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Bradykinin activates ADP-ribosyl cyclase in neuroblastoma cells: intracellular concentration decrease in NAD and increase in cyclic ADP-ribose

Haruhiro Higashida[a,b,c,*], Alla Salmina[d], Minako Hashii[a], Shigeru Yokoyama[a], Jia-Sheng Zhang[a], Mami Noda[e], Zen-Guo Zhong[f], Duo Jin[a]

Departments of Biophysical Genetics[a] and Cellular Neurophysiology[b], Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan, [c]The Kanazawa University 21st Century COE Program on Innovative Brain Science for Development, Learning and Memory, Japan, [d]Department of Biochemistry and Medical Chemistry, Krasnoyarsk State Medical Academy, Krasnoyarsk 660022, Russia, [e]Department of Pathophysiology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan, [f]Senile Brain Disease Institute, Guangzhou University of Traditional Chinese Medicine, China

*Correspondence should be addressed to H. Higashida: Department of Biophysical Genetics, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan.
Tel 81-76-265-2455; Fax 81-76-234-4236, E mail, haruhiro@med.kanazawa-u.ac.jp

Abbreviations: BK, bradykinin; PLC, phospholipase C; $InsP_3$, inositol-1,4,5-trisphosphate; CICR, Ca^{2+}-induced Ca^{2+} release; cADPR, cyclic ADP-ribose; mAChR, muscarinic acetylcholine receptor; ADPR, ADP-ribose; ACh, acetylcholine; CCh, carbamyocholine; $[\beta\text{-}NAD^+]_i$, intracellular β-NAD^+ concentrations; $[cADPR]_i$, intracellular cADPR concentrations.

Abstracts

ADP-ribosyl cyclase activity in the crude membrane fraction of neuroblastoma x glioma NGPM1-27 hybrid cells was measured by monitoring [^{3}H]cyclic ADP-ribose (cADPR) formation from [^{3}H]NAD$^+$. Bradykinin (BK) at 100 nM increased ADP-ribosyl cyclase activity by about 2.5-fold. Application of 300 nM BK to living NGPM1-27 cells decreased NAD$^+$ to 78% of the prestimulation level at 30 s. In contrast, intracellular cADPR concentrations were increased by 2–3 fold during the period from 30-120 s after the same treatment. Our results suggest that cADPR is one of the second messengers downstream of B_2 BK receptors.

Key words: Cyclic ADP-ribose; NAD ; ADP-ribosyl cyclase; Bradykinin

1. Introduction

Bradykinin (BK) plays an important role in blood pressure control, inflammation, edema, pain, and neuronal signaling [1]. Recently, BK was shown to be involved in the pathogenesis of particular conditions, such as cardiovascular diseases and glomerular injury in diabetes [2], prostate cancer [3], breast cancer [4], tumor-associated angiogenesis [5], and Alzheimer's disease [6]. These physiological and pathophysiological effects of BK are exerted through BK receptors, B_1 and B_2 [1]. Stimulation of BK receptors leads to activation of phospholipase C (PLC) [1,7], focal adhesion kinase [8], Pyk2 [9], protein kinase C [10], and Ras or mitogen-activated protein kinase [1,9]. BK also activates Ca^{2+} processes due mainly to intracellular Ca^{2+} mobilization by inositol-1,4,5-trisphosphate ($InsP_3$) from the endoplasmic reticulum [1,7,10].

Ca^{2+} mobilization is triggered not only by $InsP_3$ but also by Ca^{2+} itself, *i.e.* Ca^{2+}-induced Ca^{2+} release (CICR) via ryanodine receptors [11]. CICR is co-activated by a putative second messenger, cyclic ADP-ribose (cADPR) [11-13]. cADPR is synthesized from β-NAD^+ by both membrane-bound and cytosolic ADP-ribosyl cyclases in mammalian tissues, including the nervous system [14]. The cADPR synthetic activity is regulated by receptor stimulation with several different mechanisms [12-14]. In one such mechanism, ADP-ribosyl cyclase seems to be coupled directly with neurotransmitter or hormone receptors *via* different G proteins on the membrane surface [15]. However, the same control of ADP-ribosyl cyclase by BK receptors has not been reported previously. Recently, it was demonstrated that BK increased intracellular calcium, nitric oxide, and cADPR levels, and ADP-ribosyl cyclase activity in coronary arterial endothelial cells subsequently causing smooth muscle dilatation [16]. If this is true, it should be proved that BK decreases intracellular NAD^+ levels as a consequence of ADP-ribosyl cyclase activation. To address this question, we used a neuronal model cell line of NGPM-1 neuroblastoma x glioma hybrid cells, expressing endogenous B_2 BK receptors and exogenous M1 muscarinic acetylcholine receptors (mAChRs) [17,18]. We measured ADP-ribosyl cyclase activity in crude membrane fractions of NGPM1-27 cells and showed changes in the content of [^{3}H]NAD^+ and [^{3}H]cADPR in reaction mixtures. Furthermore, to confirm the signaling from B_2

to ADP-ribosyl cyclase *in vivo*, the substrate and product concentrations, *i.e.*, intracellular NAD^+ and cADPR levels, were measured before and after application of BK onto NGPM1-27 cells.

2. Materials and methods

Membrane preparation

NGPM1-27 cells were cultured as described previously [17]. The cells harvested were suspended in 10 mM Tris-HCl solution, pH 7.3, with 5 mM $MgCl_2$ at 4 °C for 30 min. The suspension was homogenized in a glass homogenizer. The resultant homogenate was centrifuged at 4 °C for 5 min at 1,000 x *g* to remove unbroken cells and nuclei. Crude membrane fractions were prepared by centrifugation (twice) of homogenates at 105,000 x *g* for 15 min. The supernatant was removed, and the final pellet was dispersed in 10 mM Tris-HCl solution, pH 7.0. In each experiment, membranes were freshly prepared and used immediately for enzymatic reactions.

ADP-ribosyl cyclase assay

Each 20-μl reaction mixture contained 50 mM Tris-HCl (pH 7.0); 100 mM KCl; 10 μM $CaCl_2$; 2 μM β-NAD^+; 0.1 μM β-[2,8 adenine-^{3}H]NAD^+ (0.06 μBq) and 0.40-7.16 μg of membrane proteins, according to a formula reported previously [15]. Reaction mixtures were incubated for 0.5-4 min at 37 °C. Reactions were stopped by adding 2 μl of 10% trichloroacetic acid, and aliquots were centrifuged for 1 min at 2100 x g, and 2 μl of the supernatant were spotted on silica gel plastic thin layer sheets (20 x 10 cm). The layers were developed in the ascending direction for 40-70 min at 23 °C with a mixture of water/ethanol/ammonium bicarbonate (in the ratio 30%: 70%: 0.2M or 36%: 64%: 0.3 M. The positions of authentic cADPR, ADP-ribose (ADPR) and β-NAD^+ were detected by UV illumination and of [^{3}H]-labeled products were autoradiographically confirmed in each by Fuji Bas 1000 (Tokyo, Japan). Corresponding areas (about 1 x 0.7 cm) were cut out and the radioactivity was counted in a liquid scintillation counter.

Intracellular NAD^+ content

NGPM1-27 cells were cultured on polyornithine-coated dishes (35-mm in diameter) for 4 days. The NAD^+ content in the supernatant of the heat-inactivated cell homogenate was determined by a slight modification of an enzyme cycling method described as reported previously [15].

Cycling assay for cADPR

Intracellular cADPR concentrations were measured according to the enzyme cycling assay method described by Graeff and Lee [19]. Briefly, NGPM1-27 cells were cultured in polyornithine-coated 35-mm dishes. The cells were extracted with 100 μl of 0.6 M perchloric acid at 4 °C. In order to observe the agonist effect on cADPR levels, agonists were applied on cells in dishes with serum-containing growth medium with no prior change to a fresh experimental medium without serum.

3. Results

Effects of bradykinin on ADP-ribosyl cyclase activity in NGPM1-27 cells

[^{3}H]cADPR and [^{3}H]ADPR were produced from β-[^{3}H]NAD$^+$ by preparation of the crude membrane fraction of NGPM1-27 cells. During an incubation period of 4 min, the majority of radioactivity of β-NAD$^+$ was converted to ADPR and/or cADPR, as shown in Fig. 1. The accumulation of radioactivity was greater in the spot of ADPR than cADPR (Fig. 1A). The average specific activity of ADP-ribosyl cyclase, as the rate of [^{3}H]cADPR formation, was 204±43 pmol/min/mg protein (mean±S.E.M., n=27).

Addition of 100 nM BK to the reaction mixture at zero time increased the rate of [^{3}H]cADPR formation to a greater extent than [^{3}H]ADPR production (Fig. 1C). The average activation by 100 nM BK was 248±47% (n=4) of the control activity (Student's *t* test, $p<0.01$).

Next, we confirmed the response to muscarinic receptors. A similar level of stimulation (321±41% (n=4)) by 1 μM carbamylcholine (CCh) of ADP-ribosyl cyclase was obtained (321±41%, n=3; Figs. 1B and 1C).

BK-induced decrease in intracellular β-NAD$^+$ concentration

To confirm the above effects of BK *in vivo*, we examined agonist-stimulated changes in substrate levels (Fig. 2). Fig. 2A shows the time course of changes in intracellular β-NAD$^+$ concentrations ($[\beta\text{-NAD}^+]_i$) in NGPM1-27 cells challenged with 300 nM BK. $[\beta\text{-NAD}^+]_i$ was significantly decreased for 15–60 s after application of BK. The decrease in $[\beta\text{-NAD}^+]_i$ at 30 s was 77.3±3.0% (n=9, $p<0.01$) of the pre-stimulation level, and showed partial recovery at 120 s.

Intracellular cADPR concentrations in NGPM-1 cells

Intracellular cADPR concentrations ($[cADPR]_i$) were measured by enzyme recycling assay. $[cADPR]_i$ in NGPM1-27 cells was 539±54 fmol/mg protein (n=4) before stimulation. $[cADPR]_i$ increased to 1225±116 fmol/mg protein after 120 s with application of 300 nM BK

onto NGPM1-27 cells (n=4, $p<0.01$) (Figs. 2B), and recovered to the control level after 5 min. The BK-induced increase was blocked by prior incubation of cells for 2 min with 1 μM [Thi^{5,8},D-Phe^{7}]-bradykinin or D-Arginyl-[Hyp^{3},Thi^{5},D-Tic^{7},Oic^{8}]-bradykinin (Hoe140), both of which are B_2-selective antagonists (Fig. 3).

4. Discussion

The results of the present study indicated that BK and ACh activates ADP-ribosyl cyclase in crude membrane preparations of NGPM1-27 cells where B_2 and M1 and M4 muscarinic receptors are expressed [18]. In addition, we showed that BK increased the intracellular cADPR production associated with the intracellular decrease in NAD^+. This is the first demonstration of the converse of the substrate and product concentration changes as a result of ADP-ribosyl cyclase activation after receptor stimulation in living cells. The results suggest that cADPR is one of the second messengers acting downstream of BK receptors, in addition to $InsP_3$ [7], at least in neuroblastoma hybrid cells.

B_2 BK receptors as well as M1 mAChRs are expressed in intact sympathetic neurons, and stimulation of these two types of receptor leads to PLC-mediated production of $InsP_3$ [1]. In neurons it has been shown that stimulation of B_2 receptors raises intracellular Ca^{2+} more efficiently than mAChRs, suggesting the presence of a special membrane-transducing microdomain [20]. In such microdomain, $InsP_3$-dependent Ca^{2+} activates neuronal calcium sensor 1, phosphatidyl-4-kinase or phosphatidyl-5-kinase, and thus produces more inositol-4,5-bisphosphate in the membranes [21,22]. Since we showed that B_2 receptors couple with ADP-ribosyl cyclase, it is possible that the cADPR/ryanodine receptor signal may also make a contribution in the microdomain complex. It will be necessary to demonstrate the effects of cADPR/ryanodine receptor-dependent Ca^{2+} on neuronal function, such as Ca^{2+} and K^+ currents, in future studies.

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Figure legends

Fig. 1. Time course of ADP-ribosyl cyclase activity in NGPM1-27 cell membranes. Aliquots (20 μl) were withdrawn at the indicated times from 140-μl reaction mixtures containing membrane protein from NGPM1-27 cells in the absence (A) and presence (B) of 1 μM CCh. Radioactivity in spots migrating with authentic NAD^+ (open squares), cADPR (closed square), and ADPR (open diamond) on thin-layer chromatogram sheets was measured. Values are the means of two determinations from one representative of three experiments giving similar results. (C) Time course of changes in ADP-ribosyl cyclase activity (rate of formation of [^{3}H]cADPR) in membranes prepared from NGPM1-27 cells. Reaction mixtures were incubated with or without (open circle) 100 nM BK (closed square) or 1 μM CCh (closed diamond) for the indicated times. Values are the means of two determinations from one representative of three experiments giving similar results.

Fig. 2. BK-induced change in $[NAD^+]_i$ and $[cADPR]_i$ in intact NGPM1-27 cells. (A) Time course of intracellular concentrations of NAD^+ ($[NAD^+]$i). The growth medium was replaced with 2 ml of 10-mM Tris-buffered Dulbecco's modified Eagle's medium and incubated for 40 min at 37°C. The preincubated cells were then stimulated by gently adding 1 ml of medium alone (none) or with 300 μM BK for the indicated periods. Incubation was stopped by replacing the medium with 1 ml of cold PBS and washed again with 1 ml PBS with 10 mM nicotinamide. Cells were scraped, and the homogenates were heat-inactivated. $[NAD^+]_i$ was measured as described in the Methods section. The control value for the NAD^+ level in NGPM1-27 cells was 4.9±0.4 nmol/10^6 cells. (B) Time course of intracellular concentrations of cADPR ([cADPR]i). Cells were grown in 35-mm culture dishes for 4 days. Two ml of the growth medium was replaced one day before the experiments. Aliquots of 20 μl of 30 μM BK or phosphate buffer (PBS) were added to the medium and incubated for the indicated periods. The medium was removed and incubation was stopped by adding 200 μl of 0.6 M perchloric acid. Cell lysate was collected and centrifuged. The supernatant was used for measurement of cADPR concentrations as described in the Methods section. Values represent the means±S.E.M of three dishes in

triplicate cultures. *, #, Significantly different from the control value at time 0 at $p<0.05$ and 0.01, respectively.

Fig. 3. Effects of B_2 antagonists on bradykinin-induced elevation of intracellular cADPR concentrations in intact NGPM1-27 cells. Intracellular cADPR concentrations ([cADPR]i) were measured in the presence or absence of 100 nM BK with or without 1 μM or D-Arginyl-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe) or [$Thi^{5,8}$,D-Phe^7]-bradykinin ([The]). Each data point represents the mean±S.E.M. of 4 determinations. *, # Significantly different from the control value or the value with BK at $p<0.01$, respectively.

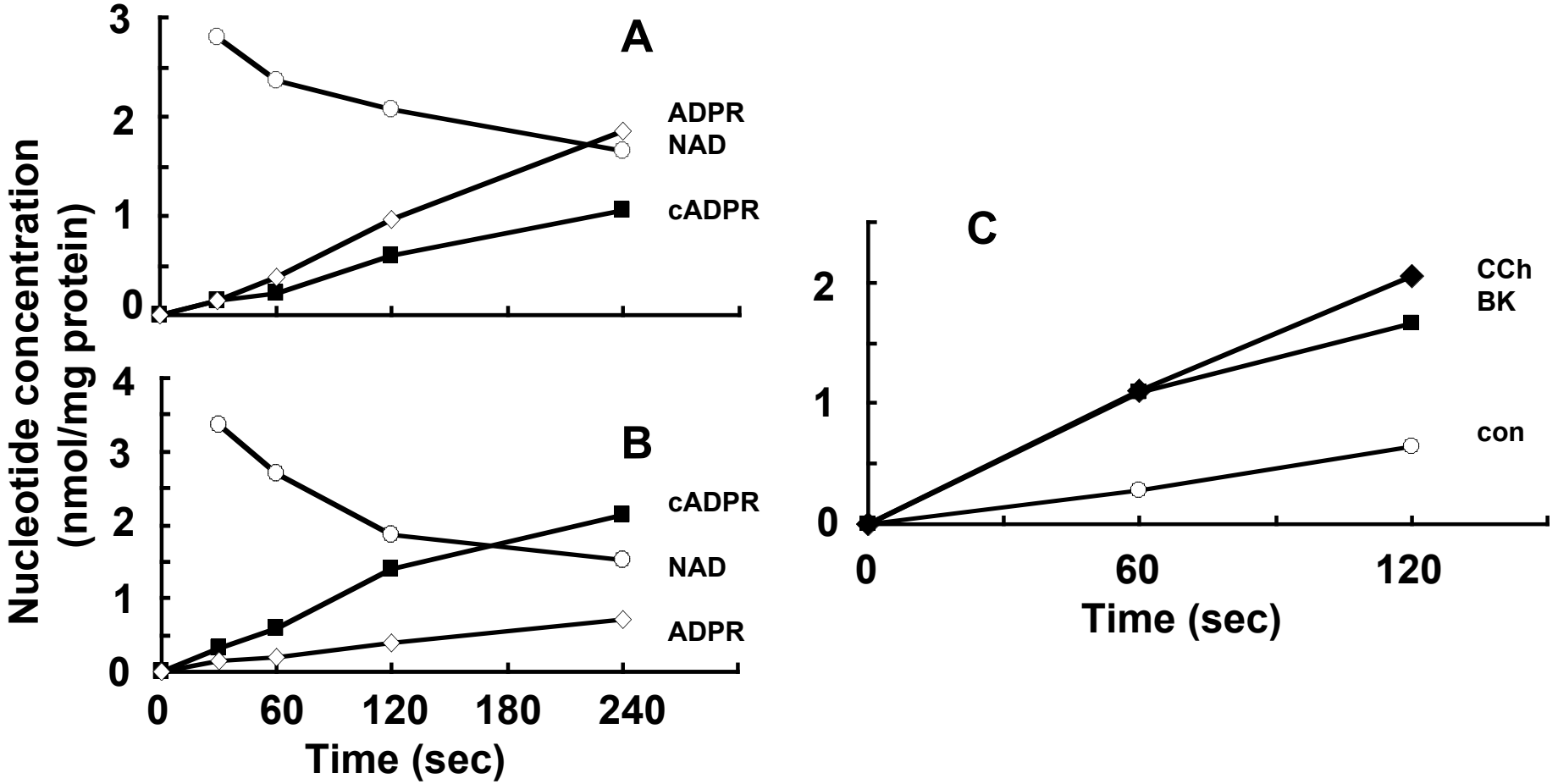
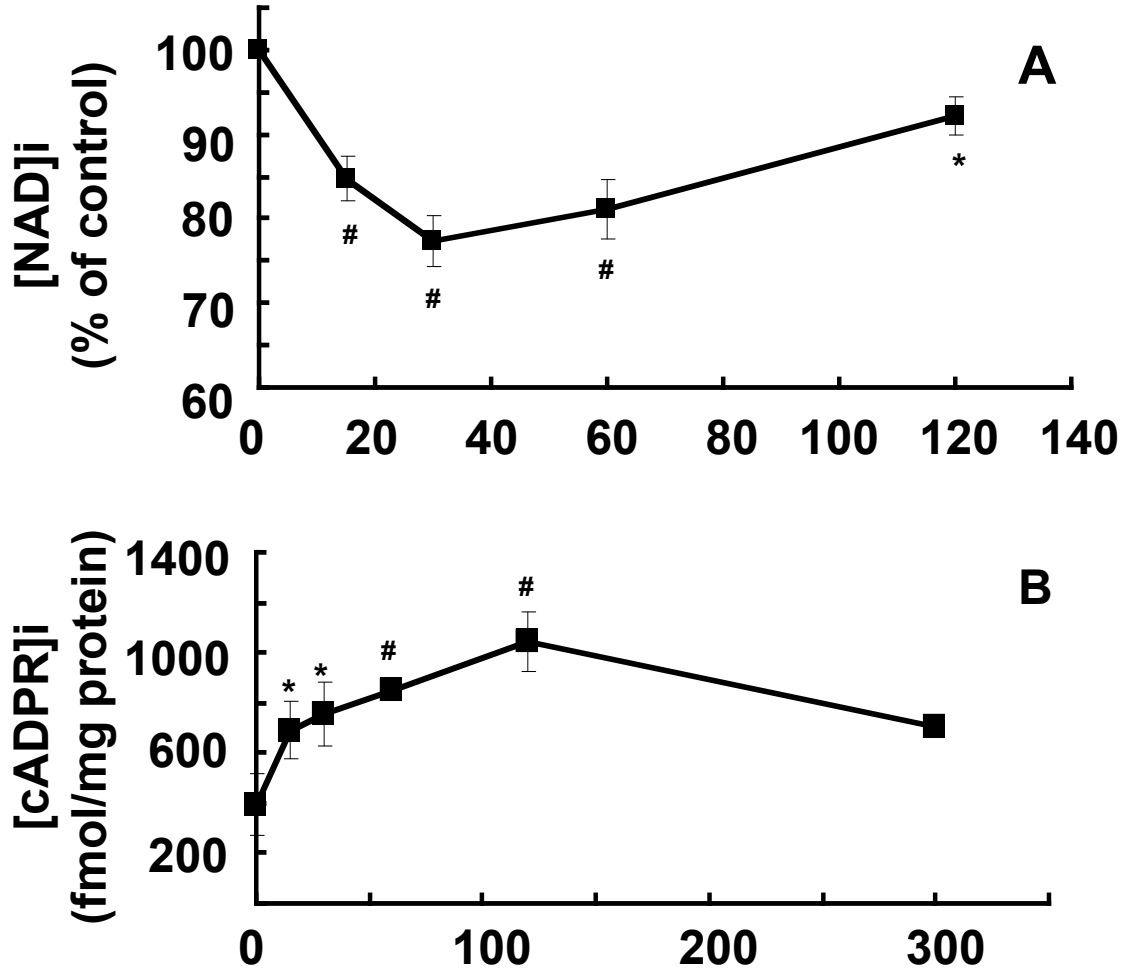

A
B
C
Nucleotide concentration
(nmol/mg protein)
ADPR
NAD
cADPR
cADPR
NAD
ADPR
CCh
BK
con
Time (sec)
Time (sec)
0
60
120
180
240

Fig. 1


A
[NAD]i
(% of control)
100
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80
70
60
0
20
40
60
80
100
120
140
#
#
#
*
B
[cADPR]i
(fmol/mg protein)
1400
1000
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200
0
100
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Time after BK stimulation (sec)

Fig. 2

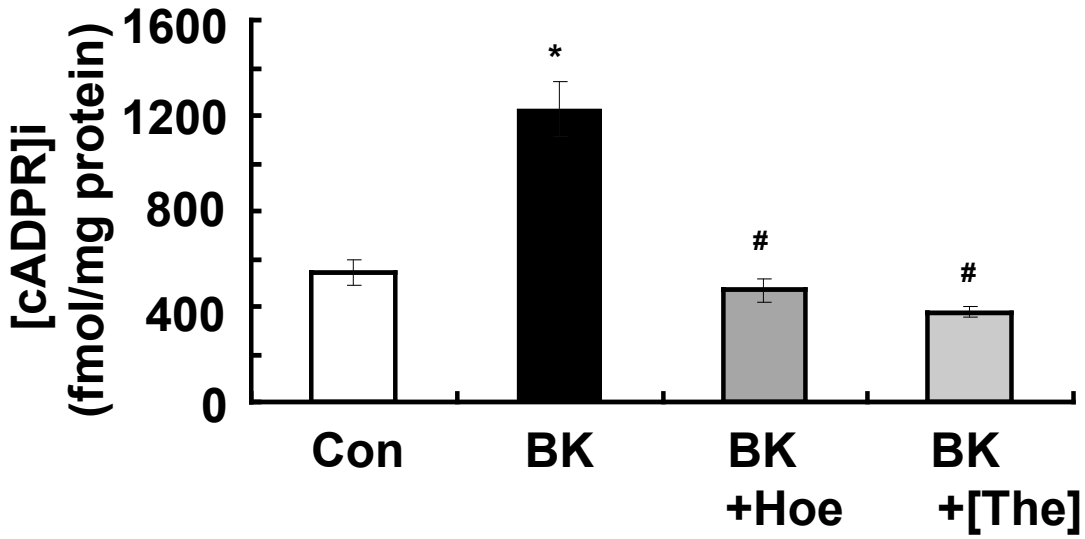

Fig. 3