Beryllium toxicity

The selective inhibition of casein kinase 1

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1. Cyclic AMP-independent casein kinase 1 in liver cytoplasm and nuclei was inhibited by Be^{2+} *in vitro* (K_i 2.5 μM and 29 μM respectively). Casein kinase 2 (phosvitin kinase) and cyclic AMP-dependent protein kinase were unaffected. 2. The inhibition of casein kinase 1 by Be^{2+} was competitive with respect to the protein substrate; at non-saturating concentrations of casein, inhibition was non-competitive with respect to ATP. 3. In rats given LD₅₀ doses of Be^{2+} 24 h before death, the activities of cytoplasmic and nuclear casein kinase 1 in livers from partially hepatectomized animals were diminished approx. 50%; with intact rats, nuclear casein kinase 1 was inhibited at concentrations of casein less than the K_m .

The basis of beryllium toxicity to mammals is not yet understood. Be^{2+} , given to rats intravenously as BeSO_4 /sulphosalicylic acid (1:1, mol/mol), is taken up into the liver lysosomes and then redistributed through the cells (Skilleter & Price, 1980). Enzyme induction in normal and regenerating rat livers (Witschi & Marchand, 1971; Witschi, 1970; Ord & Stocken, 1981a) and soluble cytoplasmic protein kinases (Kaser *et al.*, 1980) are inhibited, as is the phosphorylation of histone H2A in normal liver and of histone H2A and other histones at the transition from G₁- to S-phase in regenerating livers (Kaser *et al.*, 1980). Nuclear non-histone proteins extracted in 0.35 M-NaCl did not show diminished [^{32}P]P_i uptake in rats given 40 μmol of Be^{2+} /kg *in vivo*, but [^{32}P]P_i incorporation was decreased into proteins from liver Hn-RNP particles from partially hepatectomized rats or from those that had been given dexamethasone 3 h previously (Ord & Stocken, 1981a).

In the method of assay (Chen & Walsh, 1974) then used for the kinases, 80% of the activity was attributable to cyclic AMP-dependent kinase (EC 2.7.1.37), suggesting that this was the enzyme sensitive to Be^{2+} . This has now been re-examined with partially purified enzyme preparations from ox heart and rat liver nuclei and cytoplasm and found to be incorrect, a cyclic AMP-independent kinase which uses casein rather than phosvitin or protamine

as substrate (casein kinase 1; Huang *et al.*, 1982) being the Be^{2+} -sensitive enzyme. The Be^{2+} -sensitivity of this enzyme in liver cytoplasm and nuclei may facilitate the identification of the kinase(s) involved in nuclear-protein phosphorylation in normal and growing cells.

Materials and methods

Enzyme preparations from ox heart

Trimmed frozen ox heart was minced, suspended in 6 vol. of 4 mM-EDTA/0.1% (w/v) 2-mercaptoethanol, pH 7.5, and homogenized for 1 min in a Waring Blender. After centrifuging for 45 min at 3000 g the supernatant was adjusted to 40% saturation with $(\text{NH}_4)_2\text{SO}_4$, pH 7.5, and the precipitate used as a source of cyclic AMP-dependent protein kinase holoenzyme. The catalytic subunit obtained from the 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ supernatant was purified by passage over DEAE-Sephadex A-25 (Chen & Walsh, 1974).

Rat liver soluble cyclic AMP-independent casein kinases 1 and 2

Casein kinases 1 and 2 were prepared from liver cytoplasm and nuclei (rats 300–400 g body wt.) as shown in Fig. 1.

Protein kinase assay system

Protein substrate, 40 μl (concentrations as given in Tables and Figures); 1 M-Tris/HCl/15 mM-2-mercaptoethanol, pH 7.0, 25 μl ; 10 mM-magnesium

Abbreviations used: Hn-RNP, heterogeneous ribonucleoprotein; SDS, sodium dodecyl sulphate.

Fig. 1. Separation of soluble rat liver cyclic AMP-independent protein kinases

acetate/10 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, pH 7.0, 20 μl ; enzyme solution to 150 μl . The specific radioactivity of ATP was 0.3–1 c.p.m./pmol. Tubes were kept at 0° until the reaction was started by the addition of ATP; normally the incubation was for 15 min at 30°C. At the end of the incubation, 50 μl of the mixture was transferred to a filtering apparatus (GF/C glass-fibre filters) and the filters washed in 10% (w/v) trichloroacetic acid and acetone before determining the ^{32}P transferred. In experiments when Be^{2+} was present, the Be^{2+} was used as a 1:1 molar mixture with sulphosalicylic acid; unless otherwise stated the Be^{2+} was added to the enzyme preparation before the substrates.

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$

This was prepared by the method of Post & Sen (1967).

Chemicals

Cyclic AMP-dependent protein kinase (crude and the catalytic subunit) and protein kinase inhibitor were obtained from Sigma (London) Chemical Co. (catalogue nos. P4890, P2645 and P8140). $[\text{}^{32}\text{P}]\text{P}_i$ was obtained from Amersham International. Histones were prepared from the rat liver nuclei by standard methods (Ord & Stocken, 1975).

Results and discussion

Of the soluble protein kinases examined (Table 1), only the cyclic AMP-independent casein kinase 1

from liver nuclei and cytoplasm appeared to be sensitive to Be^{2+} inhibition *in vitro*. The inhibition by Be^{2+} was competitive with respect to the protein substrate (Figs. 2a, 2b and 2c) and at non-saturating substrate concentrations, non-competitive with respect to ATP (Fig. 2d). The inhibition studies were performed with enzymes, eluted from Whatman DEAE-cellulose DE-52 columns (thereafter termed simply 'DE-52 columns'), which were not homogeneous on SDS/polyacrylamide-gel electrophoresis. Casein kinase 1 from the cytosol had a K_m for casein of 6.25 mg/ml and $K_i(\text{Be}^{2+})$ of 2.5 μM ; the nuclear enzyme had a K_m (casein) of 5 mg/ml and $K_i(\text{Be}^{2+})$ of 29 μM . K_i values of 1–5 μM have been reported for alkaline phosphatase and phosphoglucomutase, which are among the most Be^{2+} -sensitive enzymes known (Reiner, 1970).

The effects of changing the order of addition of components in the assay system gave results consistent with the inhibitor kinetics; normally Be^{2+} was incubated with the enzyme for 5 min before the addition of casein, and the reaction was then started with ATP. If the order of addition of Be^{2+} and casein was reversed, inhibition of Be^{2+} was markedly decreased. If casein was added last, the order of addition of ATP and Be^{2+} did not effect the extent of inhibition.

The natural substrate(s) of casein kinases in the liver are not known; our partially purified cytoplasmic casein kinase 1 phosphorylated histone H1 at about 10% of the rate for casein. With protein

Table 1. *Properties of soluble protein kinases from ox heart and rat liver*

Final concentrations: casein, 6.25 mg/ml; phosvitin, 13.3 mg/ml; protamine, 3.3 mg/ml. Abbreviation used: NA, not assayed. The results are the means from at least two enzyme preparations.

Enzyme source	Sensitivity to:		Relative activity towards:			Inhibition by 0.1 mM-Be ²⁺ (%) (substrate)
	10 μM-Cyclic AMP	Protein kinase inhibitor	Casein	Phosvitin	Protamine	
Ox heart						
Holoenzyme	+	+	1	NA	23	<10 (protamine)
Catalytic subunit	—	+	1	13	27	<10 (protamine)
Rat liver						
Cytoplasm						
Casein kinase 1	—	—	1	0.45	NA	100 (casein)
Casein kinase 2	—	—	1	3.6	NA	<10 (phosvitin)
Nuclei						
Casein kinase 1	—	—	1	0.32	0.03	98 (casein)
Casein kinase 2	—	—	1	1.17	0.35	30 (phosvitin)

Fig. 2. *Be²⁺ inhibition of casein kinase 1 from rat liver cytoplasm and nuclei*

The enzymes were isolated and partially purified as outlined in Fig. 1. Where appropriate, Be²⁺ (BeSO₄/sulphosalicylic acid, 1:1) was incubated with enzyme for 5 min at 30°C before addition of the casein substrate. (a) Liver cytoplasmic enzyme was precipitated between 25 and 50% saturation with (NH₄)₂SO₄; 1.5 mg of enzyme protein was used per assay. Concentrations of reactants were as follows: ATP, 2.6 mM; Be²⁺, 10 μM; casein, various. (b) Liver cytoplasmic enzyme was the void-volume fraction off the DE-52 columns; 85 μg of enzyme protein was used per assay. Concentrations of ATP, Be²⁺ and casein were as in (a). The results in the presence of Be²⁺ have been transposed on the same scale as for the controls. (c) Liver nuclear enzyme was void-volume fraction off the DE-52 columns; 64 μg of enzyme protein was used per assay. Concentrations of reactants were as follows: ATP, 2.6 mM; Be²⁺, 20 μM; casein, various. (d) Liver cytoplasmic enzyme was void-volume fraction off the DE-52 columns; 85 μg of enzyme protein was used per assay. Concentrations of reactants were as follows: casein, 6.25 mg/ml; Be²⁺, 10 μM; ATP, various. ●, Control; ○, + Be²⁺.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of ^{32}P incorporation by nuclear casein kinase into non-histone proteins and histones

Equal amounts of material were analysed (Weber & Osborn, 1969) from control preparations (a,c) and from those incubated with $50\mu\text{M-Be}^{2+}$ (b,d). Slices (2mm) were digested and their ^{32}P radioactivity determined. The position of molecular-mass markers (values in kDa) is indicated by the arrows. (a and b) Crude nuclear enzyme [25–50% saturation with $(\text{NH}_4)_2\text{SO}_4$; see Fig. 1]. No substrates were added. (c and d) Nuclear enzyme (void-volume fraction from the DE-52 columns) incubated with non-histone proteins extracted from rat liver nuclei in 0.35 M-NaCl.

substrates at 0.25 mM, $10\mu\text{M-Be}^{2+}$ inhibited the phosphorylation of casein by 33% and histone H1 by 44%. The crude nuclear enzyme applied to the DE-52 columns still had associated with it phosphorylatable non-histone proteins and histones (Figs. 3a and 3b; Table 2); ^{32}P uptake into the non-histone proteins was 58% inhibited by $50\mu\text{M-Be}^{2+}$ and 94% inhibited into the histones. The enzyme that was eluted in the void volume from the DE-52 columns did not contain significant amounts of endogenous substrate; its capacities to phosphorylate non-histone proteins and histone H1 (extracted together from liver nuclei in 0.35 M-NaCl) were inhibited about 45% by $50\mu\text{M-Be}^{2+}$ (Figs. 3c and 3d).

Equilibrium dialysis had shown strong and specific Be^{2+} binding to organelles and proteins of the liver (Aldridge, 1966; Witschi & Aldridge, 1968). It was also observed (Parker & Stevens, 1979) that most of the beryllium was bound to a highly phosphorylated fraction of the non-histone proteins and there was no binding to the histones. Our equilibrium-dialysis studies of $^{7}\text{Be}^{2+}$ binding on cytoplasmic casein kinase 1 in void-volume fractions from the DE-52 columns gave Scatchard plots which indicated the presence of material with a high-affinity binding for Be^{2+} (K_d approx $0.2\mu\text{M}$). Control experiments showed that the casein also bound Be^{2+} at $50\mu\text{M}$; one Be^{2+} ion being bound per molecule of casein (25kDa). In spite of the expectation that SDS would probably dissociate Be^{2+} from protein, we have also examined its binding by the cytoplasmic casein kinase fraction by using SDS/polyacrylamide-gel electrophoresis. $^{7}\text{Be}^{2+}$ was detected (Fig. 4a) on four main proteins (67.5, 62, 45 and 42kDa) as well as on a number of minor ones. Void-volume material eluted from the DE-52 columns was enriched in 39kDa Be^{2+} -binding protein, relative amounts of the other binding materials being greatly decreased (Fig. 4b). Molecular masses of 40–45kDa have been reported for the α - and α' -subunits of casein kinase 2, which was present in the material applied to the DE-52 columns (Fig. 4a); the molecular mass of casein kinase 1 is in the range 34–40kDa (Huang *et al.*, 1982), consistent with the main $^{7}\text{Be}^{2+}$ -binding component in the void-volume fraction (Fig. 4b).

Casein kinase 1 was next isolated from intact or partially hepatectomized rats 24 h after they had received $40\mu\text{mol}$ of BeSO_4 /sulphosalicylic acid per kg body wt. intravenously. To diminish casein phosphorylation by Be^{2+} -insensitive kinases, partial purification of casein kinase 1 was required. Isolation and purification steps were performed in parallel with control and Be^{2+} -treated animals. There was no evidence that Be^{2+} treatment caused detectable differences in the amounts of protein recovered at different stages of purification. In our previous

Table 2. Effects of Be²⁺ *in vivo* on liver casein kinase 1 activity

Five to six intact rats or twelve to fifteen animals partially hepatectomized 22–24 h before death were used per group. Be²⁺ [40 μmol/kg body wt. intravenously as BeSO₄/sulphosalicylic acid (1:1, mol/mol)] was given 24 h before death. The enzymes were prepared as shown in Fig. 1.

Enzyme	[Casein] (mg/ml)	Enzyme activity (nmol of [γ - ³² P]ATP transferred/min per mg of protein)			
		Intact rats		Partially hepatectomized rats	
		Controls	+ Be ²⁺	Controls	+ Be ²⁺
Cytoplasmic	6.25	0.3 ± 0.02	0.49	0.42	0.26
	1.50	0.06	0.12, 0.18	—	—
Nuclear	6.25	0.56 ± 0.04	0.68 ± 0.02	3.9	2.1
	1.50	0.06, 0.07	0.01 ± 0.01	0.40 ± 0.05	0.21 ± 0.02

hepatectomized rats given Be²⁺ 17 h previously. Such iso-osmolar extracts contain predominantly cyclic AMP-dependent kinase and casein kinase 2, neither of which has now been found to be sensitive to Be²⁺ *in vitro*.

Casein kinase 1 activity in nuclei and cytoplasm was diminished by 40–50% from the Be²⁺-treated partially hepatectomized rats (Table 2). With intact rats, no inhibition of casein kinase 1 activity was found with cytoplasmic preparations, but was detected with nuclei from Be²⁺-treated animals if competition from the substrate was decreased by lowering the casein concentration from 6.25 to 1.5 mg/ml (Table 2). Beryllium diminishes inducible protein synthesis in liver. The effects of Be²⁺ on nuclear casein kinase 1 from normal livers were found only if substrate competition was decreased, so demonstrating the direct effect of Be²⁺ on the catalytic site of the kinase *in vivo*.

The proteins phosphorylated by liver casein kinase 1 *in vivo* are unknown, as is the essentiality of such phosphorylation. Casein kinase 1 preparations used here phosphorylated nuclear non-histone proteins and showed some activity towards histones. Histone phosphorylation, including that of histone H2A in resting liver nuclei, and the incorporation of [³²P]P_i into non-histone protein in Hn-RNP particles, had previously been found to be inhibited in livers from Be²⁺-treated rats; the possibility now arises that the diminished phosphorylation is attributable to the selective inhibition of casein kinase 1 by Be²⁺.

Since constitutive protein synthesis in liver is unaffected by Be²⁺ (Witschi, 1970), the inhibitory effects of the metal ions resemble the actions of ionizing radiation, α-blockers and procarcinogens (see Ord & Stocken, 1981b) in selectively affecting induced rather than normal protein synthesis, and suggest that casein kinase 1, which is firmly bound to the nuclear structure, may be involved in facilitating the induction process.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of ⁷Be²⁺ binding by cytoplasmic casein kinase I. Crude enzyme [30 μg; 25–50% saturation with (NH₄)₂SO₄] (a) or void-volume fraction off the DE-52 columns (b) were incubated for 15 min at 30 °C with ⁷Be²⁺ (5 mM-⁷BeSO₄/sulphosalicylic acid) before electrophoresis (Weber & Osborn, 1969). After staining, the gels were sliced (1 mm) and the ⁷Be radioactivity measured in a γ-radiation counter.

studies (Kaser *et al.*, 1980), liver nuclei had been extracted with iso-osmolar solutions and the kinase activity measured. No diminution in activity had been detected in nuclei from normal or partially

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