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Nano-analysis Reveals High Fraction of Serotonin Release during Exocytosis from a Gut Epithelium Model Cell

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Abstract: Electrochemical methods were used to explore the exocytotic nature of serotonin (5-HT) release in human carcinoid BON cells, an *in vitro* human enterochromaffin cell model, to understand the mechanisms operating the release of gut-derived 5-HT in the intestinal mucosal epithelium. We show that the fractional vesicular 5-HT release in BON cells is 80% compared to previous work in pancreatic beta cells (34%). The fractional release increased from 80% in control BON cells to 87% with 5-HT preincubation and nearly 100% with the combination of 5-HT and the 5-HT₄ autoreceptor agonist, cisapride. Thus, partial release is the primary mechanism of exocytosis in BON cells, resulting in a variable amount of the vesicular content being released. Factors that control secretion of 5-HT from enterochromaffin cells or BON cells are important as partial release provides a mechanism for development of effective therapeutic strategies to treat gastrointestinal diseases.

Serotonin (5-hydroxytryptamine (5-HT)) is well known as an important neurotransmitter in the central nervous system that modulates many behavioral and neuropsychological processes, including aggression, mood, and memory.^[1] However, 95% of 5-HT in the human body is synthesized in enterochromaffin (EC) cells in the gastrointestinal (GI) tract and 5-HT signaling is ubiquitous in the enteric nervous system (ENS) in modulation of normal GI motility and inflammation. EC cells act as important signal-transducers that transfer information from the GI lumen to afferent nerve terminals, enabling them to detect and transduce information directly to the nervous system.^[2] The release of 5-HT in the GI tract, however, is not always beneficial. High levels of 5-HT release are associated with inflammatory bowel disease and diarrhea,

while low 5-HT levels are one of the main causes of constipation.^[3] In addition, gut-derived 5-HT outside the gut also plays an important role in regulation of glucose homeostasis, bone density, and diseases associated with metabolic syndrome including obesity and type 2 diabetes.^[4] This has led to a great deal of interest in the regulatory effect of EC cells and abnormal levels of 5-HT in various diseases. However, a detailed understanding of 5-HT exocytosis is not clear, and it is crucial to dissect the molecular mechanism for numerous human diseases as this could be beneficial for the development of novel therapeutic strategies.

It has been proposed that during exocytosis, following vesicle fusion, the fusion pore closes without full dilation, a scenario referred to as partial release. This partial release vesicle fusion results in a large fraction of vesicular content being released, followed by vesicle recycling.^[5] Compared to full release, partial release enables economical transmitter release and vesicle recycling, as well as high efficiency of transmitter storage and loading in vesicles. Electrochemical techniques that employ microelectrodes or nanoelectrodes have been recognized as crucial tools to understand the mechanism of exocytosis, as they allow real time monitoring of transmitter release with high temporal resolution. Single cell amperometry (SCA) allows quantification of the amount of transmitters released during single vesicle exocytosis and provides detailed information about the frequency of exocytotic events and release dynamics.^[6] Intracellular vesicle impact electrochemical cytometry (IVIEC), introduced by our group, allows direct quantification of transmitter storage in individual vesicles inside a single cell.^[7] The combination of SCA and IVIEC thus enables valuable information related to the mechanism of exocytosis as well as cellular communication to be obtained.^[5,8]

In this study, we investigated the release mechanism of 5-HT in the human neuroendocrine carcinoid BON cell line, an *in vitro* human enterochromaffin cell model that synthesizes and secretes 5-HT and various peptides,^[9] to understand the mechanisms operating the release of 5-HT inside and outside the gut using SCA and IVIEC. For SCA, a disk carbon fiber microelectrode was placed in close proximity to a BON cell and held at +650 mV versus an Ag/AgCl reference electrode (Figure 1A). The cell was stimulated with 10 μM ionomycin, a Ca²⁺ ionophore used to increase intracellular calcium levels, to evoke 5-HT secretion and exocytotic events were recorded as amperometric current spikes at the electrode. Figure 1B shows a typical amperometric trace for exocytotic 5-HT release obtained with SCA. For IVIEC, a flame-etched carbon fiber nanotip electrode was used to penetrate the cell membrane and intracellular vesicles then adsorb and rupture on the electrode surface to release their content

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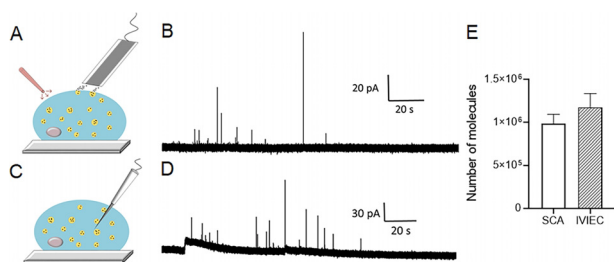


Figure 1. Exocytosis and vesicular content in BON cells. A) Schematic of single cell amperometry (SCA) and B) a representative amperometric trace obtained from SCA in BON cells. C) Schematic of intracellular vesicle impact electrochemical cytometry (IVIEC) and D) a typical amperometric trace obtained from IVIEC. E) Average number of molecules detected by SCA and IVIEC from BON cells ($p = 0.23$). $n > 100$ cells.

(Figure 1C). Figure 1D shows a typical amperometric trace for vesicular content of 5-HT obtained with IVIEC. The number of 5-HT molecules is quantified with Faraday's law ($N = Q/nF$). The average number of 5-HT molecules per exocytotic event is 984000 ± 106000 in BON cells and the average total vesicular content of 5-HT is 1174000 ± 159000 molecules (Figure 1E). The fraction of release is calculated by the number of molecules released during exocytosis over the total number of molecules stored in single vesicles. On average, $80 \pm 2.4\%$ of 5-HT is released, indicating vesicles in BON cells release a large fraction of 5-HT during individual exocytosis events. BON cells release a much larger fraction of their vesicular 5-HT content than 5-HT released in beta cells, where beta cells only release about 34% of 5-HT during exocytosis.^[10] In addition, the fraction of release in BON cells is also larger than catecholamine released from PC12 and chromaffin cells (from 58% to 74%),^[8] as well as glutamate released in hippocampal neurons (from 30% to 50%) and octopamine released from neuromuscular neurons (from 4.5% to 11%).^[5d,11]

Importantly, only a limited number of exocytotic events were detected with SCA (Figure S1), indicating some BON cell vesicles cannot secrete an adequate amount of 5-HT for amperometric detection. In order to increase the vesicular 5-HT loading to enhance the detection possibility by amperometry, BON cells were preincubated in $300 \mu\text{M}$ 5-HT supplemented medium for 96 h. 5-HT modulates physiological effects by binding to different types of 5-HT receptors (from 5-HT₁ to 5-HT₇). Among these, 5-HT₄ autoreceptors have been highlighted as an attractive drug target to modulate gut motility and synaptic plasticity in the ENS.^[12] Thus, we also investigated the effect of a 5-HT₄ receptor agonist, cisapride, to have real insight into the therapeutic effects on exocytotic release and vesicular storage of 5-HT. For cisapride treatment, BON cells were first incubated in $300 \mu\text{M}$ 5-HT supplemented medium for 48 h, and then in $300 \mu\text{M}$ 5-HT and 100 nM cisapride supplemented medium for additional 48 h. Figures 2A–D show examples of the amperometric traces obtained with SCA and IVIEC from 5-HT treated cells (Figures 2A,B) as well as the combination of 5-HT and cisapride (5-HT + cisapride) treated cells (Figures 2C,D). The amount of 5-HT released during exocytosis as well as the

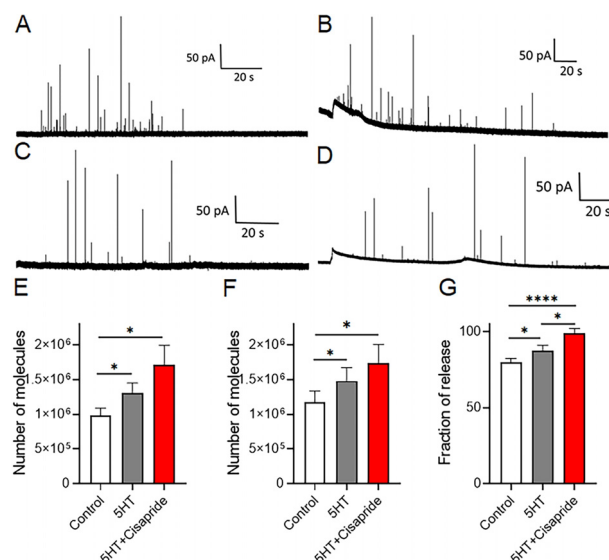


Figure 2. Effect of 5-HT and 5-HT + cisapride on the amount of exocytotic release, vesicular content, and fraction of release. Typical amperometric traces from A) SCA and B) IVIEC in cells treated with 5-HT. Typical amperometric traces from C) SCA and D) IVIEC in cells treated with 5-HT and cisapride. E) Average number of molecules released per exocytotic release measured by SCA. F) Average number of molecules stored in single vesicles measured by IVIEC. G) Fraction released. $n > 35$ cells. p values are listed in Tables S1–3.

total vesicular 5-HT content are increased after 5-HT or 5-HT + cisapride treatment (Figures 2E,F). In addition, a control experiment was also performed by incubating cells with only 100 nM cisapride for 48 h, where similar effects were observed as for 5-HT + cisapride treated cells (Figures S2,3). Our results show that the exocytotic release of 5-HT is altered in treated cells, with a 33% increase in the 5-HT preincubation group and a 74% increase in the 5-HT + cisapride treated cells compared to the control cells. The vesicular 5-HT content is also altered with a 26% increase in the 5-HT preincubation group and a 48% increase in the 5-HT + cisapride treated cells. With 5-HT treatment, vesicles release a larger fraction of its transmitter load during exocytosis (Figure 2G, $87 \pm 3.5\%$ versus $80 \pm 2.4\%$ in the control cells), while the fraction of release from the 5-HT + cisapride treated cells is nearly 100% ($98.9 \pm 3.1\%$), suggesting that vesicular release during exocytosis is augmented from partial release towards full release by the combination of 5-HT and cisapride.

A smaller increase of 5-HT storage in vesicles coupled to a larger increase in release per event leads to a higher fraction released. Thus, the opening of the fusion pore and rate of release is a key process during exocytosis. To determine how exocytotic release is increased more than vesicular storage, we analyzed amperometric spikes obtained from SCA to determine the dynamic information describing exocytotic release (Figure 3A). Significant increases in peak current, I_{max} , were observed after 5-HT preincubation and 5-HT + cisapride treatment (Figure 3B), indicating more 5-HT flux through the open fusion pore. The peak half time, t_{half} , corresponds to the duration of exocytotic events. The t_{rise} and t_{fall} represent the opening and the closing processes of the

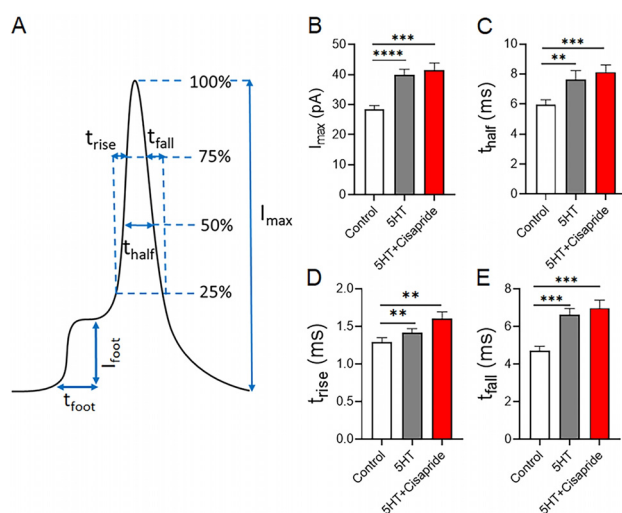


Figure 3. Amperometric peak analysis. A) Schematic of amperometric spike with different parameters. Comparisons of B) peak current, I_{max} , C) half peak width, t_{half} , D) rise time, t_{rise} , E) fall time, t_{fall} , from SCA from control (white), 5-HT treated (gray), and 5-HT + cisapride treated (red) BON cells. $n > 35$ cells. p values are listed in Tables S4–7.

fusion pore. Figures 3C,E show that the opening and closing time of the fusion pore have been significantly slowed down, allowing the pore to stay open for a longer time in both 5-HT and 5-HT + cisapride treated cells. This allows more 5-HT to be released during each exocytosis event. In addition, slight but not significant increases in t_{rise} , t_{half} , and t_{fall} were observed in the 5-HT + cisapride treated cells compared to cells only treated with 5-HT. The prespike foot, which is a small shoulder-like current increase prior to the main exocytotic peak,^[13] was also investigated to gain more insight into the opening of the fusion pore during exocytosis and this is shown in Figure S4. The information from amperometric spikes and prespike foot analysis indicates that cisapride induces a longer lasting and more stable fusion pore to release a larger amount and higher fraction of 5-HT.

The primary role of 5-HT released by EC cells is to target 5-HT receptors in the mucosal projections of primary afferent neurons, including extrinsic nerves and intrinsic primary afferent neurons (IPANs).^[14] In addition, 5-HT can also trigger intestinal smooth muscle contraction and activates dendritic cells in the context of gut inflammation.^[15] Thus, the diffusivity of serotonin in the ENS is far greater than at the synapse. In addition, the reuptake process of 5-HT is predominantly present on adjacent cells, where extracellular 5-HT is taken up by serotonin reuptake transporter (SERT) into enterocytes and epithelial cells.^[16] In this respect, a large fraction of release (80%) favors the spread of 5-HT into the extracellular space. One possible explanation is that there is probably a combination of a large fraction of full release events and a small fraction of partial release events, which makes up on average a release fraction of 80%.

The 5-HT₄ autoreceptor has been highlighted as an attractive therapeutic target due to its potential role in GI motility disorders, including irritable bowel syndrome and chronic constipation. Activation of 5-HT₄ receptors activates adenylyl cyclase, which converts ATP to cAMP and thereby

increases intracellular levels of cAMP.^[17] The vesicular monoamine transporter (VMAT) transports serotonin from the cytosol into vesicles, which is coupled to the movement of two protons in the opposite direction. This process depends on the proton electrochemical gradient generated by the vacuolar H⁺-ATPase (V-ATPases)^[18] and V-ATPase utilizes the energy released from ATP hydrolysis to drive the proton pump. During heightened V-ATPase activity, transport of 5-HT by VMAT increases through an increase in the chemical H⁺ gradient (ΔpH), which further drives 5-HT loading into vesicles. This thus explains the increases in vesicular content of 5-HT in both 5-HT and 5-HT + cisapride treated cells. In addition, cAMP has been shown to regulate exocytosis by facilitating widening of the fusion pore and prolonging its opening.^[19] This in turn leads to an increase in t_{half} , t_{rise} and t_{fall} for the exocytotic events in both 5-HT and 5-HT + cisapride treated cells and a greater fraction of 5-HT is released during exocytosis. 5-HT and activation of 5-HT₄ receptors have been reported to enhance intracellular calcium levels.^[20] An increase in intracellular calcium level is expected to induce a higher number of exocytotic events in 5-HT + cisapride treated cells than only 5-HT treated cells. However, our results suggest that cisapride mainly affects the vesicular content and exocytotic release, while has no effect on the number of exocytotic events. Figure 4 shows proposed mechanisms for the action of 5-HT and cisapride on exocytotic release and vesicular content of 5-HT. Even though 5-HT treatment activates 5-HT₄ receptors, 5-HT can of course bind to all types of 5-HT receptors and cisapride is a potent 5-HT₄ receptor agonist. Thus, exocytotic release and vesicular content of 5-HT increase more with the 5-HT + cisapride treated cells compared to the cells only treated with 5-HT. It is also possible that part of the function of cAMP is via activation of protein kinase A as demonstrated in

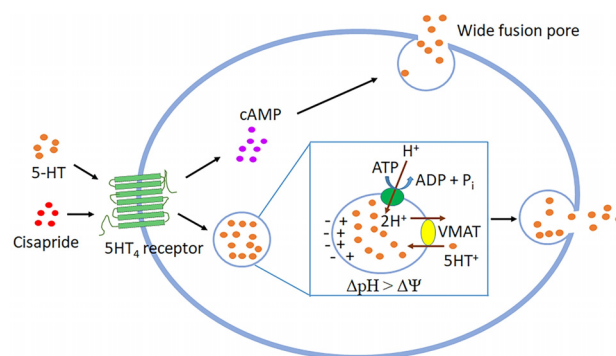


Figure 4. Proposed mechanisms for action of 5-HT and cisapride on exocytotic release and vesicular content of 5-HT. Activation of 5-HT₄ autoreceptors by cisapride increases intracellular levels of cAMP. Vesicular content: VMAT transports 5-HT from the cytosol into vesicles and is coupled to the movement of two protons in the opposite direction. This process depends on the proton electrochemical gradient generated by V-ATPases ($\Delta\mu_{H^+}$), where $\Delta\mu_{H^+}$ = chemical H⁺ gradient (ΔpH) + electrical potential ($\Delta\Psi$). cAMP increases ATP hydrolysis and V-ATPases activity. During heightened V-ATPases activity, transport of 5-HT by VMAT increases through increase in ΔpH , which further drives 5-HT loading into vesicles. Exocytotic release: cAMP regulates exocytosis by stabilizing the vesicle fusion pore with relatively wider diameters.

pancreatic beta cells,^[21] and this regulates the rate of dissociation of 5-HT from the dense core.

As incubation with 5-HT increases the fraction of release in partial release events during exocytosis. This fraction might be important in understanding treatment with 5-HT₄ receptor agonists for how they alleviate symptoms associated with GI disorders.^[15] Cisapride increases the fractional release to almost 100%, switching partial release events to nearly all full release events during exocytosis and thus strengthening the signal provided. This is a powerful use of the cells ability to modulate communication within single and collections of events. However, future studies are needed to support the hypothesis of the existence of both partial release events and full release events during exocytosis of 5-HT from BON cells.

In summary, electrochemical methods, SCA and IVIEC, were used to investigate exocytotic release and vesicular 5-HT storage in human carcinoid BON cells. Our results suggest that the release of 5-HT is predominantly partial, but that vesicles release a large fraction of 5-HT during each exocytosis event, especially when compared to 5-HT release in beta cells. Gut-derived 5-HT exerts its effects by targeting 5-HT receptors from the lumen of the gut to intrinsic and extrinsic sensory neurons and therefore, a large amount of release is sometimes needed to maximize its availability in the extracellular space and the ability to modulate this by changing fraction released is potentially important. Both addition of 5-HT and agonist to 5-HT receptors elevate the fraction of release during exocytosis, where cisapride treated cells release nearly 100% of the transmitter load. Thus, partial release results in a variable amount of vesicular content being released, which can influence the amplitude and the kinetics of the resulting synaptic current. It is possible to differentially regulate gut-derived 5-HT signaling and this might shed light on the development of potential therapeutic strategy for diseases inside and outside of the gut. In the brain, a possible switch between partial release and full release might help to utilize exocytosis for serotonergic neurotransmission and thus regulate synaptic strength.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: BON cells · exocytosis · gut-derived 5-HT · nano-analysis · partial release

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