ARTICLES

Teaching the Fundamentals of Biological Research with Primary Literature: Learning from the Discovery of the Gastric Proton Pump

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Abstract: For the purpose of teaching collegians the fundamentals of biological research, literature explaining the discovery of the gastric proton pump was presented in a 50-min lecture. The presentation included detailed information pertaining to the discovery process. This study was chosen because it demonstrates the importance of having a broad range of knowledge, performing technique with precision, and thinking creatively, in the context of an interesting story about an enzyme which is important in our daily lives. Kasbekar and Durbin were the first team that tried to purify a gastric proton pump for characterization. They isolated the wrong ATPase because of inaccurate technique. Forte et al. improved the technique and isolated the gastric proton pumps. More importantly, for the first time Forte et al. demonstrated that the proton pump is a potassium ion dependent ATPase, or H^+,K^+-ATPase. The primary literature used here served as a valuable tool to demystify both the process of formulating a testable hypothesis and the pathway for a scientific discovery.

Key words: undergraduate; research article; H^+,K^+-ATPase.

INTRODUCTION

Research articles have been incorporated into undergraduate biological education for a variety of purposes. Unlike textbooks, research articles usually feature unanswered and controversial questions that can stimulate students' curiosity and motivate them to learn. Therefore, research articles have been used to facilitate the teaching of biological courses, such as genetics (Pall, 2000, Wu, 2009), neuroscience (Lynd-Balta, 2006) and biochemistry (Zhu, 2008). Research articles are also introduced to undergraduates in the forms of journal clubs and seminars to demystify scientific research and retain these students in a biological research career (Kozeracki *et al.*, 2006). One difficulty in using research articles for this purpose is that the critical thinking leading to the discovery is usually buried deep in the introduction section, and not readily identifiable by the students and instructors who are not in this particular field of research. Working with one of the greatest contemporary scientists, John G. Forte (University of California, Berkeley), allowed me unique opportunities to understand these "hidden" details of some revolutionary discoveries.

The State University of New York at Buffalo offers college students the opportunity to participate in a Discovery Seminar Program. The program provides students an opportunity to engage in a thought-provoking and challenging topic with a research faculty member. In preparing a lecture designed to demonstrate the fundamentals in biological research to my undergraduate students, I found that Forte et al.'s discovery of the proton pump (Forte *et al.*, 1967) was well-suited for this purpose. Forte *et al.*'s discovery can be used as a tool to demonstrate the importance of a broad range of knowledge, performing technique with precision, and thinking creatively in biological research.

With the proton pump inhibitor being the second most prescribed medicine in the United States (Mullin *et al.*, 2009), the topic presented in this paper quickly caught the students' attention. In addition, the anticipation of the unveiling of the mystery of this great discovery was another enticement for them, especially for those considering graduate education or medicine-related education.

Being a simple story, this article (Forte *et al.*, 1967) is easy for students and biology instructors from different sub-specialties to comprehend. The critical experiments involved in the discovery of the proton pump are simply differential centrifugation and measurement of the enzymatic activity of ATPase, both being conventional methods for most biology instructors. With the differential centrifugation experiment alone the critical thinking leading to this discovery and the importance of performing technique with precision in biological discovery can be demonstrated. The simplicity of this 1967 article allows time for the inclusion of another related article (Limlomwongse & Forte, 1970) demonstrating the association of the proton pump molecule and the function of gastric acid

secretion. The second article is important for this lecture as it completes the discovery story introduced by the initial article, provides another example of critical thinking in biological discovery, and introduces additional interesting elements (tadpoles, gene knockout, etc.).

This module is designed for a 50-min lecture in any given class size. To use this study for the purpose described here, the instructor need only familiarize himself/herself with the material detailed in this report. To attract the attention of the students, I would begin by explaining the importance of gastric acid in our daily lives and the importance of the parietal cell as a productive model system in research. This part could be personalized according to the instructor's preferences and the students' academic level.

RESULTS

Introduction of the topic

The discovery and consequential study of the gastric proton pump has had many significant effects on science and our daily lives. This discovery is one of the most important cornerstones for the physiology of the stomach. Through the efforts of the scientists represented by John Forte, Catherine Chew, and James Goldenring etc., the study of the proton pump has evolved into a wonderful cellular model for the elucidation of many fundamental questions in general biology as well as stomach physiology, such as: the remodeling of the cytoskeleton (Forte *et al.*, 1998), PKA signaling (Chew *et al.*, 2002), the mechanisms

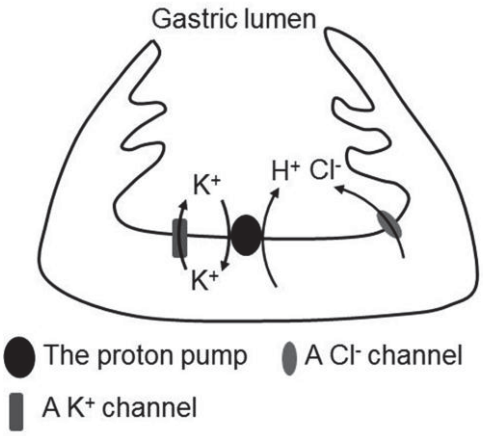

Fig. 1. Schematic representation of the mechanism for the production of gastric acid by oxyntic cells (or parietal cells in mammals). The production of hydrochloric acid by the gastric oxyntic cells is a combined function of the proton pump (gastric H^+,K^+-ATPase), a K^+ channel and a Cl^- channel. At the expense of one ATP, the proton pump pumps out one proton and takes in one K^+. This K^+ is then recycled back to the gastric lumen through a K^+ channel. A Cl^- channel is responsible for the concomitant outflow of Cl^-. The identities of the K^+ and the Cl^- channel are the subjects of current research.

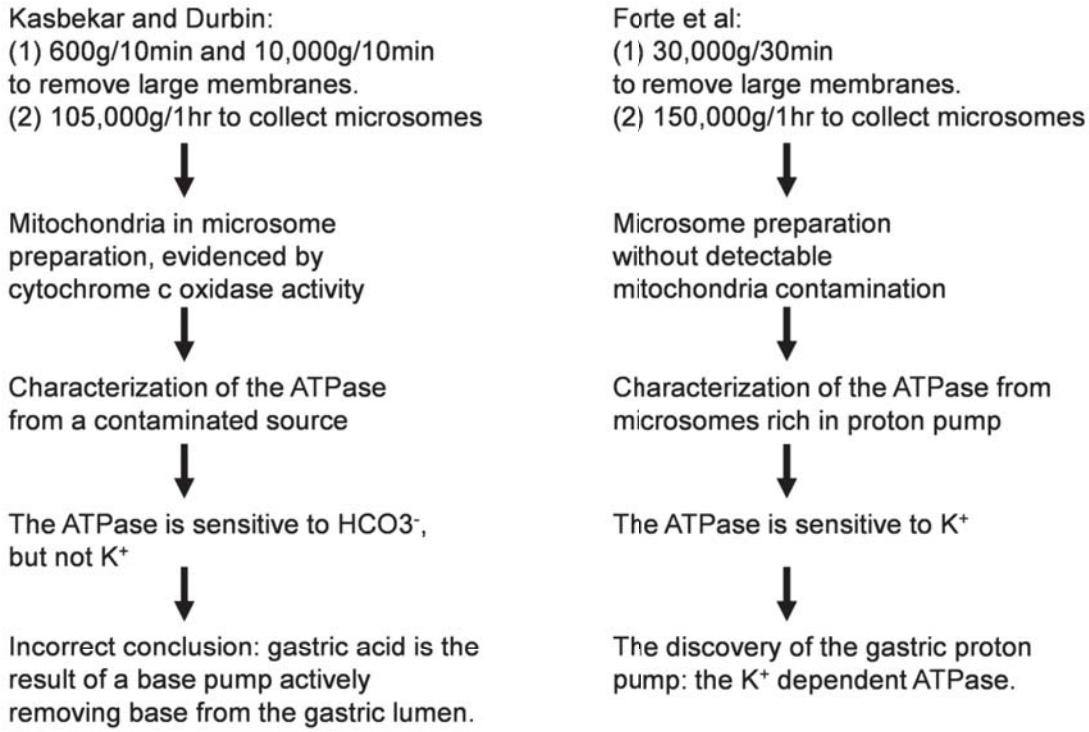

Fig. 2. An example that more accurate technique leads to a great discovery. A side-by-side comparison of the techniques employed by two different research teams to identify the mechanism for the production of gastric acid. The less accurate technique led to a wrong conclusion that the acid was produced by a base pump; while the more accurate technique led to the discovery of the gastric proton pump.

for membrane trafficking (Goldenring *et al.*, 1996), vesicle docking and fusion (Calhoun & Goldenring, 1997), and the establishment of cellular polarity (Zhu *et al.*, 2010). The proton pump is responsible for the production of isotonic acid in gastric juice. Therefore, our ongoing understanding of this pump may also lead to a better management of acid-related diseases such as GERD, a disease affecting roughly 7 million Americans (Everhart, 1994). In addition, this acid is the first powerful barrier separating the body from foreign invaders that are unavoidable in the food we consume. It is notwithstanding, of course, that the acid also activates pepsinogen, allowing our foods to be properly digested.

For all the aforementioned reasons, the discovery of the gastric proton pump (Forte *et al.*, 1967) by John Forte, Gertrude Forte, and Paul Saltman made a substantial contribution to medicine and science, and to the general public as well. In addition, this discovery is an excellent example of the process of scientific discovery. Here I dissect the procedure of this discovery in hopes that this example will inspire future generations of scientists to engage in the search for the secrets of life.

Background of the discovery

Previous hypotheses on the mechanism of gastric acid production.

The discovery of the gastric proton pump resulted from a simple curiosity about the origin of gastric acid, and a large number of hypotheses were raised to explain the production mechanism. For example, Martin Hanke (Hanke, 1926) (University of Chicago) hypothesized that gastric hydrochloric acid is produced by the hydrolysis of organic chlorides. This was disproved soon after, however, by the fact that "the gastric secretion consists mainly of mine salts (sic)" (Davenport, 1992). The redox hypothesis of Conway (University College, Dublin) (Conway, 1949) claimed that yeast in the stomach is responsible for the production of gastric acid. This argument was based on in vitro experiments which showed that yeast can secrete acid in exchange for potassium. Ultimately, this hypothesis faded away due to a lack of any further supporting evidence. Notably, however, one hypothesis in particular was very close to the truth; in fact, it even partially led to the truth. Kasbekar and Durbin isolated microsomes from frog gastric oxyntic cells, which are equivalent to mammalian parietal cells in terms of acid secretion (Kasbekar & Durbin, 1965). From this preparation, a large amount of ATPase activity was detected and was found to be stimulable by HCO_3^- but not K^+. We now know that H^+,K^+-ATPase has the very opposite character. However, Kasbekar and Durbin claimed that the ATPase drives the exchange of HCO_3^- and Cl^- (against the gradients of HCO_3^-). Kasbekar and Durbin believed that this HCO_3^- pump facilitated the dissociation of H_2CO_3 to form secreted HCl. In other words, Kasbekar and Durbin hypothesized that a base pump actively removed HCO_3^- from gastric lumen and thereby produced gastric acid.

Formulation of a new hypothesis for the mechanism of acid secretion: The importance of critical analysis of the previous literature, a broad range of knowledge and creative thinking.

Unfortunately, Kasbekar and Durbin's work was inconsistent with the well-established fact that K^+ is an essential requirement for acid secretion. Forte et al. were well aware of this inconsistency and were critical of Kasbekar and Durbin's work. Forte et al. eventually identified the problem with Kasbekar and Durbin's experiment, namely contamination (Forte *et al.*, 1974, Soumarmon *et al.*, 1974). Kasbekar and Durbin's microsome preparation was contaminated by mitochondria. Following Kasbekar and Durbin's procedure, the microsome preparations consistently exhibited high activity of cytochrome c oxidase (Forte *et al.*, 1974, Soumarmon *et al.*, 1974), a mitochondrial enzyme. The characterization of microsomal ATPase done by Kasbekar and Durbin was actually a characterization of mitochondrial enzymes. Although Kasbekar and Durbin's work led to an inaccurate conclusion, the idea to purify microsomal ATPase for further study proved to be beneficial and did contribute to Forte et al.'s discovery two years later. Consequently, having proved the old hypothesis inaccurate, the natural impulse to formulate a new hypothesis emerged.

In addition to Kasbekar and Durbin's work, important observations made by other investigators had a strong impact on Forte's new hypothesis. Firstly, earlier work by Sedar (Sedar, 1961) demonstrated that the microsomal membrane is implicated in acid secretion. The major evidence was that upon stimulation by histamine, the frog gastric oxyntic cells showed an apparent decrease in the tubular elements of the smooth-surfaced endoplasmic reticulum. Evidence also suggested that these tubular membrane elements incorporated onto the apical membrane of the oxyntic cells. Secondly, previous studies by Forte et al. also demonstrated that the rate of gastric acid secretion is directly correlated with the cellular concentration of ATP (Forte *et al.*, 1965). In addition, Skou's discovery of Na^+,K^+-ATPase in 1957 (Skou, 1957) also had an influence on the discovery of H^+,K^+-ATPase. (Skou won the 1997 Nobel Prize in chemistry because of his work on Na^+,K^+-ATPase.) His findings revealed the first ion-stimulated ATPase activity.

All of these studies catalyzed the development of the new hypothesis that the gastric acid production is powered by a K^+ driven ATPase, or H^+,K^+-ATPase on the microsomal membrane of oxyntic cells. In addition to critical analysis of Kasbekar and Durbin's work, a broad range of knowledge contributed to this new hypothesis. The creative thinking behind this hypothesis merely consisted of compiling all the information from: Kasbekar and Durbin's work;

Sedar's work on microsomal membrane (Sedar, 1961); and Forte's work on ATPase (Forte *et al.*, 1965). With this example, the pathway from the critical analysis of literature and gathering a broad range of knowledge to a "great idea" (a new hypothesis) is revealed.

The test of the new hypothesis led to a great discovery

Next, Forte et al. used Skou's methods and strategy on Na^+,K^+-ATPase (Skou, 1957) to test their hypothesis. To do this, Forte et al. first needed to isolate the microsomal membrane from the oxyntic cells. Isolation and purification are often critical steps in the discovery process. Knowing that Kasbekar and Durbin's approach led to mitochondrial contamination, Forte et al. revised the differential centrifugation technique for the isolation of microsomal membrane from gastric parietal cells (Figure 2). In Kasbekar and Durbin's experiments, the lysate of gastric oxyntic cells was run at 10,000g for 10min to remove large membranes before "microsomes" collected at 105,000g for 1h. As demonstrated by cytochrome c oxidase assays, 10,000g/10min did not remove all mitochondria. Therefore, Forte et al. revised the method for the isolation of microsomes from parietal cells. They ran the lysate at 30,000g for 30 min to remove large membranes before collecting microsomes at 150,000g for 1hr. Cytochrome c oxidase assays indicated that 30,000g/30 min did effectively remove mitochondria. Without mitochondria contamination, this microsomal membrane preparation allowed Forte et al. to discover the gastric proton pump. Here the power of utilizing more accurate technology in scientific discovery is made apparent.

With the right material, Forte et al. could then test whether the microsomal ATPase is dependent on K^+. To this end, the isolated microsomal ATPase was incubated with different concentrations of K^+ and the ATPase actitvity was analyzed. Forte et al. observed a 5-fold increase in enzymatic activity when K^+ was increased from 0 to 1mM. The enzymatic activity continued to increase and eventually plateaued when K^+ was approximately 20mM. For the first time, it was demonstrated that the gastric microsomal ATPase is K^+ dependent. And this is the discovery of the first K^+ dependent ATPase.

The conclusion and supportive follow-up studies

Based upon their 1967 experiments, Forte et al. concluded: "A model system might be devised where K^+ serves not only as an enzyme activator, but also as the locally exchangeable cation in the secretion of HCl" (Forte *et al.*, 1967). Unlike previous hypotheses, this K^+-ATPase hypothesis has survived more than 40 years and all follow-up molecular biological, structural biological, and biophysical

Table 1. The gene "knockout" tadpole study and the gene knockout mouse study: a comparison.

	The tadpole study[a]	The mouse study[b]
When was it performed?	1970	2000
The procedure of "gene deletion".	Examination of the proton pump expression with tadpoles of different developmental stages	1, Analysis of the mouse proton pump gene structure; 2, Generation of the plasmid vector targeting mouse proton pump; 3, Transfection of embryonic stem cells and selection for recombinants 4, Generation of chimeric mice by blastocyst-mediated transgenesis. 5, Genotyping by Southern blot to identify homozygous and heterozygous mice from the offsprings of the chimeric mice.
The result	Tadpoles before stage XXIV do not express proton pump in their stomachs and their stomachs do not product acid; tadpoles beyond stage XXIV start to express proton pump in their stomach and their stomachs produce acid.	The homozygous proton pump knockout mice do not produce gastric acid; while the wildtype and heterozygous animals do.

[a] (Limlomwongse and Forte, 1970)

[b] (Spicer et al, 2000)

studies of the proton pump support this hypothesis.

One piece of data in particular, presented in the 1967 study, may seem odd. Since the Na^+,K^+-ATPase is sensitive to both Na^+ and K^+, one would expect the microsomal H^+,K^+-ATPase to be sensitive to H^+, or pH; however, this is not shown in the data, and such hopeful speculation is, in fact, incorrect. By inhibiting carbonic anhydrase, it has been shown that the gastric H^+,K^+-ATPase is able to produce protons out of water (Davenport, 1946), indicating that this pump is fully functional even at extremely low proton concentrations. Therefore, a higher concentration of protons may not actually accelerate the enzymatic activity.

There are many fascinating follow-up studies on H^+,K^+-ATPase from the labs of Forte and other investigators. One in particular interests me for its ingenuity and creativity: an in vivo study that associates the gastric microsomal ATPase with acid secretion (Limlomwongse & Forte, 1970). It is an equivalent of a gene knockout study with less labor and a more intriguing procedure (See Table 1 for a comparison). This "knockout" system makes use of tadpoles at different metamorphosis stages. Forte et al. carefully characterized the stomachs of tadpoles at different metamorphosis stages and found that tadpoles start to produce gastric acid at stage XXIV (Forte *et al.*, 1969). Therefore, tadpoles before stage XXIV are equivalent to "knockout" animals as the function of gastric acid secretion is absent, while animals beyond stage XXIV are "normal controls." Having established this elegant system, it must have been very exciting to analyze the ATPase activities at the different metamorphosis stages of the tadpoles. This ingeniously designed experiment yielded simple yet beautiful results: microsomal ATPase activities are at background levels all the way to stage XXIV, after which they experience a significant jump at stage XXV (Limlomwongse & Forte, 1970). The proton pump gene knockout mouse study performed in 2000 reported essentially similar results: the deletion of the proton pump gene caused achlorhydria (Spicer *et al.*, 2000).

DISCUSSION

The purpose of this lecture is not to make students blindly memorize; rather, its purpose is to encourage students to contemplate the procedure of scientific discovery and to take in these essential ingredients for scientific research while enjoying an interesting subject.

Here is the take-home message for the students. A good scientist develops a broad range of knowledge through the experience of other knowledgeable scientists in his/her field. In this case, the works of Kasbekar, Durbin, and Sedar were the foundation for Forte's hypothesis on a K^+-stimulated ATPase, while Skou's work provided the methodology to test this hypothesis. Also, a good scientist must be a master of techniques, particularly of those involved in his/her research. Inaccurate technique for the isolation of membrane vesicles was the major reason that Kasbekar and Durbin missed the enzyme for gastric acid production. Accurate technique for membrane isolation by Forte et al. was the key to the purification, characterization and discovery of proton pump. In addition, a successful scientist needs to think creatively. Usually great ideas were not generated from scratch. A compilation of selected literature and relevant knowledge could shed light on an innovative idea. Forte's idea about K^+-driven ATPase came into being during active reading when he related his laboratory findings to literature.

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