



Coordination between glycogen metabolism and pH regulation in stingray gill cells

Jinae N. Roa^{a,*}, Tsuyoshi Morita^b, Martin Tresguerres^{c,**}

^a Department of Biological Sciences, California State University Los Angeles, USA

^b Oral and Maxillofacial Anatomy, Tokushima University Graduate School, Japan

^c Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, USA

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ABSTRACT

Elasmobranchs (shark, rays and relatives) have specialized epithelial cells in their gills that maintain blood pH homeostasis that are functionally analogous to distal tubule cells in the mammalian kidney. The gill cells are either enriched with the Na⁺/K⁺-ATPase (NKA) and responsible for acid secretion or enriched for the vacuolar-type V-H⁺-ATPase (VHA) and responsible for base secretion; both cell types are enriched in mitochondria and glycogen stores that support their high metabolic rates. In this study, we isolated and cultured stingray gill cells and found that the subcellular localization of glycogen coincides with that of NKA and VHA. Under control conditions, glycogen and NKA were located at the cell membrane of acid-secreting cells while glycogen and VHA were in the cytoplasm of the base-secreting cells. During an alkalosis, NKA and glycogen localization in the acid-secreting cells remained unchanged; however, both VHA and glycogen localized to the cell membrane of base-secreting cells. Interestingly, this process was prevented by pharmacological inhibition of the acid-base sensing enzyme, soluble adenylyl cyclase (sAC). This suggests a novel mechanism coupling glycogen metabolism with acid-base regulation that sustains ATPase function during sudden periods of increased energy demand. Since VHA, sAC, and glycogen are evolutionarily widespread, this mechanism may apply broadly to other organisms and systems including mammalian kidney cells.

1. Introduction

Animals must maintain blood pH homeostasis because excess hydrogen ions (H⁺) (i.e. low pH) can impair multiple essential physiological functions and health. Conversely, certain conditions including chronic kidney disease and kidney failure result in improper blood pH regulation and associated complications in humans [1–3]. To maintain acid-base homeostasis, animals developed specialized epithelia where polarized cells mediate absorption and excretion of H⁺ and HCO₃⁻ between the blood and the external environment. In mammals, these roles are fulfilled by the acid-secreting (A-type) and base-secreting (B-type) intercalated cells found in the renal distal tubule and collecting duct [4, 5]. These cells rely on several mechanisms, including acid-base sensing by soluble adenylyl cyclase (sAC) and subsequent activation of trans-epithelial acid-base transport [6]. For example, a blood acidosis activates sAC to produce the second messenger cyclic adenosine monophosphate (cAMP), which induces the insertion of the

vacuolar-type V-H⁺-ATPase (VHA) from cytoplasmic vesicles into the cell apical membrane [7]. From that location, VHA pumps H⁺ into the renal tubule lumen, thereby counteracting the blood acidosis. But while these processes are presumably energy-intensive, little is known about the metabolic mechanisms used to sustain ATPase function during this increased energy demand.

In eukaryotic cells, the two main sources of ATP are glycolysis in the cytosol and oxidative phosphorylation within mitochondria. The former can operate at rapid rates but has a lower comparative ATP yield whereas the latter is slower but is much more efficient. The main fuel for both pathways is glucose, which cells can import from the blood circulation and use right away, or store as glycogen for future breakdown into useable glucose via glycogenolysis. In vertebrates, the main glycogen storing organs are the liver and skeletal muscles; however, small amounts are also stored in other tissues such as the brain and kidneys. Interestingly, most of the glycogen in the kidney is in the collecting duct [8], and certain types of diabetes are linked with glycogen accumulation

* Corresponding author.

** Corresponding author.

E-mail addresses: jroa5@calstatela.edu (J.N. Roa), mtresguerres@ucsd.edu (M. Tresguerres).

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in the kidney [9], in some cases in the distal convoluted tubule [10]. While little is known about the regulation of glycogenolysis and glycogenesis in kidney cells, sAC has been shown to regulate glycogen metabolism in astrocytes [11] and liver cells [12].

The gills of elasmobranchs (sharks, rays, and relatives) are a convenient system for studies on blood acid-base regulation that are relevant for both aquatic animals and the mammalian kidney [13]: (1) they have two distinct cell populations responsible for blood acid-base homeostasis that are functionally analogous to the renal A- and B-type intercalated cells: the acid-secreting Na^+/K^+ -ATPase (NKA)-rich cells and base-secreting VHA-rich cells [14–18]; (2) both gill cell types also express sAC, which becomes activated during a blood alkalosis and triggers the translocation of VHA from cytoplasmic vesicles into the basolateral membrane of the base-secreting cells [17,19]; and (3) both cell types are found at relatively high abundance and can be isolated and plated in primary culture for short-term functional studies [17]. Moreover; (4) shark gill acid- and base-secreting cells contain abundant glycogen stores that are preferentially used during an acidosis in the acid-excreting cells and during an alkalosis in the base-excreting cells [20].

The goal of this current study is to take advantage of isolated and cultured ray gill cells to study a potential role of sAC in coordinating glycogen metabolism and acid-base relevant ion-transporting processes in acid- and base-secreting cells.

2. Materials and methods

2.1. Experimental animals and tissue sampling

Male and female round stingrays (*Urolophus halleri*) were collected from La Jolla Shores, CA, near Scripps Institute of Oceanography at the University of California San Diego, and all experiments described were conducted under the Institutional Animal Care and Use Committee protocol number S10320.

2.2. Antibodies and reagents

Anti-NKA antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) and custom-made anti-VHA antibodies both specifically recognize NKA and VHA in round rays [17]. The custom-made anti-glycogen antibody was a kind gift from Dr. Otto Baba [21] and was previously used to identify glycogen-rich cells in fish and shark gills [20,22,23]. Fluorescent secondary antibodies goat anti-mouse Alexa Fluor 555 and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Grand Island, NY, USA). MitoTracker Orange (Thermo Fisher Scientific) was used to stain mitochondria in live cells prior to fixation.

2.3. Immunohistochemistry

Whole gills were extracted, placed in fixative (3.2 % paraformaldehyde, 0.3 % glutaraldehyde, 0.2 M cacodylate buffer) for 6 h, and sequentially dehydrated in a series of ethanol buffers, as previously described [17,18]. Fixed gills were embedded in paraffin and sectioned as previously described [20]. Rehydrated sections were incubated in blocking buffer for 1 h at room temperature and primary antibodies overnight at 4 °C (12 g/ml anti-NKA, 6 g/ml anti-VHA, and 1:20 anti-glycogen in blocking buffer), followed by incubation in secondary antibodies + nuclear stain Hoechst 33342 (Invitrogen, Grand Island, NY; 5 g/ml) at room temperature for 1 h and mounted in FluoroGel with Tris buffer (Electron Microscopy Sciences, Hatfield, PA). Images were taken using a Zeiss AxioObserver Z1, with Axiovision software and Adobe Photoshop used to adjust digital images for brightness and contrast only.

2.4. Colocalization of NKA, VHA, and glycogen in gill sections

For double immunolabeling of NKA and glycogen, or VHA and

glycogen, sections were incubated in a solution of rabbit anti-NKA or rabbit anti-VHA antibodies combined with mouse anti-glycogen antibody overnight and then in a mixture of goat anti-rabbit and anti-mouse secondary antibodies. Similarly, colocalization with all three antibodies was achieved by incubating sections in a mixture of anti-NKA, anti-VHA, and anti-glycogen antibodies overnight, followed by a mixture of anti-rabbit and anti-mouse secondary antibodies. Sections were then completed as described above.

2.5. Glycogen, NKA and VHA subcellular localization in isolated gill cells

Isolated ray gill cells were obtained using a collagenase digestion protocol previously described for ray gills [17]. Isolated cells were plated onto 35 mm glass bottom dishes (cat no. D35-14-1.5-N, Cellvis, Mountain View, CA) and allowed to settle for a 2 h period at 4 °C. Cells were then exposed for 30 min to MitoTracker Orange (200 nM) and one of three treatments: control ray saline (+DMSO), high bicarbonate ('Bic') ray saline containing additional 40 mM NaHCO_3 (pH 8.0) (+DMSO), or high bicarbonate ray saline containing 50 μM KH7 dissolved in DMSO ('Bic + KH7'). Cells were then fixed (ray saline, 3.2 % paraformaldehyde, and 0.3 % glutaraldehyde for 20 min), and processed for immunocytochemistry as described above. HCO_3^- and pH values of the incubation medium were confirmed using a CO_2 analyzer (Corning, NY) and UltraBASIC pH meter (Denver Instrument, Bohemia, NY), respectively.

2.6. Quantification of VHA and glycogen subcellular localization

VHA and glycogen translocation was determined in 20 isolated gill cells from three round rays, for a total of 60 cells per treatment. Starting from the upper-right corner of the field of view, cells were imaged at 600 magnification based on the following criteria: 10–20 μm in size, positive for NKA or VHA, with strong mitochondrial staining [17]. Cells with strong signal in the cell membrane and lack of signal in the cytoplasm were counted as cells with "membrane VHA/glycogen localization" while cells without distinct membrane staining were grouped together as cytoplasmic staining.

2.7. Statistical analyses

Individual cells were considered experimental replicates ($n = 60$ total cells from three rays); which is similar to previous studies with ray gill cells [17], mammalian kidney intercalated cells [7], and mammalian epididymal clear cells [24]. Quantitative cell data were arcsine-transformed and experimental groups were analyzed for significant differences using a one-way ANOVA and Tukey's multiple-comparison test. All data are given as mean $\pm$ SEM and statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Glycogen-rich cells in ray gills

Acid-secreting NKA-rich cells and base-secreting VHA-rich cells were exclusively present along the gill filament and further colocalization with anti-glycogen antibodies revealed both cell types had conspicuous glycogen stores (Fig. 1), which resembles leopard shark gills [20]. However, ray gills additionally had glycogen-rich cells along the length of the entire gill lamellae (Fig. 1), unlike sharks that exclusively had the glycogen stores in acid- and base-regulatory cells [20].

In yet another pattern found in fish gills, the NKA-rich cells in tilapia gills have been reported to lack any noticeable glycogen, and to be directly adjacent to other cells that are glycogen-rich [22,23]. While these differences might be related to metabolic demand (e.g. rays are less active than sharks and tilapia and therefore may have a greater opportunity to store excess glucose as glycogen), the presence of

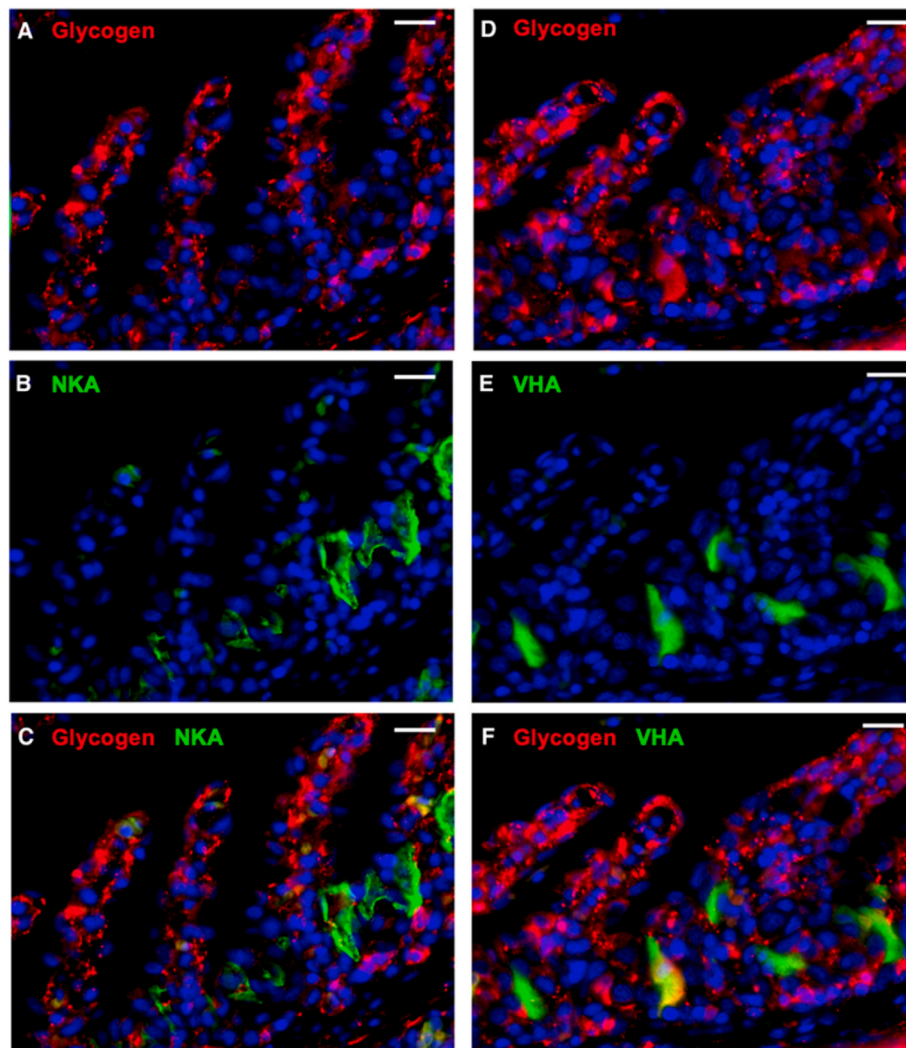


Fig. 1. Glycogen stores in ray gill cells. A–F: Glycogen (red) was highly abundant throughout the gill epithelium, including in acid-secreting NKA (green)-rich cells (A–C) and base-secreting VHA (green)-rich cells (D–F). Nuclei are shown in blue. Scale bar = 20 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

additional energy-demanding gill cell types in ray gills cannot be ruled out.

Primary cultures of isolated ray gill cells allowed us to investigate regulation of glycogen utilization in the acid- and base-secreting cells, which were identified following exposure to different conditions and fixation based on their abundant number of mitochondria and strong NKA or VHA immunostaining (Figs. 2 and 3). While the images show both NKA and mitochondria in red due to imaging equipment limitation, observation on the microscope allowed us to determine that the NKA immunolabeling was exclusively on the membrane (seen in red through the ocular piece) while the mitochondria immunolabeling was predominantly in the cytoplasm and concentrated around the nucleus (seen in orange).

3.2. Glycogen localization in isolated ray gill acid-excreting cells

Under control conditions, isolated acid-secreting cells had NKA in the basolateral membrane (Fig. 2A) that matched their subcellular localization in the native gill epithelium (Fig. 1). Interestingly, glycogen was exclusively present at the basolateral membrane colocalized with NKA, and the cytoplasm was devoid of glycogen. Short term *in vitro* exposure to alkalosis did not induce any noticeable changes in NKA or glycogen localization patterns in acid-secreting cells, and these patterns

were not affected by the sAC selective pharmacological inhibitor KH7 (Fig. 2B and C).

3.3. Glycogen localization in isolated ray gill base-excreting cells

In contrast to acid-excreting cells, base-excreting cells had both VHA and glycogen throughout the cytoplasm under control conditions; we also noticed small portions of the basolateral membrane where VHA and glycogen colocalized (Fig. 3A). These results indicate that glycogen, which is the main storage of short-term metabolic fuel in cells, has a different subcellular localization in the two subtypes of ray gill acid-base regulatory cells. Furthermore, the subcellular localization of glycogen in each cell subtype matched that of the respective main energy consumer ATPase. As previously reported [17], exposure to alkalosis induced the translocation of VHA to the basolateral membrane of base-secreting cells in sAC-dependent as evidenced by the inhibitory effect of KH7 on VHA translocation (Fig. 3B and C). Strikingly, glycogen also shifted to the basolateral membrane of the base-secreting cells in response to an alkalosis in a sAC-dependent manner (Fig. 3C and D). The rapid nature of the exposures (30 min) and relatively low temperature (4 °C) suggest that glycogen translocation to the basolateral membrane in response to alkalosis was mediated by sAC activity and microtubule movement as firmly established for VHA [17,19,25]. However, our experimental

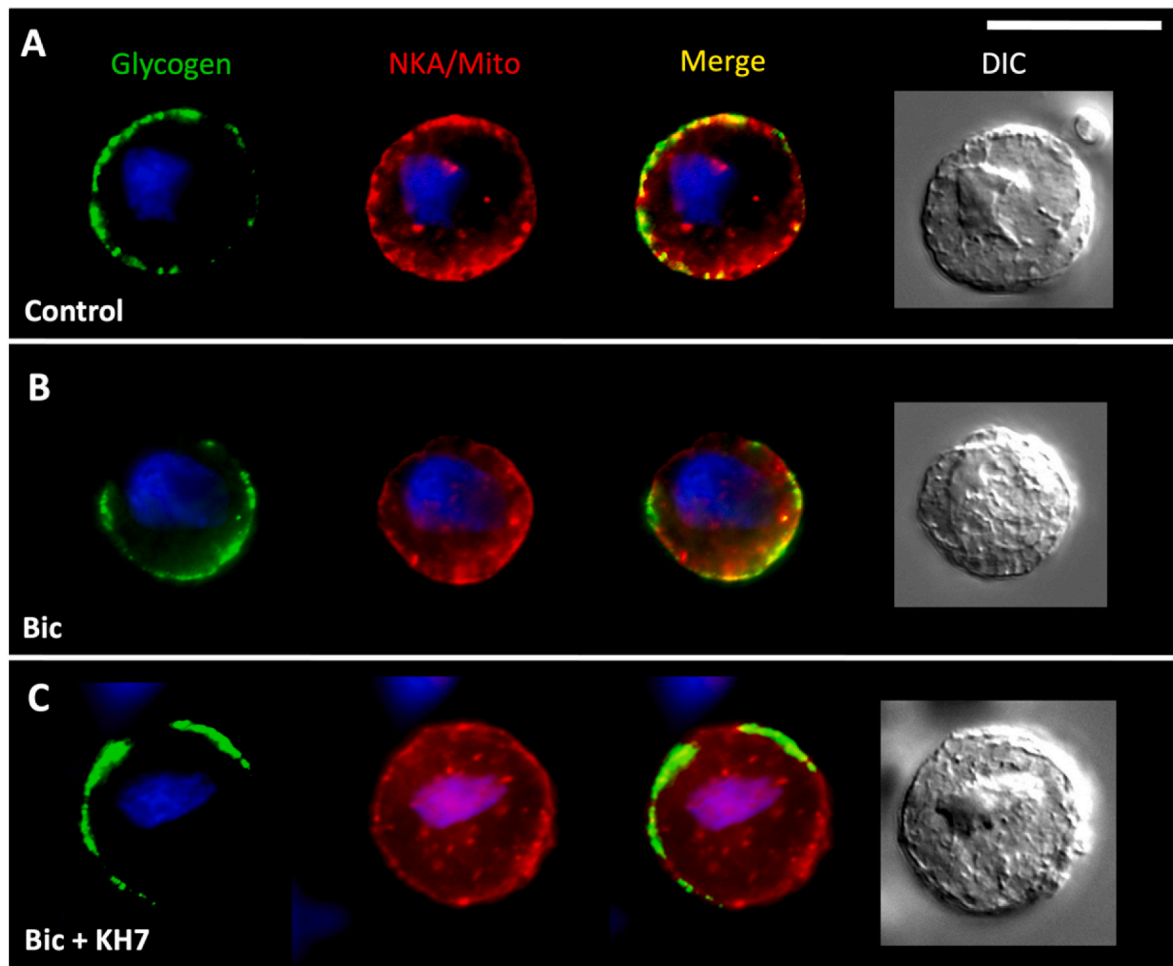


Fig. 2. Glycogen localization in isolated acid-secreting ray gill cells. Cells were exposed to control (5 mM HCO_3^- , pH 7.75), high bicarbonate (Bic; 40 mM HCO_3^- , pH 8.0), and high bicarbonate plus the sAC inhibitor KH7 (Bic + KH7; 40 mM HCO_3^- + 50 μM KH7, pH 8.0). A-C: Representative images of glycogen (green), NKA (red) and mitochondria (Mito, red) and the corresponding DIC image. Nuclei shown in blue. Scale bar = 20 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

design cannot rule out a preferential and rapid utilization of cytoplasmic glycogen stores over those at the plasma membrane, or a combination of glycogen translocation and utilization. Altogether, our results suggest a sAC-dependent spatial and functional coupling of glycogenolysis with active acid-base regulatory ion transport to sustain ATPase function during sudden periods of increased energy demand like those induced here by exposing the gill cells to an alkaline saline.

4. Conclusions and future directions

We previously reported that the acid-base regulatory cells in gills of leopard shark are glycogen-rich and that glycogen utilization increased in active acid-base regulatory cells *in vivo* [20]. Here, we confirmed a similar process occurs in isolated and cultured ray gills indicating that the underlying sensing and signal transduction mechanisms occur at the cellular level and without the need for central hormonal or nervous input.

Glycogen metabolism in human liver cancer HepG2 cells is modulated by the interplay between two sources of cAMP: transmembrane adenylyl cyclases (tmACs) at the plasma membrane that promotes glycogenolysis and sAC in the cytoplasm and mitochondria that promotes glycogenesis [12]. This coordination is achieved by the differential association of tmACs with protein kinase A (PKA) that stimulates glycogen phosphorylase and of sAC with exchange protein activated by cAMP 1 (Epac1) that inhibits it. In the presence of extracellular glucose,

AMP-activated kinase (AMPK) adds another regulatory step through the sensing of the intracellular ATP:ADP ratio [12]. In mammalian muscle fibers, glycogenolysis, glycogenesis, and glycogen granule intracellular relocation are also modulated by cAMP, PKA, and AMP-activated kinase (AMPK) [26–28]. While PKA phosphorylation leads glycogen synthase to translocate to areas of high glycogenolysis at the active contraction sites, AMPK phosphorylation leads it to translocate between myofibrils to increase glycogenesis for storage and later use. Interestingly, this antagonistic relationship between PKA and AMPK is also seen in mammalian kidney acid-base regulatory cells, where PKA triggers VHA translocation to the apical membrane and AMPK blocks this translocation [29].

Like HepG2 cells, regulation of glycogen metabolism in shark and ray gill acid-base regulatory cells likely involve and interplay between tmACs and sAC, both of which are present in both NKA- and VHA-rich cells [17,19,30,31]. However, blood acid-base regulation by shark and ray gill NKA- and VHA-rich cells requires simultaneous activation of ion transport and glycogenolysis in one cell type and deactivation of ion transport and activation of glycogenesis in the other cell type (current study and [20]). We tentatively propose that this differential regulation may be achieved by a functional coupling between tmACs with PKA and sAC with Epac1 in the NKA-rich cells, and between sAC and PKA and tmACs with Epac1 in the VHA-rich cells. However, this hypothesis requires experimental confirmation.

Glycogen, sAC and VHA, are evolutionarily widespread, as are the

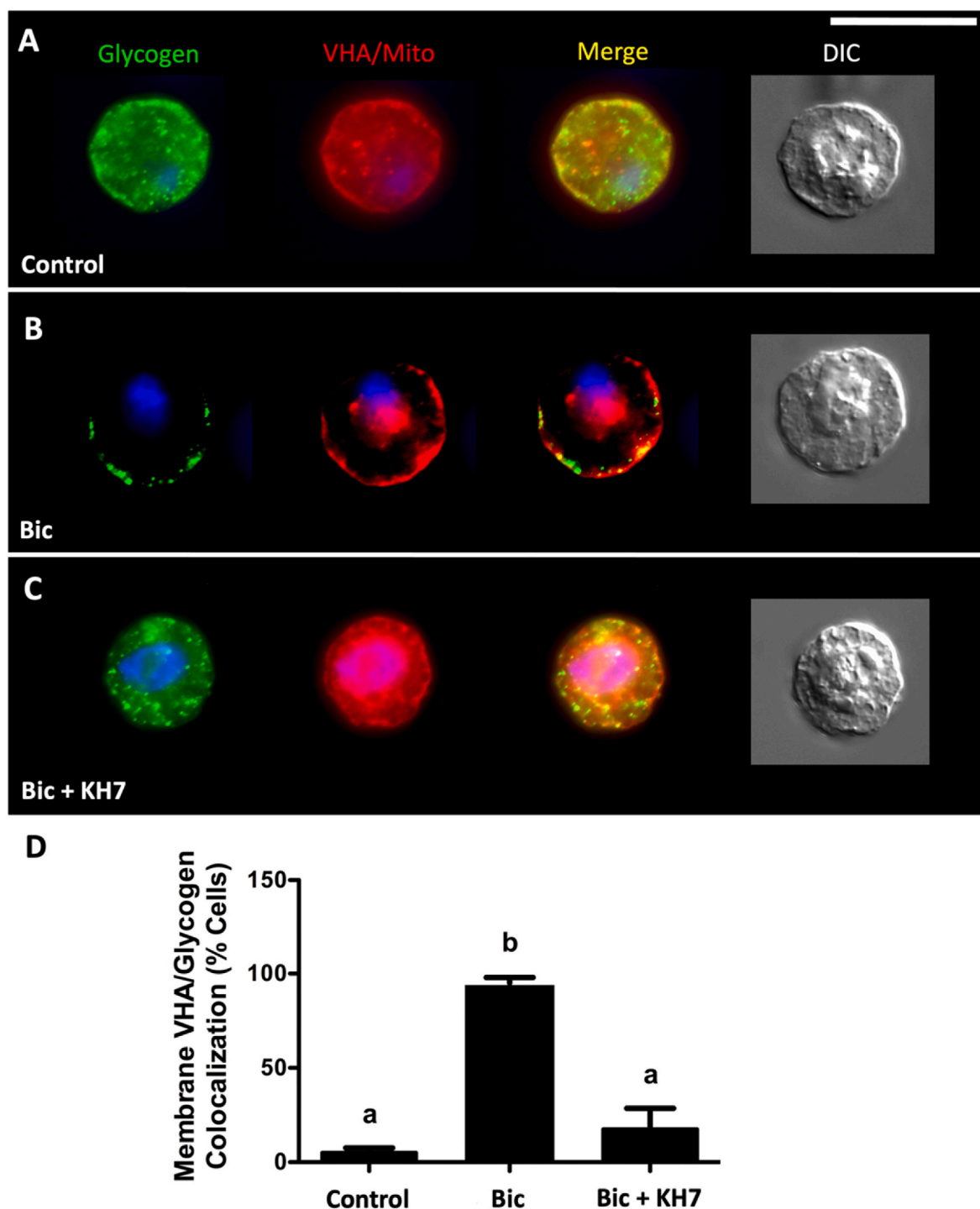


Fig. 3. Glycogen localization in isolated base-secreting ray gill cells. Cells were exposed to control (5 mM HCO_3^- , pH 7.75), high bicarbonate (Bic; 40 mM HCO_3^- , pH 8.0), and high bicarbonate plus the SAC inhibitor KH7 (Bic + KH7; 40 mM HCO_3^- + 50 μM KH7, pH 8.0). A-C: Representative images of glycogen (green), VHA (red) and mitochondria (Mito, red) and the corresponding DIC images. Nuclei shown in blue. Scale bar = 20 μm . D: Effect of elevated bicarbonate and SAC inhibition with KH7 on the colocalization of VHA and glycogen in the plasma membrane. 60 cells per treatment, the letters indicate different levels of statistical significance (one-way ANOVA and Tukey's multiple-comparison test on arcsine-transformed data; $P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

modulation of glycogen metabolism and VHA activity via the cAMP pathway [13,32–34]. Thus, it is likely that the mechanism we proposed here applies broadly to other organisms and systems with regulatory differences arising from the preferential association of SAC and tmACs with distinct signaling enzymes in specific intracellular microdomains. Furthermore, dysregulation of these pathways might be linked to certain types of diabetes that cause glycogen accumulation in the human

kidney, highlighting a potential biomedical significance to our results.

CRediT authorship contribution statement

Jinae N. Roa: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tsuyoshi Morita:** Resources. **Martin**

Tresguerres: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare no competing interests.

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