# $Ca^{2+}$-dependent inhibition of branched-chain α-ketoacid dehydrogenase kinase by thiamine pyrophosphate

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## Abstract

Catabolism of the branched-chain amino acids (BCAAs: leucine, isoleucine, and valine) is regulated by the branched-chain α-ketoacid dehydrogenase (BCKDH) complex, which in turn is regulated by phosphorylation catalyzed by BCKDH kinase (BDK). Thiamine pyrophosphate (TPP) is required as a coenzyme for the E1 component of the BCKDH complex and can also bring about activation of the complex by inhibiting BDK. The present study shows that free $Ca^{2+}$ in the physiological range greatly increases the sensitivity of BDK to inhibition by TPP ($IC_{50}$ of 2.5 μM in the presence of 1 μM free $Ca^{2+}$). This novel mechanism may be responsible for the stimulation of BCAA oxidation by conditions that increase mitochondrial free $Ca^{2+}$ levels, e.g. in skeletal muscle during exercise.

**Key words:** branched-chain amino acids, branched-chain α-ketoacid dehydrogenase complex, branched-chain α-ketoacid dehydrogenase kinase, thiamine pyrophosphate, calcium ion

Leucine, isoleucine, and valine are categorized as the branched-chain amino acids (BCAAs) which are indispensable for life in mammals. Taken together, they are very abundant in the body, comprising about 35% of the indispensable amino acids in muscle proteins and about 40% of the required preformed amino acids by mammals [1]. BCAAs also exist free in the amino acid pools in animal tissues and blood. Human plasma and muscle contain 400-650 μM BCAAs [2,3], which are low amounts relative to the muscle protein content. Free BCAAs play important roles in protein metabolism, especially leucine, a positive nutritional signal for protein synthesis [4]. Preservation of free BCAA levels by regulation of the rate of their catabolism is required for synthesis and maintaining lean body mass.

The main catabolic pathways of BCAAs are located in mitochondria. The first step, common to the three BCAAs, is transamination to form branched-chain α-ketoacids (BCKAs), catalyzed by branched-chain aminotransferase (BCAT). The second step, also common to the BCAAs, is oxidative decarboxylation of the BCKAs to produce the corresponding CoA esters, catalyzed by the BCKA dehydrogenase (BCKDH) complex [5]. Since the latter catalyzes the committed step, regulation of activity of the BCKDH complex plays an important role in maintaining appropriate concentrations of the free BCAAs [5].

The BCKDH complex is subject to covalent modification. BCKDH kinase (BDK) phosphorylates and inactivates the E1 component of the complex [6]. BCKDH phosphatase catalyzes the opposite effect on the complex [7]. The activity state of the BCKDH complex is thought to be controlled primarily by regulation of the activity of the kinase rather than the phosphatase [5,8].

Several inhibitors of the BDK have been reported [9]. α-Ketoisocaproate (KIC), derived from leucine by transamination, is considered the most important physiological inhibitor [10,11]. However, BDK is also sensitive to inhibition by thiamine pyrophosphate (TPP), which is also required as the coenzyme of the E1 component of the complex [1]. Although there is no doubt about the role of TPP as a coenzyme for the complex, the physiological significance, if any, of

TPP inhibition of BDK is uncertain. Nevertheless, an $IC_{50}$ for TPP of 8.0 μM has been reported, indicating a strong inhibitory effect that could be physiologically important [12]. The TPP-binding site of the E1 component is associated with the active site of the enzyme, which is also the region phosphorylated by BDK [13]. Dietary thiamine deficit and excess influences the mitochondrial TPP levels and also the activity state of BCKDH complex in rat livers [14], suggesting that the level of TPP may impact BDK activity and therefore the activity state of the BCKDH complex. However, this has not been studied in detail and the mechanism responsible for the inhibition has not been defined.

It is well known that exercise increases BCAA oxidation by activation of the BCKDH complex [5]. Although KIC is a strong inhibitor of BDK and clearly is involved in the regulation of the activity state of the complex in response to dietary protein intake [15], no changes in KIC concentration are induced in muscle by exercise [16]. Since mitochondrial $Ca^{2+}$ concentration is increased by exercise and since $Ca^{2+}$ activates pyruvate dehydrogenase (PDH) complex, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase [17], $Ca^{2+}$ may signal the activation of the BCKDH complex. However, prior to the current study, there has been no evidence presented for such a mechanism.

In the present study we found that the inhibition by TPP of the BDK in the BCKDH-BDK complex was greatly enhanced by the physiological range of free $Ca^{2+}$, suggesting a novel $Ca^{2+}$-dependent mechanism for the regulation of BCAA catabolism.

## Materials and Methods

### Materials

Polyclonal antibody against BCKDH complex was prepared as described previously [5]. Polyclonal antibody against phosphorylation site 1 (Ser293) on the E1α subunit of BCKDH was prepared by Sigma-Aldrich Japan (Tokyo, Japan) according to the method described previously [18]. Monoclonal antibodies against BDK and E2 subunit of the BCKDH complex were

generated using recombinant BDK and the isolated E2 from purified rat liver BCKDH complex, respectively, by a standard protocol [19]. TPP chloride (cocarboxylase) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and polyethylene glycol 6,000 (PEG) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Animals**

All procedures for animal experiments were approved by the Animal Care Committee of Graduate School of Bioagricultural Sciences, Nagoya University. Male Sprague-Dawley rats (400-500 g) were housed two per cage. The animal room was maintained at 22 ±1°C with a 12-h light-dark cycle (lights on from 0800 to 2000). Rats were fed a commercial chow diet CE2 (CLEA Japan, Tokyo) and tap water *ad libitum*. On the final day of the experiment, rats fasted overnight were killed by exsanguination under isoflurane anesthesia. Livers were immediately removed and rinsed with cold saline.

**Preparation of mitochondria and extraction of BCKDH-BDK complex from the mitochondria**

All procedures were performed at 0-4°C, unless otherwise stated. Mitochondria were prepared from rat livers by the method reported previously [20] with a slight modification that the mitochondrial pellets obtained by first 7,000*g* centrifugation were washed once. The final mitochondrial pellets were suspended in the isolation medium (70 mM sucrose, 220 mM D-mannitol, 2 mM HEPES (pH 7.4 with KOH), and 0.5 mg/ml bovine serum albumin) at 44 mg protein/ml.

The mitochondrial preparation (~60 ml) was diluted with 6 volumes of an extraction buffer (50 mM HEPES (pH 7.35 with KOH), 3% (w/v) TritonX-100, 2 mM EDTA, 5 mM dithiothreitol (DTT), 0.01 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 0.1 mg/ml trypsin inhibitor, and 20 μg/ml leupeptin), incubated for 20 min, and centrifuged at 10,000*g* for 20 min. The supernatant obtained was mixed with half volume of 27% PEG to give a final concentration

of 9%, incubated for 20 min, and centrifuged at 10,000*g* for 20 min. The precipitate obtained was suspended with ~70 ml of a suspending buffer (25 mM HEPES (pH 7.35 with KOH), 0.1% (w/v) TritonX-100, 50 mM KCl, 3 mM DTT, and 20 μg/ml leupeptin) and was applied to high-speed centrifugation at 230,000*g* for 2 h to precipitate BCKDH-BDK complex. The pellet obtained was suspended at 8.7 mg/ml with the suspending buffer and was stored at –80°C until use. This preparation was used as "partially purified BCKDH-BDK complex", which had a specific activity of BCKDH at 45 mU/mg protein and a BDK activity at 0.65 per min (first order rate constants for BCKDH inactivation).

**Enzyme assay**

BCKDH activity was assayed spectrophotometrically at 30°C as described previously [21]. One unit of BCKDH activity refers to 1 μmol NADH formed/min. BDK activity of the partially purified BCKDH-BDK complex was assayed by measuring the ATP-dependent inactivation of the BCKDH complex [21]. Kinase activity is calculated as the first-order rate constant of BCKDH complex inactivation with time.

**Treatment of the partially purified BCKDH-BDK complex with various doses of free $Ca^{2+}$ and TPP**

In the treatment of the partially purified BCKDH-BDK complex with $Ca^{2+}$ and TPP, free concentrations of $Ca^{2+}$ were controlled by the use of EGTA buffers [17] calculated as described in http://maxchelator.stanford.edu/index.html. The preparation of the partially purified BCKDH-BDK complex (150 μl containing ~60 mU BCKDH) was made to 300 μl with 100 μl of the suspending buffer, 30 μl of a pH-adjusted $CaCl_2$-EGTA solution (final concentrations of 0-25 μM free $Ca^{2+}$ and 5 mM EGTA; pH adjusted to 7.35 with NaOH), and 20 μl of a TPP solution (final concentration of 0-50 μM), and the mixture was incubated at 37°C for 20 min. The BCKDH-BDK complex was precipitated by 9% PEG precipitation as described above, and the pellet obtained was suspended in 100 μl of the BDK assay cocktail (final concentrations of

20 mM HEPES (pH 7.35 with NaOH), 1.5 mM $MgCl_2$, 50 mM KF, 50 mM KCl, 2 mM DTT, and 0.5 mM ATP) for measurement of the BDK activity. All of the enzyme assays were conducted 3 times and the results were expressed as mean ± SD.

**Immunoprecipitation of BCKDH in the partially purified BCKDH-BDK complex**

Immunoprecipitation of BCKDH in the partially purified BCKDH-BDK complex was performed as previously described [22]. The preparation obtained was used in the Western blot analyses for determination of BDK bound to the BCKDH [22].

**Western blot analysis**

SDS-polyacrylamide gel electrophoresis and Western blot analysis were carried out as previously described [8,21]. The specific antibody against the target protein and ECL Western blotting detection reagents (GE Healthcare UK, Buckinghamshire, UK) were used for visualizing the band on the membrane.

**Protein assay**

Protein of the mitochondrial preparation was determined using a biuret reagent, and that of the partially purified BCKDH-BDK complex was using a Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) using bovine serum albumin as a standard.

## Results

**Inhibition of the BDK activity of the partially purified BCKDH-BDK complex by various doses of TPP in the absence or presence of 1 μM free $Ca^{2+}$**

Prior to assay the partially purified BCKDH-BDK complex was treated with EGTA to remove contaminating $Ca^{2+}$. The effect of TPP on BDK activity was determined in the presence of an EGTA buffer [17] to control the free concentration of $Ca^{2+}$.

BDK activity of the complex was titrated with 0-50 μM TPP in the absence of $Ca^{2+}$ and the presence of calculated 1 μM free $Ca^{2+}$ (Fig. 1). The presence of this level of free $Ca^{2+}$ greatly enhanced the inhibition of BDK by TPP ($IC_{50}$ 2.5 μM). In the absence of $Ca^{2+}$, BDK activity was only decreased about 10% by 50 μM TPP.

The increased sensitivity of BDK to TPP by free $Ca^{2+}$ was also examined by the Western blotting analysis, in which phosphorylated Ser293 of the E1α subunit of BCKDH was determined after the BDK reaction with ATP. The results showed that the combination of 5 μM TPP and 1 μM free $Ca^{2+}$ causes great inhibition of ATP-dependent phosphorylation of the E1α subunit (Fig. 2).

**Inhibition of the BDK activity of the partially purified BCKDH-BDK complex by 0-25 μM free $Ca^{2+}$ with and without addition of 5 μM TPP**

The BDK activity of the complex under the conditions with addition of 5 μM TPP was significantly decreased to 66% only in the presence of 0.1 μM free $Ca^{2+}$ and reached to 33% in the presence of 1 μM free $Ca^{2+}$ (Fig. 3). This decreased BDK activity was not so much changed even in the presence of 25 μM free $Ca^{2+}$. On the other hand, the effect of free $Ca^{2+}$ on the BDK activity was minor under the condition without addition of TPP; ~20% decrease in the BDK activity in the presence of 25 μM $Ca^{2+}$ (Fig. 3). From these results, we confirmed that the BDK activity was almost not affected by free $Ca^{2+}$ alone, but was greatly inhibited by TPP in the presence of physiological concentration of free $Ca^{2+}$.

**Effect of 5 μM TPP supplementation on the amount of the bound form of BDK in the partially purified BCKDH-BDK complex in the presence of 1 μM free $Ca^{2+}$**

We have reported that the amount of the BDK bound to BCKDH is associated with the activity of BDK, suggesting that the bound form, but not the free form, of the BDK may be active [22]. It has been reported that TPP is a potent inhibitor of BDK but has no effect on the

amount of the bound form of BDK [23]. However, free $Ca^{2+}$ was not considered in the previous study. In the present study, we examined the effect of incubation with 5 μM TPP in the presence of 1 μM free $Ca^{2+}$ on the amount of bound BDK in the partially purified BCKDH-BDK complex. Under these conditions, the amount of BDK that remained bound to the complex was remarkably decreased (Fig. 4). This decrease in the bound form of BDK was similar to that caused by incubation of the BCKDH-BDK complex with 0.2 mM α-chloroisocaproate (ClIC, pan BDK inhibitor), as observed previously [23].

## DISCUSSION

TPP is an inhibitor of the BDK with a reported $IC_{50}$ of 8.0 μM when measured with the purified BCKDH-BDK complex under the physiological concentration of $K^+$ at 100 mM [12]. This value was determined without consideration of $Ca^{2+}$. In the present study, we examined the inhibitory effect of TPP against the BDK activity of the BCKDH-BDK complex under the conditions of controlled free $Ca^{2+}$ concentrations with an EGTA buffer [17,24] and found that the $IC_{50}$ of TPP was 2.5 μM in the presence of 1 μM free $Ca^{2+}$, although the inhibition by 50 μM TPP was negligible in the absence of free $Ca^{2+}$, suggesting that the inhibition of BDK by TPP is $Ca^{2+}$-dependent under the physiological conditions. Since phosphorylation of the E1α subunit at Ser293 is responsible for the inactivation of the BCKDH complex [13], we determined the phosphorylation of Ser293 of the subunit after BDK reaction with ATP. The results showed that the phosphorylation of Ser293 was greatly suppressed by the combination of 5 μM TPP and 1 μM $Ca^{2+}$. The $IC_{50}$ of free $Ca^{2+}$ for inhibition of the BDK in the presence of 5 μM TPP was as low as 0.36 μM, which is within the range of the physiological free $Ca^{2+}$ concentration in muscle cells, reported to be 0.25 μM under resting conditions and 1-2 μM during tetanic stimulation [25]. Although it has been reported that TPP inhibits BDK activity without dissociation of the bound BDK from the BCKDH complex when examined without consideration of $Ca^{2+}$, we show here that TPP decreased the bound form of BDK in the

BCKDH-BDK complex after incubation with 5 μM TPP supplementation and 1 μM free $Ca^{2+}$, similar to that of the pan BDK inhibitor ClIC [23].

Three other key intramitochondrial dehydrogenases (PDH, $NAD^{+}$-isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase) are also activated by free $Ca^{2+}$ within the concentration range of 0.1-10 μM [17,26]. These enzymes play critically important roles in mitochondrial energy metabolism. The results obtained in the present study indicate that the BCKDH complex can be added to this list of key intramitochondrial dehydrogenases that are activated by $Ca^{2+}$. We propose that the $Ca^{2+}$-dependent regulation of the BDK by TPP is a novel mechanism that is responsible under some conditions for the regulation of the BCAA catabolism.

The BCKDH complex is structurally and functionally similar to the PDH complex [27]. However, the activities of these complexes in especially muscle tissues are inversely regulated in the fed and fasted states. In the fed state, the PDH complex is active to produce acetyl-CoA from pyruvate generated by glycolysis [27], and BCKDH complex is inactive to conserve BCAAs for protein synthesis. This relationship is reversed in the fasted state. Inactivation of the PDH complex conserves pyruvate, lactate, and alanine for gluconeogenesis [28] while activation of the BCKDH complex generates substrates for gluconeogenesis and ketogenesis from BCAAs [29,30]. On the other hand, when the energy expenditure is extremely enhanced by exercise, both complexes are simultaneously activated in skeletal muscles [31,32]. During muscle contractions, $Ca^{2+}$ released from sarcoplasmic reticulum is incorporated into mitochondria [25,26], which may activate the BCKDH complex through inhibition of the BDK by TPP and the PDH complex through $Ca^{2+}$-dependent activation of PDH phosphatase 1 [33], enabling the harmonized activation of both complexes to meet the high energy demand.

BCAAs, especially leucine, activate protein synthesis in skeletal muscles. $Ca^{2+}$-dependent inactivation of BDK with subsequent activation of BCKDH complex, may induce acceleration of the BCAA catabolism, presumably resulting in inhibition of protein synthesis and promotion of proteolysis. Elevated mitochondrial $Ca^{2+}$ concentration in dystrophic muscle triggers fiber apoptosis necrosis in skeletal muscle [34]. $Ca^{2+}$-dependent upregulation of the BCAA

catabolism may contribute to dystrophic muscle atrophy.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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## Figure Legends

**Figure 1. Dose-dependent inhibition of the BDK activity of partially purified BCKDH-BDK complex by TPP in the absence or presence of 1 μM $Ca^{2+}$**

BDK activity of the partially purified BCKDH-BDK complex was measured with addition of 0-50 μM TPP in the absence of $Ca^{2+}$ and the presence of 1 μM free $Ca^{2+}$. BCKDH activity was measured 0.25, 1, 2, and 3 min after addition of ATP. BDK activity was calculated as the first-order rate constant for the inactivation of BCKDH. The BDK activity for 100% was 0.83/min for the condition without free $Ca^{2+}$; 0.71/min for the condition with 1 μM free $Ca^{2+}$. The experiments were conducted 3 times and the data are expressed as mean ± SD.

**Figure 2. Effect of free $Ca^{2+}$ on the inhibition of Ser293 phosphorylation of BCKDH E1α by TPP.**

The ATP-dependent BDK reaction of the partially purified BCKDH-BDK complex was measured for 2 min to examine the effects of addition of 5 μM TPP and 1 μM free $Ca^{2+}$. Lane 1, the control sample prepared without addition of ATP, $Ca^{2+}$ or TPP; lane 2, the sample with ATP and without addition of TPP or $Ca^{2+}$; and lane 3, the sample with addition of ATP, TPP and $Ca^{2+}$. The phosphorylation site 1 (P-Ser293) of the E1α subunit of the BCKDH and total protein of the BCKDH complex were analyzed by Western blotting. The same amount of the partially purified BCKDH-BDK complex (60 μg protein) was applied on each lane of Western blotting. Immunoblotting was performed with the polyclonal antibody against the phosphorylation site 1 (P-Ser293), and the blots were stripped and the membrane was re-probed with polyclonal antiserum against the BCKDH complex.

**Figure 3. Inhibition of the BDK activity of partially purified BCDKH-BDK complex in the presence of 0-25 μM free $Ca^{2+}$ with or without addition of 1 μM TPP**

BDK activity of the partially purified BCKDH-BDK complex was measured in the presence of 0-25 μM free $Ca^{2+}$ with (●) or without (○) addition of 5 μM TPP. BCKDH activity was measured 0.25, 1, 2, and 3 min after addition of ATP, and BDK activity was calculated as the first-order rate constant for the inactivation of BCKDH. The BDK activity for 100% was 0.83/min for the condition without addition of TPP; 0.81/min for the condition with addition of 5 μM TPP. The experiments were conducted 3 times and the data are expressed as mean ± SD.

**Figure 4. The amount of BDK bound to BCKDH in the partially purified BCKDH-BDK complex after treatment of 5 μM TPP supplementation in the presence of 1 μM free $Ca^{2+}$**

The partially purified BCKDH-BDK complex was incubated with 5 μM TPP supplementation in the presence of 1 μM free $Ca^{2+}$ and then immunoprecipitated with the antibody against BCKDH complex. The amount of bound BDK in the precipitate was visualized by Western blotting with a monoclonal antibody against BDK. The blots were stripped and the membrane was re-probed with monoclonal antibody against the E2 of BCKDH. As a positive control, the partially purified BCKDH-BDK complex was treated with 200 μM α-chloroisocaproate (ClIC) in the absence of EGTA, as reported previously [23].

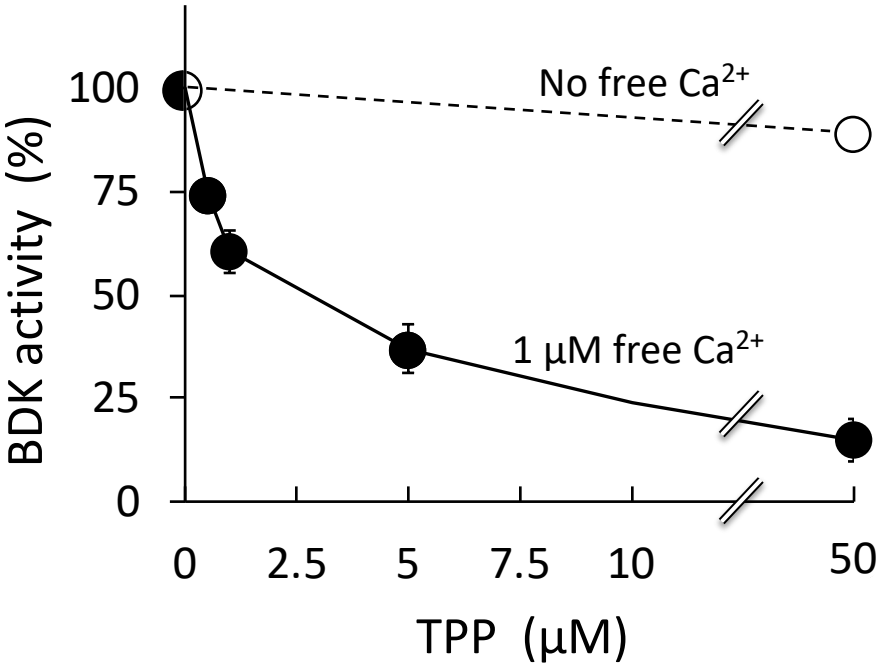
100
75
50
25
0
BDK activity (%)
No free Ca2+
1 µM free Ca2+
0
2.5
5
7.5
10
50
TPP (µM)

Figure 1

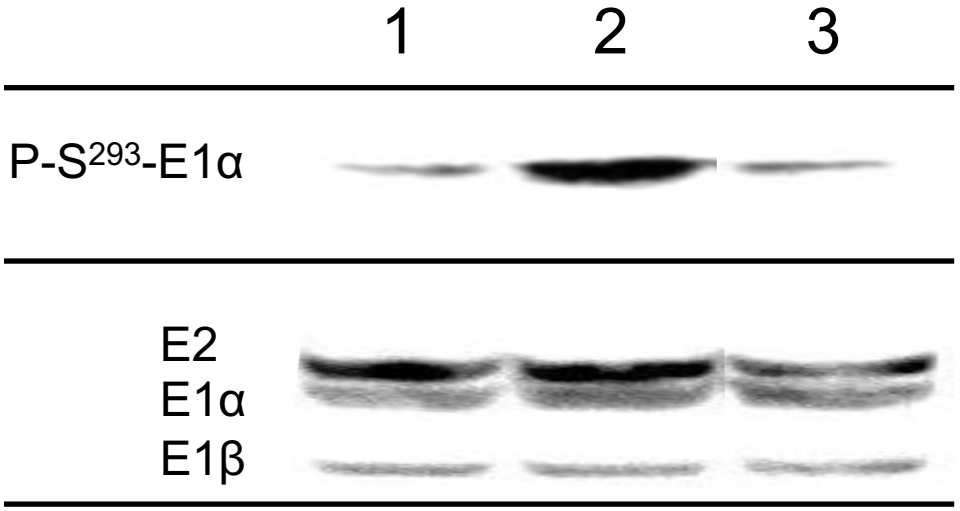


Figure 2

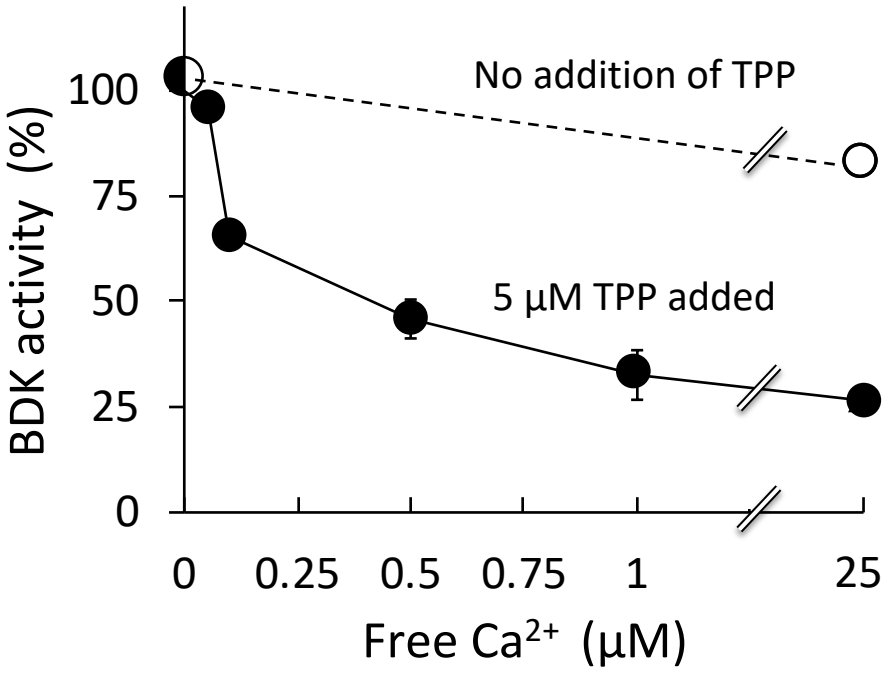
No addition of TPP
5 μM TPP added
BDK activity (%)
100
75
50
25
0
0
0.25
0.5
0.75
1
25
Free Ca2+ (μM)

Figure 3

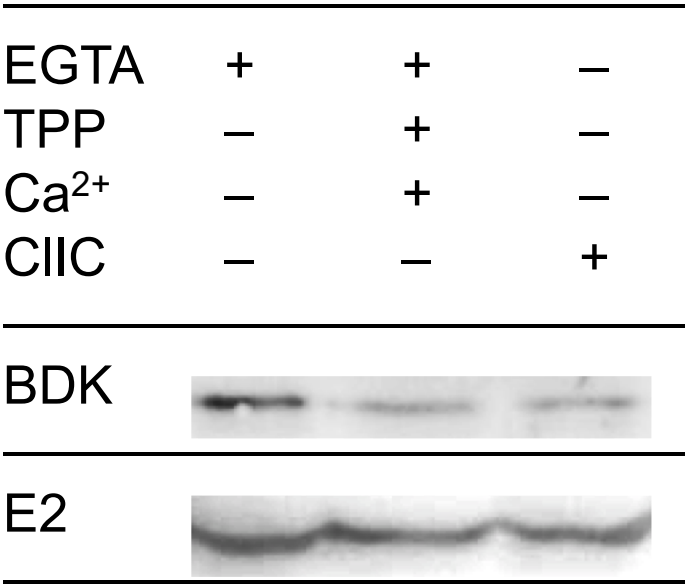


Figure 4