Factors responsible for the Ca^{2+}-dependent inactivation of calcineurin in brain

Paul M. Stemmer**,****, Xutong Wang****, Marie H. Krinks***, Claude B. Klee*

Laboratory of Biochemistry, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Received 24 August 1995

Abstract The Ca^{2+}-dependent protein phosphatase activity of crude rat brain extracts measured in the presence of okadaic acid, exhibits the characteristic properties of the calmodulin-stimulated protein phosphatase, calcineurin. It is stimulated more than 200-fold by Ca^{2+} and inhibited by the calmodulin-binding peptide, M13, and by the immunosuppressive drug, FK506. It is insensitive to rapamycin at concentrations up to 1 μM. Its specific activity, based on calcineurin concentration determined by quantitative analysis of Western blots exposed to anti-bovine brain IgG, is ten to twenty times that of purified rat brain calcineurin assayed under similar conditions. Unlike the purified enzyme it is rapidly and irreversibly inactivated in a time-, temperature-, and Ca^{2+}/calmodulin-dependent fashion without evidence of extensive proteolytic degradation. The enzyme is converted to a state which does not lose activity by removal of low molecular weight material by gel filtration. Reconstitution of a labile enzyme is achieved by the addition of the low molecular weight-containing fraction eluted from the gel filtration column. These observations indicate that calcineurin in crude brain extracts is under the control of Ca^{2+}/calmodulin-dependent positive and negative regulatory mechanisms which involve unidentified endogenous factor(s).

Key words: Calmodulin; Calcineurin; Protein phosphatase; Ca^{2+}

1. Introduction

The specific inhibition of the calmodulin-stimulated protein phosphatase, calcineurin[1], by the immunosuppressive drugs, FK506 and cyclosporin A, complexed with their respective binding proteins, cyclophilin and FKBP, suggests that these agents may be competing with endogenous ligands modulating the activity of calcineurin in vivo [10,12]. In an attempt to demonstrate the presence of such regulatory factors we analyzed the activity of calcineurin in crude brain extracts. Brain extracts were selected because the concentration of calcineurin in this tissue, determined by Western blot analysis, is ten to twenty times that of skeletal muscle [8] whereas the protein phosphatase activities of calcineurin in brain and skeletal muscle are apparently similar [6]. Endogenous inhibitory ligands could be responsible for the relatively low specific activity of calcineurin in crude brain extracts. The high concentration of the FK506-binding protein, FKBP, in brain [14] also suggests that it may be accompanied by equally high concentration of these putative regulatory ligands.

[1]Calcineurin is the sole identified member of the protein phosphatase-2B family defined by Ingebritsen and Cohen [5].

*Corresponding author. Laboratory of Biochemistry, National Cancer Institute, Building 37, Room 4E28, Bethesda, MD 20892, USA. Fax: (1) (301) 402 3095.

**Present address:* University of Nebraska Medical Center, Department of Pediatrics, Omaha, NE 68198-6055, USA.

***Present address:* Food and Drug Administration, Center for Biologics Evaluation and Research, Building 29, Room 208, National Institutes of Health, Bethesda, MD 20892, USA.

****Paul M. Stemmer and Xutong Wang contributed equally to this project.

2. Materials and methods

2.1. Materials

Ram testis calmodulin was purified as described by Newton et al. [13]. FK506 and rapamycin were generous gifts from S. Schreiber (Harvard University) and okadaic acid from A. Takai (Nagoya University). The synthetic peptide used as a substrate for calcineurin [1] purchased from Peninsula Laboratories, was purified by HPLC and phosphorylated with cAMP-dependent kinase as described by Hubbard and Klee [4]. The synthetic peptide corresponding to the calmodulin-binding domain of myosin light chain kinase (M13), used as a specific calmodulin inhibitor [1], was purchased from Peptide Technologies (Rockville, MD). The antibody against the two subunits of bovine brain calcineurin was raised in rabbits and the IgG fraction purified by ammonium sulfate fractionation. ^{125}I-labeled protein A (30 mCi/mg) was a product of Amersham.

2.2. Crude brain extract

A normally fed Sprague–Dawley rat was injected with a lethal dose of pentobarbital and decapitated. The brain was excised (approximately 1.5 g of tissue) and immediately homogenized in 2 volumes of 250 mM sucrose containing 2 mM EDTA, 0.1% mercaptoethanol, and a cocktail of protease inhibitors (100 μg/ml phenylmethyl sulfonyl fluoride, 10 μg/ml leupeptin and 100 μg/ml soybean trypsin inhibitor) with a 15 ml Dounce ball homogenizer at 0°C (6–7 strokes) followed by a 15-s homogenization in a Polytron Model PT 10/35 homogenizer (setting #5). The homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant fluid (crude extract) was separated from low-M_r material by passage through a 1.5 × 10 cm Sephadex G-50 column equilibrated with 50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 0.1% mercaptoethanol and a cocktail of protease inhibitors as above. Fractions containing most of the A_{280nm}-absorbing material eluted at the void volume of the column were pooled (fraction A, 4 ml) and used to measure the protein phosphatase activity of calcineurin. A second peak of UV-absorbing material was eluted after one column volume (fraction B, 7 ml). Fraction B was tested for its ability to alter the calcineurin activity of fraction A. The crude extract and the two G-50 fractions were stored in 50-μl aliquots at −70°C for up to six months without significant loss of activity.

2.3. Quantification of calcineurin in crude extracts

The crude brain extract was mixed with a denaturing solution containing 6 M urea, 1% sodium dodecylsulfate (SDS), 0.8 M dithiothreitol and 50 mM Tris-Cl, pH 8.0. The samples (7.5 μg protein) were boiled for 1 min and subjected to SDS-PAGE on 5–10% gradients of acrylamide. The resolved protein bands were transferred electrophoretically (20 h at 0°C and 200 mA) to 0.1-μm nitrocellulose filters as described [16]. The nitrocellulose filters were blocked with 1% ovalbumin and incubated for 90 min in a solution of anticalcineurin IgG

SSDI 0014-5793(95)01095-5

(20 mg/ml) diluted 1000-fold in phosphate buffered saline (PBS) containing 1% ovalbumin and rinsed once with PBS, twice with PBS containing 0.05% NP40 and once again with PBS. Calcineurin-bound IgG was detected with [^{125}I]protein A (0.1 μCi/ml in PBS/1% ovalbumin). The radioactive bands identified by autoradiography were cut out and counted in a gamma counter. Linear responses were obtained between 5 and 75 ng calcineurin per lane. Bovine brain calcineurin was used as a standard. Protein concentration was determined by the method of Lowry et al. [11] with bovine serum albumin as standard.

2.4. Protein phosphatase assay

The assays were carried out essentially as described by Hubbard and Klee [4]. Briefly the crude extract or fraction A (10 μg protein) in 40 μl of 40 mM Tris-Cl, pH 8.0, 100 mM KCl, 0.4 mg/ml bovine serum albumin (Buffer I) containing 1.5 mM dithiothreitol, and 0.45 μM calmodulin, 0.75 μM okadaic acid (except when indicated otherwise), was incubated at 30°C in the presence of Ca^{2+} or EGTA at the concentrations and for the times indicated in legends to the figures. Appropriate dilution of a stock solution of okadaic acid (4 mM in dimethylsulfoxide) in Buffer I was used to achieve a final concentration of dimethylsulfoxide in the phosphatase assay of 0.2% (v/v). Reactions were started by addition of 20 μl phosphopeptide dissolved in Buffer I to reach a final concentration of 1 μM and the incubation continued for up to 2 min. Except when indicated otherwise, the Ca^{2+}-dependent activity was measured in the presence of 0.66 mM Ca^{2+} and 0.33 mM EGTA. The enzyme activity was calculated as described [7].

3. Results and discussion

The immunosuppressant FK506, a specific inhibitor of the protein phosphatase activity of calcineurin, inhibits selectively the Ca^{2+}-dependent protein phosphatase activity of a crude brain extract (Fig. 1, panel A). Because of a rapid loss of enzyme activity following exposure of the extract to Ca^{2+} the incubation with FK506 was limited to 2 min, followed by a 1-min incubation with substrate. Under these conditions maximal effects of FK506 were observed. Almost complete inhibition (>90%) is observed at 1 μM FK506. As expected from the high concentration FK506-binding protein (FKBP) reported in brain by Steiner et al. [14] the inhibition does not require addition of exogenous FKBP. The high concentration of FK506 (1 μM) needed for full inactivation compared to that required to inhibit calcineurin activity of Jurkat cell lysates (< 1 nM) [3] may reflect the presence of competing ligands, a 10–20-fold higher concentration of calcineurin in brain than in other tissues or both. Attempts to prevent the inhibitory effect of

Table 1
Lack of correlation between proteolysis and loss of enzyme activity[a]

Additions	% Residual Activity	% Residual calcineurin	
		A subunit	B subunit
None	7–8	53	102
Mg^{2+}	14–16	78	103
Mg^{2+} + leupeptin	14–17	74	103

[a]Crude brain extract was incubated for 20 min at 30°C in the presence of 0.15 mM $CaCl_2$ in the presence or absence of 6 mM $MgCl_2$ and 10 mg/ml leupeptin as indicated. Duplicate samples were frozen in dry ice and analyzed by SDS gel electrophoresis while the remaining enzyme was tested for enzyme activity by addition of substrate and incubation continued for 1 min. Calcineurin A levels and activity, determined as described in methods, were compared to that of a sample kept at 0°C in the presence of EGTA. Calcineurin constitutes 0.7–1% of the total protein in crude brain extract. Calcineurin B was detected with an antibody raised against recombinant calcineurin B to avoid overestimation of calcineurin B levels due to contamination by calcineurin A proteolytic fragments.

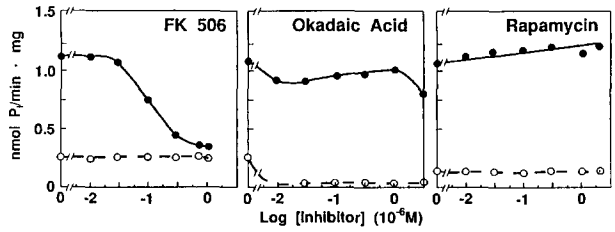

Fig. 1. Inhibition of protein phosphatase activity in crude brain extracts by FK506, okadaic acid, and rapamycin. Crude brain extract was incubated with FK506, okadaic acid and rapamycin as described in Section 2 for 2 min at 30°C in the presence of 0.1 mM Ca^{2+} (●) or 0.2 mM EGTA (○). Appropriate dilutions of stock solutions of okadaic acid (4 mM in dimethylsulfoxide), FK506 or rapamycin (5 mM in methanol) in Buffer I were used to achieve the concentrations of drug indicated in the abcissa and to maintain constant final concentrations of methanol (0.2%, v/v) or dimethylsulfoxide (0.2%, v/v) that did not significantly inhibit enzyme activity. The protein phosphatase assay was started by addition of substrate and the incubation continued for 2 min. Protein phosphatase activity is expressed as nmol/min·mg of total protein.

FK506 by rapamycin, as reported for other tissues, were inconclusive probably because of high concentrations of rapamycin needed to titrate high concentrations of FKBP and to compete with equally high concentration of FKBP-FK506 complexed with its target protein, calcineurin. FK506 has no effect on the Ca^{2+}-independent protein phosphatase activity measured in the presence of EGTA.

Okadaic acid, a specific inhibitor of protein phosphatases-1 and 2A, has no effect on the Ca^{2+}-dependent dephosphorylation of the peptide substrate at concentrations up to 1 μM but completely inhibits Ca^{2+}-independent dephosphorylation at concentration below 0.03 μM (Fig. 1, panel B). The contribution of protein phosphatase-2C to the Ca^{2+}-independent and okadaic acid-resistant activity (0.05–0.07 nmol/min·mg of total protein), measured in the presence of 6 mM Mg^{2+}, was very low (data not shown). As expected, rapamycin at concentrations up to 5 μM fails to inhibit either the Ca^{2+}-independent or Ca^{2+}-dependent phosphatase activities (Fig. 1, panel C).

Thus, the Ca^{2+}-dependent protein phosphatase activity in crude brain extract (70% of the total protein phosphatase activity measured in the absence of Mg^{2+} with this substrate) exhibits the characteristic properties of calcineurin. It is therefore puzzling that the specific activity of the enzyme (100–250 nmol/min·mg calcineurin) based on calcineurin levels determined by quantitative Western blots is 10 to 20 times that of the purified calcineurin measured under similar conditions but in the presence of added Mg^{2+}. Following the affinity chromatography step the purified enzyme becomes dependent on Mn^{2+} [15] or Mg^{2+} (C.B. Klee and M.H. Krinks, unpubl. obs.).

As shown in Fig. 2A, the Ca^{2+}-dependent protein phosphatase activity of crude brain extract is inactivated in a time- and Ca^{2+}-dependent fashion. Extracts incubated at 30°C in the presence of EGTA for up to 20 min do not lose activity but a similar treatment in the presence of 1 mM Ca^{2+} results in more than 90% loss of activity[2]. The inactivation of calcineurin also

[2]Similar inactivation patterns were observed in the presence of 1 μM free Ca^{2+}.

requires the presence of calmodulin. Crude calcineurin dissociated from endogenous calmodulin by EGTA and preincubated in the presence of Ca^{2+} with a peptide corresponding to the calmodulin-binding domain of myosin light chain kinase, M13, is not inactivated (Fig. 2B). The loss of activity is not the result of extensive proteolysis of calcineurin. As summarized in Table 1 only 28–47% loss of calcineurin A and no loss of calcineurin B were detected by Western blot analysis of calcineurin samples which had lost 87–93% of their enzymatic activity. The inactivation can also not be explained by dephosphorylation of calcineurin by protein phosphatase-1, 2A or 2C since the incubation was performed in the presence of okadaic acid and in the absence of Mg^{2+}.

To test the possibility that endogenous analogs of FK506 in crude brain extracts are responsible for this inactivation process the crude extract was subjected to a gel filtration on a

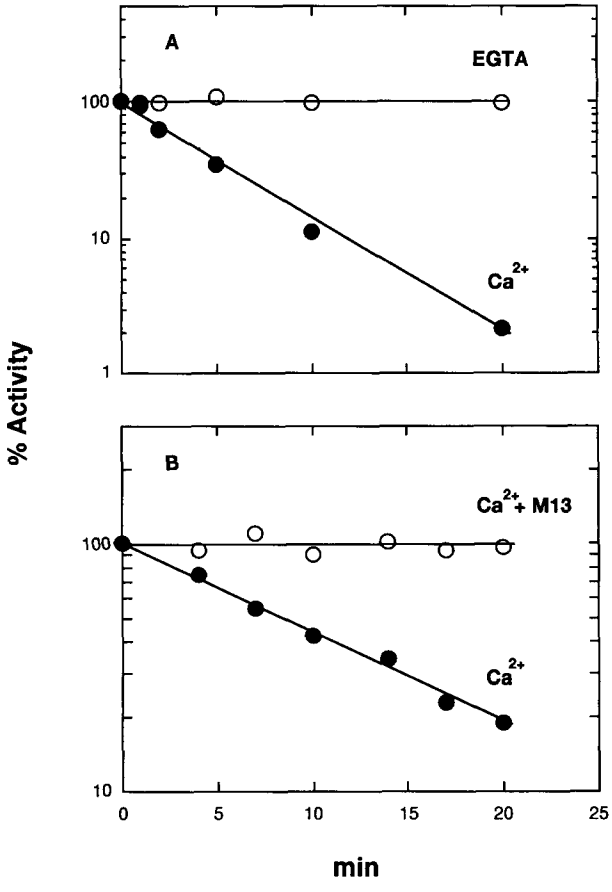

Fig. 2. Ca^{2+}- and calmodulin-dependent inactivation of calcineurin in crude brain extracts. (A) Crude brain extract was incubated as described in Section 2 in the presence of 1 μM added calmodulin, 1 mM Ca^{2+} (●) or 0.5 mM EGTA (○) for the times indicated. (B) The crude extract was first incubated in the presence of 0.5 mM EGTA and without added calmodulin for 10 min in the absence (●) or presence (○) of 1 μM M13. Ca^{2+} (1 mM total concentration) was added and the incubation continued for the times indicated. The enzymatic reactions were started by addition of substrate dissolved in Buffer I containing enough EGTA or Ca^{2+} to insure identical final concentrations of Ca^{2+} (0.66 mM) and EGTA (0.33 mM) in the assays. In panel B, calmodulin (1.3 μM, final concentration) was added with the substrate to measure enzyme activity. The specific activity of calcineurin in the crude extract assayed without prior incubation was 150 (○) and 120 (●) nmol/min · mg calcineurin (panel A) and 170 (●) and 120 (○) nmol/min · mg calcineurin (panel B).

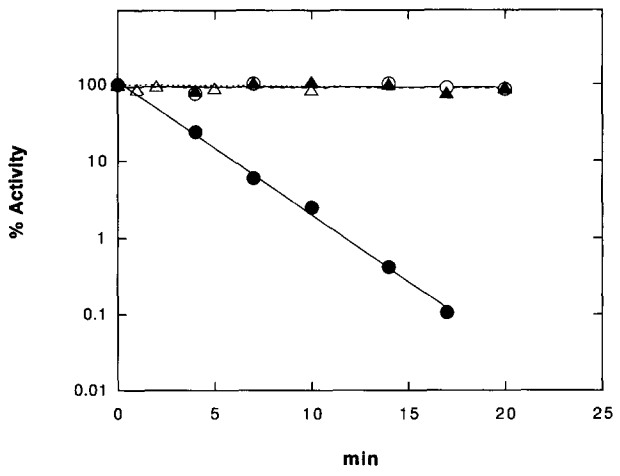

Fig. 3. Phosphatase activity of calcineurin separated from endogenous inactivating factor(s) by gel filtration of Sephadex G-50. Fraction A eluted from the Sephadex G-50 column (4 μl) was incubated in the presence of Ca^{2+} with (●) or without (○) fraction B (8 μl) for the times indicated and the reaction was started by the addition of substrate as described in legend to Fig. 2A. Also shown is the activity of Fraction A incubated in the presence of EGTA without (△) and with fraction B (▲). All enzymatic assays were performed in the presence of 0.66 mM Ca^{2+} and 0.33 mM EGTA. The specific activity of the enzyme in fraction A assayed without prior incubation (120 nmol/min · mg in the absence of fraction B and 94 in its presence) was taken as 100% activity.

Sephadex G-50 column to separate the protein phosphatase from the putative endogenous ligand(s). Following gel filtration the specific activity of calcineurin assayed in the presence of added calmodulin remains high (100–250 nmol/min · mg of calcineurin) and is still Mg^{2+}-independent. More than 90% inhibition of the Ca^{2+}-dependent phosphatase activity was observed in the presence of 6 μM M13 or FK506 (0.7 μM). After gel filtration complete inhibition by FK506 requires the addition of 0.7 μM FKBP, indicating that most of the endogenous FKBP eluting between the two major UV-absorbing peaks was also removed by gel filtration (data not shown).

As shown in Fig. 3 the enzyme, freed of low-M_r material by gel filtration, is no longer inactivated during the incubation with Ca^{2+}. Thus, the stability of calcineurin activity after gel filtration indicates that autodephosphorylation is not responsible for the inactivation process of the crude enzyme prior to gel filtration. It is therefore likely that small factor(s), responsible for the inactivation, have been removed by gel filtration. Reconstitution of a labile enzyme is achieved by mixing fractions A and B from the G-50 column (Fig. 3). As was observed in the case of the crude extract the inactivation induced by fraction B is dependent on Ca^{2+} but does not require addition of exogenous FKBP. The low-M_r inactivating factor(s) in fraction B, that are responsible for the time- and temperature-dependent inactivation process, are heat stable and dialyzable (Xutong Wang, unpubl. obs.). It is unlikely that they are non-specific inhibitors of calcineurin such as inorganic phosphate or endogenous substrates because the crude enzyme or fraction A mixed with fraction B incubated in the presence of EGTA and assayed in the presence of Ca^{2+} retains full activity.

The relationship of the inactivating factor(s) reported in this study to FK506 and cyclosporin A remains to be ascertained. The endogenous factor(s) has no effect on purified calcineurin

in the presence or absence of added FKBP. This lack of effect contrasts to the pronounced inhibition of the purified enzyme by FK506 and cyclosporin A in the presence of their respective immunophilins. Inactivation of fraction A by fraction B does not require FKBP but the presence of cyclophilin in fraction A has not been ruled out. It is possible that inactivation by the endogenous ligand, like the one observed with the immunosuppressive drugs, requires the presence of a specific binding protein. Alternatively, the inactivating factor may regulate a yet unidentified enzyme activating or stabilizing calcineurin. Purification and characterization of the inactivating factor(s) is presently under way in the laboratory.

References

[1] Blumenthal, D.K., Takio, K., Edelman, A.M., Charbonneau, H., Titani, K., Walsh, K.A. and Krebs, E.G. (1985) Proc. Natl. Acad. Sci. USA 82, 3187–3191.
[2] Blumenthal, D.K., Takio, K., Hansen, R.S. and Krebs, E.G. (1986) J. Biol. Chem. 261, 8140–8145.
[3] Fruman, D.A., Klee, C.B., Bierer, B.E. and Burakoff, S.J. (1992) Proc. Natl. Acad. Sci. USA 89, 3686–3690.
[4] Hubbard, M.J. and Klee, C.B. (1991) in: Molecular Neurobiology, A Practical Approach (Chad, J. and Wheal, H., Eds.) pp. 135–157, IRL Press, Oxford.
[5] Ingebritsen, T.S. and Cohen, P. (1983) Science 221, 331–338.
[6] Ingebritsen, T.S., Stewart, A.A. and Cohen, P. (1983) Eur. J. Biochem. 132, 297–307.
[7] Klee, C.B., Krinks, M.H., Manalan, A.S., Cohen, P. and Stewart, A.A. (1983) Methods Enzymol. 102, 227–244.
[8] Klee, C.B., Krinks, M.H., Manalan, A.S., Draetta, G.F. and Newton, D.L. (1985) Adv. Protein Phosphatases 1, 135–146.
[9] Liu, J., Albers, M.W., Wandless, T.J., Luan, S., Alberg, D.G., Belshaw, P.J., Cohen, P., MacIntosh, C., Klee, C.B. and Schreiber, S.L. (1992) Biochemistry 31, 3896–3901.
[10] Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) Cell 66, 807–815.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] McKeon, F. (1991) Cell 66, 823–826.
[13] Newton, D.L., Krinks, M.H., Kaufman, J.B., Shiloach, J. and Klee, C.B. (1988) Prep. Biochem. 18, 247–259.
[14] Steiner, J.P., Dawson, T.M., Fotuhi, M., Glatt, C.E., Snowman, A.M., Cohen, N. and Snyder, S.H. (1992) Nature 358, 584–587.
[15] Stewart, A.A., Ingebritsen, T.S. and Cohen, P. (1983) Eur. J. Biochem. 132, 289–295.
[16] Towbin, H.T., Stehelin, T.S. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.