Characterization of Calcium-dependent Phosphatase Activity of Calcineurin in Cultured Cortical Neurons

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Abstract

Calcineurin (CaN) is a serine/threonine phosphatase enzyme that is expressed in many types of tissues such as immune cells, muscle cells and neurons. It plays important roles in regulating immunological responses in lymphocytes cells. In neurons, CaN is involved in synaptic plasticity associated with learning and memory formation. It is known that the phosphatase activity of CaN required small increase in intracellular Ca^{2+} concentration due to its high affinity to Ca^{2+} ions. Various types of CaN inhibitors are widely used to indirectly determine CaN activity *in vivo* and in intact individual neurons. In *in vitro* experiments, phosphatase activity of CaN from cell lysate can be directly measured by using CaN substrates. Most of these *in vitro* assays were conducted in buffer solutions containing Ca^{2+} at the physiological level of extracellular fluid (mM). Due to high concentration of Ca^{2+}, this condition might not be the optimal assay condition for measuring CaN activity. Thus, the objective of this study was to investigate Ca^{2+}-dependent pattern of CaN activity in cell lysate of cultured cortical neurons. Phosphatase activity of CaN was determined by using RII phosphopeptide substrate, a specific substrate for CaN. The results from this study showed that CaN was highest activated in the presence of low concentrations of free Ca^{2+} (80-100 nM) in the reaction. In contrast, high level of Ca^{2+} appeared to reduce CaN activity *in vitro*. This study also showed non-specific dephosphorylation of RII phosphopeptide by other phosphatases which was probably due to high concentration of substrate used in the assay. Therefore, titrating free Ca^{2+} and substrate concentrations seems to be an important process for CaN activity measurement in test tubes. In addition, this study suggests that *in vitro* CaN activity requires low amount of Ca^{2+} as well as previous *in vivo* studies.

Keyword: calcineurin, phosphopeptide, calcium

Introduction

Calcineurin (CaN) is a serine/threonine phosphatase enzyme that is abundantly expressed in various brain regions and other types of tissues including immune and muscle cells. It plays essential roles in cell proliferation, differentiation, and death (Klee et al., 1998). Calcineurin is a heterodimer consisting of a 60 kDa catalytic A subunit (CaN A) and a 19 kDa regulatory B subunit (CaN B). The catalytic activity of CaN is regulated by Ca^{2+} binding to CaN B and to calmodulin (CaM). Calcium-CaM complex displaces an autoinhibitory domain in the A subunit, allowing access of substrates to the catalytic domain (Rusnak and Mertz, 2000).

In the immune system, CaN has received much attention since it was inhibited by the immunosuppressive drugs cyclosporine A (CsA) and FK506. The regulatory pathway involving CaN in immune cells is well established that CaN controls gene expression through nuclear factor of activated T cells (NFAT) activation (Rao et al., 1997). Inhibition of CaN blocks the transcriptional activity of NFAT, which leads to immunosuppression in T cells. In T cells, CaN/NFAT signaling is responsible for inducing transcription of genes such as interleukin 2, 3, 4 (IL-2, 3, 4), interferon-γ (IFN-γ), tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor (GM-CSF) during the immune response (Feske et al., 2000; Luo et al., 1996).

In the central nervous system (CNS), CaN influences neuronal plasticity by regulating variety of proteins, including ion channels, neurotransmitter receptors, enzymes, and transcription factors (Groth et al., 2003). It has shown that neuronal Ca^{2+} triggers specific responses based on the characteristics of the Ca^{2+} signals such as amplitude and duration of Ca^{2+} rises. A brief but large Ca^{2+} increase induces the activation of protein kinases, whereas a more prolonged but modest Ca^{2+} increase enhances protein phosphatase activity (Cho et al., 2001; Hansel et al., 1996; Yang et al., 1999). The phosphorylation status in neurons is, therefore, strictly dependent on the changes in intracellular Ca^{2+} concentrations.

The phosphatase activity of CaN can be indirectly determined by using CaN inhibitors CsA and/or FK506 in both *in vivo* and *in vitro* experiments. Direct measurement of CaN activity can be performed in test tubes by incubating cell lysate with CaN substrates. One of the specific substrates for calcineurin is phosphorylated regulatory subunit (RII) of cAMP-dependent protein kinase (PKA) (Vega et al., 2002). This phosphopeptide was used in the present study to measure CaN activity in cortical cell lysate. Previously, such *in vitro* assay was mostly performed in the assay solution contained fixed Ca^{2+} concentration, which is in the range of the extracellular Ca^{2+} concentration (1-1.3 mM). Such a high concentration of Ca^{2+} might not be the optimal condition for testing CaN activity. Thus, the present study was conducted to determine *in vitro* phosphatase activity of CaN of cultured cortical neurons at various Ca^{2+} concentrations to test the hypothesis that the activity of CaN in neuronal cell lysate required low concentration of Ca^{2+} in the reaction.

Materials and Methods

Preparation of primary cortical cell cultures

Primary cortical cell cultures were prepared from 18-day old Sprague Dawley rat fetuses. The cerebral cortex was dissected under a stereomicroscope. Tissue was cut into small pieces and suspended in 25 ml of 0.25% trypsin for 15 minutes and then was mechanically dissociated with a fire-polished pasteur pipette. Neuronal cells were collected by centrifugation 1,000 *g* for 5 min and resuspended in defined medium F12/DMEM supplemented with 10% FBS. Cells were resuspended in defined medium and plated in 96 well plate at $5x10^4$ cells/well. At 20-24 h after plating, the medium was replaced with serum-free F12/DMEM. Plates were coated one night before cell preparation with 0.001% poly-D-lysine and washed with sterile water. All experiments were performed with cells 6-8 days in culture and repeated several times in independent cultures.

Phosphatase activity assay

Free phosphates (Pi) released from the phosphopeptide were measured by the following procedure. After removing cultured medium, cortical cells were washed with 0.9% NaCl. Cells were lysed in 50 µl lysis buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 50 µg/ml tripsin inhibitor, 50 µg/ml PMSF, 10 µg/ml aprotinin, 1 mM sodium orthovanadate and 10 mM β-glycerophosphate. Various Ca^{2+} concentrations were added and concentrations of free Ca^{2+} were controlled by EGTA chelation, determined by the computer program Max Chelator. Dephosphorylation reaction was performed in assay buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM DTT, 100 µg/ml BSA) in the absence and presence of 1 µM CsA and 1 µM FK506 used as CaN inhibitors. Phosphopeptide was added at last at 50 µg/ml final concentration. After 1 h at 37 ° C, malachite green solution and 19.5% H_2SO_4 were added, respectively. The absorbance at 650 nm was measured after 45 min incubation. Malachite green solution was freshly prepared from solution A (4 ml of 4.2 N HCl + 3 ml of 0.42 M Na_2MoO_4) and solution B (7 ml of 0.09% malachite green in 1.1 % Polyvinyl alcohol). Protein concentration of cell lysate was determined by using protein assay kit (Pierce®).

Results

The aim of the first experiment was to test the sensitivity and reproducibility of the malachite green assay. Various concentrations of standard phosphate (Pi) were prepared and reactions were conducted as described in method section. Standard curve of Pi was graphed and the slope was calculated using linear regression (Fig. 1). The result showed linear correlation between free Pi concentrations (< 10 µM) and the absorbance at 650 nm. This assay appeared to be quite a sensitive assay that can detect free Pi in the micro-molar range. Slope of this standard curve was used to calculate concentrations of free Pi from tissue samples in later experiments.

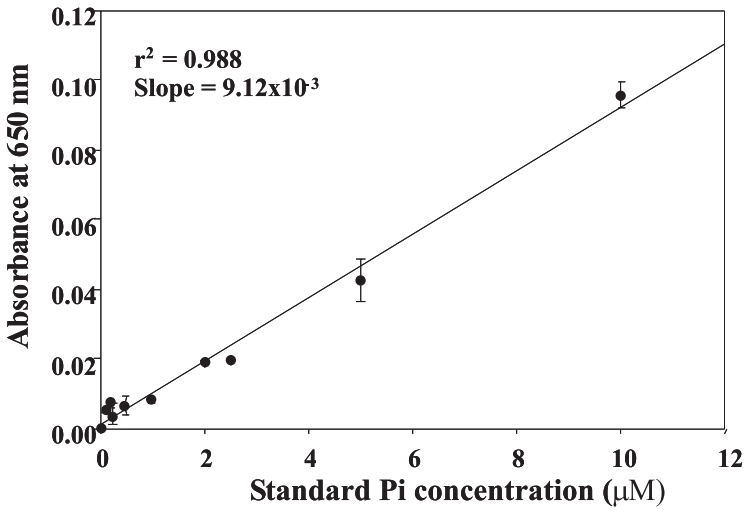

Figure 1 Standard curve of free phosphate (Pi). Various concentrations of phosphate solutions (0.1-10 μM) were prepared and used to test the malachite green assay. The absorbances from 4 experiments were averaged andlinear regression was performed to calculate slope and correlation.

To determine the phosphatase activity of CaN in cultured cortical neurons, phosphopeptide of RII subunit of PKA was used as a specific substrate of CaN. Basal CaN activity was measured from cell lysate of cultured cortical neurons. To test Ca^{2+} dependency, dephosphorylation reactions were conducted at various concentrations of free Ca^{2+} (Fig. 2). The result showed an increase in the phosphatase activity or the release of free Pi following increasing Ca^{2+} concentrations up to 80 nM. The activity reached the plateau at 80-120 nM of free Ca^{2+} levels. The release of free Pi gradually declined at Ca^{2+} concentration higher than 120 nM. To test whether this phosphatase activity was the result of CaN activity, dephosphorylation reactions were performed in the presence of CsA and FK506, specific inhibitors of CaN (Fig. 2). CsA and FK506 were chemically very different immunosuppressants that bound to unrelated intracellular receptors, cyclophilin and FK506 binding protein (FKBP), respectively (Ho et al., 1996). CsA/cyclophilin and FK506/FKBP formed inhibitory complexes that blocked phosphatase activity of CaN. Thus, the combination of CsA and FK506 was expected to completely inhibit CaN phosphatase activity. The result here showed that CsA and FK506 completely inhibited the release of free Pi suggesting the CaN-dependent pathway (Fig. 2). These results suggest that *in vitro* phosphatase activity of CaN is enhanced at low Ca^{2+} concentrations (80-100 nM), whereas it is suppressed at high Ca^{2+} concentrations (> 120 nM).

To ensure specificity of the assay for CaN activity, okadaic acid was added to the reaction buffer to inhibit dephosphorylation reactions induced by serine/threonine protein phosphatase 1 and 2A (PP1 and PP2A). Dephosphorylation reactions were tested in the presence of different concentrations of okadaic acid. The data in figure 3 showed that okadaic acid at all tested concentrations significantly blocked the release of free Pi from phosphopeptide substrate. This result indicates that not only CaN but also other protein phosphatases such as PP1 and PP2A might be able to dephosphorylate this phosphopeptide.

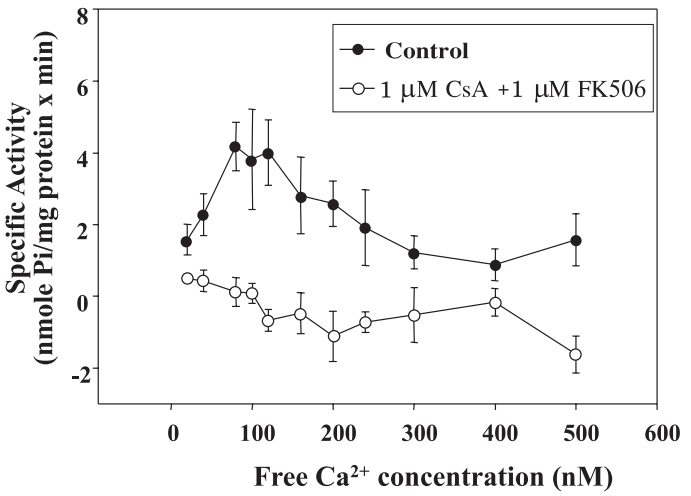

Figure 2 Dose-response curve of Ca^{2+}-induced CaN activation. CaN phosphatase activity was determined from celllysate of primary cortical cell cultures in the absence and presence of 1 μM CsA plus 1 μM FK506. Phosphatase activity was conducted at different concentrations of free Ca^{2+} (0-500 nM). Each concentrationrepresents mean+standard error of 4 independent experiments. Statistical analysis was performed by ANOVA. All comparisions were significant at $p < 0.05$.

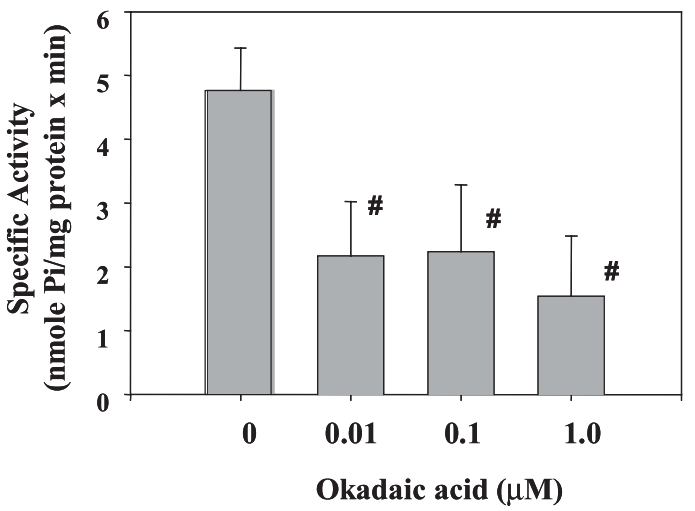

Figure 3 Phosphatase activity in the presence of okadaic acid. CaN phosphatase activity was determined in primarycortical cell cultures in the absence and presence of 0.01-1 μM okadaic acid. The reaction was conducted at 100 nM of free Ca^{2+}. The result represented the data from 4 independent experiments. Statistical analysis was performed by ANOVA, # $p < 0.05$.

Discussions

To measure phosphatase activity of CaN, various types of substrates can be used. One of the substrates, p-nitrophenyl phosphate (PNPP) has been used to test the activity of CaN activity. The generation of p-nitrophenol from dephosphorylation of PNPP can be easily determined by colorimetric technique. It should be noted that PNPP can be dephosphorylated not only by CaN, but also by other phosphatase emzymes (Sparks and Brautigan, 1986). Thus, PNPP is not useful in distinguishing among various types of phosphatases. Since PNPP is not specific to CaN, most recent studies have used phosphopeptide of regulatory subunit (RII) of PKA as a specific substrate for CaN (Vega et al., 2002). This peptide can be labeled with [^{32}P] radioactive to increase the sensitivity of the measurement of free Pi. To avoid the use of radioactive in the present study, colorimetric technique by using malachite green assay was initially tested to determine the level of

free Pi released from the phosphopeptide substrate. The result from this study showed that malachite green assay was quite a sensitive assay. Taken together with its simplicity, the malachite green assay was used for further experiments to test phosphatase activity of CaN in cultured neuron samples.

It is known that CaN activation in neurons requires small increase of intracellular Ca^{2+} because CaN has high affinity to Ca^{2+} ions (0.3 nM) (Kasai, 1993). Therefore, low level of stimulus, for example low frequency stimulation can activate CaN to induce long term depression (LTD) in neurons (Yasuda et al., 2003). In the intact neurons, the CaN-dependent activity can be determined indirectly by estimating inhibitory effect of CaN inhibitors, CsA and/or FK506. In cell lysate, phosphatase activity of CaN can be measured directly in test tubes using CaN phosphopeptide substrate. The result from this study showed that the maximal activity of CaN appeared at 80-120 nM of free Ca^{2+} concentrations. This observation agrees to previous studies that CaN is activated by low level of Ca^{2+} (Yasuda et al., 2003). Interestingly, this study showed a suppression of CaN activity at high Ca^{2+} levels. It was possible that high Ca^{2+} concentration which preferentially promoted the activity of kinase enzymes (Fukunaga et al., 1996), which indirectly reduced the release of free Pi. Increased kinase activity counteracted to the phosphatase activity of CaN by promoting re-phosphorylation of the phosphopeptides. This finding indicates that the phosphatase activity of CaN in the *in vitro* assay is enhanced at relatively low level of free Ca^{2+}, whereas high Ca^{2+} levels suppressed CaN activity. Thus ones should concern of adjusting the assay condition to maximize CaN activity. Titration of free Ca^{2+} in the assay solution might be necessary to obtain the optimal CaN activity assay. Choosing the right concentration of free Ca^{2+} can increase the sensitivity of the assay. Ones can avoid using such a sensitive but dangerous radioactive assay by maximizing the assay conditions of an available colorimetric technique.

Although the phosphopeptide used in this experiment was specific to CaN and the result showed that CsA and FK506 inhibited the release of free Pi from this peptide, non-specific dephosphorylations by other phosphatases should not be overlooked. Thus, okadaic acid, a non-selective phosphatase inhibitor, was used to inhibit the activity of protein phosphatase type 1 and type 2A (PP1 and PP2A). Low concentration of okadaic acid ($< 1\ \mu M$) can inhibit PP1 and PP2A activity, whereas high concentration ($> 1\ \mu M$) is required to block CaN activity (Bialojan and Takai, 1988). Unexpectedly, all concentrations of okadaic acid used in the present study inhibited the dephosphorylation of RII phosphopeptide. This suggested that the release of free Pi was the result of PP1 and PP2A activity in addition to CaN activity. Non-specific dephosphorylation of the RII phosphopeptide found in this study might be the result of high concentration of the phosphopeptide substrate in the assay reaction. High density of substrate might enhance the accessibility of substrate to enzyme PP1 and PP2A leading to non-specific dephosphorylation. Therefore, specificity of CaN activity determined by using phosphopeptide as a substrate might be limited by substrate concentration in the reaction. To test this hypothesis, future experiments are needed to test whether high concentrations of phosphopeptide induce non-specific dephosphorylation reactions.

Conclusions

CaN is a phosphatase enzyme enriched in many types of tissues. The phosphatase activity of CaN in cell lysate can be measured directly by using specific phosphopeptide substrate for CaN. The release of free Pi was determined by malachite green colorimetric technique. The results from this study suggest that free Ca^{2+} concentrations are critical for the *in vitro* measurement of CaN activity and the concentration of phosphopeptide, a substrate of CaN, may have an impact on the specificity of the substrate to phosphatase enzymes. This finding indicates an importance of the experimental conditions of the enzymatic assay. Ones can develop a sensitive, specific and simple assay for CaN phosphatase activity by choosing the proper reaction conditions.

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