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科学研究費助成事業　　研究成果報告書

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研究課題名（和文）A new concept of salt handling by Na-binding proteins that immobilize excess Na+ to ease salt stress of seawater teleost fish

研究課題名（英文）A new concept of salt handling by Na-binding proteins that immobilize excess Na+ to ease salt stress of seawater teleost fish

研究代表者

黄　國成（Wong, Kwok Shing）

東京大学・大気海洋研究所・特任助教

研究者番号：４０５２６９０１

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研究成果の概要（和文）：本研究は、イオン輸送体を主とした既知の浸透圧調節とは異なる新しい概念、すなわち、「イオン結合分子による浸透圧調節」を確立した。申請者は、固定法や蛍光標識法を開発し、ナトリウム結合分子の存在を細胞レベルで観察することに成功した。さらに、その分子が特殊な粘液細胞（クラブ細胞）で作られ、精製や質量分析の結果から、糖タンパク、グリコサミノグリカン、2次代謝物である可能性が示唆された。この分子の同定は、基礎生理学（浸透圧調節）や病態生理学（高血圧）の分野に大きなインパクトを与えると期待されている。
本研究を通じ、国内外に新たな共同研究先を開拓し、その成果はすでに国際誌や国内外の学会で発表している。

研究成果の学術的意義や社会的意義
Hypertension is related to Na dysregulation and bound-Na in skin and muscle are the cause of high blood pressure. The Na-binding molecule has not been identified in human, so my work on its identification may contribute to the medical cure for hypertension by solving the mechanism on Na-binding.

研究成果の概要（英文）：The project established new concept of osmoregulatory strategy by ion-binding in addition to traditional ion transporting. I developed new fixation and florescent methods to observe the Na-binding phenomenon in specific mucus-secreting cell called club cells. I showed the existences of Na-binding molecules at cellular levels and partially purified the molecules from mucus. No consistent peptide sequences could be identified via MS/MS analysis, suggesting the Na-binding molecules could be glycoproteins, glycoaminoglycans, or secondary metabolites. This unexpected results required further investigation and confirmation before publishing. I continue to investigate the molecules after the funding period and I am positive that it will lead to a breakthrough in osmoregulatory physiology and hypertension in general.
I have established domestic and international collaboration, published the results in international journals and, present the topics in domestic and international conferences.

研究分野：生物学

キーワード：Na-binding molecules　osmoregulation　hypertension　protein purification

様　式　C－１９、F－１９－１、Z－１９、CK－１９（共通）

１．研究開始当初の背景

Eels are migratory species that are capable of acclimating to freshwater (FW) and seawater (SW) transfer directly (i.e. euryhaline). I have studied osmoregulatory physiology in eels over three decades but there is a basic and yet unanswered phenomenon when the eel is facing a FW to SW challenge. FW eels drink copiously in SW but the switch from FW to SW epithelia in gill, esophagus, and intestine takes more than a day to accomplish. A delayed increase in plasma Na^+ suggested that FW eels can tolerate the initial Na^+ influx from drinking by a mechanism other than transporter-mediated osmoregulatory mechanism.

I hypothesized that binding molecules could be produced by eel to inactivate the osmolality of Na^+. In this project, I have discovered that "organic-bounded Na^+" are present in eel intestine, gill, and skin, where their contents changed rapidly upon SW transfer. This Na-binding is similar to the osmotic inactive Na found in the skin of human patients with hypertension. The Na-binding that may slow down the ion influx and contribute to the delay of plasma Na^+ changes observed in eel, and could be the feature determining why eels and some other fish models are stronger euryhaline species.

２．研究の目的

The aims are to demonstrate the Na-binding phenomenon using newly developed methods including EPMA and Na-green dye, and also identify the nature of Na-binding molecules by purification and/or bioinformatics methods. With the identification, physiological roles of the Na-binding molecules are to be examined using eel and other animal models

３．研究の方法

My research methods include the use of physiological response of euryhaline eels and their secretions which may contain the Na-binding molecules. I used bioinformatics methods to identify proteins that may have potentials to bind to Na^+, according the negative charge that the proteins process. I used 2-dimensional gel electrophoresis to identify possible Na-binding proteins. I use chromatography methods including HPLC to purify Na-binding molecules from the eel mucus secretion. I used EPMA to localize the Na-binding molecules in eel intestinal mucus and confirmed the Na-binding phenomenon. I use MS/MS system in collaboration with Jichi University to identify the possible molecules that may bind to Na+ in the purified fractions after chromatography. I use histology methods to identify mucus cells that produce Na-binding molecules and study the mucus layer formation in eel esophagus using a novel fixation method.

４．研究成果

(1) I used EPMA and Na-green to demonstrate the Na-binding phenomenon in eel tissues and mucus. I have identified mucus cells and club cells in the eel esophagus which are potentially secreting the Na-binding molecules. Figure 1 (left) showed the Na-binding molecules in the mucus cells of the eel esophagus. I used Na-green of cell-penetrative type to locate the Na-binding in vitro, and have demonstrated that the club cells are containing Na-binding molecules (Figure 1, right). The findings have been published in international journals (Wong et al, 2017).

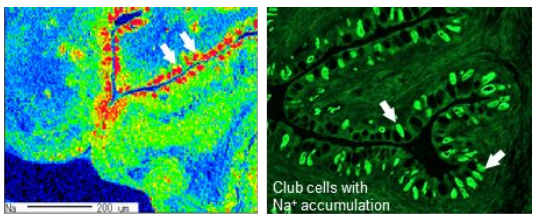

Figure 1. Insoluble Na^+ accumulations in eel esophagus demonstrated by EMPA (left) and Na-green (right).

(2) I use Carnoy's solution as fixative and discover mucus compartments in eel esophagus. Mucus cannot be fixed by ordinary fixative with crosslinking properties, namely formaldehyde-containing reagents. I use Carnoy's solution to fix the eel esophagus and found that the mucus structure and integrity can be maintained. With various histological and immunocytochemical staining technique, I found that different types of mucus cells are present and they secrete immiscible muci that form layers or compartments. This allow functional specialization among different mucus compartments (Figure 2). We identify some potential Na-binding mucus cells with this study and the findings have been

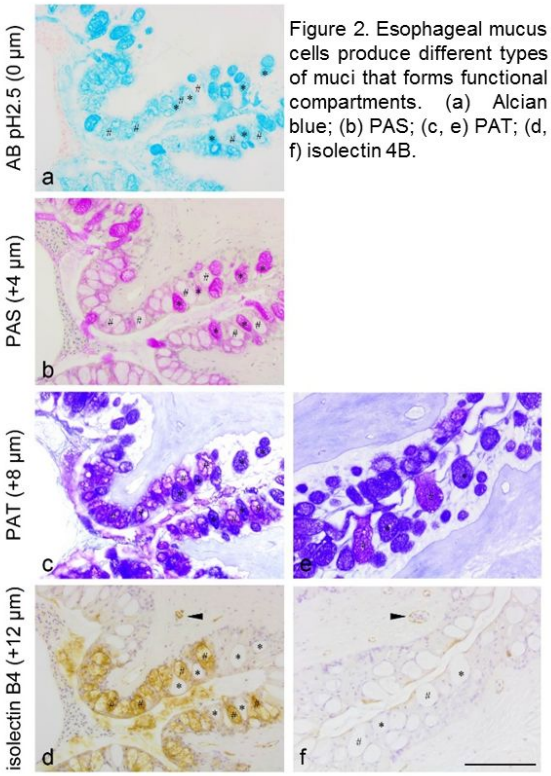

Figure 2. Esophageal mucus cells produce different types of muci that forms functional compartments. (a) Alcian blue; (b) PAS; (c, e) PAT; (d, f) isolectin 4B.

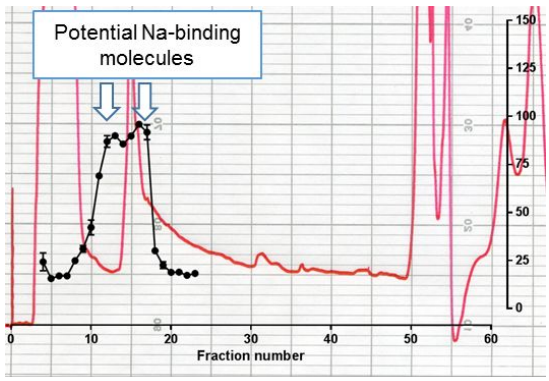

Figure 3. Representative HPLC chromatograph from reverse-phase phenyl column showing the Na-binding molecules containing fractions (Arrows). Red line indicates the OD at 215 nm.

submitted to international journals for reviews (Wong et al, 2019).

(3) I use various HPLC technique to purify the Na-binding molecules in eel mucus and have preliminary tried to identify the molecules by MS/MS. I developed HPLC technique focusing the use of Na-free, non-acidic, non-denature mobile phase for the purification of Na-binding molecules. The molecule could not be detected in the past because Na^+ is a major source of ionic strength present in mobile phases. Moreover, acidic mobile phases are common in HPLC but they can dissociate the Na-binding. I used neutral and non-denature conditions to resolve the Na-binding molecules from the mucus of eel (Figure 3, unpublished). The identification of the molecules in the fractions are still underway.

The novelty of this topic has attracted lots of attention from peer fish physiologists and was welcomed in the International Congress on the Biology of Fish held in Canada in 2018. With the funding supported through the past 3 years, I have seeded this topic in the research field and will continue to investigate this topic.

５．主な発表論文等

〔雑誌論文〕（計　1　件）

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3. Wong MKS, Tsukada T, Ogawa N, Pipil S, Ozaki H, Suzuki Y, Iwasaki W, Takei Y (2017) Japanese eels regulate osmolality by uncharacterized sodium-binding proteins: identification via transcriptome. Advanced Genome Science International Symposium "The Start of New Genomics", Tokyo, Japan.
4. Wong MKS, Ogawa N, Pipil S, Ozaki H, Suzuki Y, Iwasaki W, Tsukada T, Takei Y(2106) Sodium binding proteins in fish. 41st Japanese Comparative Endocrinology Meeting 2016, Kitasato, Japan.

〔図書〕（計　0　件）

〔産業財産権〕
○出願状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
出願年：
国内外の別：

○取得状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
取得年：
国内外の別：

〔その他〕
ホームページ等

６．研究組織

(1)研究分担者
研究分担者氏名：
ローマ字氏名：
所属研究機関名：
部局名：
職名：
研究者番号（8 桁）：

(2)研究協力者
研究協力者氏名：
ローマ字氏名：