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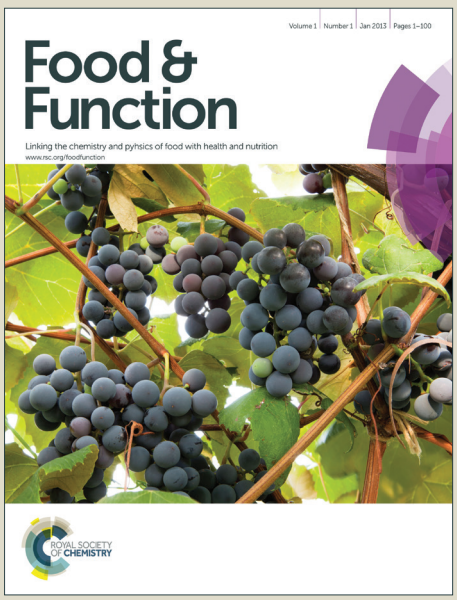

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PAPER

Analysis of free ACh and 5-HT in milks from four different species and their bioactivity on 5-HT$_3$ and nACh receptors

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Milk is one of the most beneficial aliments and is highly recommended in normal conditions; however, in certain disorders, like irritable bowel syndrome, cow milk and dairy products worsen the gastric symptoms and their use is not recommended. Among the most recognized milk-induced gatrointestinal symptoms are abdominal pain, nausea and vomiting, which are processes controlled by cholinergic and serotonergic transmission. Whether the presence of bioavailable ACh and 5-HT in milk may contribute to normal peristalsis, or to the developing of these symptoms, is not known. In this work we attempt to determine whether the content of free ACh and 5-HT is of physiological significance in milks from four different species: cow (bovine), goat, camel and human. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to identify and quantify free ACh and 5-HT in milk, and activation of the serotonergic and cholinergic ionotropic receptors was investigated using electrophysiological experiments. Our principal hypothesis was that milk from these four species had sufficient free ACh and 5-HT to activate their correspondent receptors expressed in a heterologous system. Our results showed a more complex picture, in which free ACh and 5-HT and their ability to activate cholinergic and serotonergic receptors are not correlated. This work is a first step to elucidate whether 5-HT and ACh, at the concentrations present in the milk, can be associated to a direct function in the GI.

Keywords: Acetylcholine receptor, serotonin receptor, milk bioactives, protein membrane receptor, ligand gated ion-channel, gastrointestinal tract

Introduction

Acetylcholine (ACh) and serotonin (5-HT) are classical neurotransmitters that are indispensable to the normal physiology of the digestive system. They exert their actions through the activation of G coupled protein receptors (metabotropic) and ion channel coupled receptors (ionotropic) located in epithelial, endocrine and neuronal tissue along the gastrointestinal (GI) tract.[1-3] The functional roles of ACh and 5-HT are cell and organ specific and depend on the type or receptors expressed (see reviews[4-6] for molecular function and classification of ACh and 5-HT receptors). The main source of ACh in the GI tract is a network of myenteric neurons which control peristalsis and muscle tone trough the activation of metabotropic M2 and M3-type muscarinic receptors.[7-9] Serotonin is also produced in the GI tract. About 95% of all 5-HT in the human body is found in the mucosa and in the myenteric plexus of the GI tract.[1, 2] Serotonergic activation modulates peristalsis, internal secretion, sensation, nausea, and vomiting.[10] 5-HT$_3$-type ionotropic receptors are an important pharmacological target used to reduce intestinal motility and to control nausea and vomiting.[1] Likewise, pharmacological modulation of cholinergic neurotransmission is commonly used to treat abdominal pain and exacerbated peristalsis.[11] For these reasons it is not surprising that alterations of the cholinergic and serotonergic systems are also involved in gastrointestinal disorders that can be worsened by food ingestion.

Milk is one of the most beneficial aliments and is highly recommended in normal conditions; however, in certain disorders, like irritable bowel syndrome, cow milk and dairy products worsen the symptoms and therefore their use is not recommended.[12, 13] Although lactose intolerance have been associated with milk-induced GI symptoms,[12, 13] milk has neuroactive substances that may affect neuro-communication in the GI.[14, 15] Among the most recognized milk-induced GI symptoms are abdominal pain, nausea and vomiting, which are processes controlled by cholinergic and serotonergic transmission. Whether the presence of bioavailable ACh and 5-HT in milk may contribute to the developing of these symptoms is not known. ACh has been previously measured in milks;[16-19] however, it is not clearly understood whether the ACh measured is available to activate neurotransmitter receptors in the lumen of the GI tract. Similarly, measurements of 5-HT are scarce. To our knowledge only Feldman and Lee have measured 5-HT in milk by using a radio-enzymatic analysis and thin layer chromatography.[20] Moreover, there are no reports in the literature evaluating the effect of milk on 5-HT$_3$ receptors, which are the main target for antiemetic therapies.

In this work we attempt to determine whether the content of free ACh and 5-HT is of physiological significance in milks from

four different species: bovine, goat, camel and human (BMk, GMk, CMk, HMk; respectively). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to identify and quantify free ACh and 5-HT in milk, and activation of the receptors was investigated using electrophysiological experiments. Our principal hypothesis was that milk from these four species had sufficient free ACh and 5-HT to activate their correspondent receptors expressed in a heterologous system; our results showed a more complex picture. This work is a first step to elucidate whether 5-HT and ACh, at the concentrations present in the milk, can be associated to a direct function in the GI.

Materials and Methods

2.1 Chemical and Reagents

5-hydroxytryptamine (Cat. H-7752) and acetylcholine (Cat. A-6625) were bought from Sigma-Aldrich, St. Louis, MO. L-ascorbic acid (Cat. A61-25) was acquired from Fisher Scientific, Fair Lawn, NJ. Water and methanol were OPTIMA LC-MS grade from Fisher scientific, Fair Lawn, NJ.

2.2 Milk samples

Milk samples from 9 camels, 5 goats and 5 cows were purchased from commercial vendors (See table 1). Milk samples from women were purchased to Mother's Brain Bank. This milk bank followed the recommendations for donor screening and for milk collection and storage published by the Human Milk Banking Association of North America. In all cases providers were instructed to freeze the milk immediately after milking. After arrival to the laboratory, milk samples were thawed and then centrifuged at 10000x g for 60 minutes at 4 ^{0}C to separate the fat. Samples were filtered first through gauze then 0.8-µm and 0.45-µm syringe filters (Fisherbrand, FisherScientific). Milk was collected, labelled as "source milk", and stored at -20 ^{0}C until analysis.

2.3 Analysis of acetylcholine and serotonin by LC-MS/MS

Our interest was to evaluate the concentration of free ACh and 5-HT without the contribution of possible bound forms that may be released by sample preparation, therefore a non-aggressive method was used. Briefly, 1 mL of each source milk sample was centrifuged at 10000x g for 1 h at 4 ^{0}C. Supernatant was removed carefully and transferred to an empty vial. From the supernatant, two aliquots of 50 µL were used for quantification using the standard addition method to avoid matrix effects as much as possible.[21, 22] In chemical analysis, matrix refers to the components of a sample other than the analyte which might have a considerable effect on the quality of the results obtained; such effects are called matrix effects and could lead to erroneous quantitative results by signal suppression or enhancement. The use of the standard addition method helps to minimize these effects.[23] Stock solutions of ACh and 5-HT were made in ascorbic acid 0.8 mg/mL (AA8) to avoid degradation of 5-HT and working solutions of both compounds were diluted from the stock solutions with AA8. One of the aliquots from each milk sample was added with a volume of 50 µL of AA8, the other aliquot was spiked with 50 µL of working solution containing a known concentration of ACh and 5-HT. Two replicates were analysed for each sample and the quantification was made by LC-MS/MS as it is described next.

All LC-MS/MS experiments were conducted on an Ultra Performance Liquid Chromatography (UPLC) ACQUITY with a UPLC BEH C18, 1.7 µm particle diameter, 2.1 x 50 mm column (Waters, Mildford, MA. USA). The UPLC was coupled to an electrospray (ESI)/Tandem quadrupole Quattro PremierXE (Micromass, UK) mass analyser. Chromatographic conditions were set using a binary gradient elution with Water (0.2% v/v acetic acid) and acetonitrile (0.2 % v/v acetic acid). ACh and/or 5-HT concentration was determined by the injection of 10 µL of sample.

Mass spectrometry data were collected in positive ion mode by Selected Reaction Monitoring (SRM). Precursor ions are selected by the first quadrupole (Q1) and allowed to pass into the collision cell which is a quadrupole in rf-only mode (q). The second quadrupole (Q2) was set to allow only product ions of a specific m/z value. Optimization parameters were performed automatically by infusing a 100 ng/µL solution of ACh or 5-HT. Monitored transitions were 145 -> 87 and 177 -> 160 for ACh and 5-HT respectively. Areas under the curves, in the extracted ion current chromatograms, were used to quantify the analytes of interest.

2.4 Molecular Biology

Rat α4 and β2 nicotinic acetylcholine receptors (nAchRs) subunits were kindly provided by Professor Katumi Sumikawa (University of California, Irvine). The human 5-HT$_{3A}$ cDNA was purchased from Missouri S&T cDNA Resource Center. The plasmids were transformed into *Escherichia coli* DH5α strain for storage and amplification. Linearized plasmids were used as templates for cRNA synthesis using the Ambion's mMessage mMachine kit (Ambion, Austin, TX). Fifty nanoliters of cRNA (concentration 1 mg/mL) were injected into stage V−VI *Xenopus* oocytes, which were prepared as described below.

2.5 Electrophysiological assay

The activation of ACh and 5-HT receptors by milk was done on membrane receptors heterologously expressed in mRNA-injected *X. laevis* oocytes. For this, *X. laevis* frogs were anesthetized with 0.17% 3-aminobenzoic acid methylester (MS-222) for 20–30 min. The follicles were manually removed, enzymatically defolliculated (2 µg/µL collagenase type I at room temperature for 2 h) and then kept at 16 °C in Barth's medium. The next day, 50 nL of cRNA of nAChRs subunits α4 and β2 (1 µg/µL) or 5-HT$_{3A}$ subunits were injected into the oocytes and electrophysiological recordings were obtained 2-4 days after injection.[24, 25] The oocytes were recorded with the Two Electrode Voltage (TEV) clamp method.[26] This method measures the electrical current generated by the flux of ions when the membrane receptors are activated by the agonist. Thus, the electrical currents elicited by agonists are a direct measurement of the activation of the ion channel-coupled receptor. To do this the oocytes were impaled with two microelectrodes filled with 3 M KCl, which allows the electrical access and control of the cell, and voltage clamped at −80 mV.[26] Oocytes were continuously perfused with gravity-driven frog Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 5 mM HEPES) adjusted at pH 7.4 at room temperature (19–21 °C). Data acquisition was performed using WinWCP V2.0.[27]

The activity of milk on ACh or 5-HT receptors was determined by measuring the amplitude of the ion currents elicited by

different concentrations of the milk samples. Standard solutions were used to corroborate the channel activation.

2.6 Statistical analysis of acetylcholine and serotonin in milk samples

Chemical ACh and 5-HT concentrations were determined for all the milk sample groups (see table 1). Each individual sample was analyzed by duplicate and the average concentration of these two independent replicates was used for the statistical analysis. First, the Grubb's test was applied to detect outliers within milk species sample groups ($P < 0.05$). Only one concentration of 5-HT within the CMK-USA group was considered an outlier and therefore was not included in further analysis. After the outlier analysis a Brown-Forsythe test ($P < 0.05$) to detect unequal variances among groups was performed. Finally mean differences were determined using ANOVA with the Tukey-Kramer Multiple Comparison test, results were considered significant at $P < 0.05$.[22, 28]

Results and discussion

3.1 Quantification and bioactivity of ACh in milks from different species

ACh was detected by LC-MS/MS in milks of all species and all groups. Table 2 shows that milks from humans had the lowest concentration of ACh (1.1 ± 0.4 μM; mean ± SEM; n = 5) and CMk-USA had the maximum concentration (8.1 ± 2 μM; n = 5); CMk-SA, BMk and GMk milks had intermediate ACh concentrations. ANOVA analysis followed by Tukey-Kramer Multiple Mean Comparison test did not show significant differences between milks from Saudi Arabian camels, bovines and humans ($P > 0.05$ all). Although ACh concentration in milks from USA camels was slightly larger than that from Saudi Arabian farms the difference was not statistically significant ($P = 0.7$). The only significant difference was between ACh content in milks from humans and USA camels ($P = 0.0034$). Thus, statistically the milk species groups can be classified at two levels with no significantly mean difference. Level A is formed by CMK-USA, CMk-SA, BMk and GMk and level B by CMk2, BMk, GMk and HMk (see table 2). The concentration of ACh in our study is comparable to values previously reported for cow and human milks (range from 0.68 – 3.41 μM). [16-19] To the extent of our knowledge this is the first time that the concentration of free ACh has been determined in milks from camels and goats.

It is unknown whether free Ach in milks are able to activate ACh receptors in the lumen of the GI tract. Even though cholinergic receptors are widely distributed in muscle and neurons of the gastrointestinal tract,[1, 2] most of these receptors are activated by ACh released from enteric neurons. The presence of cholinergic receptors in the lumen of the GI tract and thus able to be activated by foods and milks is less studied. So far the muscarinic receptors M2 and M3 and the nicotinic receptors containing the α3 subunit are found to be expressed in the lumen of the GI tract.[29-31] These receptors are involved in a series of physiological processes that are still not clearly understood; however, the absence of α3 is correlated with Megacystis-Microcolon-Intestinal Hypoperistalsis Syndrome[30] and the overexpression of M3 is thought to be an important factor in Sjögren's syndrome.[29] Because muscarinic and nicotinic receptors can be activated by nanomolar concentrations of ACh[32] the concentration of ACh reported in our study is theoretically sufficient to activate cholinergic receptors in the lumen of the GI tract; however, whether this ACh is bioactive is not known. This is of particular important in the milk where many other compounds may affect the bioactivity of ACh.

To determine if ACh in the milks studied was available to activate cholinergic receptors we first express rat nAChRs in *Xenopus* oocytes. We chose as a biosensor the α4β2 subtype because is an ionotropic receptor that combines high sensitivity to ACh, shows slow desensitization and is not endogenously expressed in *Xenopus* oocytes.[33, 34] The expression of α4β2 nicotinic receptors was confirmed by the application of ACh standard solutions from 10 to 100 nM ACh. 30 nM was the lowest concentration of ACh that our bioassay could detect and therefore that is considered the limit of detection (LOD) for the biological experiment. Interestingly, none of the milk samples (see table 2), each diluted to 10%, elicited responses in oocytes expressing α4β2 nicotinic receptors. According to LC-MS/MS experiments the lowest concentration of ACh is near 1 μM, the minimum concentration applied was 100 nM, which is a concentration high enough to elicit measurable responses in our bioassay. Therefore, the lack of responses elicited by milk samples is puzzling. Whether unidentified compounds that form part of the milk matrix may reduce ACh bioactivity[35] or antagonize α4β2 nicotinic in our bioassay needs further research.[16, 19]

3.2 Quantification and bioactivity of 5-HT in milks from different species

The concentration of 5-HT determined by LC-MS/MS in milk is shown in table 2. The mean concentration of 5-HT in milks was in the range of 27 to 122 nM. ANOVA analysis did not detect statistically significant differences among 5-HT concentration in the different milk groups ($P > 0.05$ all). Reports in the literature about 5-HT content in milks are very scarce. To our knowledge only Feldman and Lee[20] have reported that 5-HT content in milk is less than 1 μg/mL (or lower than about 5 μM). Since they did not report the source of milk used for their studies (e.g. goats or cows) we cannot make a direct comparison with our data.

Whether the amount of 5-HT in milk is able to have a physiological role in the GI tract will depend on the type of receptors present at the lumen of the GI tract. To date it has been reported several luminal 5-HT receptors. These receptors are expressed in epithelial and enterochromafin cells and include the metabotropic receptors 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 and, the ionotropic receptors 5-HT_3. Among those, 5-HT_3 receptors are involved in peristalsis and are an important pharmacological target in treating postoperative and chemotherapy-induced nausea and emesis[36-38] and in improving pain and discomfort associated with irritable bowel syndrome.[39, 40]

To determine if milks from the different species have an effect on 5-HT_3 receptors we followed the strategy used to detect the bioactivity of milks on nicotinic receptors shown above. First, we expressed human 5-HT_{3A} receptors in *X.* oocytes. We chose 5-HT_{3A} as a biosensor because it is the ionotropic receptor with more clinical relevance in the GI tract, it is present in the lumen of the intestine, exhibits high sensitivity to 5-HT and slow desensitization and, is not endogenously expressed in *X*

oocytes.[24, 41-44] The expression of 5-HT_{3A} receptors on the surface of the oocyte was confirmed by the application of 5-HT at concentrations of 10 nM, 30 nM, 60 nM and 100 nM. The detection limit was 30 nM as 10 nM was not sufficient to induce responses in any of the oocytes.

Because the concentration of 5-HT in whole milk is near or below the limit of detection of our bioassay (see table 2) we tested the highest dilution of milk that can be used without unspecific effects and instability in the electrophysiological recording; that is 10% milk in Ringer's solution.[14] Most the amount of 5-HT present in 10% milk dilutions was below the limit of detection of our bioassay and thus expected not to elicit responses. Interestingly, 4 out of 5 samples (4/5) in the GMk group, 3/4 in CMk-USA, 2/5 in BMK and 1/5 in HMK elicited activation of 5-HT_{3A} receptors. Because the activation of 5-HT_{3A} receptors is concentration dependent we used a dose-response analysis to evaluate the equivalent concentration of milk bioactivity on 5-HT_{3A} receptors [5-HT_{fc}], which is defined as the concentration of 5-HT that elicits a response of the same amplitude than the response elicited by milk.[14] Table 2 shows the [5-HT_{fc}] for the different groups. For statistical purposes a concentration value of LOD/√2 was given to non-responsive samples.[45]

We also evaluated whether the amplitude of 5-HT_{3A} responses, or lack thereof, elicited by milks was correlated with the concentration of free 5-HT measured by LC-MS/MS. Figure 1 shows that the serotonergic activity of milks was not correlated with the mean average of the concentration of free 5-HT per group. Moreover, the responses elicited by milk were higher than the concentrations determined chemically. These results suggest that activation of 5-HT_{3A} is not exclusively due to free 5-HT. Additionally, even though we used milk from the same batch for LC-MS/MS and the electrophysiological determination of serotonergic activity, we cannot discard that heterogeneity between aliquots may alter the quantification of free 5-HT producing lower concentrations in LC-MS/MS determinations. The higher equivalent concentration of 5-HT observed in the electrophysiological experiments compared to the concentrations found by the chemical analysis, call attention to the possible presence of molecules that could enhance the response of 5-HT in a synergic mechanism. Alternatively, the presence of bounded 5-HT to proteins or other milk components may also produce a functional response. Recently, Nongonierma et al., 2013[15] demonstrated that milk protein hydrolysates have serotonergic activity on 5-HT_{2C} receptors. It may be possible that these substances may also activate 5-HT_{3A} receptors if some of these peptides are found in native milk conditions. Further studies analysing the bioactivity of compounds in milk are encouraged.

Conclusions

The presence of ACh and 5-HT in milk was confirmed by LC-MS/MS. Even though there are some differences for the concentrations of ACh and 5-HT in milk among species, they do not seem to be significant from a biological point of view. Electrophysiological data suggest that 5-HT in milk could participate in activation of 5-HT receptors; meanwhile, ACh seems not to have a relevant role in nicotinic receptors activation. We have demonstrated that milk contains significant quantities of 5-HT and is able to activate the 5-HT_{3A} receptor. This effect may contribute to the beneficial effect of milk on enteric reflexes and peristaltic movements in the gut. This does not discard that other compounds with activity over 5-HT_{3A} receptors may also participate in activation of the receptors. The presence in native milk of other bioactives implied in agonist or antagonist effects in 5-HT or ACh receptors needs to be further studied.

Notes and references

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Table 1. Description of the origin of the milk samples evaluated.

Species	Breed	Company	Location
Camel	Dromedary camel	Camel milk association (CMk-USA) KACST (CMk-SA)	Marion, MI Riyadh, Saudi Arabia
Goat	America Saanen	Camel milk association	Marion, MI
Cow	Holstein Jersey/Holstein Brown Swiss, Montebeliarde.	Organic Pastures Dairy Company, LLC	Los Angeles, CA
Human		Human milk from Mother's Milk Bank	San Jose, CA

Human milk was obtained from 1 Hispanic, 1 Asian and 4 Caucasian 29-38 year old donors; with 1-3 lactation periods. Bovine milk was from cows with 1-6 lactation periods. Goats used for milking were 2 years olds and all except one was their first lactation, for one goat was its 4th lactation period. We do not have information of the breed and age of the camels from Saudi Arabian Farms. Camels from USA had 9-16 years old and milk was from its 3rd to 8th lactation. All milk samples were frozen immediately in glass containers and shipped in dry ice.

Table 2. ACh and 5-HT determination by LC-MS/MS and bioctivity.

Species	Origin	N	[ACh] (µM)	Range [ACh] (µM)	CLR* for ACh	ACh Bioactivity	N	[5-HT] (nM)	Range [5-HT] (nM)	[5-HT$_{fc}$] Bioactivity (nM)
CMK	USA	5	8.1 ± 2.3	4.2-16.5	A	UD	4	122 ± 39	34-224	299 ± 139
CMK	SA	4	5.9 ± 0.8	4.1-7.4	AB	NT	4	114 ± 98	11-409	NT
BMK	USA	5	3.7 ± 0.5	3.0-5.4	AB	UD	5	27 ± 6	8-41	99 ± 61
GMK	USA	5	3.5 ± 0.4	2.7-4.7	AB	UD	5	62 ± 14	32-113	501 ± 126
HMK	USA	5	1.1 ± 0.4	0.2-2.2	B	UD	5	27 ± 7	9-45	138 ± 138

UD, undetectable; NT, not tested. Data represents mean ± SEM. *CLR; connecting letter report: means that are not sharing a letter are significantly different. Concentrations were determined by LC-MS/MS using the addition standard method. Excluding outliers, the average of two replicates of each individual samples were used for statistical analysis. In the case of 5-HT, one value was considered anomalous in CMk-USA. The [5-HT$_{fc}$] denotes the concentration of 5-HT based on electrophysiological responses. Neither 5-HT nor [5-HT$_{fc}$] values were statistically different between milks.