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Specificity of protein phosphatases in the dephosphorylation of protein kinase C

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Protein kinase C can autophosphorylate *in vitro* and has also been shown to be phosphorylated *in vivo*. In order to investigate the factors that may determine the phosphorylation state of protein kinase C *in vivo*, we determined the ability of the ATP+Mg^{2+}-dependent phosphatase and the polycation-stimulated (PCS) phosphatases to dephosphorylate protein kinase C *in vitro*. These studies show that all the oligomeric forms of the PCS phosphatases (PCS_{H1}, PCS_{H2}, PCS_M and PCS_L phosphatases) are effective in the dephosphorylation of protein kinase C, showing 34–82% of the activity displayed with phosphorylase *a* as substrate. In contrast both the catalytic subunit of the PCS phosphatase and that of the ATP+Mg^{2+}-dependent phosphatase showed only weak activity with protein kinase C as substrate. All these phosphatases, however, were activated by protamine (K_a 14–16 μg/ml) through what appears to be a substrate-directed effect. The relative role of these phosphatases in the control of protein kinase C is discussed.

INTRODUCTION

Post-translational modification, in particular protein phosphorylation, is commonly employed to permit the rapid control of cellular functions. The phosphorylation state of cellular regulatory proteins is determined by the activity of protein kinases and protein phosphatases. It is through these modifying enzymes that many hormones and growth factors elicit biological responses.

In studies on protein phosphatases it has become evident that a relatively small number of multifunctional enzymes exist to control the dephosphorylation of regulatory proteins. Four different classes of protein phosphatases can be distinguished according to their regulatory properties (reviewed by Merlevede, 1985): an ATP+Mg^{2+}-dependent phosphatase, the polycation-stimulated phosphatases (PCS phosphatases), an Mg^{2+}-dependent phosphatase and calcineurin (a Ca^{2+}+calmodulin-dependent phosphatase). Most of these enzymes are subject to complex regulation, and various forms of the ATP+Mg^{2+}-dependent phosphatase (Merlevede *et al.*, 1984; Tung & Cohen, 1984; Stralfors *et al.*, 1985) and the PCS phosphatases have been described (Tung *et al.*, 1985; Waelkens *et al.*, 1986). Although the interconversion between active and inactive forms of the ATP+Mg^{2+}-dependent phosphatase has been clearly demonstrated (Resink *et al.*, 1983; Merlevede *et al.*, 1984; Goris *et al.*, 1984) the relationship and possible interconversion between the PCS phosphatases remains to be clarified. An operational subclassification of the PCS phosphatases has been made based on their apparent native M_r values: PCS_H, PCS_M, PCS_L and PCS_C, the catalytic subunit (Merlevede, 1985; Waelkens *et al.*, 1986; Goris *et al.*, 1986). The PCS_H phosphatase has a characteristically high deinhibitor phosphatase activity (Goris *et al.*, 1985, 1986); the PCS_M phosphatase has a low K_a for protamines (Waelkens *et al.*, 1986) and shows a specific increase in inhibitor-1 phosphatase activity on treatment with a Ca^{2+}-dependent proteinase (Waelkens *et al.*, 1985); PCS_L phosphatase is stimulated only poorly by protamines. These various classes of protein phosphatases possess different specificities and cofactor-dependencies, and this permits a selective control of dephosphorylation *in vivo*.

Studies on purified protein kinase C have demonstrated that the isolated protein will autophosphorylate in the presence of Ca^{2+}, phospholipid and Mg–ATP. We have previously found that protein kinase C is phosphorylated *in vivo* (Fry *et al.*, 1985), although it is not yet clear whether this is due to autophosphorylation or whether this is a heterologous event (or perhaps both). Nevertheless these observations have prompted an investigation into the factors that may control the phosphorylation state of protein kinase C. We have thus investigated the specificities of a series of protein phosphatases with respect to the dephosphorylation of protein kinase C.

MATERIALS AND METHODS

Chemicals

Biochemicals were purchased from Sigma Chemical Co., Poole, Dorset, U.K.; they include histone III-S, which is a histone HI-rich fraction. [γ-^{32}P]ATP was obtained from Amersham International, Amersham, Bucks., U.K. Other commercial reagents were of the highest quality available.

Purification of proteins

Rabbit skeletal-muscle phosphorylase *b* kinase (Pickett-Gies & Walsh, 1985), PCS_{H1}, PCS_{H2}, PCS_M and PCS_L phosphatases (Waelkens *et al.*, 1986), PSC_C phosphatase and the active catalytic subunit of the ATP+Mg^{2+}-dependent phosphatase (Ramachandran *et al.*, 1986), the catalytic subunit of the cyclic AMP-dependent protein kinase (Beavo *et al.*, 1974) and protein kinase C (Parker *et al.*, 1984) were purified to homogeneity according to

Abbreviation used: PCS, polycation-stimulated.
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Table 1. Comparison of the substrate specificities of the PCS phosphatases

Activities were determined as described in the Materials and methods section. For protein kinase C (PKC) as substrate the concentration of protamine was 15 μg/ml. The dephosphorylation of histone II-AS (II-AS) is not affected by protamine. With phosphorylase *a* (PHOSa) as substrate the concentrations of protamine were 5 μg/ml (PCS_{H1} and PCS_{H2}), 1 μg/ml (PCS_M), 10 μg/ml (PCS_L) and 20 μg/ml (PCS_C).

	Phosphatase activity (units/ml)					Activity ratio	
Substrate ...	PKC		II-AS	PHOSa		PKC/II-AS	PKC/PHOSa
Protamine ... / Phosphatase	−	+	−	−	+	−	−
PCS_{H1}	5.88	20.2	0.25	7.54	45.2	24	0.78
PCS_{H2}	3.37	15.3	0.17	6.74	30.3	20	0.50
PCS_M	11.2	54.9	0.54	13.7	123	21	0.82
PCS_L	4.38	18.8	0.22	12.9	32.3	20	0.34
PCS_C	0.83	16.2	0.24	10.4	15.6	3.5	0.08

published methods. Phosphorylase *b* kinase was kindly given by D. Walsh (Davis, CA, U.S.A.). The specific activities of phosphatases in the absence and in the presence of optimal concentrations of protamine with 1 mg of phosphorylase *a*/ml as substrate were: 200 and 800 units/mg (PCS_{H1}), 400 and 1300 units/mg (PCS_{H2}), 750 and 5800 units/mg (PCS_M), 350 and 1100 units/mg (PCS_L), 8750 units/mg (PCS_C) and 14000 units/mg (catalytic subunit of the ATP+Mg^{2+}-dependent phosphatase).

Assays

Phosphorylase *a* phosphatase was assayed as described previously (Yang *et al.*, 1980) at 1 mg of substrate/ml either in the absence or in the presence of an optimal concentration of protamine: 5 μg/ml (PCS_{H1}, PCS_{H2}), 1 μg/ml (PCS_M) and 10 μg/ml (PSC_L).

Protein kinase C phosphatase and histone phosphatase activities were assayed at 30 °C at 0.5 μM and 10 μM substrate respectively in a final volume of 30 μl containing 20 mM-Tris/HCl buffer, pH 7.5 (25 °C), 1.2 mM-EDTA and 30 mM-2-mercaptoethanol in the presence or in the absence of protamine as indicated in the text or legends. Protein phosphatases were diluted in medium containing 20 mM-Tris, 0.5 mM-dithiothreitol and 1 mg of bovine serum albumin/ml. One unit of phosphatase activity is defined as 1 nmol of phosphate released/min under the conditions described.

Phosphorylation of proteins

^{32}P-labelled phosphorylase *a* (1 mol of phosphate/mol of enzyme) was prepared as described by Krebs *et al.* (1958). ^{32}P-labelled histone type II-AS was labelled with the catalytic subunit of the cyclic AMP-dependent protein kinase to a stoichiometry of between 0.1 and 0.15 mol/mol. The labelled protein was precipitated with 20% (w/v) trichloroacetic acid, redissolved in 20 μl of 1 M-NaOH at 4 °C, diluted to 1 ml with 20 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA and dialysed for 12 h against the same buffer.

Protein kinase C was autophosphorylated by incubation of 10–20 μg (50–100 units) for 30 min at 30 °C in the presence of 1.2 mM-Ca^{2+}, 200 μg of phosphatidylserine/ml, 10 mM-Mg^{2+}, 0.5 mM-EDTA and 1 mM-[γ-^{32}P]ATP (specific radioactivity 1000–2500 c.p.m./pmol) in 20 mM-Hepes/NaOH buffer, pH 7.5. The reaction mixture (200 μl) was then made 0.01% in Triton X-100 and passed through a Sephadex G-50 column equilibrated with 20 mM-Tris/HCl buffer, pH 7.5, containing 2 mM-EDTA, 50 mM-2-mercaptoethanol and 0.01% Triton X-100. Labelled protein kinase C (0.9–1.2 mol of phosphate/mol of enzyme) was pooled and stored at 4 °C; no detectable dephosphorylation occurred on storage for up to 10 days.

RESULTS

Dephosphorylation by the PCS phosphatases

In order to assess the relative activities of the PCS phosphatases against protein kinase C, activities against histone IIA-S and phosphorylase *a* were also determined. As shown in Table 1, all forms of this phosphatase were

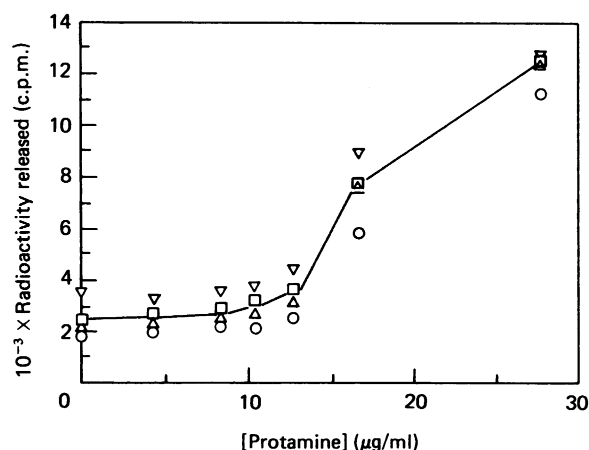

Fig. 1. Activation of the PCS phosphatases by protamine

Protein kinase C (0.5 μM) was dephosphorylated as described in the Materials and methods section. The final phosphorylase *a* phosphatase concentrations of the PCS phosphatases were: PCS_{H1}, 0.06 unit/ml (□); PCS_{H2}, 0.04 unit/ml (○); PCS_M, 0.03 unit/ml (△); PCS_L, 0.07 unit/ml (▽). Results are expressed as radioactivity (c.p.m.) released (total of 30330 c.p.m./assay) as a function of the protamine concentration (μg/ml).

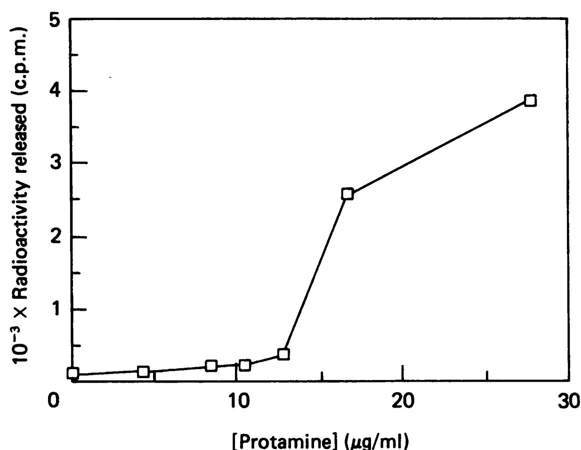

Fig. 2. Activation of the catalytic subunit of the ATP+Mg^{2+}-dependent phosphatase by protamine

The catalytic subunit of the ATP+Mg^{2+}-dependent phosphatase (0.04 unit/ml final concentration with phosphorylase *a* as substrate) was used to dephosphorylate protein kinase C (0.5 μM) as described in the Materials and methods section. Results shown are radioactivity (c.p.m.) released (total of 48864 c.p.m./assay) plotted as a function of protamine concentration (μg/ml).

effective in the dephosphorylation of protein kinase C. Indeed, the oligomeric forms of the PCS phosphatase showed protein kinase C phosphatase activities comparable with the phosphorylase *a* phosphatase activities (34–82%). However, the purified 34000-M_r catalytic subunit that is common to all these PCS phosphatases was itself relatively poor in the dephosphorylation of protein kinase C in the absence of protamine (Table 1).

It can be seen in Table 1 that all the PCS phosphatases were stimulated by protamine. Fig. 1 shows the dose-dependent activation of various forms of the PCS phosphatases by protamine. The apparent K_a values for PCS_{H1}, PCS_{H2}, PCS_M and PCS_L phosphatases were 15.3, 16.0, 15.8 and 14.5 μg/ml respectively. The similarity of these activation constants contrasts with the situation observed with phosphorylase *a* as substrate, where protamine yields a very different series of activation constants (Waelkens *et al.*, 1986); this suggests that for protein kinase C there may be substrate-directed effects involved. This interpretation is clearly consistent with the 20-fold stimulation of PCS_C phosphatase by protamine (see Table 1); with either phosphorylase *a* or histone IIA-S as substrate no stimulation by protamine is observed for PCS_C phosphatase (Waelkens *et al.*, 1986, and results not shown).

Dephosphorylation of protein kinase C by the ATP+Mg^{2+}-dependent protein phosphatase

The catalytic subunit of the ATP+Mg^{2+}-dependent protein phosphatase showed very little activity against protein kinase C even at high phosphatase concentrations (> 20 units/ml). However, significant activity against protein kinase C could be expressed in the presence of protamine (Fig. 2). The catalytic subunit of the ATP+Mg^{2+}-dependent protein phosphatase is not known to be activated directly by polycations; indeed, with phosphorylase *a* as substrate, inhibition is observed (Waelkens *et al.*, 1986). As with the PCS phosphatases (see above), this effect of protamine would appear to be

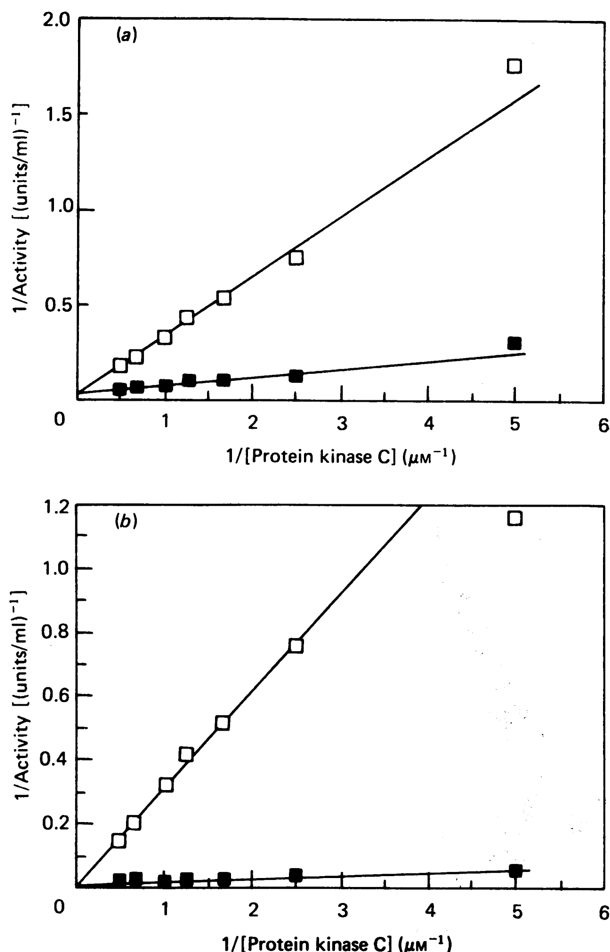

Fig. 3. Kinetic analysis of the activation of PCS_{H1} phosphatase (*a*) and the catalytic subunit of ATP+Mg^{2+}-dependent protein phosphatase (*b*) by protamine

The dephosphorylation of protein kinase C was carried out with PCS_{H1} phosphatase (*a*) or the catalytic subunit of ATP+Mg^{2+}-dependent protein phosphatase (*b*) either in the absence (□) or in the presence (■) of protamine (30 μg/ml). Results are expressed as double-reciprocal plots of activity against substrate concentration. In (*a*) the K_m and $V_{max.}$ values are 4.2 μM and 41.5 units/ml (−protamine) and 0.8 μM and 43.6 units/ml (+protamine). In (*b*) the K_m and $V_{max.}$ values are 25 μM and 28.9 units/ml (−protamine) and 0.9 μM and 32.7 units/ml (+protamine).

substrate-directed. The apparent K_a for protamine in this instance is 15.0 μg/ml. This value is similar to that determined for the PCS phosphatases, and this would be consistent with the protamine effect being substrate-directed.

Effect of protamine on apparent K_m and $V_{max.}$ values

The effects of protamine on the kinetic parameters for the dephosphorylation of protein kinase C by PCS_{H1} phosphatase and the catalytic subunit of the ATP+Mg^{2+}-dependent protein phosphatase are shown in Fig. 3. In both cases the apparent K_m for protein kinase C is decreased but there is no significant change in $V_{max.}$ values. For PCS_{H1} phosphatase protamine decreases the K_m from 4.2 μM to 0.8 μM, and for the catalytic subunit

Table 2. Effects of protamine, polylysine and histone on the dephosphoryation of protein kinase C

Assays were carried out at 30 °C for 5 min with PCS_{H1} phosphatase diluted to 0.5 unit/ml before assay (phosphorylase *a* phosphatase activity).

	Phosphatase activity (units/ml)
Control	0.36
Protamine (20 μg/ml)	1.19
Polylysine (25 μg/ml)	1.55
Histone III-S (200 μg/ml)	1.35

of the ATP + Mg^{2+}-dependent phosphatase it is decreased from > 20 μM to 0.9 μM.

Specificity of polycation stimulation

Protamine is an unusual substrate for protein kinase C in that its phosphorylation does not require Ca^{2+} and phospholipid (Wise *et al.* 1982). In order to determine whether the stimulation of dephosphorylation of protein kinase C by protamine was related to this characteristic, two other polycations were tested. Histone HIII-S (histone HI-rich) is the preferred histone substrate for protein kinase C, and its phosphorylation is dependent upon Ca^{2+} and phospholipid. In contrast, the polycation polylysine cannot be phosphorylated by protein kinase C. Table 2 shows that all three polycations were effective in stimulating the dephosphorylation of protein kinase C by PCS_{H1} phosphatase. Similarly, activation by these polycations is obtained with PCS_C phosphatase (results not shown).

DISCUSSION

In order to probe the dephosphorylation of protein kinase C various purified protein phosphatases were tested on the autophosphorylated kinase. It is evident that among the PCS phosphatases there is some selectivity with respect to protein kinase C dephosphorylation, since, in contrast with the higher-M_r species, the isolated catalytic subunit is only weakly active (in the absence of protamine). It is presumably the presence of the associated subunit(s) that permits efficient dephosphorylation by PCS_H, PCS_M and PCS_L phosphatases. The substantial influence of the subunits on the catalytic unit has been noted elsewhere (Goris *et al.*, 1985; Agostinis *et al.*, 1986). For example the deinhibitor phosphatase activity observed with the PCS_C phosphatase is negligible when compared with the PCS_H phosphatase activity, and with a basic synthetic hexapeptide substrate a 50-fold difference in efficiency was observed between the PCS_C and the PCS_H phosphatases (Agostinis *et al.*, 1986).

Interestingly the response of the PCS phosphatases to polycations with protein kinase C as substrate differs markedly from that observed with phosphorylase *a* as substrate. With protein kinase C the apparent K_a values for protamine were all between 14 and 16 μg/ml. In contrast, with phosphorylase *a* as substrate protamine-dependent activation of, for example, PCS_M and PCS_H phosphatases yields K_a values differing by nearly two orders of magnitude (Waelkens *et al.*, 1986). These observations suggest that the effect of protamine seen here is likely to be substrate-directed. With phosphorylase *a* as substrate there is evidence to indicate enzyme-directed activation by polycations (Waelkens *et al.*, 1986). Since no activation is observed at these lower protamine concentrations, it would appear that protamine is not having a direct influence on the PCS phosphatases when assayed with autophosphorylated protein kinase C, although such direct effects cannot be completely excluded.

The above conclusion is reinforced by the finding that the ATP + Mg^{2+}-dependent protein phosphatase (catalytic subunit) shows very poor activity in the dephosphorylation of protein kinase C (0.5 μM) in the absence of protamine although it shows significant activity in its presence. The ATP + Mg^{2+}-dependent protein phosphatase is not known to be stimulated directly by polycations, again suggesting a substrate-directed effect of protamine. The apparent K_a for protamine (15 μg/ml) was similar to that observed for the PCS phosphatases, consistent with this conclusion. The kinetic analysis with respect to protamine effects further reinforces this conclusion, since the effect of protamine is to decrease the apparent K_m for protein kinase C.

The interaction of protein kinase C with protamine is unusual in that protamine is the only protein substrate for this kinase that is readily phosphorylated in the absence of Ca^{2+} and phospholipid (Wise *et al.*, 1982); this suggests that protamine may interact with protein kinase C in a unique fashion. However, with respect to dephosphorylation, the effect of protamine was reproduced by two other polycations, lysine-rich histone and polylysine. This histone fraction is a substrate for protein kinase C, but phosphorylation in this instance requires Ca^{2+} and phospholipid. Polylysine cannot be phosphorylated by protein kinase C. At high concentrations polylysine will partially stimulate protein kinase C activity (K_a > 100 μg/ml; results not shown); however, this effect appears to be unrelated to the stimulation of dephosphorylation (K_a approx. 10 μg/ml). The similar effects of these different polycations in stimulating the dephosphorylation of protein kinase C must therefore be assumed to be due to non-specific interaction of these positively charged polypeptides with the kinase.

The autophosphorylation of protein kinase C *in vitro* has a significant effect upon its rate of cleavage *in vitro* into a catalytically active fragment (P. J. Parker, unpublished work); the generation of such a fragment *in vivo* has been shown to occur in response to agonists (Tapley & Murray, 1984). It remains to be determined to what extent this mode of activation is of physiological significance. Nevertheless, the influence of the phosphorylation state may represent an important mechanism for controlling the expression of this activity. In view of the relative activities it would appear that the ATP + Mg^{2+}-dependent protein phosphatase does not play a significant role in the regulation of the protein kinase C phosphorylation state. In contrast, activation (or inhibition) of the PCS phosphatases may exert a controlling effect on protein kinase C phosphorylation and therefore may play a role in the generation of an active protein kinase C species *in vivo*.

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