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Molecular cloning of a bovine MSH receptor which is highly expressed in the testis

Mirko Vanetti[a], Christiane Schönrock[b], Wolfgang Meyerhof[b], Volker Höllt[c,*]

[a]*Physiologisches Institut, Ludwig Maximilian Universität München, Munich, Germany*
[b]*Institut für Zellbiochemie und klinische Neurobiologie, Universität Hamburg, Hamburg, Germany*
[c]*Institut für Pharmakologie und Toxikologie, Otto von Guericke Universität, Leipzigerstraße 44, Magdeburg, Germany*

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Abstract

A novel G protein-coupled receptor (BDF3) was isolated from bovine genomic DNA by a combined approach of polymerase chain reaction (PCR) and hybridization techniques. The predicted amino acid sequence is 317 amino acids in length and displays 80% homology to the human α-MSH receptor MC1. Stably transfected into CHO-K1 cells, BDF3 mediates an increase of intracellular cAMP-levels following incubation with NLE-α-MSH, a potent α-MSH analog. The stimulation with $ACTH_{1-10}$ is only moderate and γ-MSH is ineffective. Northern blot analysis of bovine tissues revealed that the BDF3 gene is higly expressed in the testis as a single 2.3 kb mRNA species, suggesting an involvement of the BDF3 receptor in spermatogenesis.

Key words: G-protein coupled receptor; α-MSH; POMC; Testis; Adenylate cyclase; PCR; spermatogenesis

1. Introduction

Proopiomelanocortin (POMC) is a precursor for β-endorphin, ACTH and MSH occurring in the pituitary, in the brain and in the periphery [1]. Tissue-specific proteolytic cleavage of POMC leads to a cell specific mixture of bioactive peptides called melanocortins displaying a wide array of biological effects. Beside the known actions on glucocorticoid production in the adrenals and melanocyte growth and pigmentation, melanocortins have been shown to affect learning, attention and memory [2], analgesia [3], thermoregulation [4] and release of PRL and LH from the pituitary [5]. Little is known about the role of melanocortins in the periphery. However, melanocortins which have been found in testis [6,7] are supposed to act in a paracrine/autocrine way as growth factors and to play a role in maintaining testis cell function and differentiation [8]. MSH and ACTH act through G protein-coupled receptors resulting in an increase of adenylate cyclase activity [9], increase of intracellular calcium and activation of protein kinase C [10]. Four subtypes (MC1–MC4) belonging to the melanocortin receptor subfamily have been cloned from human and mouse tissues [11–14]. However, one or two more members of this melanocortin subfamily are predicted from Southern blot analysis [11]. Here we report the cloning and stable expression in CHO-K1 cells of the bovine melanocortin receptor BDF3 which is expressed in large amounts in bovine testis.

*Corresponding author. Fax: (49) (391) 67 34 40.

2. Materials and methods

2.1. Polymerase chain reaction, cloning and sequencing

Fragments of putative G protein-coupled receptors between transmembrane domain (TM) III and VI were cloned as follows: 100 ng of bovine genomic DNA (Stratagene, Heidelberg, Germany) was amplified in a total volume of 50 ml containing 50 mM KCl, 10 mM Tris pH 8.3, 2 mM $MgCl_2$, 200 mM dNTPs, 0.01% gelatine, 25 ng each degenerate primer against TM III and VI [15] and 1 unit Taq I Polymerase (Perkin-Elmer, Überlingen, Germany) by 60 cycles of PCR. Cycles conditions were 1 min 30 s 95°C, 2 min 55°C and 3 min 72°C with additional 6 s of extention time each cycle of PCR. PCR products were purified with Primerase colums (Stratagene), blunted with Klenow polymerase and cloned into pBluescriptSK$^-$ (Stratagene). Sequencing of the cloned DNA was performed by the chain termination method [16] with a Sequenase 2.0 kit (USB, Bad Homburg, Germany).

2.2. Library screening and subcloning

Clone 517-3 was ^{32}P-labelled by the random primer technique [17] and used as a probe to screen a bovine genomic library constructed in phage λDASH II (Stratagene, Heidelberg, Germany). Hybridization conditions were 16 h at 37°C in 20% formamide/5 × SSC/5 × Denhart's solution/1% SDS and 100 mg/ml salmon sperm DNA. Filters were washed 30 min at 42°C in 2 × SSC/1% SDS and 15 min at 60°C in 0.5 × SSC/1% SDS. Out of 5×10^5 phage clones screened, two identical clones containing ~ 20 kb inserts were isolated and verified by Southern blotting [18]. The DNA region of interest was subcloned into pBluescriptSK$^-$ by a one-step procedure: 500 ng DNA of the genomic clone were cleaved with *Hin*dIII together with 250 ng DNA of the recipient vector pBluescriptSK$^-$. The enzyme was heat-inactivated at 70°C for 10 min, the DNA precipitated, resuspended and ligated o/n at 22°C. Bacterial clones containing 1.9 kb *Hin*dIII fragments (pBDF3) were detected by hybridization using clone 517-3 as a probe.

2.3. Stable expression of BDF3 receptors in CHO-K1 cells

A 1180 bp *Eco*RI/*Stu*I fragment of pBDF3 was subcloned into expression vector pcDNA I (Invitrogen, San Diego, USA) at *Eco*RI/*Eco*RV sites. 2 μg of the derived expression plasmid pBDF3-2E was contransfected with 0.1 μg of the plasmid pcDNA I neo (Invitrogen) into CHO-K1 cells (ATCC, MD, USA)) using Lipofectin (BRL, Eggenstein, Germany). Stable transfectants were selected in NUT-F12 medium containing 400 μg/ml G418 (BRL) and 10% foetal calf serum (FCS). Clones expressing the highest levels of specific mRNA were

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selected and tested for cAMP-accumulation following incubation of the cells with melanotropic peptides.

2.4. cAMP assay

Accumulation of cAMP was measured as follows: 1.5×10^5 stably transfected CHO-K1 cells were seeded in 22 mm twelve-well dishes with NUT-F12 medium containing 10% foetal calf serum. The cells were then washed twice with serum-free NUT-F12 medium (SFM) and pretreated with SFM containing 200 μM isobutyl-methyl-xanthine for 30 min at 37°C. Thereafter, the melanotropic peptides [Nle^4,D-Phe^7]-α-MSH, γ-MSH and ACTH$_{1-10}$ (Saxon, Hannover, Germany) were added in a concentration of 10^{-6} M and the incubation continued for 30 min at 37°C. The reaction was terminated by removing the medium, scraping and sonicating the cells in 1 ml ice-cold HCl/EtOH (1 vol 1 N HCl/100 vol EtOH). After evaporation of the solution, the residue was dissolved in TE buffer and the cAMP content measured by a radioassay kit (Amersham, Braunschweig, Germany).

2.5. RNA preparation and Northern blot analysis

Total RNA was isolated by the acid guanidinium isothiocyanate method [19]. Poly(A) RNA was isolated from total RNA using the QuickPrep mRNA purification kit from Pharmacia (Freiburg, Germany). Aliquots of 10 μg poly(A) RNA were denatured by glyoxal, separated by electrophoresis, transferred to Nytran membranes (Schleicher and Schuell, Dassel, Germany) and hybridized under high-stringency conditions according to standard methods [18] with a [^{32}P]UTP-labeled cRNA probe transcribed from clone 517–3. Washing conditions were 30 min at 50°C in 1 × SSPE/0.1% SDS. Nylon membranes were exposed to X-ray films for 3–72 h.

3. Results and discussion

Bovine genomic DNA was subjected to PCR amplification using degenerate primers to transmembrane domain III and VI of G protein-coupled receptors [15]. PCR products were cloned and sequenced. Out of 40 clones analyzed, clone 517–1 of 450 bp was found to be the bovine variant of the orphan receptor dRDC4 previously cloned from dog [15], and clone 517–40 of 500 bp was identical to the bovine β_2 adrenergic receptor [21]. In addition, 9 clones were found to encode fragments of novel putative G protein-coupled receptors.

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1   GCTTGGGGCCATGCCTGGGCCGACATTTGTCCAGCCAGGGAGGGGAGGTGTGAGGCCCCTCCCAGGGGAGCCATGAGTTGAGCAGGACCCT   91

                                          M  p  a  l  g  s  Q  r  r  L  l  G  S  L     14
GAGAGCAAGCACCCCTTCCTGCTCCCTGCGGGACG ATG CCT GCA CTT GGC TCC CAG AGG CGG CTG CTG GGT TCC CTT   168

N# c  t  p  p  a  t  l  p  f  t  L  A  p  N# r  t  g  p  q  C  L  e     37
AAC TGC ACG CCC CCA GCC ACC CTC CCC TTC ACC CTG GCC CCC AAC CGG ACG GGG CCC CAG TGC CTG GAG   237

v  S  s  l  D  G  L  F  L  S*  L  G  L  V  S  L  V  E  N  V  L  V  V     60
GTG TCA TCC CTG GAC GGG CTC TTT CTC AGC CTG GGG CTG GTG AGT CTC GTG GAG AAC GTG CTG GTA GTG   306

a  a  I  a  K  N  R  N  L  H  S  P  M  y  Y  F  I  C  C  L  A  V  S     83
GCT GCC ATT GCC AAG AAC CGC AAC CTG CAC TCC CCC ATG TAC TAC TTT ATC TGC TGC CTG GCT GTG TCT   375

D  L  l  V  S  v  s  n  V  L  E  T  a  v  m  p  L  L  E  a  G  V  L     106
GAC TTG CTG GTG AGC GTC AGC AAC GTG CTG GAG ACG GCA GTC ATG CCG CTG CTG GAG GCC GGT GTC CTG   444

a  t  q  a  A  V  v  Q  Q  L  D  N  v  I  D  V  l  i  C  G  S  M  v     129
GCC ACC CAG GCG GCC GTG GTG CAG CAG CTG GAC AAT GTC ATC GAC GTG CTC ATC TGC GGA TCC ATG GTG   513

S  S  L  C  F  L  G  a  I  A  v  D  R  Y  I  S*  I  F  Y  A  L  R  Y     152
TCC AGC CTC TGC TTC CTG GGT GCC ATT GCT GTG GAC CGC TAC ATC TCC ATC TTC TAC GCC CTG CGG TAC   582

H  S  v  V  T*  l  P  R  A  W  R  I  i  a  a  I  W  v  a  S  i  l  t     175
CAC AGT GTT GTG ACA CTG CCC CGA GCG TGG AGG ATC ATT GCG GCC ATC TGG GTG GCC AGC ATC CTC ACC   651

S  l  L  F  I  t  Y  Y  n  H  k  v  i  L  L  C  L  V  g  l  F  i  A     198
AGC CTG CTC TTC ATC ACC TAC TAC AAC CAC AAG GTC ATC CTG CTG TGC CTC GTT GGC CTC TTC ATA GCT   720

M  L  a  L  M  A  v  L  Y  v  H  M  l  a  R  A  C  Q  H  a  r  G  I     221
ATG CTG GCC CTG ATG GCC GTC CTC TAC GTC CAC ATG CTG GCC CGG GCC TGC CAG CAT GCC CGG GGC ATT   789

A  r  L  q  K  R  q  R  p  i  h  Q  G  F  G  L  K  G  A  a  T  L  T     244
GCC CGG CTC CAG AAG AGG CAG CGC CCC ATT CAT CAG GGC TTT GGC CTC AAG GGC GCT GCC ACC CTC ACC   858

I  L  L  G  v  F  F  L  C  W  G  P  F  F  L  H  L  s  L  I  V  L  C     267
ATC CTG CTG GGC GTC TTC TTC CTC TGC TGG GGC CCC TTC TTC CTG CAC CTC TCG CTC ATC GTC CTC TGC   927

P  Q  H  P  T  C  G  C  I  F  K  N  F  N  L  F  L  a  L  i  i  c  n     290
CCC CAG CAC CCC ACC TGT GGC TGC ATC TTC AAG AAC TTC AAC CTC TTC CTG GCC CTC ATC ATT TGC AAC   996

a  i  v  D  P  L  I  Y  A  F  r  S*  Q  E  L  R  k  T*  L  Q  E  V  L     313
GCC ATT GTG GAC CCC CTC ATC TAT GCC TTC CGC AGC CAG GAG CTC CGG AAG ACG CTC CAA GAG GTG CTG   1065

q  C  S*  W  OPA                                                                    317
CAG TGC TCC TGG TGA GGGTGGCAGTGCCGTCGTGTGCCCCAGGCCTGTGAGGCCAGGGCAGTCCCTTGACAAAGAGGATCGGCTAG  1151
ACCATCCCTGAAGGTGAGGGTGCACAGGCCT                                                      1182
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Fig. 1. Nucleotide and predicted amino acid sequence of the *Eco*RI/*Stu*I fragment encoding the bovine receptor BDF3. The presumptive transmembrane domains (I–VII) are underlined and are assigned on the basis of a hydrophobicity plot [27]. Amino acid residues of BDF3 that differ from human and murine MSH (MC1) receptors [11] are indicated with small letters. Potential N-linked glycosylation sites at the N-terminal region (N#), phosphorylation sites at the C-terminal region and cytoplasmatic loops (S* and T*) and a potential palmitoylation site at the C-terminus (C) are indicated.

Match %/aa	BDF3	hMC1	hMC2	hMC3	hMC4
BDF3	100/317				
hMC1	80/317	100/317			
hMC2	40/288	40/290	100/297		
hMC3	50/295	47/305	48/283	100/360	
hMC4	54/272	50/292	48/285	63/285	100/333

Fig. 2. Alignment of amino acid sequences of the bovine receptor BDF3 and other members of the melanocortin receptor subfamily cloned from man [14]. Identical amino acid residues are boxed. The positions of transmembrane regions (I–VII) are indicated by bold lines over the sequences.

One of these fragments, clone 517–3, was used to isolate from a bovine genomic library a 1.9 kb *Hin*dIII fragment named BDF3 with an open reading frame of 951 bp encoding a protein of 317 amino acids (Fig. 1). Hydrophobicity analysis revealed seven highly hydrophobic regions characteristic of G protein-coupled receptors. The predicted protein contains two potential N-linked glycosylation sites (Asn^{15} and Asn^{29}) at the N-terminus, seven potential phosphorylation sites [20] at the intracellular loops and C-terminus (Ser^{71}, Ser^{145}, Ser^{154}, Thr^{157}, Ser^{302}, Thr^{308} and Ser^{316}) and a conserved Cys^{315} at the C-terminus which serves as a potential palmytoylation site and probably anchors the C-terminus at the cell membrane [22].

The bovine receptor BDF3 has a very short C-terminus and lacks some amino acid residues which are conserved among most members of the G protein-coupled receptors [23]: thus, the cystein residue at the first extracellular loop is missing, thought to form a disulfide bond with a cystein residue at the second extracellular loop; moreover, conserved prolin residues at transmembrane domain (TM) IV and V are missing and the amino acid motif NP--Y at TM VII which is strongly conserved in all cloned G protein-coupled receptors is replaced by the motif DP--Y which occurs also in the human thrombin receptor and the RH3 and RH4 opsins from fruit flies [24].

A homology search on an EMBL protein database reveals the highest similarity of BDF3 to MC1, the α-MSH receptor recently isolated from human melanoma cells [11,12], and to the murine MSH-R [11] indicating that the bovine receptor BDF3 is closely related to this subtype of the melanocortin subfamily. The homology between the bovine BDF3 receptor and other

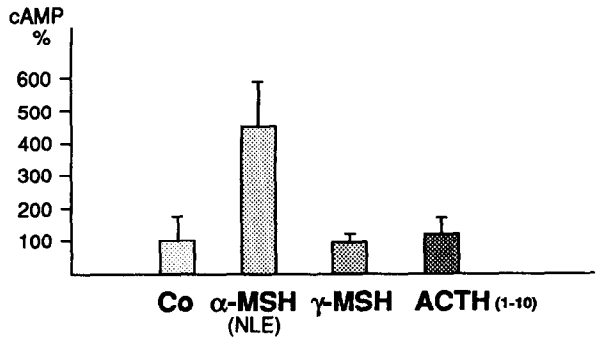

Fig. 3. cAMP accumulation in CHO-K1 cells expressing the bovine receptor BDF3 following stimulation with melanotropic agonists. Cells stably expressing the bovine receptor BDF3 were treated for 30 min with serum-free medium containing 200 μM isobutyl-methyl-xanthine to inactivate cellular phosphodiesterase activities and cAMP accumulation was measured in the absence (CON) or presence of 10^{-6} M [Nle^4,D-Phe^7]-α-MSH (NLE-α-MSH), γ-MSH and $ACTH_{1-10}$ for a further 30 min. Values are means of triplicate determinations and expressed in percent of control. Wild-type CHO cells did not respond to any of the peptides (data not shown).

members of the human melanocortin family [14] is weaker, as depicted in Fig. 2.

Two CHO cell clones, stably expressing high levels of BDF3 mRNA were tested for cAMP accumulation following exposure to melanotropic peptides (Fig. 3). Consistent with previous reports of cloned human MSH receptor stably transfected into HEK 293 cells [11], treatment of transfected cells with 10^{-6} M [Nle^4,D-Phe^7]-α-MSH [25] a potent α-MSH analog produced a 4,6-fold increase of intracellular cAMP level, whereas γ-MSH was not effective. However, in contrast to the human MSH receptor, the elevation of cAMP levels following $ACTH_{1-10}$ was only moderate. Thus, these results, as well as the homology to the human α-MSH receptor confirm that BDF3 is a bovine α-MSH receptor.

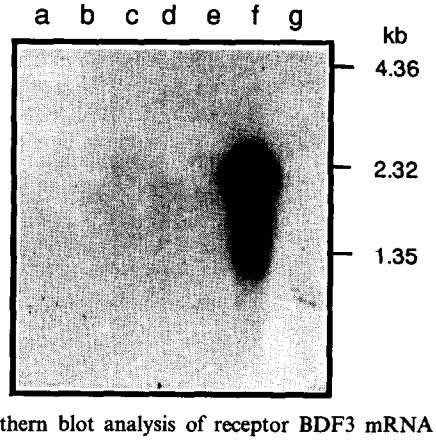

Fig. 4. Northern blot analysis of receptor BDF3 mRNA in bovine tissues. 10 μg aliquots of poly(A) RNA were subjected to electrophoresis, blotted on nylon membranes and hybridized with a ^{32}P-labelled 517-3 cRNA probe. (a) kidney (medulla), (b) brain, (c) endometrium (day 0), (d) thymus, (e) thyreoidea, (f) testis, (g) corpus luteum.

Northern blot analysis revealed that high levels of BDF3 mRNA are present in bovine testis, but none in kidney (medulla), endometrium, thyreoidea and corpus luteum. Faint signals were seen in brain and thymus (Fig. 4). The occurrence of POMC mRNA and POMC-derived peptides in testis [6,7] and the ability of ACTH/MSH to stimulate cell growth in cultured Sertoli cells [26], suggests that melanocortins may be involved in the maintenance of spermatogenesis in the adult testis and may explain the high level of BDF3 receptor mRNA in this tissue.

In contrast, no transcripts for MSH receptors could be detected in human testis [11]. Future experiments should be done to find out if this reflects a real species-specific difference or may be due to age-dependent testicular activity.

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