INHIBITION OF RAT LIVER CYTOSOL CASEIN KINASES BY HEPARIN

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Received 22 March 1982

1. Introduction

Most organisms and tissues contain two distinct types of cAMP-independent protein kinases active on casein (casein kinases), differing for both molecular structure and nucleotide specificity and affecting different phosphorylation sites (review [1]): unlike the monomeric type I casein kinase, using only ATP and affecting only Ser residues of casein, the oligomeric type II casein kinases can utilize also GTP and affect both Thr and Ser residues; consequently they are also termed casein kinases A and G [2] or casein kinases S and TS [3,4], respectively.

An additional peculiar feature of the type II casein kinases from rabbit reticulocytes [5] and erythrocytes [6], bovine adrenal cortex [2], Morris hepatoma [7] and rat liver nuclei [8] appears to be their capability of undergoing a very efficient and specific inhibition by heparin. Apparently no other protein kinases, including the type I casein kinases, are affected by heparin [5], with the exception of phosphorylase kinase which, however, is stimulated rather than inhibited [9].

The mechanism by which heparin inhibits the type II casein kinases is not yet elucidated. A competition between heparin and the protein substrate casein was reported in [2,5,8] but not in [6] and the concentrations of heparin inducing 50% inhibition (I_{50}) were found to be significantly different. Heparin inhibition might occur through an allosteric mechanism [1].

Here, we have studied the effect of heparin on two rat liver cytosol casein kinases, Ck-TS and Ck-S, corresponding to the type II and type I, respectively [10]. The results obtained indicate that Ck-TS is efficiently inhibited by very low heparin concentrations (5–20 nM) in a reversible way and by a mechanism which is apparently competitive with respect to the protein substrate and non-competitive toward ATP. The inhibitory power, however, is not only dependent on the concentration of the substrate but also on its nature and, at fixed concentrations of heparin and protein substrate, on the amount of added casein kinase which can overcome the inhibition so that the I_{50} values are significantly variable as a function of Ck-TS concentration.

Our experiments also disclosed an inhibition by heparin of the monomeric type I casein kinase, Ck-S, which was expected to be insensitive to it: such an inhibition however requires concentrations of heparin 200–300-times higher than those effective on Ck-TS and occurs through a different mechanism, as it can be apparently reversed by high ATP concentration but neither by casein nor by phosvitin.

2. Experimental

Casein kinases TS and S (Ck-TS and Ck-S) were both prepared from rat liver cytosol as in [10]. Ck-TS was purified to homogeneity, as judged by SDS–polyacrylamide gel electrophoresis. Under non-denaturing conditions it approximated 130 000 M_r. Ck-S was subjected to a second Sepharose 6B gel filtration in the presence of 0.01% Brij 35 in order to remove from it any traces of contaminating Ck-TS. It phosphorylated casein exclusively at Seryl residues and exhibited ~35 000 M_r both by gel filtration under non-denaturing conditions and by SDS–polyacrylamide gel electrophoresis.

Casein kinase activity was routinely tested on whole casein as in [10]. In the experiments with Ck-TS, 100 mM NaCl was also added to the incubation medium. In some experiments phosvitin was replaced for casein. In all the experiments the reaction rate was linear with time. Heparin, when present,

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

was added with the protein substrate and the reaction was started by the addition of the enzyme. The concentrations of the protein substrates, heparin and Ck-TS are detailed in the legends of the figures.

Phosphorylation of endogenous proteins was studied by substituting model substrates with a preparation of lyophilized rat liver cytosol (105 000 × *g* clear supernatant dialyzed against water before lyophilization). The concentration of cytosolic proteins in the incubation medium was 1.25 mg per ml, and the incubation time was 15 min. The separation and autoradiography of the labeled proteins was performed by 11% SDS–polyacrylamide gel electrophoresis according to [11], essentially as in [10].

Materials: Whole casein was kindly provided by Dr B. Ribadeau-Dumas (Jouy-en-Josas). Phosvitin was from Sigma. Heparin (from hog intestinal mucosa) was from Serva. Synthetic peptides $(Arg)_4$–Gly–Ala–$(Arg)_4$ and $(Arg)_4$–Tyr–Gly–Ser–$(Arg)_6$, reversing heparin inhibition, were kindly provided by Dr F. Marchiori (Centro Biopolimeri, CNR, Padova). All other chemicals were from either Merck or Serva.

3. Results

The inhibition of rat liver cytosol Ck-TS and Ck-S by increasing concentrations of heparin under different conditions is illustrated in fig.1A,B. While 100–300 ng heparin/ml are usually sufficient for depressing the Ck-TS activity on casein quite significantly, they have no effect, or maybe a slightly stimulatory effect, on Ck-S. However if the concentration of heparin is raised to 50 μg/ml or more also Ck-S is drastically inhibited. With both enzymes and especially with Ck-S the phosphorylation of phosvitin is more sensitive than that of casein to heparin inhibition. A complete prevention of heparin inhibition is obtained upon addition of Arg-rich synthetic deca and dodecapeptides, as occurs with polyamines and histones [9]. However, the addition of 0.02% SDS, which is expected to dissociate the oligomeric structure of Ck-TS and depresses its activity to ~50% does not prevent but actually enhances quite significantly the inhibitory effect of heparin (not shown).

While casein can reverse in a competitive way the inhibition of Ck-TS by heparin, no competition between casein and heparin could be evidenced with Ck-S (fig.2). Quite similar results were obtained by replacing casein with phosvitin (not shown).

The effect of increasing ATP was also different with the 2 enzymes as a reversal of heparin inhibition by high ATP concentrations could be detected with Ck-S but not with Ck-TS (fig.2B,D). However, the ATP kinetics with Ck-S and heparin constantly gave rise to irregular biphasic double reciprocal plots hindering a clear-out definition of the kinetic constants (fig.2D).

The extent of Ck-TS inhibition by heparin is not

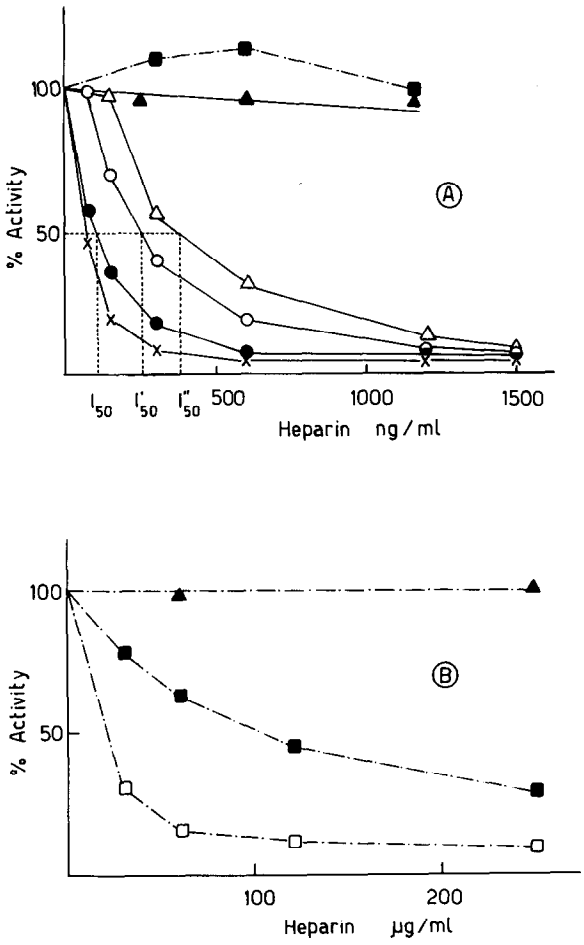

Fig.1. Effect of low (A) and high (B) concentrations of heparin on the activity of rat liver casein kinases. (——) Ck-TS (2.2 μg/ml) plus casein (●); Ck-TS (2.2 μg/ml) plus casein plus synthetic peptide $(Arg)_4$–Gly–Ala–$(Arg)_4$ (▲); Ck-TS (2.2 μg/ml) plus phosvitin (×); Ck-TS (8.8 μg/ml) plus casein (○); Ck-TS (17.6 μg/ml) plus casein (△). (. . .) Ck-S plus casein (■), plus casein plus $(Arg)_4$–Gly–Ala–$(Arg)_4$ (▲), plus phosvitin (□). The concentration of Ck-S was identical in all experiments (~2.2 μg/ml) and that of the protein substrates was 2.5 mg/ml. The peptide $(Arg)_4$–Gly–Ala–$(Arg)_4$ was 0.2 mM in A and 0.3 mM in B.

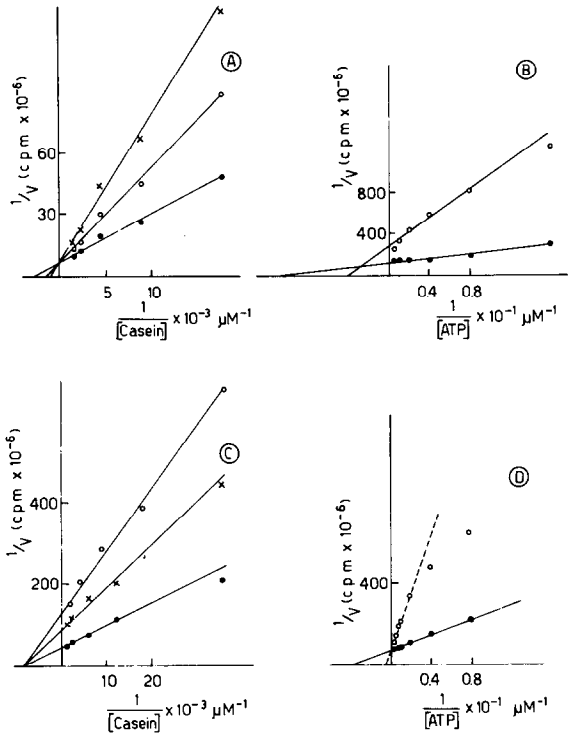

Fig.2. Double reciprocal plots for the inhibition of Ck-TS (A,B) and Ck-S (C,D) by low and high concentrations of heparin, respectively. In A and C casein was varied from 1.25–15 mg/ml while ATP was held constant at 60 μM. In B and D ATP was varied from 6.2–200 μM while casein concentration was held constant at 2.5 mg/ml. The molarity of whole casein was calculated assuming av. M_r = 24 000. In A and B the heparin concentrations were: 0 ng/ml (•), 150 ng/ml (○) and 300 ng/ml (×), while in C and D the heparin concentrations were: 0 μg/ml (•), 30 μg/ml (×) and 50 μg/ml (○).

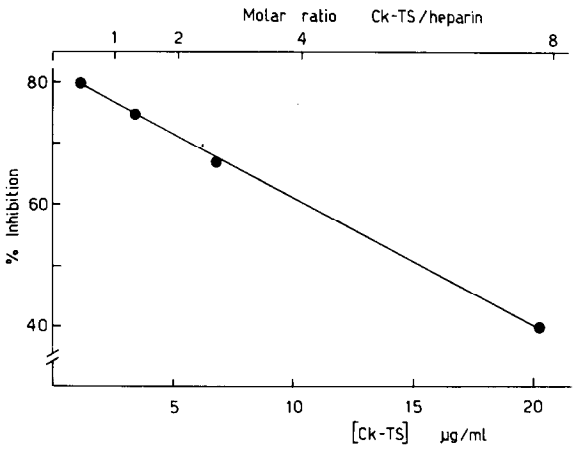

Fig.3. Decrease of heparin inhibitory efficiency by increasing Ck-TS. Casein and heparin concentrations were held constant at 2.5 mg/ml and 300 ng/ml, respectively, while the concentration of Ck-TS was varied from 8.6–154.8 nM, assuming M_r = 130 000 [10]. The molar ratio Ck-TS/heparin was calculated assuming for heparin av. M_r 14 000 [12].

only dependent on the concentration and the nature of the protein substrate but also on the amount of enzyme. Inhibition curves are significantly shifted toward higher heparin concentrations by increasing the concentration of Ck-TS (fig.1A). Consequently the I_{50}-values are not constant but they also increase with the concentration of the enzyme. Such an effect was expectable as the amount of heparin effective on Ck-TS are nearly stoichiometric with the enzyme. By keeping unchanged the concentrations of heparin and casein, the inhibitory effect can be gradually overcome by increasing the amount of Ck-TS (fig.3). From this experiment it was calculated that with 104 μM casein an 80% inhibition occurs when the heparin/Ck-TS molar ratio approaches 2.3 (assuming M_r-values for heparin and Ck-TS of 14 000 [12] and 130 000 [10], respectively). However, considering that Ck-TS is a type II casein kinase with 2 catalytic subunits/molecule [10,13], the molar ratio heparin/catalytic subunit promoting an almost complete inhibition turns out to be slightly higher than one. Inhibition is half-maximal with an heparin/catalytic subunit molar ratio as low as 0.1, suggesting that a single heparin molecule interacts with more than one catalytic subunit.

The competition between heparin and casein and the enhancement of heparin inhibition by replacing phosvitin for casein supported the view that the inhibitory power of heparin might be critically dependent also on the nature of the endogenous protein substrates of Ck-TS. To check this point the effect of heparin on the phosphorylation of rat liver cytosolic proteins by Ck-TS has been studied. A heparin concentration nearly abolishing the phosphorylation of 104 μM casein, under comparable conditions depresses only the phosphorylation of a 25 000 M_r band, corresponding to the β-subunit of Ck-TS [10], while the intensities of the remaining radiolabeled bands are quite unaffected (fig.4). By increasing the concentration of heparin to 30 and 90 μg/ml also the other bands either disappear or become fainter: a couple of bands however are still detectable at a heparin concentration which is already effective on the 'heparin-resistant' Ck-S when tested on phosvitin (see fig.1B).

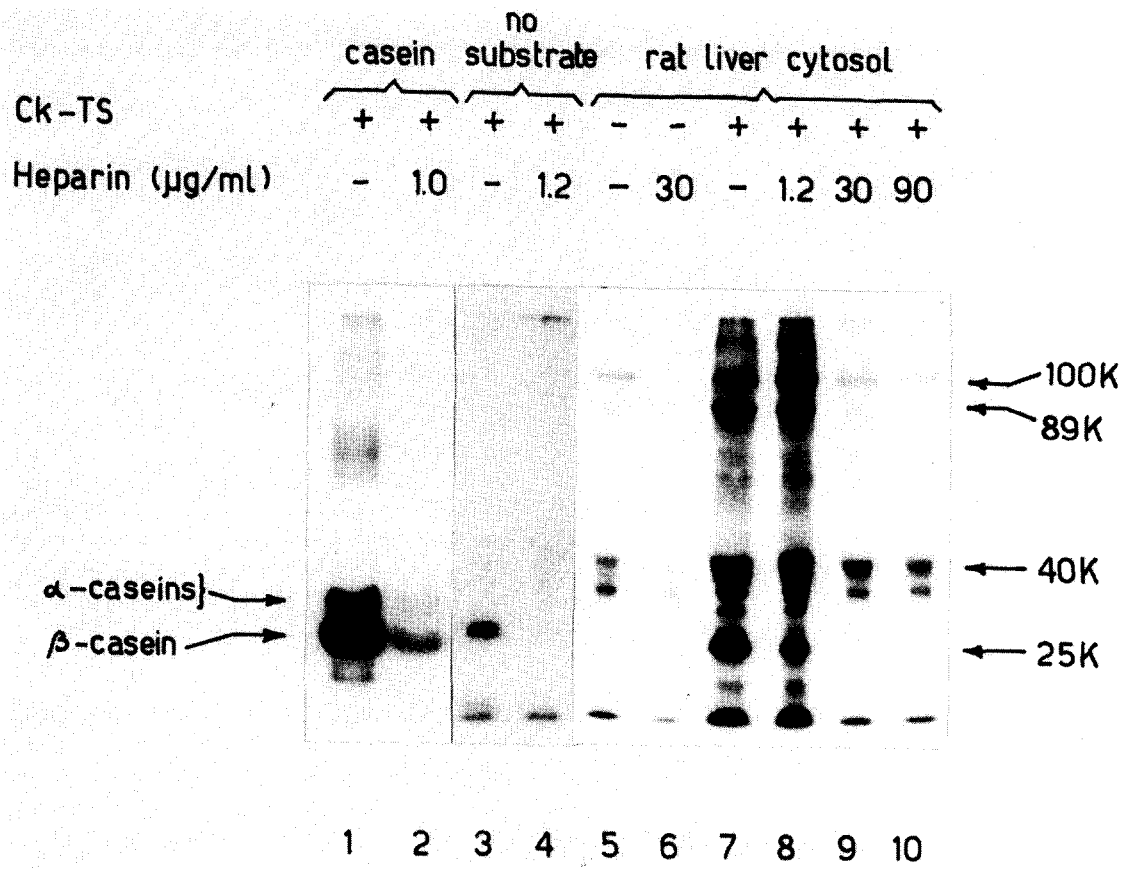

Fig.4. Effect of heparin on the phosphorylation of rat liver cytosol proteins by Ck-TS. The conditions for the phosphorylation experiments and subsequent gel electrophoresis in SDS are either detailed or quoted in section 2. The autoradiograms of the dried gels are presented. In the casein phosphorylation experiments (lanes 1–2) rat liver cytosol (1 mg/ml) was also added besides casein (2.5 mg/ml) and only 1/5 of the whole radiolabeled protein was subjected to gel electrophoresis and autoradiographed for 5 h. In all other experiments (lanes 3–10) the whole amounts of radiolabeled proteins were subjected to gel electrophoresis and the exposure time of autoradiographies was 36 h. The M_r-values of the main radiolabeled proteins were estimated by calibration of the SDS gels with the following marker proteins: glycogen phosphorylase (98 000), bovine serum albumin (67 000), ovalbumin (45 000), lactate dehydrogenase (36 000), trypsinogen (24 000) and ribonuclease (13 700). A linear plot was obtained of subunit M_r-values (log scale) *vs* relative migration (percentage).

4. Discussion

These data support the following main conclusions:

(1) Rat liver cytosol casein kinase TS (Ck-TS) is efficiently and reversibly inhibited by nM levels of heparin which are quite ineffective on casein kinase S (Ck-S) from the same source.

(2) A competition between heparin and casein (or phosvitin) for the protein substrate binding site of Ck-TS is suggested by kinetic experiments. In the presence of 100 μM casein, however, an almost complete inhibition can be promoted by just 20 nM heparin. Under these conditions the heparin/Ck-TS catalytic subunit molar ratio approaches one, suggesting that virtually all the heparin molecules are interacting with an equivalent number of catalytic subunits. Accordingly, by increasing the Ck-TS concentration the inhibition is gradually overcome. The finding that a 50% inhibition is still observed at an heparin/catalytic subunit molar ratio ~0.1 would indicate that

whenever Ck-TS is in molar excess, a single heparin molecule can interact with more than one catalytic subunit of the enzyme*.

(3) By increasing heparin to 4–8 μM (which is still compatible with physiological conditions) also the type I monomeric Ck-S is drastically inhibited. Such an inhibition however is no more competitive with respect to the protein substrate. However, the inhibition patterns at variable ATP concentration exhibited remarkable irregularities hindering an unambiguous definition of the inhibition type. Nevertheless, by increasing the [ATP] over 100 μM a reproducible reversal of heparin inhibition was constantly observed (see fig.2D).

(4) The extent of Ck-TS inhibition by heparin is largely dependent also on the nature of the protein substrate: under comparable conditions the phosphorylation of phosvitin is depressed more than that of casein, while, as an average, the endogenous cytosolic substrates are much less sensitive to heparin than are the model substrates, with the noticeable exception of a 25 000 M_r band corresponding to the 'autophosphorylatable' β-subunit of Ck-TS itself.

The above data while confirming that in vitro heparin provides a quick and very suitable tool for discriminating between the 'heparin-sensitive' type II and the 'heparin-insensitive' type I casein kinases, also disclose the possibility that under physiological conditions the specificity of heparin inhibition may not be so clearcut, giving possibly rise to a discrimination among the several potential targets of casein kinases rather than to the mere switching off of type II casein kinases.

Of course any such involvement of heparin and/or heparin-like compounds in the regulation of casein kinase activities in vivo preliminarily requires that these acidic glycosaminoglycans can actually reach the subcellular compartments harbouring the enzyme. To our knowledge such an assumption is not proven: however, some evidence about the intracellular location of dextran sulphates and their internalization by cultured cells are available (see [14]).

It should be finally pointed out that the mechanisms by which heparin affects the two rat liver casein kinases are likely to be different since heparin, acting at low concentrations as a competitive inhibitor of Ck-TS with respect to the protein substrate, behaves at higher concentrations as a non-competitive inhibitor of Ck-S with respect to the same protein substrates. This finding by the way would be consistent with the view that the protein substrate binding sites of Ck-TS and Ck-S are different, as suggested by the reports that these two kinases recognize different sites in casein fractions [4,10,15]. However, the finding that high ATP concentrations can reverse the inhibition of Ck-S suggests that heparin might also interact with the nucleotide binding site of this kinase, which has been shown to be also different from that of Ck-TS [17]. To sum up therefore, the heparin molecules might display a double binding capacity toward both the protein substrate site of Ck-TS and the nucleotide site of Ck-S, exhibiting however a much higher affinity for the former than for the latter.

Alternatively, the heparin inhibition of Ck-TS might occur through an allosteric mechanism, still consistent with the observed competition by casein and phosvitin, assuming either a reciprocal influence between the catalytic and regulatory sites or even a mere subtraction of heparin by the protein substrate itself (though a positive interaction between 2 polyanions like phosvitin and heparin would be quite unexpected). Such a possibility would be consistent with the oligomeric structure of Ck-TS and with the finding that relatively huge amounts of protein substrate are required for overcoming the effect of catalytic amounts of heparin, whose molecules lack any close similarities with casein as expected for a typical competitive inhibitor of a casein kinase [1]. However, no kinetic evidence supporting the allosteric hypothesis is available. Moreover, in the presence of SDS the heparin inhibition was not abolished, as expected if it had required the integrity of the quaternary structure, but actually it was significantly enhanced.

* The finding that antithrombin III, a protein with high affinity for heparin [14] reversing also the effect of heparin on phosphorylase kinase [9], cannot prevent, under comparable conditions, the inhibition of Ck-TS (unpublished), argues that the heparin molecules or domains responsible for the inhibition of Ck-TS are distinct from those involved in its anticoagulant effect. In particular, it is possible that the anti-Ck-TS activity of heparin resides in the so-called Low Affinity fraction (LA-heparin) rather than in the fraction exhibiting high affinity for antithrombin III (HA-heparin, corresponding to ~1/3rd of the molecules) [14]

Acknowledgements

This work was supported by a grant from the Consiglio Nazionale delle Ricerche (CT 81.00313.04). The skillful technical assistance of Mr G. Tasinato and the excellent secretarial aid of Ms M. Cuccia are gratefully acknowledged.

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