Maximakinin: an amphibian bradykinin homologue integrated into fusion proteins that bind to the bradykinin B₂ 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 (DLPKINKPRPPGFSPFR). 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
Results and Discussion

- *Bombina maxima*, source of *maximakinin* (MK)
- 19-mer DLPKINRKGPRPPGFSPFR (C-terminal 9-mer = BK)
competition of 3 nM [\(^3\)H]BK binding to rabbit B\(_2\)R-GFP (\(n = 4\)–\(7\))

rat myc-B\(_2\)R (stably expressed)

hu myc-B\(_2\)R (stably expressed)
anti-GFP mAb

EGFP-MK ‡*

maximakinin †

B_{2}R C

BK

N N
Lysates of HEK 293a producer cells

Producer HEK 293a cells
EGFP-MK

anti-GFP

anti-BK
72 55 cFos 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

non-transfected 1-h treatments

1-h treatments

3-h

myc-B\textsubscript{2}R

cFos

$\beta$-actin

$n = 2$ or $3$
HEK 293a cells stably expressing myc-B₂R + anti-myc tag 9E10-AF594

30-min stimulation

control

EGFP-MK 1:1250

EGFP-MK 1:250

EGFP-MK 1:50
rb wt B_2R

h B_1R

h ACE

HEK 293a lysate

EGFP-MK

10 \mu m
Recipient HEK 293a cells stably expressing rat myc-B2R transduction

control

EGFP-MK 1:250 -30 min

icatibant 1 μM -45 min + EGFP-MK 1:250 -30 min
B2R

Gαq

β-arrestin1,2

EGFP-MK 30 min

β-arrestin1-mCherry

Rab5-GTP-locked-mCherry

EGFP-MK 30 min

dynK44N

cointransfection

EGFP-MK 30 min

Tubulin

actin

GRKs

1

2

3

4

5

EGFP-MK 30 min

+ paclitaxel 1 μM

EGFP-MK 30 min

LAMP1

EGFP-MK 24 hrs

Tubulin

actin

nucleus

extra

intracellular

ATP

P

P

LAMP1
EGFP-MK ‡*

maximakinin †

BK

B₂R

APEX2-(NG)₁₅-MK ‡*

H₂O₂
luminol,
TrueBlue,
TMB

anti-Flag mAb

anti-GFP mAb
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

control

none

rt myc-B_2R

hu myc-B_2R

icatibant

1 µM

APEX2-(NG)_{15}-MK

1:100

icatibant +
APEX2-(NG)_{15}-MK
A. HEK 293a cells: luminol detection

- Control
- APEX2-(NG)_{15}-MK (1:100)
- Icatibant 1 µM + APEX2-(NG)_{15}-MK

B. HEK 293a cells: TMB detection

- Control
- APEX2-(NG)_{15}-MK (1:100)
- Icatibant 1 µM + APEX2-(NG)_{15}-MK
human myc-B$_2$R (stably expressed)

residual specific binding (%)

unlabeled competitor (M)

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