The bovine mammary gland expresses multiple functional isoforms of serotonin receptors

Laura L Hernandez1,2, Sean W Limesand1, Jayne L Collier1, Nelson D Horseman2 and Robert J Collier1

1Department of Animal Sciences, University of Arizona, Tucson, Arizona 85721, USA
2Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, Ohio 45267, USA

(Correspondence should be addressed to R J Collier who is now at William Parker Agricultural Research Center, University of Arizona, 1650 Limberlost #2019, Tucson, Arizona 85719, USA; Email: rcollier@ag.arizona.edu)

Abstract

Recent studies in dairy cows have demonstrated that serotonergic ligands affect milk yield and composition. Correspondingly, serotonin (5-HT) has been demonstrated to be an important local regulator of lactational homeostasis and involution in mouse and human mammary cells. We determined the mRNA expression of bovine 5-HT receptor (HTR) subtypes in bovine mammary tissue (BMT) and used pharmacological agents to evaluate functional activities of 5-HT receptors. The mRNAs for five receptor isoforms (HTR1B, 2A, 2B, 4, and 7) were identified by conventional real-time (RT)-PCR, RT quantitative PCR, and in situ hybridization in BMT. In addition to luminal mammary epithelial cell expression, HTR4 was expressed in myoepithelium, and HTR1B, 2A, and 2B were expressed in small mammary blood vessels. Serotonin suppressed milk protein mRNA expression (α-lactalbumin and β-casein mRNA) in lactogen-treated primary bovine mammary epithelial cell (BMEC) cultures. To probe the functional activities of individual receptors, caspase-3 activity and expression of α-lactalbumin and β-casein were measured. Both SB22489 (1B antagonist) and ritanserin (2A antagonist) increased caspase-3 activity. Expression of α-lactalbumin and β-casein mRNA levels in BMEC were stimulated by low concentrations of SB22489, ritanserin, or pimozone. These results demonstrate that there are multiple 5-HT receptor isoforms in the bovine mammary gland, and point to profound differences between serotonergic systems of the bovine mammary gland and the human and mouse mammary glands. Whereas human and mouse mammary epithelial cells express predominately the protein for the 5-HT2 receptor, cow mammary epithelium expresses multiple receptors that have overlapping, but not identical, functional activities.

Journal of Endocrinology (2009) 203, 123–131

Introduction

Milk secretion is regulated by both systemic and local feedback mechanisms. Suckling-induced prolactin (PRL) surges comprise the major positive feedback that drives elevated milk secretion in response to increased nursing demand in most species (Wilde et al. 1995). Local negative feedback processes are initiated by poorly understood mechanisms that monitor the state of filling within each gland. These mechanisms regulate alveolar distension, milk synthesis and secretion, and epithelial cell mass (Peaker 1995). Acting in concert, systemic and local hormonal factors adjust mammary gland physiology not only to meet the demands of the offspring, but also to compensate for circumstances such as local mastitis, nutrient supply, and metabolic demand.

Even in the face of continued endocrine stimulation, mammary glands that are unsuckled will halt milk synthesis and undergo partial involution, resulting in loss of epithelial cell mass (Peaker 1995). In many species, including rodents and humans, involution is characterized by massive apoptosis.

In other species, including dairy cattle, the glands become quiescent during involution, but do not undergo apoptosis and remodeling on a large scale (Capuco & Akers 1999). Consequences of milk stasis have been known for many years, but mechanisms responsible for stasis-induced involution have remained obscure. One line of research that explored this issue proposed the presence of a ‘feedback inhibitor of lactation’ compound in milk whose identity and physiological activity have not been confirmed. This factor was proposed to decrease milk yield, milk protein synthesis, and milk protein mRNA expression, both in vivo and in vitro and in a variety of species (Wilde et al. 1988).

Serotonin (5-HT) has been proposed to be an autocrine/paracrine regulator of lactation in the mouse, human, and more recently in the bovine, and the enzymatic machinery necessary for 5-HT biosynthesis has been detected in the mammary epithelium (Matsuda et al. 2004, Stull et al. 2007, Hernandez 2008, Hernandez et al. 2008, Pai & Horseman 2008). Expression of tryptophan hydroxylase 1 (TPH1) in mammary epithelial cells, the rate-limiting enzyme in 5-HT
biosynthesis, was induced by PRL in mouse mammosphere cultures, and by milk stasis in nursing dams (Matsuda et al. 2004). Serotonin content was measured in the mammary gland of virgin, lactating, and 5-hydroxytryptophan-treated mice. Serotonin content of the mammary gland was increased in lactating mice compared with virgin and, when treated with 5-hydroxytryptophan, 5-HT levels in the mammary gland were further increased (Stull et al. 2007). In cultures of primary mouse mammary epithelial cells, expression of β-casein mRNA was attenuated by increasing concentrations of 5-HT. Furthermore, when methysergide (METH), a non-selective 5-HT antagonist, was added to cultures, expression of β-casein mRNA was increased (Matsuda et al. 2004). Non-transformed human mammary epithelial cells (MCF10A) were demonstrated to express mRNA for the human 5-HT7 receptor (HTR7) on basolateral membranes, and 5-HT reuptake transporter (SERT) on the apical membrane (Stull et al. 2007). Additionally, it was determined that 5-HT was involved in the regulation of tight junction (TJ) status in the MCF10A cells. Addition of METH or metergoline, both broad-spectrum 5-HT receptor antagonists, resulted in increases in transepithelial resistance (Stull et al. 2007). The TJ scaffolding proteins, ZO-1 and ZO-2, were also decreased in 5-HT-treated MCF10A cells.

Studies in three-dimensional collagen cultures of bovine mammary epithelial cell (BMEC) have shown TPH1 to be expressed in a PRL-dependent manner (Stiening 2005, Hernandez et al. 2008). Furthermore, BMEC treated with 5-HT had decreased milk protein gene expression, and treatment with METH or parachlorophenylalanine (TPH1 enzyme inhibitor) increased milk protein gene expression (Hernandez et al. 2008). To date, no information is present on the identity of 5-HT receptors in bovine mammary tissue (BMT). Therefore, the objectives of these studies were to determine 1) which 5-HT receptor(s) are present in the bovine mammary gland and BMEC, 2) which selective antagonists for 5-HT receptors affect β-casein and α-lactalbumin mRNA expression in BMEC, and 3) determine whether 5-HT and selective 5-HT receptor antagonists affect caspase-3 activity in cultures of BMEC. The results of these studies point to profound functional activities of 5-HT in the bovine mammary epithelium and substantial complexity in the bovine 5-HT system, which has not yet been reported in the human or mouse 5-HT systems.

Materials and Methods

Expression of HTR subtypes in bovine brain, mammary tissue, and mammary epithelial cells

The characterization of expression for serotonergic components was initially performed on a pool of isolated bovine mammary cells representing four multiparous, non-lactating, pregnant cows. The expression patterns for 5-HT receptors in the pooled sample were confirmed in assays of four pBMEC isolates from individual cows. Additionally, expression patterns for 5-HT receptors were determined on a bovine mammary epithelial cell line (BME–UV). The morphological distribution of receptors was documented using bovine mammary and hypothalamic tissues from an individual lactating cow.

Mammary epithelial cells were collected from four multiparous, non-lactating (~30 days dry), pregnant Holstein cows. Holstein hypothalamic (BB) and BMT were collected at slaughter from a multiparous lactating cow in the first trimester of pregnancy, and fixed at 4 °C in 4% paraformaldehyde (PFA) for 14–24 h. The tissues were submerged in 30% sucrose at 4 °C overnight, then submerged in a 1:1 v:v 30% sucrose:OCT (Tissue Tek, Sakura Finetek, Torrance, CA, USA) mixture for 24 h at 4 °C. Tissues were embedded with OCT (Tissue Tek) and frozen at −80 °C. Eight micron tissue sections were then cut with a cryostat (Microm HM 520).

Other portions of BB and BMT were used for RNA isolation and snap frozen in liquid nitrogen. Tissue was stored at −80 °C until RNA extraction.

Total RNA was isolated from BB, BMT, BMEC, and BME–UV samples using TRIzol reagent (Invitrogen) in triplicate. For primary epithelial cell culture experiments, two wells of a 24-well culture plate served as one sample and there were four samples for each treatment. In a given experiment, each treatment was represented in each culture plate, resulting in four culture plates per study. Samples were stored at −80 °C until extraction. Extraction was conducted as described previously (Hernandez et al. 2008).

Quantitative real-time (RT)–PCR analysis was conducted using the iCycler IQ RT-PCR Detection System (Bio–Rad). Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was utilized as the internal control gene following standard curve analysis across all treatment group samples. Several other housekeeping genes were evaluated (ribosomal protein S18, glyceraldehyde 3 phosphate, and β-actin) and HPRT1 did not alter its expression based on treatment. Resulting gene expression data were calculated and analyzed based on the 2−ΔΔCt method (Livak & Schmittgen 2001). Amplification efficiencies of primers were evaluated prior to conducting experiment to determine equality of internal control compared with primers. All primers utilized met criteria for analysis by the 2−ΔΔCt method, with efficiencies between 95 and 105% (Livak & Schmittgen 2001). Amplicons were sequenced from RT-PCR products prior to the use of primers for quantitative PCR (qPCR). Primer sequences are shown in Table 1.

BMT was counterstained after in situ hybridization for smooth muscle α-actin (SMA) to identify myoepithelial cells and for Griffonia simplicifolia lectin I isoelectin B4 (GS-I) to identify vasculature in BMT. Primary biotinylated antibody was obtained (#B–1205; Vector Laboratories, Burlingame, CA, USA) for biotinylated GS-I. Primary antibody for SMA (#MS–113–P0; Thermo Scientific, Fremont, CA, USA) was visualized with a biotinylated goat anti-rabbit secondary antibody (#170–6401; Bio–Rad Laboratories) for immunohistochemical detection in BB and BMT following in situ

Journal of Endocrinology (2009) 203, 123–131 www.endocrinology-journals.org
Table 1 Primers utilized for RT-PCR and qPCR analyses. All 5-HT receptors were run at an annealing temperature of 64 °C. Primers for 5-HT receptors 1A–4 were obtained from Reist et al. (2003) and specific to the bovine genome. The 5-HT7 was designed using primer 3 (Rozen & Skalesky 2000) and the sequence was obtained from GenBank (NIH, Bethesda, MD, USA), accession number XM_580794. HPRT1 was used as the housekeeping gene and was also run at an annealing temperature of 64 °C. β-Casein was run at a 64 °C annealing temperature and α-lactalbumin was run at a 62 °C annealing temperature. 

| Primer | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|--------|----------------------------|--------------------------|
| HTR1A  | TCAGCTACAAAGTATGACCATCTCT  | GCCACTTGGTACGACCTG       |
| HTR1B  | TGGCTCTCAAGGCCGCTCTATG     | CTTAGGGCCATGATGGTTCCTCT  |
| HTR1D  | CTCCTCAAGATCTCCCTGATG      | CAGAACATGACGAGAGAGAAGT   |
| HTR2A  | AAGCTGAGAATGCGAACACTATF    | TGGGATGAGAGAGCTGGATTATGG |
| HTR2B  | ACAAAGTCCACAGTCACCCT     | GTCCGAATGGTCTTATGAGAG    |
| HTR2C  | TTCTTAATGGCCCTAGCCAT      | GCAATCTCTGATGGCTTATG     |
| HTR4   | ATGGGATGAACTGTCAAGTGA      | TCACCAAGCGGCAACCAGCA     |
| HTR5a  | ACAACGGGGGCACTAGGG         | TTGGGATCAGTGAATCATAGGG   |
| HTR7   | GCTTATATCCCCATGTCGCTCA   | TTTGCACTCCTCCTCCTCCT    |
| β-Casein | GCTATGGCTCCTAAGCACAAGA   | GGAAATCGACAGTGGGAAGAG    |
| α-Lactalbumin | CTTGCTCTCCTGTGACATC  | ACAGACCCATTCAGGCAACC      |
| HPRT1  | GAGAACTCGGAGTGGTGGGAGG     | GGCTCTGATGGCAAATGAAGAT    |

hybridization for 5-HT receptors. The Vectastain Elite ABC Kit (Vector Laboratories) was used for staining following the manufacturer’s instructions, and the following primary antibody dilutions were utilized: 3 μg/ml GS-I; 1:800 SMA. Proteins were visualized using DAB peroxidase substrate per manufacturer’s instructions (Vector Laboratories).

In situ hybridization for HTR in BMT

HTR1B, 2A, 2B, 4, and 7 cDNA clones were generated by PCR amplification from total RNA extracted from BB. Primers for specific genes of interest were designed against bovine sequences for HTR1B, 2A, 2B, and 4 receptors as reported in National Center for Biotechnology Information (NCBI; Bethesda, MA, USA). The HTR7 receptor was designed using human sequences for the receptor and compared with bovine genome (NCBI). The HTR7 receptor primer was generated from primer 3 (Rozen & Skalesky 2000). The following primer sequences were used for probe generation: HTR1B were 5’-ATGGGAGAAGACCCACAC-3’ and GTGATTGCCACTGTGTACCG-3’; HTR2A were 5’-TTCTCCCTGACTGCTCCTAAGAAGCTCCAGCACACAC-3’ and GCCATCTTCCCCATGCTTCTCTCTC-3’; HTR2B were 5’-CTCTGAGATGTGCTGCTCACA and GTAGCGATGAGCTGATGTGCT-3’; and HTR2C were 5’-CTCTGAGATGTGCTGCTCACA and GTAGCGATGAGCTGATGTGCT-3’. Amplified DNA products for 5-HT receptors were inserted into the TOPO TA cloning expression vector pCRII (Invitrogen) and chemically transformed into MACH1-T1 Escherichia coli (Limesand et al. 2005). Plasmids positive for PCR inserts were transformed and plasmid DNA was isolated (Qiagen) and sequenced. Chromas and BLAST (NIH) were utilized to analyze DNA sequences generated from PCR and confirm sequence identity.

RNA probes were synthesized using plasmid DNA that was linearized using endonuclease restriction enzymes and extracted. Digoxigenin (DIG)-labeled RNA probes (Roche Diagnostics) were generated with SP6 or T7 RNA polymerase (Promega), precipitated with 70% ETOH, and resuspended. Probe absorbance was measured with a NanoDrop Spectrophotometer ND-1000 to determine concentration and integrity.

In situ hybridization was conducted on frozen sections fixed in 4% PFA for 10 min, washed in 1×PBS, digested with 10 μg/ml proteinase K for 10 min, and refixed in 4% PFA for 5 min. Tissues were washed in PBS and then acetylated (102.2 mM triethanolamine, 0.01 mM 6 M HCl, and 26.9 mM acetic anhydride) for 10 min at room temperature, washed in PBS, and then blocked with 55 °C pre-hybridization buffer (50% formamide, 5X sodium chloride/sodium citrate (SSC), pH 4.5, 50 μg/ml yeast tRNA, 1X SDS, and 50 μg/ml heparin) for 2 h at 55 °C in a humidified chamber. The DIG-labeled RNA for sense (negative control) and anti-sense strands for each receptor was then separately added to hybridization buffer (HTR1B: anti-sense 66-80 ng/μl, sense 92-01 ng/μl; HTR2A: anti-sense 19.5 ng/μl, sense 103-1 ng/μl; HTR2B: anti-sense 84-59 ng/μl, sense 150-40 ng/μl; HTR4: anti-sense 56-15 ng/μl, sense 78-40 ng/μl; HTR7: anti-sense 68-30 ng/μl, sense 63-09 ng/μl), heat denatured at 80 °C for 5 min, cooled, then hybridization buffer with DIG-RNA for sense and anti-sense strands for each receptor was added to separate slides on tissue sections and incubated overnight at 70 °C in a humidified chamber. Sections were washed in 70 °C 5×SSC, pH 7.0, for 30 min at room temperature, incubated in 0-2×SSC, pH 7.0, for 3 h at 75 °C, then washed at room temperature in 0-2×SSC for 5 min. Tissues were subsequently washed in 1X malic acid buffer (MAB), pH 8.0, then incubated in blocking buffer consisting of 2% blocking reagent in MAB (Roche Diagnostics), 10% heat inactivated FBS (Gibco), 0.1% Tween-20 for 1 h at room temperature. Anti-DIG-AP Fab
Fragments antibody (Roche Diagnostics) was diluted 1:100 in blocking buffer and incubated at 4°C overnight in the dark. Following incubation, BB and BMT sections were washed in MAB with 0.1% Tween-20, then in DI water with 0.1% Tween-20 and then developed with BM Purple AP Substrate (Roche Diagnostics) containing 0.1% Tween-20 for 3–36 h until maximum intensity was achieved.

**Effects of serotonergic drugs on primary BMECs in collagen gel cultures**

Tissue dissociation, BMEC isolation, and preparation of type 1 collagen were performed according to (McGrath 1987, Hernandez et al. 2008, Stiening et al. 2008). Epithelial cells in the form of organoids clumps composed of ductal and alveolar cells were isolated from four multiparous, non-lactating (~30 days dry), pregnant Holstein cows. Organoids were thawed, resuspended in DMEM/F-12, mixed with neutralized collagen at 4–6 x 10^5 organoids/ml of collagen mixture, and cultured in 24-well plates as described previously (Hernandez et al. 2008, Stiening et al. 2008). The average DNA content of one vial of BMEC is 486±7±84±91 µg/ml (mean±s.d.). This provided sufficient cells for 72 wells or ~6.8 µg DNA/well.

Mammary cell organoids were allowed to grow for 8 days in serum-free media (100 ng/ml insulin-like growth factor-I [IGF1] and 25 ng/ml epidermal growth factor [EGF]), and then treated with a lactogenic hormone complex (100 ng/ml IGF1, 100 ng/ml PRL, and 10 ng/ml hydrocortisone, without EGF) plus gel release from the plastic by rinsing of the gels from the plastic, simultaneously, for 48 h in combination with and without 5-HT receptor antagonists, as previously described (Hernandez et al. 2008). The lactogenic hormone complex in serum-free media served as the control, and 5-HT antagonists in combination with the lactogenic complex served as treatments, with the entire experiment being replicated an additional time. At termination of lactogenic treatments, gels were dissolved in 1 ml TRIzol for 5 min (Invitrogen) and stored at −80°C until RNA extraction. Two gels were utilized per replicate and four replicates per treatment were completed. For caspase activity assays, collagen gels were digested with 0.1% collagenase I (collagenase type 1, Worthington Biochemical Corp., Lakewood, NJ, USA) in Hank's balanced salt solution with 4% BSA for 20 min at 37°C (Rocha et al. 1985). This mixture was centrifuged for 4 min at 100 g and supernatant removed. Cells were washed with M199, centrifuged for 4 min at 100 g, and supernatant removed. Cell pellets were stored at −80°C until caspase-3 activity assay was conducted. Two wells of a 24-well plate served as an N=1, and an N=4 was completed (eight total gels) for each treatment group for the two experimental replicates.

The effects of 5-HT (Sigma; 200 µM) and selective 5-HT receptor antagonists/reverse agonists for the 1B (SB224289; Sigma), 2A (ritanserin; Sigma), 2B (SB204741; Sigma), four (SB204070; Sigma), and seven (pimozide; Sigma) receptors were investigated during the lactogenic period of culture (48 h) in combination with lactogenic hormones. A 200 µM concentration of 5-HT was utilized as this was determined to be an effective concentration in other mammary epithelial cell models (Matsuda et al. 2004, Hernandez et al. 2008, Pai & Horsemman 2008). Concentrations of 5-HT receptor antagonists ranged from 0.5 to 10 µM, except for SB204070, which ranged from 0.0001 to 1.0 µM. Cultures treated with lactogenic media only, plus gel release served as the control. Effects of specific 5-HT receptor antagonists/reverse agonists on expression of mRNA for milk protein genes α-lactalbumin and β-casein during lactogenesis were also investigated.

For caspase-3 activity measurements, the following concentrations of 5-HT and 5-HT receptor antagonists were utilized during lactogenic treatment: 200 µM 5-HT (Sigma), 0.1 µM pimozide, 1.0 µM ritanserin, 0.0001 µM SB204070, 0.1 µM SB204741, and 1.0 µM SB224289. Cultures treated with lactogenic media only, plus gel release served as the control. At termination of lactogenic treatments, gels were harvested and treated for protein isolation for caspase-3 activity and stored at −80°C.

Activation of caspase-3 was quantified using BioMol QuantiZyme Colormetric Assay kit (BioMol, Plymouth Meeting, PA, USA), measuring the rate of Ac-DEVD-pNA cleavage. Caspase-3 is a committed step for caspase activity in cells that undergo apoptosis. Methods utilized were based upon (Limesand et al. 2003).

**Statistical analysis and 5-HT nomenclature**

Statistical analysis was conducted using a one-way ANOVA on qPCR data using gene expression relative to the control (lactogenic media + gel release) in a respective sample, with the PROC MIXED procedure of SAS (SAS, 9.3, SAS Institute, Cary, NC, USA). Graphical representation of data is represented by expression of treatments relative to the control (2^ΔΔCt). The ΔΔCt was calculated as ΔCt of a respective treatment minus ΔCt of the control. A one-way ANOVA was conducted on results from caspase-3 activity assay, with the PROC MIXED procedure of SAS (SAS, 9.3, SAS Institute).

Nomenclature utilized for the mRNA 5-HT receptors (HTR humans/HTR non-human species) was derived from the NCBI. Nomenclature utilized for the protein form of the 5-HT receptors (5-HTR) was derived from the International Union of Basic and Clinical Pharmacology (www.iuphar.org, IUPHAR, Kansas City, KS, USA).

**Results**

**Expression of 5-HT receptors in the bovine mammary gland**

Serotonin exerts its cellular actions through multiple receptor subtypes belonging to seven different families. Determination of 5-HT receptor subtypes present in BMEC and BMT was a necessary first step for establishing the basis of 5-HT actions in
BMT development and lactation after having determined that BMEC expresses TPH-1, the rate-limiting enzyme for 5-HT biosynthesis and the serotonin re-uptake transporter, necessary for 5-HT recycling into the cell (Hernandez 2008, Hernandez et al. 2008). To validate detection of receptor subtypes by RT-PCR, BB served as a control because all 5-HT receptors are present in brain tissue (Kroeze et al. 2002). Receptor types that were validated by amplifying BB cDNA included HTR1A, 1B, 1D, 1F, 2A, 2B, 2C, 4, 5A, and 7 (Fig. 1 and data not shown). These primers were used for detecting mRNA expression in BMT. Other receptors that were not included in these assays (e.g. types 3, 6, and a variety of subtypes) may be expressed, but further validation studies would be necessary to develop adequate assays.

Expression of genes encoding the proteins for 5-HT1B, 2A, 2B, 4, 7 receptors was detected in BMT and BMEC (Fig. 1). The mRNAs for HTR1A, 1D, 1F, 2C, and 5A receptors were not detected in BMT or BMEC (data not shown). Additionally, expression of genes encoding the proteins for 5-HT1B, 2A, 2B, 4, 7 receptors was detected in a BME–UV (data not shown). Additionally, mRNA for HTR4 was detected in BME–UV cells (data not shown).

Relative expression levels for each receptor of the five subtypes that were expressed in BMT were determined using qPCR. Levels of each receptor mRNA were quantified as the ratio of BMT expression to the level in BB extracts. The expression ratio of receptor mRNA relative to BB for each type in BMT, highest to lowest, was as follows: HTR4, 389; HTR2B, 89; HTR1B, 19·35; HTR7, 18; and HTR2A, 3·8. In BMEC, the expression ratio relative to BB (highest to lowest) was: HTR4, 230; HTR2B, 44; HTR7, 37; HTR1B, 30; and HTR2A, 1·6. The rank order of expression ratios was similar for most of the receptors in both BMT and BMEC, with the exception of HTR7, which was enriched in BMEC. The relative enrichment of 5-HT7 in epithelial cells is consistent with expression of this receptor in the mammary epithelium in mice and humans (Stull et al. 2007, Pai & Horserman 2008).

Having determined that genes encoding proteins of several 5-HT receptor subtypes were expressed in BMT, it was necessary to determine what cell types expressed each. Therefore, in situ hybridization assays for HTR1B, 2A, 2B, 4 and 7 were conducted. BB was used as a positive control and sense probes for receptors were used as negative controls (Fig. 2). Sections for each receptor probe were counterstained with GS-I or SMA to identify vascular endothelial and myoepithelial cells respectively (Fig. 2). Expression of HTR1B, 2A, 2B, 4, and 7 was each localized in BMT (Fig. 2, row A, columns 1–5). Counterstaining with GS-I (vascular endothelium) indicated that HTR1B, 2A, and 2B were present in the endothelium in addition to epithelium (Fig. 2). Myoepithelium expressed HTR4 (Fig. 2).

Agonist effects on milk protein mRNA expression and apoptosis
Serotonin has been demonstrated to decrease milk protein mRNA expression in primary mouse mammary epithelial cells and to induce apoptosis human and mouse mammary epithelial cells (Matsuda et al. 2004, Stull et al. 2007). Therefore, it was pertinent to determine whether 5-HT treatment of BMEC would also result in decreased milk protein mRNA expression and increased apoptosis.

Treatment of lactogenic collagen gel cultures of BMEC (lactogenic hormones, plus gel release) with exogenous 5-HT depressed β-casein mRNA expression by >90% compared with BMEC treated with lactogenic conditions only (P<0·0001; data not shown). Similarly, 5-HT suppressed α-lactalbumin mRNA by >80% in comparison with lactogenic control (P<0·0001; data not shown).

When lactogenic cultures of BMEC were treated with exogenous 5-HT, there was no significant increase in apoptosis as measured by caspase-3 activity (P=0·10).

Effects of 5-HT receptor antagonists on milk protein mRNA expression and apoptosis in primary BMECs
The effects of selective 5-HT antagonists/inverse agonists were tested in collagen gel cultures of BMEC under lactogenic conditions (lactogenic hormone stimulation plus gel release). For each receptor, an antagonist was chosen based on its relative selectivity for the target receptor compared with other receptors that were identified in BMEC. Antagonists utilized were as selective for a specific 5-HT receptor of interest as possible. Concentration–response curves for each antagonist were performed using a range of concentrations previously documented to encompass established IC_{50} for the target receptor. The antagonists and their respective receptors included SB224289,

---

Figure 1 5-HT receptor expression in bovine hypothalamic tissue, lactating mammary tissue, and primary bovine mammary epithelial cells. The mRNA expression of the HTR was detected by RT-PCR and electrophoresed through a 2% agarose gel and stained with EtBr (primers listed in Table 1). Lane (A) Holstein hypothalamic tissue, Lane (B) lactating Holstein mammary gland tissue from a first trimester pregnant animal, and Lane (C) primary bovine mammary epithelial cells isolated from three multiparous, non-pregnant (~30 days dry), Holstein cows treated for 7 days with serum-free proliferation media (IGF-I, EGF and INS).
(5-HT₁B), SB204070 (5-HT₄), ritanserin (5-HT₂A), SB204741 (5-HT₂B), and pimozide (5-HT₇).

In the case of 5-HT₁B, SB224289, at 0.5, 1.0, and 2.0 μM, up-regulated α-lactalbumin mRNA expression 10, 11, and 22-fold respectively (P!0.05; Fig. 3A) relative to lactogenic control. Exogenous 1.0 and 2.0 μM ritanserin (5-HT₂A receptor antagonist) increased α-lactalbumin mRNA levels relative to lactogenic control 17 and 11-fold respectively (P!0.001 respectively; Fig. 3B). Pimozide (5-HT₇ receptor antagonist) at 0.2 μM increased α-lactalbumin mRNA 16-fold compared with lactogenic control (P!0.001; Fig. 3C). We failed to detect changes in α-lactalbumin mRNA expression when BMEC was treated with exogenous antagonists to 5-HT₂B and 5-HT₄ receptor subtypes (data not shown).

β-Casein mRNA expression was up-regulated relative to the lactogenic control when treated with 1.0 and 2.0 μM SB224289 (P<0.05; Fig. 4A). Additionally, antagonism of the 5-HT₂A,7 receptors with exogenous ritanserin (2.0 μM) and pimozide (0.1 and 0.2 μM) significantly increased β-casein mRNA expression (Fig. 4B and C). We were unable to detect significant effects on β-casein mRNA expression when BMEC was treated with antagonists to 5-HT₂B,4 receptor subtypes (data not shown).

Treatment of BMEC with 1.0 μM 5-HT₁B and 5-HT₂A receptor antagonists increased caspase-3 activity (P<0.05 and P<0.001 respectively; Fig. 5). All other 5-HT receptor antagonists did not affect caspase-3 activity, a marker of apoptosis (P>0.05; Fig. 5).

**Discussion**

Recently, the 5-HT₇ receptor has been discovered in human and mouse mammary epithelium (Stull et al. 2007). Furthermore, it was demonstrated that the mammary gland epithelial cells contain the enzymatic machinery to produce...
5-HT (TPH1, aromatic amino acid decarboxylase, and SERT), and that more 5-HT is detectable in the mammary gland during lactation (Matsuda et al. 2004, Stull et al. 2007, Hernandez 2008, Hernandez et al. 2008). Additionally, it was demonstrated that 5-HT disrupts mammary epithelial TJ, and this occurs through action of 5-HT7 (Pai & Horseman 2008). In cattle, 5-HT has been demonstrated to reduce milk production in late lactation and to negatively impact milk protein gene expression in BMEC cultures (Hernandez et al. 2008).

We tested BB and BMT isolated from a single pregnant, lactating Holstein cow, and BMEC isolated from four non-lactating, pregnant Holstein cows, cultured in collagen gels, as well as a BME-UV, for mRNA for the HTR1A, 1B, 1D, 1F, 2A, 2B, 2C, 4, 5A, and 7. We detected mRNA of all 5-HT receptors in BB. mRNA for the HTR1B, 2A, 2B, 4, and 7 was detected in both BMT and BMEC by RT-PCR (Fig. 1). All five receptors were also localized to the mammary epithelium by in situ hybridization (Fig. 2), suggesting a very complex local regulation of the bovine mammary gland by 5-HT. We have previously demonstrated that in vivo intramammary infusions of 5-HT resulted in a 10% decrease in milk yield in late-lactation Holstein cows, and METH resulted in a 10% increase in milk yield (Hernandez et al. 2008).

Herein, we report that 5-HT suppressed transcription of milk protein genes (P<0.0001). Serotonin has also been reported to increase apoptosis in mouse mammary explant cultures (Matsuda et al. 2004). In three-dimensional lactogenic collagen cultures of BMEC treated with 200 μM 5-HT, we saw no significant increase in caspase-3 activity relative to lactogenic controls (P=0.10). Although contrary to the extensive apoptosis seen in mice upon treatment with 5-HT, there may be a solid biological basis for this difference. Dairy cows do not undergo extensive remodeling of BMT to a virgin-like state during their involution/dry period (Akers 2002). No evidence of a net loss of mammary epithelial cells is apparent in lactating versus non-lactating cows. Furthermore, dairy cows undergo more extensive cell turnover during lactation than mice or humans (Capuco et al. 1997, 2006).

---

**Figure 4** Effects of selective 5-HT receptor antagonists on β-casein mRNA expression in primary bovine mammary epithelial cells. Mean with s.e.m. of (A) β-casein mRNA expression in lactogenic BMEC cultures treated with SB224289, a 5-HT-1B antagonist; (B) β-casein mRNA expression in lactogenic BMEC cultures treated with ritanserin, a 5-HT-2A receptor antagonist; and (C) β-casein mRNA expression in lactogenic BMEC cultures treated with pimozide, a 5-HT-7 receptor antagonist. Columns with *Indicate statistical significance of *P<0.05, **P<0.001, ***P<0.0001 compared with lactogenic control.

**Figure 5** Caspase-3 activity in primary bovine mammary epithelial cells treated with 5-HT receptor antagonists. Mean with s.e.m. of caspase-3 activity in lactogenic cultures of BMEC treated with 1.0 μM SB224289, 1.0 μM ritanserin, 0.1 μM SB204741, 0.0001 μM SB204070, and 0.1 μM pimozide. Columns with * indicate statistical significance of *P<0.05; ***P<0.0001.
Therefore, the lack of extensive apoptosis in BMEC treated with 5-HT is not entirely surprising.

We determined that HTR1B mRNA was enriched in BMEC and in BMT, suggesting that it is predominately found in BMEC, although it appears to be also expressed in the vasculature of the mammary gland. We demonstrated that SB224289 (1B antagonist) up-regulated α-lactalbumin and β-casein mRNA expression in BMEC exposed to a lactogenic treatment (Figs 3A and 4A). Increasing cyclic AMP enhances secretion in mammary epithelial cells, and potentially 5-HT1B could be blocking this pathway because it is negatively coupled to cAMP production (Boisgard et al. 2001). In these studies, we demonstrated that treatment of BMEC with SB224289 increased apoptosis compared with lactogenic controls (Fig. 5). In the bovine, a considerable amount of cell renewal occurs, such that by the end of lactation, the majority of cells present has been produced during the lactation cycle (Capuco et al. 2001, 2003).

Apoptosis is crucial to turnover of cells in BMT during lactation (Capuco et al. 2003, Hadsell et al. 2007). Additionally, it has been suggested that increasing cell turnover in BMT could aid in increasing secretory capacity in individual BMEC or could increase replacement of less functional cells (Capuco et al. 2006).

The 5-HT2A has low affinity for 5-HT and has been shown to activate phospholipases (A2, C, and D), and therefore increase intracellular Ca2+ levels, activate Janus kinase/signal transducers and activators of transcription (Jak/Stat) pathway, and increase glucose uptake by skeletal muscle by increasing glucose transporters (GLUT) 1, 3, and 4 in the plasma membrane (Hadjuch et al. 1999, Raymond et al. 2001, Banes et al. 2005). Glucose uptake by BMT is a required step in producing lactose, the primary carbohydrate and major osmolyte in milk. The major GLUT in the mammary gland is GLUT1 (Akers 2002). PRL stimulates the Jak/Stat pathway (Jak2/Stat5) in the mammary gland, which is responsible for the induction of milk protein expression in BMEC as well as other mammary epithelial cells due to the presence of Stat5-binding sites on the epithelial cells, and this could be a potential regulatory pathway by which 5-HT acts through 5-HT2A (Horsemann 1999). Our studies indicate that the mRNA for the HTR2A is present in BMT and potentially in cells of the vascular system of BMT (Fig. 2). Expression of mRNA for this receptor was 3.77-fold in BMT and 1.64 in BMEC relative to BB. We observed that treatment of BMEC with ritanserin increased α-lactalbumin and β-casein mRNA compared with lactogenic controls (Figs 3B and 4B). The 5-HT2A is involved in contraction of vascular smooth muscle cells through ERK/MAPK pathway (Watts 1998). This action could be involved in the regulation of blood flow to the mammary gland, which is important in supporting lactation (Collier et al. 1984).

We detected the presence of HTR2B mRNA in BMT (Fig. 2). These receptors are also involved in regulation of morphogenesis and mitogenesis, and stimulate ERK and cell cycle components (Raymond et al. 2001). We did not detect any effects on milk protein gene expression or milk protein levels when treating lactogenic cultures of BMEC with SB204741 (2B antagonist). Thus, this receptor may not be involved in the regulation of milk protein genes in the bovine, but other processes not measured.

We identified the HTR4 mRNA in BMT as well as BMEC, and potentially in the myoepithelium (Fig. 2). However, no effects were observed on milk protein gene expression or apoptosis when BMEC was treated with a 5-HT4 receptor antagonist. This may also not be surprising since we did not measure the milk ejection reflex in these studies.

The 5-HT7 receptor subtype is positively coupled to adenyl cyclase through Gq, and has been detected in both human and mouse mammary epithelial cells on the basolateral side of the membrane (Stull et al. 2007). We also detected HTR7 mRNA in BMT and BMEC (Fig. 2). Additionally, it has been demonstrated that 5-HT, through cAMP, regulates TJ status in human mammary epithelial cells in a biphasic manner, with long-term exposure to 5-HT resulting in a disruption of TJ status through a p38 MAPK pathway (Pai & Horsemann 2008). We detected an increase in α-lactalbumin and β-casein mRNA expression when BMEC was treated with a 5-HT7 antagonist (Figs 3C and 4C). In epithelial cells, cAMP and protein kinase A have been demonstrated to stimulate apically directed transcytosis and secretion (Muniz et al. 1996). Furthermore, it has been demonstrated that an increase in cAMP results in increased casein secretion in mammary epithelial cells through protein kinase A (Boisgard et al. 2001).

Serotonin receptor activation patterns in vivo are unknown, and could effect milk protein gene expression variably. In addition to tissue-autonomous mechanisms explored in this study, some mechanisms of 5-HT action may involve extra-epithelial actions. For instance, 5-HT acts as a mammary artery vasoconstrictor, which severely decreases mammary blood flow, a correlate for decreased capacity for milk production (Linzell 1974, Collier et al. 1984).

**Conclusion**

We have demonstrated the basic properties of the serotonergic signaling system in dairy cows. In this process, we have discovered profound differences between the bovine system and that of mice and humans. Whereas human and mouse mammary epithelial cells express predominantly the 5-HT-7, which signals through the Gq- and G11-coupled pathways, raising the likelihood that cows have evolved unique signaling mechanisms. In this process, we have identified novel mechanisms that have served to alter the molecular tuning of intramammary serotonergic feedback. We have further demonstrated that 5-HT1B, 2A, and 7 appear to be directly involved in milk protein gene expression in the bovine.
Other potential mechanisms of serotonergic regulation of mammary gland function include cell turnover, the milk ejection reflex, and mammary blood flow. Further studies will be needed to delineate the exact mechanisms of serotonergic regulation of the bovine mammary gland.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the partiality of the research reported.

**Funding**

This research was supported by the NRI Competitive Grant no. 2007-35206-17898 from the USDA-CSREES.

**References**

Akers RM 2002 Lactation and the Mammary Gland, pp 66–87. Ames: Iowa State Press.

Banes AKL, Shaw SM, Tawfik A, Patel BP, Ogbi S, Fulton D & Marrero MB 2005 Activation of the JAK/STAT pathway in vascular smooth muscle by serotonin. *American Journal of Physiology. Cell Physiology* **288** 805–812.

Boisgard R, Chanat E, Lavialle F, Pauloin A & Ollivier-Bousquet M 2001 Roads taken by milk protein in mammary epithelial cells. *Livestock Production Science* **70** 49–61.

Capuco AV & Akers RM 1999 Mammary involution in dairy animals. *Journal of Mammary Gland Biology and Neoplasia* **4** 137–144.

Capuco AV, Akers RM & Smith JJ 1997 Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. *Journal of Dairy Science* **80** 477–487.

Capuco AV, Wood DL, Baldwin R, McLeod K & Paape MJ 2001 Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of BST. *Journal of Dairy Science* **84** 2177–2187.

Capuco AV, Ellis SE, Hale SA, Long E, Erdman RA, Zhao X & Paape MJ 2003 Lactation persistency: insights from mammary cell proliferation studies. *Journal of Animal Science* **81** 18–31.

Capuco AV, Annen E, Fitzgerald AC, Ellis SE & Collier RJ 2006 Mammary cell turnover: relevance to lactation persistency and dry period management. In *Ruminant Physiology: Digestion, Metabolism, and Impact of Nutrition* (pp 363–388). Eds K Segersen, T Hvelplund & MO Nielsen. Wageningen: Wageningen Academic Publishers.

Collier RJ, McNamara JP, Wallace CR & Dehoff MH 1984 A review of endocrine regulation of metabolism during lactation. *Journal of Animal Science* **59** 498–510.

Hadjuc E, Rencurel F, Balrendra A, Batty IH, Downes CP & Hundal HS 1999 Serotonin (5-hydroxytryptamine), a novel regulator of glucose transport in rat skeletal muscle. *Journal of Biological Chemistry* **274** 13563–13568.

Haddel D, George J & Torres D 2007 The declining phase of lactation: peripheral or central, programmed or pathological? *Journal of Mammary Gland Biology and Neoplasia* **12** 59–70.

Hernandez LL 2008 Characterization of the bovine mammary gland serotonergic system. PhD Dissertation. University of Arizona, Tucson, AZ.

Hernandez LL, Steining CM, Wheelock JB, Baumgard LH, Parkhurst AM & Collier RJ 2008 Evaluation of serotonin as a feedback inhibitor of lactation in the bovine. *Journal of Dairy Science* **91** 1834–1844.

Horseman ND 1999 Prolactin and mammary gland development. *Journal of Mammary Gland Biology and Neoplasia* **4** 79–88.

Kroene WK, Kristiansen K & Roth BL 2002 Molecular biology of serotonin receptors-structure and function at the molecular level. *Current Topics in Medicinal Chemistry* **2** 507–528.

Limesand KH, Barzen KA, Quissell DO & Anderson SM 2003 Synergistic suppression of apoptosis in salivary acinar cells by IGF-I and EGF. *Cell Death and Differentiation* **10** 345–355.

Limesand SW, Jensen J, Hutton JC & Hay WW 2005 Diminished β-cell replication contributes to reduced β-cell mass in fetal sheep with intrauterine growth restriction. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **288** 1297–1305.

Linzell JL 1974 Mammary blood flow and methods of identifying and measuring precursors of milk. In *Lactation: a Comprehensive Treatise*, pp 143–225. Eds BL Larson & VR Smith. New York: Academic Press.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2^−ΔΔC_t method. *Methods* **25** 402–408.

Matsuda H, Maoka T, Vomachka AJ, Gudeşky GA, Hou Z, Mistry M, Bailey JP, Nieport KM, Walther DJ, Bader M et al. 2004 Serotonin regulates mammary gland development via an autocrine-paracrine loop. *Developmental Cell* **6** 193–203.

McGraith MF 1987 A novel system for mammary epithelial cell culture. *Journal of Dairy Science* **70** 1967–1980.

Muniz M, Alonso M, Hidalgo J & Velasco A 1996 A regulatory role for cAMP-dependent protein kinase in protein traffic along the exocytic route. *Journal of Biological Chemistry* **271** 30935–30941.

Pai V & Horsman ND 2008 Biphasic regulation of mammary epithelial resistance by serotonin through activation of multiple pathways. *Journal of Biological Chemistry* **283** 30901–30910.

Peaker M 1995 Autocrine control of milk secretion: development of the concept. In *Intracellular Signaling in the Mammary Gland*, pp 193–202. Ed. CJ Wilde. New York: Plenum Press.

Raymond JR, Mukhin YV, Gelasco A, Turner J, Collinsworth G, Gettys TW, Grewal JS & Garnovskaya MN 2001 Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacology and Therapeutics* **92** 179–212.

Reist M, Pfaffl MW, Morel C, Meylan M, Hirsbrunner G, Blum JW & Steiner A 2003 Quantitative miRNA analysis of eight bovine 5-HT receptor subtypes in brain abomasums, and intestine by real-time PCR. *Journal of Receptors and Signal Transduction* **23** 271–287.

Rocha V, Rango DL & Read DR. 1985 Caesium production during differentiation of mammary epithelial cells in collagen gel culture. *Experimental Cell Research* **159** 201–210.

Rozen S & Skalesky H 2000 Primer3 on the WWW for general users and biologist programmers. *Methods in Molecular Biology* **132** 365–386.

Steining CM 2005 Genomic regulation of bovine mammary epithelial cell growth and differentiation. PhD Dissertation. University of Arizona, Tucson.

Steining CM, Hoving JB, Abdallah MB, Hoving AM, Pandey R, Greer K & Collier RJ 2008 The effects of endocrine and mechanical stimulation on stage I lactogenesis in bovine mammary epithelial cells. *Journal of Dairy Science* **91** 1053–1066.

Still MA, Pai V, Vomachka AJ, Marshall AM, Jacob GA & Horsman ND 2007 Mammary gland homeostasis employs serotonergic regulation of epithelial tight junctions. *PNAS* **104** 16708–16713.

Watts SW 1998 Activation of the mitogen-activated protein kinase pathway via the 5-HT 2A receptor. *Annals of the New York Academy of Science* **861** 162–168.

Wilde CJ, Addy CVP, Blatchford DR, Peaker M & Casey MJ 1998 Feedback inhibition of milk secretion: the effect of a fraction of goat milk on milk yield and composition. *Quarterly Journal of Experimental Physiology* **73** 391–397.

Wilde CJ, Addy CVP, Boddy LM & Peaker M 1995 Autocrine regulation of milk secretion by a protein in milk. *Biochemical Journal* **305** 51–58.