Protein phosphatase and kinase activities possibly involved in exocytosis regulation in *Paramecium tetraurelia*

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In *Paramecium tetraurelia* cells synchronous exocytosis induced by aminoethyldextran (AED) is accompanied by an equally rapid dephosphorylation of a 63 kDa phosphoprotein (PP63) within 80 ms. *In vivo*, rephosphorylation occurs within a few seconds after AED triggering. In homogenates (P)P63 can be solubilized in all three phosphorylation states (phosphorylated, dephosphorylated and rephosphorylated) and thus tested *in vitro*. By using chelators of different divalent cations, de- and rephosphorylation of PP63 and P63 respectively can be achieved by an endogenous protein phosphatase/kinase system. Dephosphorylation occurs in the presence of EDTA, whereas in the presence of EGTA this was concealed by phosphorylation by endogenous kinase(s), thus indicating that phosphorylation of P63 is calcium-independent. Results obtained with protein phosphatase inhibitors (okadaic acid, calyculin A) allowed us to exclude a protein serine/threonine phosphatase of type 1 (with selective sensitivity in *Paramecium*). Protein phosphatase 2C is also less likely to be a candidate because of its requirement for high Mg^{2+} concentrations. According to previous evidence a protein serine/threonine phosphatase of type 2B (calcineurin; CaN) is possibly involved. We have now found that bovine brain CaN dephosphorylates PP63 *in vitro*. Taking into account the specific requirements of this phosphatase *in vitro*, with *p*-nitrophenyl phosphate as a substrate, we have isolated a cytosolic phosphatase of similar characteristics by combined preparative gel electrophoresis and affinity-column chromatography. In *Paramecium* this phosphatase also dephosphorylates PP63 *in vitro* (after ^{32}P labelling *in vivo*). Using various combinations of ion exchange, affinity and hydrophobic interaction chromatography we have also isolated three different protein kinases from the soluble fraction, i.e. a cAMP-dependent protein kinase (PKA), a cGMP-dependent protein kinase (PKG) and a casein kinase. Among the kinases tested, PKA cannot phosphorylate P63, whereas either PKG or the casein kinase phosphorylate P63 *in vitro*. On the basis of these findings we propose that a protein phosphatase/kinase system is involved in the regulation of exocytosis in *P. tetraurelia* cells.

INTRODUCTION

Reversible protein phosphorylation plays an essential role in controlling many cellular processes [1,2]. Such a mechanism is also discussed for the regulation of exocytosis in different cell types [3] including *Paramecium tetraurelia* [4–6]. In this unicellular eukaryote a soluble phosphoprotein of 63 kDa (PP63) could be selectively dephosphorylated in response to picric acid-triggered trichocyst release [7]. With the non-cytotoxic secretagogue aminoethyldextran (AED) such a dephosphorylation step was restricted to exocytosis-competent strains and was shown to be very rapid (1 s) and reversible within 5–20 s [8]. An even more strict time correlation between dephosphorylation of PP63 to its dephosphorylated form (P63) and membrane fusion induced by AED was achieved by quenched-flow analysis, both processes occurring within 80 ms [9]. Moreover, this allowed the partial characterization of this exocytosis-sensitive phosphoprotein in its different phosphorylation states. PP63 exists in several isoforms with isoelectric points (pI) between 5.75 and 6.05 [9,10]. All pI forms are extensively dephosphorylated during synchronous exocytosis, including the most intensively phosphorylated form of pI 5.95, which is also most intensely rephosphorylated after exocytosis [9]. They all are sensitive to alkali treatment but insensitive to acid treatment, thus implicating serine/threonine phosphorylation [9,10].

On the other hand it also has been reported that PP63 could be phosphoglucosylated *in vitro* by addition of Glc-1-P, catalysed by αGlc-1-P phosphotransferase utilizing UDP-Glc, to a short chain of mannose residues O-linked to a serine [11,12]. Furthermore, in such *in vitro* assays the amount of glucose label incorporated in PP63 was reported to be reduced in the presence of Ca^{2+}, which caused the authors to conclude that dephosphorylation of PP63 during exocytosis may be Ca^{2+}-dependent and attributed to removal of Glc-1-P by a membrane-associated Glc-1-P phosphodiesterase [12]. This concept was also supported by the finding that, in an exocytosis-deficient mutant (nd9), the activation of the alleged phosphodiesterase may be defective [12]. At the same time, however, the same authors also maintained additional seryl-phosphorylation *via* a Ca^{2+}-dependent protein kinase, which was postulated to be involved in Ca^{2+}-dependent rephosphorylation of P63 after exocytosis [12]. In their model deglucosylation was assumed to cause redistribution of P63 to membranes where such a kinase would be present and where rephosphorylation might reverse this process causing the dissociation of PP63 from membranes [12].

However, there is no proof of the existence of such a cycle *in vivo*. Furthermore, the existence of the respective enzymes, e.g. α-Glc-1-P phosphotransferase, Glc-1-P phosphodiesterase, protein kinase C or Ca^{2+}/calmodulin (CaM)-dependent protein kinase, has so far not been shown in *Paramecium* cells. Previously several protein kinases have been identified and purified in *P. tetraurelia*, i.e. two types of cAMP-dependent protein kinase (PKA) [13–15], one cGMP-dependent protein kinase (PKG) [16] and two forms of a soluble Ca^{2+}-dependent protein kinase

Abbreviations used: AED, aminoethyldextran; CaM, calmodulin; CaN, calcineurin; P63, dephosphorylated 63 kDa phosphoprotein; PP63, phosphorylated/rephosphorylated 63 kDa phosphoprotein; PP-1, PP-2A, PP-2B and PP-2C, protein phosphatase 1, 2A, 2B and 2C respectively; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PNPP, *p*-nitrophenyl phosphate; PNP, *p*-nitrophenol.

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[17,18]. However, a direct involvement of these protein kinases in the regulation of exocytosis has not been demonstrated. The same is true for another type of second messenger-independent protein kinase, using casein as a substrate, of which three forms are found in the ciliary fraction, while a fourth form occurs in the particulate fraction of whole cells as well as in isolated cortices [19].

In contrast, we previously found evidence that, in *Paramecium*, a Ca^{2+}/CaM-dependent protein phosphatase [PP-2B; also known as calcineurin (CaN)] might be involved in the regulation of exocytosis [20]. Therefore a phosphatase of CaN type could be a candidate for PP63 dephosphorylation. However, until now there has been only restricted information on the existence of protein phosphatase PP-2B in *Paramecium* [20,21] whereas some other protein serine/threonine phosphatases have been isolated [22] and characterized, including PP-1 [23,24], PP-2A [23] and PP-2C [25].

In the present study we confirm the strict correlation between synchronous trichocyst exocytosis and dephosphorylation of the 63 kDa phosphoprotein by using an improved isolation scheme for the preservation of the three phosphorylation states of (P)P63. Beyond this, an analysis of the PP63-relevant enzymes, all occurring in the soluble fraction, strongly suggests participation of a phosphatase/kinase system, rather than of a phosphodiesterase/phosphotransferase system for Glc-1-P. We show that PP63 ^{32}P-labelled *in vivo* can be dephosphorylated by bovine brain CaN as well as by an enriched endogenous *p*-nitrophenyl phosphate (PNPP) phosphatase of similar properties. Rephosphorylation can be achieved either Ca^{2+}-independently by a cGMP-dependent protein kinase or by a new type of casein kinase that is inhibited by Ca^{2+} (T. Treptau, R. Kissmehl, H. W. Hofer and H. Plattner, unpublished work). Both were enriched from the soluble *Paramecium* fraction. Because we used (P)P63 in the actual phosphorylation state after labelling *in vivo*, our results strongly suggest the relevance of reversible protein phosphorylation, rather than of glucophosphorylation, for exocytosis regulation.

MATERIALS AND METHODS

Materials

Phosphocellulose, heparin–agarose, cAMP, cGMP, aprotinin, leupeptin, TAME, casein, histone-II-S, calcineurin and Azocoll were obtained from Sigma (Deisenhofen, Germany). DEAE-cellulose was from Whatman (Maidstone, Kent, U.K.). Affi-Gel Blue was purchased from Bio-Rad (Munich, Germany); PNPP was from Boehringer Mannheim (Germany) and pepstatin A from Serva (Heidelberg, Germany). Phenyl-Sepharose and SDS protein molecular mass markers (LMW) were purchased from Pharmacia LKB Biotechnology (Freiburg, Germany); okadaic acid and calyculin A were from Bio-Trend (Cologne, Germany) and carrier-free [^{32}P]P_i and [γ-^{32}P]ATP from Amersham-Buchler (Braunschweig, Germany). We also used [γ-^{32}P]ATP and phosvitin–Sepharose, which were both prepared as described by Thalhofer et al. [26]. Other reagents and all solvents used were of analytical grade.

Cell cultures

Paramecium tetraurelia wild-type cells (strain 7S) were grown at 25 °C to early stationary phase in a sterile synthetic medium [27]. Cells were harvested and rinsed twice in 5 mM Pipes/HCl buffer, pH 7.0, supplemented with 1 mM KCl and 0.1 mM $CaCl_2$ as previously described [28]. For isolation of endogenous protein kinases or phosphatases, cells were additionally rinsed twice in 20 mM triethanolamine/HCl buffer, pH 7.5, or in 20 mM Tris/HCl buffer, pH 7.0, respectively.

Exocytosis triggering

Synchronous exocytosis was induced by 0.01 % (equivalent to 2.5 μM) aminoethyldextran (AED; 40 kDa, one NH_2 group per kDa of dextran) as previously described [29]. To determine the trigger effect of this non-cytotoxic secretagogue, aliquots were additionally triggered with picric acid [30]. Although lethal, picric acid releases residual trichocysts not released by AED. Only cultures with an exocytosis capacity of more than 90 % in response to AED were used for phosphorylation studies *in vivo*.

Labelling of *Paramecium* cells *in vivo* with ^{32}P

The method used for labelling cells *in vivo* was a slight modification of that described previously [9]. Briefly, cells (5×10^4/ml) were incubated with 2.3–3.0 MBq/ml of carrier-free [^{32}P]P_i for 90 min at room temperature. Under these conditions PP63 and some other endogenous proteins were also intensely phosphorylated. Samples were taken either without AED-triggering (phosphorylated state) or 2 s or 1 min after AED-triggering, representing the dephosphorylated and rephosphorylated states, P63 and PP63, respectively [31].

Isolation of (P)P63

^{32}P-Labelled untriggered or AED-triggered cells were homogenized by twice freezing in liquid nitrogen (to maintain phosphorylation states) and thawing. Samples were centrifuged at 100000 ***g*** for 30 min at 4 °C in an Airfuge, rotor A-95 (Beckman, Munich, Germany). In each case P63 or PP63 was found in the supernatant.

Isolation procedure for soluble protein kinases and PNPP-phosphatases

Crude extracts

Axenically grown cells were harvested as described above and homogenized in either 50–70 ml of buffer A (20 mM triethanolamine/HCl, 10 % glycerol, 1 μM pepstatin A, 20 m-units/ml aprotinin, 42 μM leupeptin, 0.26 mM *N*-*p*-tosyl-L-arginine methyl ester, pH 7.5) or buffer B (buffer A + 1 mM dithioerythritol, pH 7.5) or 15–30 ml of buffer C (20 mM Tris/HCl, 2 μM pepstatin A, 40 m-units/ml aprotinin, 84 μM leupeptin, 0.52 mM *N*-*p*-tosyl-L-arginine methyl ester, pH 6.8) with an Ultraturrax type 18-10 (Janke & Kunkel KG, Staufen, Germany) for 30 s at 20 000 rev./min. The homogenate was centrifuged at 100000 ***g*** for 60 min at 4 °C. The supernatant was filtered through a layer of glass wool.

Isolation of PKA

For isolation of PKA, crude extracts were prepared from 17 litres of cell culture with buffer A. The 100000 ***g*** supernatant (51 ml) was applied to a chromatography column (diameter 1.4 cm, length 15 cm) filled with DEAE-cellulose by the method of Schultz and Jantzen [13]. The column was washed with 200 ml of buffer A and then eluted with a linear gradient from 0 to 300 mM NaCl in buffer A. The total elution volume was 115 ml.

Fractions of 2.4 ml were collected and assayed for PKA activity (see below). Fractions containing PKA activities, eluted with 50–60 mM NaCl, were pooled (18.6 ml), dialysed against buffer A and applied to a phosvitin–Sepharose column (diameter 1.4 cm, length 15 cm) equilibrated with buffer A. The column was washed with 135 ml of buffer A and then eluted with a linear salt gradient (130 ml) from 0 to 300 mM NaCl in buffer A. Fractions of 2.7 ml were collected and tested for PKA activity. PKA activity was eluted as a single peak at approx. 50 mM NaCl. After dialysing the pooled fractions (13.8 ml) against buffer A the PKA sample was loaded on to a heparin–agarose column (diameter 1 cm, length 15 cm) equilibrated with the same buffer. The column was washed with 70 ml of buffer A and then eluted with a linear gradient from 0 to 300 mM NaCl in a total volume of approx. 27 ml. Fractions of 0.6 ml were collected and assayed for casein kinase activity. PKA was eluted at approx. 50–60 mM NaCl.

Isolation of PKG

For isolation of PKG the purification scheme of Miglietta and Nelson [16] was modified. The 100000 ***g*** supernatant (65 ml) from 12.5 litres of cell culture was prepared in buffer B and applied to a chromatography column (diameter 1.9 cm, length 15 cm) filled with DEAE-cellulose. The column was washed with 275 ml of buffer B and then eluted with a linear gradient from 0 to 300 mM NaCl in buffer B. The total elution volume was 240 ml. Fractions of 5 ml were collected and assayed for PKG activity (see below). The flow-through fractions containing PKG activities were pooled (51 ml) and loaded on to an Affi-Gel Blue column (diameter 1.4 cm, length 15 cm) equilibrated with buffer B. The column was washed with 180 ml of buffer B and then developed in the same buffer with a linear salt gradient from 0 to 1.0 M NaCl in a total volume of 115 ml. Fractions (each 2.4 ml) containing PKG activity were pooled, dialysed against buffer B and applied (41 ml) to a phosphocellulose column (diameter 1 cm, length 15 cm) equilibrated with buffer B. After washing with 100 ml of buffer B, PKG was eluted with a linear salt gradient from 0 to 500 mM NaCl. The total elution volume was 60 ml and each fraction contained 1.4 ml. PKG-containing fractions were pooled, dialysed against buffer B and stored at −80 °C.

Isolation of casein kinase

For isolation of casein kinase we used a protocol described in detail by T. Treptau, R. Kissmehl, H. W. Hofer and H. Plattner (unpublished results; details available from R. K.). Briefly, casein kinase was enriched from the 100000 ***g*** supernatant by subsequent chromatographic steps on DEAE-cellulose and phenyl-Sepharose, followed by affinity chromatography on Affi-Gel Blue. Casein kinase was assayed as described below.

Isolation of PNPP phosphatase

The 100000 ***g*** supernatant (20 ml) from 7.5 litres of cell culture was prepared in buffer C as described above, mixed with an equal amount of buffer E (125 mM Tris/HCl, 0.005 % Bromophenol Blue, pH 6.8) and subjected to preparative non-denaturing gel electrophoresis with the Prep Cell Model 491 (Bio-Rad). Electrophoresis was performed with a 12.5 % polyacrylamide resolving gel and a 4 % stacking gel, each poured to 5 cm height in the 37 mm diameter tube of the preparative gel apparatus (volume 53.7 ml). The dye front was eluted from the gel after approx. 12 h at a constant 40 mA. Starting from the dye front, fractions of 2.5 ml were collected at an elution rate of 1 ml/min in buffer A. Fractions were collected for 14 h and assayed for PNPP phosphatase activity (see below). PNPP phosphatase was eluted as a single activity peak after 8.5 h. The fractions containing this PNPP phosphatase activity were pooled (160 ml) and loaded on to a chromatography column (diameter 1 cm, length 15 cm) filled with phosvitin–Sepharose. The column was washed with approx. 100 ml of buffer A and then eluted with a linear gradient from 0 to 500 mM NaCl in buffer A. The total elution volume was 55 ml. Fractions of 1.4 ml were collected and assayed for PNPP phosphatase activity.

Enzyme activity assays

Assay for protein kinase(s)

Standard assays for protein kinase activity 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 of substrate protein and 20 mM triethanolamine/HCl buffer, pH 7.2. Assays for cyclic-nucleotide-dependent protein kinase activities contained the substrate histone II-S (Sigma) and 0.15 μM cAMP or 0.15 μM cGMP. Assays for casein kinase activities contained casein as a substrate. Reactions were started by adding the protein kinase samples and, after 20 min at 20 °C, terminated by spotting 20 μl samples onto trichloroacetic acid-containing Whatman 3 MM filter papers. The filter papers had been prepared and washed as previously described [26].

Assay for PNPP dephosphorylation

Phosphatase activity was measured as PNPP phosphatase activity. Formation of *p*-nitrophenol (PNP) was monitored at 405 nm. PNPP was dissolved as an aqueous solution just before use. Incubations of 200 μl ($d = 0.59$ cm) were performed in micro-well modules (Nunc, Wiesbaden, Germany) at 25 °C containing 20 mM Tris/HCl, 10 mM NaCl, 20 μg/ml BSA, 1 mM $MnCl_2$ and 20 μl aliquots of *Paramecium* extracts, pH 7.4. The reaction was started by adding PNPP to a final concentration of 1 mM. The PNPP phosphatase activity was followed spectrophotometrically in a Titertek Multiscan MCC/340 ELISA-Reader (Flow Laboratories GmbH, Meckenheim, Germany) either by the time course of the reaction or after termination by the addition of 13 % K_2HPO_4. Controls were run to account for non-enzymic PNP formation. Specific activity was calculated from the molar extinction coefficient for the *p*-nitrophenolate anion (1.75×10^{-4} $M^{-1} \cdot cm^{-1}$) as previously described [32].

Assay for protease activity

Protease activity was determined by standard assays for Azocoll hydrolysis according to the method of Chavira *et al.* [33].

Phosphorylation/dephosphorylation properties of (P)P63 by endogenous enzymes

To characterize the phosphorylation/dephosphorylation of (P)P63 by endogenous enzymes in the (P)P63-containing fractions, [^{32}P]P63 was prepared after ^{32}P-labelling *in vivo* (as described above). Aliquots were taken (5–20 μg) and incubated in the presence or absence of EDTA or EGTA at 20 °C. Concentrations and incubation times were varied, ranging from 0.01 to 1 mM and from 0 to 16 min. The final volume of the assay was 80 μl containing in addition 5 mM Pipes/HCl, 1 mM KCl and 0.1 mM $CaCl_2$.

To analyse the influence of protein phosphatase inhibitors on the dephosphorylation in the presence of EDTA, ^{32}P-labelled PP63 was isolated in the phosphorylated or rephosphorylated

state and preincubated with or without okadaic acid or calyculin A at 20 °C. The concentrations used ranged from 0.4 nM to 25 μM okadaic acid and from 1.2 pM to 12.5 μM calyculin A. After 15 min, dephosphorylation of PP63 was assayed under similar assay conditions to those described above (20 min in the presence of 1 mM EDTA). Additional controls were run with heat-treated PP63 (84 °C, 5 min) or by omitting EDTA. Aliquots were subjected to SDS/PAGE and then processed for autoradiography.

Dephosphorylation of heat-denatured PP63 *in vitro*

To analyse the dephosphorylation of PP63 *in vitro* by exogenous or endogenous enzymes, either calcineurin (CaN) from bovine brain (3–5 μg) or endogenous PNPP-phosphatase (3–5 μg) was added to aliquots (10–35 μg) of heat-denatured (84 °C, 5 min) ^{32}P-labelled PP63, phosphorylated or rephosphorylated *in vivo*. Depending on the protein content of the samples the volume was between 75 and 200 μl in the different assays, which contained 5 mM Pipes/HCl, 1 mM KCl and 0.1 mM $CaCl_2$ in the presence or absence of an additional divalent cation, i.e. $NiCl_2$, $MnCl_2$ or $MgCl_2$, at 1 mM. After 30 min at 20 °C, aliquots were subjected to SDS/PAGE and then processed for autoradiography.

Phosphorylation of heat-denatured P63 *in vitro*

To analyse phosphorylation of P63 *in vitro*, endogenous PKA (100 ng), PKG (465 ng) or casein kinase (320 ng) was added to aliquots of P63-containing fractions (7–15 μg) that had previously been denatured by heating (84 °C, 5 min). In addition the phosphorylation assay (0.08 ml) contained 5 mM $MgCl_2$, [γ-^{32}P]ATP (185 MBq/μmol) and 5 mM Pipes/HCl buffer, pH 7.0 (with 1 mM KCl and 0.1 mM $CaCl_2$ added). In assays containing PKA or PKG, either 0.15 μM cAMP or 0.15 μM cGMP respectively were added. Assays with casein kinase were performed in the presence or absence of 0.1 mM $CaCl_2$. Reactions were started as indicated above. At the end of the incubation (20 min at 20 °C), aliquots were stopped in 'sample buffer' (see below), subjected to SDS/PAGE and then prepared for autoradiography.

Other methods

Protein determination

Protein determinations were performed by the method of Bradford [34] with chemicals obtained from Bio-Rad (Munich, Germany). BSA was used as a standard.

Polyacrylamide gel electrophoresis

Protein samples were denatured by boiling for 3 min in 'sample buffer' [125 mM Tris/HCl, 1.3% (w/v) SDS, 0.5% dithiothreitol, 20% (v/v) glycerol, pH 6.8] and subjected to electrophoresis on linear gradient (10–20% gel) SDS polyacrylamide gels with the discontinuous buffer system of Laemmli [35] modified by Westermeier [36]. Protein standards (low molecular mass from Pharmacia LKB included myosin, 220 kDa) were prepared in accordance with the manufacturer's directions. Gels were stained with silver, according to Heukeshoven and Dernick [37].

Isoelectric focusing

Proteins obtained by freeze–thaw solubilizing after ^{32}P-labelling were applied to polyacrylamide gels containing ampholines in a linear gradient of pH 4.0–8.0 (Pharmacia LKB). The calibration kit used (Serva) included trypsin-inhibitor (pI 4.55), β-lactoglubulin B (pI 5.13) and bovine carbonic anhydrase (pI 6.0).

Autoradiography

Autoradiography was performed with gels dried on to cellophane under vacuum and exposed to either Kodak XRP-5 X-ray film or Amersham Hyperfilm-MP in Kodak X-Omatic cassettes with an intensifier screen for 1–3 weeks at −70 °C. The molecular masses of the phosphoproteins in the autoradiograms were estimated according to Winston [38]. Autoradiograms were also evaluated densitometrically with a Quick Scan Densitometer from Desaga (Heidelberg, Germany). For quantification, relative peak areas were measured.

RESULTS

Correlation of (P)P63 dephosphorylation and rephosphorylation with exocytosis

We compared the phosphorylation pattern of PP63 after different periods of ^{32}P labelling *in vivo* (Figure 1). After freezing (to maintain phosphorylation states) and thawing for two cycles, PP63 could be recognized as a soluble phosphoprotein in the 100 000 ***g*** supernatant, in its phosphorylated (lanes 2–6), dephosphorylated (lane 7) and rephosphorylated forms (lane 8). In detail, this was analysed as follows. Some labelling *in vivo* was observed after only 4 min of incubation with [^{32}P]P_i (lane 2), whereas maximal phosphorylation occurred after approx. 30 min (lane 5), thus indicating a rapid turnover of phosphorylation sites. We used 90 min of ^{32}P labelling (lane 6) for further analysis. During synchronous exocytosis induction by AED, PP63 was totally dephosphorylated (lane 7) and 4 s later was already fully rephosphorylated (lane 8). Because quenched-flow analysis showed a correlation of the time course of dephosphorylation with membrane fusion, both occurring within 80 ms [9], manual pipetting as performed in this paper allows recognition of PP63 dephosphorylation only in the range of 1–2 s. In isoelectric focusing autoradiograms we confirmed previous results [9] that PP63, after ^{32}P labelling *in vivo*, exists in three isoforms of pI 5.85, 5.95 and 6.05 (results not shown).

Phosphorylation/dephosphorylation properties of PP63

To investigate the conditions for reversible protein phosphorylation, especially for a Ca^{2+}-dependent phosphorylation as reported by Subramanian and Satir [12], we

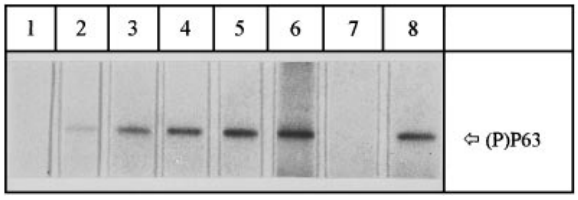

Figure 1 ^{32}P labelling of P63 *in vivo*

After different times of incubation with [^{32}P]P_i (0.4 mCi per 5 ml of cell suspension) aliquots of 200 μl (approx. 10^4 cells) were taken and processed to assay the degree of (P)P63 phosphorylation as described in the Materials and methods section. Aliquots of 5 μg of protein were subjected to SDS/PAGE and prepared for autoradiography. Lanes 1–6, incubation times of 2, 4, 8, 16, 32 and 90 min respectively; lanes 7 and 8, after 90 min of ^{32}P labelling and subsequent addition of AED for 2 s (dephosphorylation of PP63 in lane 7) or for 4 s (rephosphorylation of P63 in lane 8).

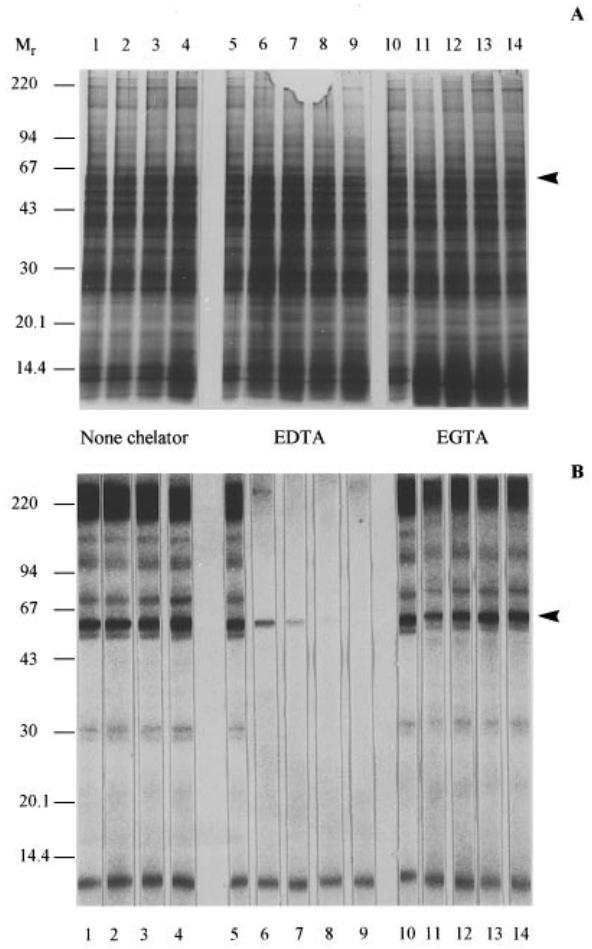

Figure 2 Protein phosphorylation and dephosphorylation in the (P)P63-containing fraction *in vitro*

The effect of chelators (EDTA and EGTA) on the phosphorylation state of proteins in the 100 000 ***g*** supernatant (including PP63) was tested after ^{32}P labelling of cells *in vivo*. Protein aliquots (16 μg) were taken and incubated in the absence (lanes 1–4) or presence of 1 mM EDTA (lanes 5–9) or of 1 mM EGTA (lanes 10–14) for different times at 20 °C. Control assays representing the starting point of each of the assays (lanes 1, 5 and 10) were compared with assays run for 30 s (lanes 6 and 11), 2 min (lanes 2, 7 and 12), 8 min (lanes 3, 8 and 13) and 16 min (lanes 4, 9 and 14). Aliquots of 5 μg of protein were subjected to SDS/PAGE and processed for autoradiography (**B**). (**A**) Silver stain of the corresponding SDS/10–20% polyacrylamide gel. In the absence of chelators (100 μM $CaCl_2$) PP63 can be slightly further phosphorylated (lanes 1–4). In the presence of EDTA a time-dependent dephosphorylation of PP63 (but also of other phosphoproteins) occurs within a few minutes (lanes 5–9). EGTA shows a biphasic response: first a slight dephosphorylation of PP63 (lanes 10 and 11) followed by a complete rephosphorylation (lanes 12–14).

analysed the PP63-containing fraction for the possible presence of protein phosphatases and kinases and their relative activities in the presence or absence of Ca^{2+} (Figure 2). PP63 was isolated in the phosphorylated state after ^{32}P labelling *in vivo*. In the presence of 100 μM $CaCl_2$ PP63 can be phosphorylated *in vitro* by supernatant components to a level exceeding that after isolation (Figure 2B, lanes 1–4). In the presence of EDTA (lanes 5–9) a time-dependent dephosphorylation of PP63, but also of some other phosphoproteins, occurs within a few minutes. EGTA causes a biphasic response, i.e. first a slight dephosphorylation (lanes 10–11) followed by rephosphorylation of PP63 (lanes 12–14). These results clearly demonstrate that the PP63 fraction (100 000 ***g*** supernatant) contains some enzymes capable of PP63

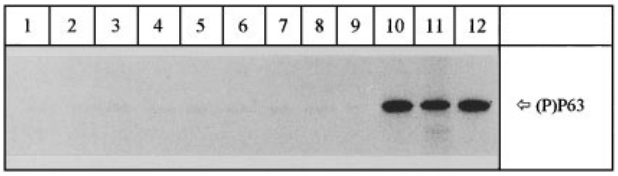

Figure 3 Lack of influence of okadaic acid on the dephosphorylation of PP63 in the presence of EDTA

Aliquots of 10 μg of [^{32}P]P63-containing fractions were preincubated for 15 min at 20 °C with (lanes 2–8) or without different concentrations of okadaic acid (lanes 1 and 9–12) and then assayed for dephosphorylation in the presence of EDTA (1 mM) as described in the Materials and methods section. After incubation for 20 min, 5 μg of samples were subjected to SDS/PAGE and processed for autoradiography. Dephosphorylation of PP63 was not affected by okadaic acid applied in concentrations of 0.4 nM (lane 2), 4 nM (lane 3), 20 nM (lane 4), 100 nM (lane 5), 500 nM (lane 6), 2.5 μM (lane 7) and 25 μM (lane 8). Controls for dephosphorylation were run in the presence (lane 1) or absence (lane 9) of 0.5% DMSO in which okadaic acid had been dissolved. In control assays either omitting EDTA (lane 11) or using heat-treated PP63 (84 °C, 5 min), no dephosphorylation of PP63 was observed whether EDTA was present (lane 10) or not (lane 12).

dephosphorylation and rephosphorylation. The EDTA effect can be explained by chelation of Mg^{2+} as a necessary cofactor for protein serine/threonine kinases. However, dephosphorylating enzymes are less affected, indicating that they also are active in the absence of divalent cations. Proteolytic digestion as an explanation of the decrease in ^{32}P labelling of PP63 can be excluded on the basis of silver staining of the corresponding gel (Figure 2A). Furthermore phosphorylation of PP63 in the presence of 1 mM EGTA demonstrates that Ca^{2+} or Ca^{2+}-dependent protein kinase were not necessary for PP63 phosphorylation. The results were all the same regardless of whether PP63 was isolated from AED-triggered or non-triggered cells (results not shown). We made analyses (results not shown) with variable concentrations of EDTA or EGTA. PP63 dephosphorylation was observed only with EDTA concentrations above 10 μM, indicating a concentration-dependent inhibition of the corresponding protein kinases. In the presence of 0.5 mM EGTA or less, results resembled those obtained in the absence of chelators (Figure 2, lanes 1–4) where rephosphorylation prevails.

Using EDTA, we have a device for decoupling phosphorylation and dephosphorylation in PP63-containing fractions. To investigate whether a dephosphorylation of PP63 could be attributed to PP-1, a type 1 protein serine/threonine phosphatase not requiring divalent cations in mammalian [39] nor in *P. tetraurelia* cells [24], we analysed this aspect with specific inhibitors. Okadaic acid or calyculin A, both potent inhibitors of protein serine/threonine phosphatases of type 1 and type 2A in mammalian cells [40], are both potent selective inhibitors of PP-1 in *Paramecium*, where IC_{50} values are 80 and 5 nM respectively [24]. Their failure to inhibit type 2A phosphatases in *Paramecium* cells [23] allowed us to analyse whether PP-1 might dephosphorylate PP63 in the presence of 1 mM EDTA. The autoradiogram in Figure 3 shows results with okadaic acid. Preincubation in concentrations between 0.4 nM and 25 μM (lanes 2–9) does not inhibit PP63 dephosphorylation which was started by adding 1 mM EDTA. Results were again the same regardless of whether PP63 was assayed in the phosphorylated (as seen here) or rephosphorylated state (results not shown) after labelling with ^{32}P *in vivo*. Similar results were obtained with calyculin A in concentrations of 1.2 pM to 12.5 μM (results not shown), thus confirming that PP-1 is not involved in the dephosphorylation of PP63 *in vitro*. PP-2C is also unlikely to be

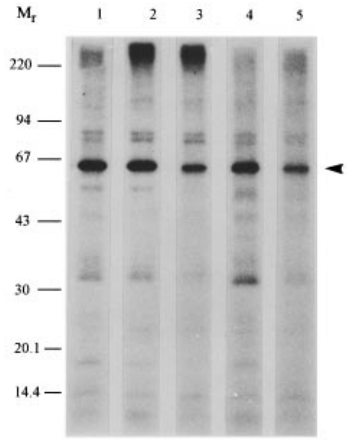

Figure 4 Dephosphorylation of ^{32}P-labelled PP63 by bovine brain CaN

A 100000 ***g*** supernatant (including ^{32}P-labelled P63 in the phosphorylated state) was prepared from cells labelled *in vivo* with [^{32}P]P_i for 90 min at room temperature as described in the Materials and methods section. Inactivation of endogenous enzymes was achieved by heat treatment for 5 min at 84 °C. Aliquots of 140 μl (35 μg of protein) were incubated in the presence (lanes 3 and 5) or absence (lanes 1, 2 and 4) of bovine brain CaN (4 μg) for 30 min at 20 °C in 0.2 ml of 5 mM Pipes/HCl, 1 mM KCl, 100 μM $CaCl_2$, pH 7.0 (lane 1). In some cases the assay contained 1 mM of either $NiCl_2$ (lanes 2 and 3) or $MnCl_2$ (lanes 4 and 5). At the end of incubation, aliquots of 10 μg were separated on linear-gradient SDS/10–20% polyacrylamide gels and then processed for autoradiography as described in the Materials and methods section. Note that dephosphorylation of PP63 *in vitro* is incomplete and depends on the divalent cation added.

involved because of its absolute Mg^{2+} requirement [25,39] in contrast with our findings in Figure 2. Such inhibitor experiments, however, would not exclude the involvement of PP-2A or PP-2B.

Dephosphorylation of PP63 by exogenous phosphatases

We next analysed in Figure 4 whether PP63 would be dephosphorylated by a type 2B phosphatase. We therefore inactivated the PP63-containing fractions, phosphorylated *in vivo*, by heat to eliminate endogenous enzyme activity. We found that 5 min at 84 °C sufficed to inactivate all enzymes without affecting the phosphorylation state of PP63 (results not shown). We also excluded any proteolytic effects (SDS/PAGE, results not shown).

In the presence of endogenous calmodulin and 100 μM $CaCl_2$ and additional 1 mM $NiCl_2$ or $MnCl_2$, bovine brain CaN largely dephosphorylates PP63 (Figure 4, lanes 3 and 5) by up to approx. 70%, depending on the divalent cation added (Table 1). Increasing CaN concentrations or prolonged incubation time did not cause more dephosphorylation (results not shown). This could mean that either the substrate property of PP63 is decreased by the heat treatment or that PP63, which exists in at least three isoforms, would also contain serine/threonine-phosphorylation sites not recognized by CaN. Furthermore, dephosphorylation on Tyr-residues is unlikely because PP63 in all its isoforms is sensitive to alkali treatment but insensitive even to strong acid treatment [9]. This suggests serine/threonine-phosphorylation sites not only for CaN but also for those not recognized by CaN. Similar results were obtained when dephosphorylation assays by CaN were performed with PP63 isolated after rephosphorylation by [^{32}P]P_i *in vivo*, i.e. shortly after AED triggering (see the Materials and methods section). Therefore results obtained by dephosphorylation of PP63 by CaN may indicate that the ^{32}P label of PP63 would be ascribed to phosphorylation on serine(s)/threonine(s) via protein kinase(s), rather than to phosphoglucosylation catalysed by a Glc-1-P phosphotransferase, as postulated by Subramanian and Satir [12].

Isolation of a PP63-relevant phosphatase

On the basis of these results we analysed the PP63-containing fraction for an endogenous phosphatase with PP-2B-like properties. Taking into account the specific requirements for enzymic activity of bovine brain CaN *in vitro*, using PNPP as a substrate [32], we could enrich such a PNPP phosphatase approx. 37-fold with preparative non-denaturing gel electrophoresis and subsequent chromatography on phosvitin–Sepharose (Table 2). More than 80% of the total PNPP-phosphatase(s) activity of whole cell homogenates could be found in the soluble fraction. Further fractionation by native preparative gel electrophoresis resulted in only a single activity peak, eluted after 8.5 h (results not shown). Most of the other proteins (approx. 96%) could be removed. In the remaining fraction (4% of the protein) approx. 45% of the activity was lost (Table 2). To analyse whether this phosphatase activity peak might contain several co-eluted enzymes, we applied the pooled fraction on to phosvitin–Sepharose (Figure 5). On elution with a linear salt gradient ranging from 0 to 500 mM NaCl, a single PNPP phosphatase activity was eluted as a sharp peak at 90–100 mM NaCl. This might indicate that the activity in this fraction was due to only one enzyme. Protease effects could be excluded by analysis with Azocoll (results not shown), an insoluble protein–dye conjugate widely used for the identification of proteolytic enzymes [33].

When we used this PNPP phosphatase (Table 2) in our *in vitro* assays with [^{32}P]P63, this enzyme was able to dephosphorylate heat-treated PP63 in a similar manner to bovine brain CaN

Table 1 Densitometric evaluation of dephosphorylation of PP63 by CaN from bovine brain

Dephosphorylation of PP63 was performed as described in Figure 4. Autoradiograms were evaluated densitometrically for quantification of relative peak areas.

Lane	*In vitro* assay for ^{32}P-labelled PP63 in the heat-treated 100000 ***g*** supernatant	Densitometric evaluation of (P)P63 in the autoradiogram (arbitrary units)	^{32}P labelling of (P)P63 (%)
1	–	784.39	100
2	+ $NiCl_2$ (1 mM)	811.05	103.4
3	+ $NiCl_2$ (1 mM) + CaN (4 μg)	436.43	56.4
4	+ $MnCl_2$ (1 mM)	739.67	94.3
5	+ $MnCl_2$ (1 mM) + CaN (4 μg)	253.35	32.3

Table 2 Summary of methods used for isolation of a PNPP phosphatase from *Paramecium* cell homogenates

Data represent a typical experiment. Isolation and assays were performed as described as described in the Materials and methods section. Specific activities were calculated after 30 min.

Purification step	Volume (ml)	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
Homogenate	20.5	170.56	184.2	1.08	1	100
100000 ***g*** supernatant	20.0	101.00	150.5	1.49	1.37	81.7
Preparative non-denaturing gel electrophoresis	160.0	3.52	85.2	24.21	22.41	46.2
Phosvitin–Sepharose eluate	4.2	0.93	37.4	40.21	37.23	20.3

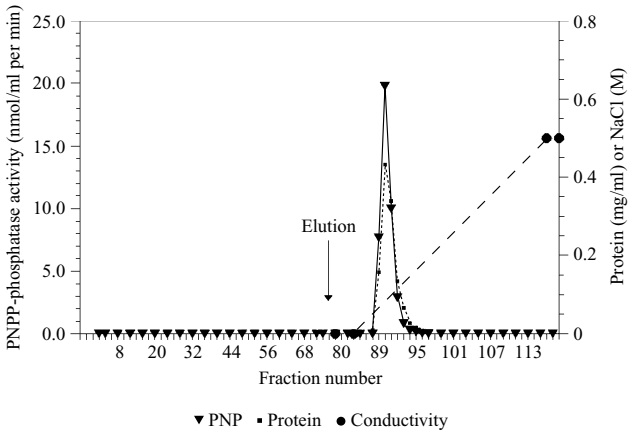

Figure 5 Chromatography of an endogenous soluble PNPP phosphatase on phosvitin–Sepharose

Isolation of a PNPP phosphatase from the 100000 ***g*** supernatant was performed by non-denaturing preparative gel electrophoresis followed by chromatography on phosvitin–Sepharose as described in the Materials and methods section. Elution was accomplished with a linear NaCl gradient ranging from 0 to 500 mM NaCl. PNPP phosphatase activities were calculated after 30 min by using the molar extinction coefficient for the *p*-nitrophenolate anion at 405 nm (1.75×10^{-4} $M^{-1} \cdot cm^{-1}$).

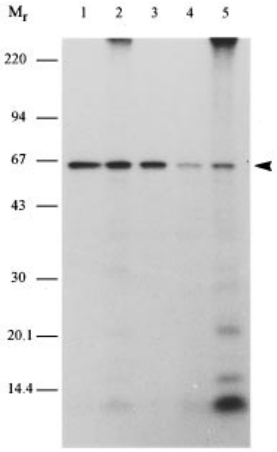

Figure 6 Dephosphorylation of ^{32}P-labelled PP63 by an endogenous PNPP phosphatase

Aliquots of 60 μl of heat-denatured 100000 ***g*** supernatant (approx. 10 μg of protein including [^{32}P]P63 in the rephosphorylated state, prepared 1 min after AED triggering from cells ^{32}P-labelled *in vivo*) were incubated in the presence (lanes 2, 4 and 5) or absence (lanes 1 and 3) of isolated PNPP phosphatase (3.2 μg per assay) for 30 min at 20 °C in 75 μl of the same buffer as described in Figure 4. The buffer was supplemented with 1 mM of either $MnCl_2$ (lanes 1 and 2) or $NiCl_2$ (lanes 3 and 4) or $MgCl_2$ (lane 5). At the end of incubation aliquots of 5 μg were subjected to SDS/PAGE and processed for autoradiography as described in the Materials and methods section. Dephosphorylation of PP63 did not occur in the presence of $MnCl_2$ (lane 2) but was observed in the presence of $NiCl_2$ (lane 4) or $MgCl_2$ (lane 5).

(Figure 6). The effect was the same whether [^{32}P]P63 was assayed in the phosphorylated (results not shown) or rephosphorylated state after labelling *in vivo* (Figure 6, lanes 4 and 5). We have shown by Western blots and ^{45}Ca overlays that CaM is present in the PP63-containing substrate fraction (results not shown). Densitometric evaluation (Table 3) confirmed that, in the presence of endogenous CaM and 100 μM $CaCl_2$, dephosphorylation of PP63 by this endogenous PNPP phosphatase was maximal in the presence of additional 1 mM $NiCl_2$ or $MgCl_2$. PP63 was then dephosphorylated by 80% or 60% respectively (Table 3). Residual ^{32}P labelling of PP63 could not be removed in this case, as with CaN, independently of whether incubation times and/or enzyme concentrations were varied (results not shown), possibly for the reasons indicated above. One remarkable difference was that PP63 dephosphorylation was achieved when $MnCl_2$ was substituted for $NiCl_2$ or $MgCl_2$ (Figure 6, lane 2). Indeed, when this endogenous PNPP phosphatase was compared with CaN, we observed some differences in enzymic activation, although most of the characteristics of a CaN-like phosphatase are fulfilled (R. Kissmehl, T. Treptau, B. Kottwitz and H. Plattner, unpublished work; details available on application to the authors).

In conclusion, these results with PP63 labelled *in vivo* showed that removal of ^{32}P label during AED-triggered exocytosis *in vivo* may be due to a dephosphorylation event by an endogenous phosphatase, rather than to dephosphoglycosylation catalysed by a phosphodiesterase [12].

Isolation of PP63-relevant protein kinases

Bacause the PP63 containing fraction probably also contained protein kinase activity (Figure 2, lanes 1–4) we also analysed this aspect. Because EGTA does not reduce phosphorylation *in vitro* (Figure 2, lanes 12–14) a Ca^{2+}-stimulated protein kinase is probably not involved. Therefore we screened for other protein kinases, including casein kinases and cyclic-nucleotide-dependent protein kinases (PKA, PKG), which also occur in *Paramecium* [13–16,19].

For the isolation of protein kinases from the 100000 ***g*** supernatant we used DEAE-cellulose chromatography, which separates PKA and PKG [13]. With histone II-S, an established substrate *in vitro* for cyclic-nucleotide-dependent protein kinases

Table 3 Densitometric evaluation of dephosphorylation of PP63 by a partly purified endogenous PNPP phosphatase

Dephosphorylation of PP63 was performed as described in Figure 6. For quantification, relative peak areas of (P)P63 in the corresponding autoradiograms were evaluated densitometrically. Data are from a typical dephosphorylation assay.

Lane	*In vitro* assay for ^{32}P-labelled PP63 in the heat-treated 100000 ***g*** supernatant	Densitometric evaluation of (P)P63 in the autoradiogram (arbitrary units)	^{32}P labelling of (P)P63 (%)
1	+ $MnCl_2$ (1 mM)	856.13	100
2	+ $MnCl_2$ (1 mM) + PNPP phosphatase (3.3 μg)	1052.49	122.9
3	+ $NiCl_2$ (1 mM)	778.78	90.9
4	+ $NiCl_2$ (1 mM) + PNPP phosphatase (3.3 μg)	171.74	20.1
5	+ $MgCl_2$ (1 mM) + PNPP phosphatase (3.3 μg)	342.36	39.9

Table 4 Isolation summary of cyclic-nucleotide-dependent protein kinases (PKA and PKG) from *Paramecium* cell homogenates

Data are drawn each from a typical experiment. Isolation and assays were performed as described in the Materials and methods section.

Purification step	Volume (ml)	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
PKA:						
Homogenate	53.0	370.7	6.74	0.0182	1	100
100000 ***g*** supernatant	51.5	176.6	6.88	0.0390	2.1	102.0
DEAE-cellulose eluate	18.6	5.9	10.99	1.8627	102.3	163.0
Phosvitin–Sepharose eluate	13.8	1.6	3.97	2.4869	136.3	58.9
Heparin–agarose eluate	0.6	0.011	0.081	7.3636	404.5	1.2
PKG:						
Homogenate	63.0	459.9	3.95	0.0086	1	100
100000 ***g*** supernatant	65.0	227.5	6.30	0.0277	3.2	159.4
DEAE-cellulose load	51.0	13.9	13.78	0.9913	115.2	348.8
Affi-Gel Blue eluate	41.0	2.3	4.09	1.7782	206.7	103.5
Phosphocellulose eluate	4.7	0.21	0.47	2.2380	260.2	11.8

in various cells including *Paramecium* [41], PKA was eluted at 50–60 mM NaCl as well as at 130–140 mM NaCl (results not shown), whereas PKG was found exclusively in the flow-through fractions (results not shown). This agrees with previous reports on PKA activities, designated PKA-I and PKA-II respectively [13,14], and PKG [16]. By the chromatography we used, both PKA (PKA-I) and PKG activities were enriched approx. 100-fold (Table 4). By this chromatographic step, however, the total activity increased up to 3–4-fold for PKG and to a smaller extent for PKA, for reasons not yet analysed. A possible explanation could be the separation of corresponding phosphodiesterases, because we have chosen a low concentration of cyclic nucleotides (cGMP, cAMP) in the assays to prevent cross-stimulation of PKA (with cGMP) or of PKG (with cAMP).

PKA-I was further enriched by chromatography on phosvitin–Sepharose (Figure 7A) and heparin–agarose (results not shown). In both cases a kinase was eluted as a single peak of activity at 50 mM NaCl with 20-fold stimulation by cAMP over basal activity (Figure 7A). We thus were able to enrich PKA-I from homogenates approx. 400-fold (Table 4).

PKG was also enriched approx. 260-fold by chromatography on Affi-Gel Blue, followed by phosphocellulose (Table 4). The specificity of this kinase is shown in Figure 7B. cAMP did not compete for the activation by cGMP. Stimulation of basal activity by cGMP was approx. 25-fold (Figure 7B).

With casein as a substrate *in vitro*, three different kinase activities could be distinguished in the 100000 ***g*** supernatant after chromatographic fractionation on DEAE-cellulose. One was identified in the flow-through fractions. The other two were eluted at 90–110 mM and at 180–200 mM NaCl respectively (results not shown). The last peak was eluted in subsequent chromatography on phosvitin–Sepharose as a single activity peak at 350 mM NaCl and on Affi-Gel Blue at 750 mM NaCl (T. Treptau, R. Kissmehl, H. W. Hofer and H. Plattner, unpublished work).

Phosphorylation of P63 *in vitro* by endogenous protein kinases

The dephosphorylated form, P63, obtained by triggering cells by AED (see the Materials and methods section), served as a substrate. To ascertain that endogenous enzymes in the P63 substrate fraction were not involved in such an assay, this fraction was heat-inactivated (84 °C, 5 min).

Figure 8 shows that P63 could be phosphorylated by endogenous PKG (Figure 8B) as well as by casein kinase (Figure 8C), whereas no phosphorylation occurred with PKA-I (Figure 8A). In detail, this was analysed as follows. None of the three enriched protein kinase fractions was contaminated by co-eluted PP63 when these fractions were assayed under conditions of autophosphorylation in the absence of a substrate (compare lanes 1–3 in each of the autoradiograms of Figure 8). These lanes were also used to evaluate the purity of the corresponding protein kinase samples. The PKA fraction contains only autophosphorylated PKA at 43 kDa (Figure 8A, lanes 1–3). In the PKG-containing fraction (Figure 8B, lanes 1–3) cGMP-dependent phosphorylation was noticed for several major proteins between 80 and 18 kDa. The 80 kDa phosphoprotein probably represents autophosphorylated PKG, because in *Paramecium*

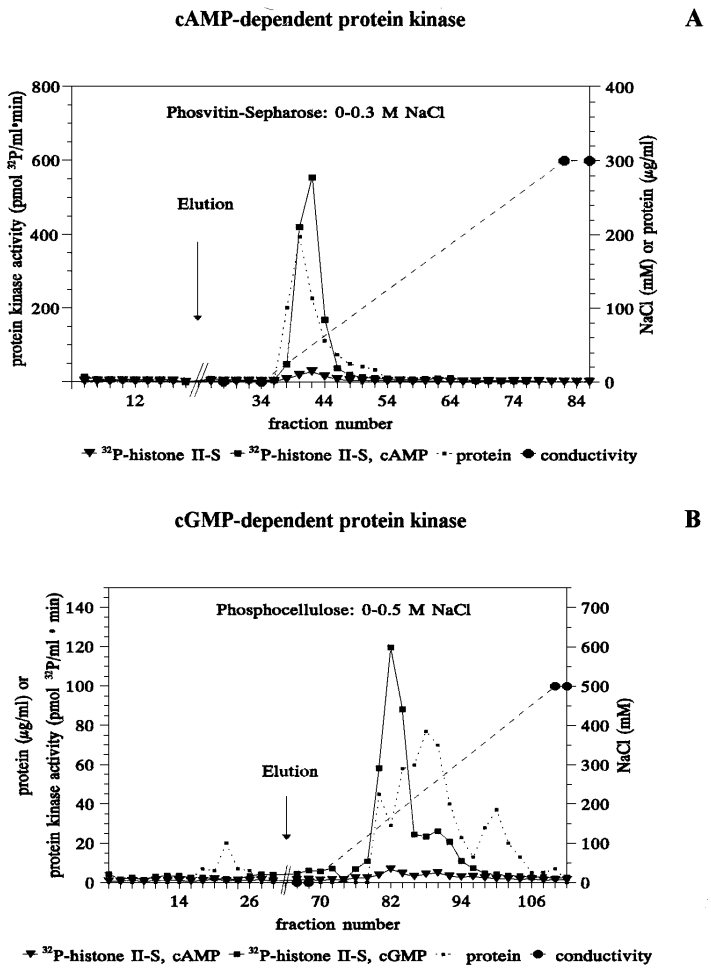

Figure 7 Isolation of endogenous cyclic-nucleotide-dependent protein kinases (PKA, PKG) by different chromatography steps

All chromatography steps were performed as described in the Materials and methods section. Separation of cyclic-nucleotide-dependent protein kinases (PKA and PKG) from the 100000 ***g*** supernatant of *Paramecium* cells was achieved by chromatography on DEAE-cellulose. With histone II-S as substrate, the resulting activities for PKA were identified in the eluate at 50–60 and 130–140 mM NaCl, whereas PKG activity was identified exclusively in the flow-through fractions. PKA activities that were eluted at 50–60 mM NaCl were enriched by two subsequent chromatography steps on phosvitin–Sepharose (**A**) and heparin–agarose. PKG activities identified in the flow-through fractions of DEAE-cellulose chromatography were further enriched by chromatography on Affi-Gel Blue followed by a chromatography step on phosphocellulose (**B**). Assay conditions for protein kinase activities were as described in the Materials and methods section.

this protein kinase has a molecular mass in this range [16]. However, the identities of the other phosphorylated bands (including at 65 kDa; arrow in Figure 8B) are unknown. The isolated P63 substrate fraction is represented in lane 4 in each of the three autoradiograms (arrowheads in Figures 8A to 8C). A very slight rephosphorylation had occurred during isolation of P63. Lack of P63 phosphorylation by PKA is documented in lanes 5–8 of Figure 8A. P63 could not be phosphorylated further by PKA in the presence of [γ-^{32}P]ATP (lane 6) supplemented with either 5 mM $MgCl_2$ (lane 7) or 5 mM $MgCl_2$/0.15 μM cAMP (lane 8). Because several other proteins were phosphorylated in a cAMP-dependent manner (lane 8), PKA was active in the assay. In contrast, a cGMP-dependent phosphorylation of P63 was observed (Figure 8B, lanes 5–7) in the presence of PKG and [γ-^{32}P]ATP (lane 5) supplemented either with 5 mM $MgCl_2$ (lane 6) or with 5 mM $MgCl_2$ + 0.15 μM cGMP (lane 7).

Figure 8C shows results obtained with the enriched casein kinase. Not only does this protein kinase not undergo autophosphorylation, this fraction does not contain any phosphoproteins (lanes 1–3) as shown when it was probed with [γ-^{32}P]ATP (185 MBq/μmol) in the absence (lane 1) or presence (lane 2) of 5 mM $MgCl_2$ or of 5 mM $MgCl_2$/100 μM $CaCl_2$ (lane 3). When the P63-containing fraction (lane 4) was incubated with casein kinase under the same conditions as in lane 3, a strong phosphorylation of P63 was observed (lane 5). Because 1 mM EGTA did not affect this strong phosphorylation of P63 (lane 6) we assume that phosphorylation is not increased by Ca^{2+}. In contrast, the phosphorylation pattern of some other phosphoproteins in the PP63-containing fraction was clearly decreased when phosphorylation occurred in the presence of 100 μM $CaCl_2$ (lane 5). Such a lack of Ca^{2+}-stimulated phosphorylation we also have shown to occur *in vitro*, i.e. in the PP63-containing fraction without heat-inactivation (Figure 2, lanes 12–14).

Isoelectric focusing autoradiograms of PP63 samples phosphorylated *in vitro* by casein kinase or by PKG revealed the same three isoforms of PP63 with pI values of 5.85, 5.95 and 6.05 [42], as occur after labelling *in vivo* [9,42]. These results we have now reproduced (results not shown). In conclusion, two of the three protein kinases tested, a cGMP-stimulated protein kinase and a casein kinase, are able to phosphorylate P63 *in vitro*.

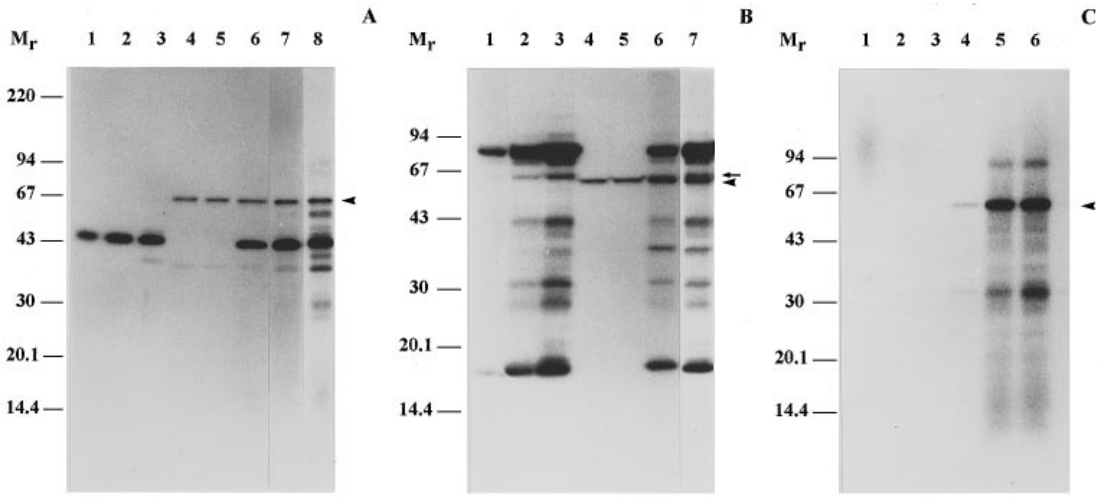

Figure 8 Phosphorylation of P63 by endogenous protein kinases *in vitro*

Phosphorylation of P63 *in vitro* was analysed by using PKA (**A**), PKG (**B**) or casein kinase (**C**) isolated and enriched from the 100 000 ***g*** supernatant of *Paramecium* homogenates. P63 was prepared after phosphorylation *in vivo* and subsequent AED-induced dephosphorylation of PP63 as described in the Materials and methods section, followed by inactivation of the containing enzymes by heat (84 °C, 5 min). Assays were performed in a volume of 80 μl containing 5 mM Pipes/HCl, 1 mM KCl and 0.1 mM $CaCl_2$, pH 7.0, in the presence or absence of 5 mM $MgCl_2$, [γ-^{32}P]ATP (185 MBq/μmol), P63 and/or the corresponding protein kinase. Phosphorylation by PKA (100 ng per assay) or PKG (465 ng per assay) was done with or without 0.15 μM cAMP (**A**) or 0.15 μM cGMP (**B**) respectively. Assays with casein kinase (320 ng each) were also accomplished in the absence of $CaCl_2$. At the end of incubations (20 min at 20 °C) aliquots of 50 μl were subjected to SDS/PAGE and processed for autoradiography. To exclude contamination of (P)P63 in the protein kinase-containing fractions, controls were run under autophosphorylation conditions in the absence of substrate. (**A**) Phosphorylation of P63 by PKA. (P)P63 was absent from the PKA-containing fraction when analysed under conditions of autophosphorylation with [γ-^{32}P]ATP in the absence (lane 1) or presence of $MgCl_2$ (lane 2) or of $MgCl_2$ + cAMP (lane 3). The 43 kDa phosphoprotein represents the autophosphorylated form of PKA. Lanes 4 and 5 show the P63 substrate fraction in the absence (lane 4) or presence of PKA (lane 5) without additives. P63 (arrowhead) was not phosphorylated by PKA in media containing [γ-^{32}P]ATP (lane 6), even when supplemented with $MgCl_2$ (lane 7) or with $MgCl_2$ + cAMP (lane 8). (**B**) Phosphorylation of P63 by PKG. cGMP-dependent phosphorylation of P63 (arrowhead) was observed when aliquots of 8 μg of the P63-containing fraction (lane 4) were assayed with PKG in the presence of [γ-^{32}P]ATP (lane 5) supplemented with $MgCl_2$ (lane 6) or with $MgCl_2$ + cGMP (lane 7). When the PKG-containing fraction was analysed for P63 contamination in the presence of [γ-^{32}P]ATP (lane 1) with $MgCl_2$ (lane 2) or $MgCl_2$ + cGMP (lane 3) added, a cGMP-dependent phosphorylation was also noticed with a phosphoprotein of about 80 kDa (PKG) and several other co-eluted phosphoproteins between 65 kDa (arrow) and 18 kDa, but not with P63. (**C**) Phosphorylation of P63 by casein kinase. Lack of autophosphorylation in the casein kinase containing fractions was demonstrated with [γ-^{32}P]ATP in the absence (lane 1) or presence of $MgCl_2$ (lane 2) or of $MgCl_2$ + $CaCl_2$ (lane 3). Lane 4 contains the P63 substrate fraction prepared after dephosphorylation *in vivo* (6.35 μg). Strong phosphorylation of P63 (arrowhead) was observed in the presence of casein kinase, [γ-^{32}P]ATP and $MgCl_2$ regardless of whether 100 μM $CaCl_2$ was present (lane 5) or chelated by 1 mM EGTA (lane 6).

DISCUSSION

Although reversible protein phosphorylation is essential in controlling exocytosis in quite different cell types, as reviewed in [3,4,43], it remains to be settled at which step phosphorylation [44–46] or dephosphorylation [47–51] events may be involved. Frequently the different steps, such as membrane fusion, contents release, membrane resealing and internalization, cannot be resolved with time and different processes could be superimposed. In *P. tetraurelia* a stimulus-dependent dephosphorylation of a 63 kDa phosphoprotein, (P)P63, occurs on exocytosis stimulation [7–9] and is reversed within 20 s or less [8]. However, there is some disagreement on whether these changes in the phosphorylation state of (P)P63 *in vivo* would involve a protein phosphatase/kinase [20] or a Glc-1-P phosphodiesterase/phosphotransferase system [12]. In our view this also implies the choice of an appropriate secretagogue, which should induce synchronous exocytosis while maintaining cell vitality, such as AED [29]. Because this does not happen with picric acid, other groups have changed to analysis *in vitro* [11,12], which may not really reflect the situation *in vivo*.

AED-triggered exocytosis in *Paramecium* [29] allows the isolation of the exocytosis-sensitive 63 kDa phosphoprotein, (P)P63, in all three phosphorylation states after ^{32}P labelling of cells *in vivo*, i.e. the phosphorylated state before adding AED, the dephosphorylated state immediately after AED triggering as well as the rephosphorylated state when samples are inactivated at later times after AED-triggered synchronous exocytosis [9], as we can confirm. This was the basis of our analysis of enzymes possibly relevant for PP63 dephosphorylation and rephosphorylation.

The present study was therefore focused on the question of whether such a protein phosphatase/kinase system could be involved in this process, rather than trying to disprove results for phosphoglycosylation obtained *in vitro* after UDP[β-^{35}S]Glc labelling of PP63 [12]. Several findings suggested an involvement of a protein phosphatase, i.e. a Ca^{2+}/CaM-dependent phosphatase of the CaN type for the following reasons. (1) Exocytosis could be inhibited *in vivo* (by microinjection) as well as *in vitro* (by using isolated cortex fragments) by polyclonal antibodies against the Ca^{2+}/CaM-dependent protein phosphatase 2B (CaN) from bovine brain [20]. (2) In contrast, an activated Ca^{2+}–CaM–CaN complex was able to trigger exocytosis *in vivo*, i.e. by microinjection, and *in vitro*, i.e. by the use of isolated cell surface complexes [20]. (3) Now we show that PP63 labelled *in vivo* is a substrate for exogenous CaN. (4) These findings are supported by further identification of an endogenous protein phosphatase of this type (R. Kissmehl, T. Treptau, B. Kottwitz and H. Plattner, unpublished work). In fact the CaN gene sequence has recently been identified in *Paramecium* [52]. As we show here, this phosphatase recognizes PNPP as a substrate and is activated by several divalent cations although it shows unusual insensitivity to Mn^{2+} during PP63 dephosphorylation. (5) Furthermore we show that PP63, prelabelled *in vivo*, could be dephosphorylated

by this PNPP phosphatase in a similar manner as by exogenous CaN. (6) Therefore it is also of some interest that CaM has been localized to trichocyst exocytosis sites by immunolabelling and affinity labelling [53]. Interestingly, exocytosis-incompetent strains become responsive when transfected with the CaM wild-type gene [54]. In sea urchin egg [55], mast cells [56] and in pancreatic acinar cells [57] CaM was considered to confer calcium sensitivity. (7) Recently the involvement of a CaN-mediated dephosphorylation step in exocytosis has been ascertained also with several other cell types, i.e. with exocrine pancreatic cells [58] or with isolated synaptosomes [59], by using the immunosuppressants cyclosporin A or FK-506, which act by binding first to their intracellular receptor proteins which then in turn bind to and inactivate CaN [60]. These compounds also inhibit degranulation of murine cytotoxic T lymphocytes [61]. All this work supports our previous observations with *Paramecium* [20], implying that CaN would be involved at a late step of exocytosis regulation.

What argues against the involvement of other protein phosphatases? In the presence of EDTA or EGTA (Figure 2), PP63 also was dephosphorylated by endogenous enzymes contained in the 100000 ***g*** supernatant. It could not be excluded from first principles that other protein phosphatases are also involved, e.g. a protein serine/threonine phosphatase of type 2A. However, PP-2A does not require divalent cations for activity [39] and in *Paramecium* it is not inhibited by okadaic acid or by calyculin A [23], both potent inhibitors of PP-1 and PP-2A in mammalian tissues [40]. Therefore PP-2A could still be involved in the dephosphorylation of PP63 in supernatants in the presence of EDTA (Figure 3), although all our evidence supports the involvement of PP-2B (see above). However, PP-1, which also does not require divalent cations for enzymic activity [39], can be excluded in our system by the fact that endogenous PP-1 is inhibited by okadaic acid and calyculin A with IC_{50} values of 80 and 5 nM respectively [24], whereas PP63 dephosphorylation is not (Figure 3).

Which protein kinases might be involved in phosphorylation of PP63? Because PP63 is sensitive to alkali treatment but insensitive even to strong acid treatment [9], tyrosine phosphorylation is unlikely, whereas serine/threonine phosphorylation is much more likely. We now show that P63 is a substrate for endogenous PKG and for a casein kinase; both of these are known to phosphorylate serine/threonine-containing substrates *in vitro* [16,19]. The occurrence of serine phosphorylation sites in PP63 has recently been confirmed by cloning and sequencing of a form with a pI of 6.5 [62]. This protein contains several potential phosphorylation sites for Ca^{2+}-dependent protein kinases, i.e. one site for protein kinase C and three sites for Ca^{2+}/CaM-dependent protein kinase [62]. However, we found that P63 phosphorylation also occurs in the absence of Ca^{2+} (Figure 2, lanes 12–14; Figure 8C, lane 6) and that either casein kinase (Figure 8C) or PKG (Figure 8B) can cause phosphorylation, none being stimulated by Ca^{2+}, whereas the existence of protein kinase C or of Ca^{2+}/CaM-dependent protein kinase in *Paramecium* is still unproved (see the Introduction section). A possible identity of the casein kinase with one of the two soluble Ca^{2+}-dependent protein kinases that would also phosphorylate casein as a substrate *in vitro* [17,18] can be excluded, because our enzyme is able to phosphorylate P63 even without Ca^{2+} (Figure 8C). Moreover the casein kinase that we isolated and used for P63 phosphorylation is inhibited by Ca^{2+} (T. Treptau, R. Kissmehl, H. W. Hofer and H. Plattner, unpublished work). The lack of autophosphorylation of casein kinase in these autoradiograms also excludes any identity with one of the two known soluble Ca^{2+}-dependent protein kinases, because they both undergo Ca^{2+}-dependent autophosphorylation. This is true for a 52 kDa protein representing type 1 [18] and for 50 and 55 kDa proteins representing type 2 [17].

Although participation of PKA in the regulation of exocytosis in quite different cells has been described, i.e. in parotid acinar cells [63], in pancreatic acinar cells [64] and in tracheal serous cells [65], PKA type 1 seems not to be involved in trichocyst exocytosis (Figure 8A). PKG could also be involved in P63 phosphorylation because cGMP concentration increases several-fold immediately after AED-stimulated exocytosis [66], i.e. within the time during which we observed rephosphorylation to take place.

Conclusions

We do not share the concept by Subramanian and Satir [12] that assumes the reversible phosphoglucosylation of (P)P63 to be relevant for trichocyst exocytosis. We also disagree on the idea that such a step would cause a redistribution of P63 to the plasma membrane or to vesicular membranes [12]. Subramanian and Satir assumed that a membrane-bound Ca^{2+}-dependent protein kinase (as yet unidentified in *P. tetraurelia*) would rephosphorylate P63 and thus cause a dissociation of PP63 from membranes [12]. Our arguments against this hypothesis are as follows. (1) After labelling *in vivo* (P)P63 in all its forms, i.e. phosphorylated, dephosphorylated or rephosphorylated, displays the same solubility properties ([9], and this paper). In all cases P63 as well as PP63, although a soluble protein, is cortex-bound [9]. (2) We find that protein kinases of possible relevance are soluble and not stimulated by Ca^{2+}, whereas the existence of any membrane-bound Ca^{2+}-dependent protein kinases in *Paramecium* is not known. (3) Also the existence of a Ca^{2+}-dependent Glc-1-P phosphodiesterase in the particulate fraction, postulated from experiments *in vitro*, has not been shown. In contrast, we can show that (P)P63, phosphorylated or dephosphorylated *in vivo*, is amenable to endogenous protein phosphatases and protein kinases. (4) Molecular cloning revealed postulated sites for serine phosphorylation by kinases, rather than for O-glycosylation [62], as suggested previously [12]. It remains to be settled whether casein kinase and/or PKG phosphorylate P63 *in vivo*. For phosphatases we present further evidence in favour of a CaN-like activity, in agreement with previous antibody studies [20].

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