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ORIGINAL PAPER

Bauhinia bauhinioides cruzipain inhibitor reduces endothelial proliferation and induces an increase of the intracellular Ca^{2+} concentration

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Abstract Proteinase inhibitors, isolated from different types of *Bauhinia*, have an effect on apoptosis, angiogenesis and inflammation. The *Bauhinia bauhinioides* cruzipain inhibitor (BbCI) is a Kunitz-type inhibitor and inactivates the cysteine proteinases cruzipain and cruzain from *Trypanosoma cruzi*. Cruzipain and tissue kallikrein have similar biochemical properties, e.g. the proteolytic cleavage of the kininogen precursor of lys-bradykinin. Tissue kallikrein stimulation in endothelial cells causes migration and capillary tube formation. The aim of this study was to examine whether the antiproliferative effect of BbCI is dependent on changes of the intracellular calcium concentration and membrane hyperpolarization. Endothelial cells were isolated from human umbilical cord veins (HUVEC). For proliferation experiments, HUVEC were incubated with BbCI (10–100 μmol/L) for 48 h. The proliferation was detected by cell counting with a Neubauer chamber. The effect of BbCI (10–100 μM) on the membrane potential was measured with the fluorescence dye $DiBAC_4(3)$ and the effect on $[Ca^{+2}]_i$ with the fluorescence probe Fluo-3 AM. The change of the fluorescence intensity was determined with a GENios plate reader (Tecan). The experiments showed that BbCI (10–100 μmol/L) reduces the endothelial cell proliferation significantly in a concentration-dependent manner with a maximum effect at 100 μmol/L (35.1± 1.8% as compared to control ($p \leq 0.05$; $n=45$)). As compared to the control, the addition of BbCI (100 μmol/L) caused a significant increase of systolic Ca^{2+} of 28.4±5.0% after 30 min incubation. HUVEC treatment with BbCI (100 μmol/L) showed a weak but significant decrease of the membrane potential of 9.5± 0.9% as compared to control ($p \leq 0.05$; $n=80$). BbCI influenced significantly the endothelial proliferation, the intracellular Ca^{2+} concentration and the membrane potential.

Keywords *Bauhinia bauhinioides* cruzipain inhibitor · Endothelial cells · Calcium · Hyperpolarization · Proliferation

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Introduction

Proteinase inhibitors (PI) are ubiquitous proteins in animals, plants and microorganisms. They are involved in a variety of biological processes, e.g. in plants defence mechanism against pests and pathogens [24, 37]. The best characterized serine PI families are the Bowman-Birk inhibitors and the Kunitz-type inhibitors, which are isolated from many leguminous plants like the genus *Bauhinia* [7, 32, 33]. Proteinase inhibitors from *Bauhinia bauhinioides* are known to effect physiological processes like blood coagulation, inflammation or cancer [29, 30, 38]. *B. bauhinioides* cruzipain inhibitor (BbCI) is a serine and cysteine proteinase inhibitor, which is isolated from the seeds of *B. bauhinioides* [8]. BbCI is an 18-kDa protein, an inhibitor of cruzipain—the main cysteine proteinase of *Trypanosoma cruzi* as well as its recombinant form cruzain and cathepsin L—a mammalian proteinase which is highly homologue to cruzipain [6, 34]. BbCI also shows inhibitory properties against some other serine proteinases: the porcine pancreatic elastase, the human neutrophil elastase and cathepsin G [2, 30]. But interestingly, BbCI does not demonstrate a significant inhibition of the serine proteinase plasma kallikrein or of papain and cathepsin B both cysteine proteinases closely related with cruzipain [8].

Cruzipain as a cysteine protease from the pathogenic parasite *T. cruzi* also displays kinin-releasing activity [10]. Tissue kallikrein differs from plasma kallikrein in genetic origin and the biochemical properties, e.g. the ability to release lys-bradykinin from its precursor kininogen (L-kininogen) [4]. Convincing evidence shows an involvement of tissue kallikrein in angiogenesis, for example, as response to ischemia or in tumour growth [5, 12, 13]. Several studies have reported that HUVECs synthesize and release functionally active tissue kallikrein. HUVECs also produce and secrete L-kininogen and express the lys-bradykinin receptor B2 [9, 43]. Tissue kallikrein stimulation in endothelial cells causes migration and formation of capillary tube-like structures over a PI3K-Akt-dependent signalling pathway [42].

Cathepsin L is a lysosomal cysteine proteinase with a basal expression in most eukaryotic cells [3, 17]. Several studies report that it is actively involved in neovascularization, tumour growth, atherosclerosis and apoptosis [18, 25, 41, 44]. Cathepsin L primarily shows potent catalytic activity against collagenase and elastase [20, 26]. Cathepsin L is also reposted to have kininogenase activity and to generate bradykinin from high and low molecular weight kinins in vitro and in vivo [11, 36]. The main aim of our study was to examine whether BbCI has an effect on the proliferation of endothelial cells and to assess a possible modulation of $[Ca^{+2}]_i$ concentration and the cellular membrane potential.

Materials and methods

Solution and chemicals

1,2-bis-(*o*-Aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) was from Merck KGaA (Darmstadt, Germany); bis-1,3-dibutylbarbituric acid-trimethine oxonol ($DiBAC_4(3)$) and Fluo-3 (AM) were both from Molecular Probes (Leiden, The Netherlands); foetal calf serum (FCS) and Hank's balanced salt solution were from PAA (Linz, Austria); endothelial basal medium and supplements were from Promo Cell (Heidelberg, Germany); trypsin–EDTA (0.05%) was from Sigma (Deisenhofen, Germany). *B. bauhinioides* cruzipain inhibitor was a gift from ML Oliva.

The HEPES-buffered bath solution was made of (in millimoles per litre): $CaCl_2$ 1.25, D-glucose 5.5, HEPES 10, KCl 5, $MgCl_2$ 0.5 and NaCl 140, and Ca^{2+}-reduced buffer was made by reducing the amount of Ca^{+2} to 0.3 mmol/L; pH was adjusted to 7.3 with 1 M NaOH.

Cell isolation and culture

HUVECs were isolated by collagenase digestion procedure and cultured as described previously [16]. Cells were cultured in endothelial cell growth medium enriched with 10% foetal calf serum. The medium was exchanged every 48 h. All experiments were performed with cells from subcultures 1 to 4.

Endothelial proliferation

The proliferation of HUVECs was investigated by cell counting with a Neubauer chamber [39]. Briefly, cells were seeded in 12-well plates (Becton Dickinson,

Heidelberg, Germany), coated with 0.2% gelatine at a density of 10,000 cells/well. Cells were grown for 24 h in EBM with 10% FCS followed by a 24-h incubation in serum-free medium containing hydrocortisone (1 μg/mL) and gentamicin (50 μg/mL). After 24 h incubation, the medium was replaced by EBM with 10% FCS and the following modifications: BbCI (10–100 μmol/L) and/or BAPTA-AM (1 μmol/L) and/or valinomycin (1 μmol/L). After 48 h, the cells were detached by trypsinization and the cell number determined with a Neubauer chamber. The mean values of two counts per well were used for statistical analysis.

Measurement of the membrane potential

Modulation of the endothelial membrane potential was investigated using the fluorescence dye $DiBAC_4(3)$. HUVECs were seeded on 24-well plates and growth 48 h to confluence. The cells incubated for 15 min with 0.5 μmol/L $DiBAC_4(3)$ at 37°C in the dark. The measurement of the fluorescence intensity started immediately after the addition of BbCI (10–100 μmol/L). Membrane potential measurements were made using a plate reader Genios (Tecan, Austria). This plate reader measures changes in fluorescence, absorbance and glow luminescence. Fluorescence can be detected in a range of 340–700 nm. Fluorescence intensity changes were measured for 30 min in intervals of 30 s with an excitation wavelength of 485 nm and detection of the emitted light at 535 nm. The data were normalized to a parallel unstimulated control. Data were analysed as $DiBAC_4(3)$ fluorescence intensity in percentage as compared untreated control.

Calcium measurement

Changes in $[Ca^{+2}]_i$ concentration were detected with the fluorescence probe Fluo-3 (AM). HUVECs cultured on 24-well plates until they were confluent (48 h). After the cells were loaded with 5 μmol/l Fluo-3 (AM) for 60 min at 37°C in the dark, the medium containing Fluo-3 (AM) was replaced by HEPES-buffered bath solution or Ca^{2+}-reduced bath solution followed by incubation of 15 min. Cells were stimulated with BbCI (100 μmol/L) and/or BAPTA-AM (10 μmol/L) and/or thapsigargin (TG; 10 μmol/L). BAPTA-AM was loaded 20–30 min before BbCI addition, to allow uptake and de-desertification. Untreated control was run in parallel. Fluorescence intensity of the calcium signal was measured using a plate reader Genios (Tecan, Austria). This plate reader measures changes in fluorescence, absorbance and glow luminescence. Fluorescence can be detected in a range of 340–700 nm. The excitation wavelength was set at 485 nm, and emission was detected at 535 nm over 60 min at intervals of 60 s. The data were normalized to parallel unstimulated control.

Statistics

Data are expressed as mean ± SEM. The significance level was $P \leq 0.05$. Results were analysed by means of ANOVA and post hoc Tukey test (SPSS version 17.0; SPSS Inc., Chicago, IL, USA).

Results

Concentration-dependent reduction of the cell growth by BbCI

To test whether BbCI has an effect on the endothelial proliferation, the cells were treated with various concentration of BbCI (10–100 μmol/L) for 48 h. BbCI caused a significant reduction in the cell proliferation in a concentration-dependent manner (Fig. 1). As compared to the unstimulated control, the cell number decreased to 81.5±0.8% (10 μmol/L),

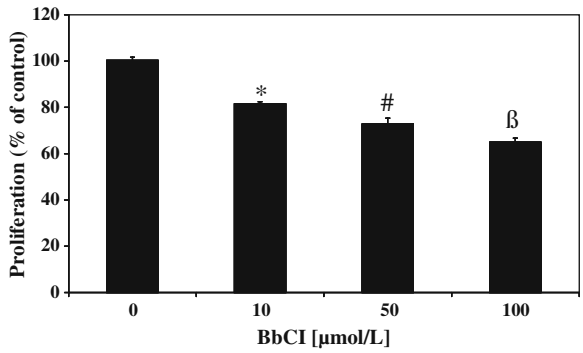

Fig. 1 Antiproliferative effect of BbCI (10–100 μmol/L) on endothelial cells. BbCI is antiproliferative in a concentration-dependent way ($n=45$). The results are shown as percentage to unstimulated control ± SEM. (*$p<0.05$ vs. 0 μmol/L; #$p<0.05$ vs. 0 and 10 μmol/L; ßp<0.05 vs. 0, 10 and 50 μmol/L), $n=45$

72.7±2.5% (50 μmol/L) and 64.9±1.8% (100 μmol/L). A concentration of 100 μmol/L BbCI was used for all following experiments.

Effect of BbCI on intracellular calcium concentration

The BbCI-stimulated signalling mechanism in endothelial cells was analysed by measuring the intracellular calcium concentration with the fluorescence dye Fluo-3 (Fig. 2). As compared to the control, the addition of BbCI (100 μmol/L) caused a significant increase of cytosolic [Ca^{2+}] concentration of 28.4± 5.0% after 30 min ($p<0.05$; $n=55$). The calcium increase was not reduced or abolished by reduction of the extracellular Ca^{2+}, indicating that the intracellular calcium rise is independent of an influx of extracellular calcium, as compared to the calcium-reduced control. This rise in $[Ca^{+2}]_i$ was completely absent in the presence of calcium chelator BAPTA (10 μmol/L).

To further analyse the calcium signal, the cells were stimulated with BbCI and TG (10 μM). There was no significant difference upon stimulation with BbCI and BbCI + TG, although there was significant increase in $[Ca^{+2}]_i$ (30±2.0%) as compared to unstimulated control. These results indicate that BbCI induced a calcium release from the endoplasmic reticulum.

BbCI-induced inhibition of cell growth is independent from rises in cytosolic Ca^{2+} concentration

The relevance of the BbCI-induced calcium increase for the reduction of the cell number was investigated using the calcium chelator BAPTA (Fig. 3). BAPTA (1 μmol/L) was not able to reverse the antiproliferative effect of BbCI (100 μmol/L). Higher concentration of BAPTA leads to a significant reduction of the cell number (data not shown).

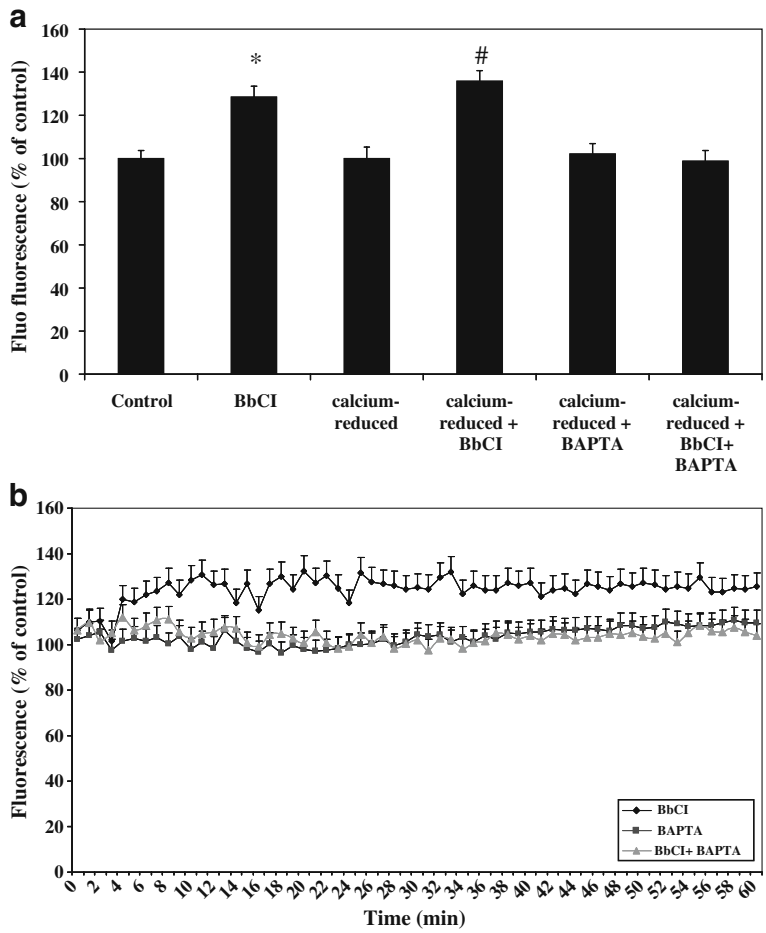

Fig. 2 **a** BbCI (100 μmol/L) induced a significant increase of $[Ca^{+2}]_i$. Calcium concentration was measured with Fluo-3. Data are expressed as percentage of unstimulated control run in parallel 30 min after addition of BbCI ($n=55$; *$p<0.05$ vs. control; #$p<0.05$ vs. calcium-reduced). **b** BbCI (100 μmol/L) induced a significant increase of $[Ca^{+2}]_i$ in calcium-reduced bath solution. Calcium concentration was measured with Fluo-3. Cells were stimulated in calcium-reduced bath solution with BbCI (100 μmol/L), and BAPTA (1 μmol/L) was added prior to stimulation. BbCI stimulation caused a significant increase in $[Ca^{+2}]_i$ over the 60-min time, while there was no significant rise in $[Ca^{+2}]_i$ as was observed when cells were pre-treated with BAPTA ($n=55$)

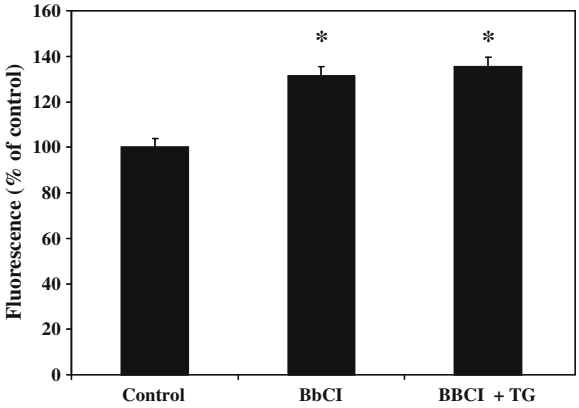

Fig. 3 BbCI (100 μmol/L) induced a significant increase of $[Ca^{+2}]_i$. Incubation of cells in the presence of thapsigargin (*TG*) 10 μM in the presence of BbCI shows similar rise in intracellular Ca^{2+}. Data are expressed as percentage of unstimulated control run in parallel 30 min after addition of BbCI (n=16; *p<0.05 vs. control)

BbCI-stimulated hyperpolarization of endothelial cells

The change of the endothelial membrane potential was analysed with the fluorescence probe $DiBAC_4(3)$. BbCI (100 μmol/L) induced a significant reduction of 9.5±0.9% of the $DiBAC_4(3)$ fluorescence intensity as compared to the parallel unstimulated control (Fig. 4; n=80; *p<0.05).

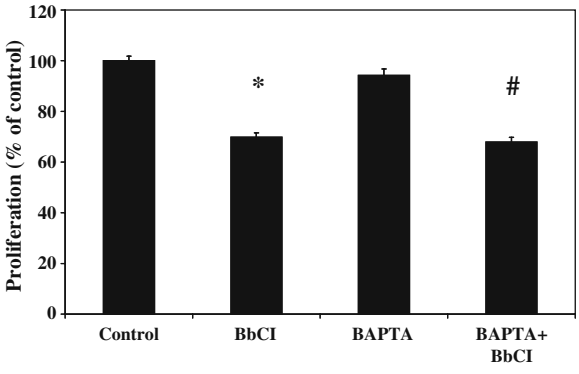

Fig. 4 Inhibition of cell growth of HUVEC by BbCI is independent of the BbCI-induced Ca^{2+} increase. Proliferation was examined by cell counting with a Neubauer chamber in the presence of BbCI (100 μmol/L), BAPTA (1 μmol/L) or BAPTA (1 μmol/L) and BbCI (100 μmol/L). Data are expressed as percentage of unstimulated control±SEM (n=12; *p<0.05 vs. control; #p<0.05 vs. control and BAPTA)

BbCI-induced inhibition of cell growth is independent from reduction in membrane potential

The relevance of the BbCI-induced membrane potential changes to the reduction of cell proliferation was investigated using valinomycin. There was no significant difference in proliferation in cells stimulated by valinomycin and BbCI to BbCI-stimulated cells, although there was significant reduction in cell proliferation as compared to unstimulated control (Fig. 5).

Discussion

The current study was aimed to explore the possible effect of the *B. bauhinioides* cruzipain inhibitor on the cell growth and proliferation of HUVEC. Furthermore, the antiproliferative effect involves rise in cytosolic Ca^{2+} concentration and changes in membrane potential in response to BbCI stimulation. BbCI is a potent inhibitor of cruzipain from *T. cruzi* and also of the highly homologue mammalian proteinase cathepsin L [6, 34]. Cruzipain is in many aspects similar to the human tissue kallikrein, e.g. in the proteolytic cleavage of kininogen to lys-bradykinin [10].

We found out that BbCI significantly inhibits the endothelial proliferation in a concentration-dependent manner with a maximum inhibition at 100 μmol/L. At this concentration, the cell proliferation was 35% lower as compared to the untreated control. Oliveira and co-workers determined the inhibition constant

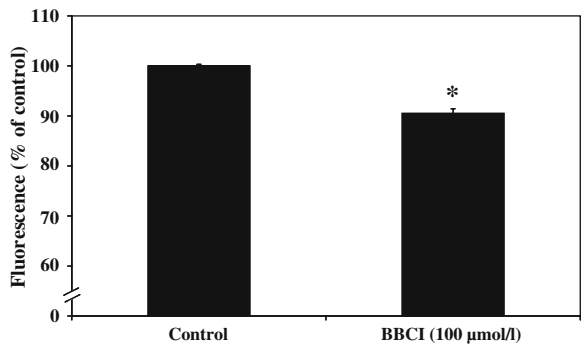

Fig. 5 BbCI caused a significant change of the endothelial cell membrane potential. Data are shown as percentage of unstimulated control (± SEM) 30 min after addition of BbCI (100 μmol/L). The membrane potential was analysed using the fluorescence probe $DiBAC_4(3)$ (n=80; *p<0.05 vs. control)

value (K_{iapp}) with 1.3 nmol/L for cruzipain and Araújo et al. measured a value of 2.2 nmol/L for cathepsin L [2, 34]. In this current cell culture study, the inhibitory constants were higher than previously presented [2, 34]. It might be possible that cell culture experiments as performed in this study requires higher amounts of inhibitors to compensate the loss by non-specific mechanisms. Neuhof et al. has shown that the pulmonary oedema in isolated rabbit lungs caused by neutrophil elastase was significantly decreased by 100 μmol/L BbCI [30]. Both proteins cathepsin L and the tissue kallikrein can regulate the cell growth. In vitro and in vivo experiments indicate that the tissue kallikrein-releasing product lys-bradykinin plays an important role in neovascularisation [14, 35, 40]. The tissue kallikrein-binding protein kallistatin, a member of the serine proteinase inhibitor (serpin) superfamily, inhibits the kininogenase activity of tissue kallikrein and was identified as an endogenous angiogenic inhibitor [45]. Kallistatin markedly inhibits angiogenesis by suppressing VEGF- and bFGF-induced proliferation, migration and adhesion of endothelial cells [28]. Aprotinin, a non-specific Kunitz-type proteinase inhibitor, is also known to inhibit tissue kallikrein [27]. Koutsioumpa and co-workers shows that aprotinin stimulates the angiogenesis of endothelial cells [22]. Cathepsin L, the second possible target of BbCI, is not only a kininogenase which release lys-bradykinin but it was also reported to be an anti-apoptotic protease [44]. Further studies are required to clarify the possible signalling mechanisms mediating antiproliferative effects of BbCI. One possible mechanism could be a BbCI-induced inhibition of tissue kallikrein-dependent angiogenesis. A further possibility might be a BbCI-induced apoptosis via inhibition of cathepsin L.

Calcium is a ubiquitous intracellular secondary messenger which plays important role in the control of different cellular processes like proliferation, migration and angiogenesis of endothelial cells [19, 21, 31]. Endothelial cells modulate the intracellular calcium concentration by a calcium release from intracellular stores or by an influx of extracellular Ca^{2+} across the cell membrane [1]. Our results show that BbCI stimulation causes an increase in $[Ca^{+2}]_i$. Additional measurements, under calcium-reduced conditions in the presence or absence of the calcium-chelator BAPTA, indicate a Ca^{2+} release from the endoplasmic reticulum. Thapsigargin addition to BbCI-stimulated cells also suggests release of calcium from intracellular store. In endothelial cells, increased cytoplasmic calcium levels stimulate the Ca^{2+}-activated K^+ channels (K_{Ca}) and lead to a hyperpolarization of the membrane [1, 31]. Our measurements also indicate that BbCI induced a significant hyperpolarization of the endothelial cell. But in this case, BbCI-induced membrane potential changes do not influence the cell proliferation. This was evident by treating the cells simultaneously with valinomycin and BbCI and BbCI treatment. There was no significant difference observed in both treatments. In previous observations, our group reported an anti-angiogenic pathway dependent from an increase of the intracellular calcium concentration [15, 23]. The presented data demonstrate that the antiproliferative effect of BbCI is not linked with the BbCI-triggered release of calcium and membrane potential changes (Fig. 6).

In summary, we have shown that BbCI modulate the electrophysiological properties of endothelial cells. BbCI induces hyperpolarization of the endothelial cell membrane and also increases $[Ca^{+2}]_i$ by releasing Ca^{+2} from intracellular stores. BbCI reduces the endothelial proliferation by a calcium-independent mechanism, and further studies might be helpful to elucidate the underlying antiproliferative and calcium signalling mechanisms of BbCI.

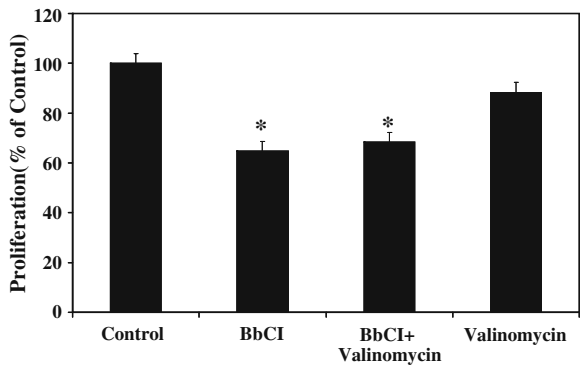

Fig. 6 Inhibition of cell growth of HUVEC by BbCI is independent of the BbCI-induced membrane potential changes. Proliferation was examined by cell counting with a Neubauer chamber in the presence of BbCI (100 μmol/L), valinomycin (1 μmol/L) or valinomycin (1 μmol/L) and BbCI (100 μmol/L). Data are expressed as percentage of unstimulated control ± SEM (n=6; *BbCI vs. control; *BbCI and valinomycin vs. control)

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