventral artery total CO_2 was 6.41 ± 0.12 mM (SEM) and dorsal artery total CO_2 was 6.03 ± 0.12 mM. The A-V difference was 0.38 mM, a statistically significant value ($p = 0.04$). After CAI with benzolamide this A-V difference fell to 0.01 mM which was not statistically significant from zero (5.74 ± 0.10 mM versus 5.73 ± 0.10 mM).

Discussion: These data show that the gills of the dogfish "pup" are capable of measurable CO_2 excretion. Total CO_2 excretion (VCO_2) calculated as perfusate flow times the A-V difference is 0.67 µmoles/min/100 g body weight. This is approximately 25% the VCO_2 measured in the normal adult dogfish (Robin et al, J. Cell. Physiol. 67:93, 1966). Our measured rates of CO_2 excretion in the pup do not match those of the whole fish and may be a consequence of the non-physiologic stress of the preparation. However it may also represent an important role to red cells apart from their carbonic anhydrase. Nevertheles it appears that gills perfused without red cells have the capacity to eliminate CO2 and confirm the work in the whole animal showing that gill CA contributes to normal CO_2 exchange (Swenson et al, Bull MDIBL, this volume).

This research was supported by NSF PCM 81-04046.

ATRIOPEPTIN: A HUMORAL MEDIATOR OF RECTAL GLAND FUNCTION IN *SQUALUS ACANTHIAS*

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The rectal gland of the shark can be stimulated *in vivo* by extracellular volume expansion (Solomon, R.J. et al. Am.J.Physiol. 246: R63-6, 1984). This stimulation of secretion results in a net loss of fluid and electrolytes from the fish that returns extracellular volume toward normal. In this negative feedback system, volume rather than osmolality is the afferent signal.

How the signal of volume expansion is translated into an increase in rectal gland secretion is unknown. The increase in secretion that follows volume expansion is accompanied by a three to fourfold increase in blood flow to the gland. This vasodilatation is observed in the absence of any change in systemic blood pressure and may contribute to the secretory response. The stimulation of rectal gland chloride secretion that follows volume expansion is also observed in denervated, transplanted glands. This suggests that the rectal gland is primarily under hormonal regulation.

The experiments described below were designed to test the hypothesis that atriopeptin, a hormone produced in cardiac tissue, is released in response to a volume load and stimulates rectal gland chloride secretion.

Male and female dogfish were prepared for *in vivo* study (Solomon, R.J., Ibid). Isolated perfused glands were also studied as previously described (Silva, P. et al. Am.J.Physiol. 233: F298-306, 1977).

Tissue extracts were prepared as follows. Specific tissues were removed by blunt dissection and placed in cold phosphate buffer (0.1 M, pH 7.6). The tissues were then blotted dry, weighed and homogenized in the same buffer (10:1;V:W) using an Ultra-Turrax for 1 minute. The homogenates were placed immediately in boiling water for 15 minutes and then cooled to room temperature. The supernatant from a single 25,000g x 30 minute centrifugation was used for infusion into the *in vitro* perfused rectal gland.

Blood was obtained from the dorsal aorta of pithed dogfish. The serum was separated from the blood and boiled for 15 minutes. It was then centrifuged at 25,000g x 30 minutes and incubated for 60 minutes at 22°C with 10U of adenosine deaminase prior to infusion into the *in vitro* perfused rectal gland. The same gland was used as the bioassay system for blood obtained before and after volume expansion.

Oxygen consumption of rectal gland slices and dispersed cells was determined by previously described methods (Silva, P. et al. Bull.Mt.Desert Isl.Biol.Lab.22: 9-11, 1982).

The effects of an intra-arterial injection of synthetic atriopeptin (10µg/kg) in the intact shark are displayed in Table 1. Significant increases in duct flow, chloride secretion, and rectal gland blood flow were observed, usually within 30 minutes of the injection. No effects on urinary flow were observed.

Table 1. The effect of atriopeptin (10µg/kg) on the *in vivo* function of the shark rectal gland and kidney.

(n=12)	CONTROL	ATRIOPEPTIN†
SYSTEMIC ARTERIAL PRESSURE (mmHg)	18.2±1.8	14.6±.5**
RECTAL GLAND		
BLOOD FLOW (ml/h/g)	11.1±3.7	19.3±7.2*
DUCT FLOW (ml/h/g)	0.67±.18	2.44±.46**
CHLORIDE SECRETION (µeq/h/g)	344±93	1241±238**
KIDNEY		
URINE FLOW (ml/h)	0.52±.16	0.67±.15

Data are mean ± SE. *P<.05 **P<.01
† Measured during peak effect.

A 1µg bolus of atriopeptin (effective concentration of 10^{-7}M) into the isolated perfused rectal gland produced a prompt increase in duct flow (231 to 969µl/h/gww, P<.01) and chloride secretory rate (96 to 428µeq/h/gww, P<.01). No effects on perfusate flow rate were observed. Atriopeptin produced approximately 50% as much stimulation of chloride secretion as an equimolar amount of vasoactive intestinal peptide given to the same gland.

Extracts of various tissues were given as a 1ml bolus (0.3-1.2mg of protein) to the *in vitro* perfused gland. Extracts of atria and ventricle but not skeletal muscle, kidney, brain, or intestine produced a significant increase in chloride secretion (Figure 1). Incubation of the extracts with trypsin (12µg) but not adenosine deaminase (20 U) completely eliminated the stimulatory effect on chloride secretion.

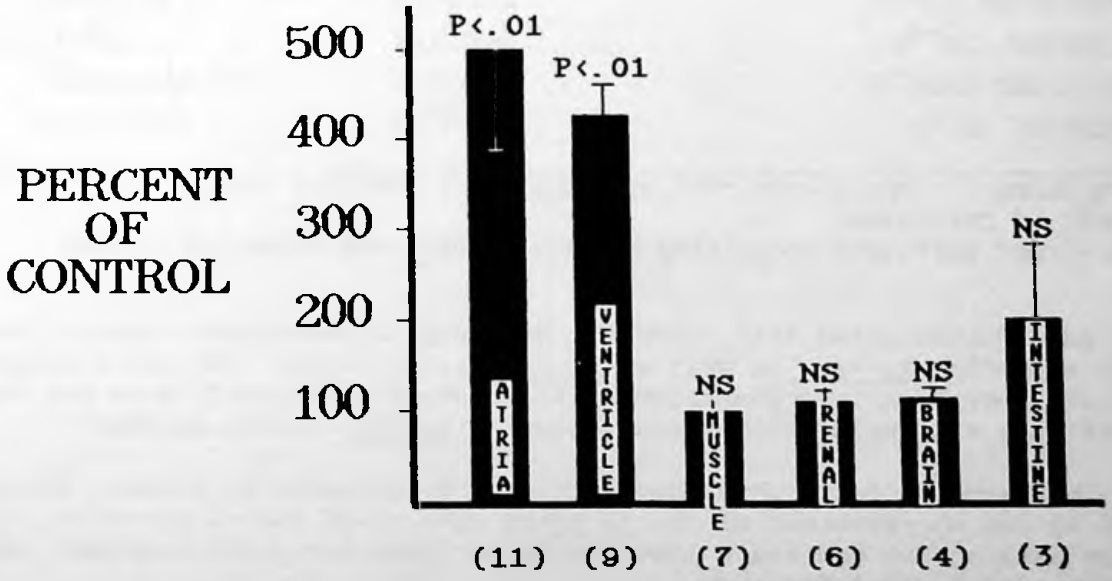

Figure 1. Stimulation of chloride secretion by tissue extracts. Tissue extracts were infused as a 1ml bolus (0.3-1.2mg of protein) into the *in vitro* perfused rectal gland after baseline measurements of chloride secretion. Extracts of atria and ventricle produced nearly fivefold increases in chloride secretion. Extracts of muscle, kidney, brain, and intestine were without effect. Data are mean ± SE.(n) = number of individual extracts tested.

Serum was obtained from intact pithed fish before and after the intravascular infusion of isotonic shark Ringer's solution (30ml/kg). Stimulation of chloride secretion was observed following the infusion of the serum contained following the volume load (188 to 294μeq/h/gww/P<.05) while no stimulation was observed from the serum obtained before volume expansion.

We were unable to find evidence for a direct cellular action of atriopeptin. Atriopeptin failed to increase oxygen consumption in slices or dispersed cells. Furthermore, no effects on chloride transport were noted in isolated perfused segments of rectal gland tubules (R.F. Greger, personal communication).

The effects of atriopeptin on the in vitro perfused gland were dependent upon calcium in the perfusate and on the entry of calcium into cells (Table 2). A perfusate containing only 0.5mM calcium and 9.0mM magnesium completely inhibited the stimulatory effects of atriopeptin but had no effect on vasoactive intestinal peptide stimulation. Similarly, perfusion in the presence of nifedipine, verapamil or diltiazem prevented the stimulatory effects of atriopeptin. Finally, perfusion in the presence of procaine also inhibited the stimulatory effect of atriopeptin but not that of vasoactive intestinal peptide.

Table 2. The effect of perfusate alterations on the stimulatory effect of atriopeptin on chloride secretion in the in vitro perfused rectal gland.

PERFUSATE (n)	% STIMULATION OF CHLORIDE SECRETION	
	ANF(n)	VIP(n)
SR†	372±53(12)**	820±283(11)*
LOW CALCIUM/HIGH MAGNESIUM SR	47±19(12)	719±210(12)*
SR + NIFEDIPINE 10^{-4}M	27±17(5)	110±62(2)
SR + VERAPAMIL 10^{-4}M	36±22(4)	615±260(4)*
SR + DILTIAZEM $5x10^{-5}$M	-7±9(4)	not determined
SR + PROCAINE 10^{-2}M	4±9(8)	508±235(6)*

Data are means. *P<.05 and **P<.01 compared to baseline levels
n = number of perfusions
†=Shark Ringer perfusate containing calcium (2.5mM) and magnesium (1.2mM)

We have demonstrated that synthetic atriopeptin stimulates rectal gland chloride secretion in vivo as well as in vitro. In intact fish the stimulation of chloride secretion is accompanied by an increase in blood flow to the rectal gland, effects similar to those seen following in vivo volume expansion.

Further support for a physiologic role of atriopeptin in volume regulation is found in the observations of the in vitro effects of tissue extracts. Only the shark cardiocytes contain a heat stable, trypsin sensitive substance which stimulates rectal gland function.

Finally, a chloride stimulatory substance is also present in the serum of the intact shark following, but not before, an intravascular saline infusion, suggesting that the hormone is released in response to expansion of circulatory volume.

While atriopeptin stimulates chloride secretion by the rectal gland, such stimulation does not appear to involve a direct effect on epithelial transport. Rather, the results suggest that atriopeptin may release an endogenous secretagogue from within the gland itself. Intracellular calcium is known to be necessary for the coupling of excitation with the release of secretory substances. Interference with the availability of intracellular calcium, either by removal of calcium from the perfusate, blockade of calcium entry channels or interference with membrane sodium channels, appears to prevent stimulation by atriopeptin. These maneuvers do not have a non-specific inhibitory effect on transport as stimulation by vasoactive intestinal peptide was unaffected.

These results are consistent with the hypothesis that atriopeptin or a similar substance is present in shark cardiocytes and is released during volume expansion. Atriopeptin stimulates rectal gland chloride secretion providing a negative feedback mechanism for the regulation of extracellular volume. The mechanism of this stimulation may involve the release of another secretagogue such as vasoactive intestinal peptide from nerve terminals within the rectal gland itself.