

Quantitative evaluation of sodium butyrate on dual coupling of human muscarinic receptor M₄

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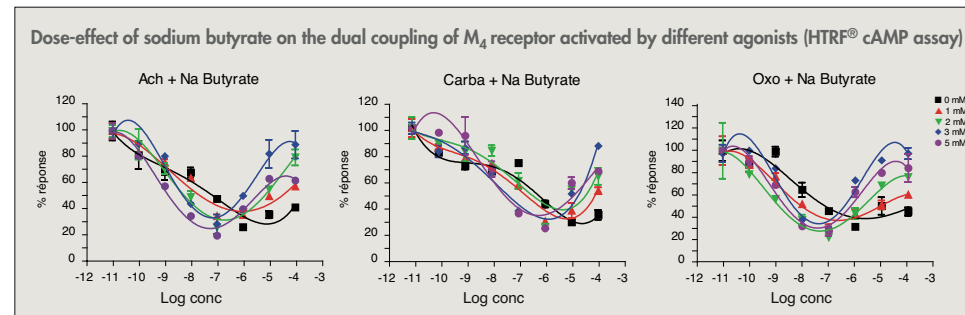
Methods – 1

Expression of recombinant human muscarinic 4 receptor in CHO-S cells

- The human genomic DNA (Clontech) was used to isolate the gene encoding human M₄ receptor (PCR with specific primers). The corresponding gene was cloned into a mammalian expression vector pCI/neo (Promega) and then stably transfected into CHO-S cells (Invitrogen).
- CHO-S M₄ cells were cultured in DMEM with 5% dialyzed FCS and G418. 1 mM to 5 mM of sodium butyrate (Sigma) were added 12-18 h prior to experimentation.

cAMP assay

CHO-S M₄ cells were harvested by EDTA and re-suspended in HBSS buffer containing 0.1% glucose and 0.5 mM IBMX. The cells were dispensed into a black 96 half-well plate at a density of 10,000 – 15,000 cells per well. After 30 min incubation at room temperature, an equal volume of assay buffer containing 3.0 μM forskolin, then acetylcholine, oxotremorine or carbachol were added. After 30 min incubation cAMP was measured via the HTRF® (homogeneous time-resolved fluorescence) technology (CisBio International).



Methods – 2

RNA extraction and cDNA synthesis

RNA was extracted from cultured CHO-S/M₄ cells using Trizol (Sigma), 1.0 μg of total RNA was reversely transcribed to cDNA using MMLV and random hexamers (Clontech).

Real-time PCR on IQ5 cyclor

- Real-time PCR was performed by SYBR Green technology using Bio-Rad Master mix.
- The condition of PCR for each pair of primer was optimized by temperature gradient amplification.

Primers used in real-time PCR (Qiagen)

Gene	Primer name	Qiagen number	Detected transcript	Amplicon size
M ₄ receptor	Hs-CHRM4	QT00214963	NM_000741	142 bp
G α3	Mm-Gnai3	QT01062278	NM_010306	109 bp
G α5	Hs-Gnas	QT00021315	NM_000516	91bp
GAPDH	Mm-Gapd	QT00309099	NM_001001303	100 bp
18S ribosome	Mm-Rn18S	QT01036875	X00686	149 bp
18S/28S ribosome	Rn-Rn1-1	QT00199374	M11188	103 bp
Cyclophilin A	Hs-PPIA	QT00052311	NM_021130	121 bp

Mm = mouse Rn = rat Hs = human

Methods – 3

Relative real-time PCR

2-^{ΔΔCt} method was used to determine the relative abundance of a studied gene expression between the control and the sodium butyrate treated cells. ΔCt is the difference in Ct values between housekeeping (used for normalisation) and specific gene.

Absolute real-time PCR

Plasmid construction

- Conventional PCR was performed to generate gene-specific amplicon using the same primers necessary for real-time PCR.
- The purified PCR products were cloned to T/A cloning pCR2.1 vector (Invitrogen). The amplicon identity was confirmed by sequencing.

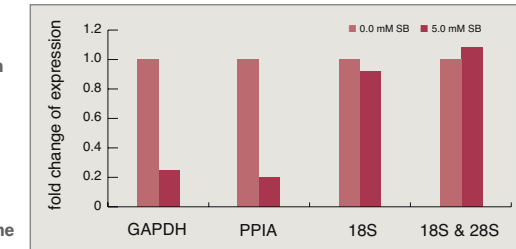
Standard curves

- Exact quantification of the DNA content for each plasmid was done in various dilution and repeats on a spectrophotometer.
- Six 10-fold serial dilutions were made starting from a plasmid concentration of 10¹⁰g/μl.
- A standard curve was drawn by plotting the threshold cycle (Ct) against the log of the concentration of each plasmid.
- The copy number (per μl) was calculated as follows: (6 × 10²³) × ADN (g/μl) / plasmid size (bp) × 660 (g/mol)

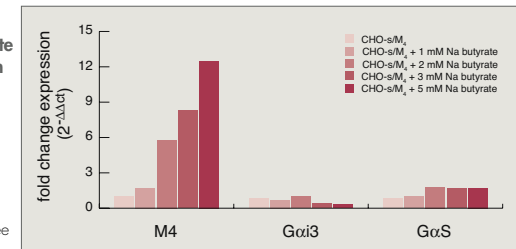
Sequence alignment between amplicon and detected transcript

Alignment between amplicon of CHO/M ₄ and NM_000741	
M4_CHO_amplicon	Sequence: ---CTA TGAGCGGTG GAATGGTCT TCATTGCCAC AGTGACAGGC
NM_ORF_000741	Sequence: TCATCATCCC ACAATCGCTA TGAGACGGTG GAATGGTCT TCATTGCCAC AGTGACAGGC
M4_CHO_amplicon	Sequence: TCCGTGAGCC TGGTGACTGT CGTGGGCAAC ATCCTGGTGA TGCTGTCCAT CAAGGTCAAC
NM_ORF_000741	Sequence: TCCGTGAGCC TGGTGACTGT CGTGGGCAAC ATCCTGGTGA TGCTGTCCAT CAAGGTCAAC
M4_CHO_amplicon	Sequence: AGGCAGTGC AGACAGTCA AACTACTTTC CTCTCAGCC TGGCGTGTGC TGATCTATC
NM_ORF_000741	Sequence: AGGCAGTGC AGACAGTCA AACTACTTTC CTCTCAGCC TGGCGTGTGC TGATCTATC
Alignment between amplicon of CHO/G α3 and NM_010306	
G alpha3_CHO_a...	Sequence: ---GAGAAAGCG GCCAAAGAG TGAAGTGTCT GGTGCTGGT
G alpha3_NM013106	Sequence: CGCAACTTGC GGGAGGACGG GAGAAAGAG GCCAAAGAG TGAAGTGTCT GGTGCTGGC
G alpha3_CHO_a...	Sequence: GGTGGAAAT CTGGTAAAG TACCATTGTG AAACAGATGA AAATCATTCA TGAGGACGGC
G alpha3_NM013106	Sequence: GGTGGAAAT CTGGTAAAG TACCATTGTG AAACAGATGA AAATCATTCA TGAGGACGGC
G alpha3_CHO_a...	Sequence: TATTCAGAGG ACGAATGTAA ACAGTATAAA GTAGTTGTCT ACAGCAATAC TATTCAGTCC
G alpha3_NM013106	Sequence: TATTCAGAGG ACGAATGTAA ACAGTATAAA GTAGTTGTCT ACAGCAATAC TATTCAGTCC
Alignment between amplicon of CHO/G α5 and NM_00516	
Galpha3_CHO_a...	Sequence: ---CGCACACA AAAAGATCGA GAAGCAGGTG CAGAGGACAC AGCAGGTCTA CCGGGCCACG
GalphaS_NM000...	Sequence: GAGCGCACACA AAAAGATCGA GAAGCAGGTG CAGAGGACAC AGCAGGTCTA CCGGGCCACG
Galpha3_CHO_a...	Sequence: CACCGCTGC TGTGCTGGG TGTGGAGAA TCTG
GalphaS_NM000...	Sequence: CACCGCTGC TGTGCTGGG TGTGGAGAA TCTG

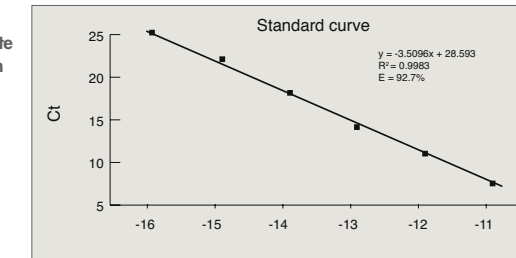
Effect of sodium butyrate (SB) on the expression of housekeeping genes – GAPDH, Cyclophilin A (PPIA), 18S ribosome and 18S/28S ribosome



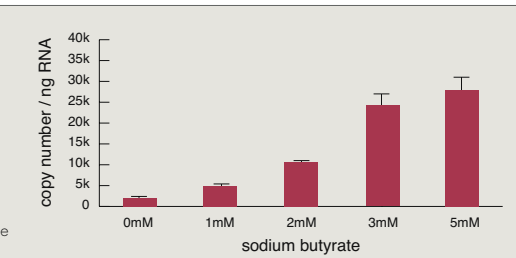
Dose-effect of sodium butyrate on the expression of M₄ receptor, G α3 and G α5 (housekeeping gene 18S ribosome)



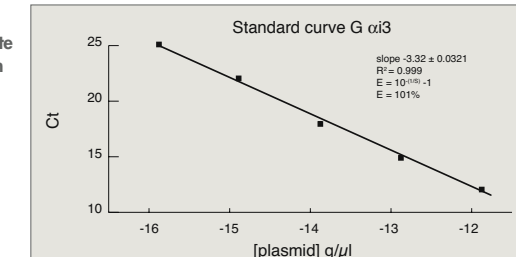
Dose-effect of sodium butyrate on the expression copy number of M₄ receptor



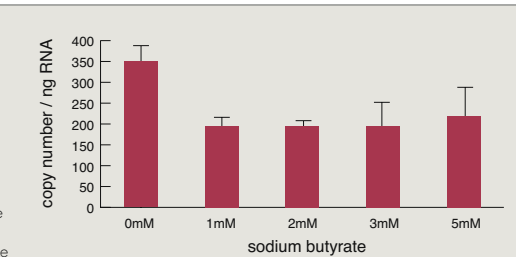
Data are representative of three experiments.



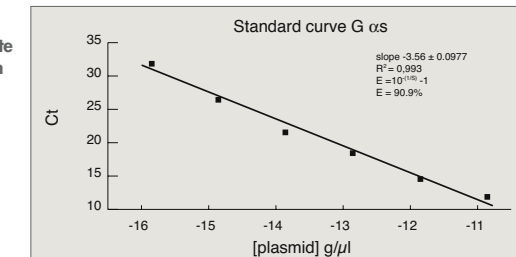
Dose-effect of sodium butyrate on the expression copy number of G α3



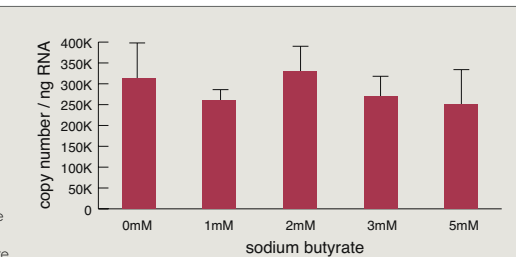
Data are shown for mean ± S.D for three experiments each performed in triplicate



Dose-effect of sodium butyrate on the expression copy number of G α5



Data are shown for mean ± S.D for three experiments each performed in triplicate



Conclusion

- Our results showed for the first time that the expression copy number of G α5 protein is much more important than G α3 (G α3 is predominantly expressed in CHO cells, data not shown).
- Dose dependant enhancement of sodium butyrate on the expression of M₄ receptor (10 to 12 fold at 5 mM) was observed. In contrast the expression of G α3 and G α5 genes was slightly affected by sodium butyrate treatment.
- M₄ receptor dual coupling (Gαi/Gαs) is dependent on the expressed receptor density and the agonist concentration. No agonist trafficking was observed.
- A high stoichiometry between G α5 and M₄ receptor gene in sodium butyrate untreated CHO-S M₄ cells does not favor the M₄/ G α5 coupling at the 10 pM to 1 μM concentration range of agonists, therefore, physiological pleiotropic G protein coupling could not exist for M₄ receptor.
- Use of sodium butyrate to manipulate the expression level of heterologous receptor and G protein might provide a new approach in the development of a robust functional assay for G αi coupling receptor.



Acknowledgements

We acknowledge Mrs Karine Cheroux, Ms Maryse Martin and Céline Billy for technical assistance. We thank Mrs Catherine Moreau and Mr Loïc Dorgeret for poster preparation.