RAT LIVER CYTOSOL CONTAINS AN INHIBITOR OF THE CASEIN KINASES 1 AND 2 FROM THE SAME SOURCE

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1. Introduction

Cyclic AMP-independent casein kinases are present in a variety of mammalian tissues, including rabbit muscle [1–3] and reticulocytes [4], pig leucocytes [5], bovine adrenal cortex [6], rat liver nuclei [7] and cytosol [8,9]. Each one of these tissues contains two types of casein kinases which can be tentatively classified according to their molecular weights: type I has M_r 34 000–42 000; type II has M_r 123 000–190 000. These two types of enzymes are also different in their kinetic properties. An important characteristic of both types of enzymes from rabbit muscle, pig leucocytes and rat liver cytosol is that they phosphorylate and inactivate I-form glycogen synthase in a cyclic AMP- and Ca^{2+}-independent manner. However, the effects promoted by the type I enzymes are greater than those promoted by the type II.

Whether or not these kinases are regulated by intracellular modulators is still unknown. In [10,11] a heat-stable protein inhibitor of the G-type (type II) casein kinase has been described in adrenal cortex, which was inactive on the A-type (type I) enzyme from the same tissue. The presence of a protein inhibitor has also been reported in rat liver nuclei [12]. The latter inhibits only the casein kinases of nuclear origin, being inactive on the cytosolic enzymes.

Here we report a heat-labile inhibitor in rat liver cytosol, of both cytosolic casein kinase 1 (CK-1 or type I) and 2 (CK-2 or type II) from the same tissue. The inhibiting effect was not due to ATPase, protein phosphatase or protease activity. The possible role of this inhibitor in the physiological regulation of the cytosolic casein kinases is discussed.

2. Methods

2.1. Protein kinase, protein phosphatase and inhibitor assays

Protein kinase assay was done at 30°C for 10 min under the standard assay conditions in [1]. One unit of casein kinase is the amount of enzyme that catalyzes the transfer of 1 nmol ^{32}P/min from [γ-^{32}]ATP to casein under these conditions.

Protein phosphatase was assayed by measuring the release of $^{32}P_i$ from [^{32}P]casein at 30°C in a 50 μl reaction mixture containing 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 5% glycerol, 8 mM magnesium acetate, 2 mg/ml of [^{32}P]casein and phosphatase. The $^{32}P_i$ and the remaining [^{32}P]casein were separated by instant thin-layer chromatography as in [13]. One unit of casein phophatase is the amount of enzyme required to release 1 nmol $^{32}P_i$/min.

The assay of the inhibitor was based on its ability to inhibit the casein kinase activity under conditions similar to those described as standard for protein kinase assay, except for the presence of the indicated amount of inhibitor. The data are indicated either as the percentage of inhibition observed when using 1.0 unit/ml of purified casein kinase, or as IP_{50}, which is the amount of inhibitor protein (μg/50 μl assay) necessary to promote a 50% inhibition of the casein kinase activity.

2.2. Preparation of casein kinases 1 and 2 (CK-1 and CK-2)

These enzymes were isolated from rat liver cytosol by chromatography on phosphocellulose and casein–Sepharose 4B as in [9]. The specific activities of the purified enzymes were 200 and 60 units/mg protein for CK-1 and CK-2, respectively.

2.3. Miscellaneous

Histone II A was bound to activated Sepharose 4B according [14]. [γ-^{32}P] ATP was prepared as in [15]. [^{32}P] Casein was prepared using casein (Merck), [γ-^{32}P]-ATP and purified CK-2. Protein was measured as in [16].

3. Results

3.1. Evidence of inhibitory activity in crude extracts

The cyclic AMP-independent casein kinase activity present in crude extracts from rat liver cytosol, obtained as in [9], varied non-linearly with respect to dilution. As can be observed in fig.1, the specific activity decreased as the amount of extract used in the assay was increased. The presence of 100 mM KF did not abolish this effect, although the activities were greater than those determined in its absence. Furthermore, when 5 μl crude extract were included in a 50 μl casein kinase standard assay mixture containing 1.3 units/ml of either purified CK-1 or CK-2,
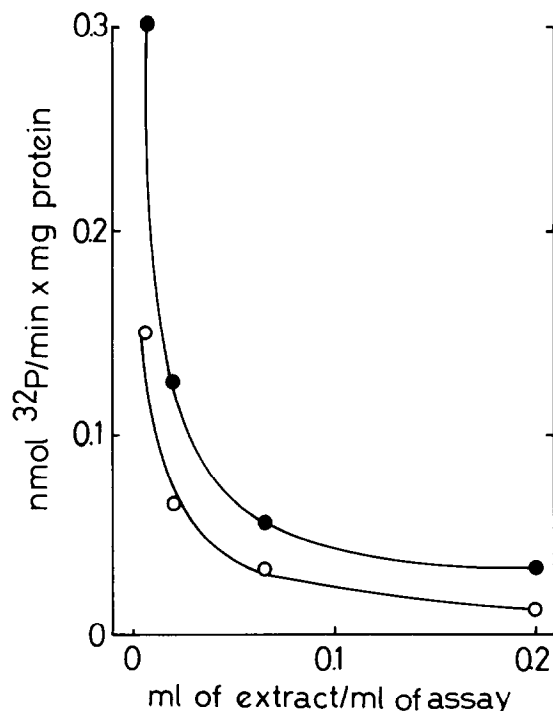

Fig.1. Effect of dilution on casein kinase activity in crude extracts. Casein kinase activity was assayed both in the absence (○) and in the presence of 100 mM KF (●) using the indicated amount of crude extract.

the activities of the kinases decreased by 88% and 85%, respectively. In fact the degree of inhibition would be even greater if the contribution by the crude extract to the total casein kinase activity in the assay (0.08 units/ml) was considered. The presence of neither 0.25 mM phenylmethylsulphonyl fluoride, 0.5 mg/ml soybean trypsin inhibitor nor 100 mM KF prevented the inactivation. However, the inhibitory activity disappeared when the extract was heated in boiling water for 3 min. These results indicated the existence of some heat-labile inhibitor of the kinases in the extracts, which was probably different from the phosphocasein phosphatases and proteases.

3.2. Partial purification of the inhibitor

The initial steps were similar to those reported for the purification of the casein kinases [9]. The flow-through fraction from the first phosphocellulose column was used in order to purify the inhibitor. Of this preparation 10 ml was applied to a 1.5 × 8 cm histone–Sepharose 4B column equilibrated with 50 mM Tris–HCl buffer (pH 7.5), containing 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride and 5% glycerol (buffer A), then the column was washed with 100 ml of the same buffer.

The inhibitor was not adsorbed to the column under these conditions whereas most of the phosphatase and histone kinase activities were retained (not shown). Fractions containing the inhibitor were pooled and applied to a 1.5 × 12 cm DEAE-cellulose column equilibrated with buffer A which was washed with 100 ml same buffer. The inhibitor was recovered in the flowthrough fraction and then it was concentrated by dialysis against buffer A containing 50% glycerol followed by dialysis against buffer A. It is difficult to quantify the extent of purification and recovery by this procedure because of the presence in the extracts of the inhibitor and also casein kinases and phosphatases not separable from the inhibitor until the phosphocellulose and histone–Sepharose 4B steps were performed (table 1). Furthermore the degree of inhibition was not linearly related to the amount of inhibitor present in the assay mixture, making it more difficult to evaluate its content in the different steps of the purification (see below).

3.3. Characteristics of the inhibitor

When incubated with ATP under conditions similar to those reported as standard for protein kinase assay except for the absence of casein and casein kinase the

Table 1
Partial purification of the inhibitor

Fraction	Total protein (mg)	Casein kinase (units/mg)	Casein phosphatase (units/mg)	ID_{50} (μg/assay)	
				CK-1	CK-2
First peak phosphocellulose	157.5	0.013	0.025	—	—
Histone—Sepharose	66.5	0.002	0.002	58	47
DEAE-cellulose (concentrated)	15.1	0	0	35	27

Assay conditions and definition of units are in section 2.1

inhibitor did not promote any release of ADP as determined chromatographically according to [17], even in the presence of 1.0 mM ATP. Furthermore the inhibitor did not inhibit hexokinase (not shown).

To test whether or not the inhibition was due to proteolytic degradation of the casein kinases by the inhibitor, a 0.5 ml reaction mixture containing 1.0 unit/ml of either CK-1 or CK-2, and 0.5 mg/ml of inhibitor was incubated at 30°C for 10 min. Each incubation mixture was then applied to a 0.3 ml phosphocellulose column equilibrated with buffer A. After washing the columns with 1 ml buffer A and 1 ml buffer A containing 0.3 M KCl the casein kinases were eluted by applying 1 ml buffer A containing 2 M KCl. Virtually all the casein kinase activity (95—98%) was recovered when either CK-1 or CK-2 were used indicating that the inhibition was easily reversible.

The effect of the inhibitor was determined at different times of incubation at 30°C (fig.2) and it was observed that the extent of inhibition remained constant for 30 min when either purified CK-1 or CK-2 were used.

The extent of inhibition promoted depended on the amount of inhibitor added (fig.3). However, there was not a linear but a hyperbolic relation between both parameters when either purified CK-1 or CK-2 were used. One can also observe that both kinases were inhibited to a similar extent by a fixed amount of inhibitor.
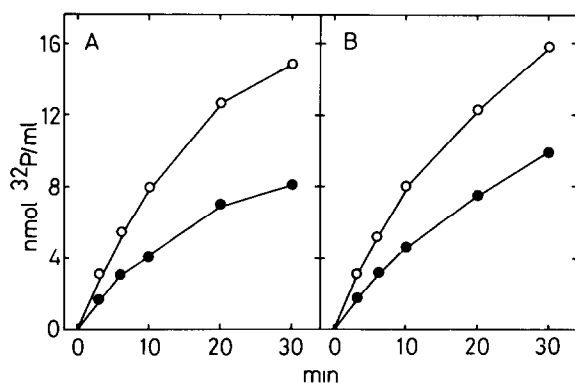

Fig.2. Time-independence of the effect of the inhibitor. A 0.2 ml reaction mixture similar to that standard for protein kinase assay, containing 1.0 unit/ml of either CK-1 (A) or CK-2 (B) was incubated at 30°C either in the absence (o) or presence (•) of 0.6 mg inhibitor/ml. At indicated times 20 μl samples were removed for the determination of the ^{32}P incorporated.
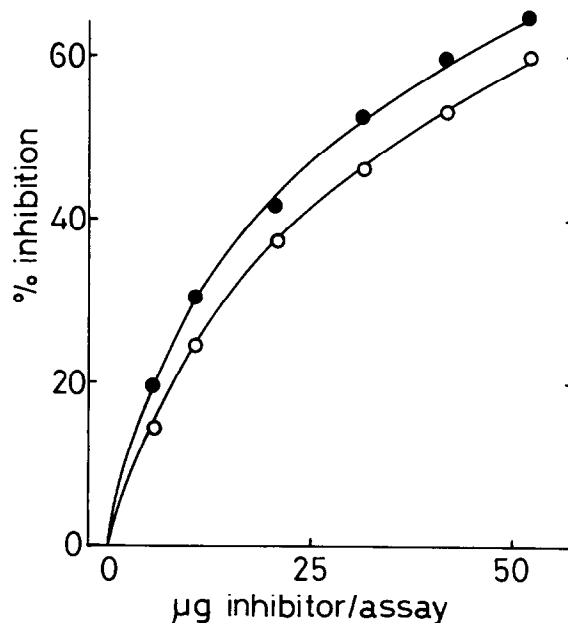

Fig.3. Degree of inhibition as a function of inhibitor concentration. Inhibitory activity was assayed in the presence of increasing amounts of inhibitor using either purified CK-1 (o) or CK-2 (•).

4. Discussion

An inhibitor of the cyclic AMP-independent casein kinases has been shown in rat liver cytosol. The inhibitory activity was not due to either destruction of the kinases or interference with the kinase assay by ATPase or phosphatase activity as indicated by:

(i)   The easy reversibility of the enzyme–inhibitor complex;

(ii) The invariance of the degree of inhibition with time;

(iii) The ineffectiveness of the inhibitor on hexo-kinase;

(iv) The lack of ATPase or casein phosphatase activity as determined by direct assay.

Furthermore, the degree of inhibition varied according to concentration in a way similar to the interaction between an enzyme and its inhibitor.

Interest has been shown in the cyclic AMP-independent protein kinases due to their putative role in the regulation of glucose and lipid metabolism. Among these kinases those denominated as casein kinases 1 and 2 (type I and II) from various sources, including rat liver cytosol [8,9], have been shown to phosphorylate and inactivate I-form glycogen synthase. However, there is little information concerning the regulation of these kinases, since they are neither inhibited by the cyclic AMP-dependent protein kinase inhibitor protein nor stimulated by Ca^{2+}, calmodulin or a combination of the two. The presence of a natural inhibitor reported herein would provide a possible regulatory mechanism for these enzymes in rat liver.

Other non-dialyzable inhibitors of casein kinases have been described in bovine adrenal cortex cytosol [10,11] and rat liver nuclei [12]. However, the first inhibitor differs from ours in that it is heat-stable and inhibits only one of the casein kinases, the so-called G-type (type II, probably). The second one is also different from ours, since it is inactive on the casein kinases from rat liver cytosol.

Evaluation of the physiological inhibitory potential of the inhibitor would be difficult because of the presence in crude extracts of various kinases and phosphatases able to act on casein and the non-linearity of the concentration dependence effect of the inhibitor. However, some information about the degree of the inhibition of the casein kinases 'in vivo' can be obtained considering that:

(i)   The casein kinase activity present in the crude extracts increased by 10-fold with dilution;

(ii) An amount of undiluted crude extract which accounted for 0.08 units of casein kinase/ml assay mixture inhibited by >85% a 16-fold greater amount of added casein kinase.

Thus the inhibitor may be of importance in the regulation of the cytosolic casein kinases in rat liver.

Acknowledgements

References

[1] Itarte, E., Robinson, J. C. and Huang, K.-P. (1977) J. Biol. Chem. 252, 1231–1234.

[2] Itarte, E. and Huang, K.-P. (1979) J. Biol. Chem. 254, 4052–4057.

[3] DePaoli-Roach, A. A., Roach, P. J. and Larner, J. (1979) J. Biol. Chem. 254, 12062–12068.

[4] Hataway, G. M. and Traugh, J. A. (1979) J. Biol. Chem. 254, 762–768.

[5] Pena, J. M., Cussó, R. and Itarte, E. (1981) Biochem. J. in press.

[6] Cochet, C., Job, D., Pirollet, F. and Chambaz, E. M. (1978) Biochimie 60, 566.

[7] Thornburg, W., Gamo, S., O'Malley, A. F. and Lindell, T. J. (1979) Biochim. Biophys. Acta 571, 35–44.

[8] Itarte, E., Mor, M. A., Pena, J. M., Salavert, A., Cussó, R. and Guinovart, J. J. (1979) FEBS Lett. 101, 347–350.

[9] Itarte, E., Mor, M. A., Salavert, A., Pena, J. M., Bertomeu, J. F. and Guinovart, J. J. (1981) Biochim. Biophys. Acta in press.

[10] Job, D., Cochet, C., Pirollet, F. and Chambaz, E. M. (1979) FEBS Lett. 98, 303–308.

[11] Job, D., Pirollet, F., Cochet, C. and Chambaz, E. M. (1979) FEBS Lett. 108, 508–512.

[12] Farron-Furstenthal, F. (1980) J. Biol. Chem. 255, 4589–4594.

[13] Huang, K.-P. and Robinson, J. C. (1976) Anal. Biochem. 72, 593–599.

[14] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.

[15] Glynn, I. M. and Chappell, J. B. (1964) Biochem. J. 90, 147–149.

[16] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.

[17] Markham, R. and Smith, J. D. (1952) Biochem. J. 52, 552–557.