Occurrence of a *para*-Nitrophenyl Phosphate-Phosphatase with Calcineurin-like Characteristics in *Paramecium tetraurelia*

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Using *para*-nitrophenyl phosphate (*p*NPP) as a substrate for enzymatic activity, we sought to identify CaN in *Paramecium*. We isolated three different *p*NPP-phosphatases from the soluble fraction of *Paramecium* cells by anion-exchange and affinity column chromatographies. One, *p*NPP-phosphatase *Peak I*, is very similar to mammalian CaN. Divalent cation dependency, inhibition by calmodulin (CaM) antagonists (trifluoperazine, calmidazolium), and insensitivity to various phosphatase inhibitors (heparin, okadaic acid, sodium vanadate, etc.) show similarity to mammalian CaN rather than to any other *Paramecium* *p*NPP-hydrolyzing enzymes tested. Polyclonal antibodies against bovine brain CaN recognizing subunits A (61 or 58 kDa) and B (17 kDa) of brain CaN cross-reacted with a 63-kDa protein in fractions containing *Peak I* *p*NPP-phosphatase activity and coeluted calmodulin. Overlay assays using biotinylated brain calmodulin indicated Ca²⁺-dependent CaM-binding by the 63-kDa protein. A Ca²⁺-binding protein with the same electrophoretic mobility as CaN B (17 kDa) was also present, though in other fractions from DEAE-cellulose chromatography. This finding strongly suggests that, in the absence of Ca²⁺, both subunits, A and B, were separated either before or during chromatographic processing. Our data support the existence of both subunits of a CaN-like phosphatase in *Paramecium* cells. © 1997

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Calmodulin (CaM)²-stimulated phosphatase, also known as calcineurin³ (CaN) (1) or protein phosphatase-2B (PP-2B) (2), is considered to occur ubiquitously in eukaryotes (3). Its concentration is especially high in mammalian brain, from where CaN has been purified and extensively characterized (for reviews see Refs. 4–6). CaN is composed of two polypeptides, CaN A and CaN B, with molecular sizes of 58–61 and of 15–19 kDa, respectively, depending on the source. CaN A is the catalytic subunit (7), which also contains regulatory domains such as the CaM-binding domain (1), the CaN B-binding domain (8), an autoinhibitory domain (9), and binding sites for Mn²⁺ and Ni²⁺ (10–13). CaN B is the Ca²⁺-binding subunit with four “EF-hand” structures (1, 14), which shows a high structural similarity to CaM. Despite this and the dual regulation of CaN by Ca²⁺ (15) the two proteins, CaN B and CaM, are not functionally interchangeable. Thus, CaN B is tightly bound to the catalytic subunit even at low Ca²⁺ concentrations (4) and cannot substitute for CaM in the activation of CaN activity. In turn, CaM cannot substitute for CaN B in reconstitution of the CaN A/CaN B dimer (16).

In comparison to other protein Ser/Thr phosphatases, CaN not only shows substrate specificity for Ser- and Thr-phosphorylated proteins (4), but it can also catalyze the dephosphorylation of *para*-nitrophenyl phosphate (*p*NPP), free phosphotyrosine, and phosphotyrosine-containing proteins (17–19).

² Abbreviations used: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BioCaM, biotinylated calmodulin; BSA, bovine serum albumin; CaM, calmodulin; CaN, calcineurin or protein phosphatase-2B or PP-2B; NBT, nitroblue tetrazolium; PP-1, PP-2A, and PP-2C, protein phosphatases-1, -2A, and -2C, respectively; *p*NPP, *para*-nitrophenyl phosphate; *p*NP, *para*-nitrophenol; TFP, trifluoperazine.

³ Enzymes used: calcineurin (EC 3.1.3.16); alkaline phosphatase (EC 3.1.3.1); acid phosphatase (EC 3.1.3.2).

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Molecular cloning in combination with immunochemical analysis demonstrated the existence of several isozymes in mammalian neuronal and nonneuronal tissues (3, 20). The existence of CaN was also demonstrated in nonmammalian (21–24), plant (25), and lower eukaryotic cells, including *Dictyostelium* (26) and several fungi (3). There is only restricted immunochemical information available on the existence of CaN in the ciliated protozoan, *Paramecium tetraurelia* (27, 28), whereas some other protein Ser/Thr phosphatases have been isolated (29) and characterized in more detail, including PP-1 (30, 31), PP-2A (30), and PP-2C (32).

In the present study we have taken into account the characteristic enzymatic properties of mammalian CaN (PP-2B) for its identification in *Paramecium*. We here report for the first time the isolation and characterization of a soluble pNPP-hydrolyzing protein phosphatase, sharing several characteristics with CaN from bovine brain when their enzymatic, physical, and immunochemical properties are compared.

MATERIALS AND METHODS

Materials

Heparin-agarose, protein A-agarose, aprotinin, leupeptin, sodium orthovanadate, levamisole, heparin, *N*-tosyl-L-arginine methyl ester (TAME), ExtrAvidin-alkaline phosphatase, Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt/nitroblue tetrazolium), histone II-S, and bovine brain CaN were obtained from Sigma (Deisenhofen, Germany). DEAE-cellulose was from Whatman (Maidstone, UK); compound R24571 (calmidazolium), bovine brain calmodulin, biotinyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester (Biocaps) were from Calbiochem (Baden Soden, Germany). Protein assay dye reagent, Kaleidoscope prestained standards, and slot blot manifold with accessories were purchased from Bio-Rad (Munich, Germany); pNPP and trifluoperazine (TFP) were from Boehringer-Mannheim (Germany); sodium potassium L(+)-tartrate and Pefabloc SC were from Merck (Darmstadt, Germany); and pepstatin A was from Serva (Heidelberg, Germany). SDS protein molecular weight markers (LMW) were purchased from Pharmacia LKB Biotechnology (Freiburg, Germany), okadaic acid was from Bio-Trend (Köln, Germany), and ^{45}Ca was from Amersham-Buchler (Braunschweig, Germany). Nitrocellulose (NC) membranes (BA-85) were obtained from Schleicher-Schuell (Dassel, Germany) and PVDF membranes (Immobilon-P) from Millipore GmbH (Eschborn, Germany). Radioactive [γ - ^{32}P]ATP and phosvitin-Sepharose were prepared as described in Ref. (33). Other reagents and all solvents used were of analytical grade.

Cell Cultures

P. tetraurelia wild-type cells (strain 7S) were grown at 25°C in a sterile synthetic medium and processed as previously described by Kissmehl *et al.* (34).

Isolation Procedure for pNPP-Phosphatase(s)

Crude extracts. Cells (~10 liters) harvested and washed were homogenized in 72 ml of buffer A (20 mM triethanolamine-HCl, 10% glycerol, 15 μM pepstatin A, 75 mU/ml aprotinin, 100 μM leupeptin,

0.26 mM TAME, 0.2 mM Pefabloc SC, pH 7.5) by ~100 hand strokes in a glass homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 100,000*g* for 60 min at 4°C. The supernatant was filtered through a layer of glass wool.

Chromatography on DEAE-cellulose. The 100,000*g* supernatant (70 ml) was applied to a chromatography column (diameter 1.6 cm, length 15 cm) filled with DEAE-cellulose. The column was washed with 170 ml of buffer A and then eluted with a linear gradient from 0 to 250 mM NaCl in buffer A. The total elution volume was 216 ml. Fractions of 4 ml were collected and assayed for pNPP-phosphatase activity (see below). Positive fractions at 40–50 mM NaCl were pooled and dialyzed against buffer A.

Chromatography on heparin-agarose. The dialyzed pNPP-phosphatase sample (24 ml) was loaded onto a heparin-agarose column (diameter 0.6 cm, length 15 cm) equilibrated with buffer A. The column was washed with 44 ml of buffer A and then eluted using a linear salt gradient ranging from 0 to 500 mM NaCl in buffer A. The total elution volume was 32 ml and each fraction contained 0.75 ml. Fractions were assayed for pNPP-phosphatase or protein phosphatase activity using the substrate pNPP or [^{32}P]histone II-S, respectively (see below). pNPP-phosphatase-containing fractions were pooled and stored at –80°C.

Chromatography on phosvitin-Sepharose. Alternatively, the dialyzed pNPP-phosphatase sample was applied to a phosvitin-Sepharose column (diameter 0.6 cm, length 15 cm) and chromatography was performed as previously described (34).

Phosphorylation of Histone

Histone type II-S was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase from bovine heart. The reaction mixture of 1.5 ml contained buffer B [10 mM triethanolamine-HCl, 15 mM 2-mercaptoethanol, 10% (v/v) glycerol, 1 μM pepstatin A, pH 7.5], 5 mM MgCl_2 , 0.1 mM ATP, [γ - ^{32}P]ATP (100 MBq/ml), 7 mg histone II-S, and 0.21 mg protein kinase. After 60 min at 30°C the mixture was dialyzed for 1 h at pH 7.5 against 3.9 M $(\text{NH}_4)_2\text{SO}_4$, 15 mM 2-mercaptoethanol, and 1 μM pepstatin A. Protein precipitated during dialysis was collected by centrifugation, resuspended in buffer B, and dialyzed against several changes of buffer B for 24 h. ^{32}P incorporation was followed by spotting an aliquot of the phosphorylated sample on chromatography paper as described in Ref. (33).

Assays for Phosphatase Activity

Assay for phosphohistone II-S dephosphorylation. Phosphoprotein phosphatase activity was measured based on the release of $^{32}\text{P}_i$ from labeled histone II-S. In routine assays, reaction was initiated by adding 0.02 ml of *Paramecium* samples (in buffer A) to a mixture of 0.02 ml containing 20 mM triethanolamine-HCl, 2 mg/ml bovine serum albumin (BSA), 2 mM MnCl_2 , and 2 μM ^{32}P -labeled histone II-S (2.6 MBq/ml), pH 7.5. Incubations at 25°C were terminated after 20 min by addition of 0.04 ml of 20% trichloroacetic acid. After centrifugation at 18,000*g* for 2 min, aliquots of 0.04 ml of the supernatant were added to 1 ml H_2O and subjected to Cerenkov counting of radioactivity.

Assay for pNPP dephosphorylation. pNPP-phosphatase activity was measured by the release of *para*-nitrophenol (pNP) (17). Standard incubations of 200 μl were performed at 25°C in microwell modules (diameter 0.59 cm, Nunc, Wiesbaden, Germany) containing buffer C (20 mM Tris-HCl, 10 mM NaCl, 20 $\mu\text{g}/\text{ml}$ BSA, pH 7.4) and 10- to 20- μl aliquots of phosphatase samples in the presence of 1 mM MnCl_2 . For further characterization assays were also run in the presence or absence of EDTA, EGTA, CaCl_2 , MgCl_2 , NiCl_2 , CuCl_2 , and ZnCl_2 , respectively, each at 1 mM. CaM dependency was analyzed with 0.1 mM CaCl_2 and 287 nM CaM (from various sources),

in either the presence or absence of an additional divalent cation. *p*NPP hydrolyzing was also probed in the presence or absence of various antagonists or phosphatase inhibitors, i.e., compound R24571 (12.5 μ M), trifluoperazine (100 μ M), sodium phosphate (10 μ M–50 mM), heparin (1–250 μ g), okadaic acid (50 pM–25 μ M), Na vanadate (0.15–150 μ M), levamisole (0.25–25 mM), or Na,K-ATPase inhibitor (0.25–25 mM) at either pH 7.4 or 8.6. All these agents were used either separately or in various combinations, as indicated. After 10 min of preincubation reaction was started by adding *p*NPP in a final concentration of 5 mM. *p*NPP was dissolved in water just before use. Formation of *p*NP was monitored spectrophotometrically in a Titertek Multiscan MCC/340 ELISA reader (Flow Laboratories GmbH, Meckenheim, Germany) at 405 nm either by the time course of the reaction or after termination by adding K_2HPO_4 at a final concentration of 13%. Controls were run to account for nonenzymatic *p*NP formation. Specific activity was calculated using the molar extinction coefficient for the *para*-nitrophenolate anion (1.75×10^4 M⁻¹ cm⁻¹) as described (18).

Reconstitution Analysis

Reconstitution analyses of *Paramecium* CaN subunits A and B were performed according to Refs. (35) and (36). Briefly, CaN subunit A- and B-containing fractions in varying molar ratios were preincubated in buffer C at 4 or 30°C in either the presence or absence of 100 μ M Ca^{2+} and/or 1 mM Ni^{2+} or Mn^{2+} . The various assay mixtures also contained 15 mM Na vanadate for reasons discussed below. At different times of preincubation (3–16 h) *p*NPP-phosphatase activity was measured as described above.

Polyclonal Antibodies against Bovine Brain CaN

A rabbit polyclonal antiserum against CaN from bovine brain was raised according to the immunization scheme of Kincaid (37). For controls 5 ml of serum was obtained prior to immunization. From both sera and preimmune sera, aliquots were taken and prepared for IgG fractions using two subsequent chromatography steps on DEAE-cellulose and protein A-agarose. Specificity of the antibodies was tested using the direct ELISA technique as described previously (38).

Protein Determination

Protein determinations were performed by the method of Bradford (39) with chemicals obtained from Bio-Rad (Munich, Germany). BSA was used as a standard.

Electrophoretic Techniques

Protein samples were denatured by boiling for 3 min in sample buffer (0.4 M Tris-HCl, 1% SDS, 0.5% DTT, 20% glycerol, pH 8.0) and subjected to electrophoresis on linear gradient (10–20%) SDS polyacrylamide gels using the discontinuous buffer system of Laemmli (40). Before electrophoresis, samples were alkylated for 30 min at 20°C by 2% iodoacetamide. Protein standards were used in accordance with the manufacturer's directions. Gels were either stained with silver or prepared for electrophoretic protein transfer onto NC membranes or PVDF membranes. Protein blotting was performed at 1 mA/cm² for 1 h either according to the technique of Kyhse-Andersen (41) using the semidry blotter from Ken-en-Tec (Copenhagen, Denmark) or in a Bio-Rad Mini Trans-blot cell equipped with a Bio-ice cooling unit. In the latter case, renaturation of proteins was promoted by the method of Dunn (42), when gels were incubated for 60 min at 20°C in 50 mM Tris-HCl, 20% glycerol, pH 7.4, and a carbonate blot buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3 , 20% methanol,

pH 9.9) was used. The molecular masses of the proteins on the gels/blots were estimated as described by Ref. (43).

Western Blots

NC membranes or PVDF membranes containing the protein replicas obtained after SDS-PAGE and electrophoretic transfer were cut in strips and blocked with 5% (w/v) nonfat dry milk in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.4) for 30 min at room temperature. The strips were incubated with polyclonal antibodies against either bovine brain CaN or *Paramecium* CaM (44). Antibodies were diluted 1:2000 in 0.25% (w/v) nonfat dry milk and TBS and applied overnight at 4°C. Controls were run using antibodies of the preimmune serum. Antibody labeling was performed by goat anti-rabbit IgGs coupled to alkaline phosphatase (Sigma) with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as substrate, in accordance with the manufacturer's directions.

Biotinylation of CaM and CaM Overlay Techniques

Bovine brain CaM was biotinylated by the method of Kincaid *et al.* (45). The molar ratio between the biotinylating agent and CaM was kept at 17:1, which covers more than twice the number of lysine sites on CaM (46). Incorporation of biotin was estimated using the molar extinction coefficient for biotin at 260 nm (2.3×10^4) and for CaM at 277 nm (0.18 for 1 mg/ml).

For specificity of CaM binding, bovine brain CaN (1 ng–2 μ g) was analyzed in a slot blot manifold according to Ref. (45). After saturation of nonspecific protein-binding sites with 5% (w/v) nonfat dry milk in TBS for 1 h, the NC membranes were cut in strips and incubated with 0.2 μ M biotinylated CaM (BioCaM) for 60 min at room temperature in the presence of either 1 mM $CaCl_2$ or, for control strips, 5 mM EGTA. BioCaM binding was visualized by ExtrAvidin-alkaline phosphatase using BCIP and NBT as substrates. For detection of CaM-binding proteins in enriched *Paramecium* extracts, samples were separated by SDS-PAGE, transferred onto NC membranes, and processed as described for bovine brain CaN.

⁴⁵Ca Overlay Technique

The ⁴⁵Ca overlay technique was carried out according to the method of Hincke (47). Briefly, NC membranes containing the electrophoretically transferred protein replicas from SDS-PAGE were soaked three times in IKM buffer (10 mM imidazole-HCl, 60 mM KCl, 5 mM $MgCl_2$, and 0.5 mM DTT, pH 6.8) at room temperature for 30 min with three changes of the solution, followed by incubations with 50 ml IKM buffer containing 20 μ g ⁴⁵Ca (0.03–0.05 MBq/ml) for 20 min at 20°C. Unbound ⁴⁵Ca was removed by incubation twice with 5% (v/v) ethanol for 2 min each. Membranes were then blotted to dryness between filter paper and exposed to Kodak X-Omat film, for 8–16 days at –70°C, in Kodak X-Omatic cassettes with intensifier screen.

RESULTS

Isolation and Enzymatic Characterization

For enzymatic screening of CaN in *Paramecium* extracts we used *p*NPP as a substrate *in vitro* (17, 18). Figure 1A illustrates the separation of two minor (*Peak I* and *Peak II*) and one major (*Peak III*) Mn^{2+} -stimulated *p*NPP-phosphatases from the soluble fraction of *Paramecium* whole-cell homogenates by DEAE-cellulose chromatography, with activity peaks at 40–50, 80–100, and 130–170 mM NaCl, respectively. By this

FIG. 1. Isolation of a soluble *pNPP*- and phosphohistone-phosphatase (*Peak I*) from *Paramecium* cells. Isolation was achieved from the 100,000*g* supernatant by two successive chromatography steps on DEAE-cellulose (A) and heparin-agarose (B and C). Aliquots of 20 μ l were used in 0.2- or 0.04 ml assays to determine *pNPP*- (A and B) or phosphohistone- (C) phosphatase activities after 30 or 20 min, respectively. By anion-exchange chromatography (A) three Mn^{2+} -stimulated *pNPP*-phosphatases, *Peak I*, *Peak II*, and *Peak III*, are separated. *Peak I* contains at least two different phosphatases, which can be separated by a subsequent affinity chromatography step on heparin-agarose (B, C). Part of the *pNPP*-phosphatase *Peak I* (from the DEAE-cellulose eluate) does not bind to heparin-agarose (B), whereas another non-*pNPP*-hydrolyzing phosphoprotein phosphatase (*Peak Ia*) can be separated on the basis of retention (C). Note that the phosphatase in the flow-through fraction (*Peak I*) dephosphorylates not only *pNPP* (B), but also ^{32}P -labeled histone II-S (C).

chromatographic step about 63% of total *pNPP*-hydrolyzing activities in the soluble *Paramecium* fraction were recovered in the course of which *Peak I* amounts to ~7% and *Peak II* and *Peak III* to ~9 and ~47%, respectively (Table I). With Ni^{2+} instead of Mn^{2+} , however, only *Peak III* *pNPP*-phosphatase could be detected (data not shown) in accordance with Ref. (48).

Further enrichment of *Peak I* was achieved by a subsequent affinity chromatography on heparin-agarose, which separates the *pNPP*-phosphatase activity of *Peak I* from another coeluted phosphoprotein phosphatase, *Peak Ia*. This protein phosphatase binds onto heparin-agarose, but does not catalyze the dephosphorylation of *pNPP* (compare Fig. 1B with 1C). With ^{32}P -labeled histone, another substrate for mammalian CaN *in vitro* (11, 12, 49), we demonstrate that *Peak I* is also able to dephosphorylate this Ser/Thr-phosphorylated substrate (Fig. 1C). Other chromatographic steps, e.g., on phosphitin-Sepharose, were insufficient to further separate *pNPP*- and protein phosphatase activities of *Peak I* (data not shown). This would be consistent with the intrinsic *pNPP*-phosphatase activity of the phosphoprotein phosphatase CaN in mammals. Although we succeeded in the separation and isolation of *pNPP*-phosphatase *Peak I*, yield and purification achieved by this isolation procedure are low (Table I).

The effects of several metal ions (Me^{2+}) on the *pNPP* hydrolysis of the three *pNPP*-phosphatases have been compared with those of bovine brain CaN (Table II). Bovine brain CaN shows little activity in the absence of divalent cations. The same is true for *Peak I* and *Peak II*, whereas *Peak III* shows a relatively high basal activity (in the absence of effectors). *Peak III* also differs remarkable by its stimulation by Me^{2+} (decreasing in the sequence $Mg^{2+} > Ni^{2+} > Zn^{2+} > Mn^{2+} \gg Cu^{2+} > Ca^{2+}$), compared to that of *Peak I* ($Mn^{2+} \gg Mg^{2+}, Ni^{2+}, Zn^{2+} > Ca^{2+}, Cu^{2+}$), *Peak II* ($Mn^{2+} > Zn^{2+} \gg Cu^{2+} > Mg^{2+} > Ni^{2+} > Ca^{2+}$), or bovine brain CaN ($Ni^{2+} \gg Mn^{2+} \gg Mg^{2+} > Ca^{2+} > Zn^{2+}, Cu^{2+}$). *Peak III* thus resembles a soluble Ca^{2+} -sensitive alkaline phosphatase in *Paramecium* (48). With exception of the lack of Ni^{2+} stimulation Me^{2+} activation of *Peak I* shows close similarity to that of bovine brain CaN, in particular with Mn^{2+} .

However, in contrast to bovine brain CaN, which requires an additional divalent cation for expression of Ca^{2+} /CaM dependency of *pNPP* hydrolysis, none of the three *Paramecium* *pNPP*-phosphatases could be significantly stimulated by addition of Ca^{2+} , separately or in combination with CaM from bovine brain or—to avoid species specific effects (50)—from *Paramecium* and independently of the presence or absence of additional Me^{2+} (data not shown). Interestingly, isolated *Paramecium* CaM and also a fraction (Fr-89) of the DEAE-cellulose eluate, far off any *pNPP*-phosphatase

TABLE I
Isolation of a Soluble *p*NPP-Phosphatase (*Peak I*) from *Paramecium* Whole-Cell Homogenates

Purification step	Volume [ml]	Total protein [mg]	Total activity [nmol/min]	Specific activity [nmol/min · mg]	Purification [fold]	Yield [%]
Homogenate	72.0	1565.2	3932.1	2.51	1.0	100.0
100,000 <i>g</i> supernatant	70.0	262.5	1020.3	3.88	1.5	25.9 (100)
DEAE-cellulose eluate						
<i>Peak I</i>	24.0	7.7	67.4	8.69	3.4	1.7 (6.6)
<i>Peak II</i>	36.0	20.2	91.4	4.51	1.8	2.3 (8.9)
<i>Peak III</i>	36.0	17.0	484.2	28.43	11.3	12.3 (47.4)
Heparin-agarose flowthrough						
<i>Peak I</i>	10.5	1.2	37.9	31.62	12.6	0.9 (3.7)

Note. Data represent a typical experiment. Isolation procedure and assay conditions were as described under Materials and Methods. Specific activities were calculated in the presence of 1 mM MnCl₂ after 30 min. Values in parentheses in last column refer to the 100,000 *g* supernatant.

activity (Fig. 1A), are able to activate bovine brain CaN in the same range as it occurs with CaM from bovine brain (data not shown), which strongly supports the presence of endogenous CaM in this fraction, Fr-89 (see Fig. 2A, lane 5).

The failure to further stimulate the activity of one of the three *p*NPP-phosphatases, especially of *Peak I* phosphatase, by added CaM might be attributed to the presence of residual CaM in this fraction. Therefore, we compared the influence of Me²⁺ chelators and of CaM antagonists on Mn²⁺-stimulated *p*NPP-phosphatase activities in either the presence or absence of additional *Paramecium* CaM (Table III). Whereas Me²⁺ chelators strongly reduce the activities of all phosphatases

tested, the response to TFP (100 μM) or R24571 (12.5 μM) depends on the type of phosphatase tested and on the presence of CaM in the assay, as we observed with brain CaN and *Peak I*, but not for *Peak II* and *Peak III*. In the presence of CaM, the *p*NPP-phosphatase activity of bovine brain CaN is clearly reduced by ~50% either by TFP or by R24571, whereas an increase of about 40–50% occurred when, under identical assay conditions, no CaM was present. This inhibition of brain CaN by CaM antagonists in the presence of CaM is in good agreement with data previously published by Gupta *et al.* (51) using phosphorylated protein substrates. Compared to these two opposite effects on CaN, the activity of *Peak I* was clearly inhibited by the same

TABLE II
Comparison of the Divalent Cation Dependency of the Three Soluble *Paramecium p*NPP-Phosphatases (*Peaks I–III*) with CaN from Bovine Brain

Additions	Bovine brain CaN specific activity [nmol/min · mg]	Soluble <i>Paramecium p</i> NPP-phosphatases		
		<i>Peak I</i> [nmol/min · mg]	<i>Peak II</i> specific activity [nmol/min · mg]	<i>Peak III</i> [nmol/min · mg]
None	0.9 ± 0.2	0.1 ± 0.05	0.1 ± 0.03	12.5 ± 2.1
EDTA	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.03
EGTA	0.8 ± 0.8	0.1 ± 0.03	0.1 ± 0.02	24.5 ± 5.5
CaCl ₂	0.3 ± 0.2	0 ± 0	0.1 ± 0.03	0.4 ± 0.07
MnCl ₂	5.6 ± 1.2	6.7 ± 0.1	3.3 ± 0.06	48.1 ± 9.1
MgCl ₂	1.9 ± 0.1	0.1 ± 0.05	0.5 ± 0.03	96.3 ± 4.4
NiCl ₂	60.6 ± 9.5	0.1 ± 0.04	0.2 ± 0.04	90.9 ± 3.8
CuCl ₂	0 ± 0	0 ± 0	0.9 ± 0.02	0.6 ± 0.01
ZnCl ₂	0 ± 0	0.1 ± 0.03	3.0 ± 0.02	62.3 ± 1.0

Note. Reaction mixtures containing 10 mM NaCl, 20 μg/ml BSA, 5 mM *p*NPP, and 20 mM Tris-HCl, pH 7.4, with one of the various *p*NPP-phosphatase fractions included [bovine brain CaN (1 μg), *Peak I* (6.4 μg), *Peak II* (10.2 μg), *Peak III* (11.9 μg)], were incubated for 60 min at 25°C, each in the presence or absence of 1 mM EGTA, EDTA, CaCl₂, MnCl₂, MgCl₂, NiCl₂, CuCl₂, or ZnCl₂, respectively. Data represent results from 2 to 12 experiments.

FIG. 2. Silver-stained proteins of chromatographically enriched *Paramecium* fractions after SDS-PAGE. (A) Samples of either 0.5 μ g of bovine brain CaN (lane 1) or *Paramecium* CaM (lane 6) were compared with 20- μ l portions of various fractions of the DEAE-cellulose eluate (see Fig. 1A). These portions contain the three pNPP-phosphatase fractions, *Peak I* (lane 2), *Peak II* (lane 3), and *Peak III* (lane 4), as well as a fraction (Fr-89) eluted at 220 mM NaCl (lane 5). Note that the *Peak I* fraction contains a 63-kDa protein (the size of CaN A in *Paramecium*) at the arrowhead. A protein with the same electrophoretic mobility as CaN B (17 kDa, arrow) can be visualized in *Peak III* fractions (lane 4). CaM may occur in Fr-89 (lane 5, lowest bands, to be compared with lane 6). In (B), a 2- μ g aliquot of the *Peak I* fraction (lane 2) after chromatography on heparin-agarose (see Fig. 1B) was compared with 0.3 μ g of bovine brain CaN (lane 1). Again, a 63-kDa protein can be visualized in fractions containing *Peak I* (lane 2, arrowhead). Lane 3 shows marker proteins.

CaM antagonists, regardless of whether *Paramecium* CaM was added or not. Without CaM added, inhibition by TFP was ~ 25 and $\sim 45\%$ by R24571. In the presence of CaM inhibition by R24571 was $\sim 20\%$. Based on this observation, we conclude that trace amounts of endogenous CaM in *Peak I* fractions are present, as we could confirm (see below).

Phosphate, a nonspecific inhibitor for phosphatases, strongly inhibits the activity of each of the pNPP-phosphatases tested, with IC_{50} values of 0.15, 0.25, 12, and 0.1 mM for *Peak I*, *Peak II*, *Peak III*, and bovine brain CaN, respectively (data not shown). Other inhibitors, e.g., heparin or okadaic acid, which are specific for protein Ser/Thr phosphatase-1 (PP-1) in mammalian and *Paramecium* cells (30, 52, 53), do not influence the activity of *Peaks I-III* (data not shown). The same is true for any identity of *Peak I* and *Peak II* with protein Tyr phosphatases by lack of vanadate inhibition (54), whereas pNPP-phosphatase *Peak III* was half-maximally inhibited at concentrations even below 100 nM (data not shown). Further analysis with levamisole and L(+)-tartrate, established inhibitors of alkaline (55) and acid phosphatase (56), also exclude any identity of the three pNPP-phosphatases with any of these phosphatases. However, L(+)-tartrate strongly inhibits pNPP-

phosphatase activities of *Peak I* in concentrations of $>100 \mu$ M at pH ~ 7 , whereas *Peak III* was not influenced (data not shown). Surprisingly, the response of bovine brain CaN strongly resembles that of *Peak I* (data not shown).

Immunobinding Assays

Specificity of the polyclonal antibodies raised against bovine brain CaN was confirmed either by indirect ELISA (results not shown) or by immunoblot analysis using original CaN from bovine brain (Fig. 3, lane 1). Both subunits are reactive with bands of 61 and ~ 58 kDa (CaN A) and of ~ 17 kDa (CaN B), in contrast to CaM either from *Paramecium* (lane 6) or from bovine brain (data not shown). Since CaN A is very sensitive to proteolytic degradation (57, 58), the presence of several minor immunoreactive bands of original CaN (lane 1) ranging from 58 to 48 kDa would imply recognition of degradation products.

Figure 3 also shows immunoblots from the three pNPP-phosphatase peak fractions of the DEAE-cellulose eluate. Anti-CaN antibodies strongly stain a band of 63 kDa and a minor band of ~ 58 kDa in *Peak I* fractions (lane 2) but also in fraction Fr-89 (lane 5). Fr-89 is far off any pNPP-phosphatase activity (see Fig. 1A) but contains CaM (see Fig. 2A, lane 5 and below).

Such an immunoreactive band at 63 kDa also occurs in *Peak I* fractions after heparin-agarose chromatography (data not shown), which we could visualize by silver staining (lanes 2 of Figs. 2A and 2B, arrowhead). Surprisingly, in these fractions we failed to detect a protein similar to CaN B. Such a reactive band of 17 kDa, however, occurs in *Peak III* fractions (Fig. 3, lane 4), far off an immunoreactive band of 63 kDa (lanes 2 and 5). A possible explanation might be the separation of both *Paramecium* CaN subunits due to the absence of Ca^{2+} during their fractionation. This is in contrast to CaN in mammalian systems (see Discussion).

Immunoblot analysis with *Paramecium* CaM specific antibodies (Fig. 4) from Ref. (44) confirms our assumption that CaM occurs in both *Peak I* fraction (lane 2) and Fr-89 (lane 3). These results strongly support our finding that Fr-89 contains CaM (Fig. 2A, lane 5) and activates bovine CaN (data not shown).

CaM and ^{45}Ca Overlays

In the presence of Ca^{2+} , biotinylation of bovine brain CaM resulted in the incorporation of ~ 6 mol biotin/mol CaM (results not shown). In slot blots biotinylated CaM (BioCaM) interacts with high affinity with bovine brain CaN in a Ca^{2+} -dependent manner (results not shown). By probing blots from the DEAE-cellulose eluate with BioCaM (Fig. 5) we also identified a 63-kDa protein in fractions of *Peak I* (lane 2, arrowhead) as well as in Fr-

TABLE III

Influence of Different Inhibitors on the Mn^{2+} -Stimulated *p*NPP-Phosphatase Activity of Either Bovine Brain CaN or the Isolated *Paramecium p*NPP-Phosphatases

Additions	Bovine brain CaN relative activity [%]	Soluble <i>Paramecium p</i> NPP-phosphatases		
		<i>Peak I</i> [%]	<i>Peak II</i> relative activity [%]	<i>Peak III</i> [%]
(A) without CaM added				
MnCl ₂ /CaCl ₂	100	100	100	100
MnCl ₂ /CaCl ₂ /EDTA	33.2	1.6	5.1	2.4
MnCl ₂ /CaCl ₂ /EGTA	11.0	2.4	3.6	39.8
MnCl ₂ /CaCl ₂ /R24571	144.4	55.2	103.5	102.7
MnCl ₂ /CaCl ₂ /ethanol	105.5	98.3	99.4	101.0
MnCl ₂ /CaCl ₂ /TFP	144.4	76.4	108.6	105.5
(B) With P-CaM added				
MnCl ₂ /CaCl ₂ /CaM	100	100	100	100
MnCl ₂ /CaCl ₂ /CaM/R24571	53.5	78.1	100	101.7
MnCl ₂ /CaCl ₂ /CaM/ethanol	106.1	93.7	101.3	101.0
MnCl ₂ /CaCl ₂ /CaM/TFP	48.2	96.1	99.4	98.1

Note. Reaction mixtures containing 10 mM NaCl, 20 µg/ml BSA, 1 mM MnCl₂, 0.1 mM CaCl₂, 5 mM *p*NPP, 20 mM Tris-HCl, pH 7.4, and various *p*NPP-phosphatases in concentrations indicated in Table II were incubated at 25°C for 30 min without (A) or with (B) 287 m CaM from *Paramecium* (P-CaM) in the presence or absence of different inhibitors, e.g., 1 mM EDTA, 1 mM EGTA, 12.5 µM R24571, or 100 µM TFP. Controls were also run in the presence of 0.5% ethanol in which calmidazolium had been dissolved. Data represent a typical experiment.

89 (lane 5) which binds CaM in a Ca^{2+} -dependent way (compare lanes a and b). The same is true for *Peak I* after further chromatographical fractionation (data not shown). Ca^{2+} -dependent BioCaM binding is also observed with the catalytic subunit of bovine brain CaN at 61 kDa (lane 1). Applying the ⁴⁵Ca-overlay technique to the same

fractions as in Fig. 5, several Ca^{2+} -binding proteins of 43 to 28 kDa occur (Fig. 6), whereas only *Peak III* (lane 4) contains a Ca^{2+} -binding protein of 17 kDa (arrow), just as original CaN B (lanes 1 and 6). Failure to detect ⁴⁵Ca binding to CaM in known CaM-containing fractions (Fr-89 and *Peak I*) might be attributed to poor retention of CaM by NC membranes (59). Since in *Peak*

FIG. 3. Western blot analysis of various DEAE-cellulose elution fractions using polyclonal antibodies against CaN from bovine brain. Aliquots (18 µl) of the three *p*NPP-phosphatase fractions, *Peak I* (lane 2), *Peak II* (lane 3), and *Peak III* (lane 4), and of another fraction (Fr-89) eluted at 220 mM NaCl (lane 5) were separated by SDS-PAGE, electroblotted onto NC membranes, and incubated with the polyclonal antibodies against bovine brain CaN (a) or with the corresponding preimmune serum (b). Control lanes containing either 0.5 µg of bovine brain CaN (lane 1) or 1 µg of *Paramecium* CaM (lane 6) demonstrate specific binding of the CaN antiserum to both subunits of bovine brain CaN (lane 1), but none to endogenous CaM (lane 6). Cross-reaction occurs with a 63-kDa protein (arrowhead) in *Peak I* fraction (lane 2) and in Fr-89 (lane 5), in addition to a faint band of 17 kDa (arrow) in fractions containing *Peak III* (lane 4).

FIG. 4. Immunoblot analysis of various DEAE-cellulose elution fractions using a polyclonal antiserum against *Paramecium* CaM. Aliquots (18 μ l) of the pNPP-phosphatase *Peak I* fraction (lane 2) and Fr-89 (lane 3) were subjected to SDS-PAGE, electroblotted onto PVDF membranes, and incubated with *Paramecium* CaM specific antibodies. Control lanes with either 1 μ g of *Paramecium* CaM (lane 4) or 0.5 μ g of bovine brain CaN (lane 1) demonstrate binding of the antibodies only to original CaM (lane 4). Note that immunobinding to CaM (arrow) occurs in both the *Peak I* fraction (lane 2) and particularly in Fr-89 (lane 3). The occurrence of additional bands in lanes 3 and 4 may be due to the different Ca^{2+} -induced conformational states of CaM.

I fraction no Ca^{2+} -binding protein of 17 kDa (CaN B) could be detected by this overlay technique (lane 2), thus confirming data obtained by immunobinding assays (Fig. 3, lane 2), we conclude that, in the absence of Ca^{2+} , the catalytic and the regulatory subunits of *Paramecium* CaN were separated during preparation and subsequent chromatography on DEAE-cellulose.

DISCUSSION

Although the existence of CaN in *Paramecium* has been postulated (27, 28), the unequivocal identification

FIG. 5. Binding of BioCaM to CaM-binding proteins present in various DEAE-cellulose elution fractions. Aliquots (100 μ l) of bovine brain CaN (lane 1), *Peak I* (lane 2), *Peak II* (lane 3), *Peak III* (lane 4), or fraction Fr-89 (lane 5) were separated by SDS-PAGE, transferred onto NC membranes, and incubated with 0.2 μ M BioCaM in the presence of either 1 mM CaCl_2 (a) or, for controls, 5 mM EGTA (b). Note Ca^{2+} -dependent binding of BioCaM to bovine brain CaN and to a protein of 63 kDa in the *Peak I* fraction as well as in Fr-89 (arrowhead).

FIG. 6. Ca^{2+} -binding proteins present in various DEAE-cellulose elution fractions. Aliquots (100 μ l) of the three pNPP-phosphatase peak fractions, *Peak I* (lane 2), *Peak II* (lane 3), and *Peak III* (lane 4), and the CaM-containing fraction Fr-89 (lane 5) and bovine brain CaN in concentrations of 0.5 μ g (lane 1) and 1 μ g (lane 6) were electrophoresed in the presence of SDS, electroblotted onto NC membranes, and assayed for ^{45}Ca binding as described under Materials and Methods. Note that the *Peak III* fraction (lane 4) contains a Ca^{2+} -binding protein of the same electrophoretic mobility (17 kDa, arrow) as subunit B from bovine brain CaN (lanes 1 and 6).

of CaN in *Paramecium* has not been accomplished. We isolated three different soluble pNPP-phosphatases, one of which shares several enzymatic, pharmacological, immunochemical, and biochemical characteristics with mammalian CaN.

Criteria for Identification of a CaN-like Phosphatase

Mammalian CaN catalyzes the dephosphorylation of a number of phosphoproteins (4, 6) and some low-molecular-weight phosphocompounds such as pNPP (17, 18). Using this substrate, we isolated three pNPP-phosphatases from the soluble fraction of *Paramecium* whole-cell homogenates, designated as *Peaks I-III*, of which only *Peak I* may represent the catalytic subunit of CaN for the following reasons. *Peak I* which also contains an intrinsic phosphoprotein-phosphatase activity was further enriched by affinity chromatography on heparin-agarose since sufficient *Paramecium* CaM was not available for coupling to Sepharose and since affinity chromatography using bovine brain CaM was ineffective. The use of heparin-agarose appears justified since this allows separation not only of type-1 phosphatases from *Paramecium* (29, 30), but also of different isozymes of mammalian CaN (60). By this step, we separated another phosphatase, subpeak *Peak Ia*, which might account for PP-1 because it lacks pNPP-

phosphatase activity in Fig. 1B and immunoreactivity with CaN specific antibodies and since it is sensitive to heparin (data not shown).

We also checked all three *p*NPP-phosphatases for possible identity with CaN on the basis of (i) Me^{2+} dependency, (ii) stimulation by CaM or of inhibition by CaM antagonists, and (iii) susceptibility to various phosphatase inhibitors. For all peaks we can exclude any possible identity with PP-1 by their insensitivity toward heparin or okadaic acid. Additionally, the absolute dependency on Mn^{2+} (but not on Mg^{2+}) for *p*NPP-phosphatase activity of *Peak I* and *Peak II* argues against PP-2A, which is also active in the absence of Me^{2+} (5, 6). The same holds for PP-2C which, in *Paramecium*, is membrane-bound and requires divalent cations, such as Mg^{2+} (32). Lack of stimulation by CaM or of inhibition by CaM antagonists excludes any identity of *Peak II* and *Peak III* with CaN, whereas the failure of stimulation of *Peak I* by adding CaM could be explained by coelution of CaM, as we have shown by CaM specific antibodies in Western blots (Fig. 4) or enzymatically by sensitivity to trifluoperazine or calmidazolium (Table III). Although other phosphatases, e.g., protein Tyr phosphatases or alkaline or acid phosphatase occurring in *Paramecium* (61, 62), also hydrolyze *p*NPP, a possible interference for *Peak I* (and for *Peak II*) can be excluded by inhibitor responses. From further enzymatic analysis of *Peak III*, we assume this *p*NPP-phosphatase to be identical with a Ca^{2+} - and vanadate-inhibited alkaline phosphatase, which was previously found in the soluble fraction of *Paramecium* cells (48). On the basis of all our results we conclude that only *Peak I* can represent the equivalent of mammalian CaN A in *Paramecium*.

These findings were further corroborated by immunobinding analysis with CaN specific antibodies and by overlay techniques to determine binding of CaM or Ca^{2+} . In the *Peak I* fraction we demonstrated immunoreactivity of a 63-kDa CaM-binding protein which we, therefore, assume to be the equivalent to mammalian CaN A. A 63-kDa CaM-binding protein in *Paramecium* also has been previously described, although its identity has not been established (63). A size of 63 kDa is not unusual since, in nonneuronal systems, CaN A ranges between 55 and 80 kDa, depending on the species and the cell type analyzed (21, 26). We also showed the existence of an immunoreactive and Ca^{2+} -binding protein of 17 kDa in another fraction, which may represent the equivalent to the regulatory subunit of mammalian CaN.

However, we must admit several unusual features, i.e., the absolute requirement for Mn^{2+} , the absence of the regulatory subunit in a catalytically active fraction, and the existence of a second immunoreactive, but catalytically nonactive form, which altogether must be rec-

onciled with our assumption of the identity of *Peak I* with CaN. Our arguments are as follows. Mammalian CaN occurs in different conformational states, depending on the presence of the regulatory subunit and on the type of stimulating Me^{2+} added (12, 36, 64). In the absence of the regulatory subunit, phosphoprotein phosphatase activity was activated only in the presence of Mn^{2+} (64). Also in other systems, this finding has been expanded to *p*NPP hydrolysis by analysis with monoclonal antibodies, which affect the various Me^{2+} -induced conformations in quite different ways (65). Therefore, it was suggested that subunit B of CaN plays a critical role for expressing Ni^{2+} -stimulated *p*NPP-phosphatase activity and this was supported by reconstitution analysis of CaN subunits A and B (66). Thus, the absolute requirement for Mn^{2+} , together with the failure to immunodetect CaN B in *Peak I* fractions, suggests that both subunits, A and B, were separated either before or during DEAE-cellulose chromatography which was performed in the absence of Ca^{2+} , although this allowed coelution of CaN A and B in mammalian samples (4). Therefore, we also performed reconstitution experiments with fractions containing the CaN B subunit (data not shown). Immunoblots or ^{45}Ca overlay analysis unfortunately revealed that subunit B of *Paramecium* CaN coelutes with *Peak III* phosphatase. Since *Peak III* contains approximately half of the total soluble *p*NPP-phosphatase activity in *Paramecium*, even after vanadate inhibition residual phosphatase activity in *Peak III* was too high to allow unambiguous detection of Ni^{2+} -stimulation of *Peak I* during reconstitution. We also failed to recover any activity of the second, i.e., the nonactive, form of CaN in the CaM-containing fraction. The existence of catalytically low active forms of CaN was recently reported for rat crude brain extracts which has been interpreted as meaning that CaN might be under the control of not only a positive, but also a negative regulation mechanism and both may involve unidentified endogenous factors (67).

Remarkably bovine brain CaN is stimulated by CaM from *Paramecium*. This was not foreseeable since *Paramecium* wild-type CaM contains 19 amino acid exchanges as well as some posttranslational modifications (68) which substantiates that only endogenous, but not mammalian, CaM can substitute for mutant CaM in repairing ciliary functions in *Paramecium* (50).

Finally, our conclusion on the existence of CaN in *Paramecium* is supported by the recent identification of part of the *Paramecium* CaN gene by molecular cloning, showing ~46% identity with the mammalian gene (69), although no detailed data have been published as yet. Taken together, these data strongly support the existence of CaN in *Paramecium*. Future reconstitution analyses of recombinant subunits may allow more detailed biochemical characterization.

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