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α_{1c}- and β_2-adrenergic receptor mRNA distribution in the bovine mammary gland detected by competitive RT-PCR

By OLGA WELLNITZ[1]*, ANDREAS ZURBRIGGEN[2], ROBERT R. FRIIS[3], JÜRG W. BLUM[1] and RUPERT M. BRUCKMAIER[1]†‡

[1] *Institut für Tierzucht*, [2] *Institut für Tierneurologie*, [3] *Department für klinische Forschung, Universität Bern, CH-3012 Bern, Switzerland*

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Milk ejection and milk removal is considerably influenced by the sympathetic nervous system. Stimulation of α-adrenergic receptors by administration of α-adrenergic agonists inhibits alveolar milk ejection and milk removal in dairy cows due to smooth muscle contraction (Blum *et al.* 1989; Bruckmaier *et al.* 1991). However, contraction of the teat in response to α-adrenergic receptor stimulation has no influence on milk flow as long as milk is available in the cistern (Bruckmaier *et al.* 1997). Therefore, α-adrenergic stimulation causes inhibition of transport of alveolar milk into the cistern. On the contrary, the stimulation of β-adrenergic receptors facilitates milk ejection and milk removal in dairy cows (Bernabé & Peeters, 1980; Bruckmaier *et al.* 1991) because of muscle relaxation. Therefore, the distribution of α- and β-adrenergic receptors plays an important role in the milkability of dairy cows. However, from these *in vivo* studies it is not possible to distinguish between the different α_1- and α_2- and β_2-receptor subtypes owing to the non-specific nature of the pharmacological agents used.

To date, the precise tissue distribution of these different subtypes, in bovine mammary tissue, is unknown. Using molecular techniques, we were interested in the expression of genes that encode α_{1c} and β_2 as a preliminary study towards the understanding of noradrenergic receptor-gene expression and regulation in this important system.

In addition, α_{1c}- and β_2-adrenergic receptors were determined in front and rear quarters of the mammary gland to investigate differences in receptor distribution within the udder and possible relations between adrenergic receptor distribution and the higher milk flow rates in rear than in front quarters (Rothenanger *et al.* 1995).

Materials and methods

Total RNA preparation

Tissue samples ($\sim$ 100 mg) of the muscular layer of the teat (teat tissue), tissue around the gland cistern including the large mammary ducts (cisternal region) and from the proximal region containing mammary parenchyma and no visible mammary

* Present address: *Institut für Anatomie, Physiologie und Hygiene der Haustiere, Universität Bonn, D-53115 Bonn, Germany.*

† Present address: *Institut für Physiologie, Technische Universität München, Weihenstephan, D-85350 Freising, Germany.*

‡ For correspondence; e-mail: bruckmaier@weihenstephan.de

ducts (parenchyma) of one front and one rear quarter of the udder of five lactating cows were taken immediately after slaughter. Samples were collected into 1 ml TRIzol® Reagent (Life Technologies AG, CH-4019 Basel Switzerland), snap frozen in liquid nitrogen, and stored at −80 °C. Samples were thawed and total RNA was extracted according to the manufacturer's protocol. RNA was diluted in diethyl-pyrocarbonate (DEPC) treated aqua bidest. RNA was treated with 10 u RNase-free DNase (DNaseI, Roche Diagnostics GmbH, D-68305 Mannheim) for 30 min at 37 °C and afterwards for 5 min at 75 °C to inactivate the DNase. To determine the amount of final RNA preparation, the optical density was measured at 260 nm and the RNA concentration calculated accordingly.

Construction of an internal RNA reference standard

RT-PCR was performed with total RNA from udder tissue of a lactating cow. For α_{1c}-receptor sense primer 5′-CGGTCACACACTACTACATCG-3′, and antisense primer 5′-GGAGATGACCAAAGAGAGC-3′ were used. The DNA product of 299 bp was sequenced and a 100 % homology with bp 175 to 474 of the open reading frame of the bovine α_{1c}-adrenergic receptor sequence (Schwinn *et al*. 1990) was established. It was cloned into the pGEM-T Easy vector (Promega, CH-8304 Wallisellen, Switzerland) containing the promotor for T7-RNA polymerase. This product was cut within the insert with the restriction enzyme BglII, blunt-ended and ligated with a DNA fragment of 64 bp. For β_2-receptor, 5′-GACAACGCAGGACCTCCAAG-3′ sense primer and 5′-CCAGGTGATATCCACTCTGTTC-3′ antisense primer was used for RT-PCR. The PCR product of 351 bp showed a 100 % homology to the bovine β_2-adrenergic receptor sequence (bp 993 to 1343; Einspanier, 1997) and was cloned into the pGEM-T Easy vector (Promega). The product was cut within the insert with the restriction enzyme Hind*II* and ligated with a DNA fragment of 64 bp.

For use as internal standards in competitive RT-PCR, RNA was synthesized with T7-RNA polymerase from these products.

Competitive RT-PCR

RT-PCR was performed with 0·5 μg total RNA in the presence of different concentrations of standard RNA (6·5, 2·16, 0·72 and 0·24 fmol α_{1c}-receptor, 3·7, 0·462, 0·058, 0·007 and 0·0009 amol for β_2-receptor; 1 : 3 or 1 : 8 serial dilutions, respectively) using the same primer as for construction of standards. SYMBOLcDNAs were synthesized using 100 units SuperScript™ RNase H⁻ Reverse Transcriptase (Life Technologies AG) and 25 pmol primer (MWG-Biotech AG, CH-4142 Münchenstein Switzerland) at 42 °C. For PCR reaction 1·25 units Taq DNA Polymerase (Life Technologies AG) were used under the following conditions: 5 min at 94 °C, followed by 40 s at 94 °C, 40 s at 58 °C (α_{1c}-receptor) or 63 °C (β_2-receptor) and 40 s at 72 °C for 32 cycles, ending 2 min at 72 °C. PCR products were analysed on an agarose gel (1·2 %) containing 0·2 μg/ml ethidium bromide. Samples of one receptor of one cow (front and rear quarter) were loaded simultaneously on one agarose gel. Mass of gel bands (299 bp-band for wild-type and 363 bp-band for internal standard in case of α_{1c}-receptor or 351 bp-band for wild-type and 415 bp-band for standard in case of β_2-receptor) were analysed with the ImageQuaNT™-System (Molecular Dynamics GmbH, D-47807 Krefeld Germany). The ratio of the masses of DNA-bands of the wild-type and standard DNA were plotted (ordinate) against the input of standard (abscissa). The point where the ratio was equal to 1 was taken as the concentration of wild-type receptor mRNA present. This concentration was corrected to allow for differences in the incorporation of ethidium bromide due to the different lengths of

Table 1. *The distribution of adrenergic receptors in mammary tissue*

(Values are amol $\times 10^{-3}$ (α_{1c}-adrenergic receptor) or amol (β_2-adrenergic receptor) mRNA/μg total RNA; means $\pm$ SEM for $n = 5$)

	Teat tissue	Cisternal region	Parenchyma
α_{1c}-adrenergic receptor			
front	$26{\cdot}4 \pm 8{\cdot}4^{A}$	$10{\cdot}0 \pm 3{\cdot}7^{AB}$	$4{\cdot}3 \pm 1{\cdot}6^{B}$
rear	$17{\cdot}3 \pm 6{\cdot}0^{AB}$	$14{\cdot}1 \pm 6{\cdot}3^{AB}$	$13{\cdot}9 \pm 9{\cdot}1^{AB}$
average	$21{\cdot}9 \pm 5{\cdot}1^{A}$	$11{\cdot}8 \pm 3{\cdot}3^{AB}$	$9{\cdot}7 \pm 5{\cdot}1^{B}$
β_2-adrenergic receptor			
front	$0{\cdot}9 \pm 0{\cdot}3^{A}$	$1{\cdot}3 \pm 0{\cdot}6^{AB}$	$0{\cdot}3 \pm 0{\cdot}1^{B}$
rear	$2{\cdot}0 \pm 0{\cdot}7^{AB}$	$1{\cdot}6 \pm 0{\cdot}5^{AB}$	$0{\cdot}2^{AB}$ (nd)
average	$1{\cdot}5 \pm 0{\cdot}4^{A}$	$1{\cdot}4 \pm 0{\cdot}4^{AB}$	$0{\cdot}3 \pm 0{\cdot}1^{C}$

^{A,B,C} Means without common superscript letters were significantly different: $P < 0{\cdot}05$.
nd, not detectable in 4 cows.

DNA (Menzo *et al*. 1992) of standard and wild-type PCR product, by dividing by 0·8 (299 bp/363 bp) for α_{1c}-receptor or by 0·85 (351 bp/415 bp) for β_2-receptor. The results are presented as mRNA concentration/μg total RNA.

To show that the RNA preparations were free from DNA, PCR was performed on each sample without reverse transcription.

Statistical evaluation

Data are presented as means $\pm$ SEM. For statistical evaluations the SAS program package, release 6·1 (SAS, 1997) was used. The mRNA concentrations in the different regions of the udder were tested for significance ($P < 0{\cdot}05$) using the General Linear Model procedure. The model $Y_{ijk} = \text{general mean} + \text{animal}_i + \text{region}_j + \text{residual error}_{ijk}$ was used. The same model was used to test differences between front and rear quarters.

RESULTS

The results presented in Table 1 show that α_{1c}-adrenergic receptor mRNA could be detected in all regions of the bovine udder (teat tissue, cisternal region and parenchyma). Similarly, the expression of β_2-adrenergic receptor mRNA was detected in the teat tissue and the cisternal region of the udder (Table 1, Fig. 1). However, in the parenchyma of the rear quarters of four cows β_2-adrenergic receptor mRNA could not be detected. DNA was not detectable in all total-RNA preparations. The α_{1c}-adrenergic receptor mRNA concentrations were 1000-fold ($\times 10^3$) those of β_2-adrenergic receptor mRNA in all three regions of the udder.

The concentration of the α_{1c}-adrenergic receptor mRNA/μg total RNA was lower ($P = 0{\cdot}09$) in the cisternal region than in teat tissue and was lower ($P < 0{\cdot}05$) in the parenchyma than in teat tissue. There were no significant differences between front and rear quarters. The statistical significance of the effect of animal was shown in the analysis of variance as $P = 0{\cdot}06$.

The concentration of the β_2-adrenergic receptor mRNA/μg total RNA was lower ($P = 0{\cdot}06$) in the cisternal region than in the teat tissue and lower ($P < 0{\cdot}05$) in the parenchyma than in teat tissue and cisternal region. There were no significant differences between front and rear quarters, but individual differences were significant. The statistical significance of the effect of animal was shown in analysis of variance as $P = 0{\cdot}08$.

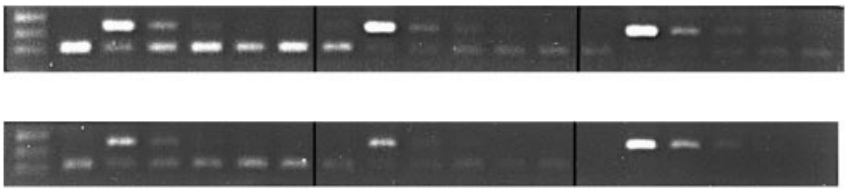

Fig. 1. Ethidium bromide stained Agarose gel electrophoresis of competitive RT-PCR products for β_2-adrenergic receptor of one representative cow. In the first lane in both pictures a DNA marker is visible. Receptor-mRNA within 0·5 μg total-RNA of teat tissue, cisternal region and parenchyma of the udder in series of one front (upper picture) and one rear quarter (picture below) was cotranscribed and coamplified with different amounts of standard-RNA (0, 3·70, 0·462, 0·58, 0·007 and 0·009 amol). The top DNA bands (415 bp) are products of the standard-RNA, the bottom DNA bands (351 bp) are products of the wild-type β_2-adrenergic receptor mRNA. In this example no β_2-adrenergic receptor mRNA could be detected in parenchyma of the rear quarter.

DISCUSSION

α_{1c}- and β_2-adrenergic receptor mRNA expression could be detected by RT-PCR in different regions of the bovine udder. The synthesized RNA reference standards were suitable for competitive RT-PCR.

The highest α_{1c}- and β_2-adrenergic receptor mRNA concentrations were found in teat tissue, whereas the lowest α_{1c}- and β_2-adrenergic receptor mRNA concentrations were found in the parenchyma. These results correspond with a previous investigation on the overall protein level based on radioligand binding studies, where highest α_1-, α_2- and β_2-adrenergic receptor concentrations were found in the muscular layer of the bovine teat with decreasing binding in the cisternal region, and the parenchyma tissue (Hammon *et al.* 1994; 126 ± 25, 39 ± 6 and 8 ± 3 fmol maximal binding capacity of ^{3}H-Prazosin/mg protein for α_1-adrenergic receptor, and 123 ± 21, 119 ± 22 and 24 ± 5 fmol maximal binding capacity of ^{3}H-Dihydroalprenolol/mg protein for β_2-adrenergic receptor, respectively).

In studies of receptor density at the protein level, there were almost no differences between α_1-, α_2- and β_2-adrenergic receptor binding (Hammon *et al.* 1994). However, at the RNA level the expression of α-adrenergic receptor gene was 1,000-fold ($\times 10^3$) that of the expression of the β-adrenergic receptor gene. This might be the consequence of the shorter half life of the α-adrenergic receptor protein than that of the β-adrenergic receptor protein. The α_{1c}-receptor is only one subtype of the α_1-adrenergic receptors (Zhong *et al.* 1999) and it may be that other subtypes have a different turnover and half life. For β_2-adrenergic receptors no subtypes are known to our knowledge.

Stimulation of α-adrenergic receptors inhibits (Bruckmaier *et al.* 1991), whereas stimulation of β-adrenergic receptor facilitates (Bernabé & Peeters, 1980; Hamann *et al.* 1980; Vandeputte-Van Messom, 1986) milk ejection and, therefore, milk removal. Milk ejection occurs mostly in the proximal region where milk is stored in the alveoles and is ejected into the milk ducts by contraction of the myoepithelial cells. This underlines that the adrenergic system cannot have a great influence on milk ejection directly, because lowest adrenergic receptor concentrations and receptor mRNA concentrations were found in this region (parenchyma). Additionally, contraction of the teat by α-adrenergic receptor stimulation does not have any influence on milk flow as long as milk is present in the cistern (Bruckmaier *et al.* 1991, 1997). Consequently, adrenergic receptors influence milk removal in the ductular system of the udder.

The relationship between milkability and adrenoceptor concentrations in the teat was described earlier by Roets *et al.* (1986). Distribution of α_{1c}- and β_2-adrenergic receptors was not different between front and rear quarters. Thus the higher milk flow rate in rear quarters (Rothenanger *et al.* 1995) is not related to differences of adrenergic receptor-gene expression. The equal distribution of α_{1c}- and β_2-adrenergic receptor mRNA between front and rear quarters might be linked with the density of the receptors in the total body. Therefore, receptor density in other regions of the body, such as on blood cells, mirror receptor density in the udder and are therefore related to the milkability, as described by Roets *et al.* (1995).

In conclusion, competitive RT-PCR is an excellent method for investigation of the physiological regulation of milk ejection by the sympathetic nervous system. The different expression levels of α_{1c}- as compared with β_2-adrenergic receptors within the bovine udder elucidates that the sympathetic nervous system influences milk removal not by modification of milk ejection, but inhibition or stimulation by α_1- or β_2-adrenergic receptors of the milk flow in the region of the large ducts and the gland cistern.

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REFERENCES

Bernabé, J. & Peeters, G. 1980 Studies on the motility of smooth muscles of the teats in lactating cows. *Journal of Dairy Research* **47** 259–275

Blum, J. W., Schams, D. & Bruckmaier, R. 1989 Catecholamines, oxytocin and milk removal in dairy cows. *Journal of Dairy Research* **56** 167–177

Bruckmaier, R. M., Mayer, H. & Schams, D. 1991 Effects of α- and β-adrenergic agonists on intramammary pressure and milk flow in dairy cows. *Journal of Dairy Research* **59** 411–419

Bruckmaier, R. M., Wellnitz, O. & Blum, J. W. 1997 Inhibition of milk ejection in cows by oxytocin receptor blockade, α-adrenergic receptor stimulation and in unfamiliar surroundings. *Journal of Dairy Research* **64** 315–325

Einspanier, Ralf 1997 EMBL/GeneBank/DDBJ databases, accession Z86037

Hamann, J. & Tolle, A. 1980 Comparison between manual and mechanical stimulation. *Milchwissenschaft* **35** 271–273

Hammon, H. M., Bruckmaier, R. M., Honegger, U. E. & Blum, J. W. 1994 Distribution and density of α- and β-adrenergic binding sites in the bovine mammary gland. *Journal of Dairy Research* **61** 47–57

Menzo, S., Bannarelli, P., Giacca, M., Manzin, A., Varaldo, P. E. & Clementi, M. 1992 Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction. *Journal of Clinical Microbiology* **30** 1752–1757

Roets, E., Burvenich, C. & Hamann, J. 1995 Relationship between numbers of α_2- and β_2-adrenoceptors on blood cells of bulls and milkability of their daughters. *Journal of Dairy Research* **62** 567–575

Roets, E., Vandeputte-Van Messom, G. & Peeters, G. 1986 Relationship between milkability and adrenoceptor concentrations in teat tissue in primiparous cows. *Journal of Dairy Science* **69** 3120–3130

Rothenanger, E., Bruckmaier, R. M. & Blum, J. W. 1995 Association and dissociation of single quarter and total milk flow in dairy cows: effects of milking with and without pre-stimulation. *Milchwissenschaft* **50** 63–67

SAS 1997 *SAS/STAT User's Guide*, release 6.12. Cary, NC: SAS Institute

Schwinn, D. A., Lomasney, J. W., Lorenz, W., Szklut, P. J., Fremeau, R. T. Jr., Yang-Feng, T. L., Caron, M. G., Lefkowitz, R. J. & Cotecchia, S. 1990 Molecular cloning and expression of the cDNA for a novel alpha-1-adrenergic receptor subtype. *Journal of Biological Chemistry* **265** 8183–8189

Vandeputte-Van Messom, G., Roets, E. & Peeters, G. 1986 Is milk flow rate associated with specific adrenoceptor pattern in the cow's teat? *Archives internationales de physiologie et de biochimie* **94** 33

Zhong, H. & Minneman, K. P. 1999 α_1-Adrenoceptor subtypes. *European Journal of Pharmacology* **375** 261–276