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RESEARCH ARTICLE

Introducing a novel mechanism to control heart rate in the ancestral Pacific hagfish

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ABSTRACT

Although neural modulation of heart rate is well established among chordate animals, the Pacific hagfish (*Eptatretus stoutii*) lacks any cardiac innervation, yet it can increase its heart rate from the steady, depressed heart rate seen in prolonged anoxia to almost double its normal normoxic heart rate, an almost fourfold overall change during the 1-h recovery from anoxia. The present study sought mechanistic explanations for these regulatory changes in heart rate. We provide evidence for a bicarbonate-activated, soluble adenylyl cyclase (sAC)-dependent mechanism to control heart rate, a mechanism never previously implicated in chordate cardiac control.

KEY WORDS: Heart rate control, Soluble adenylyl cyclase, Bicarbonate ions, cAMP production, Anoxia tolerance, Cardiac evolution

INTRODUCTION

Hagfishes are extant representatives of the craniate lineage, whose origin dates back 0.5 billion years (Ota and Kuratani, 2007). Beyond their basal position in vertebrate evolution, hagfishes are biologically intriguing because of their anoxia tolerance and their legendary ability to produce copious amounts of slime (Hansen and Sidell, 1983; Stecyk and Farrell, 2006; Cox et al., 2010; Herr et al., 2010). More recently, hagfish have been proposed as champions of CO_2 tolerance (Baker et al., 2015). Although tolerance of environmental extremes suits their habit of burrowing into dead and decaying animals for sustenance (Jørgensen et al., 1998), mechanistic explanations for sustained function under such conditions remain enigmatic. For example, when Pacific hagfish [*Eptatretus stoutii* (Lockington 1878)] are exposed to prolonged (36 h) anoxia, cardiac output is only reduced by approximately 26% because an increased cardiac stroke volume largely compensates for the halving of heart rate (10 to ~4 beats min^{-1}; Cox et al., 2010; Gillis et al., 2015). Immediately after normoxic conditions are restored, heart rate then increases to 17.5 beats min^{-1}, almost double the normoxic heart rate, and cardiac output increases. Thus, over a 1-h period when the hagfish heart switches from sustained anaerobic metabolism (Hansen and Sidell, 1983; Farrell and Stecyk, 2007; Cox et al., 2011) back to aerobic metabolism, heart rate quadruples (~4 to 17 beats min^{-1}; Cox et al., 2010). Therefore, it is surprising that the hagfish can regulate its heart rate over such a large range without any cardiac innervation. Consequently, the hagfish offers a fascinating model for the study of aneural mechanisms for controlling heart rate that contrasts with the situation for anoxia-tolerant vertebrates, such as crucian carp and freshwater turtles, which similarly slow heart rate during anoxia but use increased parasympathetic vagal tonus to the heart (Vornanen and Tuomennoro, 1999; Hicks and Farrell, 2000a,b; Stecyk et al., 2004; Stecyk et al., 2007).

Neural control of heart rate in the vertebrate lineage primarily involves sympathetic (stimulatory β-adrenergic) and parasympathetic (inhibitory cholinergic) mechanisms (Nilsson, 1983). The aneural hagfish heart, instead, is known to respond to applied catecholamines and has its own intrinsic store of catecholamines (Greene, 1902; Augustinsson et al., 1956; Jensen, 1961, 1965; Farrell, 2007). Furthermore, given that routine heart rate is dramatically slowed after injection of β-adrenergic antagonists (Fänge and Östlund, 1954; Axelsson et al., 1990; Fukayama et al., 1992), it would seem that routine, normoxic heart rate in hagfish is set by an autocrine adrenergic tonus acting presumably on the primary cardiac pacemaker cells located in the sinoatrial node, which would set the intrinsic cardiac pacemaker rate (Farrell, 2007). To date, the sinoatrial node has not been identified in any hagfish species, but is presumed to be present in *Eptatretus cirrhatus* because a V-wave that is characteristic of cardiac muscle contraction in the sinus venosus preceeds the P-wave associated with atrial contraction (Davie et al., 1987). [Note: a V-wave was not evident in the electrocardiogram of *Myxine glutinosa* (Satchell, 1986).] Combining this knowledge with the observation that stressing hagfish does not trigger the characteristic increase in circulating catecholamines shown by most vertebrates (Perry et al., 1993) has led to the hypothesis that the bradycardia observed in hagfish during anoxia represents a withdrawal of adrenergic tonus. Adrenergic tonus would presumably act by stimulating cAMP production involving transmembrane adenylyl cyclase (tmAC), a mechanism common to all vertebrate hearts (Nilsson, 1983).

Although an increased adrenergic tonus seems an attractive mechanism to explain the post-anoxia tachycardia in hagfish, routine heart rate in normoxic hagfish is notoriously unresponsive to catecholamine stimulation (Fänge and Östlund, 1954; Axelsson et al., 1990; Forser et al., 1992). Therefore, we explored another mechanism to supply cAMP to stimulate heart rate, namely the soluble adenylyl cyclase (sAC). sAC activity was first discovered in mammalian sperm cells (Buck et al., 1999), and sAC activity has subsequently been shown in the kidney, eye, respiratory tract, digestive tract and pancreas, and bone, and has also been shown to be involved in neural and immune functions (reviewed by Tresguerres et al., 2011). Intracellular sAC compartments also include the nucleus, mitochondria, mid-bodies and centrioles (Zippin et al., 2003, 2004; Acin-Perez et al., 2009; Tresguerres

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List of symbols and abbreviations

anti-dfsAC	anti-dogfish soluble adenylyl cyclase antibody
BMAC	Bamfield Marine Sciences Centre
cAMP	cyclic adenosine monophosphate
DFO	Department of Fisheries and Oceans Canada
DMSO	dimethyl sulfoxide
EC_{50}	half-maximal effective concentration
HCN	hyperpolarization-activated cyclic nucleotide-gated
I_f	cardiac pacemaker 'funny' current
I_{Kr}	rapid, delayed rectifier potassium current
NBC	Na^+/HCO_3^- cotransporters
NCX	Na^+/Ca^{2+} exchange
PVDF	polyvinylidene difluoride
sAC	soluble adenylyl cyclase
tmAC	transmembrane adenylyl cyclase

et al., 2010a). While mammalian sAC requires both Mg^{2+} and Ca^{2+} as cofactors to produce cAMP from ATP (Litvin et al., 2003), shark sAC seems to require Mg^{2+} and Mn^{2+} (Tresguerres et al., 2010b). Importantly, sAC differs from tmAC by being activated by bicarbonate ions (HCO_3^-) rather than catecholamines (Buck et al., 1999; Chen et al., 2000; Tresguerres et al., 2010b, 2011). Although hagfish sAC has not yet been cloned, sAC genes are present in cartilaginous and bony fishes (Tresguerres et al., 2010b; reviewed in Tresguerres et al., 2014; Tresguerres, 2014), as well as in a variety of invertebrate animals including *Trichoiplax*, sponges, cnidarians, molluscs, echinoderms, insects and cephalochordates (Barott et al., 2013; Tresguerres, 2014; Tresguerres et al., 2010b, 2014). Furthermore, a hallmark of sAC enzymatic activity is HCO_3^--stimulated cAMP production, which is sensitive to the small inhibitor molecule KH7 (Hess et al., 2005; Tresguerres et al., 2010b, 2011). Based on HCO_3^--stimulation and sensitivity to KH7, two independent studies concluded that sAC modulates NaCl absorption across the intestine of marine bony fishes (Tresguerres et al., 2010a; Carvalho et al., 2012). This conclusion was substantiated by detection of a protein band of the predicted ~110 kDa by heterologous antibodies against shark sAC (Tresguerres et al., 2010a).

Consequently, in addition to proposing that tmAC-mediated cAMP production is suppressed during anoxia and slows heart rate, we tested the hypothesis that sAC-mediated cAMP production plays a role in stimulating heart rate in hagfish, a cardiac control mechanism never previously tested in a chordate heart. To test these hypotheses, heart rate was measured in isolated hearts following sAC and tmAC stimulation, while sAC presence in cardiac tissue was probed using immunofluorescence, western blotting and enzyme activity assays. Immunofluorescence was also used to locate Na^+/HCO_3^- cotransporters (NBC) in the hagfish heart, which could facilitate HCO_3^- movements to modulate sAC activity.

MATERIALS AND METHODS

Animal husbandry

Pacific hagfish (112±1 g; mean±s.e.m.; n=110; male and female; wild caught) were collected using baited traps off the coast of Bamfield Marine Sciences Centre (BMSC), Bamfield, BC, Canada, where they were held until experimentation or transport. *In vivo* anoxia exposure experiments and tissue sampling were carried out at BMSC. Measurements of cAMP, western blotting and immunofluorescence were conducted on tissues shipped to the Scripps Institution of Oceanography, University of California, San Diego, CA, USA. Isolated heart experiments took place at the University of British Columbia, Vancouver, BC, Canada, which required transport to the West Vancouver Laboratory, Department of Fisheries and Oceans Canada (DFO), West Vancouver, BC, Canada, where they were housed in 4000 l tanks supplied with flow-through seawater (10±1°C) and fed frozen squid weekly. Animals were always fasted for a minimum of 1 week prior to an experimental treatment, and all experiments were conducted in accordance with the animal care policies of the University of British Columbia, BMSC and DFO (AUP 12-0001).

Isolated heart experiments

Hagfish were killed by a blow to the head, followed by immediate decapitation. The heart was rapidly excised and immersed in 25 ml chilled (10°C), aerated hagfish saline within a plastic basket containing two electrocardiogram electrodes. The composition of the hagfish saline was (in mmol l^{-1}): 410 NaCl, 10 KCl, 14 $MgSO_4$, 4 urea, 20 glucose, 10 HEPES, 4 $CaCl_2$ (Sigma-Aldrich, St Louis, MO, USA for all chemicals). The pH of the saline was adjusted to 7.9. The electrocardiogram of the excised heart was continuously monitored, stored and analyzed using Biopac and AcqKnowledge software (Goleta, CA, USA). Heart rate (an average over 10 beats) stabilized prior to (<1 h) any testing. Trial runs (data not shown) established that this control normoxic heart rate of the excised heart would remain stable for >24 h under these conditions while the saline was aerated. A different set of hearts was used for each of the following tests.

Nadolol (Tocris Bioscience, Minneapolis, MN, USA), a β-adrenoceptor antagonist, was used to study the inhibition of adrenergic stimulation of tmAC. The bath saline was changed every 30 min to incrementally increase the nadolol concentration from 1.0 pmol l^{-1} to 1 mmol l^{-1}. Then, with maximal adrenergic stimulation, anoxia was induced by bubbling the saline with N_2 for 2 h while recording changes in heart rate. Normoxia was restored by re-aerating the saline and following the change in oxygen saturation with an oxygen probe (Ocean Optics, Dunedin, FL, USA).

The tmAC agonist forskolin (Tocris Bioscience) was used to assess the role of stimulating tmAC in the increase in anoxic heart rate. Hearts that had been first subjected to a 2-h anoxic exposure were stimulated with forskolin, which was added incrementally (1.0 pmol l^{-1} to 100 μmol l^{-1}) every 30 min to the saline bath. Forskolin was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) for a final DMSO concentration of up to 0.01 μl DMSO ml^{-1} saline in the final preparation. Additions of up to 10 μl DMSO ml^{-1} saline had no effect on heart rate (data not shown).

To assess the role of HCO_3^- stimulation of sAC in the increase of the anoxic heart rate, hearts that had been first subjected to a 2-h anoxic exposure were stimulated with $NaHCO_3$ (Sigma-Aldrich), which was added incrementally (20, 40 and 60 mmol l^{-1}) every 30 min to the saline bath. The pH of the saline was readjusted to 7.9 in the stock salines with NaOH. In separate experiments with anoxic hearts stimulated with 60 mmol l^{-1} HCO_3^-, the sAC antagonist KH7 (Tocris Bioscience) was added at a concentration of 50 μmol l^{-1} (dissolved in DMSO as above).

Anoxic exposure *in vivo*

A simple way to rapidly obtain blood samples for HCO_3^- analysis without the complications of vessel cannulation described by Cox et al. (2011) is from the subcutaneous sinus. Heart samples were also taken at the same time for western blots and for assaying cAMP concentration and production by sAC. Hagfish were held overnight in one of four darkened 2.5 l respirometry chambers that were continuously flushed (0.5 l min^{-1}) with aerated seawater (10±1°C). Under these normoxic conditions, hagfish adopt a curled-up

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posture, but remain immobile and straight when made anoxic, greatly facilitating fish removal from the chamber (Cox et al., 2010; Wilson et al., 2013). The water supply was made anoxic (within <1 h) by supplying the two gas-exchange columns (placed in-series) with N_2. O_2 concentration was monitored every second throughout the experiment using MINI-DO probes (Loligo Systems, Tjele, Denmark). Different batches of four hagfish were sampled after an anoxic exposure of 3, 6, 12 and 24 h. Similarly, following a 24-h anoxic exposure, hagfish were sampled 1 and 6 h after re-aerating the water (achieved in <30 min). By the 6-h sample time, the hagfish had restored their coiled posture. Control animals were held in the same apparatus under normoxic conditions for 42 h before sampling. Each hagfish was killed by a blow to the head and was then decapitated. Blood was rapidly removed from the subcutaneous sinus in the tail and placed on ice. The heart was then rapidly excised and freeze-clamped in liquid N_2. Plasma was separated by centrifugation. All tissue samples were stored at −80°C until analyzed.

Western blots

Hearts (100±17 mg), sampled after either a 24-h anoxic exposure or a 42-h normoxic exposure as described above (n=5 per treatment), were homogenized under liquid N_2 using a pestle and mortar, and then in a glass homogenizer filled with 500 µl homogenization buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Samples were centrifuged (500 ***g*** for 15 min at 4°C) to collect the supernatant and stored at −80°C until analysis. A supernatant sample was mixed with an equal volume of 2× Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) with 5% β-mercaptoethanol and heated at 95°C for 5 min. Protein (13 µg; estimated by Bradford assay) was separated by SDS/PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After transfer, PVDF membranes were incubated in blocking buffer (Tris-buffered saline, 1% Tween, 10% milk, pH 7.4) at room temperature for 30 min and incubated in anti-dogfish sAC (anti-dfsAC) (Tresguerres et al., 2010b) or anti-rat NBC1 [Chemicon International Inc., USA (Schmitt et al., 1999); known to cross-react in fish (Parks et al., 2007)] overnight at 4°C, followed by 1 h incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies. Membranes were washed three times in TBST (Tris-buffered saline, 1% Tween) for 20 min between antibody treatments. Bands were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Waukesha, WI, USA) and imaged in a Bio-Rad Universal III Hood, with sAC protein abundance quantified using ImageQuant software (Bio-Rad).

Cardiac cAMP concentration

Hearts (mass=89±8 mg; mean±s.e.m.) were pulverized under liquid N_2 using a pestle and mortar, followed by homogenization in a glass Dounce homogenizer filled with homogenization buffer (250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 30 mmol l^{-1} Tris, pH 7.5) at a ratio of 10:1 weight (mg)/volume (µl), and held on ice for 10 min. Supernatant samples were collected after centrifugation (600 ***g*** for 15 min at 4°C) and stored at −80°C until analysis. cAMP concentration in hearts (control and anoxic exposure for 3, 6, 12 and 24 h; n=5 per treatment) was measured using the Direct Cyclic AMP Enzyme Immunoassay (Arbor Assays).

Cardiac cAMP production by sAC

Heart homogenates were incubated for 45 min at room temperature in an orbital shaker (300 rpm) in 100 mmol l^{-1} Tris (pH 7.5), 5 mmol l^{-1} ATP, 10 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} $MnCl_2$, 0.5 mmol l^{-1} isobutylmethylxanthine, 1 mmol l^{-1} dithiothreitol, 20 mmol l^{-1} creatine phosphate and 100 U ml^{-1} creatine phosphokinase. Homogenates were incubated in concentrations of HCO_3^- and KH7. cAMP production was determined using the DetectX Direct Cyclic AMP Enzyme Immunoassay (Arbor Assays).

Plasma bicarbonate concentration

The HCO_3^- concentration in the plasma sinus blood was estimated by measuring total CO_2 (Cameron, 1971) on plasma samples obtained after 3, 6, 12 and 14 h of anoxia and also after a 0.5 h and a 1 h recovery from a preceding 24 h anoxia exposure.

Cardiac immunohistochemistry

Hearts were fixed in 0.2 mol cacodylate buffer, 3.2% paraformaldehyde and 0.3% glutaraldehyde for 6 h, transferred to 50% ethanol for 6 h, and stored in 70% ethanol. Fixed hearts were serially dehydrated in 95% ethanol (10 min), 100% ethanol (10 min), xylene (3×10 min) and paraffin (55°C) (3×30 min), after which paraffin blocks were left to solidify overnight. Sections of the atrium and ventricle were cut at 7 µm using a rotary microtome; three consecutive sections were placed on slides and left on a slide warmer (37°C) overnight. Paraffin was removed in xylene (10 min×3) and sections were re-hydrated in 100% ethanol (10 min), 95% ethanol (10 min), 70% ethanol (10 min) and phosphate-buffered saline (PBS; Cellgro Corning, Manassas, VA, USA). Non-specific binding was reduced by incubating the sections in blocking buffer (PBS, 2% normal goat serum, 0.02% keyhole limpet hemocyanin, pH 7.8) for 1 h. Sections were then incubated in the primary antibody overnight at 4°C [anti-dfsAC=1:250, anti-rat NBC1=1:100). Slides were washed three times in PBS and sections were incubated in the appropriate secondary antibody (1:500) at room temperature for 1 h, followed by incubation with the nuclear stain Hoechst 33342 (Invitrogen, Grand Island, NY, USA) (1:1000) for 5 min. Slides were then washed three times in PBS and sections were permanently mounted in Fluorogel with Tris buffer (Electron Microscopy Sciences, Hatfield, PA, USA). Immunofluorescence was detected using an epifluorescence microscope (Zeiss AxioObserver Z1) connected to a metal halide lamp and appropriate filters. Digital images were adjusted, for brightness and contrast only, using Zeiss Axiovision software and Adobe Photoshop. Images of atrium and ventricle are representative of hearts from two small hagfish (∼100 g). Immunolocalization of sAC in the sinus venosus was performed on the heart of a larger hagfish (∼300 g). This was necessary in order to be able to identify the sinus venosus.

Calculations and statistics

Statistical analyses were conducted using SigmaPlot for Windows Version 11.0, Build 11.1.0.102, on a Sager NP7352-Clevo W350ST notebook running Microsoft Windows 7 Ultimate Service Pack 1. Data sets passed normality tests (Shapiro–Wilk) and values are presented as means±s.e.m. Concentration-dependent effects of nadolol (n=8) or forskolin (n=6) on the heart rate of isolated hearts were assessed using a repeated-measures one-way ANOVA followed by a Holm–Sidak test, while the effects of HCO_3^- (n=6) and KH7 (n=6) were assessed using a one-way ANOVA also followed by a Holm–Sidak test. Plasma HCO_3^- concentrations were similarly assessed with a one-way ANOVA followed by a Holm–Sidak test (n=5). Cytosolic cAMP concentrations were also evaluated with a one-way ANOVA followed by a Student–Newman–Keuls test (n=5). sAC abundance was compared between control and 24 h anoxia

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using a one-way ANOVA. Bicarbonate-stimulated cAMP production rates in hagfish heart crude homogenate were assessed using repeated-measures one-way ANOVA and Tukey's multiple comparison test (n=6). Statistical significance was assigned to a P-value <0.05.

RESULTS

Transmembrane adenylyl cyclase

The spontaneous heart rate of normoxic, isolated hagfish hearts at 10°C was 13.4±1.1 beats min^{-1}, i.e. similar to the routine *in vivo* heart rate of 10.4±1.3 beats min^{-1} previously measured for normoxic hagfish at the same temperature (Cox et al., 2010). Application of 1 mmol l^{-1} nadolol, a β-adrenoreceptor antagonist, to the normoxic, isolated hagfish heart significantly decreased (P<0.05) heart rate to 8.6±1.1 beats min^{-1}, 64% of the normoxic heart rate (Fig. 1A). A subsequent 2-h anoxic exposure further reduced heart rate to 5.1±0.5 beats min^{-1}, almost one-third of the normoxic heart rate. The anoxic bradycardia was fully reversible (Fig. 1A). Forskolin, a tmAC agonist, applied to isolated hearts did not significantly change the normoxic heart rate. Instead, 1 and 10 μmol l^{-1} forskolin applied to anoxic isolated hearts restored the depressed heart rate to, but not above, the normoxic heart rate (Fig. 1C). However, 100 μmol l^{-1} forskolin reduced heart rate back to the anoxic rate during anoxia.

Combined, these results are consistent with our suggestion that tonic adrenergic stimulation of the hagfish heart rate via tmAC is present under normoxic conditions and that this tonic stimulation is lost during anoxia. Furthermore, no support is provided for tmAC stimulation increasing heart rate beyond the routine normoxic rate, a result consistent with *in vivo* observations (see Introduction).

The cardiac cytosolic cAMP concentration measured in normoxic hagfish (8.4±0.7 pmol mg^{-1} protein) was significantly (P<0.05) reduced by 33% to 5.6±0.5 pmol mg^{-1} protein after hagfish had been exposed to anoxia for 3 h. Cardiac cAMP concentrations remained stable at this level throughout the 24-h anoxic exposure (Fig. 1B), a result that is consistent with the stable depression of heart rate during anoxia observed both here *in vitro* and previously *in vivo* (Cox et al., 2010).

Soluble adenylyl cyclase

Expression of sAC protein was demonstrated at the expected ~110 kDa band in western blotting analysis of crude homogenate cardiac cell suspensions from hagfish (Fig. 2A). No band was seen when the antibody was pre-incubated with oversaturating concentrations of purified sAC peptide prior to application to the transfer membrane. Moreover, sAC abundance, estimated from western blots, was unchanged following a 24 h anoxic challenge.

cAMP production measured in homogenized hagfish hearts in the presence of HCO_3^- and its inhibition by KH7 provides a causal linkage between sAC and cAMP synthesis. Increasing concentrations of HCO_3^- stimulated cAMP production, producing a maximum twofold increase in cAMP production with 40 mmol l^{-1} HCO_3^- (Fig. 2B). This HCO_3^--mediated cAMP production was completely blocked by 50 μmol l^{-1} KH7 (P<0.05), a small molecule that specifically inhibits sAC activity (Fig. 2C).

Addition of HCO_3^- induced a dose-dependent stimulation of spontaneously beating, anoxic, isolated hearts. Specifically, 20 mmol l^{-1} HCO_3^- significantly (P<0.05) increased the anoxic heart rate from 7.9±1.2 to 16.9±0.9 beats min^{-1}, more than restoring the normoxic heart rate (13.4 beats min^{-1}; Fig. 3A).

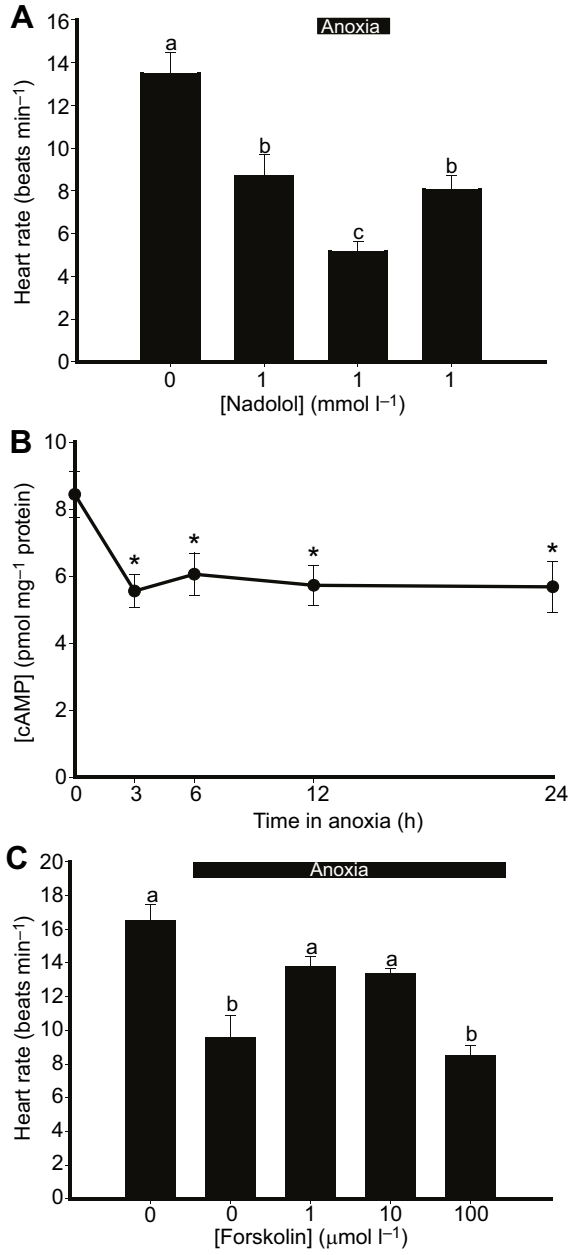

Fig. 1. Effects on hagfish heart rate and cardiac cAMP concentration during anoxia and β-adrenoreceptor blockade or stimulation. (A) Spontaneous heart rate of isolated hearts (n=8) slowed significantly when exposed to the β-adrenoreceptor antagonist nadolol and further when made anoxic (indicated by thick black bar). Removal of anoxia restored heart rate. Different letters indicate statistical differences between treatments (P<0.05, repeated-measures ANOVA). (B) Cardiac cAMP concentration decreased in intact animals (n=5) exposed to prolonged anoxia. Asterisks indicate significant differences from control group (*P<0.05, one-way ANOVA). (C) The tmAC agonist, forskolin, increased the anoxic heart rate of isolated hearts (n=6) (indicated by thick black bar) up to but not beyond routine normoxic heart rate. Different letters indicate statistical differences between treatments (P<0.05, repeated measures one-way ANOVA).

Furthermore, the anoxic heart rate could be further stimulated by 40 and 60 mmol l^{-1} HCO_3^- such that heart rate reached 22.4±2.6 and 23.1±2.6 beats min^{-1}, respectively (Fig. 3A), rates that were 75% higher than the normoxic heart rate (13.4 beats min^{-1}). Thus, maximal stimulation with HCO_3^- nearly tripled the anoxic heart rate (7.9 beats min^{-1}) in this test, and was 4.5 times

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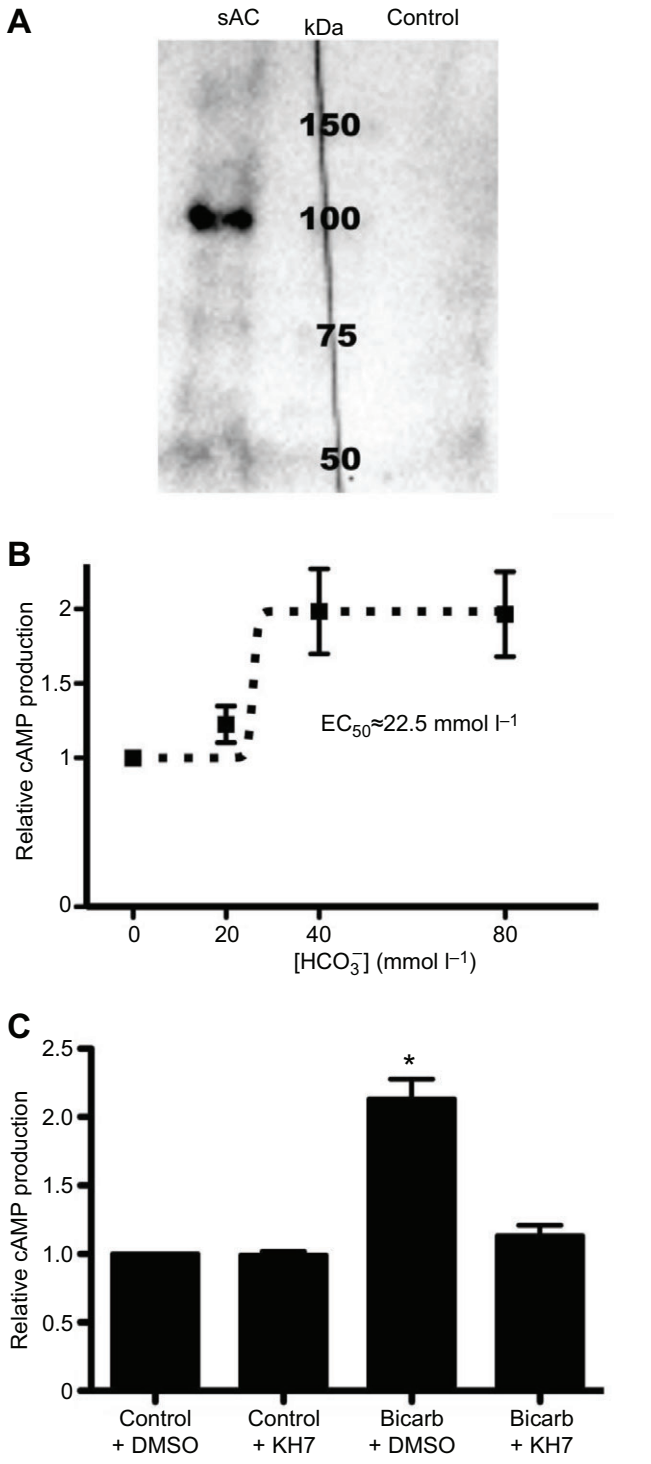

Fig. 2. Soluble adenylyl cyclase (sAC) is present in the hagfish heart. (A) Western blot of hagfish heart crude homogenate using anti-dfsAC antibody in the absence (sAC) or presence (control) of purified sAC peptide; ladder is indicated by kDa. (B) cAMP production in hagfish heart homogenates exposed to increasing HCO_3^- concentrations. Values are normalized to 0 mmol l^{-1} HCO_3^-. At 0 mmol l^{-1} HCO_3^-, cAMP production equaled 18.65 pmol mg^{-1} min^{-1}, increasing twofold at 40 mmol l^{-1} HCO_3^-. n=4. (C) Inhibition of HCO_3^--stimulated cAMP production in hagfish heart homogenates by the sAC inhibitor KH7 (50 μmol l^{-1}). Control=40 mmol l^{-1} NaCl; Bicarb=40 mmol l^{-1} $NaHCO_3$. DMSO is the vehicle for KH7. Asterisk indicates a significant difference between treatments (*P<0.05, repeated-measures one-way ANOVA, Tukey's multiple comparisons test; n=6).

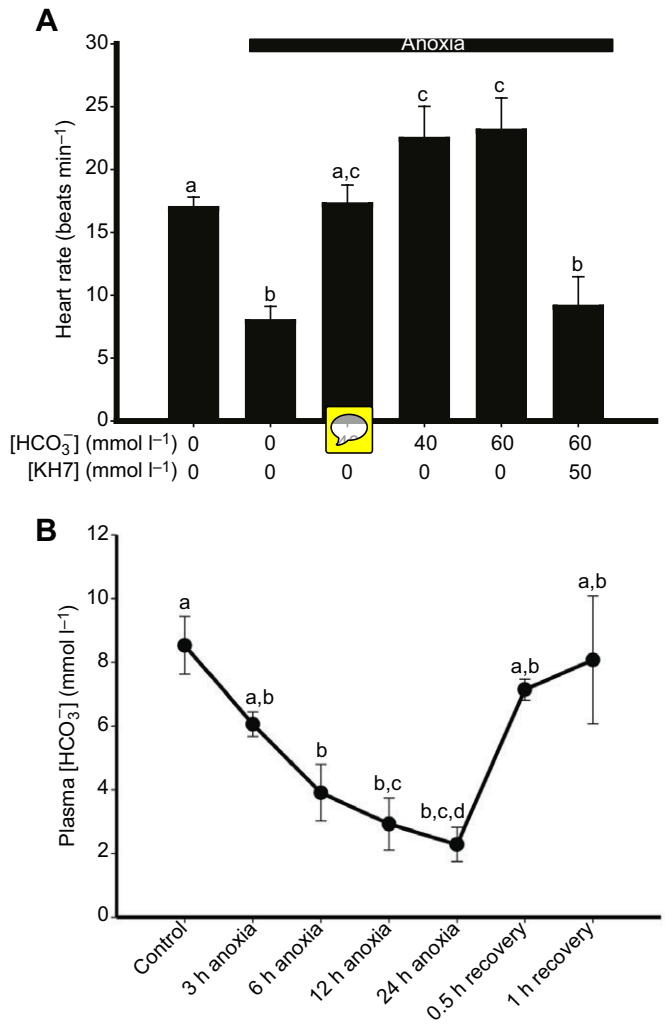

Fig. 3. Role of HCO_3^--stimulated sAC on regulating hagfish heart rate during recovery from anoxia. (A) The anoxic bradycardia of isolated hagfish hearts and the HCO_3^--stimulated tachycardia during anoxia. The sAC antagonist, KH7, completely blocked the HCO_3^--mediated tachycardia. Different letters indicate statistical differences between treatments (P<0.05, one-way ANOVA, n=6). (B) The effects of anoxia and subsequent recovery on plasma HCO_3^- concentration in the subcutaneous sinus of the Pacific hagfish (n=5) and during control normoxia, a 24-h anoxic exposure and normoxic recovery. Different letters indicate significant differences (P<0.05, one-way ANOVA).

higher than the anoxic heart rate after a 2-h anoxic exposure (5.1 beats min^{-1}).

The tachycardia stimulated by 60 mmol l^{-1} HCO_3^- was completely blocked by the sAC-specific antagonist KH7 (50 μmol l^{-1}; Fig. 3A), a finding that directly implicates sAC in the modulation of the hagfish heart rate by HCO_3^-. The normoxic heart rate of the isolated heart was unaffected by the application of either HCO_3^- or KH7.

Significant changes in plasma [HCO_3^-] were noticed in hagfish recovering from anoxia (Fig. 3B). Anoxia significantly decreased (P<0.05) plasma [HCO_3^-] in the subcutaneous sinus from 8.5±0.9 to 2.3±0.5 mmol l^{-1}. The anoxic reduction in plasma [HCO_3^-] was progressive, and reached statistical significance after 6 h. Plasma [HCO_3^-] was restored within 0.5 h of normoxia.

Immunolabelling of hagfish hearts identified sAC distributed throughout atrial and ventricular myocardial cells, with strong

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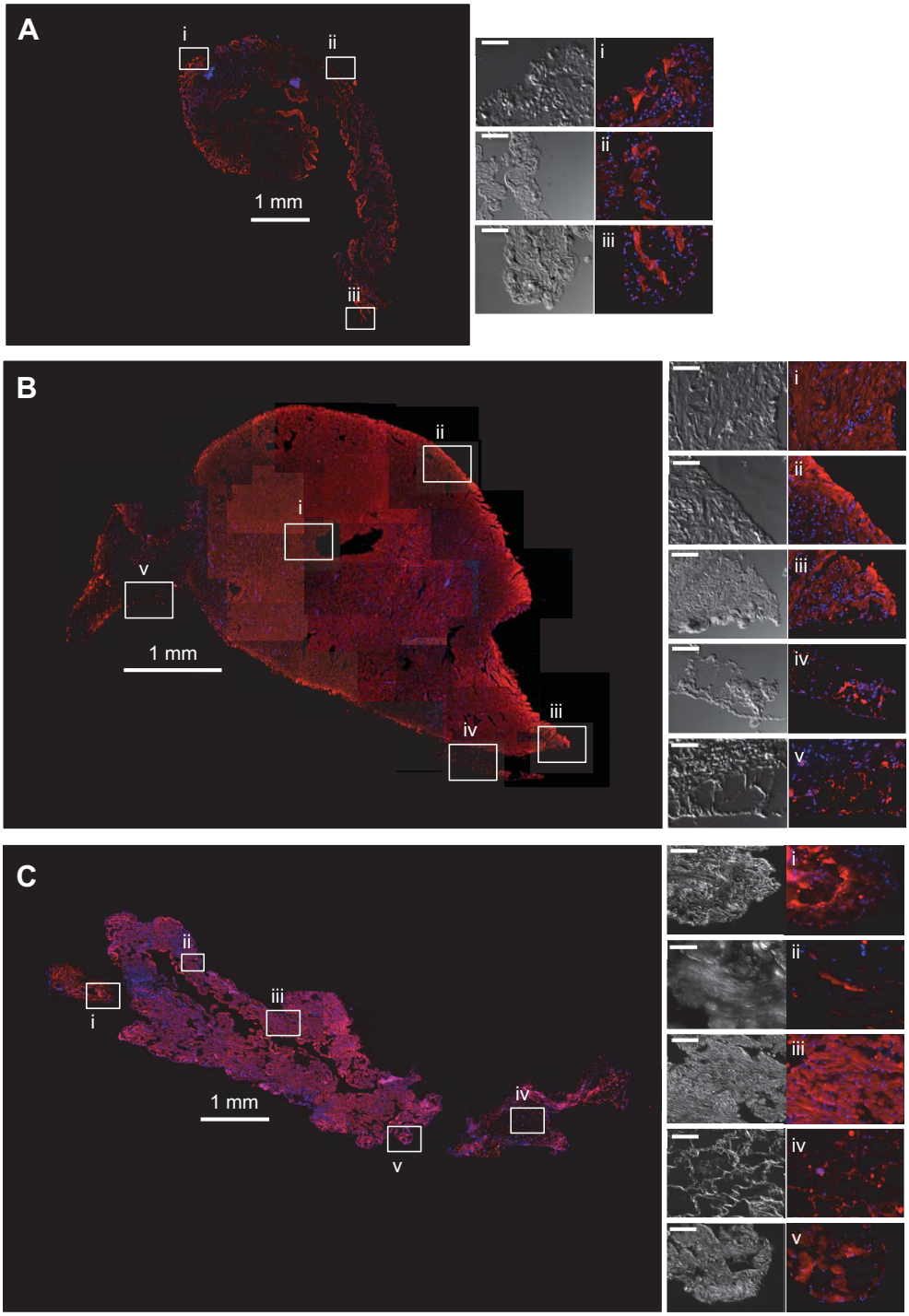

Fig. 4. Immunolocalization of sAC throughout the hagfish heart.
(A) Composite of 18 images showing sAC in the atrium; the boxed areas are shown at higher magnification in i–iii.
(B) Composite of 45 images showing sAC in the ventricle; the boxed areas are shown at higher magnification in i–v.
(C) Composite of 70 images showing sAC in the sinus venosus from a larger hagfish; the boxed areas are shown at higher magnification in i–v. In the higher-magnification images, the left panels are differential interference contrast, and the scale bars represent 50 μm. sAC immunostaining is in red; nuclei are stained in blue.

staining evident in cardiac trabeculae and in the atrium. Fig. 4 shows a composite of sections across an entire heart, with details shown for the atrium (Fig. 4Ai–iii), the ventricle (Fig. 4Bi–v) and the sinus venosus (Fig. 4Ci–v). Although widespread, sAC is not evenly distributed throughout the hagfish heart. Strong sAC immunostaining is seen in atrial areas with a high nuclear density (blue staining, Fig. 4), which could indicate the primary pacemaker region. sAC immunostaining is also most abundantly present in specific regions of cardiomyocytes, presumably A- or I-sarcomeric bands (Fig. 4Cii), but it appears absent in many unidentified cardiac cells (e.g. see Fig. 4Aiii, Biv,v, Ci,ii).

Na^+/HCO_3^- cotransporter immunoreactivity

Hagfish hearts demonstrated a band of the predicted size (~100 kDa) of the mammalian Na^+/HCO_3^- co-transporter (NBC, a member of the SLC4 family) in western blots (Fig. 5A). NBC-like immunoreactivity was observed throughout the atrium and ventricle in cells (Fig. 5B,C).

DISCUSSION

The present study documents the discovery of a novel control pathway to increase heart rate. We are not aware of any previous study on a chordate heart that has implicated HCO_3^- stimulation of

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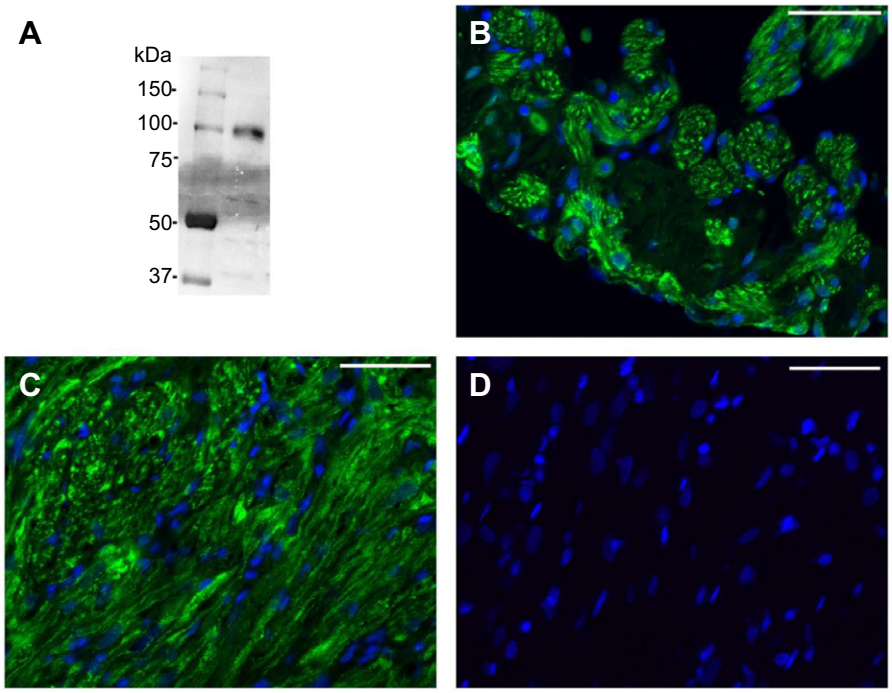

Fig. 5. Immunolocalization of Na^+/HCO_3^- cotransporters (NBC) in the hagfish heart. (A) Western blot of NBC in hagfish heart crude homogenate, with strong staining showing an immunoreactive band at 100 kDa. (B,C) Immunofluorescence of NBC (green) in hagfish atrium and ventricle, respectively, showing co-staining of nuclei (blue). As is the case for sAC, NBC is found throughout the myocardium of both atria and ventricles, with the location of trabeculae shown by the fluorescence of the antibodies. (D) Immunofluorescence control showing solely staining of nuclei in blue. Scale bars in B–D, 20 µm.

intracellular sAC to produce cAMP and stimulate the spontaneous heartbeat. This discovery should spur further research in the functional roles of sAC in vertebrate hearts to determine whether this control mechanism is more widespread in the lineage, or just another remarkable aspect of the biology of hagfishes.

Furthermore, we provide quantitative evidence for and suggest putative mechanisms that could explain the remarkable upregulation of heart rate (from ∼4 to 17.5 beats min^{-1}; Cox et al., 2010) when anoxic *in vivo* hagfish emerged from a 36-h anoxic exposure. In this regard, we note excellent quantitative agreement between the heart rates measured in spontaneously beating, isolated hagfish hearts and those measured previously *in vivo* for the normoxic state (13.4 versus 10.1 beats min^{-1}), prolonged anoxia (5.4 versus 4.0 beats min^{-1}) and a maximally stimulated state (23.4 versus 17.5 beats min^{-1}). In addition to supporting the hypothesis that the bradycardia associated with anoxia represents a withdrawal of an autocrine adrenergic tonus mediated by tmAC, a mechanism common to all vertebrate hearts, we provide support for a novel HCO_3^--mediated control of the anoxic heart rate. We further propose that sAC-mediated cAMP production plays a role in the HCO_3^- stimulation of heart rate in hagfish during anoxic recovery because tachycardia was fully blocked by the sAC-specific antagonist KH7. Additional evidence for the presence of sAC in hagfish hearts includes detection of the predicted ∼110 kDa protein band in western blots using antibodies against shark sAC, which is the same size as in shark (Tresguerres et al., 2010b) and toadfish (Tresguerres et al., 2010a), immunolabeling throughout the heart, and HCO_3^--stimulated and KH7-sensitive cAMP production by cardiac homogenates, which are hallmarks of sAC enzymatic activity. Consequently, we propose that hagfish have two mechanisms to regulate the heart rate via cAMP production, a sAC pathway working in tandem with a tmAC pathway during recovery from anoxia, and a tmAC pathway during normoxia. These potential distinct physiological functions of sAC- and tmAC-generated cAMP fit the concept of intracellular cAMP microdomains (Schwencke et al., 1999; Rich et al., 2000; Zippin et al., 2004; Tresguerres et al., 2011), which, in fish, has been empirically substantiated by differential effects of sAC and tmAC antagonists and agonists on NaCl and water absorption (Tresguerres et al., 2010a; Carvalho et al., 2012).

This study, taken together with past work, has also greatly increased our understanding of the catecholaminergic control of heart rate in hagfish. Rather than the dual sympathetic and parasympathetic controls found in certain teleosts and tetrapods (Nilsson, 1983), catecholaminergic control appears to act primarily through a paracrine action in hagfish. This is done via modulation of catecholamine release from chromaffin tissue located within the cardiac chambers themselves (Euler and Fänge, 1961; Bloom et al., 1963; Perry et al., 1993; Axelsson et al., 1990; Farrell, 2007). A tonic β-adrenergic stimulation of the hagfish heart is consistent with the bradycardic effects of sotalol *in vivo* (Hansen and Sidell, 1983; Axelsson et al., 1990) and nadolol *in vitro* (present study). Production and recycling of catecholamines involves the rate-limiting conversion of tyrosine to 3,4-dihydroxyphenylalanine, which requires oxygen (Levitt et al., 1965). Plausibly, anoxia could prevent catecholamine re-synthesis and the normal tonic paracrine β-adrenergic stimulation, which would decrease cAMP production, a suggestion consistent with the observed decrease in cardiac cAMP concentration after a 3 h anoxic exposure. Withdrawal of adrenergic stimulatory capacity in anoxia is also seen for the hearts of two other anoxia-tolerant taxa, crucian carp and freshwater turtles (Stecyk et al., 2004, 2007; Stecyk and Farrell, 2006; Hicks and Farrell, 2000b).

Additionally, we propose that tachycardia beyond the normoxic heart rate during the recovery from anoxia relies on the putative autocrine or humoral mechanism HCO_3^- stimulation of sAC identified here. Indeed, 20 mmol l^{-1} HCO_3^- produced a heart rate of 16.9 beats min^{-1} in the isolated anoxic heart, which compares well with the peak heart rate of 17.5 beats min^{-1} measured *in vivo* during normoxic recovery from anoxia in the same species and at the same temperature (Cox et al., 2010). The exact switching point between tmAC and sAC mechanisms is an interesting issue that was not addressed here. Our hypothesis is that tmAC is reactivated as soon as O_2 is available for catecholamine production (as above).

This stimulation, together with that from sAC, causes heart rate to increase beyond routine levels, and eventually the sAC mechanism decays under normoxia. Resolving this switch would involve mixtures of adrenergic and HCO_3^- stimulation at different levels of oxygenation.

The vertebrate heartbeat is initiated in sinoatrial pacemaker cells, but very little is known of pacemaker cell activity in hagfish. Icardo et al. (2016) were unable to observe specialized nodal tissue in the sinus wall or at the sinoatrial junction. Yet, electrocardiogram recordings in *E. cirrhatus* (Davie et al., 1987) showed a depolarization specific to only the central region of the sinus venosus, something not seen, however, in *M. glutinosa* (Arlock, 1975; Satchell, 1991). Therefore, we must still presume that pacemaker cells exist in hagfish hearts, and initiate the heartbeat as they do in all vertebrate hearts. Our investigation revealed areas of high nuclear density in the atrium (Fig. 2), which may represent primary pacemaker tissue, but confirmation will require electrophysiological mapping of action potentials. Therefore, until further work is performed specifically on pacemaker tissue in hagfishes, we can only presume that catecholamines and HCO_3^- act on pacemaker cells to increase heart rate. This task will be all the more difficult because the different HCN channels identified in *E. stoutii* hearts compared with mammals (Wilson et al., 2013) may make the available immunofluorescent markers for pacemaker cells less specific. Alternatively or additionally, sAC may mediate the observed increase in heartbeat rate by mechanisms at sites different from the pacemaker cells. For example, the presence of sAC in cardiomyocytes suggests a role in regulating their contractibility and therefore cardiac function more generally.

The atrium and ventricle of *E. stoutii* are rich in hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels (Wilson et al., 2013), which have been implicated in the control of pacemaker activity of the vertebrate heart (Qu et al., 2008). Application of an HCN channel blocker (zatebradine) completely stopped all spontaneous atrial contractions and almost all spontaneous ventricular contractions of isolated *E. stoutii* hearts (Wilson and Farrell, 2013), something not observed in vertebrate hearts, where the effect is just a slowing, and not a cessation, of the spontaneous heartbeat (Baruscotti et al., 2010; Monfredi et al., 2013). Thus, the most parsimonious explanation is that zatebradine blocked pacemaker HCN channels, especially because the ventricular cells have their own, slower spontaneous heartbeat (Wilson et al., 2013) and HCN channels are central to the control of intrinsic beating of atrial and ventricular chamber of *E. stoutii*. The difference in the efficacy of HCN channel blockers between *E. stoutii* and other vertebrates is perhaps because both HCN and Ca^{2+} cycling have been implicated in contributing to mammalian pacemaker rhythm (Monfredi et al., 2013). The major side effect, and concern, with using HCN blockers in humans is QRT prolongation and atrial fibrillation. However, zatebradine and ivabradine, which were initially considered to be specific blockers of HCN channels, can also inhibit the cardiac rapid, delayed rectifier potassium current (I_{Kr}) (Lees-Miller et al., 2015; Melgari et al., 2015). I_{Kr} is a major repolarizing current of fish hearts and so cardiac repolarization would be prolonged if zatebradine blocked I_{Kr} (Lees-Miller et al., 2015; Melgari et al., 2015), an action that would tend to decrease heart rate. Thus, the effect of zatebradine on hagfish heart rate could be due to either I_{Kr} or the cardiac pacemaker 'funny' current (I_f), or both. However, Wilson and Farrell (2013) did not observe atrial fibrillation in *E. stoutii* after zetabradine, but rather a cessation of all spontaneous atrial activity, an action that would require a complete blockage of I_{Kr}. Another important difference between hagfish and vertebrate hearts is the effect of ryanodine, which had no effect on normoxic heart rate in *E. stoutii*. In contrast, ryanodine and Ca^{2+} cycling have been shown to have a greater effect in lamprey (Vornanen and Haverninen, 2013) and the more active *Myxine glutinosa* (Bloom, 1962; Helle and Storesund, 1975). It would be of interest to compare the effects of zatebradine and sAC on these animals with those on other vertebrate species and *E. stoutii*.

Therefore, until additional electrophysiological studies are performed with hagfish, an approach that has up to now proven too challenging in our laboratory, we propose the following parsimonious mechanism as a working hypothesis for the control of cardiac pacemaker activity in *E. stoutii.* Removing adrenergic tonus decreases intracellular cAMP, which could primarily decrease I_f via HCN channels and, potentially, secondarily Na^+ influx through the sodium/calcium exchange channel (NCX) activated by Ca^{2+} cycling through the sarcoplasmic reticulum, as seen in mammalian hearts (Monfredi et al., 2013). Reducing these ion currents would decrease the diastolic depolarization rate of pacemaker cells, slowing heart rate. During anoxia, we suggest that gating of HCN channels by cAMP becomes minimal, and is restored with normoxia. Indeed, tmAC may be nearly fully activated in normoxia given that forskolin, which stimulates cAMP and bypasses tmAC, failed to increase heart rate in either normoxia or beyond routine rates in anoxia. Importantly, forskolin does not fit into the pseudosymmetrical site at the sAC pseudodimer interface, which in tmACs results in stimulation of cAMP, and as a result sAC is insensitive to forskolin (Chen et al., 2000; Kleinboelting et al., 2014). The explanation for the inhibition of heart rate by the highest pharmacological dose of forskolin is unknown, but it resembles biphasic effects of forskolin reported in other systems (Szabo et al., 1990; Tian, et al., 2009). Tachycardia without tmAC involvement would also explain mechanistically why all previous studies with normoxic hagfish have seen, at best, modest increases in heart rate with injections of β-adrenergic agonists (Euler and Fänge, 1961; Chapman et al., 1963; Axelsson et al., 1990; Johnsson and Axelsson, 1996).

The proposed role of sAC as a mechanism to gate HCN channels and control heart rate in hagfish may not be the only role of sAC in vertebrate hearts. Indeed, if HCN gating was its only role, sAC might not be omnipresent throughout the atrial and ventricular cardiomyocytes, as we discovered for hagfish using sAC protein expression. Furthermore, sAC has been found in mammalian cardiac myocytes and implicated in the apoptosis signalling pathway (Kumar et al., 2009; Appukuttan et al., 2012; Chen et al., 2012). Also, the roles of cAMP as a second messenger are many, especially via cAMP-dependent protein kinase A. Therefore, as this is the first time that sAC has been implicated in the control of heart rate, new roles for sAC in vertebrate hearts become a possibility. For example, hypercapnia produced tachycardia in embryonic zebrafish, a response blocked by atenolol, a β-adrenergic antagonist, and by a carbonic anhydrase inhibitor (Miller et al., 2014). Thus, it would be interesting to investigate whether KH7 might block this tachycardia, which would implicate a sAC-mediated control of heart rate in this case. As another example, the common eel, *Anguilla anguilla*, tolerates extreme hypercapnia and plasma HCO_3^- reaches 70 mmol l^{-1}, while arterial blood oxygen saturation is halved (McKenzie, 2003). How eels regulate heart rate during hypoxic hypercapnia is unknown.

Our findings regarding both tmAC and sAC have allowed us to propose a more complete model for the control of heart rate in the ancestral hagfish during anoxia. What remains very much unclear, and requiring much more study, is how cellular HCO_3^- concentrations are regulated in the hagfish heart, and whether

HCO_3^- stimulation is directed to sinoatrial pacemaker tissues. During anoxia, it is easy to envision both plasma (as observed here) and intracellular HCO_3^- concentrations being driven down by the absence of CO_2 production from aerobic metabolism and by the metabolic acidosis developing through glycolytic ATP production (Cox et al., 2011). Normoxic recovery from anoxia rapidly elevates O_2 consumption (Cox et al., 2011) and CO_2 production, which then increases HCO_3^- levels throughout the body, including blood plasma and inside cardiomyocytes. While our activity assays with hagfish heart crude homogenates suggest an apparent EC_{50} (half-maximal effective concentration) for HCO_3^- of approximately 20 mmol l^{-1}, obtaining the exact value would require cloning of hagfish sAC followed by enzymatic characterization on purified recombinant protein. As a reference, the EC_{50} for HCO_3^- varies considerably between sAC from vertebrates, ranging from ~5 mmol l^{-1} in sharks (Tresguerres et al., 2010b) to ~20 mmol l^{-1} in mammals (Chen et al., 2000; Litvin et al., 2003; Tresguerres, 2014). Of course, *in vivo* and unlike the continuously anoxic situation used here *in vitro*, there is an increase in oxygen as well as HCO_3^- during recovery, and so it would be interesting to examine the interactive effects of oxygen, catecholamines and HCO_3^- on heart rate. Minimally, we have demonstrated that heart rate can increase with HCO_3^- without the presence of oxygen.

In proposing that sAC acts as a metabolic sensor of changes in the concentration of CO_2/HCO_3^-, the heart can adjust its beating frequency reasonably rapidly without cardiac innervation. sAC could be stimulated by HCO_3^- entry into cardiomyocytes from the plasma through NBC-like transporters (which likely occurred when HCO_3^- was added to the extracellular saline), as well as by CO_2 and HCO_3^- generated within cardiomyocytes. Unfortunately, the limited information on *in vivo* tissue and plasma HCO_3^- concentrations in hagfish limits further speculation. Hagfish plasma HCO_3^- concentrations can reach a remarkable 70 mmol l^{-1} during sustained hypercapnia (Baker et al., 2015), a level well beyond that needed to obtain maximal cardiac sAC stimulation. However, hagfish are notoriously difficult to anaesthetize to reliably sample blood and tissues, and they routinely tie knots in indwelling catheters used to provide stress-free blood samples. Although sampling blood from the subcutaneous sinus of hagfish is rapid, easy and without excessive stress, we caution that subcutaneous sinus blood may not accurately reflect the rapidity or full extent of the changes in venous blood HCO_3^- concentration during the first hour of anoxic recovery because subcutaneous sinus blood takes 8–18 h to equilibrate with the venous circulation (Forster et al., 1989). Thus, intracellular and extracellular measurements of HCO_3^- within the heart are still needed to fully validate the *in vivo* regulation of heart rate by sAC activity, and to determine whether HCO_3^- is derived extracellularly or by a burst of intracellular, aerobic CO_2 production when the heart increases its work rate post-anoxia (Cox et al., 2010), or both.

In summary, the main finding of the present study is the discovery of a novel mechanism to control heart rate and a more detailed analysis of catecholamine control of heart rate. This allowed us to propose a more complete picture of the control mechanisms for heart rate in the ancestral hagfish. While putative upstream mechanisms are proposed, much work is still needed to establish the exact pathways. The implications of our discovery of sAC-mediated control of heart rate could open up a new field of cardiovascular research.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
C.M.W. was involved in study conception and design, carried out all experiments with live animals and isolated hearts, contributed to the sAC biochemical experiments and produced the first draft of the manuscript. J.N.R. performed most of the sAC and NBC biochemical and microscopy experiments. G.K.C. conducted the plasma bicarbonate measurements. M.T. designed the biochemical and microscopy portion of the study and contributed to the microscopy experiments. A.P.F. was involved in study conception and design. All authors analyzed the data, critically assessed and revised the manuscript, and gave final approval for publication.

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