Identification and characterization of the bovine G protein-coupled receptor GPR41 and GPR43 genes¹

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ABSTRACT

Volatile fatty acids (VFA), including acetate, propionate, and butyrate, are not only a primary source of energy, but also regulate rumen development, insulin and glucagon secretion, and other physiological processes in cattle and sheep. The mechanism underlying the regulatory effects of VFA is unknown. Recent “reverse pharmacology” studies identified human G protein-coupled receptors GPR41 and GPR43 as receptors for short-chain fatty acids. It is possible that proteins similar to human GPR41 and GPR43 mediate the regulatory effects of VFA in cattle. In this study, we determined first, whether the bovine genome contains genes similar to the human GPR41 and GPR43 genes; second, whether and where these genes are expressed in cattle; and third, if the proteins encoded by these genes can be activated by acetate, propionate, and butyrate. A search of GenBank revealed bovine genomic sequences and expressed sequence tags highly similar to the human GPR41 and GPR43 DNA and cDNA sequences. The protein-coding and 5′ untranslated regions of the bovine GPR41 and GPR43 mRNA were cloned and sequenced from spleen tissue. Based on these sequences, the bovine GPR41 gene contains 3 exons and its transcription is initiated at 2 leader exons, generating 2 GPR41 mRNA variants differing in the 5′ untranslated region. The bovine GPR43 gene contains 2 exons and transcription of this gene is initiated from a single start site. The amino acid sequences deduced from the bovine GPR41 and GPR43 mRNA sequences are more than 75% identical to those of the human GPR41 and GPR43 and are predicted to encode 7 transmembrane

domains, typical of G protein-coupled receptors. Both bovine GPR41 and GPR43 mRNA were detected in a variety of tissues including rumen and pancreas. In a cell system, interaction of the overexpressed bovine GPR41 or GPR43 protein with acetate, propionate, or butyrate inhibited luciferase reporter expression from a cyclic AMP-responsive promoter, suggesting that the bovine GPR41 and GPR43 proteins couple to $G\alpha_{i/11}$. In total, these results demonstrate that the bovine genome encodes functional GPR41 and GPR43 genes and suggest that GPR41 and GPR43 may play a role in the regulatory effects of VFA in cattle.

Key words: G protein-coupled receptors GPR41 and GPR43, volatile fatty acids, cattle

INTRODUCTION

Volatile fatty acids are short-chain fatty acids produced by microbial fermentation in the gastrointestinal tract (Bergman, 1990). Acetic, propionic, and butyric acids constitute approximately 95% of total VFA (Bergman, 1990). Because the pH in the gastrointestinal tract and the blood is higher than the pK of these VFA, 90 to 99% of VFA are circulating in their anion forms (Bergman, 1990). For this reason, acetic, propionic, and butyric acids are often referred to as acetate, propionate, and butyrate, respectively.

The VFA are major sources of energy for ruminant animals. It is estimated that 60 to 70% of the energy requirement of cattle and sheep is provided by rumen VFA (Siciliano-Jones and Murphy, 1989). In addition to providing energy, VFA also play a regulatory role in several important physiological processes in cattle and sheep. Intraruminal administration of VFA stimulates rumen epithelial development (Sakata and Tamate, 1978, 1979; Lane and Jesse, 1997), whereas limiting rumen production of VFA inhibits rumen epithelium growth (Warner et al., 1956; Harrison et al., 1960; Tamate et al., 1962). Intravenous and intraportal infusions of propionate and butyrate increase serum concentrations of insulin and glucagon in sheep and cattle (Horino et al., 1968; de Jong, 1982; DiCostanzo

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et al., 1999). Intraruminal and intravenous infusions of propionate also inhibit feed intake in cattle (Faverdin, 1999; Allen, 2000). The VFA can also stimulate blood flow and motility of the gastrointestinal tract (Bergman, 1990; Cherbut, 2003) and inhibit liver cholesterol synthesis (Bergman, 1990).

Little is known about the mechanisms underlying the regulatory effects of VFA in cattle and sheep. Recent studies employing a “reverse pharmacology” approach identified short-chain fatty acids, including acetate, propionate, and butyrate, as ligands for orphan human G protein-coupled receptor 41 (**GPR41**) and 43 (**GPR43**; Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). For this reason, GPR41 and GPR43 are also called free fatty acid receptor 3 (FFAR3) and 2 (FFAR2), respectively. Subsequent studies showed that GPR41 mediated the stimulation of short-chain fatty acids on leptin production in adipocytes (Xiong et al., 2004) and the effect of gut microbiota on host energy balance and adiposity (Samuel et al., 2008), and GPR43 was shown to mediate the stimulatory effect of short-chain fatty acids on adipogenesis (Hong et al., 2005) and inhibition of lipolysis (Ge et al., 2008).

It is possible that proteins similar to human GPR41 and GPR43 mediate the effects of VFA on rumen development, insulin and glucagon secretion, gastrointestinal motility, and feed intake in cattle. As a step toward testing this possibility, we determined in this study whether the bovine genome contains genes similar to the human GPR41 and GPR43 genes, whether and where these genes are expressed in cattle, and whether the protein products of these genes can interact with acetate, propionate, and butyrate.

MATERIALS AND METHODS

Tissue Collection and RNA Extraction

Various bovine tissues were collected from 2 Holstein bulls (about 5 yr old) at slaughter. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted using Tri reagent (Molecular Research Center, Cincinnati, OH), essentially according to the manufacturer's instructions. The quality of the extracted RNA was determined by standard RNA gel electrophoresis. All RNA samples except those from the pancreas were intact. The pancreatic RNA appeared to be partially degraded, likely because the bovine pancreas has extremely high levels of ribonucleases. The poly(A) RNA was isolated from spleen total RNA using Oligotex (Qiagen, Valencia, CA), according to the manufacturer's instructions.

Reverse Transcription and PCR

Reverse transcription (**RT**)-PCR was used to amplify the protein-coding regions of bovine GPR41 and GPR43 mRNA and to detect the expression of these mRNA in bovine tissues. Ten micrograms of total RNA was pooled equally from 2 animals and digested with 10 U of DNase I (Promega, Madison, WI) at 37°C for 30 min followed by standard protease K digestion and phenol-chloroform extraction. One microgram of the DNase I-digested RNA was reverse-transcribed to cDNA in a total volume of 20 μL using ImProm-II reverse transcriptase and random primers (Promega) according to the manufacturer's instructions.

To amplify the protein-coding regions of bovine GPR41 and GPR43 mRNA, 50 ng of bovine spleen cDNA was mixed with 10 μL of 2 $\times$ PCR Master Mix (Promega) and 10 pmol of primers bGPR41F1 and bGPR41R1, and primers bGPR43F1 and bGPR43R1, respectively (Table 1) in a total volume of 20 μL . The conditions of these PCR were 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The primers were designed based on the human GPR41 and GPR43 mRNA sequences in GenBank (accession numbers NM_005304 and NM_005306). The PCR products were resolved by standard agarose gel electrophoresis, and the DNA bands at expected sizes were purified from the gel using a QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The amplified GPR41 and GPR43 cDNA products were sequenced from both ends using the same primers for PCR (Table 1). The sequencing was performed by the Virginia Bioinformatics Institute Core Laboratory Facility (Blacksburg, VA).

To determine the expression of GPR41 and GPR43 mRNA in bovine tissues, DNase I-treated total RNA was reverse-transcribed in the presence or absence of reverse transcriptase, as described earlier. Twenty nanograms of cDNA representing each tissue was amplified using primers bGPR41F2 and bGPR41R2, or primers bGPR43F2 and bGPR43R2, respectively (Table 1). The conditions of these PCR were 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The “reverse transcription” in the absence of reverse transcriptase served as a control for amplification of undigested genomic DNA. In these PCR reactions, GAPDH cDNA was also amplified as a loading control, using primers bGAPDHF1 and bGAPDHR1 (Table 1).

5' Rapid Amplification of cDNA Ends

The 5' rapid amplification of cDNA ends (**5' RACE**) was used to amplify the 5' end regions of bovine GPR41 and GPR43 mRNA. This procedure was performed

Table 1. Primers used in this study

Primer	Sequence of primer (5' to 3')
bGPR41F1	TGTGCTGCCATCTCTGTCTC
bGPR41R1	CTCCAGAGCACCCACCTAGA
bGPR41F2	AACCTCACCTCTCGGATCT
bGPR41R2	GCCGAGTCTTGTACCAAAGC
bGPR41R4	ATGGATCCCTACTTGTTCATCGTCGTCCTTGTAATCGTAGCTAGCCTCT
bGPR41R5	AGATCCGAGAGGGTGAGGTT
bGPR41R6	AGTGATTGCCGAGGAAGAAG
bGPR43F1	CCACAGACTGAACCAGACCA
bGPR43R1	CCAGGAACATCCCTAGTCCA
bGPR43F2	TCATGGGTTTCGGCTTCTAC
bGPR43R2	GGGGAAGAAGAAGAGGAGGA
bGPR43R4	ATGGATCCCTACTTGTTCATCGTCGTCCTTGTAATCGTAGTCCAGGGTG
bGPR43R5	GTAGAAGCCGAAACCCATGA
bGPR43R6	GAGGATGTGGACAGGTGCAG
AAP	GGCCACGCGTCGACTAGTACGGGHHGGHHGGHHG
AUAP	GGCCACGCGTCGACTAGTAC
bGAPDHF1	GGGTCATCATCTCTGCACCT
bGAPDHR1	GGTCATAAGTCCCTCCACGA

as described previously (Wang et al., 2003). Briefly, first-strand cDNA was transcribed from 1 µg of spleen poly(A) RNA using bGPR41 mRNA-specific primer bGPR41R2 or bGPR43 mRNA-specific primer bGPR43R2 as the reverse primer (Table 1). A homopolymeric cytosine tail was added to the 3' terminus of the cDNA using dCTP and terminal deoxyribonucleotide transferase (New England Biolabs, Ipswich, MA). The dCTP-tailed cDNA was then used as template in PCR to amplify the 5' end sequence of bGPR41 or bGPR43 cDNA using primers AAP and bGPR41R5, or primers AAP and bGPR43R5, respectively (Table 1). The product of this PCR was diluted at 1:1,000, and 1 µL of the dilution was used as template in PCR to further amplify the 5' end sequence of bGPR41 or bGPR43 cDNA, using primers AUAP and bGPR41R6, or primers AUAP and bGPR43R6, respectively (Table 1). The product of this PCR was resolved by gel electrophoresis. The DNA bands were isolated and cloned into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions.

Plasmid Construction

A 214-bp bGPR41 cDNA fragment and a 323-bp bGPR43 cDNA fragment were amplified from bovine spleen total RNA by RT-PCR, using primers bGPR41F2 and bGPR41R2, and bGPR43F2 and bGPR43R2, respectively (Table 1). These PCR products were cloned into the pGEM-T Easy vector (Promega), and the resulting plasmid constructs were named pGEM-TE-bGPR41 and pGEM-TE-bGPR43, respectively. A 1,052-bp bGPR41 DNA and a 1,093-bp bGPR43 DNA, which corresponded to the predicted protein-coding regions of bGPR41 and bGPR43 mRNA, were amplified

from bovine genomic DNA using primers bGPR41F1 and bGPR41R1, and primers bGPR43F1 and bGPR43R1, respectively (Table 1). Genomic DNA instead of cDNA was used as template in these PCR because the protein-coding regions of bGPR41 and bGPR43 genes were found to be within one exon. The products of these PCR were cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA), and the resulting constructs were named pcDNA3-bGPR41 and pcDNA3-bGPR43, respectively. The same GPR41 and GPR43 protein-coding DNA regions were also amplified by PCR using primers bGPR41F1 and bGPR41R4, and primers bGPR43F1 and bGPR43R4, respectively (Table 1). These PCR products were cloned into pcDNA3.1 to generate constructs pcDNA3-bGPR41-FLAG and pcDNA3-bGPR43-FLAG. In these 2 constructs, the bGPR41 and bGPR43 protein-coding regions were fused to a FLAG peptide (DYKDDDDK) tag at their C-termini. All plasmid constructs were validated by DNA sequencing.

In Vitro Transcription and Translation

In vitro transcription and translation were used to validate the ability of the constructs pcDNA3-bGPR41 and pcDNA3-bGPR43 to express bGPR41 and bGPR43 proteins. The analysis was performed using the TNT In Vitro Transcription/Translation System (Promega). Briefly, 1 µg of pcDNA3-bGPR41 or pcDNA3-bGPR43 plasmid was transcribed and translated in a total volume of 50 µL, composed of 2 µL of TNT reaction buffer, 1 µL of TNT T7 RNA polymerase, 20 µM of amino acid mixture, 1 µL of ³⁵S-methionine (PerkinElmer Life and Analytical Sciences, Wellesley, MA), 1 µL of RNasin ribonuclease inhibitor, and 25 µL of TNT rabbit

reticulocyte lysate, at 30°C for 90 min. The translation products were resolved by electrophoresis through a 15% SDS polyacrylamide gel. The gel was fixed, dried, and visualized by autoradiography.

Cell Culture, Transient Transfection, and Luciferase Assay

Chinese hamster ovary (**CHO**) cells (ATCC, Manassas, VA) were used to express the bovine GPR41 and GPR43 proteins and to determine whether these proteins were activated by acetate, propionate, and butyrate. These cells were chosen because they are easy to transfect, provide stable and accurate posttranslational modification, and contain components for typical GPR signaling but no endogenous GPR41 or GPR43 (Itoh et al., 2003; Le Poul et al., 2003; Doupnik et al., 2004). The CHO cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma, St. Louis, MO). The cells at approximately 50% confluency were transfected with pcDNA3-bGPR41, pcDNA3-bGPR43, or pcDNA3.1 (empty vector), and the cyclic AMP (**cAMP**)-response reporter plasmid pCRE-Luc (Clontech, Mountain View, CA), using FuGene 6 as transfection reagent (Roche, Indianapolis, IN). Transfection efficiency was controlled by cotransfection of pRL-SV40 (Promega). Twenty-four hours after the transfection, the medium was replaced with serum-free minimum essential medium, and the cells were cultured for another 8 h. The cells were subsequently treated with different concentrations of sodium acetate, sodium propionate, sodium butyrate, or PBS for 16 h before being lysed for luciferase assay. The luciferase assay was done using the dual luciferase reporter assay system (Promega), essentially according to the manufacturer's instructions. Some CHO cells were also transfected with pcDNA3-bGPR41, pcDNA3-bGPR43, pcDNA3-bGPR41-FLAG, pcDNA3-bGPR43-FLAG, or pcDNA3.1. Forty-eight hours after transfection, total RNA or total cellular proteins were isolated from the cells to confirm expression of bGPR41 or bGPR43 mRNA or protein.

Ribonuclease Protection Assay

The ribonuclease protection assay (**RPA**) was used to confirm the expression of bGPR41 and bGPR43 mRNA in the transfected CHO cells. The ³²P-labeled riboprobes for bGPR41 and bGPR43 mRNA were transcribed from the pGEM-TE-bGPR41 and pGEM-TE-bGPR43 plasmids, respectively, by *in vitro* transcription in the presence of [α -³²P]CTP (PerkinElmer Life and Analytical Sciences), as described previously (Wang et

al., 2003). A ³²P-labeled riboprobe was also synthesized from a mouse GAPDH cDNA plasmid prepared in a previous study (Eleswarapu et al., 2008). This probe was included in the RPA as a control for RNA loading. The RPA was performed using a RPAII kit (Ambion Inc., Austin, TX), as described previously (Wang et al., 2003). Briefly, 1×10^5 dpm of bGPR41, bGPR43, and GAPDH riboprobes were hybridized with 20 µg of total RNA in 20 µL of hybridization buffer for about 16 h at 42°C, and then digested with ribonucleases A and T₁ at 37°C for 30 min. The undigested RNA fragments were precipitated and resolved through a 6% polyacrylamide gel containing 7 M urea. Following electrophoresis, the gel was dried, exposed to a phosphor-screen, and scanned on a Molecular Imager FX System (Bio-Rad, Hercules, CA).

Western Blotting Analysis

Total cellular protein lysates from cells were prepared as described previously (Zhou et al., 2008). Thirty micrograms of cellular protein lysates were separated through a 12% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad). After being incubated in 5% nonfat dried milk for 3 h, the membrane was incubated with 1:1,000 diluted anti-FLAG M2 monoclonal antibody (Sigma) at 4°C overnight. After being washed 3 times in Tris-buffered saline-Tween, the membrane was incubated with 1:5,000 diluted horseradish peroxidase-conjugated donkey anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The membrane was subsequently incubated in SuperSignal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL) for 5 min, and the chemiluminescent signals were visualized by exposure to x-ray films. The membrane was then stripped by incubation in Restore Western Blot Stripping Buffer (Pierce Biotechnology) for 30 min at room temperature. The stripped membrane was probed with 1:1,000 diluted rabbit anti- β -actin antibody (Cell Signaling), and 1:5,000 diluted horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Santa Cruz Biotechnology) to detect β -actin.

Statistical Analysis

The luciferase data were analyzed using the GLM procedure (SAS Inst. Inc., Cary, NC). The model included the effects of plasmid construct (pcDNA3.1, pcDNA3-bGPR41, pcDNA3-bGPR43), concentration of acetate, propionate, or butyrate, and plasmid $\times$ concentration interaction. Multiple means were compared using the Tukey analysis. All data were expressed as means $\pm$

SEM (standard error of the mean). A difference was considered statistically significant at $P < 0.05$.

RESULTS

Bioinformatic Evidence for the Presence of Functional Bovine GPR41 and GPR43 Genes

A search of the bovine genome database in GenBank using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) revealed that the bovine genome contains sequences more than 80% identical to human GPR41 mRNA (NM_005304) and GPR43 mRNA (NM_005306) sequences. The corresponding bovine GPR41 (bGPR41) and bGPR43 genes are located in tandem on chromosome 18. A BLAST search of the bovine expressed sequence tags (EST) database revealed 5 EST (GenBank accession numbers BI541693, AW632495, AW660795, BE755048, and CK773115) that appeared to be transcripts of the bGPR41 gene and 4 EST (accession numbers AW478068, CK977505, AJ813869, AJ818136) of the bGPR43 gene. The first 7 EST were sequenced from libraries MARC 1BOV, MARC 2BOV, and BARC 9BOV made from pooled bovine tissues. The last 2 EST (i.e., AJ813869, AJ818136) were sequenced from a bovine monocyte cDNA library.

Sequences of the Protein-Coding and 5' Untranslated Regions of bGPR41 and bGPR43 mRNA

The protein-coding sequences of bGPR41 and bGPR43 mRNA were amplified from spleen total RNA by RT-PCR. Spleen was chosen because preliminary experiments indicated that this tissue had a relative high-level expression of GPR41 and GPR43 mRNA. The RT-PCR generated DNA products at expected sizes. Sequencing of the DNA products revealed that they were more than 80% identical to human GPR41 and GPR43 mRNA sequences. The 5' end sequences of bGPR41 and bGPR43 mRNA were amplified by 5' RACE from spleen poly(A) RNA. Cloning and sequencing of the 5' RACE products revealed a single bGPR43 mRNA 5' end sequence and 2 different bGPR41 mRNA 5' end sequences. Aligning these 5' end sequences and the protein-coding sequences determined earlier as well as the EST sequences (BI541693, AW632495, AW660795, BE755048, CK773115, AW478068, CK977505, AJ813869, AJ818136) against the bovine genome database revealed the organization and transcription start sites of the bGPR41 and bGPR43 genes (Figure 1). The bGPR41 gene is composed of 3 exons, exon 1A, 1B, and 2 (Figure 1A). Transcription of the bGPR41 gene in the spleen is initiated at both exon 1A and exon 1B,

Figure 1. Organization and transcription of the bovine G protein-coupled receptor (bGPR) GPR41 (panel A) and GPR43 (panel B) genes. Exons are indicated by boxes and introns by lines; the sizes of the introns are indicated. Sites of transcription are indicated by horizontal arrows. The protein-coding and 5' end sequences of bGPR41 and bGPR43 mRNA were determined by reverse transcription-PCR and 5' rapid amplification of cDNA ends (RACE) of bovine spleen RNA. The bGPR41-1A, bGPR41-1B, and bGPR43 mRNA sequences are deposited in GenBank under accession numbers FJ562213, FJ562214, and FJ562212, respectively.

generating GPR41-1A mRNA composed of exon 1A and exon 2, and GPR41-1B mRNA composed of exon 1B and exon 2 (Figure 1A). The bGPR43 gene contains 2 exons (Figure 1B). Transcription of this gene in the spleen is initiated from a single start site and, hence, generates a single bGPR43 mRNA transcript (Figure 1B). The bGPR41-1A, bGPR41-1B, and bGPR43 mRNA sequences are deposited in GenBank under accession numbers FJ562213, FJ562214, and FJ562212, respectively.

Amino Acid Sequences and Features of Bovine GPR41 and GPR43 Proteins

Despite differing in the 5' end sequence, the bovine GPR41-1A and GPR41-1B mRNA are predicted to encode an identical 326-AA sequence (Figure 2A) because the translation start codons (AUG) of both mRNA sequences reside in the common exon 2 region (Figure 1A). The bGPR43 mRNA is predicted to encode a 329-AA protein (Figure 2B). The predicted bGPR41 and bGPR43 AA sequences are more than 75% identical to human GPR41 and GPR43 AA sequences (Figure 2). Analyses of the

deduced bGPR41 and bGPR43 AA sequences using the TMpred software (http://www.ch.embnet.org/software/TMPRED_form.html) indicated that both bGPR41 and bGPR43 are membrane proteins containing 7 putative transmembrane domains (Figure 2).

Expression of bGPR43 and bGPR41 mRNA in Bovine Tissues

Based on RT-PCR analyses, both GPR41 and GPR43 mRNA were expressed in various bovine tissues, including liver, heart, spleen, skeletal muscle, kidney, cerebral

cortex, hypothalamus, pituitary, adrenal gland, lung, pancreas, small intestine (duodenum), colon, rumen, omasum, and reticulum (Figure 3). Among the bovine tissues examined, adipose tissue was the only tissue that did not appear to express GPR43 mRNA (Figure 3). Adipose tissue expressed barely detectable GPR41 (Figure 3).

Interaction of bGPR41 and bGPR43 with Acetate, Propionate, and Butyrate Inhibited cAMP Signaling

We determined whether bGPR41 and bGPR43 proteins could be activated by acetate, propionate, and

A

bGPR41	-MTNPDHSFFLGNHWLFFSVYLF	TFLVGLPLNLMALVIFVGKLR	RRRPLAVDVLLLNLTLS	SDLVLLLFLPF	69
hGPR41	MDTGPDQSYFSGNHWLFVFSVYLL	TFLVGLPLNLLALVVFVGKLR	RRPVAVDVLLLNLTAS	DLLLLFLPF	70
bGPR41	RMVEAASAMHWSLPFVFC	PFSSRFLFFTTIYLTSL	FLLAAVSTERFLSVAY	PLWYKTRPRPGQAGLV	SGACW 139
hGPR41	RMVEAANGMHWP	LPFILCPLSGFIFFTTIYLT	ALFLLAAVSIERFLS	VAHPLWYKTRPRLGQAGLV	SVACW 140
bGPR41	LLAAAHCSVVYVIEFSGN	SSPSQGINGTCYLEFRED	QLALLLPVRLEMAVVLF	GVPLFISSYCYSRLV	CI 209
hGPR41	LLSAHCSVVYVIEFSGD	ISHSQGTNGTCYLEFRKD	QLAILLPVRLEMAVVLF	VVPLIITSYCYSRLV	WI 210
bGPR41	LGRGASHRRRKRVAGL	AAATLLNFLVCFGPYNM	SHIVGYIQKSP	TWRSYVLLLSTLNS	CDPLVYFSS 279
hGPR41	LGRGGSRRQR	RVAGLLAATLLNFLVCFGPYNV	SHVVGYICGESPAWRI	YVTLLSTLNS	CDPFVYFSS 280
bGPR41	SGFQADWRFHGLL	GRLTGSWGPQENGVT	SKKSEG-----	EGPPQELFNIEAS-----	326
hGPR41	SGFQADWQFHELL	LRRLCGLWGQQESSMELKE	QKGEGEEQRADRP	AEKRTSEHSQCGTG	GGQVACAES 346

B

bGPR43	-MPDWDS	SLIITAYIIILLTGLPANLL	LALRAFLGRV	RQHPAPVHILL	SLTLADVLLLLLL	LPFKIEAA 69
hGPR43	MLPDWK	SSLIIMAYIIIFLTGLPANLL	LALRAFLVGRIR	QPPAPVHILL	SLTLADLLLLLLL	LPFKIEAA 70
bGPR43	SDFRWEL	SNLACALMGFGFYG	SIYCSTLLLAGIS	VERYLGVAFPVQYKLS	RPRVYGVIAALIA	WVMSFGH 139
hGPR43	SNFRWYL	PKVVCALTSFGFYSS	IYCSTWLLAGISIERY	LGVAFPVQYKLS	RPLVGVIAALVA	WVMSFGH 140
bGPR43	GTVVIVQYLN	STQRAPKENETTCYENFT	QEQRLRL	LLPIRLELC	LLFFFP	MVTTFCYSRFVWIMLSQP 209
hGPR43	CTIVIVQYLN	TTEQVRSGNEITCYENFT	DNQLD	VLPVRLELC	IVLFFIPMAVT	IFCYWRFVWIMLSQP 210
bGPR43	HMGAKQ	QRRAMGLAIVSLLN	FLLCFGPYNISHLV	GFYMKASPKWR	REAVVFGSLNAS	LDPLLFFYFSSSVV 279
hGPR43	LVGAQ	RRRRVGLAVVTLN	FLVCFGPYNVSHLV	GYHQRKSPW	RSIAVVFSSLNAS	LDPLLFFYFSSSVV 280
bGPR43	RRSFGKGLQ	ALYHWGSSLLGRRCKE	TAEAA	NEDPGVSQAEGAP	SSDFTLD	329
hGPR43	RRAFGRGLQ	VLNRQSSLLGRRGKD	TAEGT	NEDRGVQGEGMP	SSDFTTE	330

Figure 2. Alignments of bovine G protein-coupled receptor (bGPR) 41 (bGPR41) and human GPR41 (hGPR41) amino acid sequences (panel A) and bGPR43 and hGPR43 amino acid sequences (panel B). The bovine GPR41 and GPR43 amino acid sequences are deduced from their mRNA sequences determined in this study. The GenBank accession numbers for the human GPR41 and GPR43 amino acid sequences are NP_005295 and NP_005297, respectively. The identical amino acid residues between the bovine and human sequences are shaded. The predicted 7 transmembrane domains of bovine GPR41 and GPR43 are underlined.

Figure 3. Expression of bovine G protein-coupled receptor (bGPR) 41 and GPR43 mRNA in bovine tissues. The expression of GPR41 and GPR43 mRNA in 18 bovine tissues was detected by reverse transcription-PCR. Total RNA pooled from 2 bulls was digested by DNase I before reverse transcription. The “reverse transcription” in the absence of reverse transcriptase (–RTase) was used to control for amplification from incompletely digested genomic DNA. The GAPDH mRNA served as a loading control. The sizes of GPR41, GPR43, and GAPDH (internal control) PCR products were 214, 323, and 176 bp, respectively. Sk = skeletal; Cer = cerebral; Sm = small.

butyrate to mediate cAMP signaling. In the CHO cells without bGPR41 or bGPR43 overexpression, acetate, propionate, and butyrate each increased ($P < 0.05$) luciferase activity expressed from the cAMP-response reporter plasmid pCRE-Luc in a dose-dependent manner (Figure 4A). However, when bGPR41 was overexpressed in these cells, the stimulatory effects of acetate, propionate, and butyrate on luciferase activity were attenuated compared with when the cells were transfected with the empty vector pcDNA3.1 ($P < 0.05$; Figure 4A). Overexpression of bGPR43 in the CHO cells similarly reduced the stimulation of acetate and propionate on luciferase activity from pCRE-Luc ($P < 0.05$, Figure 4A). Overexpression of bGPR43 reduced the stimulatory effect of low concentrations (0.1, 1, and 10 mM) of butyrate, but, paradoxically, enhanced the effect of high concentration (100 mM) of butyrate ($P < 0.05$, Figure 4A). Overexpression of bGPR41 or bGPR43 had no effect on luciferase activity in the absence of short-chain fatty acid treatment ($P > 0.1$; data not shown).

Ribonuclease protection analysis confirmed the expression of bGPR41 and bGPR43 mRNA in the transfected CHO cells (Figure 4B). Because of the lack of antibodies that cross-react with bGPR41 and bGPR43 proteins (Western blotting images not shown), expression of bGPR41 and bGPR43 proteins in the transfected CHO cells was confirmed indirectly by analyzing the FLAG peptide fused to the C-terminus of bGPR41 or bGPR43 protein. The bGPR41-FLAG and bGPR43-FLAG fusion proteins were detected in the CHO cells transfected with their respective expression plasmids but not in the cells transfected with the empty vector (Figure 4C). The ability of the bGPR41 and bGPR43 expression plasmids to express bGPR41 and bGPR43

proteins was also confirmed by in vitro transcription and translation analyses, which showed translated protein products at expected sizes (Figure 4D).

DISCUSSION

It has been known for decades that VFA, including acetate, propionate, and butyrate, regulate rumen development, insulin and glucagon secretion, and other physiological processes in cattle and sheep (Bergman, 1990). However, little is known about the mechanism underlying these regulatory effects. The orphan human G protein-coupled receptors GPR41 and GPR43 were recently identified as receptors for VFA (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). In this study, we have shown that the bovine genome encodes genes similar to the human GPR41 and GPR43 genes, that these genes are expressed in many bovine tissues including rumen and pancreas, and that the proteins encoded by these genes can mediate an inhibitory effect of acetate, propionate, and butyrate on cAMP signaling. These results together support a potential role of GPR41 and GPR43 in mediating the regulatory effects of VFA in cattle.

Before this study, the sequences of GPR41 and GPR43 mRNA and, hence, the organization of the GPR41 and GPR43 genes had not been carefully characterized in any species. Most of the GPR41 and GPR43 mRNA sequences in GenBank resulted from computational predictions. In this study, we cloned and sequenced the protein-coding and the 5' untranslated regions of the bovine GPR41 and GPR43 mRNA. Based on these sequences, the bovine GPR41 gene contains 3 exons and the bovine GPR43 gene 2 exons; transcription of the bovine GPR43 gene is initiated from a single

Figure 4. Bovine G protein-coupled receptor (bGPR) 41 and 43 inhibited acetate-, propionate-, and butyrate-activated cyclic AMP (cAMP) signaling. A) Effects of bGPR41 and bGPR43 on acetate-, propionate-, and butyrate-activated luciferase expression from pCRE-Luc, a cAMP-response luciferase reporter plasmid, in Chinese hamster ovary (CHO) cells; bGPR41 or bGPR43 expression plasmid or the empty vector pcDNA3 was cotransfected with pCRE-Luc and pRL-SV40 (a transfection efficiency control plasmid) into CHO cells. The transfected cells were treated with indicated concentrations of acetate, propionate, and butyrate for 16 h before luciferase assay; * indicates $P < 0.05$ ($n = 3$) compared with pcDNA3 at the same concentration of acetate, propionate, or butyrate. B) Confirmation of bGPR41 and bGPR43 mRNA expression in transfected CHO cells. The bGPR41 and bGPR43 mRNA were detected by a ribonuclease protection assay. Endogenous GAPDH mRNA and yeast RNA (yRNA) served as a loading control and a negative control, respectively. C) Confirmation of bGPR41 and bGPR43 protein expression in the transfected CHO cells by Western blotting analysis of FLAG fused to the C termini of bGPR41 and bGPR43. Total protein lysates from CHO cells transfected with the bGPR41-FLAG, the bGPR43-FLAG fusion-protein expression plasmid, or the empty vector pcDNA3 were analyzed by a Western blotting analysis using the anti-FLAG antibody; β -actin served as a loading control for this analysis. D) Confirmation of the ability of the bGPR41 and bGPR43 expression plasmids to express bGPR41 and bGPR43 proteins by in vitro transcription and translation. The bGPR41 and bGPR43 expression plasmids were transcribed and translated in the presence of ^{35}S -methionine. Transcription and translation in the absence of a plasmid (no plasmid control) and transcription and translation of a plasmid encoding chloramphenicol acetyltransferase (CAT) served as a negative and a positive control, respectively. The radiolabeled protein products were separated by gel electrophoresis and visualized by autoradiography. The translated bGPR41, bGPR43, and CAT proteins were expected to be 36.5, 36.8, and 25.5 kDa, respectively.

nucleotide; transcription of the bovine GPR41 gene is initiated from 2 different leader exons, generating mRNA variants differing in the 5' untranslated region. The 5' end nucleotide sequence of bovine GPR41 and GPR43 mRNA and the deduced N-terminal amino acid sequences of bovine GPR41 and GPR43 proteins determined in this study are different from those predicted by computational analyses (XM_605910, XM_600712). This underscores the need to validate experimentally the results of computational analyses.

Based on the deduced AA sequences, both bovine GPR41 and GPR43 proteins are membrane proteins containing 7 putative transmembrane domains, characteristic of G protein-coupled receptors (Wettschureck and Offermanns, 2005). In response to ligand binding, G protein-coupled receptors typically mediate changes in intracellular levels of second messengers cAMP, cyclic guanosine monophosphate, inositol trisphosphate (IP3), diacylglycerol (DAG), and Ca^{2+} , depending on the type of G protein coupled to the receptor (Wettschureck and Offermanns, 2005). In this study, we have shown that interactions between bovine GPR41 and GPR43 and VFA inhibit reporter gene expression from a cAMP-response promoter in cells. This result demonstrates a functional link between bovine GPR41 and GPR43 proteins and acetate, propionate, and butyrate, and suggests that bovine GPR41 and GPR43 might couple with $\text{G}\alpha_{i/11}$, because activation of $\text{G}\alpha_{i/11}$ normally leads to reduced cAMP production, and reduced cAMP would explain reduced reporter gene expression from the cAMP-responsive plasmid (Wettschureck and Offermanns, 2005). In response to VFA, human GPR41 and GPR43 couple with $\text{G}\alpha_{i/11}$ to reduce intracellular cAMP production (Le Poul et al., 2003). The functional similarity between bovine and human GPR41 and GPR43 is not surprising because they share more than 75% identity in AA sequence. This study has also shown that in CHO cells without GPR41 or GPR43 overexpression, acetate, propionate, and butyrate stimulate cAMP signaling. The mechanism underlying this stimulatory effect of VFA on cAMP signaling is not clear, but apparently is not related to GPR41 or GPR43 because the CHO cells do not express endogenous GPR41 and GPR43 (Le Poul et al., 2003). In addition to activating GPR41 and GPR43, VFA (in particular, butyrate) are also known to function as histone deacetylase inhibitors and to affect gene expression through sequence-specific transcription factors and regulatory DNA sequences (Davie, 2003). Whether acetate, propionate, and butyrate stimulate cAMP-mediated gene expression by inhibiting histone deacetylase or by activating sequence-specific transcription factors remains to be determined.

This study has shown that GPR41 and GPR43 mRNA are widely expressed in cattle. This mirrors both human and rodent GPR41 and GPR43 mRNA results (Brown et al., 2003; Le Poul et al., 2003; Hong et al., 2005). Among the bovine tissues that express GPR41 and GPR43 mRNA are pancreas and rumen. Expression of GPR41 and GPR43 mRNA in these tissues supports the possibility that GPR41 and GPR43 might mediate the well-established effects of VFA on rumen development and insulin and glucagon secretion in cattle (Warner et al., 1956; Harrison et al., 1960; Tamate et al., 1962; Horino et al., 1968; Sakata and Tamate, 1978, 1979; Lane and Jesse, 1997). Adipose tissue is the only bovine tissue examined that does not express GPR41 or GPR43 mRNA. Absence of GPR41 and GPR43 expression in adipose tissue seems to be unique for cattle because these mRNA are expressed in human and rodent adipose tissue, where GPR41 and GPR43 have been further shown to mediate stimulation of VFA on leptin production and adipogenesis (Hong et al., 2005; Xiong et al., 2004) and inhibition of VFA on lipolysis (Ge et al., 2008). However, lack of GPR41 and GPR43 expression in bovine adipose tissue may explain why infusion of propionate does not change serum leptin concentration but does increase serum insulin concentration in cattle (Bradford et al., 2005).

In summary, this study has shown that the bovine genome encodes GPR41 and GPR43 genes, that the protein products of these genes can interact with acetate, propionate, and butyrate to inhibit cAMP signaling in vitro, and that these genes are expressed in various bovine tissues, including rumen and pancreas. These results demonstrate a novel functional link between GPR41 and GPR43 and VFA, suggesting that GPR41 and GPR43 might mediate the regulatory effects of VFA in cattle such as those on rumen development and insulin and glucagon secretion. In this study, we also determined the organization and transcription start sites of the bovine GPR41 and GPR43 genes. This information should be useful for studying how GPR41 and GPR43 genes are regulated in bovine tissues and has implications for the organization of the GPR41 and GPR43 genes in other species.

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