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Maximakinin: an amphibian bradykinin homologue integrated into fusion proteins that bind to the bradykinin B_2 receptor

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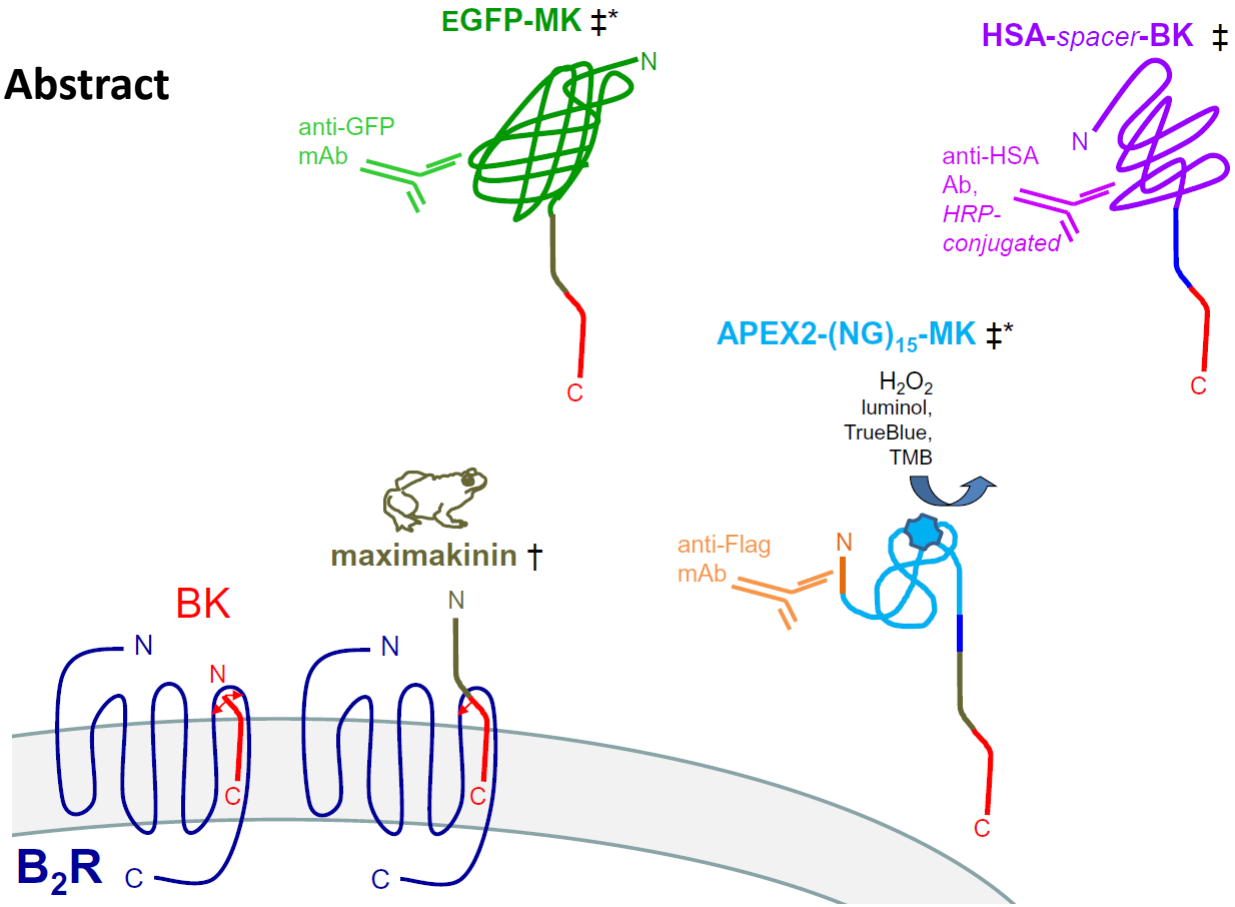
Graphical Abstract

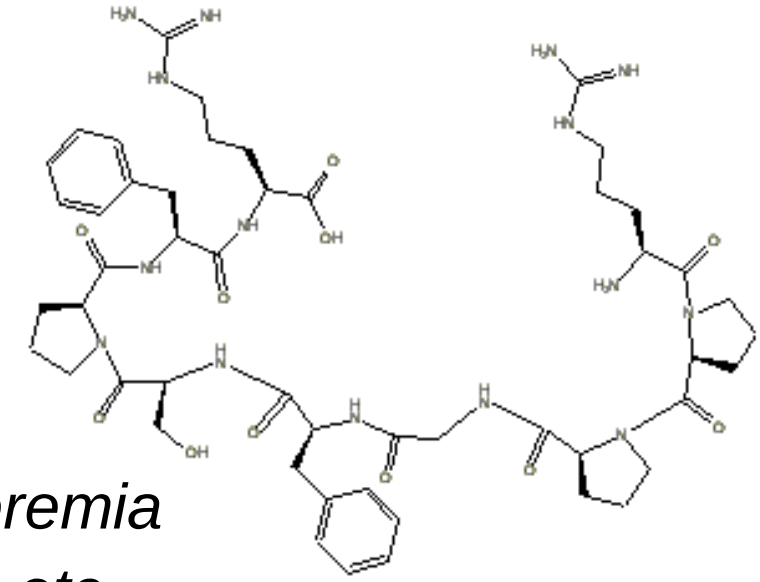


Abstract: Bradykinin (BK), a blood-derived nonapeptide, is a vasodilator, increases microvascular permeability and stimulates nociceptors mostly via receptors (B2Rs). Maximakinin (MK), discovered in the skin of an amphibian, has the full BK sequence extended by 9 residues at its N-terminus (DLPKINRKGPRPPGFSPFR). MK has a good affinity for the rat and rabbit B2R and is more resistant to inactivation than BK. Fusion proteins consisting of MK positioned at the C-terminus of functional proteins (enhanced green fluorescent protein (EGFP), the peroxidase APEX2) were produced as lysates of HEK 293a cells transfected with the corresponding expression vector; they are agonists of the B2R as judged from the receptor-mediated signaling in cells expressing the recombinant receptors. EGFP-MK is endocytosed along with the B2Rs and colocalized with various molecular partners (β-arrestins, Rab5, LAMP1) during its slow transition towards lysosomes (epifluorescence microscopy). It does not bind to angiotensin converting enzyme or kinin B1 receptors. The peroxidase APEX2-(Asn-Gly)15-MK, containing a further spacer sequence, detects B2Rs with cytochemistry reagents, luminol or TMB. However, MK and the fusion proteins that include MK have little affinity for the human form of the B2R. Effects of changes in the spacer sequence support the feasibility of alleviating this limitation. Positioning MK or BK with spacers at the C-terminus of human serum albumin failed to produce B2R ligands. Fusion protein ligands of the B2R are subjected to slow intracellular inactivation, species specificity and possible steric hindrance between the receptor and large proteins.

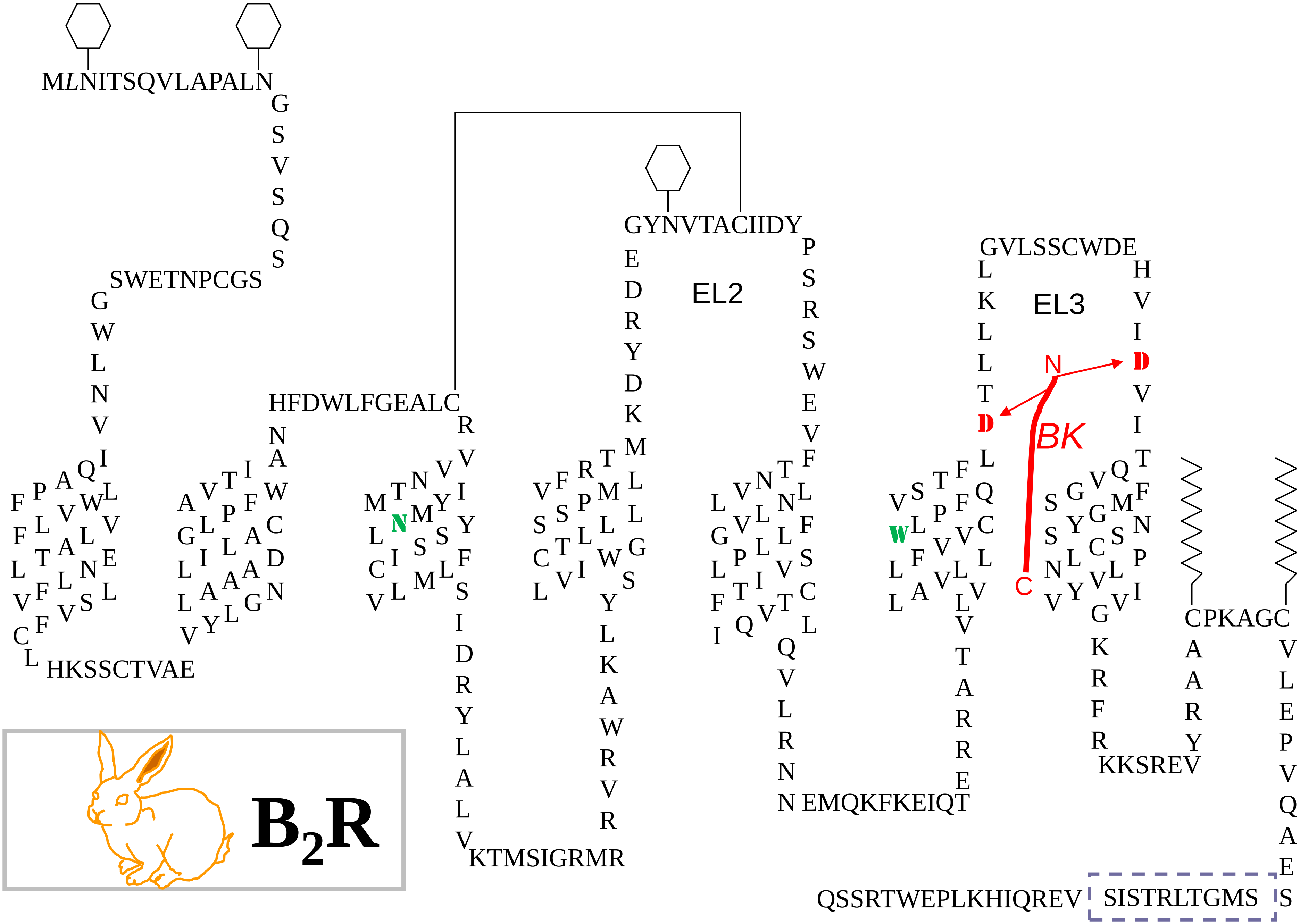
Keywords: bradykinin; B2 receptors; peptide ligands; fusion protein design

Introduction
Importance of bradykinin (BK)

- Derived from kininogens via the action of kallikreins
- A small and unstable peptide

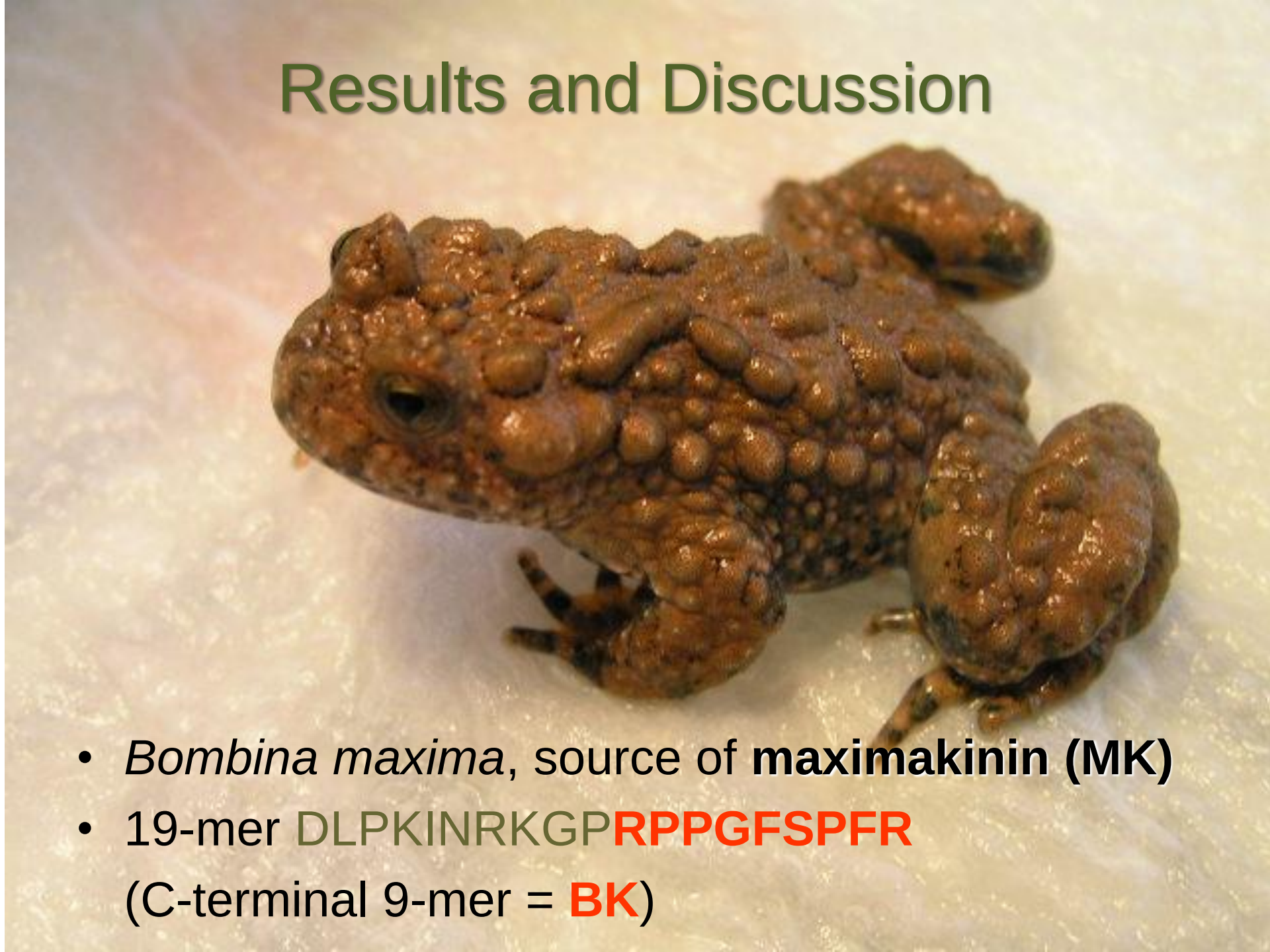


- Target cell types:
  - Endothelial cells: *edema, hyperemia*
  - Sensory nerve terminals: *pain, etc.*
  - Epithelial cells: *various inflammatory consequences*
  - Smooth muscle cells

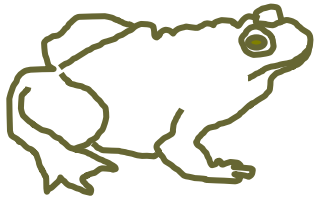


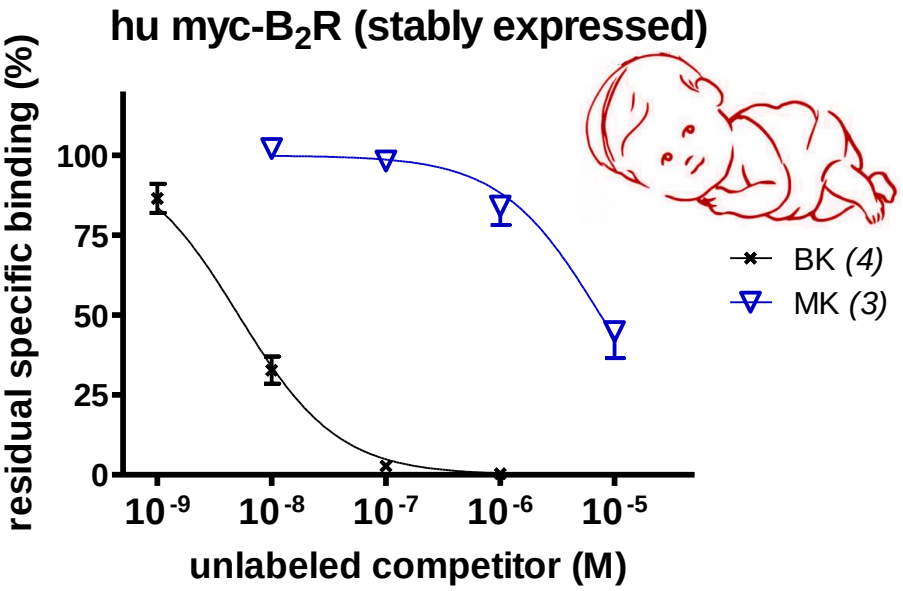
MLNITSQVLAPALN
SWETNPCGS
HFDWLFGEALC
HKSSCTVAE
KTMSIGRMR
GYNVTACIIDY
EL2
GVLSSCWDE
EL3
N
D
D
BK
C
CPKAGC
KKSREV
N EMQKFKEIQT
QSSRTWEPLKHIQREV
SISTRLTGMS
B2R

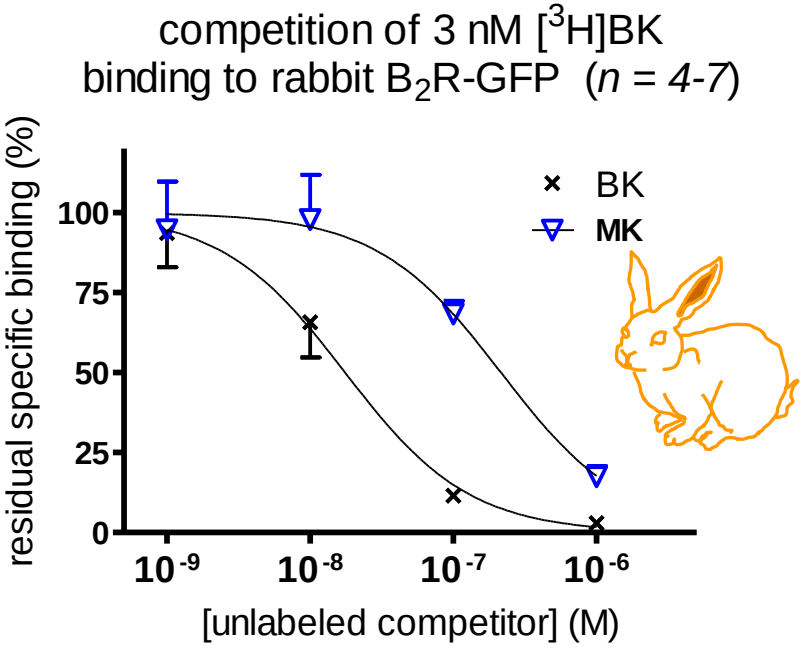
Results and Discussion

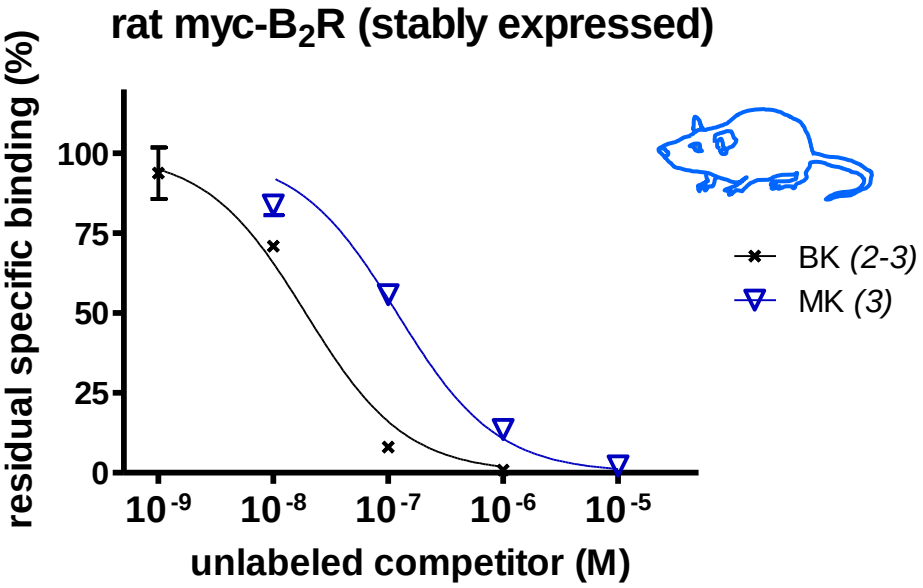


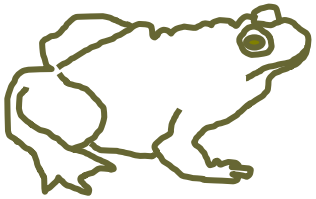
- *Bombina maxima*, source of **maximakinin (MK)**
- 19-mer DLPKINRKGP**RPPGFSPFR**
  (C-terminal 9-mer = **BK**)

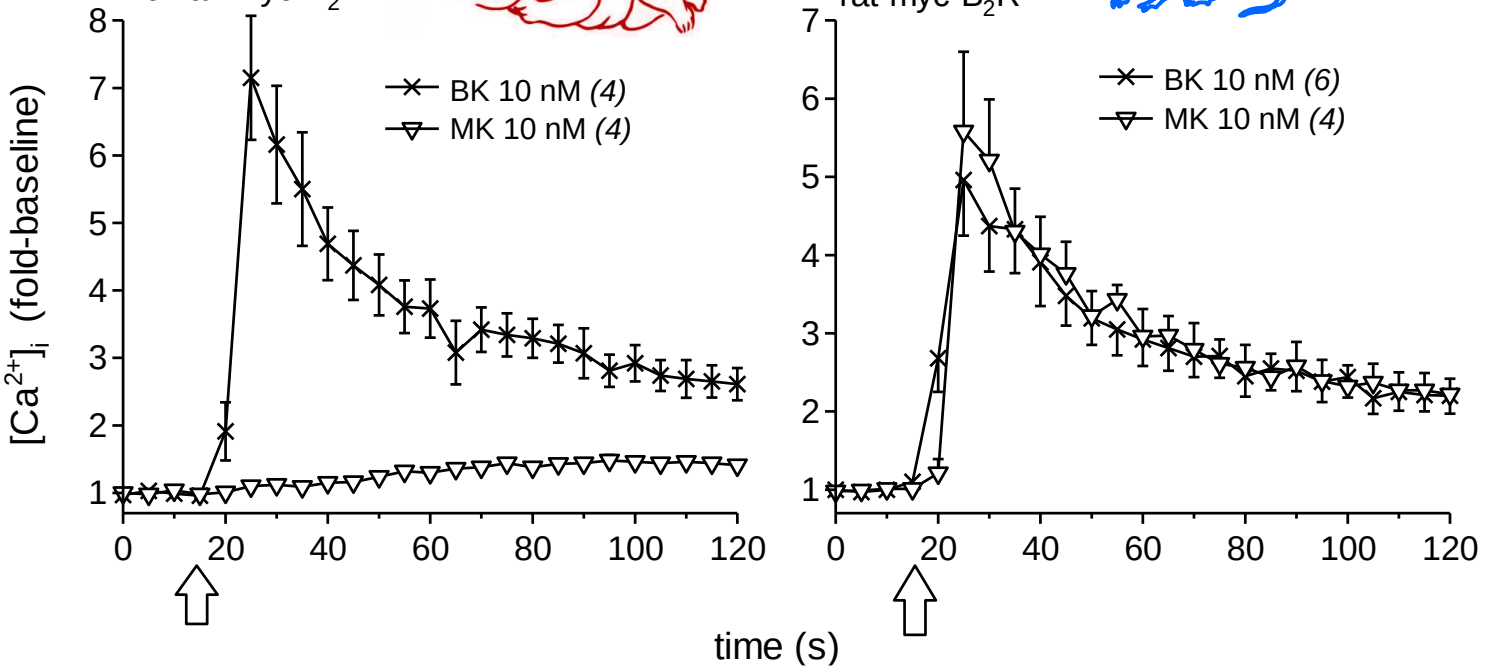


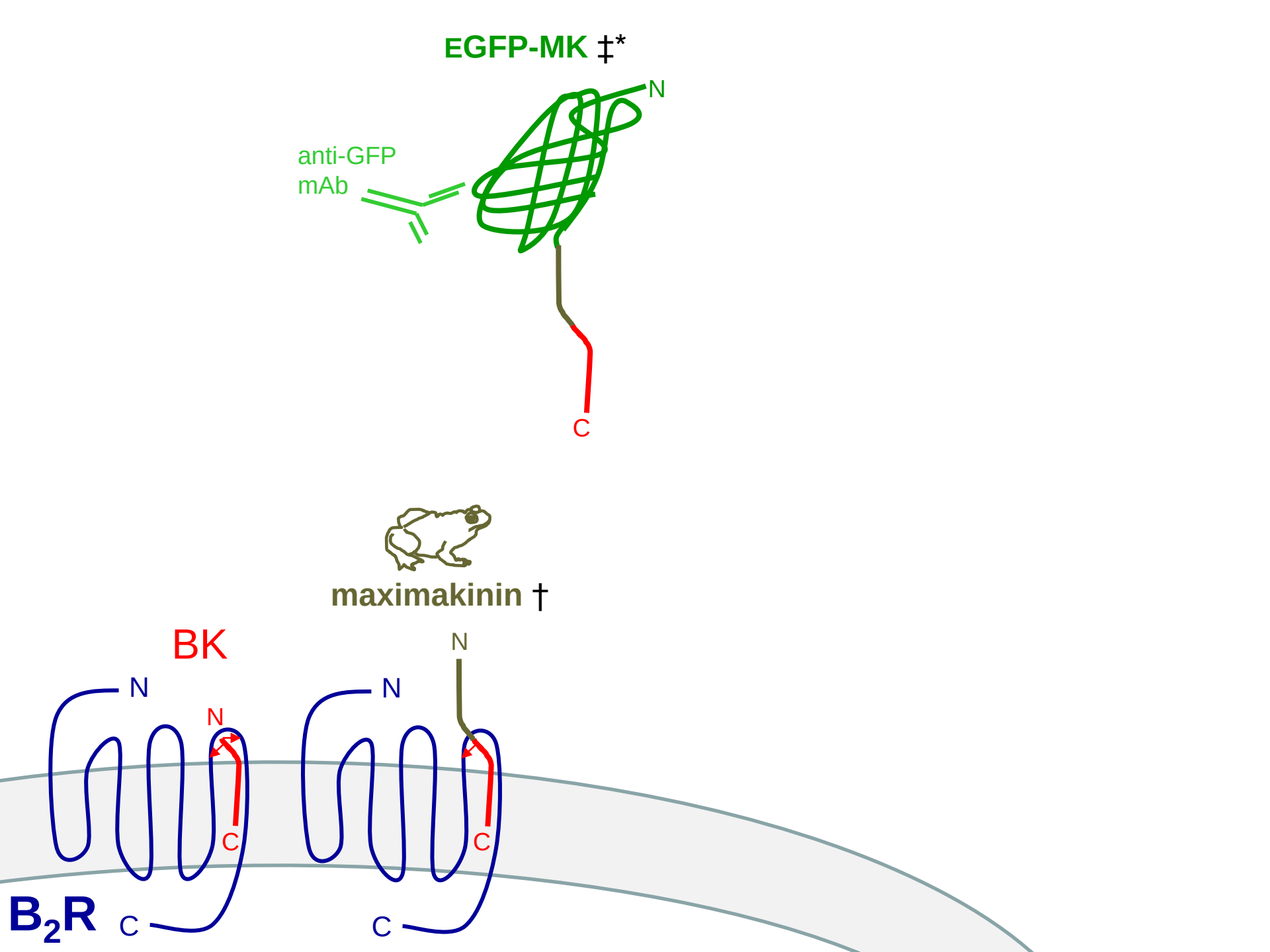

hu myc-B2R (stably expressed)
residual specific binding (%)
100
75
50
25
0
10-9
10-8
10-7
10-6
10-5
unlabeled competitor (M)
BK (4)
MK (3)


competition of 3 nM [3H]BK
binding to rabbit B2R-GFP (n = 4-7)
residual specific binding (%)
100
75
50
25
0
10-9
10-8
10-7
10-6
[unlabeled competitor] (M)
BK
MK


rat myc-B2R (stably expressed)
residual specific binding (%)
100
75
50
25
0
10-9
10-8
10-7
10-6
10-5
unlabeled competitor (M)
BK (2-3)
MK (3)

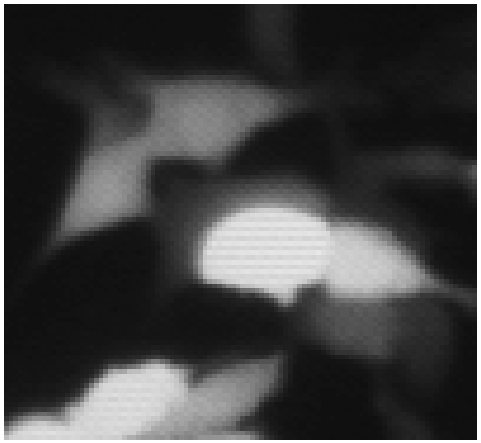



human myc-B2R
rat myc-B2R
BK 10 nM (4)
MK 10 nM (4)
BK 10 nM (6)
MK 10 nM (4)
[Ca2+]i (fold-baseline)
time (s)
0
20
40
60
80
100
120
1
2
3
4
5
6
7
8


EGFP-MK ‡*
N
anti-GFP
mAb
C
maximakinin †
BK
N
N
N
N
C
C
B2R
C
C

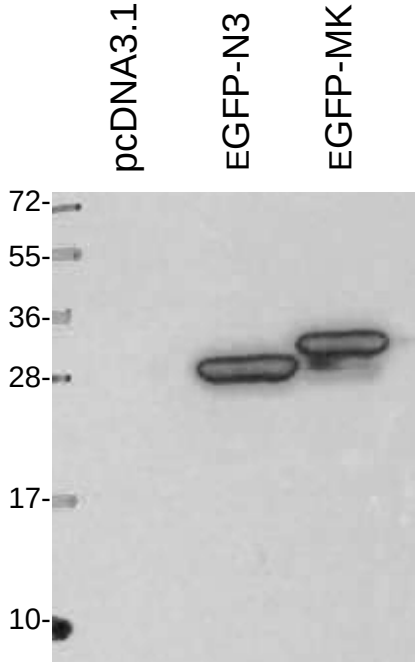
Lysates of HEK 293a producer cells

Producer HEK 293a cells
EGFP-MK

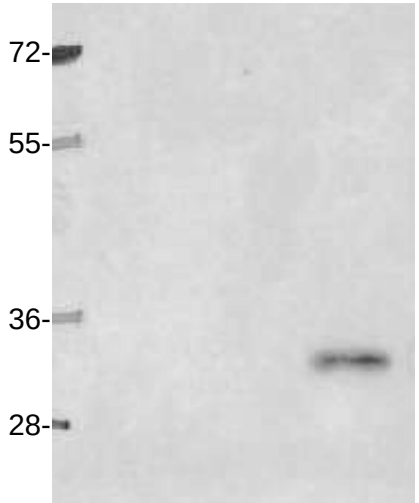


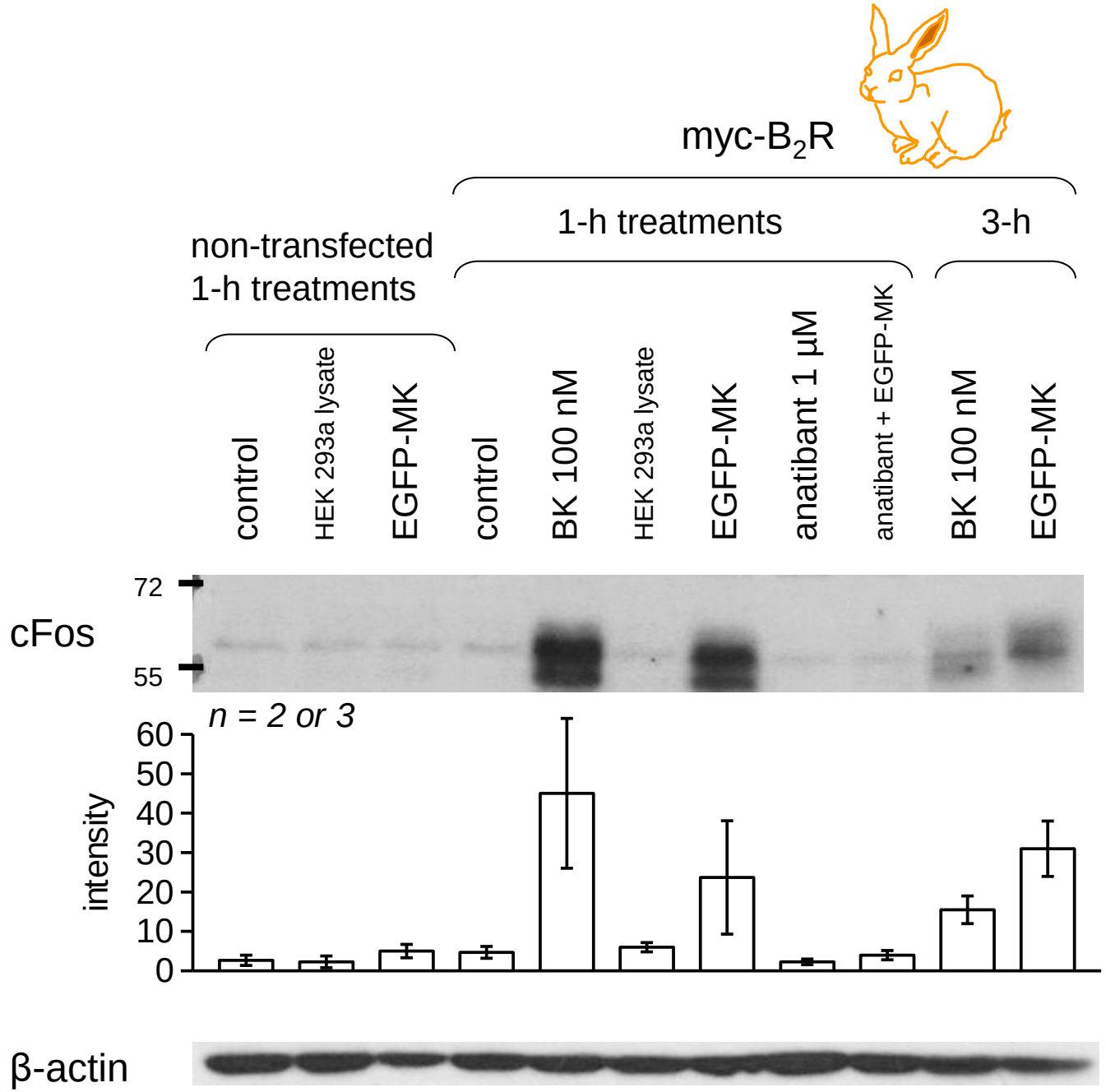
10 µm

anti-GFP



anti-BK




myc-B2R
1-h treatments
3-h
non-transfected
1-h treatments
control
HEK 293a lysate
EGFP-MK
control
BK 100 nM
HEK 293a lysate
EGFP-MK
anatibant 1 µM
anatibant + EGFP-MK
BK 100 nM
EGFP-MK
cFos
72
55
n = 2 or 3
intensity
60
50
40
30
20
10
0
β-actin

HEK 293a cells stably expressing myc-B_2R
+ anti-myc tag 9E10-AF594

human myc-B_2R | rat myc-B_2R

composite | red | green | composite | red | green

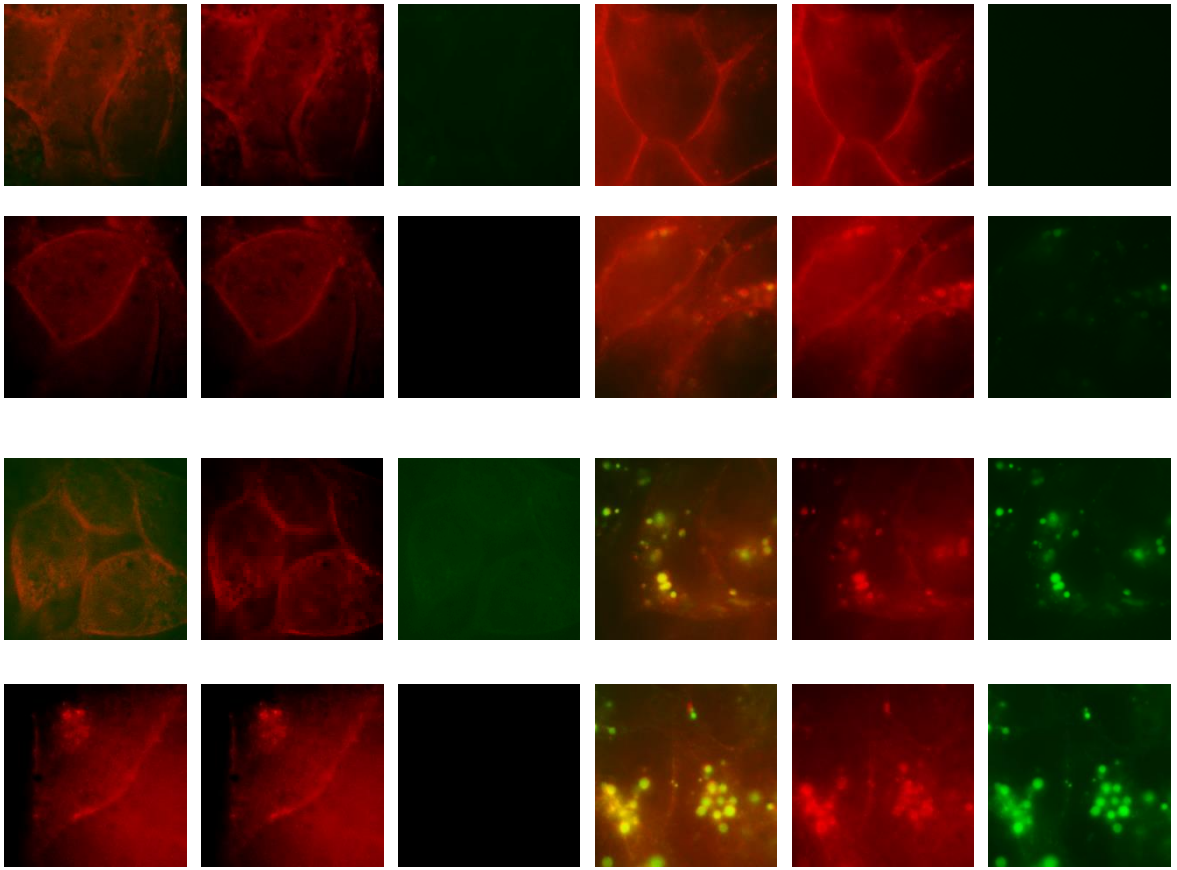
30-min stimulation

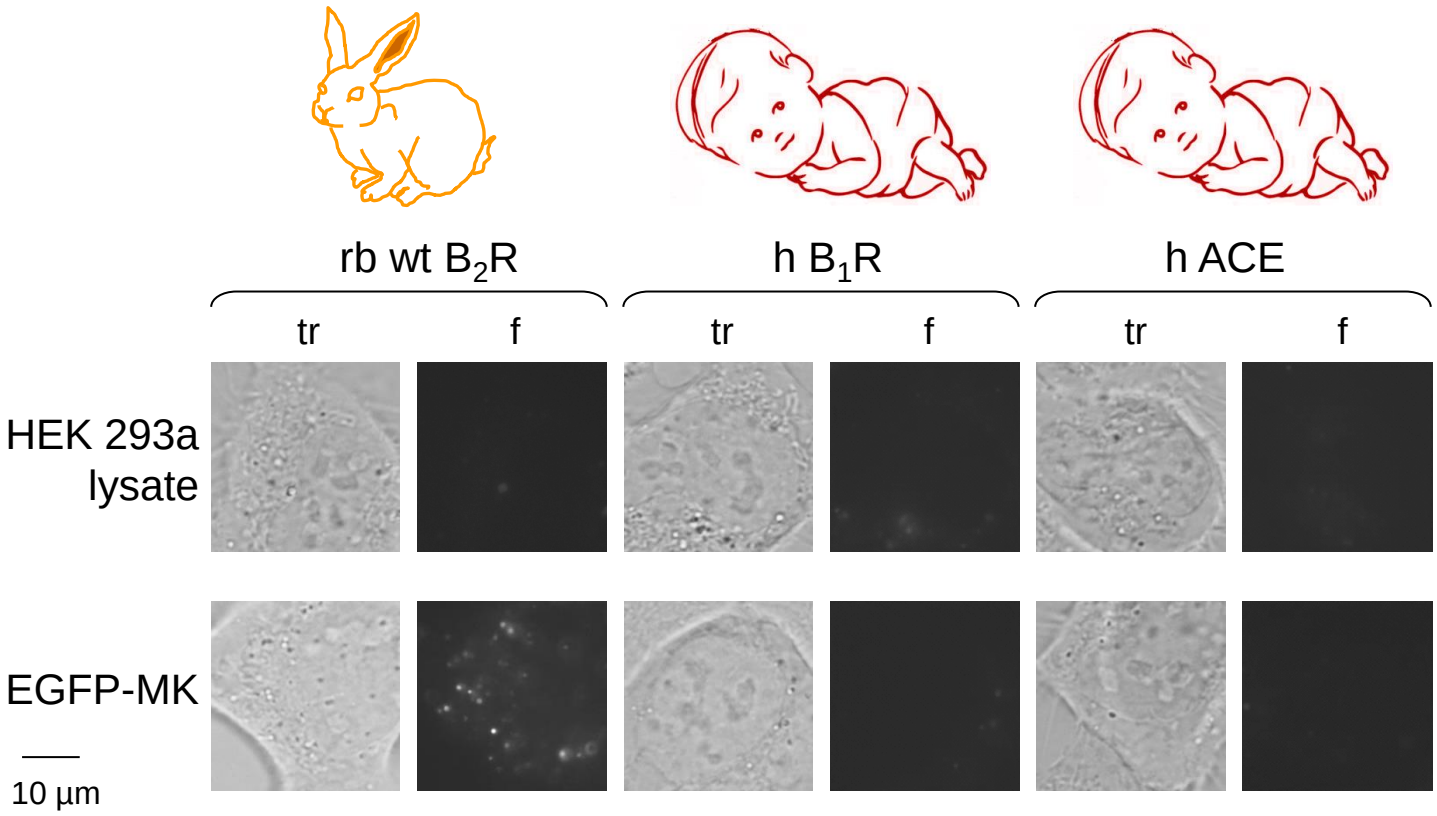
control

EGFP-MK 1:1250

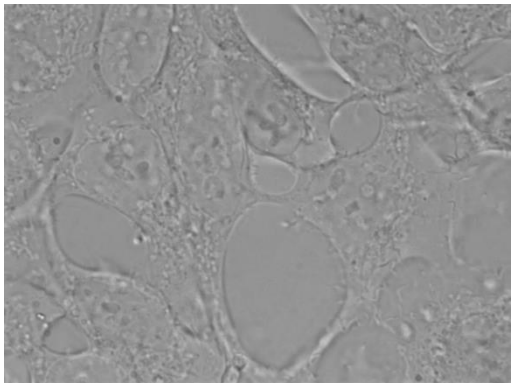
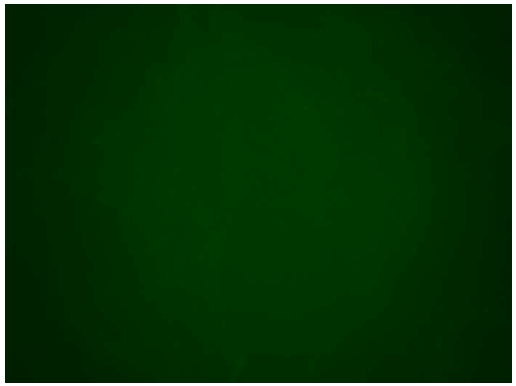
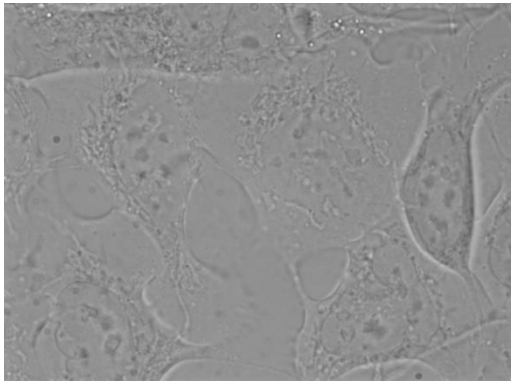
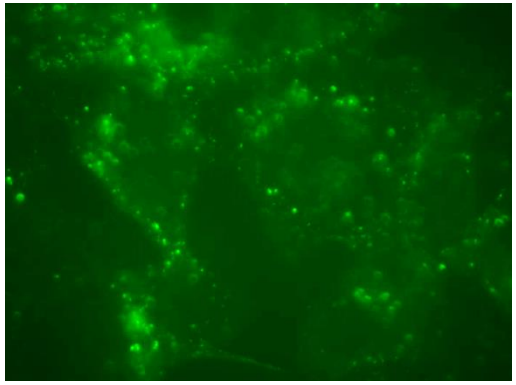
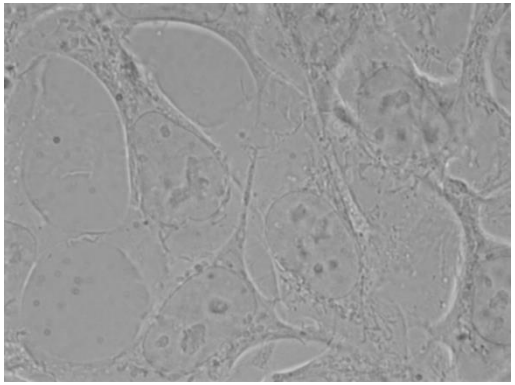
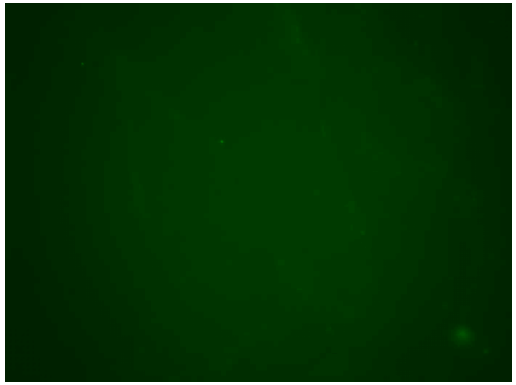
EGFP-MK 1:250

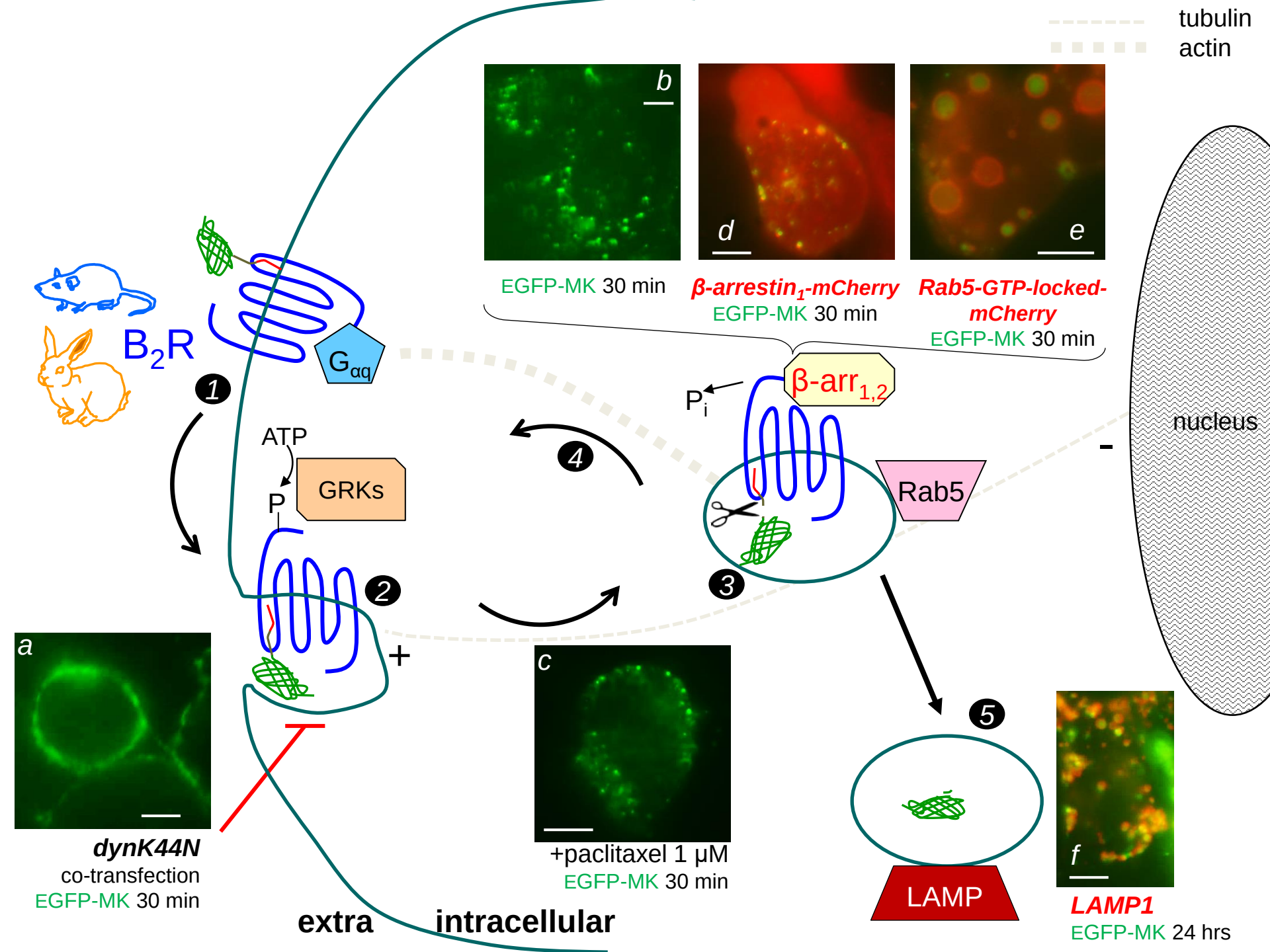
EGFP-MK 1:50

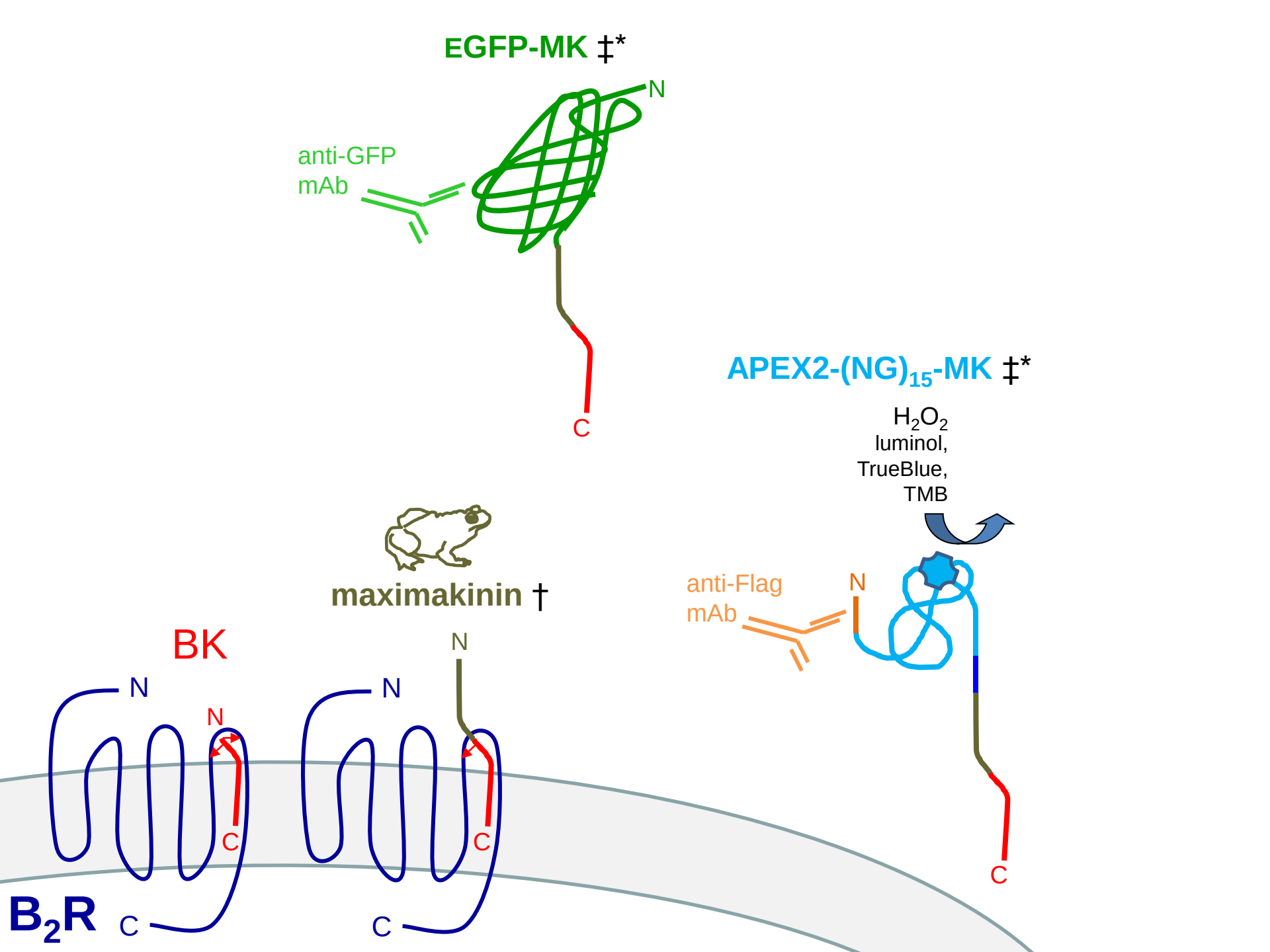



rb wt B2R
h B1R
h ACE
tr
f
tr
f
tr
f
HEK 293a lysate
EGFP-MK
10 µm

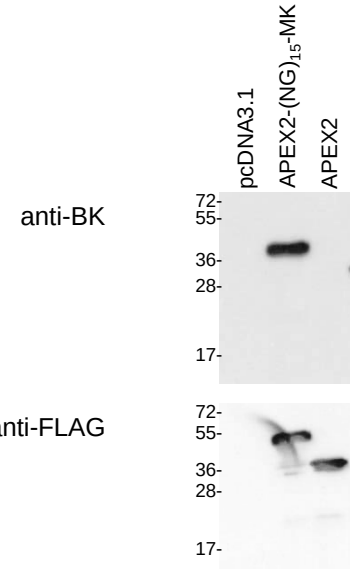
Recipient HEK 293a cells stably expressing rat myc-B_2R

	transmission	fluorescence
control		
EGFP-MK 1:250 -30 min		
icatibant 1 µM -45 min + EGFP-MK 1:250 -30 min		

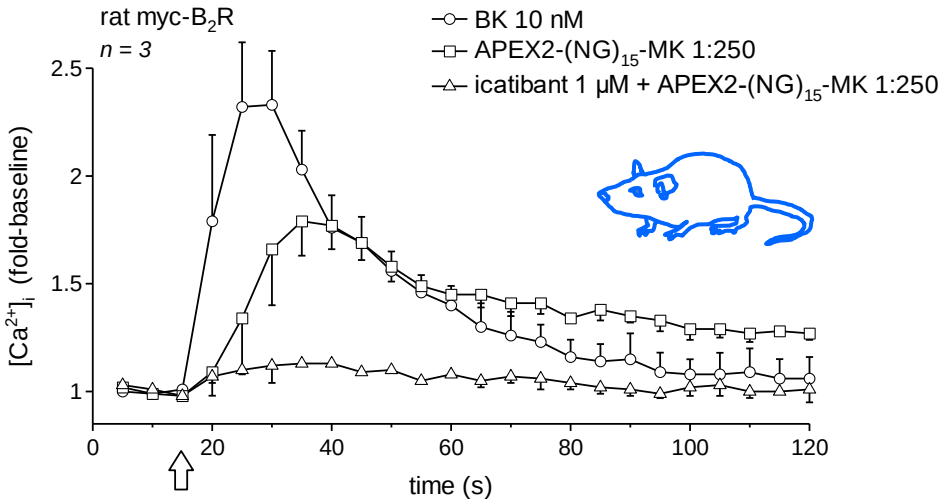

tubulin
actin
b
d
e
EGFP-MK 30 min
β-arrestin1-mCherry
EGFP-MK 30 min
Rab5-GTP-locked-mCherry
EGFP-MK 30 min
B2R
Gαq
1
ATP
P
GRKs
2
+
a
dynK44N
co-transfection
EGFP-MK 30 min
extracellular
intracellular
4
Pi
β-arr1,2
Rab5
3
-
nucleus
c
+paclitaxel 1 μM
EGFP-MK 30 min
5
LAMP
f
LAMP1
EGFP-MK 24 hrs


EGFP-MK ‡*
N
anti-GFP
mAb
C
APEX2-(NG)15-MK ‡*
H2O2
luminol,
TrueBlue,
TMB
anti-Flag
mAb
N
C
maximakinin †
BK
N
N
C
N
N
C
B2R
C
C

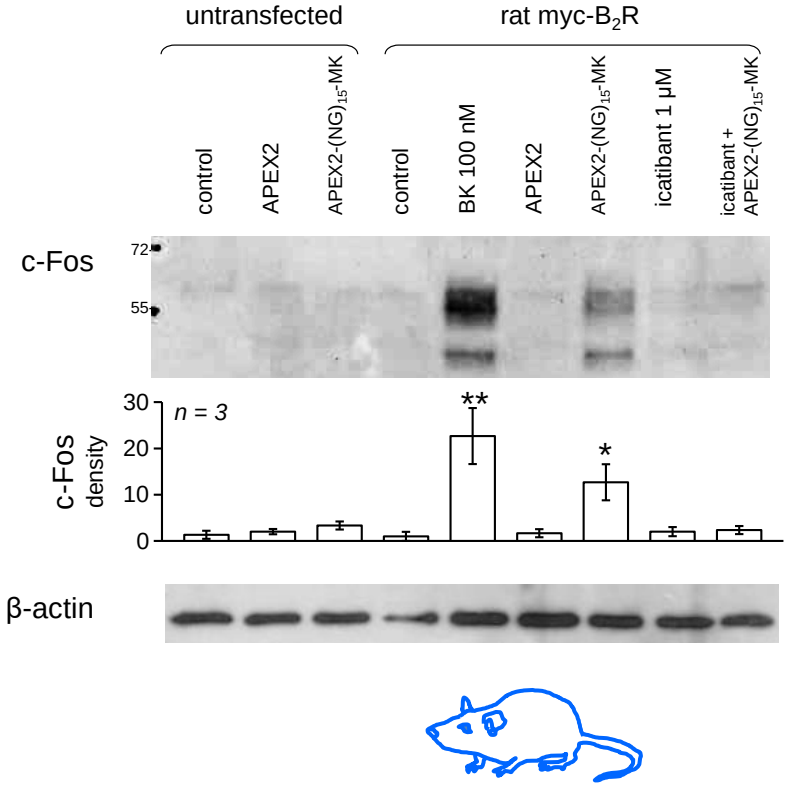
A. Lysates of HEK 293a producer cells



B. Effect of lysate (1:250) on recipient HEK 293a cells

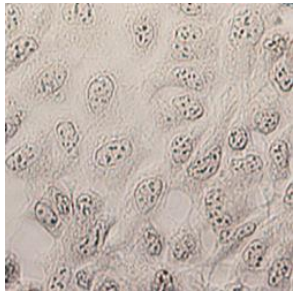
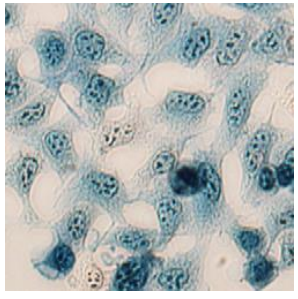


C. Effect of lysates (1:250) on recipient HEK 293a cells








vector

ligand

none

rt myc-B_2R

hu myc-B_2R

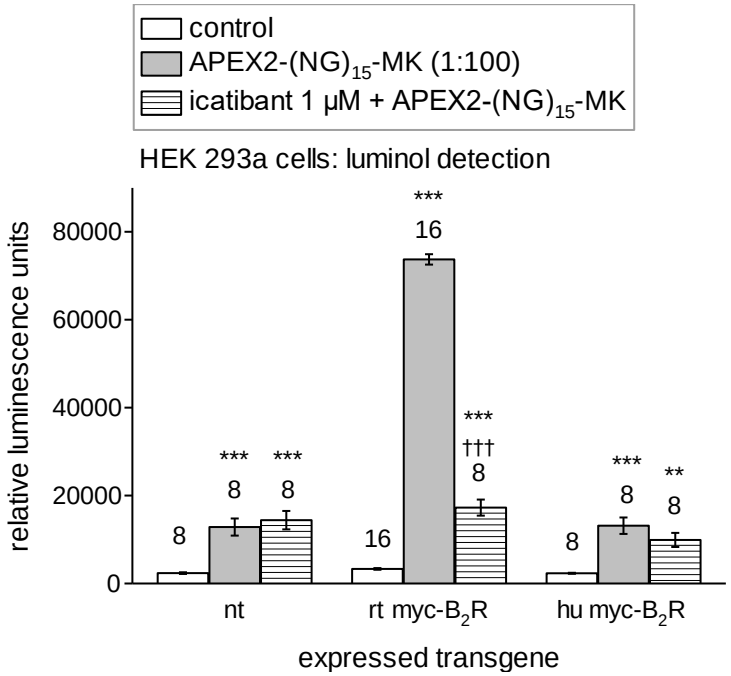
control

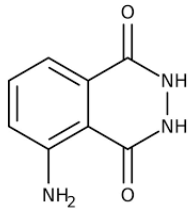
icatibant
1 µM

APEX2-$(NG)_{15}$-MK
1:100

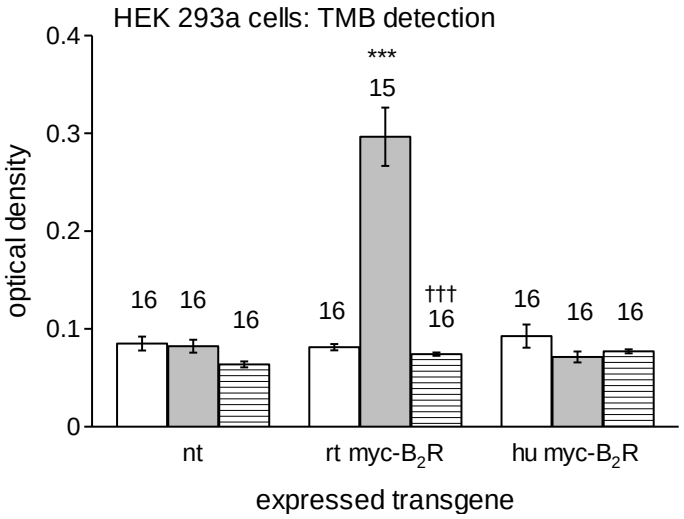
icatibant +
APEX2-$(NG)_{15}$-MK

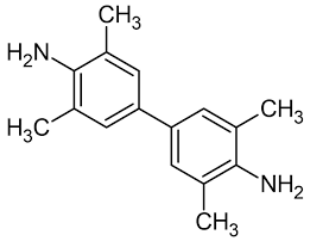
A.

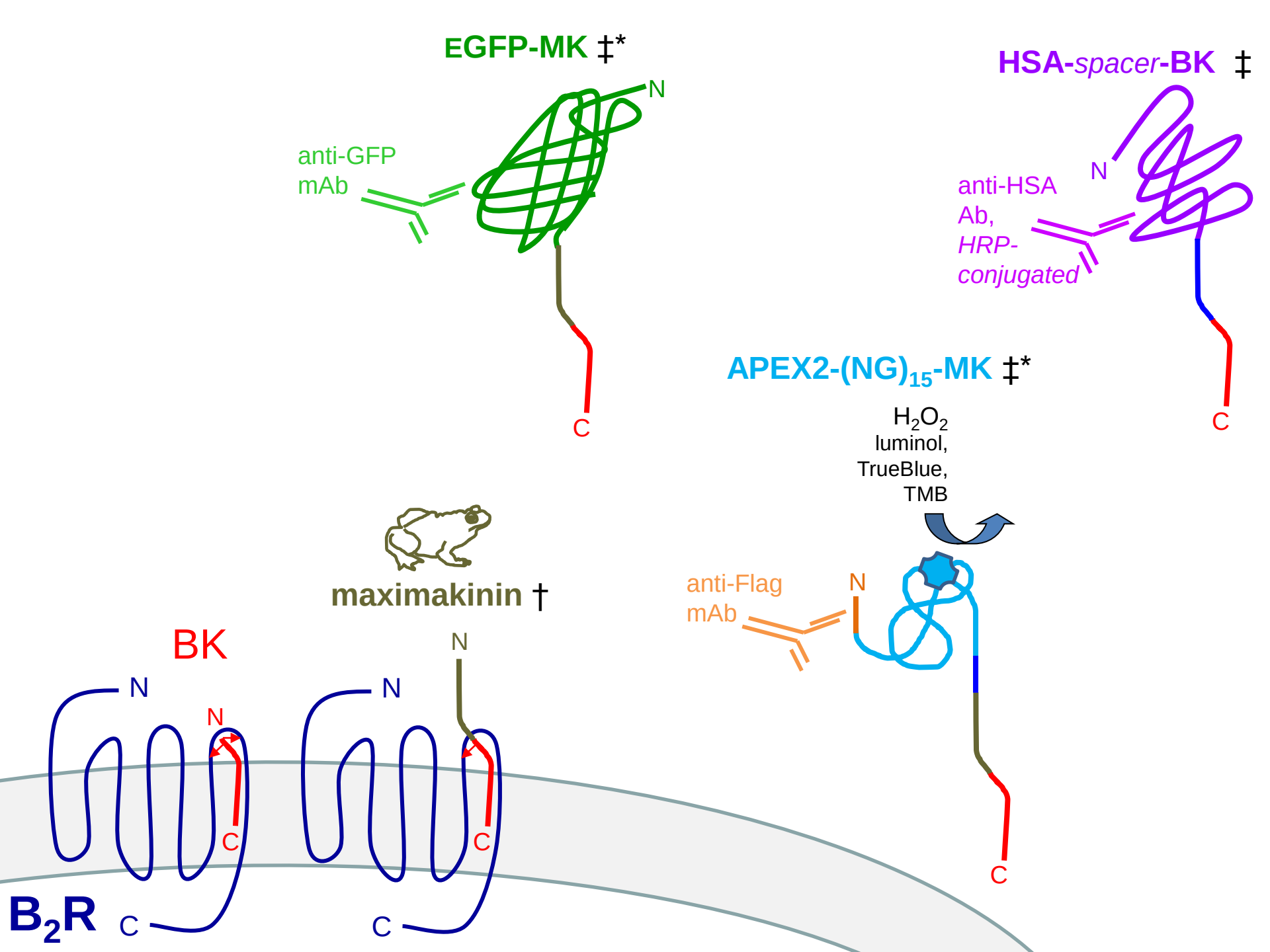

control
APEX2-(NG)15-MK (1:100)
icatibant 1 µM + APEX2-(NG)15-MK
HEK 293a cells: luminol detection
relative luminescence units
80000
60000
40000
20000
0
***
16
***
†††
8
***
***
8
8
8
16
8
***
8
**
8
nt
rt myc-B2R
hu myc-B2R
expressed transgene

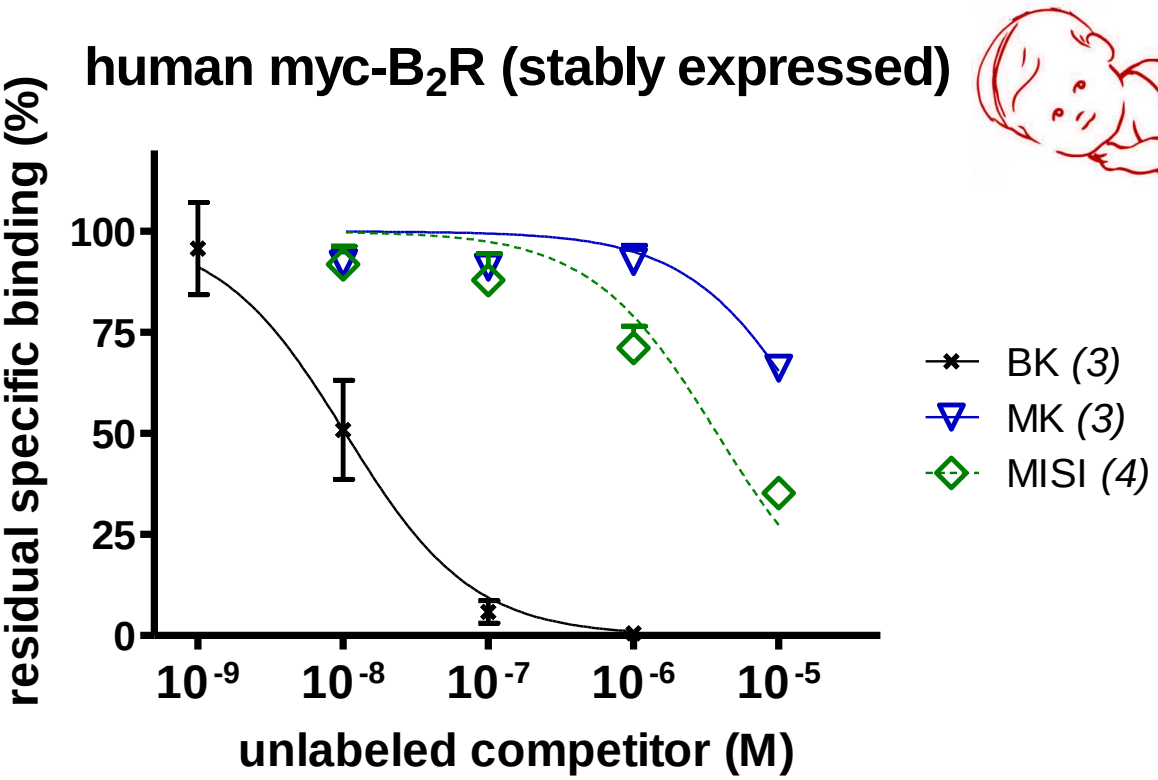

O
NH
NH
NH2
O

B.


HEK 293a cells: TMB detection
optical density
0.4
0.3
0.2
0.1
0
***
15
†††
16
16
16
16
16
16
16
16
16
nt
rt myc-B2R
hu myc-B2R
expressed transgene


CH3
H2N
H3C
CH3
NH2
CH3


EGFP-MK ‡*
N
anti-GFP
mAb
C
HSA-spacer-BK ‡
N
anti-HSA
Ab,
HRP-
conjugated
C
APEX2-(NG)15-MK ‡*
H2O2
luminol,
TrueBlue,
TMB
anti-Flag
mAb
N
C
maximakinin †
BK
N
N
C
N
N
C
C
B2R



MISI = MK with Ile^{-2}-Ser^{-1} insert

conclusions

Fusion protein ligands of the B2R are stable but subjected to slow intracellular inactivation, strong species specificity and possible steric hindrance between the receptor and large proteins.

They provide direct, antibody-independent detection of the receptor in intact cells.

Such fusion proteins may support diagnostic and perhaps therapeutic applications in the future.

funding

acknowledgements

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