Initial bradykinin triggers calcium-induced calcium release in C6 glioma cells and its significance

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Abstract: Objective To investigate the underlying mechanism for the selective modulation of the permeability of blood-tumor barrier (BTB) by small dose of bradykinin (BK). **Methods** C6 glioma cells were treated with BK, and changes of intracellular nitric oxide (NO) and intracellular calcium level were measured with fluorescent spectrophotometer. **Results** The initial application of BK easily triggered extracellular calcium influx, which resulted in intracellular calcium store release in C6 glioma cells. The above mechanism was also named ryanodine mediated calcium induced calcium release (CICR). We also detected a long-lasting intracellular NO elevation in C6 glioma cells upon BK treatment. Further study showed that ryanodine mediated CICR contributed greatly to the secondary NO elevation induced by BK treatment. **Conclusion** These results suggested that BK triggered CICR in C6 glioma cells and the associated NO generation might be the underlying mechanism for the selective modulation of BTB permeability by BK.

Keywords: bradykinin; blood brain barrier; glioma

1 Introduction

The blood-brain barrier (BBB) protects brain by restricting the entry of circulating non-lipid molecules that are not recognized by specific transporters on the luminal side of the brain capillaries. Brain tumor capillaries have different structural and functional characteristics from the normal brain capillaries, and constitute the blood-tumor barrier (BTB), which, despite being more 'leaky' than the BBB, also limits delivery of therapeutic agents to the tumor^[1]. It was found that a small dose of bradykinin (BK) could selectively open BTB therefore enhance the delivery of large particles which were normally impeded by BTB^[1,2]. The selective modulation

of BTB provides a promising tool for efficient chemotherapy.

Calcium-induced calcium release (CICR) is a mechanism by which calcium (Ca^{2+}) influx can stimulate Ca^{2+} release from internal stores^[3,4]. Astrocytes, like many other cell types, express Ca^{2+} -sensitive ryanodine receptors (RyRs) in their smooth endoplasmic reticulum (ER) membrane^[5,6]. Binding of Ca^{2+} to RyRs can activate Ca^{2+} -permeable channels allowing Ca^{2+} to exit from ER and enter the cytosol. Ryanodine is a CICR inhibitor that can block Ca^{2+} -sensitive RyRs and limit the passage of Ca^{2+} from smooth ER into the cytoplasm. Previously, it is demonstrated that small doses of BK could selectively activate intracellular calcium release in C6 glioma cells and BK could easily elicit peak-after plateau, shoulder formation and sometimes a second Ca^{2+} peak in C6 glioma cells when compared to astrocytes^[7]. In this study we found that the above observation resulted from a delayed calcium transient following calcium peak and that RyRs mediated calcium store release contributed to this delayed calcium

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transient, which contributed to long-lasting intracellular nitric oxide (NO) elevation in C6 glioma cells after BK treatment.

2 Materials and Methods

2.1 Cell culture and drug treatment Rat C6 glioma cells (obtained from the Cell Biology Department of China Medical University), primary astrocytes, and brain microvascular endothelial cells (BMECs) were maintained in complete culture media, containing DMEM, 20% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL heparin, and 10 µg/mL endothelial cell growth supplement (BD Biosciences).

Fura-3/AM and 4, 5-diaminofluorescein diacetate (DAF-2/DA) were purchased from Sigma and dissolved in DMSO. BK was purchased from Sigma and dissolved in PBS. Cells were treated with 1×10^{-6} mol/L BK. Ryanodine was purchased from Calbiochem.

2.2 Measurement of intracellular Ca^{2+} and NO before and after BK was applied The intracellular Ca^{2+} was labeled by Fluo-3. NO was monitored by labeling with DAF-2/DA that was de-esterified intracellularly to DAF-2. NO provided the third nitrogen to form a triazo ring from the two amino groups of the nonfluorescent DAF-2 and converted it to diaminotriazolo fluorescein (DAF-2T) that could be excited at 490 nm excitation and monitored at 530 nm emission^[8,9]. Cells were incubated with 10 µmol/L DAF-2/DA or 5 µmol/L Fluo-3/AM at 37 °C for 30 min. The living cell real-time fluorescence change before and after BK treatment was measured by fluorescent spectrophotometer (F-2000, HITACHI, Japan). Central part analysis stands for both intracellular store calcium release and extracellular calcium influx, while peripheral analysis stands for mainly extracellular calcium influx. Both Fluo-3 and DAF-2 were excited at 488 nm laser and emissions between 515-560 nm were obtained. Images of 512×512 pixels were acquired with a 40× objective.

The artificial cerebrospinal fluid (ACSF, pH 7.4, in mmol/L: NaCl 126, KCl 3.75, CaCl_2 2, MgCl_2 1, NaHCO_3 26, KH_2PO_4 1.25, glucose 10) was employed as the extracellular balance solution. The cells were designed into 3 groups: (1) control group (normal ACSF); (2) Cd^{2+} group (Ca^{2+} was removed from ACSF and 0.5 mmol/L EGTA was added); (3) ryanodine group.

2.3 Statistical analysis The differences among groups were determined using unpaired Student *t*-test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Extracellular calcium contributed to peak formation after BK treatment

We monitored intracellular calcium under conditions without extracellular calcium. It showed that removing calcium from extracellular solution resulted in no calcium peaks in response to 1×10^{-6} mol/L BK application [(3±2)% increase in fluorescence at 180 s after BK treatment, $n = 17$ cells; Fig. 1A]. This result suggested that influx of calcium from the outside was essential to calcium peaks in C6 glioma cells after BK treatment, consistent with signaling through ligand-gated ion channels. Moreover, we noticed that there was a much slower and low attitude calcium elevation after BK treatment without extracellular calcium. It maintained more than 90 s, especially in the central part of the cell (Fig. 1A). Because extracellular calcium influx was excluded, this slow central part calcium elevation should be released from intracellular calcium stores, which was verified by the central part analysis and cell peripheral analysis. The central part analysis showed that there was a significant fluorescence increase around the central part of the cell, which increased more than 20% of the baseline [(25±5) % in 17 cells] at 180 s after BK treatment (Fig. 1Bb); while peripheral analysis showed that there was no significant fluorescence elevation [(3±5) % in 17 cells] around peripheral part of the cell (Fig. 1Bc). To test whether calcium store release could contribute to the long-lasting calcium elevation for the first application of BK (Fig. 2C), we took peripheral and central part analysis of repeated BK stimulation in a single glioma cell. The results showed that other than the first application, the peak time of central part was closely correlated with the corresponding peripheral part and that there was a central part calcium elevation peak with a 5-10 s delay to the peripheral part peak (Fig. 2A, B).

3.2 BK triggered CICR via ryanodine-sensitive receptors

1×10^{-6} mol/L BK could only trigger calcium peak in C6 glioma cell incubated with 100 µmol/L ryanodine for 30 min before BK was applied (Fig. 3B), which formed in 30 s. Compared to

Fig. 1 A: Without extracellular calcium, 1×10^{-6} mol/L BK could only trigger a delay and low attitude intracellular calcium elevation, lacking peak formation in the whole C6 glioma cell analysis. B: A regional fluorescence analysis in a C6 glioma cell showed under no extracellular calcium, in contrast to peripheral part (c), the fluorescence enhancement was concentrated around the central part of the C6 glioma cell (b), which mainly stood for intracellular calcium store release. Each line represents one cell. Figure a, b and c are the regions of interest of one cell, respectively. Vertical line in x-axis indicates the time point when BK was applied.

Fig. 2 A: Contrast to repeated treatment of 1×10^{-6} mol/L BK, initial BK could trigger the long-lasting calcium elevation such as peak-after plateau, second peak or shoulder formation in C6 glioma cells. B: The peripheral (line a) part and central part (line b) analysis of a single C6 glioma cell under treatment of repeated 1×10^{-6} mol/L BK every 4 min. It showed that beside the first application, the peripheral fluorescence was correlated with the central part fluorescence very well in the last four applications. In the first application, there was a 10 s delay peak formation in the central part fluorescence. In Fig. A, each line represents one cell. Line a and b are the regional analyses of one cell shown in Fig. A. Vertical line in x-axis indicates the time when BK was applied.

a wide range of calcium elevation (Fig. 3A), the time of calcium elevated more than 50% of the base line was less than 50 s. In all of the 40 cells with only calcium peak upon BK treatment, BK triggered peak-after plateau and shoulder formation disappeared. The observed calcium influx peak, which occurred ahead of the intracellular calcium store release peak (Fig. 2), suggested that the BK treatment initially caused an influx of extracellular calcium, possibly mediated by receptor operated calcium channel, which was followed by a calcium release from ryanodine-sensitive calcium stores induced by

the calcium influx. This experiment showed that an application of 1×10^{-6} mol/L BK resulted in a ryanodine mediated CICR in C6 glioma cells.

3.3 CICR contributed to BK related NO release Different from the rapid elevation of intracellular calcium in C6 glioma cells upon BK application, there was a slower and long lasting elevation of intracellular NO. The increase in NO maintained more than 20 min, the ratio of NO at 20 min to NO at 0 min ranged from 1.3-1.7, average 1.5 ± 0.2 in 35 out of 40 cells (Fig. 4A). The generation of NO was significantly reduced

Fig. 3 A: 1×10^{-6} mol/L BK triggered a long lasting accumulation of intracellular calcium in C6 glioma cells; most of the cells underwent peak-after plateau or shoulder formation. B: 100 μ mol/L ryanodine incubated 30 min before 1×10^{-6} mol/L BK treatment abolished peak-after plateau and shoulder formation in C6 glioma cells, with only a calcium peak formation just like repeated BK stimulation. Each line represents one cell. Vertical line in x-axis indicates the time point when BK was applied.

Fig. 4 A: In 35 out of 40 C6 glioma cells, there was a long-lasting intracellular NO release after 1×10^{-6} mol/L BK was applied. B: 100 μ mol/L ryanodine incubated 30 min before 1×10^{-6} mol/L BK treatment abolished intracellular NO elevation. Each line represents fluorescence change of one cell after BK treatment. Vertical line in x-axis indicates the time point when BK was applied.

by $(70 \pm 5)\%$ ($P < 0.05$, $n = 40$ cells) during the 30 min pretreatment with $100 \mu\text{mol/L}$ ryanodine, which could disrupt CICR just as we previous described (Fig. 4B). This observation indicated that CICR contributed greatly to the secondary NO generation after BK treatment in C6 glioma cells.

4 Discussion

In the present study we have two principle findings. First of all, C6 glioma cells, similar to astrocytes^[6,10], have functional ryanodine-sensitive receptors. An extracellular calcium influx upon BK treatment can trigger ryanodine-sensitive receptor mediated calcium store release, which is also named CICR. BK triggered CICR is largely tumor cell specific. Secondly, BK can trigger long-lasting intracellular NO release in C6 glioma cells and astrocytes, which mainly results from the intracellular CICR.

Bradykinin, when used at high doses, induces breakdown of normal BBB, but intracarotid infusion of low doses of BK can selectively increase the permeability of capillaries in brain tumors without any effect on the permeability of normal brain capillaries^[1,2]. In our previous *in vivo* animal study, we showed that B_2 receptors were expressed mainly in the tumor cells, but not in normal or tumor capillary endothelial cells^[1,11]. Based on the intracellular calcium change after BK treatment, we found that low doses of BK selectively increased intracellular calcium in C6 glioma cells. Importantly, the initial BK triggered a long-lasting calcium elevation in C6 glioma cell compared to astrocytes. However, BMECs could not express B_2 receptor and was not the direct target of BK^[7]. Lots of *in vivo* researches showed that BK could enhance the transport vesicles of capillary endothelial cells, which facilitated transferring of chemicals to tumor cells^[1,2]. Regarding that BMECs are not the direct target of BK, we speculated that there might be certain intercellular messengers participating in the BK modulation of the permeability of BMECs. In this study, we observed a long-lasting NO release in C6 glioma cells upon application of $1 \times 10^{-6} \text{ mol/L}$ BK (Fig. 4A), but $1 \times 10^{-5} \text{ mol/L}$ BK, which could trigger intracellular calcium elevation in astrocytes^[7], could not trigger NO release. Similarly, repeated $1 \times 10^{-6} \text{ mol/L}$ BK stimulation in C6 glioma cells also could not enhance further elevation of NO release

(data not presented). However, such doses of BK could effectively trigger intracellular calcium elevation as shown in our previous study^[5]. These results showed that intracellular calcium elevation could not always trigger secondary NO release.

We also noted that initial $1 \times 10^{-6} \text{ mol/L}$ BK application could elicit a wider calcium peak in C6 glioma cells with features such as peak-after plateau, shoulder formation and second peaks, whereas repeated $1 \times 10^{-6} \text{ mol/L}$ BK in C6 glioma cells (Fig. 2C) and $1 \times 10^{-5} \text{ mol/L}$ BK in astrocytes could not trigger such calcium response^[7]. Therefore we proposed that this wider calcium peak might contribute to the secondary NO release after initial BK treatment. Further study showed that calcium influx accounted for peak formation with BK application, and calcium store release contributed greatly to this wider calcium peak. This was indicated by the fact that ryanodine, which could block ryanodine-sensitive calcium store release, could completely abolish BK triggered peak-after plateau, shoulder formation and second peaks in C6 glioma cells. These data revealed that intracellular calcium store release accounted for this long time intracellular calcium elevation of initial BK treatment and contributed to the peak-after plateau, shoulder formation and second peak after initial application of BK application. Since extracellular calcium influx is the prerequisite for the intracellular calcium peak formation, it seems that BK causes extracellular calcium influx, which triggers ryanodine-sensitive mediated calcium store release. This type of intracellular calcium elevation is called CICR^[6,7], a mechanism for calcium release from internal stores. C6 glioma cells and astrocytes, like many other cell types, express Ca^{2+} -sensitive RyRs in their smooth ER membrane. Binding of Ca^{2+} to RyRs activates Ca^{2+} permeable channels, allowing Ca^{2+} to exit from the ER and enter the cytosol. Ryanodine, when incubated for 30 min, could disrupt CICR triggered by $1 \times 10^{-6} \text{ mol/L}$ BK in C6 glioma cells (Fig. 3B), which also could effectively lower the secondary NO release triggered by $1 \times 10^{-6} \text{ mol/L}$ BK (Fig. 4). This suggested that CICR was a prerequisite for the secondary long-lasting NO release.

Based on *in vitro* intracellular calcium change study after BK treatment^[7], we found that CICR was BK concentra-

tion dependent. With the elevation of BK concentration, CICR in C6 glioma cells and astrocytes increased rapidly. However, CICR is very rare in astrocytes, and very common in C6 glioma cells. Because CICR was a prerequisite for NO release and NO contributed a lot to BK modulation of BTB permeability^[12], as shown in this study, BK selectively triggered CICR in C6 glioma cells might be the underlying basis for BK selective modulation of BTB permeability.

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缓激肽触发 C6 胶质瘤细胞内 Ca^{2+} 内流诱发的 Ca^{2+} 释放

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摘要: **目的** 探讨小剂量缓激肽选择性开放血肿瘤屏障的内在机制。**方法** 通过免疫荧光探针实时测定缓激肽刺激前后C6胶质瘤细胞内的钙离子和一氧化氮(nitric oxide, NO)的变化。**结果** C6胶质瘤细胞上存在尼诺丁敏感受体,小剂量缓激肽可以引起肿瘤细胞外的钙离子内流,导致细胞内钙离子水平升高,特别是首次刺激后,细胞外钙离子内流可以选择性的引起C6胶质瘤细胞内的钙库释放,即 Ca^{2+} 内流诱发的 Ca^{2+} 释放(calcium-induced calcium release, CICR)。同时,缓激肽刺激后,在C6胶质瘤细胞内存在NO的持续升高,且NO是继发于细胞内尼诺丁受体介导的CICR之后。**结论** 小剂量缓激肽选择性触发C6胶质瘤细胞内钙库释放,可能是小剂量缓激肽可以选择性开放血肿瘤屏障的原因。

关键词: 缓激肽; 血脑屏障; 胶质瘤