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Detection and quantification of mRNA expression of α- and β-adrenergic receptor subtypes in the mammary gland of dairy cows

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Abstract

Adrenergic receptors are pharmacologically classified into the receptor types α_1, α_2, β_1, β_2, and β_3. Structural differences and varying affinities in radioligand binding studies lead to a further classification of α_1- and α_2-receptors into subtypes which are termed α_{1A} (formerly α_{1C}), α_{1B}, and α_{1D} (formerly α_{1AD}), and α_{2AD}, α_{2B}, and α_{2C}, respectively. mRNA expression of all but one α-adrenergic receptor subtypes and of all β-adrenergic receptor types was measured quantitatively in total RNA extracted from mammary tissue of 10 lactating dairy cows by real-time reverse transcription (RT) polymerase chain reaction (PCR). mRNA expression of α_1-adrenergic receptors was highest for the α_{1A}-subtype followed by α_{1B}, whereas the α_{1D}-subtype could not be detected. The highest mRNA expression of α_2-adrenergic receptors was found for the α_{2AD}-subtype, followed by α_{2B} and α_{2C}. Within the β-adrenergic receptors, the β_2-receptor type was most highly expressed, followed by β_1 and β_3.

In conclusion, eight of nine adrenergic receptors classified to date were detected and relatively quantified in the mammary gland of dairy cows.
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Keywords: mRNA expression; Real-time RT-PCR; Adrenergic receptor types and subtypes; Bovine mammary gland

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1. Introduction

The various physiological effects of the adrenergic system in different tissues are mediated by binding of catecholamines to alpha (α)- and beta (β)-adrenergic receptors. Adrenergic receptors belong to the family of G-protein-coupled receptors which are located in the cell membrane. The primary structure of these proteins is remarkably similar; most characteristic are seven stretches of hydrophobic transmembrane spanning domains which are connected by alternating intracellular and extracellular loops, with the amino terminus located on the extracellular side and the carboxy terminus on the intracellular side. The binding sites for adrenergic ligands are contained in a binding crevice formed by transmembrane spanning domains [1]. So far, α- and β-adrenergic receptors have been pharmacologically classified into the receptor types α_1 and α_2, and β_1, β_2 and β_3, respectively. Between these receptor types there are functional differences and often antagonistic effects, e.g. vasoconstriction mediated by α_1- and α_2-receptors and vasodilation mediated by β_2-receptors [2].

Structural differences and varying affinities in radioligand binding studies within the α_1- and α_2-adrenergic receptor type show heterogeneity and lead to a further classification into receptor subtypes [3]. Several molecular cloning studies suggest that there are three distinct subtypes of α_1-adrenergic receptors, originally classified as α_{1AD}, α_{1B} and α_{1C} [4,5] and four distinct subtypes of α_2-adrenergic receptors, α_{2A}, α_{2B}, α_{2C} and α_{2D} [6,7]. Recently, α_{2A}- and α_{2D}-adrenergic receptors were combined to one receptor subtype α_{2AD} [8,9] as they seem to be variants of the same adrenergic receptor between species, the α_{2A} in human and porcine and the α_{2D} in rat, murine and bovine, differing in one amino acid only [10].

According to a recent classification of adrenergic receptors in human pharmacological research α_1-adrenergic receptors were subdivided into α_{1A} (formerly α_{1C}), α_{1B} and α_{1D} (formerly α_{1AD}), whereas the α_2-subclassification remained as described above [2]. In this study, we kept to the new classification into three α_1- and α_2-adrenergic receptor subtypes each, which are designated A, B, and D (α_1), and AD, B and C (α_2), respectively. The former nomenclature of the α_1-adrenergic receptor subtypes is additionally shown in brackets.

So far, expression of α_1- and α_2-adrenergic receptor subtypes has been studied mainly in rat but also in human, rabbit and other species by Northern blot analysis. Diversity of tissue distribution of α_1- and α_2-adrenergic receptor subtypes indicate that different species may express different receptor subtypes in different tissues [3].

Especially vascular adrenergic receptor subtypes play an important role in human pharmacological research [11]. In veterinary research, however, functional purposes of α_1- and α_2-adrenergic receptor subtypes have not been discussed yet whereas pharmacological functions of the adrenergic receptor types α_1 and α_2, and β_1, β_2 and β_3, respectively, are well known. In the bovine mammary gland, stimulation of β-adrenergic receptors facilitates milk removal [12,13] whereas α-adrenergic receptor stimulation reduces total milk yield and peak flow rate [14,15]. The presence of α_1-, α_2- and β-adrenergic receptor binding sites in the bovine mammary gland has been demonstrated previously [16–19]. On the level of mRNA expression, only two adrenergic receptor (sub)types, the $\alpha_{1A(C)}$- and β_2-adrenergic receptor, were detected in the bovine mammary gland [20].

We tested the hypothesis that those six α-adrenergic receptor subtypes ($\alpha_{1A(C)}$, α_{1B}, $\alpha_{1D(AD)}$, α_{2AD}, α_{2B}, α_{2C}) and three β-adrenergic receptor types (β_1, β_2, β_3) are present in

the mammary gland of dairy cows. Therefore, a highly sensitive and quantitative method, a real-time reverse transcription (RT) polymerase chain reaction (PCR), was developed to detect and quantify the mRNA expression of these receptors.

2. Materials and methods

2.1. Total RNA preparation and cDNA synthesis

Mammary tissue samples of the udder of 10 lactating cows were taken immediately after slaughter, snap frozen in liquid nitrogen, and stored at −80 °C. For RNA preparation, 200 mg of mammary tissue was homogenized with an Ultra-Turrax homogenizer (T 25, Janke & Kunkel, Staufen, Germany) using 2 ml TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) followed by 5 min of incubation at room temperature (rt). After addition of 0.4 ml chloroform, 10-min incubation at rt, and centrifugation for 15 min at 13,500 × *g* and 4 °C, the RNA being in the upper aqueous phase was carefully pipetted and precipitated by adding 0.5 ml *iso*-2-propanol and centrifuged for 10 min at 13,500 × *g* at 4 °C. RNA pellets were washed with 75% ethanol and again centrifuged at 8000 × *g* for 8 min at 4 °C. The supernatant was decanted completely, and after drying for 10 min at 37 °C, the remaining pellet was diluted in 30 μl RNase-free water.

In order to quantify the extracted total RNA, optical density of the RNA stock solution was determined at 260 nm. Additionally, optical density of the $OD_{260\,nm}/OD_{280\,nm}$ (nucleic acid/protein) absorption ratio was measured which lay in an optimum range between 1.8 and 2.0. The stock solution was diluted into a work solution of 100 ng/μl by RNase-free water and again optically determined at 260 nm.

Synthesis of first strand complementary DNA (cDNA) was performed with 200 units reverse transcriptase (MMLV-RT, Promega, Madison, WI, USA) and 100 pmol random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany). Final concentration of reversely transcribed total RNA was 25 ng/μl.

2.2. Oligonucleotide primers

Nine different primer pairs (sense and antisense primer) were designed using published bovine or human nucleic acid sequences [8,21–28]. To demonstrate the amplification of only the cDNA but not the genomic DNA (gDNA) the sense and antisense primers of every target gene were placed in two different exons of the gene. So the length of the PCR products of cDNA and gDNA differed.

Oligonucleotide primers for each subtype were designed by multiple sequence alignments of published nucleotide sequences of different species (HUSAR program, DKFZ, Heidelberg, Germany). In regions of high homology primers were designed from nucleotide sequences of about 20 bp long. Another multiple sequence alignment between sequences of the subtypes within one receptor type (e.g. α_{2AD}, α_{2B}, α_{2C}) showed very low homology and verified that regions of designed oligonucleotide primers were subtype specific.

Primer information, product length, and the EMBL ac. numbers are listed in Table 1.

Table 1
Sequence of PCR primers, position of the primers (f = forward; r = reverse) in the coding sequence (CDS), PCR product length, and EMBL ac. no. (species in brackets) of the used published nucleic acid sequences

Primer	Sequence (5′→3′)	CDS	Length	EMBL ac. no.
$\alpha_{1A(C)}$ f	GTGAACATTTCCAAGGCCAT	61–80	310	J05426 (bovine)
$\alpha_{1A(C)}$ r	GGTCGATGGAGATGATGCAG	351–370		
α_{1B} f	ACTTCACTGGCCCCAACCAG	71–90	388	L31773 (human)
α_{1B} r	TACTGCAGAGAGTAGCGCAC	439–458		
$\alpha_{1D(AD)}$ f	ACCTGCAGACCGTCACCAACTA	392–413	191	D29952 (human)
$\alpha_{1D(AD)}$ r	GGTGCAGAGGCTGAGGA	566–582		
α_{2AD} f	TCATCTCGGCCGTCATCTCCTT	476–497	178	U79030 (bovine)
α_{2AD} r	CGCACATAGACCAGGATCATGAT	631–653		
α_{2B} f	TTGCTGGGCTACTGGTACTTC	220–239	296	Y15944 (bovine)
α_{2B} r	TACCAGGCCTCTTGGTTGAGCT	494–515		
α_{2C} f	TGCGCGCCCCGCAGAACCTCTTCCT	245–269	403	NM_000683 (human)
α_{2C} r	ATGCAGGAGGACAGGATGTACCA	625–647		
β_1 f	TCGCCCTTCCGCTACCAGA	566–584	160	AF188187 (bovine)
β_1 r	ACTCGGGGTCGTTGTAGCA	707–725		
β_2 f	TCATGTCGCTTATTGTCCTGG	116–136	202	Z86037 (bovine)
β_2 r	CACCAGAAGTTGCCAAAAGTCC	296–317		
β_3 f	ACCGTGGGAGGCAACCTG	151–168	155	X85961 (bovine)
β_3 r	TGGCCGGTCAGCGCCAA	289–305		
UbC f	AGATCCAGGATAAGGAAGGCAT	86–107	654	Z18245 (bovine)
UbC r	GCTCCACCTCCAGGGTGAT	721–739		
GAPDH f	GTCTTCACTACCATGGAGAAGG	265–286	197	U85042 (bovine)
GAPDH r	TCATGGATGACCTTGGCCAG	442–461		

2.3. *Quantification by real-time PCR*

Polymerase chain reaction was performed in the LightCycler (Roche Diagnostics) with 25 ng reversely transcribed total RNA (25 ng/μl). Further reaction components for the LightCycler reactions were 1.0 μl LightCycler FastStart Mastermix (Roche) (containing Taq DNA Polymerase, reaction buffer, 10 mM $MgCl_2$, dNTP mix, and SYBR Green I dye), 4 mM $MgCl_2$, 4 pmol forward primer, 4 pmol reverse primer, and sterile water up to a final volume of 10 μl.

Prior to amplification an initial denaturation step (10 min at 95 °C) ensured complete denaturation of the cDNA. All PCR reactions were performed with a total of 40 amplification cycles. Product-specific PCR cycle conditions for all receptor (sub)types and the housekeeping genes are summarized in Table 2. After the last amplification cycle, PCR products were specified in a melting curve analysis as each product has its specific melting temperature depending on the GC-content (Table 2). In this way it could be demonstrated that there were no primer dimers or unspecific amplification products.

To verify the specificity of each PCR quantification method the PCR amplicons were sequenced (MWG BIOTECH, Ebersberg, Germany). For each product three PCR runs

Table 2
Product-specific LightCycler-PCR conditions of adrenergic receptor (sub)types and housekeeping genes (hkg) ubiquitin (UbC) and glyceraldehyde-phosphate-dehydrogenase (GAPDH)

Adrenergic receptor (sub)type and hkg	Denaturation		Primer annealing		Elongation		Melting temperature (°C) mean ± S.E.M.; $n = 10$
	(°C)	(s)	(°C)	(s)	(°C)	(s)	
$\alpha_{1A(C)}$	95	600	60	10	72	20	90.3 ± 0.0
α_{1B}	95	600	60	10	72	20	91.0 ± 0.1
$\alpha_{1D(AD)}$	95	600	60	10	72	10	86.9 ± 0.1
α_{2AD}	95	600	65	10	72	20	89.1 ± 0.1
α_{2B}	95	600	65	10	72	20	91.3 ± 0.1
α_{2C}	95	600	65	10	72	20	93.4 ± 0.1
β_1	95	600	60	10	72	10	92.8 ± 0.0
β_2	95	600	60	10	72	10	88.8 ± 0.1
β_3	95	600	60	10	72	10	91.9 ± 0.1
UbC	95	600	62	10	72	25	87.5 ± 0.0
GAPDH	95	600	58	10	72	20	88.4 ± 0.1

Total number of cycles: 40.

were performed to gain sufficient amounts of PCR amplicons. Comparison between the sequenced PCR product and the corresponding published coding sequence resulted in a nucleotide sequence identity of at least 95–100% (HUSAR program, DKFZ, Heidelberg, Germany).

Ubiquitin (UbC) and glyceraldehyde-phosphate-dehydrogenase (GAPDH) were chosen to confirm constant housekeeping gene (hkg) expression levels in the investigated cDNA samples [29,30]. Given the possibility that these genes, too, are regulated to a certain extent, the mean value of UbC and GAPDH for each sample was chosen to gain an optimum of constant expression level.

2.4. Mathematical and statistical evaluations

mRNA expression was evaluated by amplification curve analysis of the LightCycler real-time RT-PCR. After incorporation into double stranded DNA (dsDNA), SYBR Green I (DNA binding dye) shows fluorescence emission and increases according to target amplification with cycle number. The exponential phase of the PCR becomes detectable when the fluorescence signal from accumulated PCR product is greater than the background fluorescence. To eliminate uniformative fluorescence background points, a fixed fluorescence threshold line is set to the exponential portion of the amplification curve as low as possible (in this case at a fluorescence level of 3) without including any background points. The intersection of the threshold line and the amplification curve represents the crossing point value [31].

Crossing points (CP) for each receptor (sub)type were determined at a constant fluorescence threshold line for all receptor (sub)types (fit points method; [31]). CP results are shown as raw data of the PCR amplification program of the LightCycler for each receptor (sub)type and for the hkg UbC and GAPDH. The hkg mean value of each sample was used

for a normalization of the amount of every target gene which results in the ΔCP value. The ΔΔCP value represents the difference between the ΔCP of the target gene and of the lowest expressed adrenergic receptor subtype within each receptor type (=α_{1B}, α_{2C}, β_3) or within all nine receptor (sub)types (=β_3), respectively. Relative expression levels within each receptor type are given by the arithmetic formula $2^{-\Delta\Delta CP}$ [32]. The logarithm dualis (log 2) is based on an optimum efficiency (E) of PCR which is $E = 2$ where the PCR product is replicated every cycle.

Data are presented as means ± S.E.M. For statistical evaluations the SAS program package [33] was used. Effects of the animal and the adrenergic receptor type were tested for significance ($P < 0.05$) by ANOVA using the General Linear Model (GLM) procedure. Differences between receptor subtype gene expression within each receptor type (α_1, α_2, and β) were localized with the least significance difference test (LSD).

3. Results

3.1. Confirmation of primer and PCR-product specificity

Specificity of the desired products in bovine mammary gland total RNA was documented with melting curve analysis and additionally with high resolution gel electrophoresis. Derived mean melting temperatures of PCR products were product-specific and are listed in Table 2.

3.2. Bovine α_{1B}-, $\alpha_{1D(AD)}$-, and α_{2C}-sequences

Three of the nine distinct adrenergic receptor (sub)types were previously not described as bovine nucleotide sequences: α_{1B}, $\alpha_{1D(AD)}$, and α_{2C}.

The $\alpha_{1D(AD)}$-adrenergic receptor subtype was not detectable in the bovine mammary gland on the level of mRNA expression. There was a definite product peak in the real-time

Table 3
Crossing points (CP), shown as raw data (mean ± S.E.M.; $n = 10$) of the PCR amplification program of the LightCycler for each adrenergic receptor (sub)type and for the housekeeping genes (hkg) UbC and GAPDH

Adrenergic receptor (sub)type and hkg	CP	Significance within the receptor types α_1, α_2, and β ($P < 0.05$)
$\alpha_{1A(C)}$	28.19 ± 0.45	a
α_{1B}	30.74 ± 0.51	b
α_{2AD}	27.11 ± 0.23	a
α_{2B}	31.34 ± 0.17	b
α_{2C}	32.39 ± 0.12	c
β_1	29.94 ± 0.25	b
β_2	28.51 ± 0.30	a
β_3	32.81 ± 0.42	c
UbC	19.80 ± 0.29	–
GAPDH	22.96 ± 0.21	–

(a–c): means without common letters are significantly different ($P < 0.05$).

PCR but the PCR product length was lower than required. Complete sequencing of this nucleotide fragment was not possible. A 388 bp fragment for the α_{1B}-adrenergic receptor subtype, which is 94.3, 91.0, and 89.6%, respectively, homologue with corresponding regions of human, rat and mouse nucleotide sequences was identified (Fig. 1), and a 403 bp fragment

```
bovine                               ACTTCACTGG CCCCAACCAG ACCTCGAGCA
human   51 ~~~~~~~~~~ ~~~~~~~~~~ ---------- ---------- ----------
rat     51 ~~~~~~~~~~ ~~~~~~~~~~ ---------- ---------- ----------
mouse   51 ~~~~~~~~~~ ~~~~~~~~~~ ---------- ---------- ----------

bovine     ACTCCACACT GCCTCAGCTG GACATCACCA GGGCCATCTC CGTGGGCCTG
human      ---------- ---C------ ---------- ---------- T---------
rat        ---------- ---C------ ---G------ ---------- T---------
mouse      ---------- ---C------ ---G------ ---------- T------TGT

bovine     GTGCTGGGCG CCTTCATCCT CTTTGCCATC GTGGGCAACA TTCTAGTCAT
human      ---------- ---------- ---------- ---------- -C--------
rat        ---------- ---------- ---------- ---------- -CT-G-----
mouse      ---------- ---------- ---------- ---------- -CT-G-----

bovine     CTTGTCTGTG GCCTGCAACC GTCACCTGCG GACGCCCACC AACTACTTCA
human      ---------- ---------- -G-------- ---------- ----------
rat        -C----G--- ---------- -G-------- ---------- --------T-
mouse      -C----G--- ---------- -G-------- ------A--- ----------

bovine     TCGTCAACCT GGCCATTGCC GACCTGCTGC TAAGCTTCAC CGTTCTGCCC
human      -T-------- ------G--- ---------T -G-------- ---C------
rat        ---------- ---------T ---------T -G--T----- A--A------
mouse      -T-------- ---------T ---------T -G-------- A-AC------

bovine     TTCTCTGCTG CCCTGGAGGT GCTTGGCTAC TGGGTGCTGT TCTGGATCTT
human      -----A--G- ----A----- ---C------ ---------G GGC-------
rat        -----C---A ----A--A-- ---------- ---------- -GA-TT----
mouse      -----C---A -------A-- ---C------ ---------G GGC-C-----

bovine     CTGTGACATC TGGGCCGCCG TGGATGTCCT GTGCTGCACA GCCTCCATTC
human      ---------- -----A---- ---------- ---------- --G-------
rat        ---------- -----A--G- -A-------- ------T--G --------C-
mouse      ---------- -----A--G- -T-------- ---T-----G --------C-

bovine     TAAGCCTGTG CGCCATCTCC ATCGACCGCT ACATTGGGGT GCGCTACTCT
human      -G-------- ---------- -----T---- ----C----- ----------
rat        -G-----A-- T--------- --T------- ---------- ---A------
mouse      -G-----A-- T--------- --T------- ---------- ---T------

bovine     CTGCAGTA~~ 460
human      --------~~ 460
rat        --------~~ 460
mouse      --------~~ 460
```

Fig. 1. Partial nucleotide sequence of bovine α_{1B}-adrenergic receptor subtype (new EMBL ac. no. AJ488280) and its alignment to human (EMBL ac. no. L31773), rat (EMBL ac. no. M60655), and mouse (EMBL ac. no. Y12738) α_{1B}-adrenergic receptor. Primer localisations are framed.

for the α_{2C}-adrenergic receptor subtype, which is 94.5, 93.1, and 92.3%, respectively, homologue with corresponding regions of human, rat and mouse nucleotide sequences (Fig. 2). The new nucleotide fragments have been submitted to the EMBL Nucleotide Sequence Databases and assigned the accession numbers AJ488280 (α_{1B}) and AJ488281 (α_{2C}).

```
bovine     ---------- ---------- ---------- ---------- ----TGCGCG
human 201  ---------- ---------- ---------- ---------- ----------
rat   201  ---------- ---------- ---------- ---------- ----------
mouse 201  ---------- ---------- ---------- ---------- ----------

bovine     CCCCGCAGAA CCTCTTCCTG GTGTCGCTGG CCTCCGCTGA CATCCTGGTG
human      -G--A----- ---------- ---------- ----G--C-- ----------
rat        ---------- ---------- -----T---- ----A----- ----------
mouse      ---------- ---------- -----T---- ----A----- ----------

bovine     GCCACACTGG TCATGCCCTT CTCGCTGGCC AACGAGCTCA TGGCCTACTG
human      -----G---- ---------- ----T----- ---------- ----------
rat        ---------- ---------- T--------- --T------- ----------
mouse      ---------- ---------- T-T------- --T------- ----------

bovine     GTACTTCGGG CAGGTGTGGT GCGGCGTGTA CCTGGCGCTA GACGTCCTCT
human      ---------- ---------- ---------- ---------C --T--G--G-
rat        ------T--- --A------- -T--T----- ------A--G -----G----
mouse      ---------- --A------- -T--T--A-- ------A--G -----G----

bovine     TCTGCACCTC GTCCATCGTG CACCTGTGCG CCATCAGCCT GGACCGCTAC
human      -T-------- ---G------ --T-----T- ---------- ----------
rat        ---------- ---------- --------T- ----T----- ----------
mouse      ---------- ---------- --------T- ----T--T-- ----------

bovine     TGGTCCGTGA CCCAGGCCGT CGAGTACAAC CTGAAGCGCA CGCCGCGCCG
human      -----G---- -G-------- ---------- ---------- -A--A-----
rat        -----G---- -G--A--G-- A--------- ---------- ----A-----
mouse      -----G---- -G--A--G-- A--------- ---------- ----------

bovine     CGTCAAGGCC ACCATCGTGG CCGTGTGGCT CATCTCGGCC GTCATCTCCT
human      ---------- --------C- ---------- ---------- ----------
rat        T--------- ---------- ---------- ---------T ----------
mouse      T--------- ---------- ---------- ------C--T ----------

bovine     TCCCGCCGCT AGTCTCGCTC TACCGCCGGC CCGACGGCGC CGCCTACCCG
human      ---------- G-----G--- -------A-- ---------- ----------
rat        -------T-- C-----TT-- ---------- ---------- ----------
mouse      -------T-- C------T-- ---------- ------T--- ------T---

bovine     CAGTGTGGCC TCAACGACGA GACCTGGTAC ATCCTGTCCT CCTGCAT---
human      -----C---- ---------- ---------- ---------- ---------- 650
rat        -----C---- -------T-- ---------- ---T------ ---------- 650
mouse      -----C---- -------T-- ---------- ---T------ ---------- 650
```

Fig. 2. Partial nucleotide sequence of bovine α_{2C}-adrenergic receptor subtype (new EMBL ac. no. AJ488281) and its alignment to human (EMBL ac. no. NM_000683), rat (EMBL ac. no. M58316), and mouse (EMBL ac. no. NM_007418) α_{2C}-adrenergic receptor. Primer localisations are framed.

Table 4
Relative mRNA expression of α_1-, α_2- and β_2-adrenergic receptor (sub)types and hkg ($n = 10$)

Adrenergic receptor (sub)type	ΔCP (target − mean hkg)	$\Delta\Delta$CP (ΔCP_{target} − $\Delta CP_{lowest\ expressed\ (sub)type}$)	x-fold mRNA expression ($2^{-\Delta\Delta CP}$)	$\Delta\Delta$CP (ΔCP_{target} − $\Delta CP_{lowest\ expressed\ adr.\ rec.}$)	x-fold mRNA expression ($2^{-\Delta\Delta CP}$)
$\alpha_{1A(C)}$	$+6.81 \pm 0.37^{a}$	-2.55 ± 0.37	5.86 (4.53–7.57)	-4.62 ± 0.37	24.59 (19.03–31.78)
α_{1B}	$+9.36 \pm 0.44^{b}$	0.00 ± 0.44	1.00 (0.74–1.36)	-2.07 ± 0.44	4.20 (3.10–5.70)
α_{2AD}	$+5.74 \pm 0.18^{a}$	-5.27 ± 0.18	38.59 (34.06–43.71)	-5.69 ± 0.18	51.63 (45.57–58.49)
α_{2B}	$+9.97 \pm 0.27^{b}$	-1.04 ± 0.27	2.06 (1.71–2.48)	-1.46 ± 0.27	2.75 (2.28–3.32)
α_{2C}	$+11.01 \pm 0.21^{c}$	0.00 ± 0.21	1.00 (0.86–1.16)	-0.42 ± 0.21	1.34 (1.16–1.55)
β_1	$+8.56 \pm 0.26^{b}$	-2.87 ± 0.26	7.31 (6.11–8.75)	-2.87 ± 0.26	7.31 (6.11–8.75)
β_2	$+7.13 \pm 0.17^{a}$	-4.30 ± 0.17	19.70 (17.51–22.16)	-4.30 ± 0.17	19.70 (17.51–22.16)
β_3	$+11.43 \pm 0.42^{c}$	0.00 ± 0.42	1.00 (0.75–1.34)	0.00 ± 0.42	1.00 (0.75–1.34)

(a–c): corresponding means (within one receptor type) without common superscript letters are significantly different ($P < 0.05$). Column 2: ΔCP (CP difference) presents each receptor (sub)type CP value normalized to an internal reference (mean hkg CP value). Columns 3 and 5: $\Delta\Delta$CP values are evaluated as the normalized target genes (ΔCP values) are relative to a calibrator, which is the lowest expressed receptor (sub)type within each of the three receptor types α_1, α_2 and β ($=\alpha_{1B}$, α_{2C}, β_3) (column 3), or within all adrenergic receptor (sub)types ($=\beta_3$) (column 5). Columns 4 and 6: Relative mRNA expression levels within each of the three receptor types α_1, α_2 and β (column 4) and within all nine adrenergic receptor (sub)types (column 6) are given by the arithmetic formula $2^{-\Delta\Delta CP}$ assuming that the efficiency (E) of the PCR is $E = 2$ where the PCR product is replicated every cycle ($\Delta\Delta$CP − S.E.M. and $\Delta\Delta$CP + S.E.M. are shown in brackets giving a range for the mRNA expression).

Sequence alignments (to verify a subtype specific product amplification) between the amplified product sequence and sequences already known showed high homology within one (sub)type ($\alpha_{1A(C)}$: 88–100%, α_{1B}: 88–95%; α_{2AD}: 82–100%, α_{2B}: 86–100%, α_{2C}: 84–95%; β_1: 87–100%, β_2: 87–100%, β_3: 86–99%). Sequence alignments between the amplified product sequence and sequences of other (sub)types resulted in low homology (between α_1-adrenergic receptor subtypes: 67–73%; between α_2-adrenergic receptor subtypes: 68–72%; between β-adrenergic receptor types: 55–71%).

3.3. Distribution of mRNA expression

mRNA of almost all adrenergic receptor types and subtypes classified to date could be detected. mRNA expression levels of the various receptor (sub)types are shown as uncorrected CP values in Tables 3 and 4 within each of the three receptor types α_1, α_2 and β (column 4), and within all receptor types and subtypes (column 6).

The α_{2AD}-receptor subtype shows highest expression levels not only among the α_2-receptors, followed by α_{2B} and α_{2C}, but also in comparison with all adrenergic receptor (sub)types. Within the α_1-receptor type mRNA of the $\alpha_{1A(C)}$-subtype is most frequent, followed by α_{1B}, and within the β-receptors the β_2-receptor type is most highly expressed, followed by β_1 and β_3.

On the level of mRNA expression the receptor (sub)types are distributed as follows: $\alpha_{1A(C)} > \alpha_{1B}$, $\alpha_{2AD} > \alpha_{2B} > \alpha_{2C}$, and $\beta_2 > \beta_1 > \beta_3$. Expression levels of the adrenergic receptor subtypes show noticeable differences within each of the receptor types α_1, α_2 and β (Tables 3 and 4).

4. Discussion

In this study, mRNA expression of almost all established adrenergic receptor types and subtypes were detected in the mammary gland of dairy cows. Adrenergic receptors are classified into nine adrenergic receptor types and subtypes. Within the α_1- and α_2-adrenergic receptor type there are three subtypes each: $\alpha_{1A(C)}$, α_{1B}, $\alpha_{1D(AD)}$, and α_{2AD}, α_{2B}, α_{2C}, and within the β-adrenergic receptor there are three receptor types: β_1, β_2, and β_3 [9,11].

For six of those nine receptor (sub)types, bovine nucleotide sequences had been published; for the remaining three factors (α_{1B}, $\alpha_{1D(AD)}$, and α_{2C}) human sequences were used for the primer design. The new bovine nucleotide fragments (α_{1B} and α_{2C}) could be amplified by PCR and sequenced.

For the $\alpha_{1D(AD)}$-adrenergic receptor subtype the primer design seemed to be successful looking at the real-time PCR results showing a specific product peak which was reproducible in many different mammary gland and also liver cDNAs. The length of this PCR product, however, was approximately 80 bp shorter than expected and sequencing of this fragment was only partially successful. Thus, it can be concluded that the amplified PCR product of the $\alpha_{1D(AD)}$-adrenergic receptor subtype is not the one required and, even more, that the $\alpha_{1D(AD)}$-adrenergic receptor subtype perhaps does not occur in the bovine mammary gland.

Different mRNA expression levels could be shown by variation of crossing points (CP) of all receptor (sub)types in the LightCycler RT-PCR. The CP indicates the cycle number

at which the fluorescence signal of the amplified target reaches a fixed threshold line. The higher the initial amount of genomic DNA, the sooner accumulated PCR product is detected in the PCR process, and the lower is the CP value.

The amount of target gene was normalized to an internal reference, which was the mean of the housekeeping genes UbC and GAPDH. Housekeeping genes are expected to be unaffected by experimental treatments, i.e. show constant expression levels and thus serve as control genes. In this study, UbC as well as GAPDH showed similar but not identical mRNA expression patterns in the investigated samples ($n = 10$). Working with the mean values of both housekeeping genes perhaps mirrors best a constant expression level that can be used for a normalization of the target genes.

Values of the normalized target genes (=ΔCP values) were given relative to a calibrator gene which is an arbitrary constant [34]. In this study, the lowest expressed receptor subtype within each of the three receptor types (=α_{1B}, α_{2C}, β_3) and on the other hand the lowest expressed receptor subtype of all adrenergic receptor (sub)types (=β_3) were chosen as calibrators. The resulting values are termed as $\Delta\Delta$CP as the calibrator value itself is a normalized target gene, i.e. a ΔCP value.

mRNA expression was evaluated by the formula $2^{-\Delta\Delta CP}$ assuming that the efficiency (E) of the PCR is $E = 2$, which means that the PCR amplicons were duplicated every cycle.

On the level of mRNA expression, the β_2-receptor was the most abundant β-receptor type in the bovine mammary gland. Expression levels of β_1- and β_3-receptor mRNA were not surprisingly lower than of the β_2-receptor; β_1-adrenergic receptors were described to mediate mainly cardiovascular responses to endogenous catecholamines [11]. The β_3-receptor type is the most capable adrenergic receptor for inducing lipolysis and thus is present mainly in fat tissues [35]. In earlier investigations receptor binding sites [19] as well as receptor mRNA [20] of the β_2-receptor were found in the mammary gland of cows. In receptor binding studies varying affinities of β-specific antagonists indicated a predominant β_2-receptor population and a small number of β_1-adrenergic receptors in teat muscles [16].

Distribution of α_2-adrenergic receptor subtypes-mRNA in the bovine mammary gland corresponded with earlier studies that described the α_{2AD}-adrenergic receptor subtype as the most abundant one located post- as well as presynaptically in different tissues and species [2]. Measuring mRNA-levels by Northern blot—also in different tissues and species—indicated a higher distribution of the $\alpha_{1A(C)}$- and α_{1B}-receptor subtype than of the $\alpha_{1D(AD)}$-receptor subtype [2] which was also matching with our results obtained by RT-PCR.

Mean receptor density found in teat tissue by radioligand binding studies was highest for α_2-receptors, and slightly lower for α_1-receptors [17,18]. Those earlier findings are difficult to compare with the distribution of α_1- and α_2-adrenergic receptor subtypes on the level of mRNA expression because the available ligands were not subtype selective.

In conclusion, eight of nine known adrenergic receptor types and subtypes were detected in the mammary gland of dairy cows on the level of mRNA expression despite their low abundance. Expression levels of adrenergic receptor subtypes vary considerably.

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