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A novel, calcium-inhibitable casein kinase in *Paramecium* cells

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Abstract **This is the first identification of a Ca^{2+}-inhibitable casein kinase (CPK) which we have isolated from the 100 000 × *g* supernatant of *Paramecium* cell homogenates. The 1000-fold enriched CPK activity depends on millimolar Mg^{2+} and is inhibited by low concentrations of heparin or by ≥100 μM Ca^{2+}. Enzyme activity is stimulated by polylysine or polyarginine with either casein or with specific casein kinase-2 (CK-2) peptide substrates (RRRDDDSDDD and RREEETEEE). The enzymic properties are similar with GTP instead of ATP. CPK does not undergo autophosphorylation. In gel kinase assays, enzyme activity is associated with a 36 kDa band. Calmodulin as another characteristic substrate for mammalian CK-2 has not been phosphorylated by this protein kinase. Besides casein, CPK phosphorylates in vitro the catalytic subunit of bovine brain calcineurin (CaN), a typical substrate of type 1 mammalian casein kinase (CK-1) in vitro. Again this phosphorylation is significantly reduced by Ca^{2+}. Thus, CPK combines aspects of different casein kinases, but it is clearly different from any type known by its Ca^{2+} inhibition. Since CPK also phosphorylates the exocytosis-sensitive phosphoprotein, PP63, in *Paramecium*, which is known to be dephosphorylated by CaN, an antagonistic Ca^{2+}-effect during phosphorylation/dephosphorylation cycles may be relevant for exocytosis regulation.**

Key words: Calcineurin; Calmodulin; Casein kinase; Exocytosis; *Paramecium*; Phosphorylation

1. Introduction

Casein kinases (EC 2.7.1.37) are multipotential cyclic nucleotide-independent Ser/Thr protein kinases which were originally defined by their ability to phosphorylate acidic proteins such as casein and phosvitin in vitro. These enzymes are ubiquitous in eukaryotes and control many cellular processes (for reviews see [1–3]). They are generally divided into two classes according to different parameters, such as subunit structure, chromatographic behavior, catalytic properties and substrate specificity [2,3].

Type 1 casein kinases (CK-1) from mammalian cells have been typically described as monomeric cytosolic polypeptides of 30–37 kDa, whereas a larger range of molecular mass values exists for nuclear forms ranging from 25 to 55 kDa [3]. Heterogeneity in size is also due to isoforms not only in mammals [4–8] but also in lower eukaryotes such as *Dictyostelium* [9], yeast [10,11] or plants [12]. Typically, members of the CK-1 family are identified chromatographically by their order of elution from DEAE-cellulose at relatively low ionic strength [3] or, enzymatically, by their strict requirement for ATP as a co-substrate, moderate heparin sensitivity (IC_{50} ~ 20 μg/ml) and preferential use of phosphoserine/phosphothreonine [2,13].

In contrast, type II casein kinase (CK-2) is unique among the protein kinases since it can use ATP as well as GTP as a phosphoryl donor, is extremely sensitive to heparin inhibition and can be activated by polyamines and by basic polypeptides (reviewed in [2,3]). Human CK-2 as a typical form of mammalian CK-2 is composed of subunits, α, α′ and β with molecular masses of 43, 38 and 28 kDa, the latter showing autophosphorylation [1,2]. Catalytic activity resides in the α subunit [2], while the β subunit is regulatory, stimulating catalytic activity, mediating interactions with basic polypeptides and polyamines and participating in substrate recognition [14–16].

In *Paramecium tetraurelia* several protein kinases have been isolated from cilia and cell bodies, such as cyclic nucleotide-dependent [17–20] and Ca^{2+}-dependent protein kinases [21,22]. Recently, three monomeric forms of CK-1 in *Paramecium* were reported [23], with molecular masses of 28–45 kDa, as well as an additional casein kinase of 65 kDa enriched in particulate fractions of whole cells and in cell cortices. However, the physiological role of most of these kinases is not known.

In *Paramecium* protein phosphorylation/dephosphorylation cycles are involved in exocytosis regulation [24–26]. Exocytosis capacity is strictly paralleled by the capacity to dephosphorylate a 63 kDa phosphoprotein (PP63), followed by rapid rephosphorylation on Ser/Thr residues [24,25]. A casein kinase analyzed in detail in the present paper phosphorylates PP63 in vitro [26] while dephosphorylation was catalyzed by calcineurin (CaN) [26,27], a protein Ser/Thr phosphatase requiring Ca^{2+} and calmodulin (CaM). Mammalian CaN is a heterodimer composed of a ~61 kDa CaM-binding catalytic subunit (CaN A) and a ~17 kDa Ca^{2+}-binding subunit (CaN B) [28,29]. The catalytic subunit of mammalian CaN is phosphorylated in vitro by several protein kinases on multiple Ser/Thr phosphorylation sites [30–34], including a glycogen synthase (casein) kinase-1 (CK-1) [35]. CaM itself, a highly conserved acidic polypeptide of ~17 kDa [36,37], can be phosphorylated in vitro by CK-2 on Thr-79, Ser-81, Ser-101 and Thr-117, all sites being consensus sequences characteristic of CK-2 [38], but not of CK-1 or PKA [39]. In the context of PP63 dephosphorylation by CaN it is interesting that, in *Paramecium*, CaM has been localized to trichocyst exocytosis sites [40]. Furthermore, exocytosis incompetent strains be-

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Abbreviations: CaM, calmodulin; CaN, calcineurin or protein phosphatase-2B; CaN A, catalytic subunit of CaN; CaN B, regulatory subunit of CaN; CPK, casein kinase isolated from *P. tetraurelia*; CK-1, type 1 casein kinase; CK-2, type 2 casein kinase; HM, homogenization medium; PAGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PLA, poly(L-arginine); PLL, poly(L-lysine); PP63, exocytosis-sensitive phosphoprotein of 63 kDa; SP, spermine; TAME, *N*-*p*-tosyl-L-arginine methyl ester.

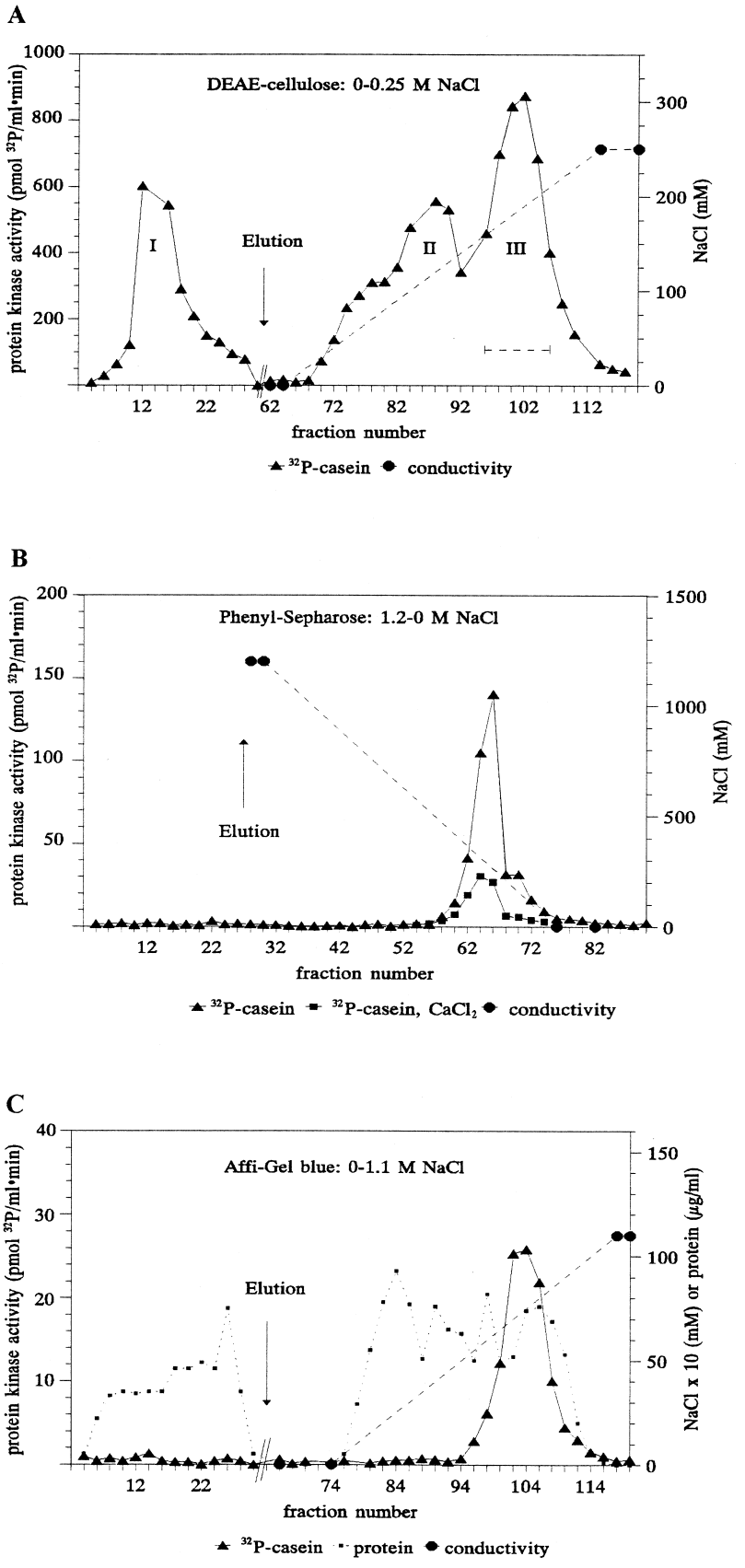
A
DEAE-cellulose: 0-0.25 M NaCl
protein kinase activity (pmol 32P/ml•min)
NaCl (mM)
Elution
I
II
III
fraction number
32P-casein
conductivity
B
Phenyl-Sepharose: 1.2-0 M NaCl
protein kinase activity (pmol 32P/ml•min)
NaCl (mM)
Elution
fraction number
32P-casein
32P-casein, CaCl2
conductivity
C
Affi-Gel blue: 0-1.1 M NaCl
protein kinase activity (pmol 32P/ml•min)
NaCl x 10 (mM) or protein (µg/ml)
Elution
fraction number
32P-casein
protein
conductivity

Fig. 1. Isolation of CPK from *Paramecium*. For isolation from the soluble fraction of whole cell homogenates we used three successive chromatography steps on DEAE-cellulose (A), phenyl-Sepharose (B) and Affi-Gel blue (C) as described under Section 2. Aliquots of 10 μl were used in 0.03 ml assays to determine casein kinase activity after 20 min. By chromatography on DEAE-cellulose and with casein as a substrate, separation of at least three different protein kinase activities, peaks I–III, was achieved (A). Peak III (CPK) containing fractions, eluted at 180–200 mM NaCl, were submitted to subsequent chromatography steps on phenyl-Sepharose (B) and Affi-Gel blue (C). Note a significant decrease in CPK activity, when Ca^{2+} (2 mM) was included in the reaction mixture (B).
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come responsive when transfected with the CaM wild type gene [41].

We report the isolation of a novel type of a soluble casein kinase (CPK) from *Paramecium* whole cell homogenates. It shares several properties with either type of mammalian casein kinases, CK-1 and CK-2, but differs in its sensitivity to Ca^{2+}. Considering its possible relevance for exocytosis regulation, enriched protein kinase fractions were tested for their ability to phosphorylate several exogenous and endogenous substrates, including CaN and CaM from bovine brain as well as CaM and PP63 from *Paramecium*.

2. Materials and methods

2.1. Materials

Aprotinin, leupeptin, TAME, phosphocellulose, polylysine (mean size 36.6 kDa), polyarginine (mean size 45.5 kDa), spermine, heparin, casein from bovine milk (Sigma C4765), histone II-S from calf thymus, and CaN from bovine brain were obtained from Sigma (Deisenhofen, Germany). Bovine brain CaM was from Calbiochem (Bad Soden, Germany), DEAE-cellulose from Whatman (Maidstone, UK), Affi-Gel blue from BioRad (Munich, Germany) and pepstatin A from Serva (Heidelberg, Germany). Casein kinase II peptide substrates $R_2E_3TE_3$ and $R_3D_3SD_3$ were obtained from Biomol (Hamburg, Germany) and from Boehringer (Mannheim, Germany), respectively. Phenyl-Sepharose and SDS protein molecular weight markers (LMW) were from Pharmacia Biotechnology (Freiburg, Germany), [γ-^{32}P]ATP and [γ-^{32}P]GTP from Amersham-Buchler (Braunschweig, Germany). [γ-^{32}P]ATP was also prepared as previously described [42]. *Paramecium* CaM was a gift from Dr. J.E. Schultz, University of Tübingen, Germany. Other reagents and solvents used were of analytical grade.

2.2. Recombinant P63

PP63 cDNA was isolated from a λZAP cDNA library of *P. tetraurelia* and after changing all codons deviating from the universal genetic code into the universal codons the cDNA was expressed in *E. coli*. The recombinant protein contained a His_{10} tag and was purified by Ni^{2+}-affinity chromatography [43].

2.3. Cell cultures

P. tetraurelia wild type cells (strain 7S) were grown at 25°C in a sterile synthetic medium and processed as previously described [26].

2.4. Crude extract

Cells were harvested, concentrated and homogenized on ice in 50 ml of homogenization medium (HM) containing 20 mM triethanolamine-HCl, 10% glycerol, 1 mM dithioerythritol (DTE), 1 μM pepstatin A, 20 mU/ml aprotinin, 42 μM leupeptin, 0.26 mM TAME, pH 7.5, using an Ultraturrax type 18-10 (Janke & Kunkel KG, Staufen, Germany) for 30 s at 20 000 rpm. The homogenate was centrifuged at 100 000 × *g* for 60 min at 4°C. The supernatant was filtered through a layer of glass wool.

2.5. Isolation of casein kinase (CPK)

Crude extracts were prepared from 7.5–10 l cells using HM. The 100 000 × *g* supernatant (70 ml) was applied to a DEAE-cellulose column (diameter 1.9 cm, length 15 cm). The column was washed with 370 ml of HM and eluted with a linear gradient from 0 to 250 mM NaCl in HM. The total elution volume was 210 ml. Fractions of 5.0 ml were collected and assayed for casein kinase activity (see below). CPK containing fractions eluted at 180–200 mM NaCl were pooled (41 ml) and dialyzed against HM containing 1.2 M NaCl. Samples were applied to an equilibrated phenyl-Sepharose column (diameter 1.4 cm, length 15 cm). The column was washed with 71 ml of 1.2 M NaCl in HM and then eluted using a linear salt gradient (118 ml) ranging from 1.2 to 0 M NaCl. Fractions of 3.8 ml were collected and tested for CPK activity. Activity was eluted as a single peak at 300 mM NaCl. After dialyzing the pooled fractions (50 ml) against HM the CPK sample was loaded onto a Affi-Gel blue column (0.9 cm diameter, length 15 cm) equilibrated with the same buffer. After washing with 47 ml of HM the column was eluted with a linear salt gradient from 0 to 1.1 M NaCl in a total volume of 45 ml. Fractions of 1.1 ml were collected and assayed for CPK activity.

Alternatively, CPK, which eluted at 180–200 mM NaCl on DEAE-cellulose, was applied to an Affi-Gel blue column (diameter 1.4 cm, length 15 cm). The column was washed with 140 ml of HM and then eluted with a linear salt gradient (115 ml) ranging from 0 to 1.0 M NaCl. Fractions of 2.7 ml were collected and tested for CPK activity. Activity eluted as a single peak at 800 mM NaCl. After dialyzing the pooled fractions (13.2 ml) against HM the CPK sample was loaded onto a phosphocellulose column (diameter 0.6 cm, length 15 cm) equilibrated with HM. After washing with 10 ml of HM the column was eluted with a linear salt gradient from 0 to 500 mM NaCl in a total volume of 21 ml. Fractions of 0.5 ml were collected and assayed for CPK activity.

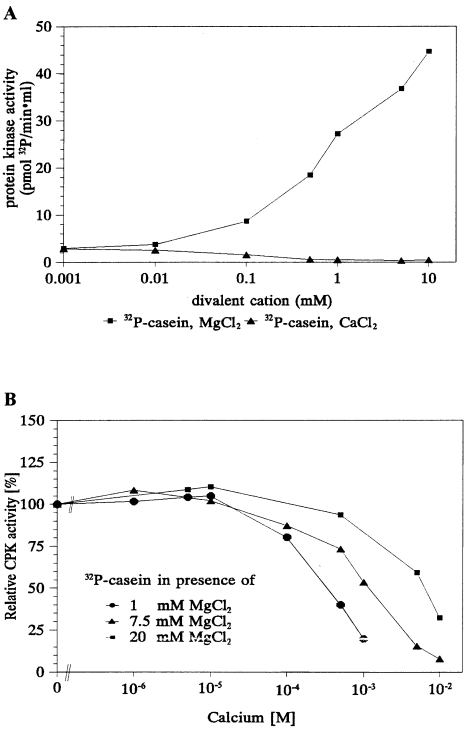

Fig. 2. Influence of Ca^{2+} on CPK activity. Influence of Ca^{2+} on the phosphorylation of casein by CPK was analyzed in either the absence (A) or presence of fixed concentration of Mg^{2+} (1, 7.5 or 20 mM) (B). Aliquots of chromatographically enriched CPK fractions (560 ng) were incubated for 25 min at 20°C in assays (0.04 ml) containing 120 μM ATP including [γ-^{32}P]ATP (10–25 MBq/μmol), 26.4 μg casein and 50 mM triethanolamine-HCl buffer, pH 7.2 in the presence of different concentrations of either $CaCl_2$ or $MgCl_2$ separately (A) or in combination as indicated (B). Note that Ca^{2+} cannot substitute for Mg^{2+} (A), whereas the Mg^{2+}-stimulated activity of CPK was inhibited by Ca^{2+} (B).

2.6. Assays for protein kinase activity

Standard assays for protein kinases were performed in a volume of 0.03 ml containing 5 mM $MgCl_2$, 120 μM ATP, [γ-^{32}P]ATP (10–25 MBq/μmol), 19.8 μg mixed casein and buffer (20 mM triethanolamine-HCl buffer, 10% glycerol, 1 mM DTE, pH 7.5) with or without Ca^{2+} as indicated. In some cases, ATP was replaced by 120 μM GTP including [γ-^{32}P]GTP (10–25 MBq/μmol). Reactions were started by adding protein kinase samples and, after 20 min at 20°C, terminated and measured by spotting 20 μl aliquots either onto trichloroacetic acid-prepared Whatman 1 Chr 3 MM filter papers [42] or, in the case of phosphate incorporation into peptide, by binding to phosphocellulose paper (Whatman P81) as described previously [44]. For further characterization standard assays were also run in the presence or absence of either 1 mM EGTA, 1 μM to 1 mM of polylysine, polyarginine, and spermine or 1–25 μg/ml of heparin. For determination of substrate specificity, casein was replaced by equal amounts of either histone II-S, histone III-S or the synthetic CK-2 peptides, RRRDDDSDDD [45] and RREEETEEE [46].

Casein phosphorylation was also analyzed in an assay (0.04 ml) containing different concentrations of divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} and Ni^{2+}, all as chlorides), 120 μM ATP including [γ-^{32}P]ATP (10 to 25 MBq/μmol), 26.4 μg casein and 50 mM triethanolamine-HCl buffer, pH 7.2. Reactions were started by adding 0.3–1.3 μg of chromatographically enriched CPK fractions and incubated for 25 min at 20°C.

2.7. Substrate specificity

Substrate specificity of CPK was analyzed either as described above or in a reaction mixture (0.08 ml) containing 5 mM $MgCl_2$, [γ-^{32}P]ATP (185 MBq/μmol), 2 μg of a protein substrate, 50 ng of chromatographically enriched CPK fractions and buffer (20 mM triethanolamine-HCl, 10% glycerol, 1 mM DTE, pH 7.5) in the presence or absence of either Ca^{2+} (0.1 to 2 mM), EGTA (1 mM) or polylysine (100 μM). Protein substrates tested were from either *Paramecium* (calmodulin, recombinant PP63), bovine brain (calmodulin, calcineurin) or bovine milk (mixed casein). At the end of incubation (20 min at 20°C) aliquots were stopped in Laemmli sample buffer, subjected to SDS-PAGE and then prepared for autoradiography (see below).

2.8. In situ gel kinase assay

The method described for CaM-dependent protein kinase II by Kameshita and Fujisawa [47] was modified to detect casein kinase activity. Aliquots of CPK fractions from the various chromatographic isolation steps were electrophoresed on 7.5% SDS polyacrylamide gels containing 1 mg/ml of dephosphorylated casein. After electrophoresis, SDS was removed by washing the gels with two changes of 20% 2-propanol in 50 mM Tris-HCl, pH 8.0, followed by two changes of buffer A (50 mM Tris-HCl, 5 mM 2-mercaptoethanol, pH 8.0), each for 30 min. The proteins in the gel were denatured with 6 M guanidine-HCl in buffer A for 1 h and then renatured with the same buffer containing 0.04% Tween 40 at 4°C for 16 h. Phosphorylation was performed by equilibrating the gels first in reaction buffer (20 mM triethanolamine-HCl, 10 mM $MgCl_2$, pH 7.5) at 20°C for 2 h and then in the same buffer (100 ml) supplemented with 9.25 MBq of [γ-^{32}P]ATP. After 2 h at 20°C gels were washed extensively in 5% trichloroacetic acid containing 1% sodium pyrophosphate, and prepared for autoradiography (see below). Prestained molecular mass standards were used to estimate the size of the phosphorylated bands.

2.9. Other methods

2.9.1. Protein determination. Protein determinations were performed according to Bradford [48] with chemicals obtained from Bio-Rad (Munich, Germany). BSA was used as a standard.

2.9.2. 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 either linear gradient (10–20%) or 7.5% SDS polyacrylamide gels using a discontinuous buffer system [49]. In some cases samples were alkylated for 30 min at 20°C by 2% iodoacetamide prior to electrophoresis. Protein standards (low molecular mass from Pharmacia LKB including myosin, 220 kDa, or Kaleidoscope prestained standards from BioRad) were used according to the manufacturer's directions. Gels were either stained with silver [50] or prepared for electrophoretic protein transfer onto nitrocellulose (NC) membranes (BA-85). This was performed at 1 mA/cm^2 for 1 h according to the technique of Kyhse-Andersen [51] using the semi-dry blotter from Ken-en-Tec (Copenhagen, Denmark).

2.9.3. Immunoblot analysis. Immuno-binding analysis was as described previously [52] using chromatographically enriched CPK fractions and a monoclonal antibody to the recombinant α-subunit of human CK-2.

2.9.4. Autoradiography. After SDS-PAGE gels were dried onto cellophane sheets under vacuum and exposed to either Kodak XRP-5 X-ray film or Amersham Hyperfilm-MP in Kodak X-Omatic cassettes with intensifier screens for 1–3 weeks at −70°C. The molecular weights of the radiolabeled proteins were estimated according to Winston [53]. Autoradiograms were also evaluated densitometrically using a Quick Scan Densitometer from Desaga (Heidelberg, Germany). For quantitation, relative peak areas were measured.

3. Results

3.1. Isolation of CPK

With mixed casein as a substrate, at least 3 different casein kinase activities were identified in the 100 000 × *g* supernatant of *Paramecium* cell homogenates by their separation on DEAE-cellulose (Fig. 1A). One activity peak (peak I) was found in flow-through fractions, whereas the other two peaks (peaks II and III) eluted at 90–110 mM NaCl and at 180–200 mM NaCl, respectively. Using mixed histones as a substrate, the peaks were distinct from those obtained with casein (data not shown). The third peak, also designated as CPK and showing enrichment by a factor of ∼50 after DEAE-cellulose chromatography (Table 1), was further purified by subsequent chromatography steps on phenyl-Sepharose (Fig. 1B) and Affi-Gel blue (Fig. 1C). When 2 mM Ca^{2+} was added, the activity of CPK, eluted at 300 mM NaCl from the phenyl-Sepharose column, was inhibited by ∼80% (Fig. 1B). Subsequent chromatography on Affi-Gel blue again resulted in a single activity peak at 750 mM NaCl (Fig. 1C).

Alternatively, CPK (peak III after DEAE-cellulose chroma-

Table 1
Isolation of CPK from *Paramecium* cell homogenates

Purification step	Volume (ml)	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (-fold)	Yield (%)
Homogenate	70.0	894.53	2.905	0.00325	–	–
100 000 × *g* supernatant	66.0	513.08	11.768	0.02294	1.0	100.0
DEAE-cellulose eluate	42.0	21.25	27.302	1.28467	56.0	232.0
Affi-Gel blue eluate	12.2	0.61	6.376	10.45302	455.7	54.2
Phosphocellulose eluate	2.5	0.04	0.997	24.93600	1087.2	8.5

Data are from a typical experiment. Isolation and assays were performed as described under Section 2. Specific activities were calculated in the presence of 5 mM Mg^{2+}, after 20 min of incubation, without Ca^{2+}.

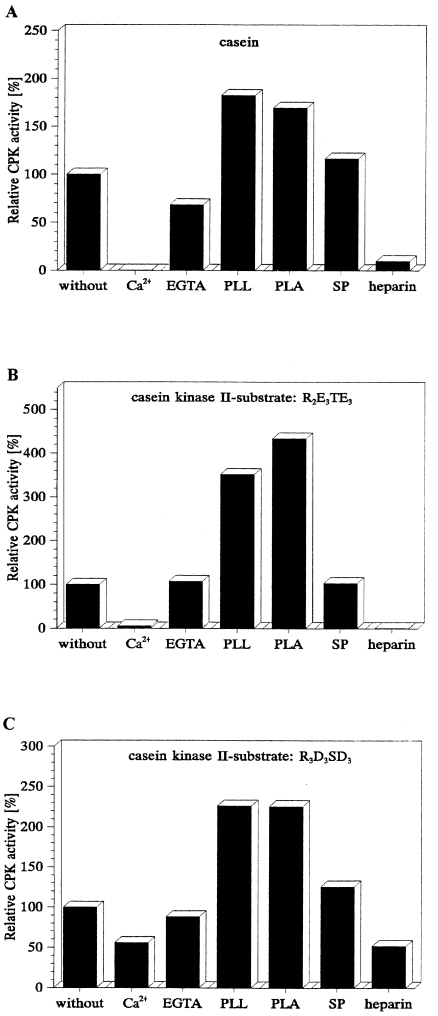

Fig. 3. Stimulatory and inhibitory effects of various ligands on the CPK activity towards casein and synthetic CK-2 peptides. Chromatographically enriched CPK fractions (280 ng) were analyzed in standard assays which were supplemented either with 2 mM $CaCl_2$, 1 mM EGTA, 1 μM polylysine (PLL), 1 μM polyarginine (PLA), 100 μM spermine (SP) or 1 μg/ml of heparin, respectively. Substrates tested were mixed casein (A), and synthetic CK-2 peptides, RREEETEEE (B) and RRRDDDSDDD (C), each in amounts of 19.8 μg. Note that with each substrate CPK activity can be stimulated by basic polypeptides such as polylysine and polyarginine, in contrast to polyamines (spermine). Ca^{2+} and polyanionic compounds, such as heparin, are inhibitory. CPK activity is not or only weakly affected by EGTA.

tography) was enriched by factor of ~1000 using two subsequent chromatography steps on Affi-Gel blue and phosphocellulose (Table 1). Via these steps CPK activity was separated from most of the other co-eluted proteins (Table 1).

3.2. Dependence of CPK activity on divalent cations

CPK activity isolated in these two different ways (Fig. 1 and Table 1) was completely dependent on Mg^{2+}, but was depressed, rather than stimulated, by Ca^{2+} (Fig. 2, Table 2). Rising Mg^{2+} concentrations resulted in an increase in CPK activity (Fig. 2A), with maximal stimulation at 20 mM (data not shown). Table 2 shows that no other divalent cation could substitute for Mg^{2+}. At concentrations of 1 mM, Ca^{2+}, Mn^{2+}, Ni^{2+} or Zn^{2+} resulted only in a small percentage of the activity obtained with Mg^{2+}. When CPK activity was assayed in

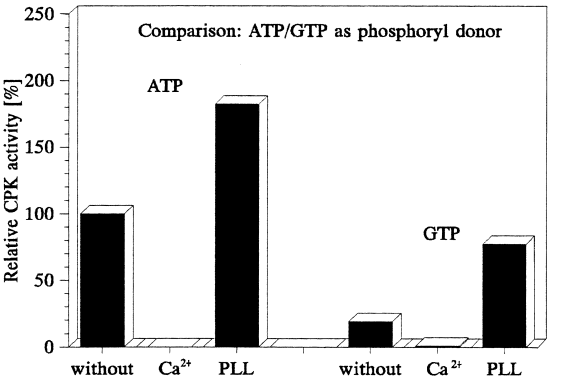

Fig. 4. Effect of phosphate donor on the phosphorylation of casein by CPK. Chromatographically enriched CPK fractions (280 ng) were analyzed for casein phosphorylation (19.8 μg) in standard reaction mixtures containing either 120 μM ATP including [γ-^{32}P]ATP (10–25 MBq/μmol) or with replacement of ATP by equal amounts of GTP including [γ-^{32}P]GTP. The effects of Ca^{2+} (2 mM) or polylysine (1 μM) were compared with control assays (without additives). Note that GTP can substitute for ATP as phosphate donor, although GTP is by factor ~5 less efficient than ATP. Inhibitory or stimulatory effects by Ca^{2+} or polylysine also occur with GTP as phosphate donor.

the presence of different concentrations of Mg^{2+} we consistently observed inhibition with rising concentrations of Ca^{2+} (Fig. 2B). With Mg^{2+} at concentrations of 1, 7.5 and 20 mM, the Ca^{2+} concentrations necessary for half-maximal inhibition of CPK activity were 300 μM, 1.2 mM and 7 mM, respectively. These data explain the significant inhibition of CPK activity during phenyl-Sepharose chromatography using 5 mM Mg^{2+} and 2 mM Ca^{2+} (Fig. 1B).

3.3. Influence of CK-2 ligands on CPK

Further enzymic characteristics of chromatographically enriched CPK are summarized in Fig. 3. Basic polypeptides such as polylysine and polyarginine stimulate CPK activity in vitro by factor of 2–5, not only with casein (Fig. 3A), but also with CK-2 specific synthetic peptide substrates, RREEETEEE (Fig. 3B) and RRRDDDSDDD (Fig. 3C). Thereby, the phosphorylation capacity of CPK does not discriminate between phosphorylation sites on Thr or Ser residues. Spermine, also known to stimulate CK-2 activity in vitro [2], does not or only weakly increases the activity of CPK towards all substrates tested (Fig. 3A–C), even when tested up to 1 mM (data not shown). In contrast, Ca^{2+} and heparin are inhibitory. However, the extent of inhibition depends on the substrate used.

Table 2
CPK activity as a function of different divalent cations

Assay	Specific activity (pmol ^{32}P/min per mg)	Relative activity (%)
1.0 mM Ca^{2+}	7.9	1.8
1.0 mM Mn^{2+}	85.5	20.1
1.0 mM Ni^{2+}	63.8	14.9
1.0 mM Zn^{2+}	19.6	4.6
1.0 mM Mg^{2+}	425.4	100

CPK activity was measured in a reaction mixture (0.04 ml) containing 50 mM triethanolamine-HCl, pH 7.2, 120 μM ATP including [γ-^{32}P]ATP (10–25 MBq/μmol), 26.4 μg casein, 280 ng CPK, and 1 mM of different divalent cations. Assays were for 25 min at 20°C. Note a strong dependency of CPK on Mg^{2+}.

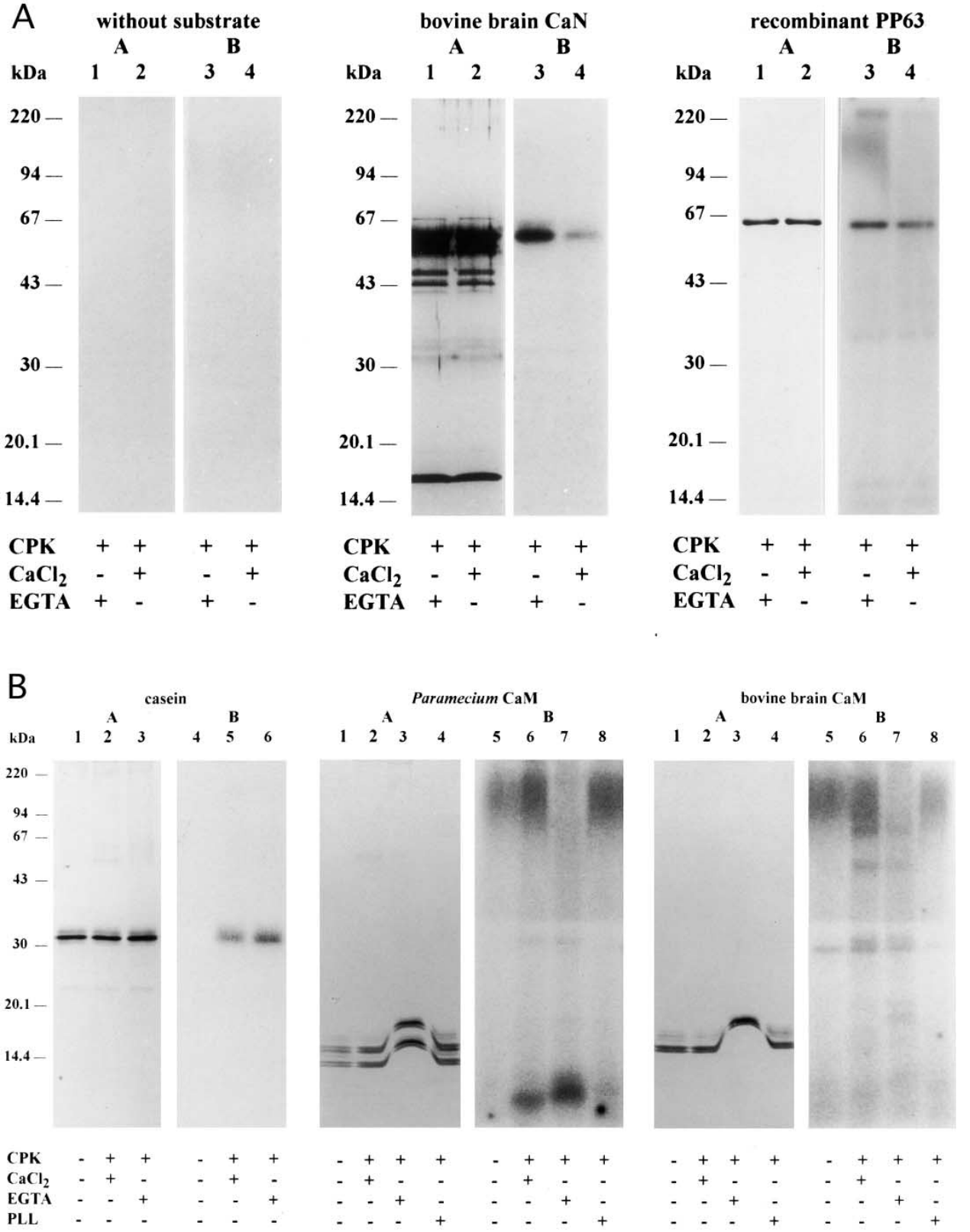

Fig. 5. Substrate specificity of CPK. CPK partially purified from the 100 000 × *g* supernatant of whole cell homogenates was assayed either for its capacity of autophosphorylation (panel A, left) or of phosphorylating substrates in vitro, i.e. bovine brain CaN (panel A, middle), recombinant PP63, (panel A, right), casein from bovine milk (panel B, left) and CaM either from *Paramecium* (panel B, middle) or from bovine brain (panel B, right). Assays were performed in a volume of 80 µl containing 20 mM triethanolamine-HCl, 10% glycerol, 1 mM DTE, 5 mM $MgCl_2$, and [γ-^{32}P]ATP (185 MBq/µmol), pH 7.5 in the presence or absence of either 2 µg protein substrate, 50 ng CPK fractions, 0.1 or 2 mM Ca^{2+}, 1 mM EGTA or 100 µM polylysine (PLL), as indicated. Incubations at 20°C were terminated after 20 min by adding 80 µl 'sample buffer'. Aliquots of 80 µl were subjected to SDS-PAGE, silver stained (lanes A) and processed for autoradiography (lanes B) as described

Whereas phosphorylation of casein (Fig. 3A) and of the synthetic peptide RREEETEEE (Fig. 3B) has been almost completely inhibited by these ligands, only ~50% inhibition is observed with peptide substrate RRRDDDSDDD (Fig. 3C) suggesting that inhibitory effects of Ca^{2+} and heparin on CPK activity are also influenced by the structure of the protein or peptide substrate. In addition to ATP, CPK also utilized GTP in the phosphotransferase reaction. However, GTP has only 20% of the effectivity of ATP when assayed under otherwise identical conditions (Fig. 4). Inhibitory or stimulatory effects by either Ca^{2+} or polylysine also occur with GTP as phosphate donor (Fig. 4).

3.4. Substrate specificity

Casein and the CK-2 specific synthetic peptide substrates, RREEETEEE and RRRDDDSDDD, were phosphorylated in vitro by CPK (Fig. 3A–C), whereas other protein substrates, such as mixed histones (histones II-S and III-S), were not (data not shown). To assign CPK to one of the two established casein kinase families, CK-1 and CK-2, another in vitro-phosphorylation study, shown in Fig. 5, was performed. We used several CPK effectors and established substrates which are reportedly specific either for CK-1 (bovine brain CaN), CK-2 (bovine brain CaM), or for both, CK-1 and CK-2 (mixed casein). Beyond that, we also assayed CPK for possible *Paramecium* substrates involved in exocytosis regulation such as CaM and PP63 (see Section 1), the latter in recombinant form.

With bovine brain CaN as substrate (Fig. 5, panel A, middle), we observed a strong phosphorylation of the catalytic subunit (61 kDa) in the absence of Ca^{2+} (lane 3) which was decreased by ~80%, when Ca^{2+} (100 μM) was present (lane 4). In this case, inhibition by Ca^{2+} is much more pronounced than with casein as a substrate (Fig. 5, panel B, left, lane 5). Although CPK was not fully stimulated in presence of 5 mM $MgCl_2$ (Fig. 2A), the catalytic subunit of bovine brain CaN seems to be a good substrate for this *Paramecium* casein kinase.

PP63, which previously has been demonstrated to be a substrate for CPK in *Paramecium* crude cell extracts [26] is now shown to be phosphorylated by CPK as a recombinant protein in a Ca^{2+}-inhibitable manner (Fig. 5, panel A, right, bars 3,4). These data indicate that the inhibitory effect of Ca^{2+} on CPK activity was not restricted to casein but also occurs with recombinant PP63 or with the catalytic subunit of bovine brain CaN.

The regulatory subunit of CaN (17 kDa) was not phosphorylated (Fig. 5, panel A, middle, lanes 3,4). Beyond that, neither CaM from wild type *Paramecium* cells (Fig. 5, panel B, middle) nor that from bovine brain (Fig. 5, panel B, right) was phosphorylated by CPK regardless of whether Ca^{2+} was present (lanes 6) or absent (lanes 7). As shown in the corresponding silver stain (lanes 1–4), both calmodulins underwent a mobility shift when EGTA was present in the assay mixtures (lanes 3). Although the phosphorylation of most of the substrates by mammalian CK-2 occurs in the absence of any effectors, the phosphorylation of CaM is strongly dependent upon the addition of polycationic effectors [54,55]. Therefore, polylysine was included in the assay. Even in the presence of 100 μM polylysine, CPK failed to phosphorylate CaM from both sources (lanes 8). However, the *Paramecium* CaM fraction (Fig. 5, panel B, middle) also contains several other Ca^{2+}-binding proteins below the migration band of CaM, of which one of them – not analyzed in detail – was phosphorylated by CPK, and underwent a characteristic mobility shift in the presence of EGTA (lane 7).

We also assayed bovine brain CaN as well as CaM from the two species (bovine brain and *Paramecium*) with PKA or with PKG, both of which were isolated from the $100\,000 \times g$ supernatant of *Paramecium* whole cell homogenates [26]. Like CPK, PKA, but not PKG, was able to phosphorylate the catalytic subunit of CaN (data not shown). Also like CPK, PKA could phosphorylate neither the regulatory subunit of CaN nor CaM from both species (data not shown). However, any interference of PKA with CPK can be excluded in our experiments.

CPK does not undergo autophosphorylation (Fig. 5, panel A, left) in either the presence or absence of Ca^{2+} (lanes 3,4). The same is true when assays contained additional polylysine (data not shown). Although we employed a very sensitive silver staining procedure [50], CPK could not be stained in the corresponding SDS-polyacrylamide gel (lanes 1,2). Therefore, we used an in situ gel kinase assay to detect the protein band possessing the catalytic activity for CPK. As shown in Fig. 6, radioactive phosphate was incorporated into casein with all CPK fractions obtained during the isolation procedure, forming a band of 36 kDa (lanes 4–6). Additional bands of ~150 kDa, 45 kDa and predominantly below 36 kDa occur with CPK fractions of the DEAE-cellulose eluate (lane 4). Incorporation of radioactive phosphate was not observed in any of the CPK fractions when assayed under conditions of autophosphorylation without casein (lanes 1–3).

To explore the relationship of CPK to mammalian type 2 casein kinase, a monoclonal antibody raised against recombinant α-subunit of human casein kinase 2 (clone 1AD9, Boehringer-Mannheim) was used to probe immunoblots of various CPK fraction obtained during chromatographic isolation. However, the antibody did not react in any of the CPK fractions tested (data not shown).

4. Discussion

4.1. Biochemical aspects

The CPK we isolated shares several properties with both types of mammalian CK-1 and CK-2, but differs in its inhibition by Ca^{2+}. Thus, this kinase is different from any of the Ca^{2+}-dependent protein kinases from *Paramecium* which also phosphorylate casein [21,22]. These types also differ from

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under Section 2. Note that CPK does not undergo autophosphorylation (panel A, left), irrespective of whether the assay contained EGTA (lane 3) or 2 mM $CaCl_2$ (lane 4). In contrast, both the catalytic subunit of bovine brain CaN (panel A, middle), and recombinant PP63 (panel A, right), were phosphorylated by CPK (lanes 3 vs. 4), however, significantly less in the presence of 0.1 mM Ca^{2+} with CaN (lane 4, middle panel) or 2 mM Ca^{2+} with recombinant PP63 (lane 4, right panel). In the case of casein (panel B, left), phosphorylation by CPK (lane 6) is also reduced when 2 mM Ca^{2+} was present in the assay (lane 5). However, the regulatory subunit of bovine brain CaN was not phosphorylated by CPK (panel A, middle), in the presence of either EGTA (lane 3) or 100 μM Ca^{2+} (lane 4). The same is true for CaM from *Paramecium* (panel B, middle) or from bovine brain (panel B, right), not even when the reaction mixtures contained 100 μM polylysine (lanes 8).

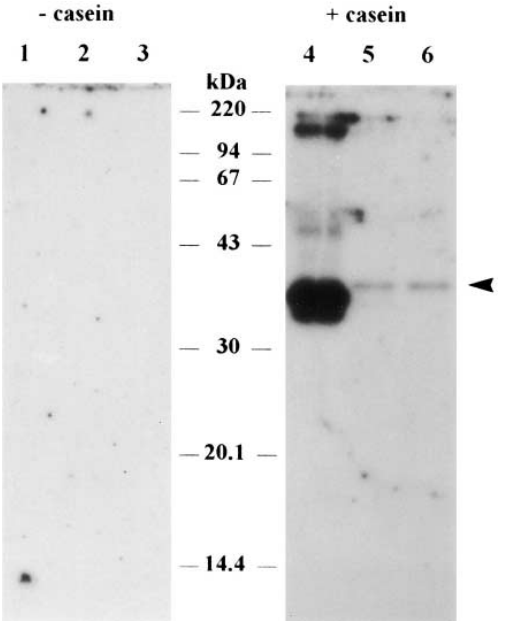

Fig. 6. In situ gel kinase assay for CPK. Aliquots (10 μl) of CPK fractions obtained during chromatography on DEAE-cellulose (lanes 1,4), Affi-Gel blue (lanes 2,5) and phosphocellulose (lanes 3,6) were electrophoresed on 7.5% SDS polyacrylamide gels, in which either 1 mg/ml of casein was added to the matrix prior to polymerization (lanes 4–6) or not (lanes 1–3). After SDS-PAGE, CPK was processed for in situ phosphorylation of casein. Note that radioactive phosphate was incorporated into casein, forming a band at 36 kDa (lanes 4–6). In lane 4, showing casein phosphorylation by CPK fractions obtained after the first chromatographic step, a strong band of unknown identity occurs below the 36 kDa CPK band, with additional bands of 150 and 45 kDa. No incorporation of radioactive phosphate was observed in any of the CPK fractions when assayed under conditions of autophosphorylation (lanes 1–3).

mammalian protein kinase C and Ca^{2+}/CaM-dependent protein kinases [21,22] which have not yet been identified in *Paramecium*. Also, in contrast to known *Paramecium* protein kinases (all being Ser/Thr protein kinases), our CPK does not undergo autophosphorylation, in either the presence or absence of Ca^{2+}. This is in contrast to the two endogenous Ca^{2+}-dependent protein kinases of 50 and 55 kDa [21] and of 52–53 kDa [22], respectively, as well as to PKA and PKG from *Paramecium* showing autophosphorylation bands of 44–52 kDa [19] and 77 kDa [17], respectively. The four second messenger-independent protein kinases known from *Paramecium*, probably of the casein kinase type I family, are also not autophosphorylated [23]. Three of these casein kinases (CKS1, CKS2 and CKA) which were isolated from cilia and one form (65 kDa) from a particulate fraction [23] differ from CPK in (i) their subcellular localization, (ii) their molecular masses by in situ gel kinase analysis, (iii) their heparin sensitivity, and (iv) by their failure to be inhibited by Ca^{2+}.

Since in *Paramecium* CK-2 members are unknown, the properties of CPK should be compared with those from mammalian and other species. Similarity to CK-2 is based on the following aspects. (i) Whereas CK-1 elutes from DEAE-cellulose at relatively low ionic strength, CK-2 elutes between 0.15 and 0.25 M NaCl [3] as does CPK. (ii) CK-2 is unique among the protein kinases in also using GTP as a phosphoryl donor instead of ATP [2,3], just like CPK. (iii) CPK can phosphorylate in vitro CK-2 specific synthetic peptides, RREEETEEE [46] and RRRDDDSDDD [45], on either Thr- or Ser-residues. (iv) Members of the CK-2 family show extreme sensitivity towards heparin ($IC_{50} \leq 1$ μg/ml) and can be activated by basic compounds such as polyamines and polylysine [2,3]. For CPK activity, heparin and polylysine fulfil these expectations, whereas spermine does not. However, it is problematic to apply only these criteria to discriminate between type 1 and type 2 casein kinases, since all these ligands affect the activity not only of CK-2 [2] but also of CK-1 [6,56,57].

Properties arguing against CPK as a member of the CK-2 family are summarized as follows. (i) Identification of a 36 kDa catalytic subunit by in situ gel kinase analysis and (ii) the lack of autophosphorylation resembles CK-1, rather than CK-2, the latter undergoing autophosphorylation of the β-subunit in the absence of polybasic peptides [2]. (iii) This is also supported by the lack of binding of CK-2 specific antibodies by CPK enriched fractions. iv) Substrate specificity, especially the capacity to phosphorylate mammalian CaN, is in favor of CPK membership to casein kinase type 1, rather than to type 2, since only type 1 is able to phosphorylate mammalian CaN [35]. However, no data are available on their inhibition by Ca^{2+}. (v) CPK failed to phosphorylate not only CaM from *Paramecium* but also CaM from bovine brain, even in the presence of polybasic polypeptides up to 100 μM. Since phosphorylation of mammalian CaM in vitro was restricted to tyrosine kinases [38] and type II casein kinases [39,55], our data argue rather against CPK being a member of the CK-2 family.

Since CPK activity depends on the absence of Ca^{2+}, the question arises as to whether Ca^{2+} inhibition is unique among the (casein) kinases identified thus far. Micromolar Mn^{2+}, Zn^{2+} Co^{2+}, Ni^{2+} and Fe^{2+} inhibit the Mg^{2+}-stimulated activity of CK-1 from bovine kidney, whereas Ca^{2+} does not [58]. CK-2 from bovine brain is also inactivated by millimolar Mn^{2+}, Co^{2+} and Mg^{2+} [59]. 50 μM Ca^{2+} antagonizes the polycation-dependent phosphorylation of calf brain CaM by CK-2 from rat liver [55]. However, the authors assumed a reciprocal antagonism between phosphorylation and Ca^{2+}-binding capacity of CaM without interfering with the activity of CK-2 [55]. Since Ca^{2+} inhibition of *Paramecium* CPK is not only restricted to casein or CaN, but also occurs with all other substrates tested, Ca^{2+} probably directly interacts with the enzyme, rather than with the substrate.

Taken together CPK seems to be a new type of casein kinase which shares several properties with either type of mammalian casein kinases, CK-1 and CK-2, but which also differs in its characteristic inhibition by Ca^{2+}. The presence of at least one other type of casein kinase has also been shown for several other cell types, such as bovine kidney [60], HeLa cell nuclei [61] or yeast [62] combining properties of type 1 and type 2 casein kinases. However, the significant Ca^{2+} inhibition observed with CPK in *Paramecium* has not been described for these types.

4.2. Possible implications for exocytosis regulation

CaM is considered to be involved in exocytosis regulation not only in *Paramecium* [27,40,41] but also in mammalian cells [63]. From our results we can exclude any effect of CaM phosphorylation on trichocyst exocytosis. CaN is also involved in trichocyst exocytosis [27] and bovine brain CaN dephosphorylates PP63 in vitro [26]. Concomitantly, an endogenous protein phosphatase sharing several important char-

acteristics with CaN from mammalian species (R. Kissmehl, T. Treptau, B. Kottwitz and H. Plattner, unpublished results) dephosphorylates PP63 in vitro [26]. We now could show that both CaN from bovine brain and recombinant PP63 (this paper) as well as original PP63 [26], can be fully phosphorylated in vitro by *Paramecium* CPK when Ca^{2+} is absent.

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