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RHEINISCHE FRIEDRICH-WILHELMS-UNIVERSITÄT BONN

Institut für Pharmakologie und Toxikologie

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PHARMACOLOGICAL CHARACTERIZATION OF THE MAS-RELATED G PROTEIN-COUPLED RECEPTOR D

Diploma thesis

Supervisors: Dr. Kristina Hoffmann

PharmDr. Jan Zitko, Ph.D.

Hradec Králové 2015 Michaela Navrátilová

UNIVERZITA KARLOVA V PRAZE

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FARMAKOLOGICKÁ CHARAKTERIZACE MRGPRD

Diplomová práce

Vedoucí diplomové práce: Dr. Kristina Hoffmann

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Hradec Králové 2015

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Abstract

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Title of diploma thesis: PHARMACOLOGICAL CHARACTERIZATION OF THE MAS-RELATED G PROTEIN-COUPLED RECEPTOR D

The human MRGPRD (Mas-related G protein-coupled receptor D) belongs to the big family of GPCRs (G Protein-coupled receptors). Signaling pathways mediated by GPCRs regulate a high number of vital body functions and approximately 30 % of all modern clinical drugs target GPCRs (Overington *et al*., 2006). The MRGPR subfamily was discovered 10 years ago and still remains mainly unexplored and considered "orphan" (Solinski *et al*., 2014).

Several ligands such as β-alanine, GABA, β-aminobutyric acid (Shinohara *et al*., 2004; Ajit *et al*., 2010; Uno *et al*., 2012), angiotensin and alamandine (Gembardt *et al*., 2008; Lautner *et al*., 2013) are able to bind to the human MRGPRD.

The hMRGPRD is specifically expressed in the primary sensory trigeminal ganglia neurons. Activation of the receptor by β-alanine has shown to elicit pruritogenic sensation and to contribute to normal mechanical and thermal pain thresholds. The restricted expression pattern suggests that the hMRGPRD could be a new specific target for the development of antinociceptive drugs.

In the present study, I tested 16 compounds (including β-alanine) to find out whether they can activate the hMRGPRD receptor. The strategy involved searching for ligands by monitoring signaling transduction pathways of the hMRGPRD, such as changes in cellular cAMP production by the activation of Gi-proteins or Gq-mediated changes in Ca^{2+} - related signaling in CHO Flp-In cells stably expressing the recombinant hMRGPRD receptor. As a positive control, β-alanine was used to activate the receptor protein.

In experiments, measuring Gi-mediated changes in cellular cAMP formation, β-alanine caused an inhibition of forskolin-induced cAMP production by 36.63 %. 10 of the 16 tested compounds showed no effect, but (*R)*-3-amino-2-methylpropionic acid and also its diastereomer *(RS)*-3-amino-2-methylpropionic acid caused an inhibition of forskolin induced cAMP production by 41. 31 % and 43.11 %, respectively. In contrast, 3-aminopropan-1-ol increased intracellular cAMP levels (56.1 %) and showed the same effect also in nontransfected CHO Flp-In cells, suggesting this effect to be nonspecific. 1 mM concentration of 1-(3-piperidinopropyl)piperazin also caused a nonspecific 34.4 % increase of forskolin-induced cAMP production.

For the measurement of Gq-mediated effects, the Nuclear Factor of Activated T-cells (NFAT)-driven luciferase reporter gene assay was used. β-alanine was again used as a positive control in these experiments, causing an increase of the luciferase activity by 28.00 %. The addition of 1-(3-piperidinnopropyl)piperazin caused an increase in luciferase activity by 99.40 %. Fenpropidin [100 μM] caused 57.6 % increase in RLU and 1 mM concentration of this compound showed an inhibition of 56.1 %. (*R)*-3-amino-2-methylpropionic acid was also tested in this assay and increased the luciferase activity by 29.40 %.

Abstrakt

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Název diplomové práce: Farmakologická charakterizace MRGPRD

MRGPRD (Mas-příbuzný receptor spřažený s G proteinem) patří do velké skupiny GPCR (Receptory spřažené s G proteinem). Signální dráhy zprostředkované GPCRs regulují velký počet životních funkcí lidského těla a zhruba 30 % všech moderních klinicky používaných léčiv cílí právě na tyto receptory (Overington *et al*., 2006). MRGPR podskupina byla objevena před deseti lety, je stále do velké míry neprobádána a označena za „sirotčí". (Solinski *et al*., 2014).

Několik ligandů jako β-alanin, GABA, β-aminobutyrová kyselina (Shinohara *et al*., 2004; Ajit *et al*., 2010; Uno *et al*., 2012), angiotensin a almandin (Gembardt *et al*., 2008; Lautner *et al*., 2013) mají schopnost se vázat na lidský MRGPRD.

MRGPRD je specificky exprimován pouze v neuronech primárních senzorických trojklaných ganglií. Aktivace tohoto receptoru β-alaninem způsobila snížení pruritogenní citlivosti a přispěla k normalizaci prahu pro vnímání mechanických a tepelných stimulů. Fakt že se MRGPRD nacházejí v rámci centrálního systému pouze v periferních gangliích spolu s faktem, že jejich vazebné místo je velmi strukturně specifické, nám dávají naději, že by MRGPRD mohl sloužit jako dobrá cílová struktura pro vývoj nového specifického antinociceptivího léku s minimálním množstvím nežádoucích účinků.

V mé práci jsem testovala 16 sloučenin (včetně β-alaninu), abych zjistila, jestli mohou aktivovat MRGPRD receptor. Moje strategie zahrnovala hledání ligandů monitorováním dvou signálních cest. V první z nich jsem měřila změny v buněčné

cAMP produkci způsobené aktivací Gi-proteinem a v druhé Gq-zprostředkované změny v Ca^{2+} závislých signálních drahách. K měření jsem používala CHO Flp-In buňky, které stabilně exprimovaly testovaný receptor. Jako pozitivní kontrolu jsem používala β-alanin u kterého je jeho schopnost aktivovat receptor potvrzena.

První metoda zahrnovala měření změn v buněčné cAMP produkci zprostředkované Gi. β-alanin jako kontrolní sloučenina způsobil 36.63 % inhibici forskolinem indukované cAMP produkce. Deset z celkového počtu testovaných látek neukázaly žádný efekt, ale (*R)*-3-amino-2-methylpropionová kyselina a také její diastereomer DL-3-aminoisobutyrová kyselina způsobily forskolinem indukované snížení produkce cAMP o 41. 31 % a 43.11 %. 3-Aminopropan-1-ol překvapivě zvýšil nitrobuněčnou produkci cAMP o 56.1 % a tento efekt zopakoval i u buněk, které neexprimovaly testovaný receptor. Z toho můžeme usuzovat, že efekt 3-aminopropan-1-olu je nespecifický. 1-(3-Piperidinopropyl)piperazin [1 mM] způsobil také nespecifické zvýšení produkce nitrobuněčného cAMP o 34.4 %.

V druhé metodě, kdy jsme měřili Gq-zprostředkované změny v Ca^{2+} závislých signálních drahách, jsme použili metodu založenou na NFAT-promotorem řízené expresi luciferázového genu. β-alanin, opět použitý jako pozitivní kontrola způsobil zvýšení luciferázové aktivity o 28.00 %. Přidání 1-(3-piperidinnopropyl)piperazinu způsobilo zvýšení luciferázové aktivity o 99.40 %. Fenpropidin v100 μM koncentraci způsobil zvýšení RLU o 57.6 % a v 1 mM koncentraci snížení o 56.1 %. *(R)*-3-Amino-2-methylpropionová kyselina byla také testována použitím této metody a mohli jsme vidět zvýšení luciferázové aktivity o 29.40 %.

CONTENTS

1. LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ATP	Adenosine triphosphate
BP	Binding protein
BSA	Bovine serum albumine
CaM	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
DPBS	Dulbecco's phosphate buffered saline
CHO cells	Chinese hamster ovary cells
Cn	Calcineurin
DAG	Diacylglycerol
EC_{50}	Half-maximally effective concentration
EDTA	Ethylendiaminetetraacetic acid
ER	Endoplasmatic reticulum
GABA	Gamma-aminobutyric acid
GPCR	G Protein-coupled receptors
GDP	Guanosine-5´-diphosphate
GTP	Guanosine-5´-triphosphate
HBSS	Hank's balanced salt solution
hMRGPCRD	Human Mas-related G Protein-coupled receptors subfamily D
IP_3	Inositol 1,4,5-trisphosphate
LB-Agar (medium)	Luria Bertani Agar (medium)
MRGPR	Mas-related G Protein-coupled receptors
MRGPRD	Mas-related G Protein-coupled receptors subfamily D
MRGPR A-H, X	Mas-related G Protein-coupled receptors subpopulation A-H and X
NFAT	Nuclear factor of activated T cells
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PLC	Phospholipase C
PKA	Protein kinase A
PKC	Protein kinase C
RLU	Relative light units
SNSR	Sensory neuron specific receptor
TrkA+	Tyrosine kinase
WT	Wild type

2. INTRODUCTION

Mas-related G Protein-coupled receptors (MRGPRs) belong to the group of G-protein-coupled receptors (GPCR).

GPCRs represent the largest family of membrane-bound receptors in the human genome with over 1000 members (Cherezov *et al*., 2007). They are involved in a wide variety of physiological and pathophysiological processes. As these receptors are playing a role in many diseases, a therapeutic potential of drugs targeting GPCRs can be assumed. Approximately 30 % of all clinically relevant drugs target GPCRs (Overington *et al*., 2006). Despite the large number of GPCRs which are already used as drug targets, hundreds still remain unexplored.

The members of the subfamily of MRGPRs are considered to play a role in nociception, pruritus, sleep, cell proliferation, circulation and mast cell degranulation (Solinski *et al*., 2014). Unfortunately, the structure, function and location of most MRGPR members are not completely characterized.

This work focuses on the pharmacological characterization of the human Mas-related G Protein-coupled receptor subfamily D (hMRGPRD), which is not very well explored. Improved characterization of the hMRGPRD may in the future facilitate development of compounds specifically interacting with this receptor protein.

3. THEORETICAL BACKGOUND

3.1. GPCR

3.1.1. General information

G Protein-coupled receptors (GPCRs) are seven-transmembrane domain receptors, which transduce extracellular signals into intracellular reaction *via* coupling with heterotrimeric G Proteins. GPCRs are the main information flow into cells. To the extracellular signals belong physical and chemical stimuli, such as amino acids, biogenic amines, lipids, peptides, proteins, light, odorants and pheromones.

GPCRs consist of seven transmembrane α-helices, three extracellular and three intracellular loops, C-terminal intracellular domain and N-terminal extracellular domain (see Fig. 1) (Cherezov *et al*., 2007). The first crystallographic structure of a GPCR, that of bovine rhodopsin, was resolved in 2000 (Palczewski *et al*., 2000). The first structure of a human GPCR (β_2 adrenergic G-protein-coupled receptor) was solved seven years later (Rasmussen *et al*., 2007).

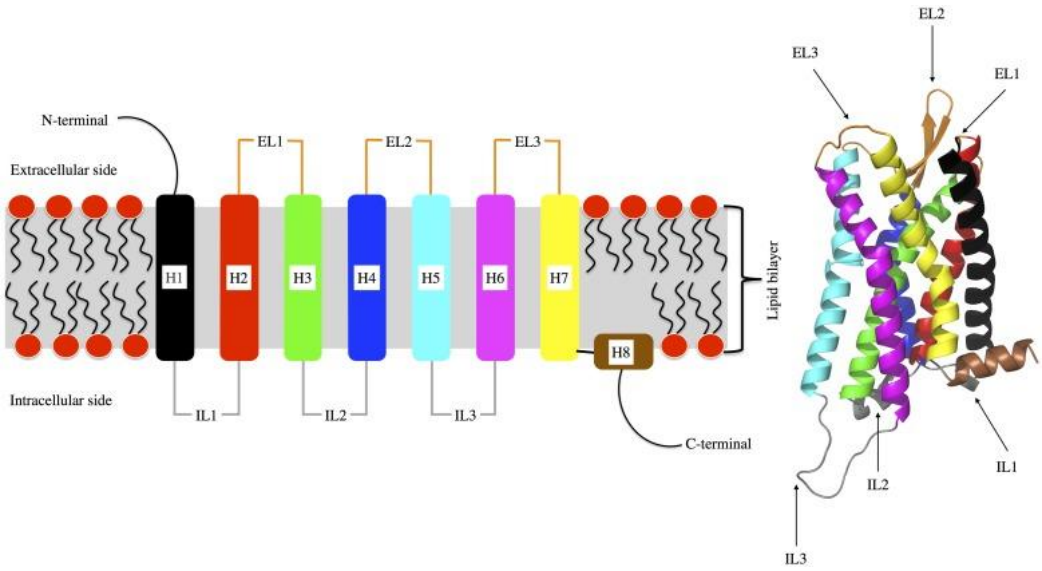

Fig. 1 Schematic view of the overall structure of a G Protein-coupled receptor. EL- extracellular loop, IL- intracellular loop, H- transmembrane helices between loops. Modified from Cherezov *et al*., 2007.

The activation of the GPCR causes a conformational change of the receptor protein resulting in the exchange of GDP by GTP. That promotes the dissociation of the heterotrimeric G Protein into α subunit and βγ subunit and subsequent modulation of the activity of adenylyl cyclase, phospholipase C activity and other effectors.

Signaling transduction pathways, analyzed in this study are the cAMP-related signaling pathways mediated by inhibitory G-proteins (Gi) and stimulative G-proteins (Gs) and the phosphatidylinositol signaling pathway mediated by Gq.

3.1.2. cAMP signaling pathway

In the cAMP signaling pathway (see Fig. 2), two types of G-proteins play a role: stimulative G-proteins (Gs) and inhibitory G-proteins (Gi). GPCRs are able to activate more than one subtype of G-proteins and also may have a preference for one subtype over another. In the case of Gs, the α subunit with bound GTP activates the adenylyl cyclase (AC), in the case of Gi, the α subunit with bound GTP inhibits AC from generating cAMP. The level of cytosolic cAMP may then determine the activity of various enzymes and ion channels. Activation or inhibition of AC is terminated by hydrolysis of the bound GTP to GDP, which returns the system to its ground state. Adenylyl cyclase is a 12-transmembrane glycoprotein that converts ATP into cAMP, which exerts its effect by activating the protein kinase A. The pathway is amplificated at this step, because a single activation of AC can produce many cAMP molecules. Protein kinase A is an important enzyme in cell metabolism due to its ability to phosphorylate other substrates, conveying the signal further to the cell. In the case of Gi, the α subunit with bound GTP inhibits the adenylyl cyclase, leading to a decrease of cellular cAMP levels. A decrease of intracellular cAMP levels changes cell metabolism. As readout for receptor function, cAMP levels are measured, because they are changed by receptor activation as part of the signal transduction of the receptor analyzed in the present study.

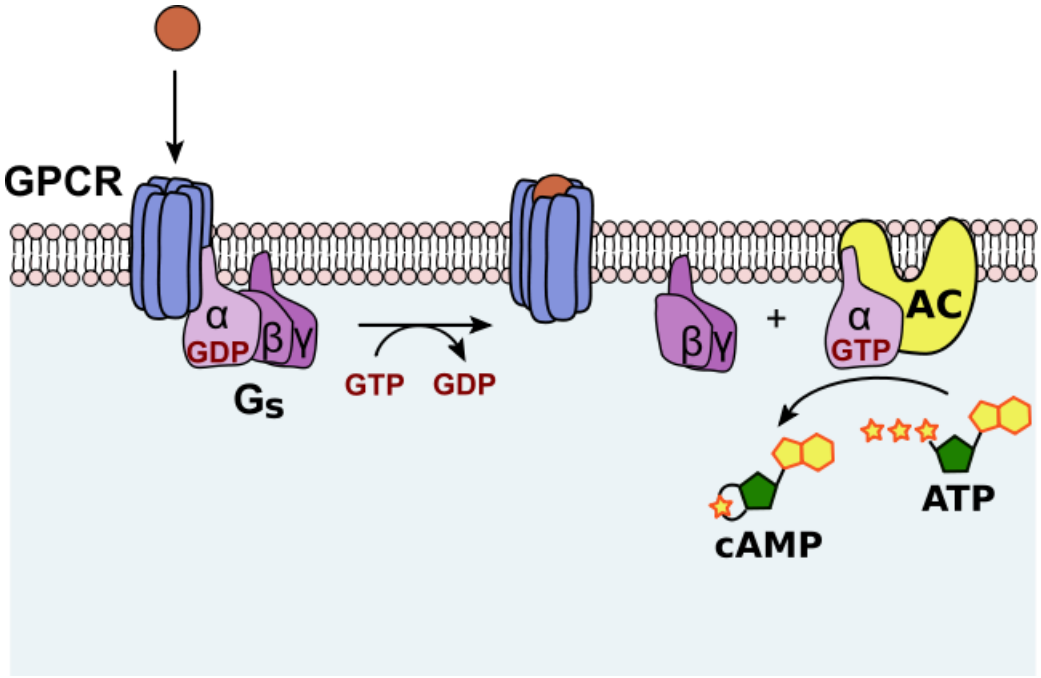

Fig. 2 cAMP signaling pathway.

Available from: http://commons.wikimedia.org/wiki/File%3AActivatoin-Adenylate_cyclase-outlined.svg, 9.1.2015

3.1.3. Phosphatidylinositol signaling pathway

The phosphatidylinositol signaling pathway is mediated by Gq proteins (see Fig. 3). The activation of the α subunit affects phospolipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 opens Ca^{2+} channels in the endoplasmatic reticulum and causes a release of Ca^{2+} ions into the cytosol. DAG and the increase in cellular Ca^{2+} cause activation of protein kinase C (PKC), which phosphorylates many other proteins, affecting cell metabolism.

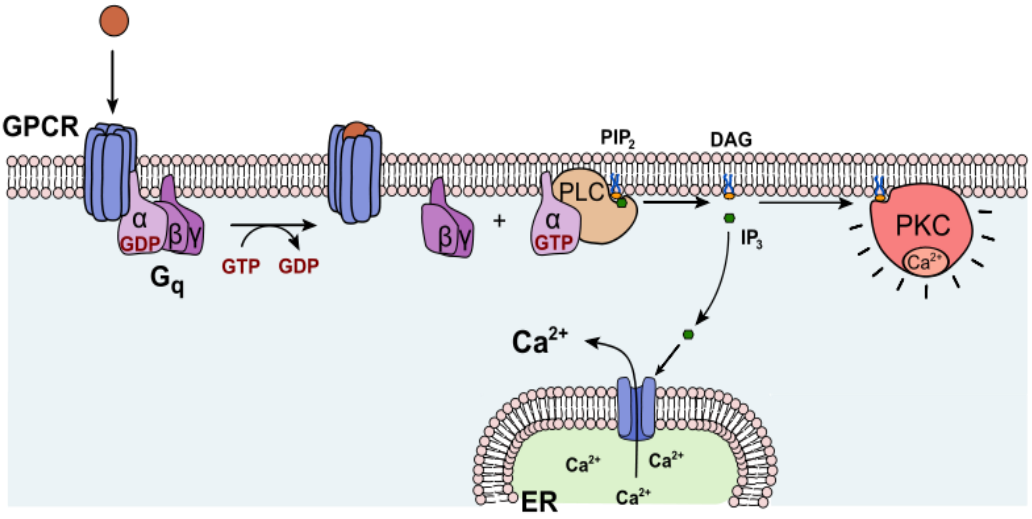

Fig. 3 Phosphatidylinositol signaling pathway. ER- endoplasmatic reticulum, PLC – Phospholipase C, PKC – Protein kinase C.

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http://commons.wikimedia.org/wiki/File%3AActivation_protein_kinase_C.svg, 9.1.2015

The activation of PKC is not the only effect of Ca^{2+}. Increase of this ion concentration propagates its signal through a wide range of proteins including protein kinases, metabolic enzymes and regulatory proteins (*e.g.* Ca^{2+}/calmodulin-dependent protein kinase pathway - see section 5.4).

3.2. MRGPR

Mas-Related G Protein-coupled receptors (MRGPRs) were discovered 10 years ago. Dong and colleagues (2001) were studying a tyrosine kinase receptor (TrkA+), which detected painful stimuli. The authors compared cDNA libraries from wild-type mice and mice lacking the transcription factor neurogenin 1. Mice without neurogenin did not develop TrkA+, so the authors postulated that genes lacking in the neurogenin-deficient mice were specific for TrkA nociceptors and called them MAS-related genes. They named them MAS-related genes, because of their homology to the GPCR MAS1, which was the first discovered member (Young *et al.*, 1986) of this subfamily of GPCRs.

The Mas-Related G Protein-coupled receptor family consists of 40 members divided into 9 groups of receptors. Human orthologs are designated by capital letters (MRGPRA to MRGPRAH and MRGPRX). All members are still formally considered "orphan", which means no endogenous agonist is clearly identified (Solinski *et al*., 2014).

No members of the MRGPR family were found outside the Tetrapods superclass, which is suggesting together with the fact that they are expressed in sensory neurons and mast cells in the skin, that pruriception is probably the major function (Bader *et al*., 2014).

MRGPRs are expressed in primary sensory neurons and mast cells and have not been detected in the central nervous system or in other tissues. That is the reason for having another name – sensory neuron specific receptor (SNSRs).There are several potential ligands such as β-alanine, angiotensin, alamandine, gamma-aminobutyric acid (GABA) and others. MRGPRs are considered to have a role in nociception, pruritus, sleep, cell proliferation, circulation and mast cell degranulation (Solinski *et al*., 2014).

Significant progress has been made recently; our knowledge about MRGPR has been extended. Several MRGPRs are analyzed as pharmacological targets for development of antihypertensive, antipruritogenic and analgesic drugs (Solinski *et al*., 2014).

3.3. MRGPRD

The MRGPRD subfamily consists of only one receptor subtype per species, which is conserved in rodents and primates. It has been discovered that several ligands are able to bind to the human MRGPRD. β-Alanine has been established as the only physiological ligand of MRGPRD and can be responsible for its endogenous effects (Shinohara *et al*., 2004). In addition, GABA, beta-aminobutyric acid (Shinohara *et al*., 2004; Ajit *et al*., 2010; Uno *et al*., 2012), angiotensin and alamandine (Gembardt *et al*., 2008; Lautner *et al*., 2013) have been shown to activate the receptor.

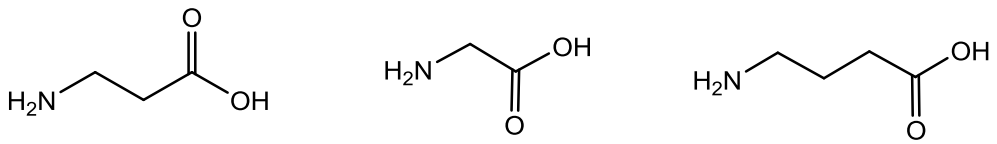
MRGPRD couples to inhibitory regulative G-proteins (Gi) that inhibit adenylyl cyclase activity and cAMP production and Gq proteins that induce intracellular calcium ion influx in response to β-alanine (Shinohara *et al*., 2004; Ajit *et al*., 2010).

The receptor can be found in primary sensory trigeminal ganglia neurons and in blood vessels. In neurons the β-alanine induced activation of MRGPRD has been shown to elicit pruritogenic sensation and to contribute to normal mechanical and thermal pain thresholds. It has been discovered that MRGPRD is expressed in a subpopulation of nonpeptidergic, nociceptive neurons that project exclusively to the skin (Ajit *et al.*, 2010). These neurons innervate a layer of the epidermis, which is distinct from that innervated by peptidergic nerve fibers (Ajit *et al*., 2010), suggesting that antagonism of MRGPRD could be beneficial for the treatment of pain and nociception. In blood vessels, MRGPRD is activated by the angiotensinogen metabolite alamandine (Lautner *et al*., 2013), which induces the production of nitric oxide that leads to vasorelaxation. Interestingly, β-alanine fails to relax aortic rings and surprisingly blocks the effect of alamandine, suggesting that the two agonists (Alamandin and β-alanine) direct receptor signaling to different pathways (Lautner *et al*., 2013).

Just like other MRGPRs, MRGPRD also specifically marks a subset of primary sensory neurons: MRGPRD-positive neurons. Currently, it is not known which physiological stimuli activate these neurons. Moreover, the role that MRGPRD activation plays in the function of these afferents is unknown. The comparison of mice lacking MRGPRD and mice with ablation of MRGPRD-positive neurons showed that the ablation of MRGPRD caused a deficit in the detection of heat, cold and mechanical stimuli. The ablation of MRGPRD-positive neurons affected only acute mechanical pain, whereas cold and heat transduction was completely intact (Cavanaugh *et al*., 2009). Thus, the deficit in mechanical pain sensation is caused by the ablation of MRGPRD-positive neurons.

3.4. β – Alanine

β-alanine is a naturally occurring non-essential amino acid and it is structurally related to the major inhibitory neurotransmitters GABA and glycine (see Fig. 4). In the human body it is built by the degradation of dihydrouracil and carnosin. After its synthesis in the liver, β-alanine is transported to the muscle, where its uptake is dependent on the same transporter for the delivery of GABA, glycin, taurin and other structure-related substances. It has been known, that β-alanine acts as a depressant in the central nervous system (DeFeudis *et al*., 1977, Usherwood, 1978), it activates glycine and GABA receptors and decreases glutamatergic excitation. These dual effects indicate that β-alanine acts as a neurotransmitter and/or neuromodulator (Wu *et al*., 1993; Rajendra *et al*., 1997).



ß-alanine glycine GABA

Fig. 4 The structural similarities of β-alanine, glycine and GABA (using CS ChemBioDraw Ultra version 14.0,CambridgeSoft, Cambridge, MA, USA).

β-alanine is a very well known and widely used molecule. It is a structural component of carnosine, anserine and also pantothenic acid (see Fig. 5). As a component of carnosine (together with histidine) it is used as a supplement for muscle building. β-Alanine improves workload and muscle resistance to fatigue (Helms *et al*., 2014). It is an effective buffer to prevent muscle cells from becoming acidic and is very important during anaerobic exercises (Liu *et al*., 2012).

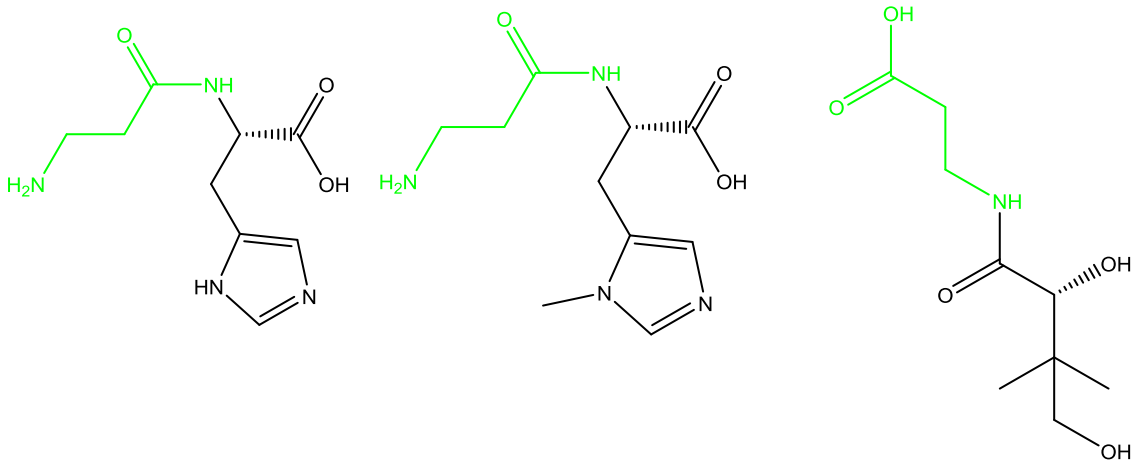


Fig.5 Structural similarities of carnosine, anserine and pantothenic acid. Structure of β-alanine distinguished by green colour (using CS ChemBioDraw Ultra version 14.0,CambridgeSoft, Cambridge, MA, USA)

Common side effects of β-alanine are skin itching and tingling. Intradermal application of β-alanine caused burning, itching and stringing in all subjects during a human pilot study that contained 11 healthy individuals. In comparison, the same dose of *L*-alanine caused a weak itch in only 5 of 11subjects (Liu *et al*., 2012). Liu and colleagues also tested if oral administration of β-alanine could also induce itching. The reaction of wild type (WT) mice fed with water supplemented with β-alanine resulted in a reaction similar to that reported in humans. Mice lacking MRGPRDs exhibited significantly less scratching than WT controls (with MRGPRDs). Taken together, these data show that oral administration of β-alanine induced itching behavior in mice and that this effect was MRGPRD-dependent (Liu *et al*., 2012).

β-alanine binds to the MRGPRD playing essential roles in mediating itching signals. Because these receptors do not respond to histamine and they are not sensitive to chloroquine (Liu *et al*., 2012), they are a candidate for mediating itching and the associated sensation elicited by histamine-independent pruritic stimuli including β-alanine, heat and mechanical stimuli.

In the present study, β-alanine was used as a standard positive control for the activation of the hMRGPRD and the effects of the new, tested compounds were compared to β-alanine.

4. AIM OF THIS WORK

The goal of this study was to identify new substances that activate the hMRGPRD.

We investigated whether potential compounds can activate MRGPRD. The activation of the receptor was measured by the quantification of changes in cellular cAMP levels or activation of Ca^{2+}-mediated nuclear factor of activated T cells (NFAT)-driven luciferase activity. For the direct quantification of cAMP, a [^{3}H]cAMP-radioaffinity assay was performed, whereas a NFAT-directed luciferase reporter gene assay was used for the analysis of Ca^{2+}mediated signals caused by receptor activation.

The determination of the effects of compounds structurally related to β-alanine on the hMRGPRD may facilitate the understanding on the structure – activity relationships of the hMRGPRD activators, contributing to the general knowledge about the function of the hMRGPRD receptor.

5. METHODS AND MATERIALS

5.1. CHO Flp-In cells stably expressing the hMRGPRD receptor

Chinese hamster ovary (CHO) Flp-In cells stably expressing the hMRGPRD receptor were cultured at 5 % CO_2 and 37 °C in F-12 nutrient Mixture (Ham) medium with GlutaMAXI, 10 % FBS and hygromycin B (500 μg/ml). We worked with cells from passages 3-30. Every manipulation was performed under sterile condition and cells were checked every day by microscopy. After 3-4 days, when cells were about 80 % confluent, cells were split using trypsin-EDTA 0.05 % solution (trypsin 0.5 g/l, EDTA 0.2 g/l).

Nontransfected CHO Flp-In cells were cultured the same way in F-12 nutrient Mixture (Ham) medium with GlutaMAXI and Zeocin (100 μg/ml).

All reagents were purchased from Gibco® by Life Technologies (INVITROGEN).

5.2. Cell culture protocol

Cell were split every 3-4 days by the following standard protocol.

1. Remove old medium from the flask.
2. Wash the cells with 10 ml of Dulbecco's phosphate Buffered Saline (DPBS) solution [(-) $CaCl_2$, $MgCl_2$].
3. Add 1.5 ml of Trypsin-EDTA solution to detach cells from the flask.
4. Incubate for 2-3 minutes.
5. Prepare a new flask with 10 ml of medium.
6. Check in the microscope to confirm that the cells are detached.
7. Remove the trypsin solution.
8. Use 2 ml of the new medium to wash off the cells from the flask wall.
9. Transfer 300 μl of cell suspension (15 % of the cells) to the prepared new flask with 10ml of fresh medium.

10. Incubate the flask in humidified incubator at 37 °C, 5 % CO_2.

5.3. Seeding cells

50 % of the cells of a 75 cm^2 cell culture flask were seeded for 24 hours in a volume of 500 µl cell suspension per well of a 24 well multiwell plate.

1. Follow the cell culture protocol (see section 5.2.).
2. Transfer 1ml of cell suspension (50 % of the cells) to conical tube with 12 ml of fresh medium and mix.
3. Pipette 500 µl into each well.
4. Shake the plate lightly and incubate at 37 °C, 5 % CO_2 for 24hours.

5.4. NFAT Luciferase assay

5.4.1. Luciferase assay description

Luciferase assay is a method analyzing receptor function by quantification of the receptor – mediated effects on luciferase gene expression. The luciferase gene is widely used as a reporter gene to study a broad range of mechanisms. Luciferase is an enzyme isolated from the luminous beetles. This enzyme catalyzes the oxygenation of the beetle luciferin into oxyluciferin by using ATP as a cofactor (see Fig. 4). The result of this reaction is photon production, which can be detected by luminescence measurements (RLU; relative light units). Luciferase expression can be driven by a variety of different promoter sequences. As an example, promoter activation and subsequent gene expression can be initiated by the increase of cAMP or Ca^{2+} caused by the activation of respective signal transduction pathways. One of the advantages is that activity of the enzyme does not depend on any posttranslational modification, which makes it available for the quantification immediately and moreover, the assay is reliable, rapid and easy to perform.

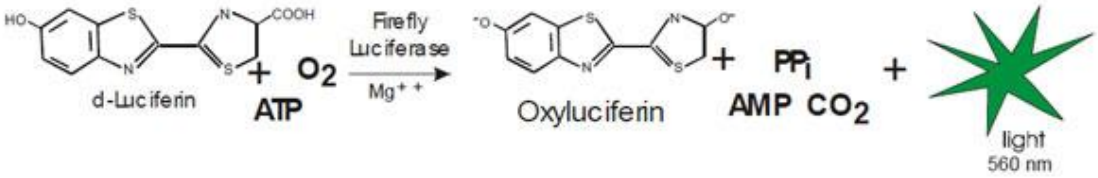


Fig. 6 Oxygenation of beetle luciferin into oxyluciferin. The oxygenation is catalyzed by luciferase in the presence of Mg^{2+} and ATP. Taken from Held P. 2006. Available from: http://www.nature.com/app_notes/nmeth/2006/063006/full/an1755.html

5.4.2. NFAT-Luciferase reportergene assay

The activation of Gq protein-coupled receptors (Gq) causes an increase of Ca^{2+}, which results in activation of the calmodulin/calcineurin complex. This complex induces dephosphorylation of NFAT (nuclear factor of activated T cells), which serves as a promoter and regulates the expression of the luciferase gene (see Fig. 5).

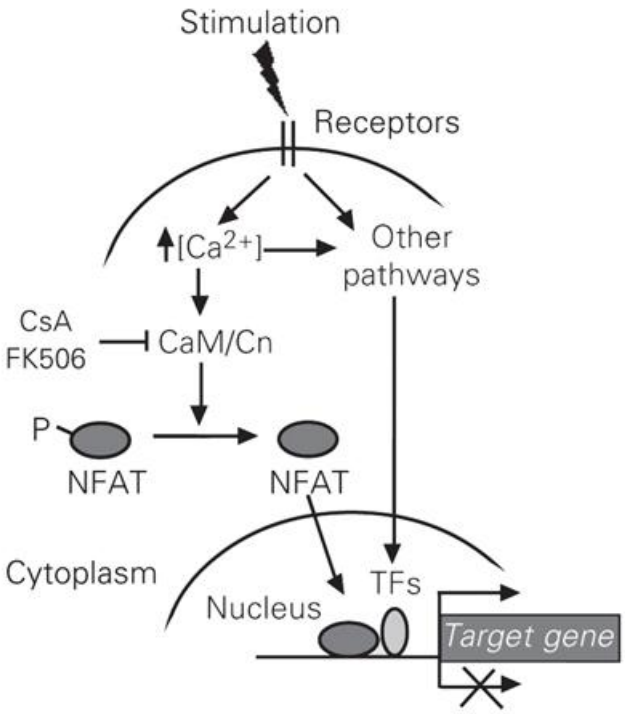


Fig. 7 Schematic view on different signal transduction pathways regulating gene expression. Stimulation of Gq-coupled receptors causes an increase of the Ca^{2+}

level, which leads to activation of CaM (calmodulin) Cn (calcineurin) and dephosphorylation of NFAT. NFAT translocates to the nucleus, where it affects the expression of target (luciferase) genes. Taken from Viola et al. (2005). Available from: http://www.scielo.br/scielo.php?pid=S0100-879X2005000300003&script=sci_arttext

5.4.3. Plasmid purification

For the luciferase assay we needed the pNFAT luc plasmid DNA. We obtained this plasmid from pre-cultivated TOP 10F *E. Coli* bacteria carrying the pNFAT-luc plasmid.

Plasmid purification is a standardized procedure. We used EndoFree plasmid purification Maxi kit (QIAGEN). The endotoxin-free Plasmid DNA will improve transfection into eukaryotic cells. We proceeded according to the manufacturer's protocol - QIAGEN Plasmid Purification Handbook (available from https://www.qiagen.com/cz/resources/resourcedetail?id=fc49b220-129b-4b0f-bab0-3de3e89c1525&lang=en). Plasmid purification protocols are based on an alkaline lysis procedure followed by purification steps. Plasmid DNA is then eluted, concentrated and desalted by isopropanol and ethanol precipitation.

After the plasmid purification, the quality and quantity of the plasmid DNA is analyzed by UV spectrophotometry (at 260 nm) and agarose gel electrophoresis.

1. Prepare culture 2 days before. Seed the bacteria into LB-Agar plate with ampicilin (100 μg/ml) and cultivate for 16 hours. Use ampicilin to select bacteria with the desirable plasmid (Plasmid also contains the gene for the resistance to ampicilin).
2. After 16 hours pick a single colony with a sterile pipette tip, resuspend it in 2 ml of Luria Bertani (LB) medium with ampicilin (100 μg/ml). Then place the colony into the incubator (37 °C, 200 rpm) and let it shake for 8 hours.
3. Take 300 μl of the preculture, mix it with 150 ml of LB medium containing ampicilin and incubate at 37 °C for 14 hours with shaking.

4. Harvest the bacteria cells by centrifugation at 5000 g for 15 min at 4 °C.
5. Resuspend pellets with medium to get an alcalic lysate.
6. Then clear lysates by filtration to remove proteins, genomic DNA and cell debris.
7. Add endotoxin removal buffer, mix and incubate on ice for 30 min.
8. Bind the DNA by applying the filtered lysate to the column.
9. Wash the column with 30 ml of buffer twice.
10. Elute DNA from the column.
11. Precipitate DNA by adding 10.5 ml isopropanol (70 % v/v).
12. Mix and centrifuge immediately at 5000 g for 60 min at 4 °C. Carefully remove the supernatant.
13. Wash with endotoxin-free ethanol ant centrifuge at 5000 g for 60 min at 4 °C.
14. Resuspend DNA in endotoxin-free resuspention buffer.
15. Check the quality by using agarose gel electrophoresis (1 % agarose gel) to determine the purity of the DNA by using ethidium bromide to mark the DNA.
16. Determine the yield of DNA by using UV spectrophotometry. For the MAXI prep the amount is around 3 µg/ml. Perform the spectrophotometry at 260 nm to quantify the amount of the DNA and build the quotient of 260/280 nm to estimate the purity.

5.4.4. Plasmid transfection

For the preparation of the NFAT luciferase reporter gene assay, CHO Flp-In cells were transfected with the NFAT-luc (nuclear factor of activated T-cells) plasmid gene. The plasmid gene contains the firefly luciferase gene, the NFAT promoter sequence and the SV40 late polyadenylation signal (to ensure proper transcription of the luciferase transcript).

Cells were seeded one day earlier using medium without antibiotics in a density that ensures 70-80 % confluency on the day of transfection.

For transfection, we used Lipofectamine 2000 reagent (Invitrogen), which is mixed with DNA in OptiMEMI medium for the formulation of micelles, which are able

to transport the plasmid inside the cells. The cells need approximately 18 hours to insert the DNA fragment.

1. One day before transfection, split the cells in the morning.
2. Next day in the afternoon prepare the Lipofectamine 2000 solution. Mix 25 µl of the Lipofectamine reagent with 500 µl of OptiMEMI medium and wait 5 min (preformation of micelles).
3. Prepare pNFAT-luc DNA solution by mixing 10 µg of DNA with 500 µl of OptiMEMI medium.
4. Mix both solutions and wait 20 min for forming the micelles.
5. Before adding DNA solution to the cells, change the medium to remove dead cells and compounds secreted. Distribute the solution equally on the cells and incubate for 18 hours. After 18 hours, seed the cells as described in the cell culturing section.

5.4.5. Assay protocol

1. After 24 hours of incubation remove the medium and wash the cells with 500 µl of warm HBSS buffer.
2. Add 900 µl of warm HBSS buffer and incubate for 1h.
3. Prepare solutions:
    a. Dilute compound solutions.
    b. Dilute ATP to a final concentration of 100 µM for the positive control.
4. After 1 hour of incubation pipette 100 µl of appropriate solutions into each well.
5. Incubate for 3 hours and then remove the reaction medium.
6. Pipette 50 µl of HBSS and 50 µl of Bright-GLO Luciferase assay reagent (Promega®) into each well. The reagent contains the luciferase substrate (luciferin) and will destroy the cells.

7. After 3 min take 90 µl from each well and pipette it into a well of a white 96 well multiwell plate (Greiner®) for luminescence measurements.
8. Measure the plate in a Polar Star Omega plate reader (BMG Labtech).

5.4.5. Quantification

Each well was measured three times in the Polar Star Omega plate reader. Luminescence signals were given in RLU (relative light units).

5.5. [^{3}H]cyclic AMP radioaffinity assay

5.5.1. [^{3}H]cAMP radioaffinity assay description

The main principle of this assay is competition between native cAMP molecules (which are created by the activation of adenylyl cyclase enzyme) and the tritium [^{3}H] labeled cAMP molecules. Both molecules compete for a binding site on the binding protein PKA (Protein Kinase A). PKA has a high specificity and affinity for cAMP. The competition of labeled and unlabeled cAMP for binding at the PKA results in an inverse correlation. This means that if there is more native cAMP in the reaction buffer, less tritium labeled cAMP can bind to the PKA. Because the concentration of the labeled cAMP is constant, we can easily identify the amount of the native cAMP by a linear regression analysis of a standard curve with defined concentrations of unlabeled cAMP.

Forskolin solution [10 µM] is used to induce intracellular cAMP production. Because we want to measure the concentration of the intracellular cAMP, we have to lyse the cells with a hot EDTA + TritonX100 solution (Na_2EDTA 4 mM, Triton X100 0.1 ‰). We use Triton to destroy the cell membrane, so cellular cAMP can be detected in the supernatant. For conservation of cAMP, we use EDTA that has an ability to bind Calcium ions. Calcium ions are very important for the function of phosphodiesterase enzymes which are responsible for breakdown of cAMP into 5'-AMP.

Separation of the cAMP bound to the binding protein from the unbound nucleotide is achieved by absorption of the unbound nucleotide by a coated charcoal

suspension (0.75 g neutralized charcoal, 1 g bovine serum albumin BSA, 25 ml of sodium-acetate buffer pH 6). Charcoal can absorb only small molecules like free nucleotides, because of the size of its pores. The next step is centrifugation (14000 rpm, 5 min, 0 °C) of the sample. Then the supernatant containing the binding protein with the bound cAMP is used for subsequent liquid scintillation counting for the quantification of radioactivity in each sample.

5.5.2. Assay protocol

1. Prepare the cells for the assay according to the cell seeding protocol (see section 5.2).
2. After 24 hours of incubation, remove the medium and wash the cells with 500 µl HBSS. Then add 800 µl of prewarmed buffer andincubate for 1 hour.
3. In the meantime prepare all needed solutions:
    a. Forskolin – dilute the forskolin stock solution to get final 10 µM concentration.
    b. Binding protein – dilute 400 µl of binding protein (purified from bovine adrenal cortex) frozen solution with 1600 µl of buffer.
    c. Agonist solutions - dilute agonist solutions with HBSS to get 1 mM, 300 µM, 100 µM, 30 µM , 10 µM and 1 µM concentrations.
    d. Standards - prepare standards of cAMP for the calibration curve. Dilute cAMP stock solution [160pm] with EDTA to get dilusion raw: 4, 2, 1, 0.25 and 0,0625.
    e. Control solutions - prepare 4 tubes with 50 µl of EDTA for the binding protein control and 1 tube with 90 µl for the negative control.
4. After 1 hour of incubation place the plate with cells to the water bath at 36.5 °C and add the agonist solution to the each well except of control wells (four positive controls with forskolin and buffer and two negative controls only with buffer).
5. Pipette 100 µl of forskolin solution to each well except two negative controls and stop 10 min. In these 10 minutes the cAMP will be produced and after that stop the reaction by removing the medium.

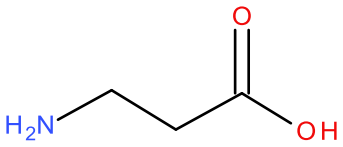
6. Add 500 µl hot (about 90 °C) EDTA solution to each well to destroy the cells and let the plate shake on ice for 1 hour.
7. After 1 hour pipette 50 µl of cell lysate from each well to reaction tubes.
8. Add 30 µl of tritium labled cAMP to every sample.
9. Add 40 µl of diluted binding protein at each sample except the negative control and mix.
10. After addition of the tritium labeled cAMP and binding protein mix the reaction medium and let it incubate on ice for 1 hour. This is important for formation of the balance between bound and unbound cAMP.
11. Add 50 µl of coated charcoal suspension and mix vials thoroughly. Reaction time is 5 min.
12. Centrifuge all vials to remove surplus cAMP. Take 100 µl of the supernatant from each vial after centrifugation.
13. Add 2 ml of the scintillation cocktail (Rotiszint ecoplus; Carl Roth®) to increase the strength of the signal and to avoid precipitation of the bound nucleotide. Mix properly.
14. Do wipe tests to detect possible radioactive contamination of the working area.
15. After minimum of two hours in the dark, measure the radioactivity in the liquid β-scintillation counter (BECKMAN COULTER LS-5000TD). Each vial is measured 5 min in the dark at room temperature.

5.5.3. Quantification of cAMP levels

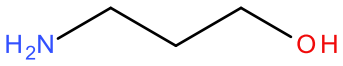
The supernatant is analyzed by β-scintillation counting. The concentration of unlabeled cAMP in the sample is then determined from regression analysis of a standard curve for each experiment. Concentration-response data are processed by non-linear regression using GraphPad Prism 4 to calculate the half-maximal effective concentration (EC_{50}). The EC_{50} is the concentration of a drug, where 50 % of its maximal effect is observed.

5.6. Tested compounds

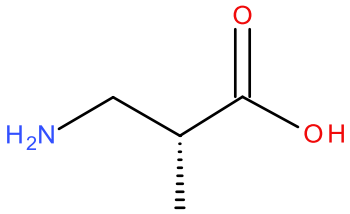
β-alanine, Molecular Weight: 89.09



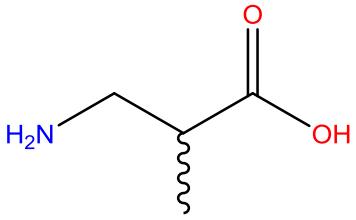
3-aminopropan-1-ol, Molecular Weight: 75.11



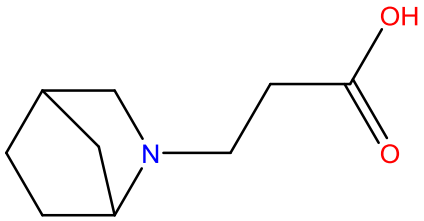
***(R)*-3-amino-2-methylpropionic acid,** Molecular Weight: 103.12



***(RS)*-3-amino-2-methylpropionic acid; DL-3-aminoisobutyric acid**, Molecular Weight: 103.12

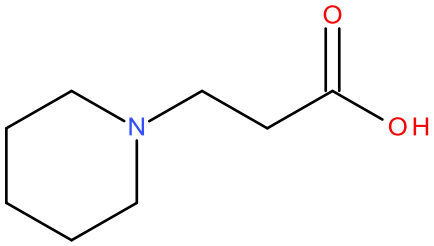


3-(2-azabicyklo[2.2.1.]hept-2-yl)propionic acid hydrochloride, Molecular Weight: 169.22

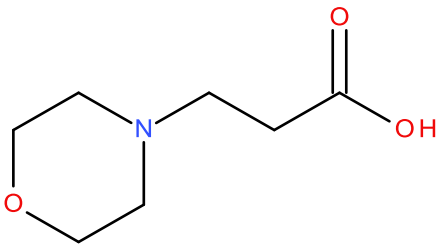


HCl

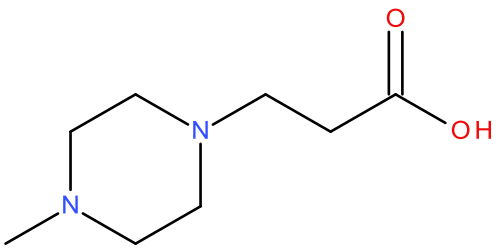
3-(piperidin-1-yl)propionic acid, Molecular Weight: 157.21



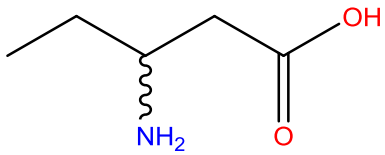
3-(morpholin-4-yl)propionic acid, Molecular Weight: 159.19



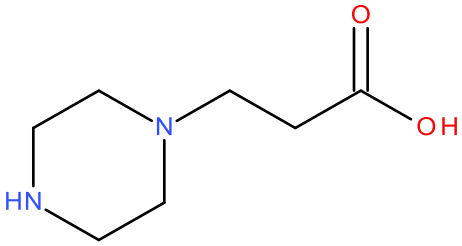
3-(4-methylpiperazin-1-yl)propionic acid, Molecular Weight: 172.23



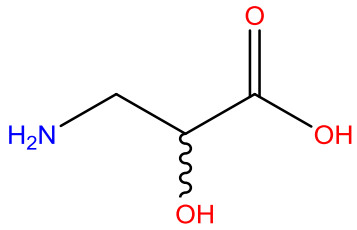
DL-3-aminopentanonic acid, Molecular Weight: 117.15



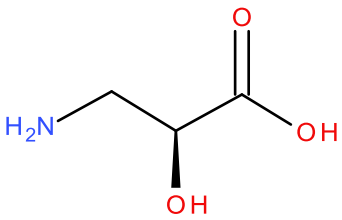
3-(1-piperazinyl)propionic acid, Molecular Weight: 158.20



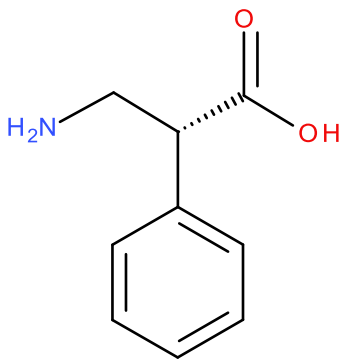
DL-isoserine, Molecular Weight: 105.09



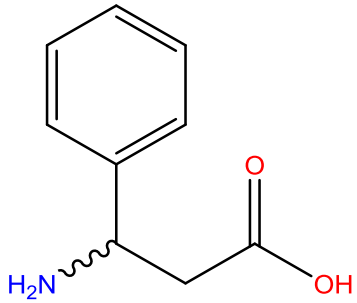
L-isoserine, Molecular Weight: 105.09



***(R)*-3-amino-2-phenylpropionic acid,** Molecular Weight: 165.19

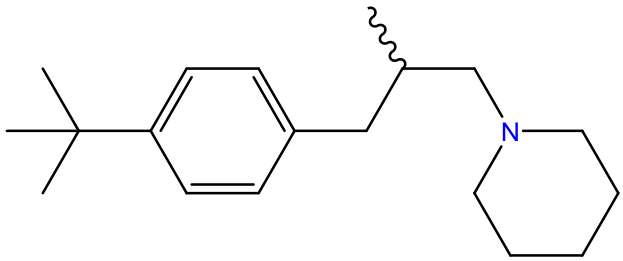


DL-3-amino-3-phenylpropionic acid, Molecular Weight: 165.19



Fenpropidin, DL-1-(3-(4-(*tert*-butyl)phenyl)-2-methylpropyl)piperidine

Molecular Weight: 273.46



1-(3-piperidinopropyl)piperazin

Molecular Weight: 211.35

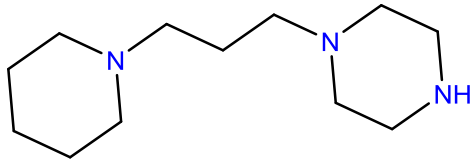


Fig. 8 Structures of tested compounds (using CS ChemBioDraw Ultra version 14.0,CambridgeSoft, Cambridge, MA, USA); β-alanine and *(RS)*-3-amino-2-methylpropionic acid were purchased from Sigma-Aldrich; all other compounds were obtained from the Prof. Müller team, department of pharmaceutical chemistry, Faculty of Pharmacy, Rheinische Friedrich-Wilhelms-Universität Bonn

6. RESULTS

6.1. [^{3}H]cAMP radioaffinity assay

The [^{3}H]cyclic AMP radioaffinity assay was used to study the effects of the tested compounds on the production of intracellular cAMP. We performed measurements on CHO Flp-In cells stably expressing the hMRGPRD receptor or on nontransfected CHO Flp-In cells for excluding unspecific effects.

6.1.1. β-Alanine

As a standard compound we used β-alanine in concentrations of 1 μM, 10 μM, 30 μM, 100 μM, 300 μM and 1 mM. Forskolin [10 μM] was used to stimulate the intracellular cAMP production. Duration of the reaction was 10 minutes. The inhibition of forskolin-induced cAMP production in by β-alanine was expressed as percentage of forskolin induced cAMP production in the absence of β-alanine (con). 1 mM concentration of β-alanine caused a 36.63 ± 5.2 % inhibition of forskolin induced cAMP production. Concentration-response data were fitted by nonlinear regression using GraphPad Prism 4 to estimate the EC_{50} value.

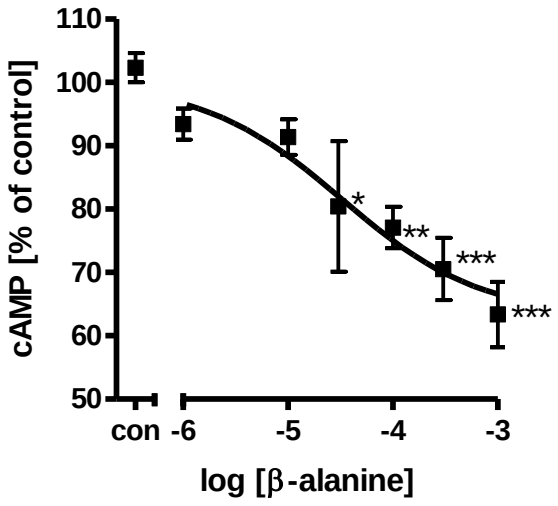


Fig. 9 Effect of β-alanine on forskolin-induced cAMP accumulation in CHO Flp-In cells stably expressing the hMRGPRD receptor. Con indicates control values with forskolin, but without β-alanine: 24.37 ± 0.9 pmol cAMP/well. n= 3-8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs.* respective control (con 100 %, t-test). EC_{50} 32.96 μM. Concentration-response data were processed by non-linear regression using GraphPad Prism 4.

6.1.2. 3-Aminopropan-1-ol

Concentrations of 10 μM, 30 μM, 100 μM, 300 μM and 1 mM were used to activate the receptor in the [^{3}H]cAMP assay. Forskolin [10 μM] was used to stimulate intracellular cAMP concentration. Duration of the reaction was 10 minutes. 1 mM concentration of 3-aminopropan-1-ol caused a 56.1 ± 14.16 % increase of forskolin-induced cAMP production. Concentration-response data were fitted by nonlinear regression to estimate the EC_{50} value.

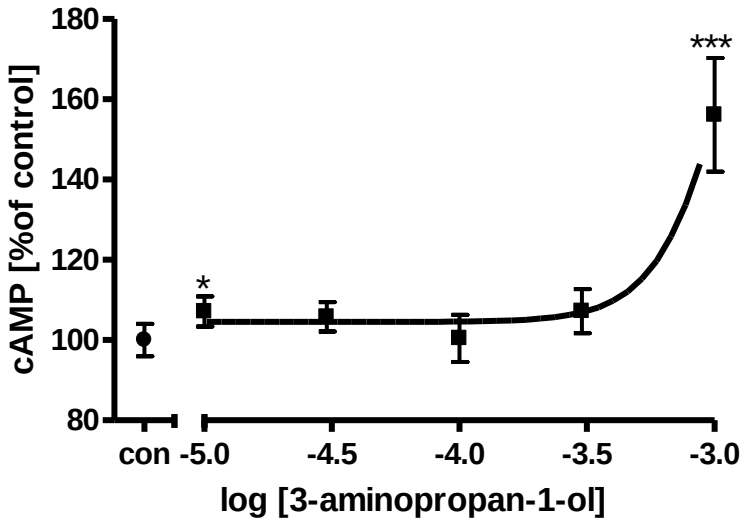


Fig. 10 Effect of 3-aminopropan-1-ol on forskolin-induced cAMP accumulation in CHO Flp-In cells stably expressing the hMRGPRD receptor. Con indicates control values with forskolin, but without 3-aminopropan-1-ol: 24.6 ± 1.3 pmol cAMP/well. n = 6 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant

differences *vs*. respective control (con 100%, t-test). The EC_{50} value amounted to 0.5953 mM. Concentration-response data were processed by non-linear regression using GraphPad Prism 4.

The measrurement with 3-aminopropan-1-ol was repeated in nontransfected CHO Flp-In cells to find out whether the effect on cAMP - production is specific. Concentrations of 10 μM, 30 μM, 100 μM, 300 μM and 1 mM were used. Forskolin [10 μM] was used to stimulate intracellular cAMP concentration. Duration of the reaction was 10 minutes. 1 mM concentration of 3-aminopropan-1-ol caused 99.3 ± 12.3 % increase of forskolin induced cAMP production in nontransfected CHO Flp-In cells. Concentration-response data were fitted by nonlinear regression to estimate the EC_{50} value.

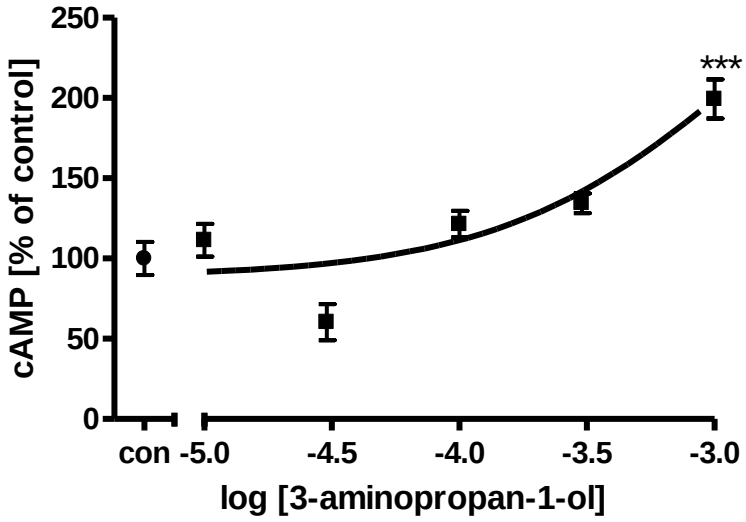


Fig. 11 Effect of 3-aminopropan-1-ol on forskolin-induced cAMP accumulation in CHO Flp-In cells. Con indicates control values with forskolin, but without 3-aminopropan-1-ol: 11.16 ± 1.4 pmol cAMP/well. n = 6 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs*. respective control (con 100%, t-test). The EC_{50} value was 0.3064 mM. Concentration-response data were processed by non-linear regression using GraphPad Prism 4.

6.1.3. (*R)*-3-Amino-2-methylpropionic acid

In previous luciferase reportergene-assays, (*R)*-3-amino-2-methylpropionic acid showed an agonistic effect at cells stably expressing the hMRGPRD. This result was confirmed by the use of the [^{3}H]cAMP radioaffinity assay. We measured concentrations of 100 µM and 1 mM. Forskolin [10 µM] was used to stimulate intracellular cAMP production. Duration of the reaction was 10 minutes. The inhibition of forskolin induced cAMP production in the presence of the compound was expressed as percentage of forskolin induced cAMP production in the absence of (*R)*-3-amino-2-methylpropionic acid. 1 mM concentration of (*R)*-3-amino-2-methylpropionic acid caused a 41.31 ± 3.1 % inhibition of forskolin-induced cAMP production.

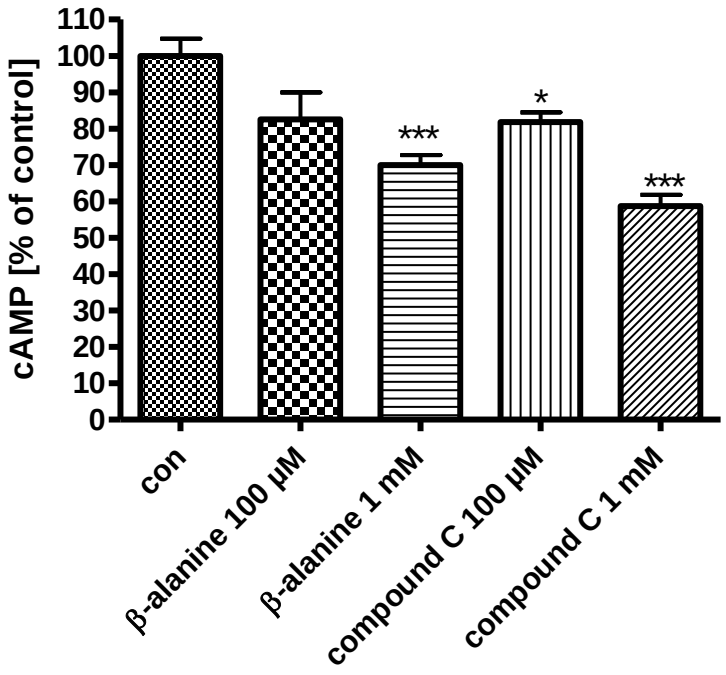


Fig. 12 Effect of (*R)*-3-amino-2-methylpropionic acid (compound C) on forskolin-induced cAMP accumulation in CHO Flp-In cells stably expressing the hMRGPRD receptor. Con indicates control values with forskolin, but without (*R)*-3-amino-2-methylpropionic acid: 21.99 ± 1.0 pmol cAMP/well. n = 4 - 6; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs*. respective control (con 100%, t-test). Data were processed using GraphPad Prism 4.

We repeated measurement with (*R)*-3-amino-2-methylpropionic acid on nontransfected CHO Flp-In cells to find out whether the decrease of cellular cAMP-production is specific. Concentrations of 100 µM and 1 mM were used to activate the receptor. Forskolin [10 µM] was used to stimulate intracellular cAMP accumulation. Duration of the reaction was 10 minutes. (*R)*-3-amino-2-methylpropionic acid [1 mM] caused a non-significant 17.12 ± 7.1 % decrease of forskolin-induced cAMP production in nontransfected CHO Flp-In cells.

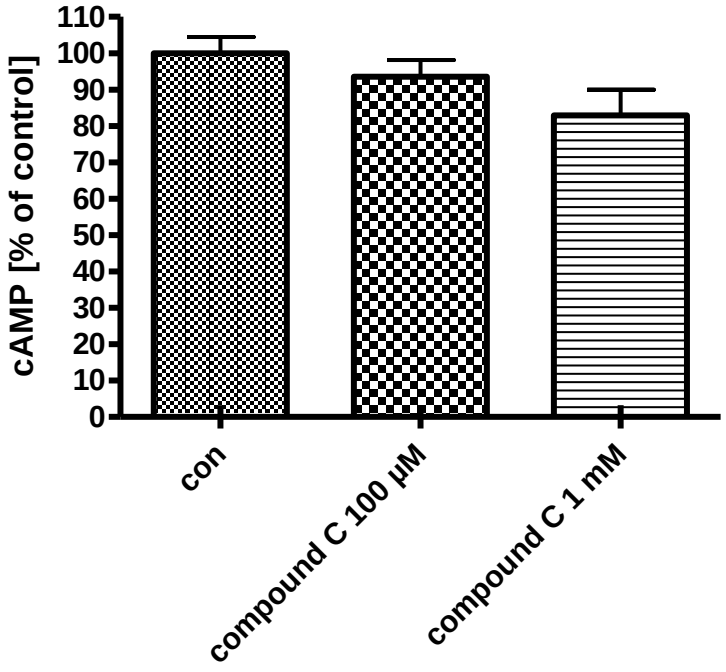


Fig. 13 Effect of (*R)*-3-amino-2-methylpropionic acid (compound C) on forskolin induced cAMP accumulation in nontransfected CHO Flp-In cells. Con indicates control values with forskolin, but without (*R)*-3-amino-2-methylpropionic acid: 15.7 ± 0.7 pmol cAMP/well. n = 4 – 6. Data were processed using GraphPad Prism 4.

6.1.4. *(RS)*-3-Amino-2-methylpropionic acid

Because of activity of the diastereomer (*R)*-3-amino-2-methylpropionic acid (DL-3-aminoisobutyric acid) we decided to test the chiral mixture of the compound *(RS)*-3-amino-2-methylpropionic acid using the same method and conditions. Addition of *(RS)*-3-amino-2-methylpropionic acid [1 mM] caused an inhibition of forskolin-induced cAMP production by 43.11 ± 7.7 %, which is in line with the activity of the *R*-enantiomer.

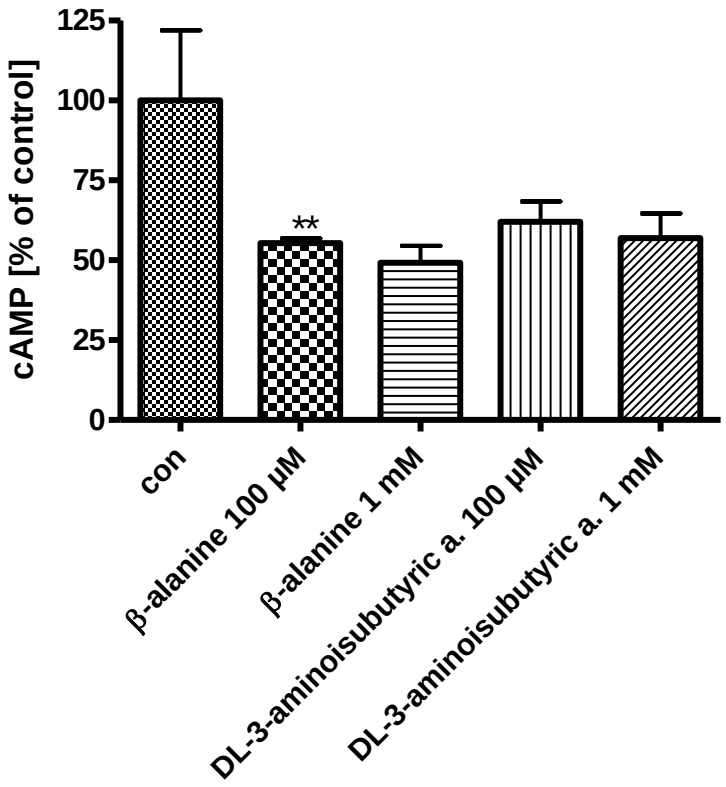


Fig. 14 Effect of *(RS)*-3-amino-2-methylpropionic *(*DL-3-aminoisobutyric acid) on forskolin-induced cAMP accumulation in CHO Flp-In cells stably expressing the hMRGPRD receptor. Con indicates control values with forskolin, but without *(RS)*-3-amino-2-methylpropionic acid: 16.5 ± 3.6 pmol cAMP/well. n = 4 - 6; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs*. respective control (con 100%, t-test). Data were processed using GraphPad Prism 4.

6.1.5. 1-(3-Piperidinopropyl)piperazin

The effect of 1-(3-piperidinopropyl)piperazin (compound M) at cells stably expressing the hMRGPRD in the previous NFAT Luciferase assay has been shown to be specific for Gq. This result was corfirmed by the use of the [^{3}H]cAMP radioaffinity assay, where no activation of the Gi has been seen. We measured concentrations of 30 μM, 100 μM, 300 μM and 1 mM. Forskolin [10 μM] was used to stimulate intracellular cAMP production. Duration of the reaction was 10 minutes. The inhibition of forskolin induced cAMP production in the presence of the compound was expressed as percentage of forskolin induced cAMP production in the absence of 1-(3-piperidinopropyl)piperazin. 1 mM concentration of 1-(3-piperidinopropyl)piperazin caused 34.4 ± 9.8 % increase of forskolin-induced cAMP production.

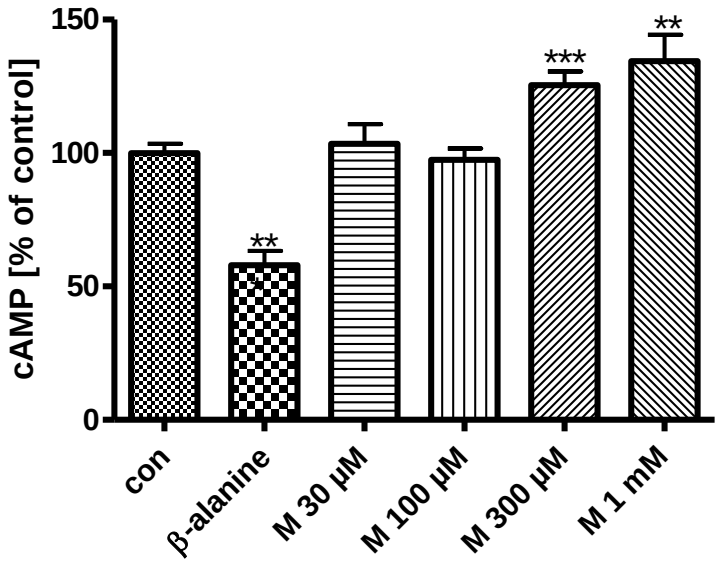


Fig. 15 Effect of 1-(3-piperidinopropyl)piperazin (compound M) on forskolin-induced cAMP accumulation in CHO Flp-In cells stably expressing the hMRGPRD receptor. Con indicates control values with forskolin, but without 1-(3-piperidinopropyl)piperazin: 20.51 ± 0.91 pmol cAMP/well. n = 6 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs*. respective control (con 100%, t-test). Data were processed using GraphPad Prism 4.

6.1.6. Fenpropidin

The effect of fenpropidin (compound L) at cells stably expressing the hMRGPRD in the previous NFAT Luciferase assay has been shown to be specific for Gq. This result was corfirmed by the use of the [^{3}H]cAMP radioaffinity assay, where no activation of the Gi has been seen. 100 μM concentration was measured. Forskolin solution [10 μM] was used to stimulate intracellular cAMP production. Duration of the reaction was 10 minutes. The inhibition of forskolin induced cAMP production in the presence of the compound was expressed as percentage of forskolin induced cAMP production in the absence of fenpropidin. 100 μM concentration fenpropidin caused 0.7 ± 11.98 % increase of forskolin-induced cAMP production.

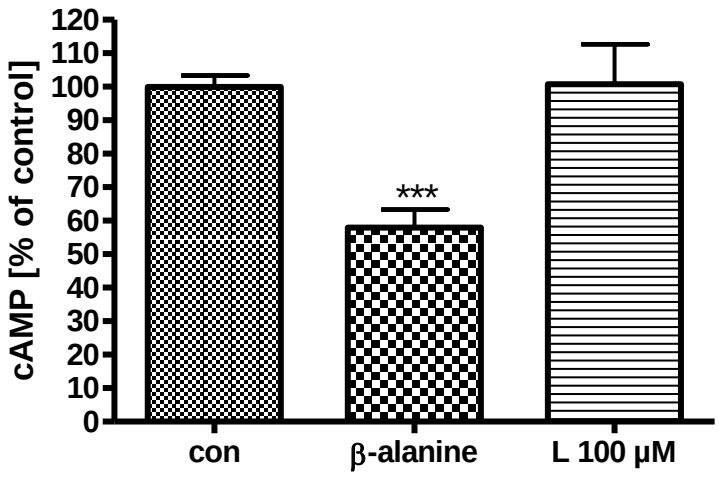


Fig. 16 Effect of fenpropidin (compound L) on forskolin-induced cAMP accumulation in CHO Flp-In cells stably expressing the hMRGPRD receptor. Con indicates control values with forskolin, but without 1-(3-piperidinopropyl)piperazin: 20.51 ± 0.91 pmol cAMP/well. n = 6 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs*. respective control (con 100%, t-test). Data were processed using GraphPad Prism 4.

6.2. NFAT Luciferase assay

6.2.1. Thirteen tested compounds

Thirteen compounds were tested in the NFAT-driven luciferase assay (see Tab. 1), as described above (see section 5.4.5). Concentrations of 1 mM of the different compounds were used to activate the hMRGPRD. Duration of the reaction was 3 hours. NFAT-driven luciferase activity is given in relative light units (RLU).

Tab. 1 Effects on NFAT-directed luciferase activity in CHO Flp-In cells stably expressing the hMRGPRD receptor by the 13 tested compounds. Results are given as relative light units (RLU) in % of respective control without any of the tested compounds (HBSS, 100 %).

	Compound	**Changes in RLU [% of control]**
	β-alanine	28.00 ± 1.5 %
A	3-(2-azabicyklo[2.2.1.]hept-2-yl)propionic acid hydrochloride	0.90 ± 1.9 %
B	3-(piperidin-1-yl)propionic acid	3.50 ± 3.2 %
C	*(R)*-3-amino-2-methylpropionic acid	29.40 ± 2.6 %
D	3-(morpholin-4-yl)propionic acid	-13.77 ± 1.6 %
E	3-(4-methylpiperazin-1-yl)propionic acid	0.30 ± 3.6 %
F	*DL*-3-aminopentanonic acid	-7.59 ± 1.5%
G	3-(1-piperazinyl)propionic acid	-1.27 ± 2.0 %
H	DL-isoserine	8.40 ± 2.9 %
I	L-isoserine	2.10 ± 3.0%
J	*(R)*-3-amino-2-phenylpropionic acid	-1.0 ± 7.2%
K	DL-3-amino-3-phenylpropionic acid	-15.73 ± 2.8 %
L	fenpropidin	-56.1 ± 2.0 %
M	1-(3-piperidinopropyl)piperazin	99.40 ± 13.8 %

When compared to the respective control (HBSS), most of the tested compounds showed no effect. Exceptions are (*R)*-3-amino-2-methylpropionic acid **(C)** that showed 29.40 ± 2.6 % increase in RLU, indicating a similar activity at the hMRGPRD when compared to β-alanine (increase 28 ±1.5%). In contrast, 1-(3-piperidinopropyl)piperazin **(M)** showed a marked increase in the NFAT-driven luciferase activity by 99.4 ± 13.8 %. The addition of fenpropidin **(L)** led to a decrease of the NFAT-directed luciferase activity by 56.1 ± 2.0 %.

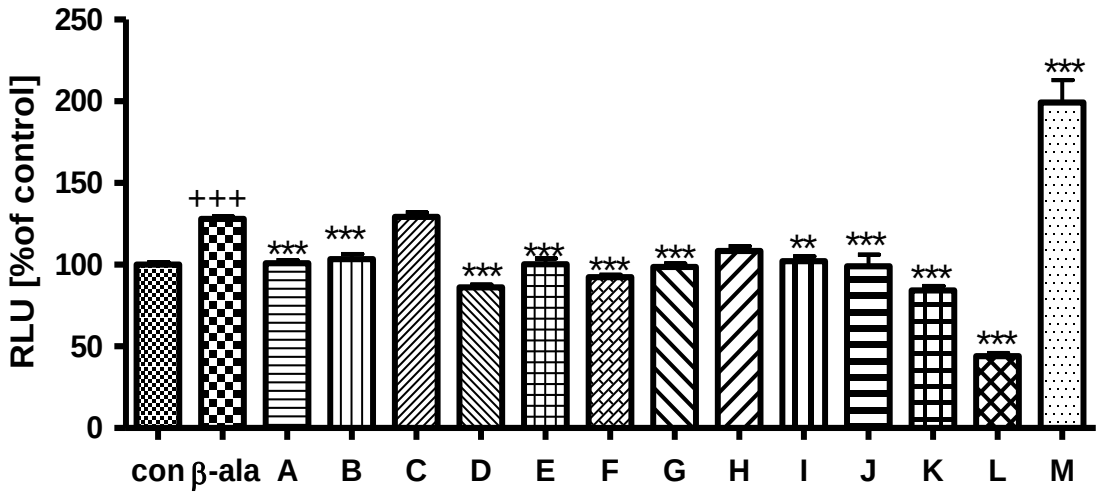


Fig. 17 Effects on NFAT-directed luciferase activity in CHO Flp-In cells stably expressing the hMRGPRD receptor by the tested compounds [1mM]. Con indicates control values in the absence of any of the tested compounds (con; 100 %; HBSS). n = 4 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs*. β-alanine [1 mM] (t-test). +++ $p \leq 0.001$ significant differences *vs*. control (t-test). Data were processed using GraphPad Prism 4.

6.2.2. Fenpropidin

In the previous luciferase reportergene-assay (see section 6.2.1), Fenpropidin (compound **L**) showed an agonistic effect at CHO Flp-In cells stably expressing the hMRGPRD. In this measurement concentrations of 100 µM and 1 mM were used to activate the hMRGPRD. Duration of the reaction was 3 hours. NFAT-driven luciferase activity is given in relative light units (RLU). When compared to the respective control (β-alanine 1 mM, increase in RLU by 19.2 ± 2.8 %) 100 µM concentration of fenpropidin caused 57.6 ± 3.6 % increase in RLU. 1 mM concentration was toxic for cells as could be seen by microscopy, resulting in a decrease of RLU by 56.22 ± 3.2 %.

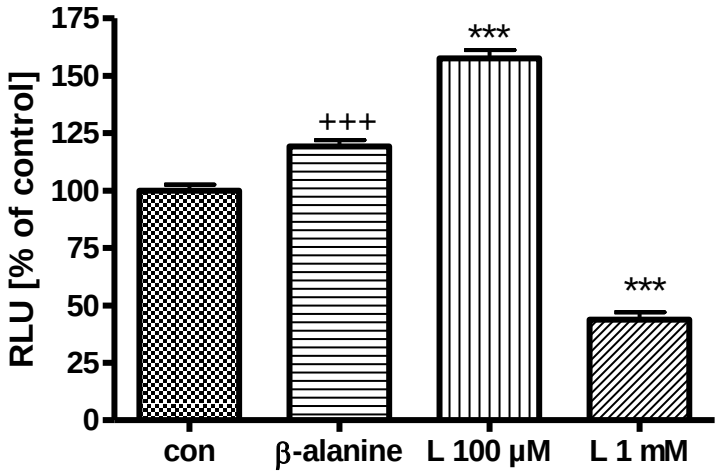


Fig. 18 Effect on NFAT-directed luciferase activity in CHO Flp-In cells stably expressing the hMRGPRD receptor caused by fenpropidin (compound **L**). Con indicates control values in the absence of fenpropidin (100 %; HBSS). n = 4 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs.* β-alanine [1 mM] (t-test). +++ $p \leq 0.001$ significant differences *vs.* control (t-test). Data were processed using GraphPad Prism 4.

We repeated the measurement with fenpropidin on nontransfected CHO Flp-In cells to find out whether the change in NFAT-driven luciferase activity is specific. Concentrations of 100 µM and 1 mM were used. Duration of the reaction was 3 hours. NFAT-driven luciferase activity is given in relative light units (RLU). When compared to the respective control (100 %, HBSS) 100 µM concentration of fenpropidin caused

67.7 ± 11.4 % increase in RLU. 1 mM concentration was toxic for cells as could be seen by microscopy, resulting again in a decrease of RLU by 93.2 ± 1.0 %. This result revealed an unspecific effect of fenpropidin on CHO Flp-In cells.

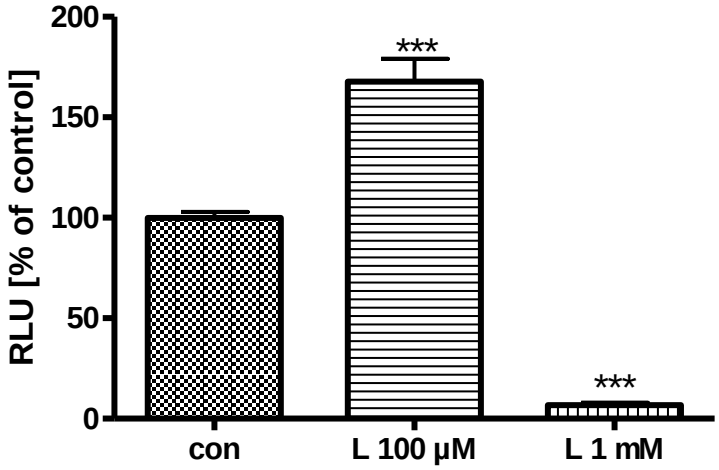


Fig. 19 Effect on NFAT-directed luciferase activity in CHO Flp-In caused by fenpropidin (compound **L**). Con indicates control values in the absence of fenpropidin (100 %; HBSS). n = 4 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs.* control (t-test). Data were processed using GraphPad Prism 4.

6.2.3. 1-(3-Piperidinopropyl)piperazin

As 1-(3-piperidinopropyl)piperazin (compound **M**) caused significant changes in NFAT-driven luciferase activity, it was tested at nontransfected CHO Flp-In cells using the same method. Concentrations of 100 µM and 1 mM were used. Duration of the reaction was 3 hours. NFAT-driven luciferase activity is given in relative light units (RLU). When compared to the respective control (100 %, HBSS), the addition of 100 µM concentration of 1-(3-piperidinopropyl)piperazin caused an increase in RLU by 30.9 ± 4.3 % and 1 mM concentration showed an increase in RLU by 325.6 ± 19.7 %. This result revealed an unspecific effect of 1-(3-piperidinopropyl)piperazin on CHO Flp-In cells.

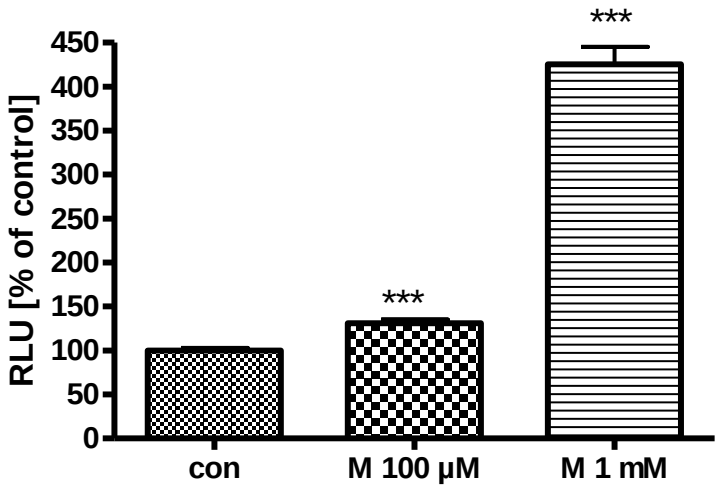


Fig. 20 Effect on NFAT-directed luciferase activity in CHO Flp-In caused by 1-(3-piperidinopropyl)piperazin (compound M). Control indicates control values in the absence of the compound (100 %; HBSS). n = 4 - 8; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs.* control (t-test). Data were processed using GraphPad Prism 4.

6.2.4. 3-Aminopropan-1-ol

In the previous [^{3}H]cAMP radioaffinity assays, 3-aminopropan-1-ol caused an unspecific increase of forskolin induced cAMP production. Concentrations of 100 µM and 1mM were used to test whether 3-aminopropan-1-ol also shows an unspecific effect on NFAT-directed luciferase activity. The measurement was performed on nontransfected CHO Flp-In cells. Duration of the reaction was 3 hours. NFAT-driven luciferase activity is given in relative light units (RLU). When compared to the respective control (100 %, HBSS), the addition of 100 µM concentration of 3-aminopropan-1-ol caused an increase in RLU by 33.5 ± 0.3 % and 1 mM concentration showed an increase in RLU of 75.5 ± 12.5 %.

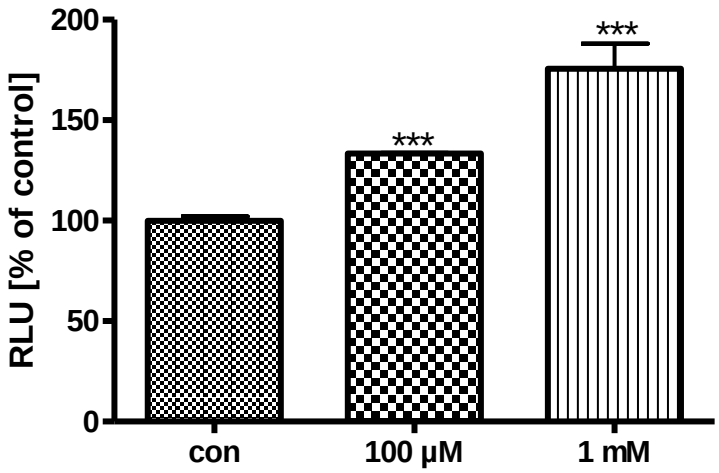


Fig. 21 Effect on NFAT-directed luciferase activity in CHO Flp-In caused by 3-aminopropan-1-ol. Con indicates control values in the absence of the compound (100 %; HBSS). n = 4 - 10; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ significant differences *vs*. control (t-test). Data were processed using GraphPad Prism 4.

7. DISCUSSION

In the last century, our knowledge about Mas-related G Protein-coupled receptors (MRGPR) has been significantly extended. New agonists have been discovered and some effects upon activation of MRGPRs have been described. Unfortunately, all information we know about the MRGPR family are still very superficial, there are some subfamilies which are not yet analyzed and no subtype of the MRGPRs has officially been declared deorphanized.

The MRGPRD subfamily belongs to the mammalian family of MRGPR, which is subdivided into 9 subfamilies (MRGPR A-H, X). Only subfamily X is specific to humans, whereas subfamilies A, B, C and H can be found only in rodents. Subfamilies D to G are conserved in some mammalian species (Dong *et al*., 2001, Lembo *et al*., 2002, Zylka *et al*., 2003). The location, structure and function of most family members are incompletely charecterized. MRGPRA3 has been characterized as itch-mediating chloroquine receptor (Liu *et al*., 2009), MRGPRC is responsible for analgesic and pain-enhancing effects and is activated by a set of structurally related peptides (Grazzini *et al*, 2004). Such a huge range of agonists is unique among the GPCR group. For MRGPRB, E, F, G and H, no ligands have been described so far. Interestingly, the MRGPRX family is very well described. This subfamily affects excitability of primary sensory neurons. Several agonists and antagonists were identified at the MRGPRX1 (BAM peptides, chlorochine, benzimidazol derivates and others) (Lembo *et al*., 2002). MRDPRX2 binds several other peptidergic ligands and also shows a broader expression pattern (Burstein *et al*., 2006).

In contrast to MRGPRX and C subfamilies, the MRGPRD receptor shows a very restricted structure-activity relationship, which was confirmed in the present study.

β-alanine is endogenously expressed in humans as a part of the degradation of dihydrouracil and carnosin in the liver (Wu *et al*., 1993; Rajendra *et al*., 1997). It is the best characterized agonist at the hMRGPRD so far. In the present study, we confirmed the MRGPRD-signaling *via* Gq and Gi proteins. Activation of the receptor by β-alanine

induced intracellular Ca^{2+}-influx and suppressed intracellular cAMP production in CHO Flp-In cells stably expressing the hMRGPRD receptor. In the [^{3}H]cAMP radioaffinity assay performed on CHO Flp-In cells stably expressing the hMRGPRD receptor, β-alanine caused 36.63 ± 5.2 % inhibition of forskolin induced cAMP production with an EC_{50} value of 32.96 μM which is in good agreement with literature data (Shinohara *et al*., 2004).

Analysis of the different structures tested in the present study, reveals a high similarity to the already known ligand β-alanine. All compounds possess the base of structure formed by aminopropionic acid only with different substituents. In the present study we have discovered that even a small change in the structure of β-alanine caused marked changes in the activity of the tested compounds. MRGPRD did not react on chloroquine, which was characterized as an agonist for MRGPRA3 (Solinski *et al*., 2014). Thus, the results indicate a very restricted binding site of the hMRGPRD receptor protein.

In experiments of the present study, from the fifteen tested compounds, only five showed an effect (3-aminopropan-1-ol, 1-(3-piperidinopropyl)piperazin, *(R)*-3-amino-2-methypropionic acid, *(RS)*-3-amino-2-methylpropionic acid and fenpropidin). Compounds showing an effect on cellular cAMP production and on luciferase production in CHO Flp-In cells stably expressing the hMRGPRD receptor were additionally analyzed at nontransfected Flp-In cells to exclude unspecific effects at CHO cells used as expression system in this study.

In the [^{3}H]cAMP radioaffinity assay performed on CHO Flp-In cells stably expressing the hMRGPRD receptor, **3-aminopropan-1-ol** caused an increase of forskolin-induced cAMP production. The same method was performed on nontransfected CHO Flp-In cells, where an increase in cAMP production was again visible, indicating an unspecific effect of this compound on CHO Flp-In cells. This effect was markedly higher on nontransfected cells (99.3%) in comparison to cells expressing the hMRGPRD receptor (56%). 3-Aminopropan-1-ol has also an unspecific effect on Gq signaling pathway. When compared to the respective control (100 %, HBSS) 1 mM concentration showed increase in RLU in nontransfected CHO Flp-In cells.

In the [^{3}H]cAMP radioaffinity assay performed on CHO Flp-In cells stably expressing the hMRGPRD receptor 1 mM concentration of **1-(3-piperidinopropyl)piperazin** caused an increase of forskolin-induced cAMP production. In the NFAT-directed luciferase assay 1-(3-piperidinopropyl)piperazin caused an increase in luciferase activity on CHO Flp-In cells stably expressing the hMRGPRD receptor (99%) and also on nontransfected CHO Flp-In cells (326%), revealing this effect to be nonspecific in the Gq signaling pathway.

In the [^{3}H]cAMP radioaffinity assay performed on CHO Flp-In cells stably expressing the hMRGPRD receptor, **(*R*)-3-amino-2-methylpropionic acid** and enantiomeric mixture of ***(RS)*-3-amino-2-methylpropionic** acid showed an inhibition of forskolin-induced cAMP production. (*R)*-3-amino-2-methypropionic acid caused an inhibition of cellular cAMP production at Flp-In cells stably expressing the hMRGPRD receptor (41%), whereas the effect was markedly lower at nontransfected CHO Flp-In cells (17%). Therefore, we cannot rule out an unspecific effect of this compound as well. The [^{3}H]cAMP radioaffinity assay also showed that both enantiomer and its enantiomeric mixture are equally active. (*R)*-3-amino-2-methylpropionic acid was also tested in the NFAT-directed luciferase assay and increased the luciferase activity, suggesting that both pathways *via* Gi and Gq are activated by this compound.

In the NFAT-directed luciferase assay, **fenpropidin** [100 μM] caused an increase in RLU and 1 mM concentration of this compound showed an inhibition of RLU probably caused by its toxicity for cells. In nontransfected cells we also saw an increase in RLU in the case of the lower concentration, wheras 1mM concentration of fenpropidin was again toxic.

When compared to the structures, (*R)*-3-amino-2-methylpropionic acid has the most similar structure to β-alanine (the only difference is one methyl- group), therefore also the effect of this compound is more similar to β-alanine than the effect of the other compounds. The effect of the other 4 compounds was shown to be unspecific for CHO Flp-In cells, confirming the very restricted structure-activity relationship of MRGPRDs in contrast to MRGPRX and C subfamilies. Thus, the strong resriction of the structure of putative ligands at the MRGPRD will complicate the development of new compounds that specifically interact with the receptor protein.

8. CONCLUSION

The aim of this work was to find new compounds which activate the hMRGPRD, contributing to a better understanding of the function of the hMRGPRD receptor.

In summary, we investigated an effect at five out of fifteen compounds structurally related to β-alanine, suggesting that a ligand must meet very strict specifications to be recognized by the hMRGPRD.

MRGPRD shows a close structure-activity relationship. The receptor eventually serves as a good target for drug development, because of its restricted expression pattern as in the central nervous system, MRGPRD can only be found in primary sensory trigeminal ganglia neurons. This restricted expression pattern may be beneficial for a reduced risk of off-target effects of new drugs that specifically affect the MRGPRD. These drugs may be used in the future to specifically treat itching and/or pain.

9. LIST OF FIGURES AND TABLES

10. REFERENCES

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