βγ-CAT, a non-lens betagamma-crystallin and trefoil factor complex, induces calcium-dependent platelet apoptosis

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Summary
In recent years, it has been reported that apoptosis may occur in platelets and play a role in the clearance of effete platelets. βγ-CAT, a newly identified non-lens βγ-crystallin and trefoil factor complex from frog Bombina maxima skin secretions, caused several in vivo toxic effects on mammals. Through determined haematological parameters of rabbits, it has been found that βγ-CAT significantly reduced the number of platelets in a time-dependent manner. Here, in order to explore the effect of βγ-CAT on platelets, washed platelets were incubated with various concentrations of βγ-CAT for 30 minutes. We found that βγ-CAT induced several apoptosis events in human platelets, including caspase-3 activation, phosphatidylserine (PS) exposure, depolarisation of mitochondrial inner transmembrane potential (ΔΨm), cytochrome c release and strong expression of pro-apoptotic Bax and Bak proteins. However, βγ-CAT did not significantly induce platelet activation as detected by P-selectin surface expression, GPIIb/IIIa activation and platelet aggregation. In addition, we observed that βγ-CAT-induced PS exposure and ΔΨm depolarisation in platelets are Ca2+-dependent. Taken together, βγ-CAT can induce Ca2+-dependent platelet apoptosis but does not cause platelet activation.

Keywords
Platelet apoptosis, non-lens βγ-crystallins, trefoil factor, βγ-CAT, Ca2+

Introduction
Platelets, whose average life span is normally just 5–9 days, are derived from fragmentation of megakaryocytes. They play a fundamental role in blood clotting and thrombosis (1). Apoptosis, or programmed cell death, is a physiological and controlled process for elimination of unwanted or useless cells (2). Over the last decade, apoptosis of anucleated cytoplasts (3) and platelets (4–14) has been recognised. Platelet apoptosis was described in a number of in vitro and in vivo models. For example, apoptosis was induced in platelets stored at 37°C (12) or when platelets were treated with agonists, including physiologic agonists like thrombin (7), and non-physiologic agonists like A23187 (15) or ionomycin (16). However, it is unknown whether other proteins except physiologic agonists can also cause platelet apoptosis. Platelet apoptosis has been documented in different models using well-defined apoptosis markers of nucleated cells, including depolarisation of mitochondrial membrane potential (ΔΨm), Bcl-2 family protein expression, cytochrome c (Cyt. c) release, caspase-3 activation and phosphatidylserine (PS) exposure (4, 10, 12). Platelet apoptosis plays an important role in clearing effete platelets from the circulation, thus to explore the mechanisms of platelet apoptosis has useful implications for thrombosis and haemostasis (17, 18).

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βγ-CAT induces platelet apoptosis

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The βγ-CAT was purified from the skin secretions of B. maxima as previously described (27). Active caspase-3 polyclonal antibody was purchased from Abcam (Cambridge, UK). The anti-human P-selectin (CD62P)-FITC mAb, anti-PAC-1-FITC (specific for the αc, anti-Bcl-XL, anti-Bax, anti-Bak and anti-Bcl-2 mAb were purchased from BD Pharmingen (San Diego, CA, USA). The ABT-737, PARP, TUNEL, apoptosis, CD95, annexin-V-FITC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-human P-selectin (CD62P)-FITC mAb and HRP-conjugated goat anti-mouse IgG were purchased from Pierce, Rockford, IL, USA). The mitochondria isolation kit was purchased from Sigma (St Louis, MO, USA). The protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA, USA).

Materials and methods

Materials

βγ-CAT was purified from the skin secretions of B. maxima as previously described (27). Active caspase-3 polyclonal antibody was purchased from Abcam (Cambridge, UK). The anti-human P-selectin (CD62P)-FITC mAb, anti-PAC-1-FITC (specific for the activated conformation of human platelet GP Ib/IIIa), anti-Cyt. c, anti-Bcl-XL, anti-Bax, anti-Bak and anti-Bcl-2 mAb were purchased from BD Pharmingen (San Diego, CA, USA). The ABT-737, anti-β-actin mAb and HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-cytochrome c oxidase subunit 4 (anti-COX4) mAb and annexin-V-FITC were purchased from Molecular Probes (Eugene, OR, USA). The mitochondria isolation kit was purchased from Pierce (Rockford, IL, USA). Human thrombin, fluo-3-AM, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocya- nine iodide (JC-1), N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), rotenone and oligomycin were purchased from Sigma (St Louis, MO, USA). The protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA, USA).

Preparation of washed platelets and lysate

Platelet-rich plasma (PRP), from the Kunming Blood Center, was centrifuged with 500 g for 5 minutes (min) at room temperature. The isolated platelets were washed twice with CGS buffer (123 mM NaCl, 33 mM D-Glucose, 13 mM trisodium citrate, pH 6.5), and resuspended in Tyrode's buffer (10 mM Heps, 137 mM NaCl, 2.8 mM KCl, 12 mM NaHCO3, 0.4 mM NaH2PO4, 5.5 mM D-glucose, pH 7.4) at the concentration of 5 × 109/ml, and supplemented with 1 μM CaCl2. In the experiments performed in Ca2+-free medium, platelets were resuspended in EGTA buffer which contains the same salts as Tyrode's buffer but without CaCl2. In caspase activity assays and Western blot experiments, platelets were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton-100, 10 mg/ml leupeptin, 10 mg/ml apro tinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM Na3VO4). The platelet lysate was then cleared by 12,000 rpm centrifugation and the protein concentration was determined by the bicinchoninic acid (BCA) protein assay.

Caspase-3 activity assay

Caspase-3 activity was measured using the tetrapeptide p-nitroanilide (pNA) substrates in a colorimetric assay (13). Platelets were treated with βγ-CAT (2–50 nM, 30 min) or ABT-737 (1–25 μM, 180 min) at 22°C, and then lysed in lysis buffer. Platelet lysates containing 30 μg protein were incubated with 50 μM Ac-DEVD-pNA. The total reaction mixture volume was 100 μl in assay buffer (50 mM Heps, pH 7.5, 10 mM DTT, 1 mM EDTA, 1% sucrose, 0.1% Chaps). Reactions were incubated at 37°C in a 96-half-well assay plate (Corning, Cambridge, MA, USA) and measured at a wavelength of 405 nm every 30 min to a 4-hour (h) final time point. The mean rate of substrate cleavage (pmol pNA/min/μg total protein) was calibrated with standard pNA.

SDS-PAGE and protein immunoblotting

The method of Western blotting was performed as described previously (27). Platelets (5 × 109/ml) were treated with βγ-CAT (5–25 nM) at 22°C for 30 min, and then lysed in lysis buffer. Proteins were separated by SDS-PAGE and electrophoretically transferred onto a PVDF membrane. Membranes were blocked with 3% BSA in TBS and then probed with primary antibodies of anti-β-actin (1:1,000), anti-Bak (1:1,000), anti-Bcl-2 (1:1,000), anti-Bcl-Xl (1:1,000), anti-COX4 (1:200) or anti-Cyt. c (1:200) overnight. The washed blots were incubated with a HRP-conjugated secondary antibody (diluted 1:3,000 in TBST) for 1 h, followed by detection using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA).

Determination of phosphatidylserine externalisation

PS exposure was measured by staining the platelets with annexin- V-FITC following the instructions given by the manufacturer. Briefly, platelets (1 x 109/ml) were treated with βγ-CAT (2–50 nM,
30 min) or ABT-737 (1–25 μM, 90 min) at 22°C, then incubated for 5 min with annexin-V-FITC and analysed immediately by flow cytometry (FACSVantage SE, BD Biosciences, San Jose, CA, USA). For the experiments performed in the absence of extracellular Ca2+, before PS exposure were the platelets were washed in Tyrode’s buffer containing 1 mM CaCl2, then incubated for 5 min with annexin-V-FITC in the appropriate binding buffer, therefore EGTA was not present to interfere in the annexin binding assay. Data were collected and analysed from a minimum of 20,000 events per sample.

**Determination of ΔΨm depolarisation**

Mitochondrial membrane potential (ΔΨm) was determined with JC-1 according to a method described previously (5). Briefly, washed platelets (1 x 10⁶/ml) were incubated with various concentrations of βγ-CAT (2–50 nM) at 22°C for 30 min, and JC-1 was added into the platelet suspension in a 2 μg/ml final concentration. Then samples were further incubated in the dark at 37°C for 20 min, and analysed by flow cytometry. Platelets were gated on the forward scatter (FSC)-side scatter (SSC) dot plots. In non-apoptotic cells, JC-1 exists as a monomer in the cytosol (green, FL1) and also accumulates as aggregates in the mitochondria (red, FL2). Whereas, in apoptotic cells, JC-1 exists in monomeric form and stains the cytosol green. Data were collected and analysed from a minimum of 20,000 events per sample.

**Determination of Cyt. c release from mitochondria**

Platelets (1 x 10⁶/ml) were treated with βγ-CAT (5–25 nM) at 22°C for 30 min, and then the mitochondrial fraction was isolated using a mitochondria isolation kit according to the manufacture’s specifications. Cyt. c release was determined by immunoblotting as described earlier.

**Determination of platelet aggregation**

Platelets (5 x 10⁸/ml) were treated with βγ-CAT (2–50 nM) or thrombin (0.1 U/ml), and aggregometry was monitored by light transmission in an aggregometer (Plis, Beijing) for 15 min at 37°C under stirring.

**Determination of P-selectin expression and PAC-1 binding**

Platelets (1 x 10⁹/ml) were treated with βγ-CAT (2–50 nM) at 22°C for 30 min, then incubated for 10 min with anti-P-selectin-FITC (1 μg/ml) or anti-PAC-1-FITC (2 μg/ml) as described previously (8). Samples were analysed by flow cytometry. Data were collected and analysed from a minimum of 20,000 events per sample.

**Determination of intracellular free Ca²⁺ concentration ([Ca²⁺])**

Optical fluorimetric recordings with fluo-3-AM were used to evaluate the [Ca²⁺]i. Washed platelets were incubated with 2 μM fluo-3-AM in Tyrode’s buffer at 22°C for 30 min, then centrifuged for 5 min at 500 g and resuspended in Ca²⁺-free or Ca²⁺-containing Tyrode’s buffer. For mitochondrial inhibition, Fluo-3-loaded platelets were pretreated with rotenone (10 μM) plus oligomycin (10 μM) in Ca²⁺-free Tyrode’s buffer. Fluo-3-Ca²⁺ complex fluorescence was measured with a Perkin-Elmer LS50B Luminescence spectrometer. Fluo-3 was excited by argon laser light at 488 nm and fluorescence was measured at wavelengths of 525 nm.

**Statistical analysis**

Data were analysed with Student’s t-test for variance. Experimental values expressed as means ± SEM. The level of statistical significance was set at the level of p < 0.05.

**Results**

**βγ-CAT induces caspase-3 activation in platelets**

Caspase-3, which is an effector caspase, plays a central role in the execution of apoptosis (31). In order to determine whether βγ-CAT had activation of the cell death pathways, caspase-3 activation was measured by Western blot and cleavage of the substrate Ac-DEVD-pNA. As shown in Figure 1A, Western blot analysis of platelet lysates revealed that the cleaved fragment of caspase-3 (active caspase-3, 17 kDa) was observed 30 min after the βγ-CAT treatment. Besides, βγ-CAT-induced caspase-3 activation in a dose-dependent manner, which achieved maximal efficacy at 50 nM (Figure 1B). To evaluate the effect of βγ-CAT on inducing platelet apoptosis events, we compared the magnitude of βγ-CAT effects with that of ABT-737. ABT-737, a potent antagonist of Bcl-2, Bcl-XL, and Bcl-w, induces apoptosis events in platelets that are distinct from platelet activation (10, 32). Table 1 demonstrates that βγ-CAT at 50 nM triggered caspase-3 activation, which can be achieved in 25 mM ABT-737-induced platelets.

**βγ-CAT induces PS exposure in platelets**

We also examined the effects of βγ-CAT on PS exposure using various concentrations. PS exposure is conveniently measured with annexin V, a Ca²⁺- and phospholipid-binding protein that preferentially binds PS. Figure 2 shows that 50 nM βγ-CAT markedly enhanced PS exposure in platelets (63.75 ± 3.57%), similar in extent to that induced by 25 μM ABT-737 (77.22 ± 4.43%) (Table 1). In the same incubation conditions, βγ-CAT-caused PS exposure was also dose-dependent, with 5 nM βγ-CAT-induced being significantly lower than 50 nM βγ-CAT induced.
βγ-CAT induces mitochondria-associated apoptotic events in platelets

Up to now, numerous studies show that mitochondria play an important role in platelet apoptosis (33). Thus, we investigated the effects of βγ-CAT on platelet ΔΨm by the lipophilic cation JC-1. Figure 3A and B show that βγ-CAT concentration-dependently induced ΔΨm depolarisation in platelets, which achieved their maximal efficacy at 50 nM.

To further characterise the mitochondria-associated platelet apoptosis induced by βγ-CAT, we examined the expression levels of Bax, Bak, Bcl-2 and Bcl-XL, all of which regulate cell apoptosis by controlling the mitochondrial membrane permeability (33). Western blot analysis demonstrated that βγ-CAT significantly upregulated the pro-apoptotic (Bax, Bak) proteins in human platelets; however, no obvious increase in Bcl-2 and Bcl-XL expression was observed (∆Fig. 3C).

Cyt. c is located in the mitochondrial intermembrane space in the resting state. The Western blot study showed that βγ-CAT triggered Cyt. c release from mitochondria to the cytosol (∆Fig. 3D).

Table 1: Comparison of βγ-CAT effects on caspase-3 activation and phosphatidylserine exposure in human platelets with the effect of ABT-737 stimulation.

<table>
<thead>
<tr>
<th>Apoptosis stimuli</th>
<th>Caspase-3 activation (pmol pNA/min/μg total protein)</th>
<th>Phosphatidylserine exposure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>0.035 ± 0.009p</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>βγ-CAT (2 nM)</td>
<td>0.049 ± 0.022p</td>
<td>2.00 ± 0.41</td>
</tr>
<tr>
<td>βγ-CAT (10 nM)</td>
<td>0.199 ± 0.033 NS</td>
<td>37.94 ± 5.88*</td>
</tr>
<tr>
<td>βγ-CAT (50 nM)</td>
<td>0.280 ± 0.041</td>
<td>63.75 ± 3.57</td>
</tr>
<tr>
<td>ABT-737 (1 μM)</td>
<td>0.095 ± 0.008*</td>
<td>8.12 ± 1.61†</td>
</tr>
<tr>
<td>ABT-737 (5 μM)</td>
<td>0.138 ± 0.012*</td>
<td>41.28 ± 3.13*</td>
</tr>
<tr>
<td>ABT-737 (25 μM)</td>
<td>0.236 ± 0.017GS</td>
<td>77.22 ± 4.43GS</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SEM (n = 4). P-values vs. 50 nM βγ-CAT-treated group are presented. NS, not significant, *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the solvent control.
These results suggest that $\beta\gamma$-CAT-induced platelet apoptosis is related to the mitochondrial apoptotic pathway.

$\beta\gamma$-CAT does not induce platelet activation

To test whether the $\beta\gamma$-CAT can induce platelet activation, we investigated changes of platelet activation markers and aggregation response to $\beta\gamma$-CAT. Thrombin (0.1 U/ml) was used as the positive control of platelet activation. P-selectin is a cell adhesion molecule on the surfaces of activated platelets and endothelial cells (34). Anti-human P-selectin-FITC mAb was used to detect P-selectin expression. Figure 4A shows that the $\beta\gamma$-CAT (2–50 nM) treatment did not significantly induce P-selectin expression. Another distinctive feature of platelet activation is PAC-1 binding. Monoclonal antibody PAC-1 binds only to activated platelets and appears to be specific for this recognition site within GPIIb/IIIa (35), and incubation of platelets with $\beta\gamma$-CAT (2–50 nM) resulted in similar PAC-1 binding level as negative control (Fig. 4B). In addition, Figure 4C shows that incubation of platelets with $\beta\gamma$-CAT (2–50 nM) did not induce platelet aggregation as determined by aggregometry. Taken together, these data indicate that platelet activation did not occur during the process of platelet apoptosis induced by $\beta\gamma$-CAT.

$\beta\gamma$-CAT induces [$Ca^{2+}$], increase

$\beta\gamma$-CAT was applied to human platelets in the absence or presence of external $Ca^{2+}$ in order to study intracellular $Ca^{2+}$ release and extracellular $Ca^{2+}$ influx. As shown in Figure 5A, a significant rise of [$Ca^{2+}$] was seen in the presence of 1 mM external $Ca^{2+}$. Moreover, in the absence of external $Ca^{2+}$, $\beta\gamma$-CAT induced a small increase in [$Ca^{2+}$]. Different inhibitors were utilised to investigate the source of the $Ca^{2+}$ released. The combination of rotenone plus oligomycin, which were used to deplete the mitochondrial pool (36), inhibited $\beta\gamma$-CAT-induced [$Ca^{2+}$] increase in the absence of external $Ca^{2+}$. However, when addition of 1 mM external $Ca^{2+}$, the

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**Figure 3:** $\beta\gamma$-CAT induces depolarisation of $\Delta\Psi_m$, release of cytochrome c and up-regulation of Bak and Bax proteins in platelets.

A) Washed platelets were treated in a medium containing 1 mM CaCl$_2$ with various concentrations of $\beta\gamma$-CAT (2–50 nM) at 22°C for 30 min, followed by the addition of JC-1 for flow cytometric analysis. B) Means ± SEM of the percentage of normal $\Delta\Psi_m$ from four independent experiments are shown. *$P <0.05$, **$P <0.01$ and ***$P <0.001$ compared with the solvent control. C and D) Washed platelets were treated in a medium containing 1 mM CaCl$_2$ with various concentrations of $\beta\gamma$-CAT (5–25 nM) at 22°C for 30 min; (C) the treated platelets were lysed and the expression of Bak, Bax, Bcl-2 and Bcl-XL was determined by Western blotting. (D) The cytosol and mitochondrial fractions were isolated and analysed by Western blotting with antibodies specific for cytochrome c. Cytochrome c oxidase subunit 4 (COX4) and actin were used as the respective internal controls. The figure is representative of three to four experiments.
**Figure 5: Ca²⁺ evaluations in βγ-CAT-treated human platelets.**

A) Washed platelets were loaded with fluo-3, then treated with βγ-CAT (10 nM) at 22°C for 30 min, followed by the addition of calcium (1 mM CaCl₂) or absence of calcium (1 mM EGTA was added) in the reaction medium. B) Fluorescence intensity and calcium activity were determined using a flow cytometric analysis. Data are presented as the means ± SEM (n = 4).

***P < 0.001 compared with the solvent control.
combination of rotenone plus oligomycin did not significantly inhibit the \([\text{Ca}^{2+}]\), increase (▶ Fig. 5B), which indicated that the extracellular \(\text{Ca}^{2+}\) influx is independent of instore-\(\text{Ca}^{2+}\) release.

**\(\beta\gamma\)-CAT inductions of PS exposure and \(\Delta\Psi_m\) depolarisation in platelets are \(\text{Ca}^{2+}\)-dependent**

To investigate the role of \(\text{Ca}^{2+}\) during \(\beta\gamma\)-CAT-induced platelet apoptosis we suppressed the influx of \(\text{Ca}^{2+}\) during stimulation by using a \(\text{Ca}^{2+}\) chelator (EGTA). In the absence of extracellular \(\text{Ca}^{2+}\), PS exposure and \(\Delta\Psi_m\) depolarisation, both induced by \(\beta\gamma\)-CAT (10 nM), were inhibited (▶ Fig. 6). These results indicate that \(\text{Ca}^{2+}\) influx is the upstream of PS exposure and \(\Delta\Psi_m\) depolarisation. Therefore, \(\text{Ca}^{2+}\) influx was required for \(\beta\gamma\)-CAT-induced platelet apoptosis.

**Discussion**

Over the past several decades, numerous studies have focused on the bioactive components existing in amphibian skin secretions (37). A lot of proteins and peptides with diverse biological activities have been identified and purified, facilitating the understanding of the physiological and envenomation mechanisms of amphibian skin (38). In our previous studies, \(\beta\gamma\)-CAT, which was purified from the skin secretions of Chinese red belly frog (\(B.\ maxima\)), was identified and characterised to be the first example of naturally existing complex of a non-lens \(\beta\gamma\)-crystallin and a trefoil factor (27).

So far, our previous studies showed that at least two forms of TFFs exist in \(B.\ maxima\) skin secretions. One is a two-domain TFF (Bm-TFF2) (25). The other is a three-domain TFF (BmC-TFF) that acts as \(\beta\)-subunit of \(\beta\gamma\)-CAT, the sequence of its domains 2–3 exhibits 69% identities with that of Bm-TFF2 (27). Interestingly, the activity of these two proteins is very different. Bm-TFF2 can not only stimulate epithelial cell migration and wound healing, as well as suppression of apoptosis, but also trigger human platelet aggregation (25, 39). BmC-TFF (the \(\beta\)-subunit) combination with a non-lens \(\beta\gamma\)-crystallin member (the \(\alpha\)-subunit) can form a naturally existing complex (\(\beta\gamma\)-CAT). \(\beta\gamma\)-CAT has been proved to stimulate cell migration and cell apoptosis in primary cultured human umbilical vein endothelial cells (HUVECs) depending on the dosages used (27). \(\beta\gamma\)-CAT was also able to stimulate cell migration and inhibit cell proliferation in melanoma A375 cells (40). Furthermore, \(\beta\gamma\)-CAT possessed lethal toxicity to mammals and reduced the number of platelets in the blood (30). In this study, we further reported that \(\beta\gamma\)-CAT markedly induces apoptotic events.
in human platelets, including ΔΨm depolarisation, Bcl-2 family protein expression, Cyt. c release, caspase-3 activation and PS exposure.

Platelet apoptosis was first reported in 1997 by Vanags et al. (16) and in the recent years it has been described in several experiments (4–16). Although platelet apoptosis and platelet activation share some common physiological features, including PS exposure, caspase activation and microparticle release (41–45), they are not equivalent and may be induced by different mechanisms or require different levels of triggering stimuli (44). Leytin et al. showed that low thrombin concentrations induced platelet activation, whereas high thrombin concentrations triggered platelet apoptosis (45). Lin et al. reported the resveratrol, which is a polyphenolic natural product, could induce platelet apoptosis without activation of platelets (5). Similar results also were observed in calmodulin agonists treated platelets (46). In this study, P-selectin expression, GPIIb/IIa activation and platelet aggregation did not occur during the process of platelet apoptosis stimulated by βγ-CAT (Fig. 4), which further suggested that platelet activation and apoptosis were different phenomena.

Caspases, which are cysteinyl proteases that can cleave after aspartic acid residues, are key effectors of apoptosis (31). In extrinsic apoptosis pathways, procaspase-8 is recruited by its Death effector domains (DEDs) to the death inducing signalling complex and then activates each other by autopropeolysis. The downstream effector caspasas such as caspases-1, -3, -6 and -7 were cleaved and activated by activated caspase-8 (31). Intrinsic apoptosis pathways involve the release of Cyt. c from mitochondria which induces the formation of the apoptosome and the activation of procaspase-9. Activated caspase-9 further mediates activation of effector caspasas (31). Platelets express the major caspasas implicated in the apoptosis of nucleated cells (13). Diverse stimuli can induce caspase-3 activation, such as ABT-737 (10), thrombin (7) and platelet storage (13); some stimuli can also induce caspase-8 activation, such as N,N,N4,N6-tetrakis(2-pyridylmethyl)ethylen-diamine (TPEN) (4) and resveratrol (8). In this study, we demonstrated that βγ-CAT induced activation of caspase-3 (Fig. 1). Furthermore, we detected caspase-8 activation by Western blot analysis (8) and cleavage of the specific colorimetric substrates Ac-IETD-pNA (47), but the results showed that caspase-8 was not activated in βγ-CAT-induced platelet apoptosis (data not shown), suggesting that extrinsic pathway was not involved in this process.

In nucleated cells, the Bcl-2 family of proteins is the key regulator of mitochondria-dependent apoptosis (33). Although human platelets lack a nucleus, they are able to express biologically relevant gene products in a timely and signal-dependent manner (48). Previous studies found that platelets expressed Bcl-2 family proteins at mRNA level, and the Bcl-2/Bax protein ratio was altered when platelets were stimulated with ionomycin (16) and thrombin (7), and platelets stored at 37°C (12). Other studies have demonstrated that Bid, Bax and Bak are activated by thrombin (6). In the present study, Western blot analysis showed that βγ-CAT enhanced the expression levels of Bax and Bak (Fig. 3C), but whether Bax and Bak are activated or not remains to be further studied.

Excessive accumulation of intracellular Ca2+ can induce apoptosis in the cell (49). We provide evidence that βγ-CAT caused significant [Ca2+]i increase in the presence of external Ca2+ (Fig. 5). We also observed a small increase in [Ca2+]i in a Ca2+-free medium, further inhibitor experiments implied that mitochondria is the source of Ca2+ released. However, in the presence of external Ca2+, even when the mitochondria Ca2+ release was inhibited by rotenone plus oligomycin, βγ-CAT could still significantly induce [Ca2+]i elevation. The results demonstrated that extracellular Ca2+ influx rather than intracellular Ca2+ release performs a major function in the βγ-CAT-induced [Ca2+]i elevation. Besides, Ca2+ influx is required for the βγ-CAT-induced PS exposure and ΔΨm depolarisation in human platelets (Fig. 6). Therefore, Ca2+ influx may play an important role in βγ-CAT-induced platelet apoptosis. There are two possible Ca2+ influx pathways, one is activation of the Ca2+-channels that already exist in platelet membrane, and the other is βγ-CAT forming the pores by itself. Previous study suggested that βγ-CAT tends to be oligomerised and may form transmembrane pores in human erythrocyte membrane (27). In addition, we found that bepridil, an effective Ca2+ channel blocking agent, did not significantly inhibit Ca2+ influx in human platelets (Gao et al., unpublished data). These findings indicate that βγ-CAT may create pores in the membrane of human platelets, but whether these pores are ion selective or not still needs to be confirmed.

In conclusion, we first demonstrate that βγ-CAT stimulates platelet apoptosis, including ΔΨm depolarisation, Bcl-2 family protein expression, Cyt. c release, caspase-3 activation and PS exposure. These apoptotic events in human platelets are dependent on Ca2+ influx. Due to its strong physiological activities, βγ-CAT should be a useful tool for the study of platelet apoptosis via its acting targets and molecular mechanisms. Furthermore, this new finding provides new clues to physiopathological roles of non-lens βγ-crystallins and trefoil factors in vertebrates.

Acknowledgements

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