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POLYAMINES AS NEGATIVE REGULATORS OF CASEIN KINASE-2: The phosphorylation of calmodulin triggered by polylysine and by the α[66-86] peptide is prevented by spermine

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Calmodulin and other protein substrates of casein kinase-2 (CK2) are not phosphorylated by CK2 holoenzyme under basal conditions. The non catalytic β-subunit of CK2 is responsible for such a down-regulation which can be overcome by the addition of polylysine [Meggio, F. et al. (1992) Eur. J. Biochem. 205, 939-945]. Here we show that the peptide CVVKILKPVKKKKIKREIKILE, reproducing the basic insert 66-86 of CK2 catalytic subunit, can mimick polylysine in triggering the latent "calmodulin kinase" activity of CK2 holoenzyme, and that spermine and, to a lesser extent, spermidine, but not putrescine, can reversibly and dose-dependently counteract such an activation. Spermine also abolishes the stimulation by polybasic peptides of basal CK2 activity. These findings disclose the possibility that spermine may act *in vivo* as a negative regulator of CK2 activity toward a category of substrates, like calmodulin and ornithine decarboxylase, whose phosphorylation is dependent on polybasic peptides.

Casein kinase-2 (CK2) is an ubiquitous Ser/Thr specific protein kinase implicated in a wide spectrum of cellular functions, with special reference to gene expression, cell proliferation and signal transduction [1, 2]. While the specificity determinants of CK2 , resting on acidic and/or phosphorylated residues in the proximity of the target aminoacid, have been enlightened [1, 3, 4], the mode of regulation of

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Abbreviations: α-peptide, CVVKILKPVKKKKIKREIKILE; CK2, casein kinase-2; ODC, ornithine decarboxylase.

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this pleiotropic protein kinase still remains elusive. Apparently in fact CK2 is not strictly dependent either on second messengers or on a phosphorylation/dephosphorylation control mechanism, since it is spontaneously active toward a variety of protein and peptide substrates.

Interestingly, however, the basal activity of CK2 is nearly silent toward a number of physiological substrates, including, among others, ornithine decarboxylase (ODC) [5] and calmodulin [6], whose phosphorylation is triggered by the addition of polybasic peptides, like protamines, some histones and polylysine. Such an intrinsic down-regulation of CK2 activity toward calmodulin is imposed by the non catalytic β-subunit, since, unlike native CK2 heterotetramers ($\alpha_2\beta_2$), the isolated α-subunit does display a spontaneous "calmodulin kinase" activity which disappears upon assemblance with the β-subunits [7]. An acidic cluster located in the N terminal moiety of the β-subunit seems to participate both in the down-regulation of CK2 activity and in the activation by polybasic peptides, since substitution of acidic residues in that cluster gives rise to mutants which are unable to suppress the "calmodulin kinase" activity (manuscript in preparation) and are poorly responsive to polylysine [8].

Intriguingly physiological polyamines, which are capable to stimulate CK2 activity under some circumstancies [9, 10], and are known to operate by binding to the β-subunit [11], proved totally unable to surrogate polybasic peptides as activators of CK2 activity toward either calmodulin [6] or ODC [5], i.e. substrates that are nearly unaffected by CK2 under basal conditions. Collectively these observations suggested that different classes of polycations could bind to the β-subunit with variable effectiveness, and disclosed the possibility that polyamines could actually counteract the capability of polybasic peptides to trigger the "calmodulin kinase" activity of CK2 by competing for the same binding site(s). Here we show that indeed physiological concentrations of spermine effectively reverse the stimulation of CK2 activity induced by polylysine and by a peptide reproducing an unique basic insert of CK2 catalytic subunit.

Materials and Methods

Calmodulin, polylysine (47,500 M_r), spermine, spermidine and putrescine were from Sigma. CK2 was isolated from rat liver cytosol and purified to near homogeneity as previously described [12] with a further purification step on FPLC Mono Q column. Its specific activity was about 35 U/mg, one unit being defined as the amount of enzyme transferring 1 nmol P to casein substrate in one minute. The peptides

RRRAADSDDDDD and CVVKILKPVKKKKIKREIKILE, used as substrate and activator of CK2, respectively, were synthesized on an Applied Biosystems peptide synthesizer (Model 431 A) with a procedure that will be described in details elsewhere. The latter peptide reproduces the 66-86 sequence of CK2 α-subunit with an N-terminal cysteinyl residue added for technical reasons, and will be henceforth termed α-peptide.

Calmodulin (8 μM) and peptide substrate (100 μM) were phosphorylated by CK2 (2 mU) by incubation at 37 °C for 15 minutes in a final volume of 30 μl containing 50 mM Tris-HCl buffer pH 7.5, 2 mM $MgCl_2$, and 50 μM [γ^{32}P]ATP (specific activity 600 cpm/pmol). ^{32}P incorporated into protein and peptide substrates was evaluated as previously described [7].

Results

A typical experiment showing that both polylysine and the synthetic peptide CVVKILKPVKKKKIKREIKILE are capable to trigger the phosphorylation of calmodulin by CK2 is illustrated in figure 1. It can be also seen that spermine, which is totally ineffective *per se*, reduces the phosphorylation of calmodulin promoted by either polylysine or the α-peptide, its effect being especially drastic with the latter.

Spermine inhibition of calmodulin phosphorylation induced by either polylysine (figure 2A) or the α-peptide (figure 2B) is dose dependent. In the presence of 50 μM α-peptide 4 mM spermine nearly

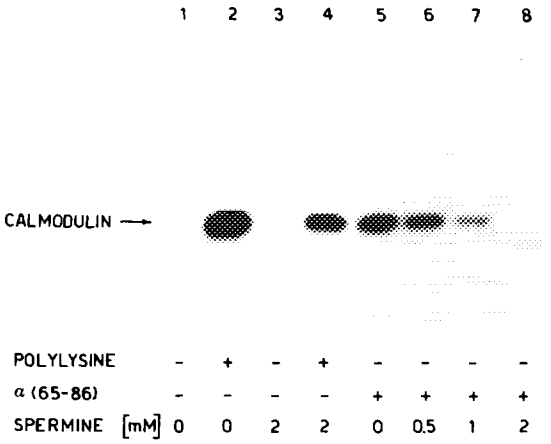

Figure 1. *Spermine counteracts the phosphorylation of calmodulin promoted by polybasic effectors.*
Phosphorylation of calmodulin by CK2 was performed as described under Materials and Methods. Polylysine and α-peptide, where present, were 40 nM and 50 μM, respectively. The autoradiography of the gel is shown.

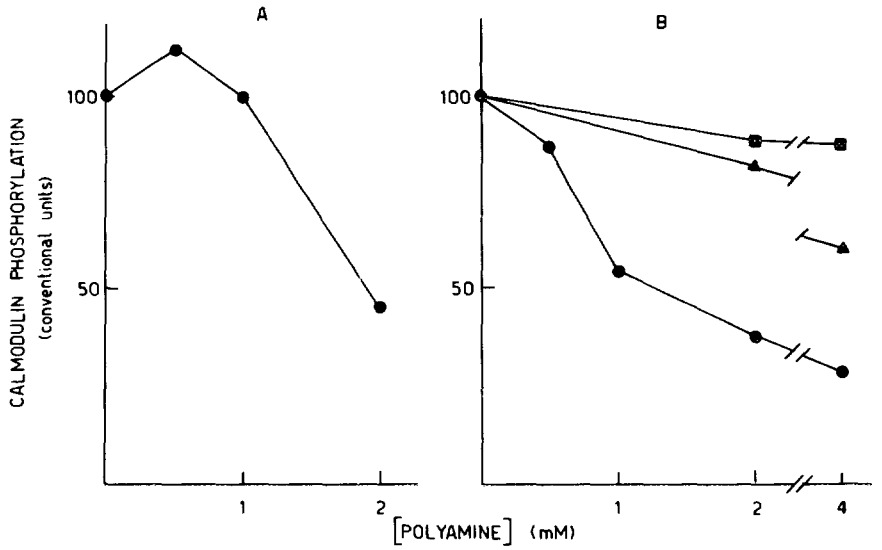

Figure 2. *Dose dependent effect of spermine (●), spermidine (▲) and putrescine (■) on calmodulin phosphorylation promoted by either polylysine (A) or α-peptide (B).*
Phosphorylation of calmodulin by CK2 in the presence of either 40 nM polylysine (A) or 50 μM α-peptide (B) was performed and assayed as described under Materials and Methods. The ^{32}P incorporated into calmodulin was quantified by excising the bands from the gel and counting them in a liquid scintillator (Packard).

suppresses calmodulin phosphorylation, with an IC_{50} value of about 1.2 mM. As also shown in figure 2, spermidine is much less effective than spermine and putrescine is nearly ineffective up to 4 mM concentration.

On the other hand spermine inhibition can be reversed by increasing the concentration of the activator α-peptide (Table 1), supporting the concept that there is a competition between these two polycationic compounds for the same binding site.

Besides triggering CK2 activity toward calmodulin and some other substrates, polybasic peptides also stimulate CK2 basal activity toward "canonical" substrates, including a variety of synthetic peptides [7], whose phosphorylation is also increased, albeit to a lesser extent, by spermine. In order to check if also in this case spermine could reduce the stimulation by polybasic peptides, the effects of increasing concentrations of spermine on either the basal or α-peptide induced phosphorylation of a CK2 peptide substrate have been investigated. As shown in figure 3, spermine alone slightly stimulates CK2 activity. Such a stimulation, however, far from being additive to that of the α-peptide, is converted into an overall dose-dependent

Table I: *Reversal of spermine inhibition of calmodulin phosphorylation by increasing concentrations of the α-peptide*

Concentration of α-peptide (μM)	Calmodulin phosphorylation (pmol P)		Inhibition by spermine (%)
	no spermine	spermine (2 mM)	
50	18.13	2.86	84.2
100	19.76	8.26	58.2
200	21.00	13.83	34.1
400	17.16	16.41	4.4

Phosphorylation of calmodulin by CK2 was performed and assayed as described under Materials and Methods and in the legend of figure 2.

inhibition, if spermine is added together with the stimulatory peptide. As a consequence, the stimulation induced by the α-peptide is gradually abolished by increasing spermine concentration, the activity assayed at 4 mM spermine being almost as low as the basal one.

Discussion

A rather paradoxical outcome of this study is that spermine, which is normally considered a stimulator of CK2 activity [9, 10], under

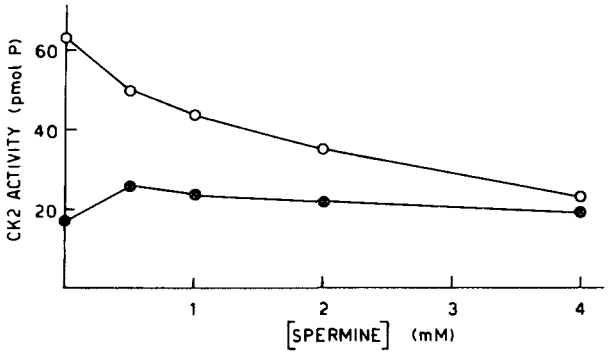

Figure 3. *Dose-dependent effect of spermine on CK2 stimulation by the α-peptide.*
The peptide substrate RRRAADSDDDDD was phosphorylated by CK2 as described under Materials and Methods either in the absence (●) or in the presence of 50 μM α-peptide (o).

certain conditions behaves as an inhibitor, since it can prevent the phosphorylation of calmodulin by CK2. Such a contraddiction is only apparent considering that under basal conditions CK2 holoenzyme is silent toward calmodulin and some other substrates, whose phosphorylation is triggered by a variety of polybasic peptides, but not by spermine. It is conceivable therefore that polyamines and notably spermine could actually reverse such an activation of CK2 specifically promoted by polybasic peptides, through a mechanism of futile competition.

The results presented here fully support this expectation by showing that physiological concentrations of spermine can efficiently and reversibly counteract the "calmodulin kinase" activity of CK2 that is induced by polylysine and by a basic peptide reproducing a unique segment of CK2 catalytic subunit. It is conceivable therefore that spermine could indeed act *in vivo* as a negative regulator of CK2 activity with special reference to those substrates whose phosphorylation is strictly dependent on polycationic effectors. Nuclei appear to be especially suitable for such a regulatory mechanism since the presence in them of CK2 [13, 14], calmodulin [15] and spermine [16] is well documented and histones could represent in nuclei the natural compounds committed with the triggering of calmodulin phosphorylation by CK2.

Although in general the identity of such activators, whose effect is mimicked *in vitro* by polylysine, remains a matter of conjecture, many potential candidates are available, besides histones, including a variety of proteins having basic stretches in their sequence. It should be noted in this connection that a definite structure of the basic cluster is required in order to trigger the "calmodulin kinase" activity of CK2, since several peptides resembling the α-peptide for their size and number of positively charged residues, but displaying different sequences, proved either less effective or totally ineffective (unpublished results).

It may also be worthy of attention that the latency of CK2 holoenzyme activity toward calmodulin under basal conditions is due to a down regulation of the intrinsic activity of the catalytic subunit (which otherwise phosphorylates calmodulin) by the β-subunit [7]. We have recently shown that an acidic cluster in the 55-64 sequence of the β-subunit is, at least partially, responsible for such a down-regulation [8]. Here we show that a peptide reproducing the highly conseved, basic insert of CK2 catalytic subunit responsible for heparin inhibition [17], is very effective in counteracting the negative

regulation imposed by the β-subunit. These findings strongly suggest that the 66-86 sequence of the catalytic subunit might specifically interact with the acidic 55-64 segment of the β-subunit.

It is conceivable therefore that polybasic effectors whose structure mimick that of the α(66-86) sequence will operate by preventing the binding to this sequence of the acidic down regulatory stretch of the β-subunit. Spermine, which is known to bind to the β-subunit [11], will in turn counteract the stimulation of CK2 by these basic peptides, thus abolishing the phosphorylation of substrates whose targeting is entirely resting on this mechanism of activation. The presence among these substrates of ODC [5], i.e. the key enzyme of the biosynthetic pathway leading to spermine, is of special interest, as it highlights a potential feedback regulation of ODC by spermine, mediated by CK2.

Acknowledgments

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