Positive cooperativity between the thrombin and bradykinin B₂ receptors enhances arachidonic acid release

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Abstract

Bradykinin (BK) or kallikreins activate B₂ receptors (R) which couple Gαi and Gαq proteins to release arachidonic acid (AA) and elevate $[Ca^{2+}]_i$. Thrombin cleaves the protease-activated-receptor-1 (PAR1) that couples $Gα_i$, $Gα_q$ and $Gα_{12/13}$ proteins. In CHO cells stably transfected with human B₂R, thrombin liberated little AA, but it significantly potentiated AA release by B₂R agonists. We explored mechanisms of cooperativity between constitutively expressed PAR1 and B₂R. We also examined human endothelial cells expressing both Rs constitutively. The PAR1 agonist hexapeptide (TRAP) was as effective as thrombin. Inhibitors of components of $Gα_i$, $Gα_q$ and $Gα_{12/13}$ signaling pathways, and a PKCα inhibitor, Gö6976 blocked potentiation while phorbol, an activator, enhanced it. Several inhibitors, including a RhoA kinase inhibitor, a $[Ca^{2+}]_i$ antagonist, and an inositol-(1,3, 4)-trisphosphate R antagonist, reduced mobilization of $[Ca^{2+}]_i$ by thrombin and blocked potentiation of AA release by B₂R agonists. Because either a non-selective inhibitor (isotetrandrine) of phospholipase A₂ (PLA₂) or a Ca^{2+}-dependent PLA₂ inhibitor abolished potentiation of AA release by thrombin, while a Ca^{2+}-independent PLA₂ inhibitor did not, we concluded that the mechanism involves Ca^{2+}-dependent PLA₂ activation. Both thrombin and TRAP modified activation and phosphorylation of the B₂R induced by BK. In lower concentrations they enhanced it, while higher concentrations inhibited phosphorylation and diminished B₂R activation. Protection of the N-terminal Ser¹-Phe² bond of TRAP by an aminopeptidase inhibitor made this peptide much more active than the unprotected agonist. Thus, PAR1 activation enhances AA release by B₂R agonists through signal transduction pathway.

Keywords

PAR1; TRAP; protein kinases; potentiation; Ca^{2+}; kallikrein

Abbreviations

PAR1, protease activated receptor type 1; TRAP, thrombin receptor activator peptide; Kallikrein, kallikrein of human plasma; BK, bradykinin; thr, thrombin; BEL, bromo-enol lactone; DAG, diacylglycerol; cPLA₂ inhib, Ca^{2+}-dependent phospholipase A₂ inhibitor; IP₃, inositol-(1,3,4)-trisphosphate; 2-APB, 2-aminoethoxydiphenyl borate; TMB-8,8-(N, N, diethylamino) octoyl-3,4,5-

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Table 1. Ineffective inhibitors: [3H]AA-labeled cells were incubated for 30 min in the presence or absence of the indicated drugs and then treated for an additional 30 min with 1 nM BK in the presence or absence of 10 nM human thrombin. After treatments, [3H]AA release was measured as described in Methods. Results were calculated by taking basal release as 1.0. All data are means ± S.E.M. of 3 separate experiments using triplicate samples. Significance is indicated by p < 0.01 (paired t-test). The release of AA by BK was significantly (p < 0.01) potentiated in spite of the presence of an inhibitor.

trimethoxybenzoate; Y27362, Rho kinase inhibitor; RGS10, regulator of G protein signaling type 10; ⊣ inhibition

INTRODUCTION

Thrombin, the potent physiologic activator of platelet aggregation, is a major factor in myocardial infarction and other thrombotic processes (15,22,32). In addition to promoting coagulation, thrombin plays a central role in the cellular response to injury or inflammation and wound repair. Thrombin initiates many of these complex cellular events by cleaving the Arg[41]-Ser[42] bond of the protease-activated receptor-1 (PAR1) to expose a new N-terminus. This terminus then acts as a tethered ligand (13,38) and activates PAR1. Various guanine nucleotide-binding (G) protein α-subunits can then couple with PAR1. When coupled to $G\alpha_q$ protein PAR1 generates diacylglycerol (DAG) and inositol-(1,4,5) trisphosphate (IP_3), which in turn binds to IP_3 Rs on the endoplasmic reticulum to trigger intracellular calcium $[Ca^{2+}]_i$ release. Interaction of IP_3 R with transient R potential channel 1 (TRPC1) is required to activate store depletion-induced Ca^{2+} entry (21,27,31). In addition to $G\alpha_q$, the stimulated PAR1 couples $G\alpha_{i/0}$ (14,25,37) and $G\alpha_{12/13}$ proteins (12,17,27). Activation of the $G\alpha_{i/0}$ protein leads to release of the prostaglandin precursor, arachidonic acid (AA).

Activation of B_2Rs for bradykinin (BK) can cause among others hypotension, bronchoconstriction, pain, or inflammation (3,16) and releases vascular mediators such as NO, or prostaglandins, and others (3,8–11). The PAR1-4 group of thrombin and trypsin Rs, as their name indicates, were thought to be unique protease-activated Rs. However, we showed that the human BK B_2R can be activated by certain proteases as well (20). Kallikreins and some other human or bacterial serine proteases, including cathepsin G or endoarginase C, can activate B2 BK Rs directly (19,20). Furthermore, activation of the ubiquitous B_2R by BK or proteolytic enzymes (20) recruits both $G\alpha_i$ and $G\alpha_q$ proteins, as indicated by the release of AA and elevation of $[Ca^{2+}]_i$ (18,26).

Burch and Axelrod found that BK and thrombin synergized prostaglandin synthesis in fibroblasts, and after prolonged stimulation, thrombin amplified responses to BK (5–7). After we discovered that kallikrein and other proteases directly activate the BK B_2Rs (19,20), we used cultured cells to determine if thrombin would enhance AA release by kallikrein via B_2R and if PAR1 activation potentiates kallikrein via signal transduction pathways. We established that the two proteases acted on a single R, either PAR1 or B2 because we obtained the same results with peptide agonists specific for the individual Rs. We used inhibitors of the major factors in signal transduction to point out the steps in the potentiation process.

MATERIALS AND METHODS

Chinese Hamster Ovary (CHO) cells were purchased from American Type Culture Collection (Rockville, MD). HPAE cells were obtained from Clonetics (Walkersville, MD). The cDNA encoding the human BK B_2R was donated by Dr. K. Jarnigan, Syntex Co (Palo Alto, CA), the human regulator of G protein signaling type 10 (RGS10) cDNA was from the University of Missouri-Rolla (Rolla, MO). Mammalian expression vectors pcDNA3 was from Invitrogen (San Diego, CA); lipofectin and geneticin (G418) were from Gibco BRL (Gaithersburg, MD). $[5,6,8,9,11,12,14,15-^3H(N)]$-arachidonic acid (AA); 100 C1/mmol) and $[^3H]$BK were purchased from American Radiolabeled Chemicals (St. Louis, MO). The fura-2/AM, anti-GFP polyclonal antibodies were obtained from Molecular Probes (Eugene, OR), and D-valyl-leucyl-arginyl-aminomethyl-coumarine (D-Val-Leu-Arg-AMC) was from Enzyme Systems Products (Livermore, CA). Bromo-enol lactone (BEL) was obtained from Cayman Chemical Company (Ann Arbor, MI) and $cPLA_2$ inhibitor (N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-

amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl), Gö6976, Y27632, H-89 dihydrochloride, 2-aminoethoxydiphenyl borate (2-APB) and 8-(N, N, diethylamino) octoyl 3,4,5-trimethoxybenzoate (TMB-8) from Calbiochem (La Jolla, CA). Human thrombin and human plasma kallikrein from Enzyme Research Lab (South Bend, IN), BK, porcine pancreatic kallikrein, thermolysin, trypsin, genistein, culture media, penicillin, phorbol-12-myristate 13-acetate (PMA), thrombin R activator peptide (TRAP) and scrambled TRAP (scTRAP), amastatin and other peptides and chemicals were purchased from Sigma Chemical (St Louis, MO). HOE 140 was a gift from Dr. B.A. Schölkens, Novartis Co., (Frankfurt, Germany). The Pro-Q diamond phosphoprotein gel stain kit was from Molecular Probes Inc. (Eugene, OR).

Cell Culture and Transfection

CHO cells were grown in Ham s F-12 culture medium supplemented with L-glutamine, penicillin-streptomycin, HEPES buffer and 10% fetal bovine serum (FBS). One day prior to transfection, cells were seeded into 60 mm dishes at 30–40 % confluence. The human B_2R and human B_2R C-terminally tagged with the green fluorescent protein (B_2-GFPct) were inserted into a pCDNA3 vector, and CHO cells were transfected with human BK B_2R (CHO/B_2), the B_2-GFPct (CHO/B_2-GFPct), or co-transfected with human BK B_2R and RGS10 (CHO/B_2+RGS10) using lipofectin. After 2 h incubation at 37°C, cells were thoroughly washed with DMEM or Ham s F-12 medium, and 2 ml of the same medium containing geneticin (G418) was added to start the selection of stably B_2 or tagged-B_2R transfected cells. Blasticidin was added to select the RGS10 co-transfected cells. Different clones were selected and propagated using cloning rings. HPAE cells, which constitutively express BK B_2Rs, were grown to confluence (4–6 passages).

[³H]BK saturation binding assay

To select the transfected clone with the highest expression of B_2 or B_2-GFPct Rs and to quantitate the effect of thrombin on BK binding to its R, we used a [³H]BK saturation binding assay (20). We found that both human BK B_2 and B_2-GFPct were expressed on CHO plasma membrane; we estimated the B_2R to be 5.8 x 104 per cell and the B_2-GFPct to be 1.4 x 105 per cell.

[Ca^{2+}]$_i$ mobilization

We assessed the effectiveness of specific [Ca^{2+}]$_i$ release inhibitors by measuring [Ca^{2+}]$_i$ levels in CHO/B_2 cells with a microspectrofluorometer (PTI Deltascan, Princeton, NJ) coupled to an inverted microscope and the Ca^{2+} sensitive fluorescent dye, fura-2/AM (20). The excitation wavelengths were 340 and 380 nm, and the emission wavelength was 510 nm. The signals are represented in figures as a ratio of bound/free Ca^{2+}, F_{340}/F_{380}.

[³H]AA release

Endothelial and transfected CHO cells were grown to confluence in 6 well dishes. The medium was replaced with 1 ml of Ham s F12 medium containing 0.5 μCi/ml of [³H]AA, and cells were incubated for 16 h at 37°C. The labeled cells were then serum starved for 3 h prior to each assay. After washing with incubation medium (Ham s F12 medium plus 0.1% bovine serum albumin, BSA), they were incubated for 30 min at 37°C with either medium alone, 1 – 50 nM BK, or enzymes with or without the B_2R blocker, HOE 140 (0.5 μM). The medium was removed and its [³H]AA content was measured by scintillation counting. The amount of released [³H]AA was expressed after subtracting the background (spontaneous release) (19). The experiments were carried out routinely in triplicate.

Thrombin and kallikrein assay

A putative direct effect of TRAP on human plasma kallikrein activity and inactivation of thrombin by DFP were followed fluorometrically by measuring cleavage of D-Val-Leu-Arg-AMC substrate by the two enzymes (1,36). Plasma kallikrein (100 nM) was pre-incubated with increasing concentrations of TRAP from 1 μM to 300 μM for 15 min. Fifty μl of kallikrein solution was then mixed with 445 μl of 0.1 M Tris-HCl (pH 8.0). Addition of 5 μl of AMC substrate to final concentration of 0.1 mM initiated the reaction. The liberated fluorogenic coumarin derivative was measured in a spectrofluorometer with excitation at 380 nm and emission at 460 nm wavelength; the increase in fluorescence was monitored with a recording spectrofluorometer. We used thrombin irreversibly inhibited by DFP as an additional control.

Human BK B_2 immunoprecipitation and phosphorylation

CHO/B_2-GFPct cultures in 6-well culture dishes were treated with 1 μM BK in the presence or absence of increasing concentrations of thrombin (3 nM - 1 μM) for 30 min at 37°C. Cells were washed with ice-cold PBS and lysed with 0.4 ml 0.5% DOC buffer (pH 7.5) containing 1% NP-40, 0.1% SDS, 1 mM PMSF, 50 mM Tris, 150 mM NaCl and 10 μl protease inhibitor mixture. After shaking for 10 min at 4°C, the lysates were sonicated, then centrifuged at 16,000g for 15 min at 4°C. Supernatants were collected and diluted with 390 μl of 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl and protease inhibitors. Samples were then incubated with 1 μg rabbit anti-GFP antibody overnight at 4°C. B2-GFPct R immune complexes were precipitated with protein A-Sepharose beads (Sigma, St. Louis, MO) at 4°C for 2 h. The beads were then washed five times with lysis buffer, and the precipitated proteins were eluted by boiling the beads in sample buffer (80 mM Tris [pH 6.8], 3% SDS, 15% glycerol, 0.01% bromophenol blue, 5% DTT). Proteins were then separated on a 4–20 % gradient SDS-PAGE gel. The phosphorylated R was selectively stained with a Pro-Q diamond phosphoprotein gel stain kit, and its 555/580 nm excitation/emission maxima was detected with a visible-light-scanning instrument (Molecular Imager FX pro plus, Bio Rad). Density of the band was quantified using Labworks software, and data were expressed as the ratio of phosphorylated protein density/total protein density.

Statistical analysis

Means and standard errors were calculated for all experiments, and statistical significance of differences between means was tested by a paired t-Test (Microsoft Excel).

RESULTS

Thrombin enhances [³H] arachidonic acid (AA) release by BK B_2R agonists

BK or kallikrein released [³H]AA from labeled CHO cells that were stably transfected to express human B_2R (CHO/B_2). Although these cells express thrombin PAR1 constitutively, direct activation of PAR1 by 10 nM thrombin for 30 min at 37°C released relatively little labeled AA. If the amount released spontaneously was taken as baseline to equal 1.0, the value for AA was 1.76 ± 0.4 fold over the baseline. Figure 1a shows that the amount of AA released by thrombin was unaffected by 0.5 μM HOE 140, an inhibitor of the BK B_2R. However, when thrombin activated PAR1 first, subsequent stimulation of B_2R with porcine pancreatic kallikrein nearly doubled [³H]AA release from 6.4 ± 1.5 to 12.6 ± 2. Similar results were obtained with human plasma kallikrein (5.9 ± 0.8 to 11.0 ± 0.7) and BK (6.3 ± 1.7 to 11.8 ± 2.6), where thrombin activated cells reacted to the agonists with a significant increase in release of AA. HOE 140 (0.5 μM) completely blocked the effects of thrombin receptor activation. We consistently recorded thrombin potentiation of B_2 agonists in numerous experiments: n = 21 for BK (1 nM) and n = 17 for tissue kallikrein (1–10 nM). HPAE cells that constitutively express both receptors confirmed positive cooperativity (p < 0.01) between PAR1 and BK

B_2Rs (Fig 1a, right panel). Ten nM human thrombin increased basal release of AA by endothelial cells slightly (1.4 ± 0.1) but also potentiated the effect of BK and plasma kallikrein on the BK B_2R significantly.

The synthetic thrombin R activating peptide SFLLRN (TRAP) is composed of the first six amino acids of the new N-terminal tethered ligand that is revealed when thrombin cleaves PAR1. This peptide functions as a PAR1 agonist that activates the R independently of thrombin and proteolysis (12,38). TRAP was used as a control for two reasons: it is specific for the PAR1 and it ensures that enhanced B_2 agonist activity results from PAR1 activation, not from thrombin acting on B_2R (Fig 1b). Like thrombin, TRAP (30 μM) released trace amounts of $[^3H]AA$ from CHO/B_2 cells (2.4 ± 0.8), and the B_2R blocker, HOE 140 (2.4 ± 0.1), did not affect it. TRAP doubled $[^3H]AA$ released by either plasma kallikrein (6.4 ± 1.5 to 13.0 ± 2) or BK (6.3 ± 1.7 to 12.9 ± 2.0), a significant elevation over control cells blocked by HOE 140. In separate experiments *in vitro*, TRAP did not inhibit substrate hydrolysis by kallikrein (not shown). Reaction to the PAR1 peptide agonist was specific, because 30 μM of a scrambled TRAP peptide (scrTRAP, LFRSLN) neither increased basal $[^3H]AA$ nor potentiated $[^3H]AA$ release induced by kallikrein or BK (Fig 1b, right panel). As an additional control, thrombin inhibited by 1 mM DFP failed to activate PAR1 and did not modify $[^3H]AA$ release by B_2 R agonists. Together these data show that activation of the thrombin PAR1 leads to a positive cooperativity with BK R agonists resulting in increased receptor-mediated activities.

We did additional experiments with wild type (WT) CHO cells that have native PAR1 Rs but lack the B_2 Rs. Fig 1c shows that treatment of WT CHO cells with BK, porcine pancreatic and human plasma kallikreins did not release $[^3H]AA$. Basal release of $[^3H]AA$ induced by 10 nM thrombin (1.2 ± 0.7) or TRAP peptide (1.2 ± 0.2) was not significantly increased by added BK, tissue kallikrein, or plasma kallikrein in three separate experiments. These data confirm that expression of both receptors are required for receptor-to-receptor crosstalk and the positive cooperativity reflected by enhanced release of labeled AA.

Inhibitors of PKCα block potentiation

In order to determine the mechanisms of thrombin potentiation, we used selective inhibitors of G proteins ($G\alpha_i$ and $G\alpha_q$) signaling pathways, phospholipases (PLA_2, PLC_β) and protein kinases that regulate G protein coupled R activity. Because the PKCα subtype phosphorylates transient receptor potential Cl (TRPC1) channel and regulates store-operated Ca^{2+} entry in endothelial cells upon PAR1 activation (2), we inhibited PKCα to see if it could block potentiation of AA release by thrombin. CHO/B_2 cells were pretreated for 30 min with either a non-specific PKC inhibitor, calphostin C (100 nM) or a specific inhibitor of PKCα, Gö6976 (100 nM) (30). PKC is not essential for regulation of the B_2R activity (4), and calphostin C and Gö6976 did not inhibit AA release by either kallikrein or BK significantly (Fig 2). Addition of either calphostin C or Gö6976 inhibited thrombin potentiation without significantly affecting basal B_2R activity, because it was only slightly less than in untreated cells (Fig 2a). Thrombin-potentiated AA release by BK was significantly reduced by calphostin C (7.3 ± 0.9) and Gö6976 (9.4 ± 1.1) compared to control cells (15.9 ± 1.4). The thrombin-enhanced response to kallikrein (KLK) was similarly blocked by calphostin C (4.1 ± 0.3) or Gö6976 (6.1 ± 0.9) v. control (10.2 ± 1.1) (Fig 2b). Gö6976 also inhibited the potentiation by TRAP (30 μM) and reduced the augmentation of either BK (15.1 ± 1.3 v. 9.7 ± 1.2) or kallikrein (10.3 ± 0.8 v. 5.5 ± 0.8) responses. Phorbol 12-myristate 13-acetate (PMA, 100 nM) had opposite effects, it enhanced thrombin potentiation of both BK (from 15.9 ± 1.4 in controls to 21.2 ± 1.3) and kallikrein (from 10.2 ± 1.1 to 16.3 ± 1.2) (Fig 2).

Because different signaling pathways resulting from PAR1 activation might be involved in enhancement of the BK B_2R agonists, we applied additional inhibitors of serine/threonine kinases: a PKG inhibitor, (RQIKIWFQNRRMKWKKLRKKKKH (0.5 μM), a PKA inhibitor,

H-89 dihydrochloride, (0.5 μM), an IP$_3$ kinase inhibitor, wortmannin (0.5 μM) and a tyrosine kinase inhibitor, genistein (100 μM). None of these agents significantly modified the thrombin potentiation of BK on the B$_2$R (Table 1), indicating that it does not require PKG, PKA, IP$_3$ kinase or tyrosine kinase. Methyl-β-cyclodextrin can be used to deplete cholesterol from cell membranes and lipid rafts. Since pretreatment of CHO/B$_2$ cells with 5 mM of methyl-β-cyclodextrin for 30 min at 37°C had no effect, it is highly unlikely that thrombin potentiates B$_2$R by heterodimerization with PAR1 (Table 1).

Role of G$\alpha_{12/13}$ in potentiation

Inhibition of PKCα, a downstream effector of Gα_q, blocked potentiation of B$_2$R agonists by thrombin. Stimulation of Gα_q generates DAG and IP$_3$, and IP$_3$ then binds to IP$_3$ R in the endoplasmic reticulum to trigger $[Ca^{2+}]_i$ release. The interaction of IP$_3$R with TRPC1 channels activates store depletion-induced Ca^{2+} entry (21,27,31). Together with DAG, Ca^{2+} mobilized from intracellular stores activates PKCα. Fig. 3 shows that activation of the IP$_3$R and the subsequent $[Ca^{2+}]_i$ release are required for the thrombin potentiation phenomenon. CHO/B$_2$ cells were pretreated for 30 min with either 75 μM of 2-APB to inhibit the R of IP$_3$ or 20 μM of TMB-8, a $[Ca^{2+}]_i$ antagonist. 2-APB blocked the IP$_3$R dependent $[Ca^{2+}]_i$ mobilization (Fig 3a) and the thrombin-induced potentiation of B$_2$ agonists (4.7 $\pm$ 0.6 compared to 9.9 $\pm$ 0.8) (Fig 3b). Potentiation by BK was also reduced in cells pretreated with the $[Ca^{2+}]_i$ antagonist TMB-8 (7 $\pm$ 0.6 compared to 9.9 $\pm$ 0.8), a weaker inhibitor of Ca^{2+} flux (fig 3a).

PAR1 agonists can activate other heterotrimeric G-proteins, the Gα_i (14,25,37) and the G$\alpha_{12/13}$ (13,17,27) that initiate cascading downstream processes. The released α subunit of G$_{12/13}$ activates P115 Rho guanine nucleotide exchange factor (p115RhoGEF) and thereby also Rho. In order to determine whether thrombin potentiates BK effects through the G$\alpha_{12/13}$ pathway as well, we pretreated CHO/B$_2$ cells for 30 min with 10 μM Y27632, a Rho kinase inhibitor. This inhibitor decreased extracellular Ca^{2+} entry into thrombin-activated cells (Fig 3a) and consequently, lessened the potentiation of B$_2$ agonist (6.8 $\pm$ 0.7 v. untreated cells 9.95 $\pm$ 0.8). It follows that the mechanism of potentiation at the B$_2$R requires Rho acting *via* IP$_3$ R to induce $[Ca^{2+}]_i$ release and activate the subsequent signaling cascade. Based on our experiments with inhibitors, thrombin appears to potentiate plasma kallikrein differently than BK. Even though 2-APB completely blocked the thrombin-induced potentiation of kallikrein just as for BK, neither TMB-8 nor the RhoA kinase blocker, Y27632, blocked it (Fig 3c). These data thus suggest that influencing the R of IP$_3$ is a focal point of interaction between the B$_2$R and PAR1 signaling pathway. Additional experiments indicated that kallikrein and BK regulate $[Ca^{2+}]_i$ mobilization through different mechanisms (D. Biyashev, unpublished observations).

Thrombin enhances cytosolic PLA$_2$ activation

Among the different isoforms of PLA$_2$, cytosolic PLA$_2$ (cPLA$_2$) is a major factor for receptor-regulated release of AA from membrane phospholipids (24). The activity of cPLA$_2$ is Ca^{2+}-dependent, and increased $[Ca^{2+}]_i$ triggers translocation from the cytosol to plasma membranes (34,35) to enhance its activity. Because the thrombin potentiation effect requires mobilization of $[Ca^{2+}]_i$, we investigated the role of cPLA$_2$. We pre-incubated CHO/B$_2$ cells for 30 min with 20 μM of isotetrandrine, a non-selective inhibitor that blocks both Ca^{2+}-dependent and Ca^{2+}-independent PLA$_2$s. When both Ca^{2+}-dependent and Ca^{2+}-independent PLA$_2$s were inhibited, basal release of [^{3}H]AA by 1 nM BK or by 10 nM plasma kallikrein was blunted (2.5 $\pm$ 0.1 v 5.9 $\pm$ 0.3 and 3.9 $\pm$ 0.5 v 7.4 $\pm$ 0.9), as was potentiation of these agonists by thrombin. A more specific inhibitor of cPLA$_2$ (20 nM; see Materials) similarly reduced the effects of B$_2$ R agonists or plasma kallikrein on untreated cells as well as their potentiation by thrombin. In cells where cPLA$_2$ was inhibited, release of AA fell to approximately half that in uninhibited cells. We used bromo-enol lactone (BEL), a specific Ca^{2+}-independent PLA$_2$ (iPLA$_2$) inhibitor, as a control. Figure 4 shows that BEL (20 μM) after 30 min preequilibration inhibited

the release of $[^3H]AA$ by BK by 32% (4.0 ± 0.5 v 5.9 ± 0.3), but not that of plasma kallikrein (7.16 ± 1.0 v 13.5 ± 1.0). However, the relative potentiation by thrombin of either B_2R agonist was not abolished by BEL. Even though the release of AA by BK in the presence of thrombin decreased, the ratio of $[^3H]AA$ released by BK in presence of thrombin (7.9 ± 0.7) over that released in absence of thrombin (4.0 ± 0.5) was similar to the ratio in control cells (9.9 ± 0.8 over 5.9 ± 0.3) (Fig. 4).

Effect of transfected Gα$_i$ negative regulator

We repeated our thrombin potentiation experiments in CHO/B_2 cells that were co-transfected with a regulator of G protein signaling type 10 (RGS10), which negatively modulates Gα$_{i/o}$ signaling upon Rs activation. Figure 4 shows that expression of RGS10 completely blocked B_2R agonists, even in the presence of thrombin. RGS10 serves as a GTPase-activating protein for Gα$_{i/o}$, but it may also regulate the Gα$_q$ subunit (33). Data from these experiments suggest that thrombin mobilizes $[Ca^{2+}]_i$ to activate the Ca^{2+} dependent cPLA$_2$ and thus potentiates the activity of BK B_2R agonists as reflected by a doubling of AA release.

Thrombin modifies the phosphorylation of the BK R

To determine how PAR1 agonists augment B_2 agonists, we investigated their actions on B_2R phosphorylation. We used 1 μM BK to detect the marked phosphorylated R better. Figs 5a and 6a show changes in $[^3H]AA$ release from CHO/B_2 cells. The response to BK alone was taken as 100 % and plotted it vs. results from increased concentrations of PAR1 agonists. Thrombin potentiated either 1 μM or 1 nM BK, but the response was more prominent at the lower BK concentration (Fig 5a). Fig 5b shows the precipitation of B_2R with antibodies to GFP in CHO/B_2-GFP transfected cells. CHO/B_2-GFPct cells were incubated for 30 min with 1 μM BK (37° C), and increasing concentrations of thrombin were added. We established that the GFP tag attached to the C terminus of the B_2R affected neither release AA nor mobilization of $[Ca^{2+}]_i$, (19). Furthermore, this manipulation did not influence potentiation by thrombin (data not shown). The phosphorylated R was quantified and normalized to the total protein level using the ratio of phosphorylated protein density/total protein density (Fig 5b). The increased basal phosphorylation of the receptor following BK challenge (4.3 ± 0.5-fold) was taken as 100 %. Fig 5b shows how B_2R phosphorylation changed with increasing amounts of thrombin. In absence of added BK, thrombin did not phosphorylate the BK B_2R (data not shown), but it enhanced BK-induced phosphorylation of B_2-GFPct R in a concentration-dependent manner, reaching a maximum of 166 % at 30 nM. Higher concentrations of thrombin decreased it. The phosphorylation of B_2R that thrombin augmented was inhibited in cells pretreated with either 75 μM of 2-APB or 100 nM of Gö6976 (Fig 5b).

Similarly to thrombin, TRAP potentiated either 1 nM or 1 μM BK on CHO/B_2 transfected cells. $[^3H]AA$ was released in a concentration-dependent manner with maximum at 10–30 nM of peptide. BK alone increased the basal level of phosphorylation; this increase was taken as 100 %, and data from additional treatments are expressed relative to this (Fig 6a). Because an aminopeptidase on the cell surface rapidly degrades TRAP, we repeated this experiment in the presence of amastatin (50 μM), an aminopeptidase inhibitor. When hydrolysis of the N-terminal Ser of TRAP was blocked by amastatin, as little as 3 μM TRAP potentiated the BK response. Increasing amounts of TRAP augmented B_2R phosphorylation by BK (1 μM); maximum phosphorylation (192 % of control) was obtained with 30–100 μM TRAP in the absence of the aminopeptidase inhibitor. When 50 μM amastatin was added to the reaction mixture, the effect of TRAP resembled that of thrombin (Fig 5b, 6b). TRAP potentiation of BK-induced B_2R phosphorylation reached a maximum (232 %) between 3 to 10 μM concentration of the peptide, and higher concentrations decreased it (Fig 6b).

DISCUSSION

We found that activation of PAR1 by either thrombin or the hexapeptide, TRAP, potentiates BK B_2R agonists to enhance release of [^{3}H]AA. AA is the precursor of prostaglandins, such as prostacyclin, a coronary vasodilator, and prostaglandin E2 that prevents platelet aggregation. It is also a precursor of thromboxane (8,35). Although thrombin releases AA from many cell types, we used CHO cells in most of our experiments because they express native PAR1. Thrombin or TRAP released relatively small amounts of AA above baseline, but these agents both potentiated B_2R agonists. In experiments repeated all together 35 – 40 times with human plasma kallikrein (10 nM) or BK (1 nM) we found that thrombin and TRAP consistently doubled AA release by these agonists. We obtained qualitatively similar results in experiments with human arterial endothelial cells that constitutively express both PAR1 and B_2Rs.

Besides BK, we also tested human plasma kallikrein, because kallikreins affect B_2R differently than BK. It is clear that kallikrein action at the B_2R does not depend upon release of a kinin (19,20). As with BK, stimulation of the B_2R by kallikrein was blocked by HOE 140, and both prokallikrein and inhibited kallikrein were inactive. Kallikrein and BK both desensitized the R, but there was no cross-desensitization. [^{3}H]AA release by BK was reduced 40% by addition of carboxypeptidase M, which removes Arg^9. Carboxypeptidase M did not influence R activation by kallikreins and other proteases. Site-directed mutagenesis of the Arg^{169} residue of the B_2R further emphasized differences in the mechanisms of activation by the proteases and BK. The mutation decreased R activation by BK but not by kallikrein. In addition, our study shows that B_2R activation by BK depends, at least in part, on Ca^{2+}-independent PLA_2 activation while activation by kallikrein does not.

Potentiation at the B_2R is not simply due to direct activation of PAR1 by the protease action of kallikrein, because we obtained similar results with the peptide agonist, BK. We further ruled out a proteolytic action of thrombin at the B_2R because TRAP, a specific agonist of PAR1, potentiated both BK and kallikrein. DFP-inhibited thrombin (inactive on PAR1) failed to enhance B_2R activation by agonists. A scrambled peptide sequence of TRAP was equally inactive, indicating that the potentiation mechanism is initiated and mediated through activation of PAR1. TRAP did not affect substrate hydrolysis by kallikrein (not shown).

Lipid rafts are believed to be essential for BK B_2R and epidermal growth factor R crosstalk (23), and methyl-β-cyclodextrin blocks this interaction by depleting cholesterol which disrupts complex formation. We found that 5 mM methyl-β-cyclodextrin failed to modify thrombin-induced potentiation in CHO/B_2 transfected cells. Thus, it seems unlikely that the potentiation phenomenon depends on PAR1/B_2R dimer formation, even though B_2 Rs can form heterodimers with angiotensin converting enzyme (ACE; Z. Chen and E.G. Erdös, unpublished observations and 28). In addition, because of the discrepancy in the ratio of PAR1 to B_2 expression in CHO/B_2 transfected cells, potentiation cannot be due simply to co-localization; it is presumably a consequence of enhanced signal transduction.

In a comparable system, thrombin first activates $G\alpha_q$/$PKC\alpha$ and the $G\alpha_{12/13}$/Rho pathways to mobilize [$Ca^{2+}]_i$ in endothelial cells (21,31). Upon stimulation, PAR1 coupled to $G\alpha_q$ protein generates DAG and IP_3; the latter then binds to IP_3 Rs on the endoplasmic reticulum to trigger [$Ca^{2+}]_i$ release. The interaction of IP_3 R with transient receptor potential channel 1 (TRPC1) activates Ca^{2+} entry by store depletion (21,27,31). Yet, beside $G\alpha_q$, stimulated PAR1 couples to $G\alpha_{12/13}$ proteins (13,17,27). The released α subunit of $G_{12/13}$ induces P115 Rho guanine nucleotide exchange factor (p115RhoGEF) activation that, in turn, activates Rho. Upon activation of $G\alpha_{12/13}$/Rho signaling cascade, activated Rho associates with IP_3 R and TRPC1 by actin filament polymerization to provoke [$Ca^{2+}]_i$ store depletion (31). Following emptying of stored Ca^{2+}, the Rho/IP_3 R/ TRPC1 complex translocates to the plasma membrane to trigger

extracellular Ca^{2+} entry (21,31). The phosphorylation of p115RhoGEF depends on PKCα (21), which is activated via $Gα_q$ signaling pathway stimulation. These experiments with endothelial cells show that increased $[Ca^{2+}]_i$ upon PAR1 activation requires cooperative interaction of both $Gα_{12/13}$ and $Gα_q$ /PKCα pathways.

Our results are consistent with those findings and indicate that $[Ca^{2+}]_i$ release indeed triggers thrombin-mediated potentiation of BK. We found that enhancement of AA release by thrombin was attenuated after inhibition of either $Gα_q$/PKCα or $Gα_{12/13}$ /Rho pathways. The inactivation of the IP_3 R-dependent $[Ca^{2+}]_i$ mobilization in CHO/B_2 cells by 2-APB, the inhibition of PKCα by Gö6976, and inhibition of Rho by a Rho kinase inhibitor Y27632 all blocked potentiation of BK. Together these findings suggest that PAR1 R stimulation causes formation of the Rho/TRPC1/IP_3 R complex, and that the resulting $[Ca^{2+}]_i$ release initiates signaling events leading to increased $[^3H]$AA release.

However, interestingly thrombin potentiates the effects of plasma kallikrein and BK on the B_2R differently. While an inhibitor of the IP_3 R completely blocked the potentiation of kallikrein by thrombin to release $[^3H]$ AA, the selective inhibitor of $[Ca^{2+}]_i$ release (TMB-8), or that of Rho kinase (Y27632) did not modify it. Thus, both the IP_3 R activation and the resulting elevation of $[Ca^{2+}]_i$ level are involved in potentiation of BK, only the IP_3 R activation is necessary for potentiation of kallikrein by thrombin. Altogether, these data are showing that IP_3 R is indeed the critical point of interaction between the pathways leading to positive cooperativity. Because PAR1 initiates enhancement of $[^3H]$AA release by B_2R agonists and Ca^{2+} stimulates cPLA2, cPLA2 is likely involved in potentiation. Indeed, a specific inhibitor of Ca^{2+}-dependent PLA2 blocked the potentiation by thrombin, while an inhibitor of the Ca^{2+}-independent isoform was ineffective, implying that $[Ca^{2+}]_i$ mediates enhanced B_2R activity by agonists. Both thrombin and TRAP modified B_2R phosphorylation by agonists in a concentration-dependent manner. Once activated by agonist, B_2R undergoes phosphorylation and desensitization. ACE inhibitors decreased the phosphorylation of B_2R by BK and thus enhanced the activity of the peptide agonist (29). Now we find that low concentrations of thrombin (10–30 nM) or TRAP (30–100 μM without addition of the aminopeptidase inhibitor amastatin and 1–10 μM with amastatin) elevate BK B2R activity and increase phosphorylation of the R. Higher amounts of enzyme or peptide reversed the process and inhibited BK-initiated phosphorylation. Blocking of these effects by the IP_3 R inhibitor, 2-APB, and by the PKCα inhibitor Gö6976 can be explained by co-activation of $Gα_{12/13}$ and $Gα_q$ /PKCα pathways by thrombin and the subsequent $[Ca^{2+}]_i$ mobilization that modulates B2R phosphorylation and activity. Increased phosphorylation is not likely due to increased expression of B2R, because we found no concomitant increase in B2-GFP total protein with the increase in R phosphorylation (Fig. 5). In addition, inhibitors would unlikely alter protein synthesis and receptor expression during the brief time course of our experiments, but only modify activity. Additional controls where we measured $[^3H]$BK binding to the cell membrane indicated no modification by thrombin (data not shown).

Fig 7 provides a scheme that summarizes pathways potentially involved in the interaction of PAR1 and B_2R agonists. We conclude that PAR1 R activation enhances the effects of BK B2R agonists in signal transduction which then results in augmented release of AA. Since AA is a precursor of several vasoactive mediators, regulation of these pathways influences many diverse physiological functions. The cooperativity between the two Rs that we described for signal transduction pathways involved in AA release may underline the cardioprotective nature of kinins *in vivo*.

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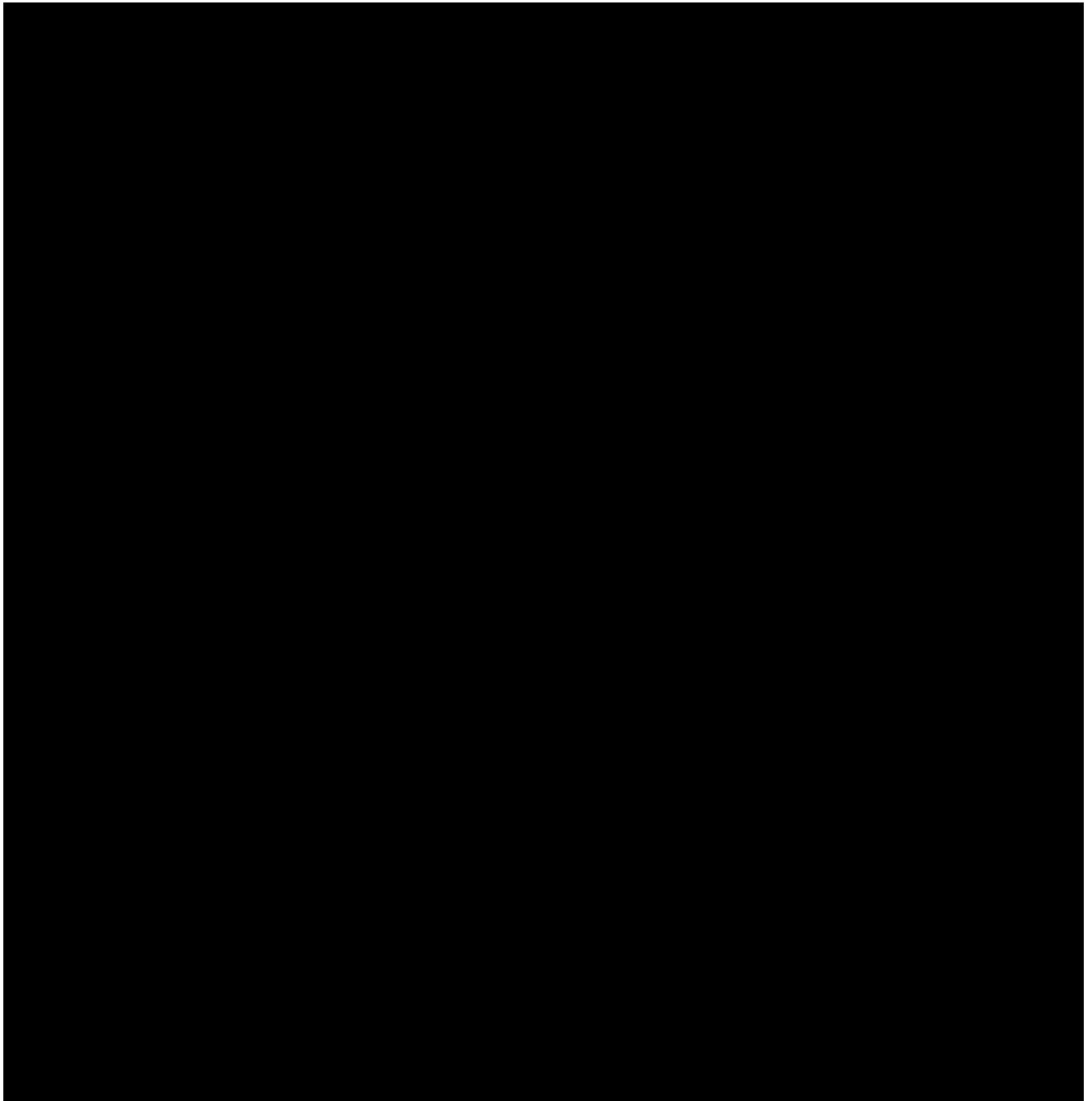
Fig 1. PAR1 activation by human thrombin and TRAP potentiates BK B$_2$ R agonists
CHO cells transfected with human B$_2$Rs (CHO/B$_2$) and human pulmonary artery endothelial (HPAE) cells were incubated 30 min at 37°C with 1 nM BK, 10 nM human plasma kallikrein (KLK) or porcine tissue KLK in the presence or absence of 10 nM human thrombin (panel a), 30 μM TRAP or 30 μM scrambled TRAP (scrTRAP: [^{3}H]AA release is expressed on the ordinate in relative units. Panel b). Thrombin and TRAP enhanced [^{3}H]AA release by B$_2$ R agonists; this response was blocked by the B$_2$ antagonist, HOE 140. Solid columns = agonist alone; diagonal lines = combination of B$_2$ R agonist and PAR1 agonist; vertical lines = combination of B$_2$ R agonist and PAR1 agonist in the presence of 0.5 μM HOE 140. All data

are means ± S.E.M. of 3–5 separate determinations. * indicates $P < 0.05$ vs control agonist alone.

Panel c: Thrombin and TRAP did not potentiate wild type (WT) CHO cells that express PAR1 but lack B_2R. Solid column = agonist alone; diagonal lines = PAR1 agonist alone; vertical lines = combination of B_2R agonist and PAR1 agonist. Data are means ± S.E.M. from 3–5 separate experiments.
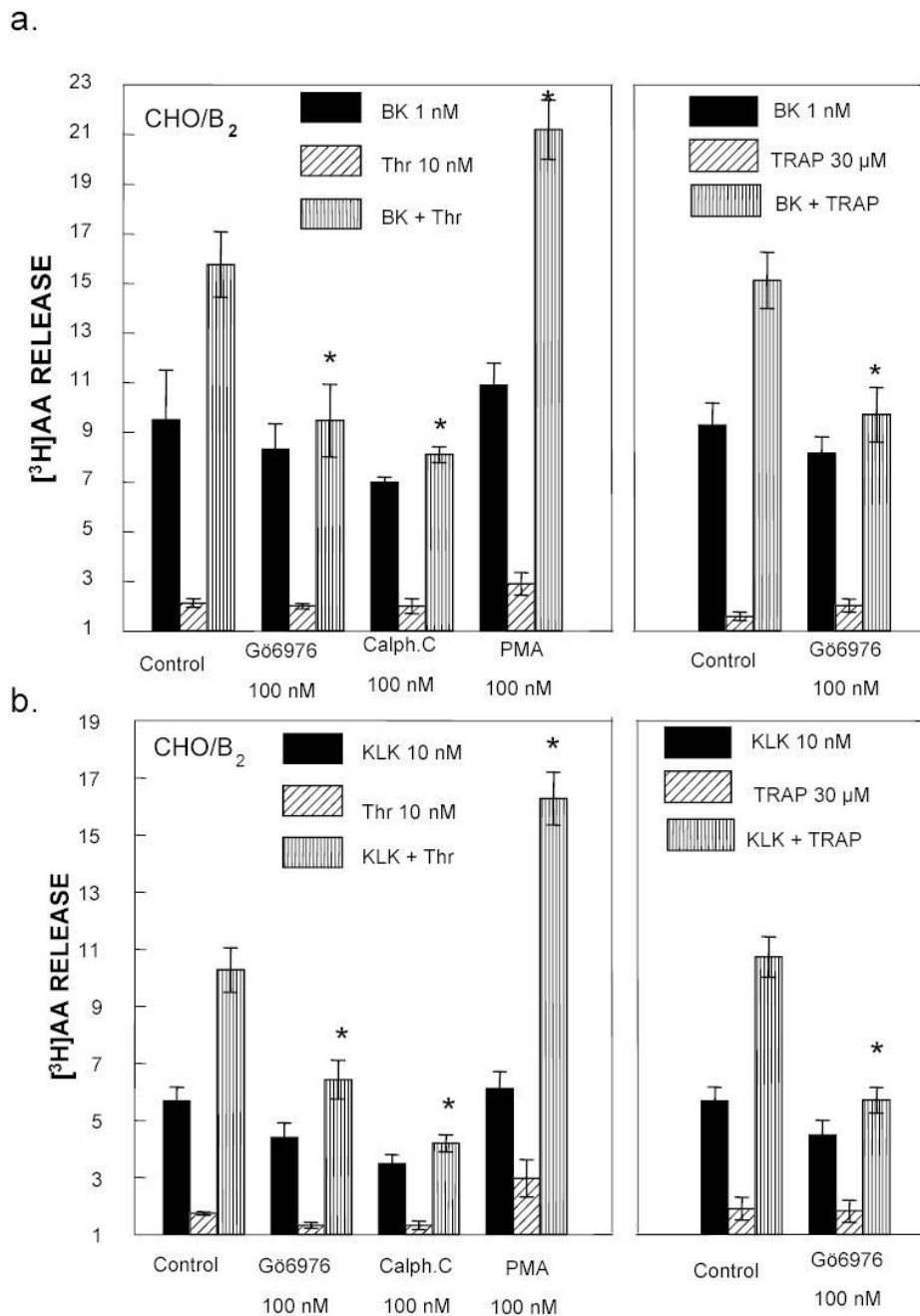
Fig 2. Inhibitors of PKCα blocked thrombin-induced potentiation
CHO/B$_2$ cells were incubated with 100 nM Gö6976, calphostin C (Calph.C) or phorbol 12-myristate 13-acetate (PMA) for 30 min. Then 1 nM BK (panel a) or 10 nM KLK (panel b) was added in the presence or absence of 10 nM human thrombin or 30 μM TRAP. Ordinate as in Fig 1. Solid column = agonist alone; diagonal lines = PAR1 agonist alone; vertical lines = combination B$_2$R agonist and PAR1 agonist. Data are means ± S.E.M from 3–5 separate experiments. * P < 0.05 vs control untreated cells. Phorbol enhanced the potentiation by PAR1 agonists, but PKC inhibitors blocked it.
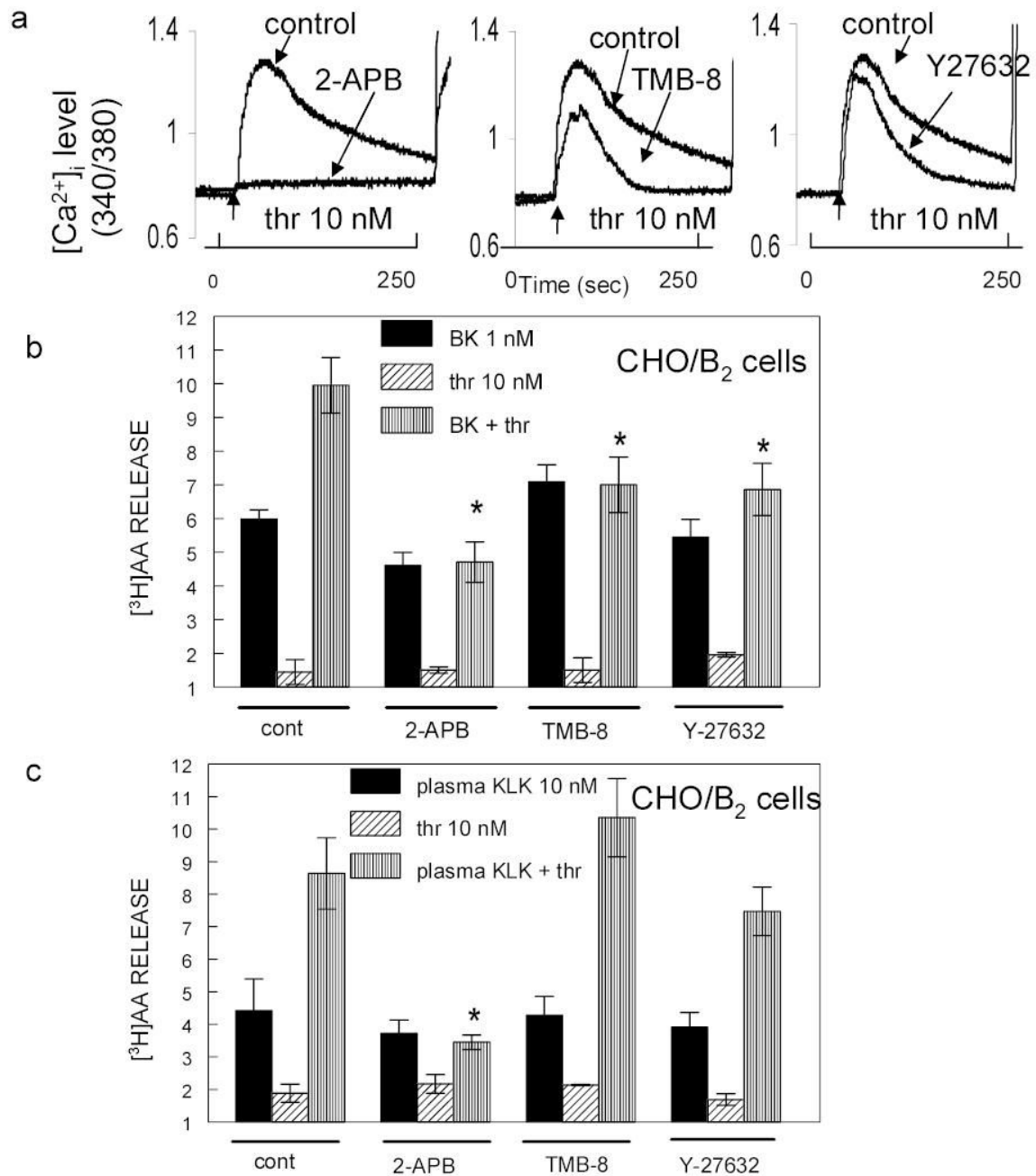
Fig 3. Role of Gα12/13 in potentiation

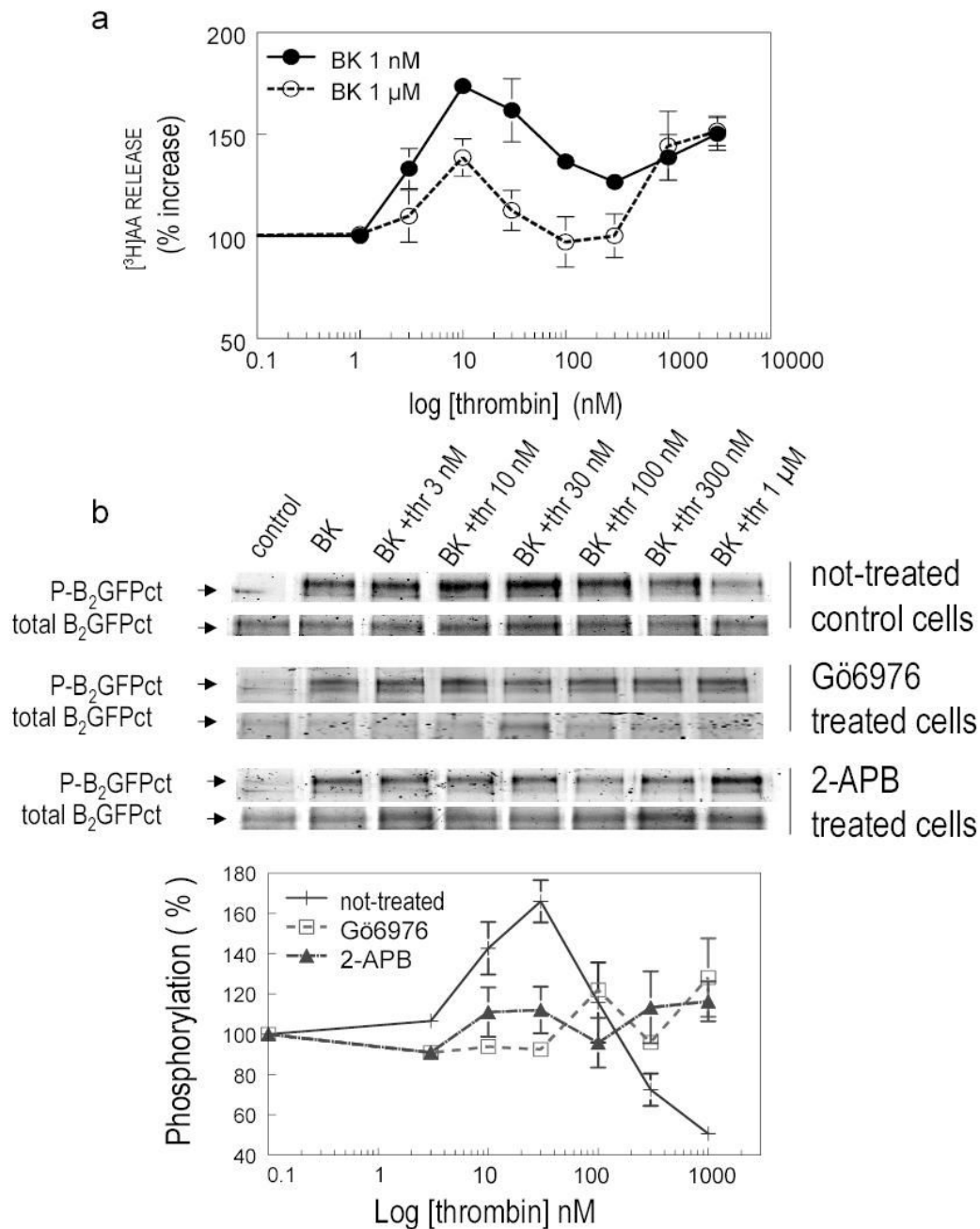*Panel a*: Inhibition of thrombin-induced $[Ca^{2+}]_i$ release. Fura-2AM loaded CHO/B$_2$ cells were incubated with 75 μM 2-APB, 20 μM TMB-8 or 10 μM Y27632 for 30 min to inhibit IP$_3$ R, $[Ca^{2+}]_i$ release or RhoA kinase. Cells were then exposed to 10 nM thrombin. Abscissa: time in seconds. Ordinate: relative $[Ca^{2+}]_i$ level. Experiments were repeated three times with similar results. *Panels b and c*: [3H]AA loaded CHO/B$_2$ cells were treated with 75 μM 2-APB, 20 μM TMB-8 or 10 μM Y27632 for 30 min at 37 °C and then exposed to 1 nM BK (panel b) or 10 nM plasma kallikrein (panel c) with or without 10 nM thrombin. Solid column = agonist alone; diagonal lines = PAR1 agonist alone; vertical lines = combination B$_2$R agonist and PAR1 agonist. Data are means ± S.E.M. from 3–5 separate experiments. * P < 0.03 comparing

B$_2$R agonists in thrombin stimulated control cells. 2-APB blocks Ca^{2+} entry into cells, the two other agents reduced it. Inhibitors abolished or reduced potentiation by thrombin. TMB-8 blocked potentiation of BK but not that of kallikrein.

a.

b.
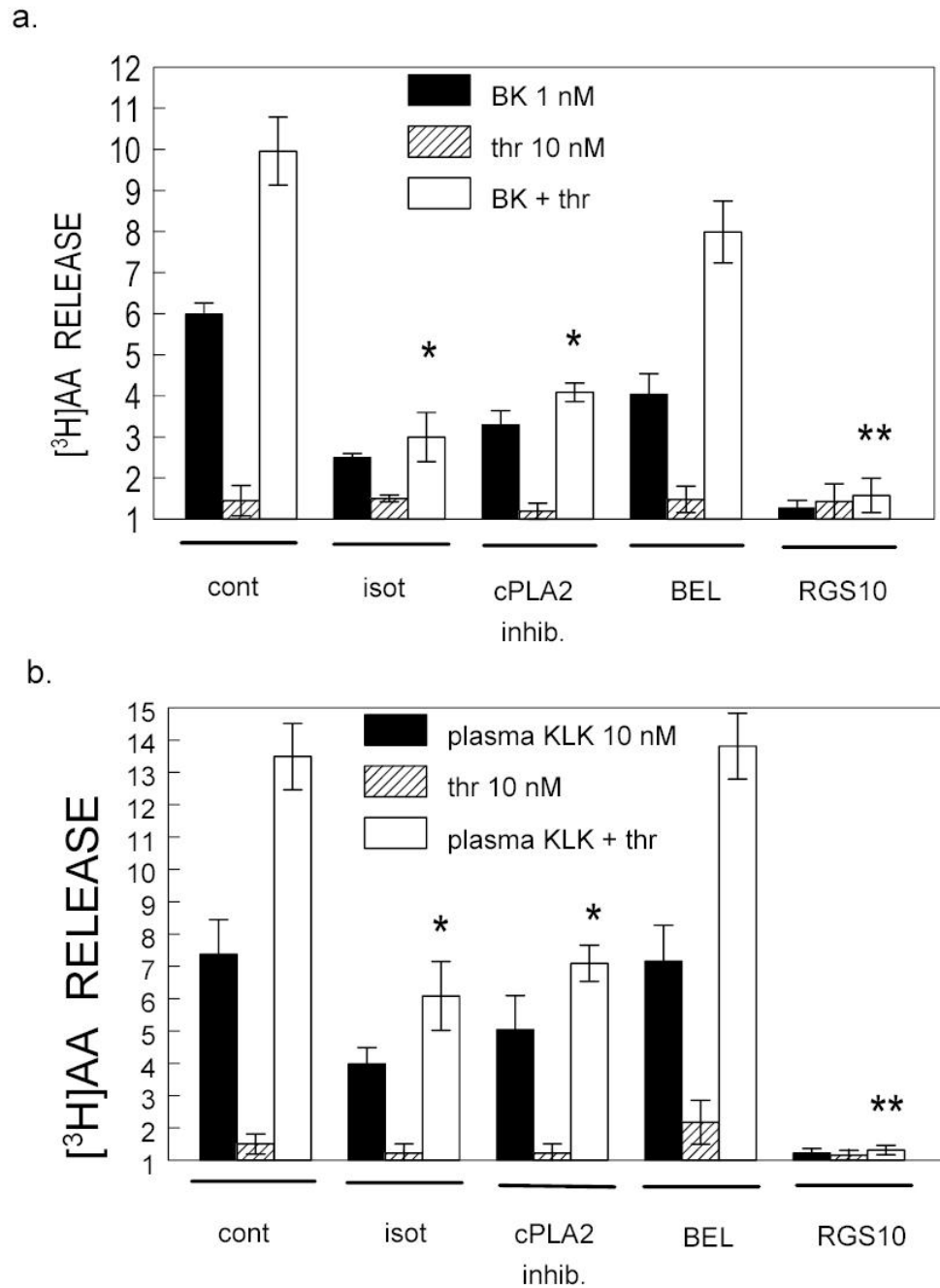
Fig 4. Thrombin enhances cytosolic PLA$_2$ (cPLA$_2$) activation by B$_2$ agonists to release AA
CHO/B$_2$ cells were treated with 20 μM isotetrandrine (ISO), cPLA$_2$inhib (100 nM), a specific
cPLA$_2$ inhibitor, or with 20 μM bromoenol lactone (BEL) for 30 min prior to incubation with
agonists for a further 30 min. Solid bars = BK 1 nM (panel a) or plasma kallikrein 10 nM (panel
b); diagonal lined bars = thrombin 10 nM; Open bars = thrombin + B$_2$ receptor agonist. Data
are means ± S.E.M. from 3–4 separate experiments. * P < 0.01 and ** P < 0.005 indicate
significant differences with untreated cells stimulated with B$_2$R agonists and thrombin. The
cPLA$_2$ inhibitors reduced AA release, abolished potentiation (*), but BEL did not. The
expression of RGS10, a negative modulator of Gα$_{i/o}$, completely blocked B$_2$R agonists even
in presence of thrombin.

Fig 5. Effect of thrombin on stimulated B$_2$R phosphorylation

Panel a: CHO/B$_2$ transfected cells were incubated with 1 nM BK (-•-) or 1 μM BK (-○-) in the presence or absence of increasing concentrations of thrombin (abscissa). The increase in [^{3}H]AA is expressed as % on the ordinate. The effect of BK without thrombin is taken as 100 %; $P < 0.05$. *Panel b*: thrombin modified BK-induced B$_2$R phosphorylation in a concentration dependent manner. CHO/B$_2$-GFPct transfected cells untreated (+) or treated with 100 nM Gö6976 (-□-) or 75 μM 2-APB(- ▲ -) were incubated with 1 μM BK (30 min) and increasing concentrations of thrombin (0.1 – 1 μM. BK). Data are from three separate determinations. BK increased the basal level of phosphorylation of the B$_2$R by 4.3 ± 0.5 fold (n=3) taken as 100%. $P < 0.05$ vs not-treated cells without thrombin. Thrombin in low concentrations enhanced

phosphorylation of B_2-GFP by BK, higher concentrations blocked it; effects were abolished by IP_3 R and $[Ca^{2+}]_i$ release inhibitors.
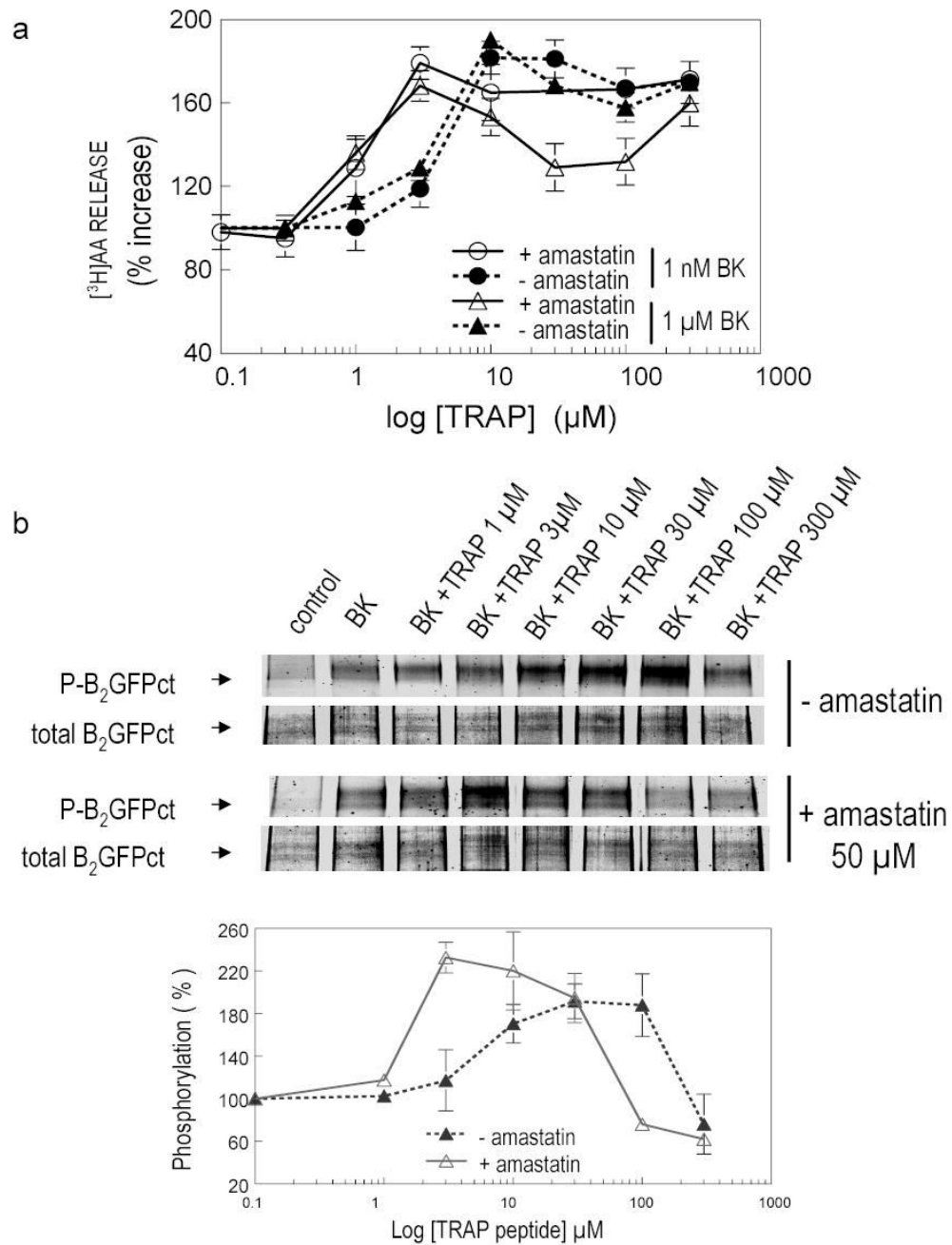
Fig 6. Effect of aminopeptidase inhibitor on [^{3}H]AA release and B$_2$ R phosphorylation stimulated by TRAP

Panel a: TRAP potentiates stimulation of CHO/B$_2$ transfected cells by 1 nM BK (-•-) or 1 μM BK (-▲-) to release AA in a concentration dependent manner. Effects of TRAP were increased by 50 μM amastatin (-○-for 1 nM BK and -◦- for 1 μM BK). Ordinate and abscissa as in Fig 5. *Panel b*: TRAP modifies BK-induced B$_2$R phosphorylation in a concentration-dependent manner. CHO/B$_2$-GFPct transfected cells were incubated with 1 μM BK (30 min) and increasing concentration of TRAP ranging from 1 – 300 μM with or without 50 μM amastatin. Details as in Fig 5. Aminopeptidase inhibitor amastatin blocked cleavage of N-terminus of TRAP and enhanced its activity.
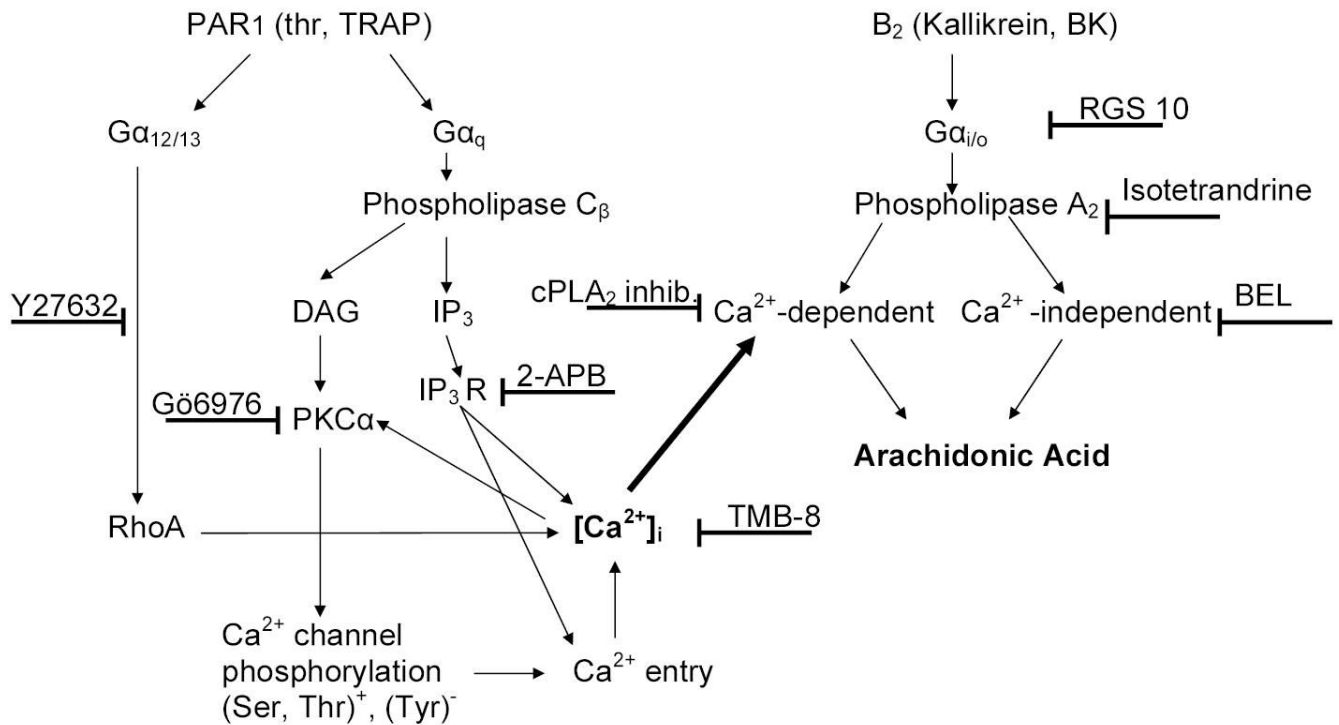
Fig 7. Scheme for the enhanced AA release
Upon thrombin activation, $G\alpha_q$ and $G\alpha_{12/13}$ signaling pathways converge to empty Ca^{2+} stores and redirect subsequent $PKC\alpha$ dependent Ca^{2+} entry. The elevation of $[Ca^{2+}]_i$ enhances Ca^{2+}-dependent PLA_2 activation initiated by B_2 R agonists and increases release of AA.

Table I

Negative Inhibitors of B$_2$R Potentiation

Treatment	[^{3}H]AA release (relative units; basal release = 1) (n = 3)	
	BK	BK + thrombin
Vehicle	6.8 ± 0.8	11.8 ± 0.7
Tyr kinase inhibitor genistein 100 μM	6.8 ± 0.4	11.6 ± 0.6
PKG inhib. 0.5 μM (RQIKIWFQNRRMKWKKLRKKKKH)	6.7 ± 0.3	11.7 ± 0.5
PKA inhib. 0.5 μM (H-89, dihydrochloride)	8.6 ± 0.4	11.7 ± 0.5
IP$_3$ kinase inhibitor wortmannin 0.5 μM	6.5 ± 0.6	11.3 ± 0.9
Methyl-β-cyclodextrin 5 mM	6.9 ± 0.6	12.0 ± 0.8