INTERACTION OF A CASEIN KINASE (G-TYPE) WITH A SPECIFIC ENDOGENOUS INHIBITOR

Possible target for the regulation of a cyclic nucleotide-independent protein kinase activity by polyamines

D. JOB, F. PIROLLET, C. COCHET and E. M. CHAMBAZ
Biochimie Endocrinienne, Université Scientifique et Médicale, CERMO, BP 53 X, 38041 Grenoble, France

Received 30 October 1979

1. Introduction

Cyclic nucleotide-independent ATP:protein phosphotransferase (protein kinases, PK) activities have been described in mammalian tissues [1]. Criteria for their characterization have been established [2] and highly purified preparations have been obtained by several research groups [3–7]. Although these PK have been suggested to be involved in the modulation of key metabolic pathways [8–10], possible regulation and intracellular effectors of their activity remain to be defined.

We have reported the characterization in bovine adrenal cortex of a soluble cyclic nucleotide-independent casein kinase (CK) activity [11]. This activity has subsequently been shown to represent several enzymatic moieties which could be classified into two groups according to their properties [12,13]: a type A casein kinase (CKA) using only ATP and a type G (CKG) using GTP as well as ATP as phosphoryl donor. It was then found that an endogenous inhibitory factor, specifically directed toward CKG activity (CKG I) was present in the same tissue. The inhibitor was isolated and characterized as a heat-stable protein moiety [14]. The presence of CKG I led to a mostly inhibited CKG activity in crude adrenal cortex cytosol, suggesting that a CKG–CKG I interaction might represent a potential regulatory pathway of CKG activity in intact cell [14].

Here we report the observation that several naturally occuring polyamine structures can reverse the inhibitory action of CKG I upon CKG activity. A CKG–CKG I association is demonstrated to occur using purified components and dissociation of the enzyme–inhibitor complex is promoted by the presence of polyamines. In addition, saturable binding of polyamines to CKG I is observed. These data allow the proposal of a model as a working hypothesis, in which polyamines appear as potential intracellular effectors regulating CKG activity via an action upon the CKG–CKG I couple, in addition to a direct stimulating effect on the enzyme.

2. Material and methods

[γ-^{32}P]ATP (20 Ci/mmol) and [γ-^{32}P]GTP (25 Ci/mmol) were purchased from the Radiochemical Center (Amersham) and [^{3}H]spermine (21 Ci/mmol) from New England Nuclear. Casein (Merck) was treated according to [15] before use. Unlabeled polyamines were obtained from Sigma.

2.1. *Casein kinase of the G-type (CKG)*

This was isolated from adrenal cortex cytosol after phosphocellulose chromatography as in [12,14]. CKG was further purified by an affinity chromatography step through a casein–Sepharose column according to [16]. Details of the procedure and properties of the purified enzymatic preparation will be reported elsewhere.

Elsevier/North-Holland Biomedical Press

2.2. *G-type casein kinase inhibitor (CKG I)*

This was obtained either from bovine adrenal cortex or lung tissues, by the procedure in [14].

2.3. *Protein kinase assay*

This was performed under the incubation conditions [17], with the trichloroacetic acid precipitation step from [18]. The 80 μl standard reaction mixture contained 50 μl CKG preparation or a 50 μl mixture of enzyme, inhibitor and/or effector as indicated and 30 μl TDG buffer (25 mM, (pH 7.5) Tris–HCl containing 1 mM dithiothreitol and 2% glycerol) containing 60 μg casein, [γ-^{32}P]ATP and $MgCl_2$ as indicated. The reaction was initiated upon addition of the enzyme and run at 30°C under linear conditions with regard to time and enzyme concentration.

2.4. *Sedimentation studies*

These were performed in linear sucrose (5–20%) density gradients in TDG buffer spun at 127 000 × g_{av} in a MSE SW-50 rotor at 4°C for 10 h. Glucose oxidase ($s_{20,w}$ 7.8) and bovine serum albumin (BSA, $s_{20,w}$ 4.3) were used as protein calibration standards.

2.5. *Protein measurements*

These were performed using the method in [19]. Radioactivity was determined in Bray's solution [20] with a Nuclear Chicago scintillation spectrometer.

3. Results and discussion

3.1. *Effect of polyamines on CKG and CKG I activities*

Increasing concentrations of spermine were introduced into a series of incubations carried out with a constant amount of CKG under two sets of conditions: (i) without CKG I; (ii) with a constant amount of CKG I in order to yield about 70% inhibition of the casein kinase activity in the absence of spermine. The enzymatic activity and the extend of its inhibition obtained when spermine was increased from 0–2 mM are plotted in fig.1. In the absence of CKG inhibitor, spermine exhibited a stimulating effect upon CKG activity, rapidly increasing at > 0.1 mM polyamine and reaching a plateau at ~2 mM. This effect has been reported with bovine adrenal cortex casein kinases [13] and various cyclic nucleotide-independent protein kinases from other sources [21–23].

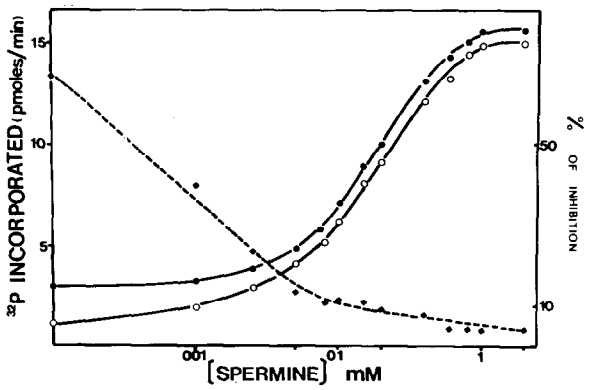

Fig.1. Effect of spermine on CKG activity in the presence and absence of CKG I. Incubation mixtures in final vol. 80 μl were: TDG buffer containing 60 μg casein, 10^{-4} M [γ-^{32}]ATP (spec. act. 100 cpm/pmol), 2 mM $MgCl_2$, spermine concentrations as indicated and either 0.09 μg CKG I (○——○) or no addition of CKG I (●——●). The reaction was initiated by the addition of 0.048 μg CKG. Corresponding % inhibition of CKG in the presence of CKG I were plotted (◆- - -◆). Each point was the mean of 3 replicates.

Most interesting was the finding that at low spermine concentrations (≤0.1 mM), the inhibitory effect of CKG I upon CKG activity was progressively abolished, falling from 70% (no spermine) to <10% (80 μM spermine) inhibition. The specificity of this effect was examined using various naturally occuring polyamines at a concentration for which the direct CKG stimulation was minimal. As shown in table 1, spermine appeared the most effective agent able to promote the reversal of the CKG I inhibitory action.

Table 1
Effect of various polyamines on CKG activity in the presence and absence of CKG I

	^{32}P incorporated (pmol/min)		% inhibition
	No CKG I	+ CKG I	
Control	2.62 ± 0.06	0.63 ± 0.01	75.9
Spermine	3.13 ± 0.03	2.42 ± 0.06	22.7
Spermidine	2.51 ± 0.05	1.01 ± 0.09	59.8
Cadaverine	2.21 ± 0.03	0.61 ± 0.05	72.4
Putrescine	2.29 ± 0.08	0.65 ± 0.07	71.6

Assay conditions were as in fig.1. Polyamines were tested at final conc. 20 μM. Each point was the mean of 4 replicates (6 replicates for the control)

The effectiveness of the other structures sharply decreased as the polyamine chain was shortened. These data suggest that polyamines may be able to reverse the effect of CKG I upon CKG activity. However, since a substrate (casein)–polyamine interaction has been suggested to explain the stimulatory effect of these polycations on protein kinase activity [24], a demonstration of a CKG–CKG I interaction was attempted and the effect of spermine on this process was examined.

3.2. *Sedimentation studies*

Purified CKG and CKG I preparations could be independently characterized upon density gradient centrifugation, as shown in fig.2. Purified CKG sedimentation behavior (fig.2a) was greatly influenced by the presence of polyamine in the medium (fig.2b), thus both conditions were included as proper control

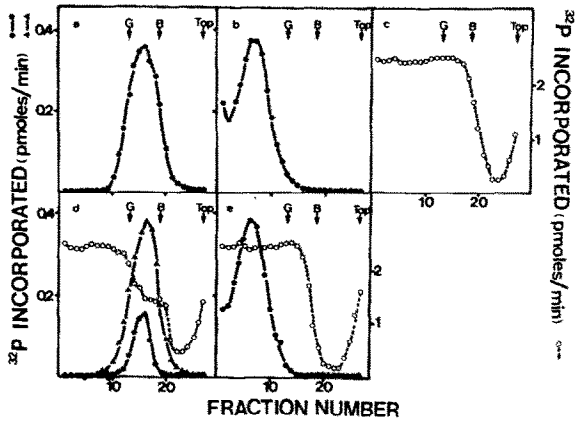

Fig.2. Analysis of the CKG–CKG I interaction by sucrose density gradient centrifugation. Sucrose gradients (5–20%, w/v) were prepared in TDG buffer containing 0.05 M NaCl and 2 mM $MgCl_2$ (a,c,d) or 2 mM $MgCl_2$ and 0.5 mM spermine (b,e). Loaded samples contained in 100 μl: (a,b) 2.4 μg of CKG; (c) 0.88 μg CKG I, (d,e) 2.4 μg CKG and 0.88 μg CKG I. Centrifugation was carried out at 127 000 × g_{av} for 10 h. CKG activity was assayed on 5 μl aliquots of collected fractions with a reaction mixture containing 10^{-5} M [γ-^{32}P]ATP (spec. act. 1000 cpm/pmol), 50 mM $MgCl_2$ and 60 μg (●——●) or 600 μg (▲——▲) of casein. Inhibitory activity (○- - -○) was assayed on 30 μl aliquots of heat-treated fractions (1 min, 100°C) in the presence of 0.048 μg CKG, 10^{-5} M [γ-^{32}P]ATP, 60 μg casein and 50 mM $MgCl_2$, in TDG buffer [14]. Glucose oxidase (G), bovine serum albumin (B) were used as marker proteins.

experiments. This effect of spermine upon the physicochemical properties of CKG may suggest that the stimulatory effect of the polyamine on the enzyme activity involves a direct effect upon the enzyme molecular organization. The inhibitor exhibited a sedimentation coefficient of ~2.5 S which was not influenced by the presence of polyamine (fig.2c). Figure 2d shows that when CKG and its inhibitor were mixed before density gradient analysis, a significant part of the CKG I activity was found associated with the enzymes. Although this observation will need quantitative studies to assess the type of interaction involved, it was interpreted as showing that CKG and CKG I associate into a molecular complex. This association might have been expected since CKG I was significantly inhibited under these conditions. However, it may be pointed out that when casein concentration was increased in the assay (fig.2d), the inhibitory action of CKG I could be reversed as the result of a substrate–inhibitor competition, as in [14]. As depicted in fig.2e, addition of spermine to the CKG–CKG I mixture promotes the dissociation of the enzyme–inhibitor complex, and restores the CKG activity to the uninhibited level, while the sedimentation behavior of the enzyme was also shifted as expected from the control experiment (fig.2b).

These data clearly demonstrate a CKG-CKG I affinity leading to a molecular association of the two components corresponding to an inhibited form of the enzyme. A polyamine is able to dissociate the complex, therefore reversing the inhibitory effect of CKG I.

The mechanism of this polyamine effect might involve an interaction with the casein kinase moiety and subsequent modification of the enzyme molecular structure with a parallel loss of its affinity for the inhibitor. Although such a process was suggested by the effect of relatively high concentrations of spermine (e.g., 0.5 mM) upon sedimentation properties of CKG (see fig.2), much lower polyamine concentration (80 μM) was sufficient to promote almost total reversion of the inhibitor effect (see fig.1). The alternative process, i.e., interaction of the polyamine with the inhibitor was examined in further experiments.

3.3. *CKG I–polyamine interaction*

The inhibitor preparation was incubated with

[^{3}H]spermine and the mixture passed through a Sephadex G-75 column. As observed in [14] CKG I was excluded from the gel whereas spermine was retained on the column. As shown in fig.3, radioactivity was found associated with CKG I eluting from the column, thus demonstrating that the inhibitor is able to bind the polyamine. That this binding represents a saturable process was demonstrated by the fact that addition of unlabeled spermine to the mixture displaced the bound radioactivity (fig.3). Quantitative study of this binding process is currently in progress, especially in order to define the affinity of various polyamines toward CKG I and CKG, respectively.

From the aforementioned data, one might propose that at low concentration (i.e., < 0.1 mM in the case of spermine) polyamines associate to CKG I; this phenomenon is concomitant with reversal of the inhibitory CKG I effect and a minimal CKG activation by a direct effect on the free enzyme (see fig.1). On the other hand, at higher polyamine concentration (i.e., > 0.1 mM spermine), the CKG–CKG I complex is dissociated (and the CKG I effect consequently abolished); a direct effect of the polyamine on the enzyme leads to further increase of CKG activity.

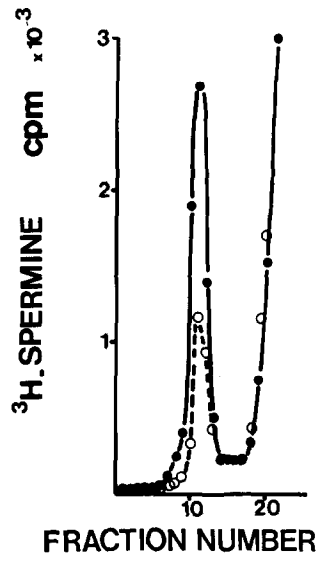

Fig.3. Spermine binding to CKG I. CKG I (4.4 μg) in 50 μl TDG buffer containing 10^5 cpm [^{3}H]spermine (•- -•) or 10^5 cpm [^{3}H]spermine and 1 mM spermine (○- -○) were incubated 5 min at 25°C before being loaded onto a Sephadex G-75 column (0.6 × 10 cm) which was eluted with TDG buffer. Radioactivity was measured in the collected 100 μl fractions.

It may thus be possible to visualize these data by the following equilibrium which is proposed as a working hypothesis:

$$\underset{\text{(inactive complex)}}{\text{CKG–CKG I} + \text{polyamine}} \rightleftharpoons \underset{\text{(active enzyme)}}{\text{CKG I} - \text{polyamine} + \text{CKG}}$$

The active enzyme, after dissociation from its inhibitor, being possibly subjected to additional stimulation by a direct effect of an excess polyamine. Further, studies are obviously needed to establish the validity of this hypothesis as a general scheme which may represent a target for polyamine action at 'physiological' concentrations. The CKG–CKG I couple would represent a possible link between polyamines and cyclic nucleotide-independent phosphorylation processes, both being potentially important factors in the control of cellular growth and differentiation [25]. Studies are in progress to define more completely the proposed model and other possible effectors before examining its validity in the intact cell.

Acknowledgements

This work was possible thanks to the support from the CNRS (ERA 478), the INSERM (ATP 52-77-84 and 82-79-114) and the Fondation pour la Recherche Médicale Française.

References

[1] Rubin, C. S. and Rosen, O. M. (1975) Ann. Rev. Biochem. 44, 831–887.
[2] Traugh, J. A., Ashby, C. D. and Walsh, D. A. (1974) Methods Enzymol. 38, 290–296.
[3] Issinger, O. G. (1977) Biochem. J. 165, 511–518.
[4] Thornburg, W. and Lindell, T. J. (1977) J. Biol. Chem. 252, 6660–6665.
[5] Dahmus, M. E. and Natzle, J. (1977) Biochemistry 16, 1901–1907.
[6] Lerch, K., Muir, L. W. and Fischer, E. H. (1975) Biochemistry 14, 2015–2023.
[7] Hathaway, G. M. and Traugh, J. A. (1979) J. Biol. Chem. 254, 762–768.

[8] Nimmo, H. G. and Cohen, P. (1977) Adv. Cyclic Nucl. Res. 8, 145–266.
[9] Datta, A., De Haro, C., Sierra, J. M. and Ochoa, S. (1977) Proc. Natl. Acad. Sci. USA 74, 1463–1467.
[10] Trachsel, H. Ranu, R. and London, I. M. (1978) Proc. Natl. Acad. Sci. USA 75, 3654–3658.
[11] Cochet, C., Job, D. and Chambaz, E. M. (1977) FEBS Lett. 83, 53–58.
[12] Cochet, C., Job, D., Pirollet, F. and Chambaz, E. M. (1978) Biochimie 60, 566.
[13] Cochet, C., Job, D., Pirollet, F. and Chambaz, E. M. (1980) Endocrinology in press.
[14] Job, D., Cochet, C., Pirollet, F. and Chambaz, E. M. (1979) FEBS Lett. 98, 303–308.
[15] Ashby, C. D. and Walsh, D. A. (1974) Methods Enzymol. 38, 350–358.
[16] Farron-Furstenthal, F. and Lightholder, J. R. (1977) FEBS Lett. 84, 313–316.
[17] Corbin, J. D. and Reiman, E. M. (1974) Methods Enzymol. 38, 287–290.
[18] Sandoval, I. V. and Cuatrecasas, P. (1976) Biochemistry 15, 3424–3432.
[19] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[20] Bray, G. A. (1960) Anal. Biochem. 1, 279–282.
[21] Mäenpää, P. H. (1977) Biochim. Biophys. Acta 498, 294–305.
[22] Ahmed, K., Wilson, M. J., Goueli, S. A. and Williams-Ashman, H. G. (1978) Biochem. J. 176, 739–750.
[23] Farron-Furstenthal, R. and Lightholder, J. R. (1978) Biochem. Biophys. Res. Commun. 83, 94–100.
[24] Yamamoto, M., Criss, W. E., Takai, Y., Yamamura, H. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 5049–5052.
[25] Greengard, P. (1978) Science 199, 146–152.