Effect of bivalent cations on rat liver cytosol casein (glycogen synthase) kinases 1 and 2

Influence of the protein substrate

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Rat liver cytosol casein kinases 1 and 2 were stimulated by free Mg^{2+} , but the optimal concentration of cation varied with both the casein kinase and the protein substrate used. Mn^{2+} , but neither Ca^{2+} nor Zn^{2+} , could efficiently substitute for Mg^{2+} in forming the bivalent-cation-ATP complex used as substrate, but free Mn^{2+} was inhibitory. The magnitude of these effects depended on the type of casein kinase and the protein substrate used. These results support the idea that, besides the effects of Mg^{2+} as a component of the Mg-ATP complex, or through interaction with the protein substrate, free Mg^{2+} is an allosteric effector of both casein kinases.

Casein kinases are a class of cyclic AMP-independent protein kinases defined initially by their ability to phosphorylate casein and phosvitin. Several reports have demonstrated the existence of two types of casein kinases, which differ in their M_r and kinetic properties, and which are widely distributed in mammalian tissues (see Hathaway & Traugh, 1982, for a review), including rabbit muscle (Itarte & Huang, 1979; Huang *et al.*, 1982) and rat liver cytosol (Itarte *et al.*, 1981). Casein kinases have been shown to be affected *in vitro* by a variety of compounds, such as polyamines, caffeine, basic and acidic peptides, heparin and 2,3-bisphosphoglycerate (Plana *et al.*, 1982; Hathaway & Traugh, 1982, 1984a), but the mechanism for the physiological control of the activity of hepatic and muscle casein kinases is still unknown.

It is well known that Ca^{2+} plays an important role in the hormonal and neural control of metabolism. The possible involvement of Mg^{2+} and Mn^{2+} in the control of cellular activity has also been suggested (Rubin, 1975; Williams, 1982; Ash & Schramm, 1982). Alterations in the cellular content of Ca^{2+} and Mg^{2+} seem to be responsible for the increased activity of some glycolytic enzymes in tumour cells (Bygrave, 1978; Bossi *et al.*, 1979). Protein kinases require the presence of a bivalent cation for activity, and in most cases Mg^{2+} fulfils this requirement. Previous studies on muscle and liver phosphorylase kinase have shown that Mg^{2+} has a regulatory role in addition to

forming part of the Mg-ATP complex (Singh *et al.*, 1982; Chrisman *et al.*, 1984).

In our search for possible modulators of the casein kinases 1 and 2 from rat liver cytosol, the activity of each casein kinase on glycogen synthase, casein and phosvitin was assayed in the presence of different bivalent cations. We have found that free Mg^{2+} affected the activity of casein kinases 1 and 2, depending on the protein substrate used.

Experimental

Materials

ATP and phosvitin were purchased from Sigma. Glycogen synthase I-form was purified to homogeneity from rabbit skeletal muscle as described by Takeda *et al.* (1975). The sources of all other chemicals, biochemicals and radiochemicals were as indicated previously (Itarte *et al.*, 1981). Where indicated, the sample of phosvitin used was dialysed first for 36 h against 50 vol. of 5 mM-Tris/HCl buffer, pH 7.5, containing 2 mM-EDTA and 0.5 mM-EGTA, with buffer changes every 12 h, and then against 50 vol. of 5 mM-Tris/HCl buffer, pH 7.5, for 36 h with buffer changes every 12 h, to remove contaminating cations.

Casein kinases

Casein kinases 1 and 2 were obtained as described previously (Itarte *et al.*, 1983; Martos *et al.*, 1985).

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Assays

In the experiments to determine the individual effects of cations, the casein kinase activity was assayed under the standard assay conditions described previously (Itarte *et al.*, 1981), except that neither EDTA nor EGTA was present in the assay. In all cases the concentration of casein or phosvitin in the assays was 4 mg/ml, whereas that of glycogen synthase was 0.2 mg/ml. Separation of ^{32}P -labelled protein from unchanged $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was accomplished by instant t.l.c. ('ITLC') as described by Huang & Robinson (1976).

The concentrations of free Mg^{2+} , free Mn^{2+} , magnesium complexes and manganese complexes were calculated from their stability constants and the acid dissociation constants reported in the literature (Smith & Alberty, 1956; Alberty, 1969). The composite stability constants for nucleotides, calculated as indicated by Veloso *et al.* (1973), were 5704M^{-1} for Mg-ATP complexes, 646M^{-1} for Mg-ADP complexes, 41M^{-1} for the Mg-AMP complex and 18482M^{-1} for Mn-ATP complexes at 30°C .

Results

Effect of Mg^{2+} on casein kinases 1 and 2

Under standard assay conditions (0.125 mM-ATP) casein kinases 1 and 2 from rat liver cytosol were activated by Mg^{2+} , but the concentration-dependence of this cation varied with the protein substrate used in the assays (Fig. 1). With casein as substrate, maximal stimulation of casein kinase 1 was observed at about 3 mM total Mg^{2+} , with

inhibition occurring at higher concentrations. On the other hand, the activity of casein kinase 2 on casein continued to increase as the concentration of total Mg^{2+} was raised to 10 mM. The estimated K_a values were 0.5 mM for casein kinase 1 and 1.3 mM for casein kinase 2.

A more complex pattern was observed with phosvitin. As observed previously with casein kinase 1 from rabbit muscle (Itarte & Huang, 1979), casein kinases 1 and 2 from rat liver cytosol were active on untreated commercial phosvitin in the absence of added Mg^{2+} , an effect that was probably due to the presence of Mg^{2+} in the preparation of phosvitin. Addition of increasing concentrations of Mg^{2+} up to 6 mM promoted a moderate stimulation of phosvitin phosphorylation by casein kinase 2, and about 50% stimulation of its phosphorylation by casein kinase 1.

The phosphorylation of dialysed phosvitin by casein kinases 1 and 2 was considerably stimulated by added Mg^{2+} , with marked effects observed at concentrations of Mg^{2+} above 1 mM. This indicates either that high concentrations of free Mg^{2+} are required for phosphorylation of phosvitin, or that this protein chelates Mg^{2+} , and the real concentration of Mg^{2+} available to form the Mg-ATP complex is much lower than that expected, since at 1 mM- Mg^{2+} and 0.125 mM-ATP more than 80% of the nucleotide should be forming a Mg-ATP complex if no interfering substances were present. Binding of bivalent cations such as Mg^{2+} , Mn^{2+} , Ca^{2+} and Fe^{2+} to phosvitin has been reported previously (Panic *et al.*, 1971; Taborsky, 1974; Osaki *et al.*, 1975).

Fig. 1. Effect of Mg^{2+} on the activity of casein kinases 1 and 2

The activities of casein kinases 1 (a) and 2 (b) were determined with 0.125 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and casein (●), untreated phosvitin (△), dialysed phosvitin (▲) or glycogen synthase (■) as substrate in the presence of various amounts of total Mg^{2+} . Other assay conditions are indicated in the Experimental section.

Fig. 2. Effect of Mn^{2+} on the activity of casein kinases 1 and 2

The general assay conditions are similar to those indicated in the legend of Fig. 1, except that MnCl_2 was used instead of MgCl_2 in the phosphorylation of casein (●), untreated phosvitin (△), dialysed phosvitin (▲) or glycogen synthase (■). The effect of MnCl_2 on the phosphorylation of casein was also assayed by using 0.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (○).

Effect of other bivalent cations

When the ability of other bivalent cations to substitute for Mg^{2+} was studied, it was observed that casein kinase 1 and casein kinase 2 were active in the presence of Mn^{2+} , whereas no activity was detected with Ca^{2+} or Zn^{2+} . As indicated for Mg^{2+} , the concentration of Mn^{2+} required for maximal stimulation of each protein kinase depended on the protein substrate used in the assay, being much lower with casein than with glycogen synthase or dialysed phosvitin (Fig. 2). When untreated phosvitin was used as substrate, both casein kinases were essentially unaffected by concentrations of total Mn^{2+} below 0.1 mM , but a clear inhibition was observed as the cation concentration increased above this value. The optimal concentration of Mn^{2+} for the phosphorylation of casein by casein kinase 1 and casein kinase 2 also depended on the concentration of total ATP present in the assay. With both casein kinases, increasing the concentration of total ATP from 0.125 mM to 0.5 mM promoted an increase of both the optimal concentration of Mn^{2+} and the maximal activity. Since at a fixed total Mn^{2+} concentration an increase in total ATP would cause an increase in the concentration of Mn-ATP and a decrease in that of free Mn^{2+} , a possible reason for this effect could be that Mn-ATP was a substrate for both enzymes, whereas free Mn^{2+} inhibited. To investigate this possibility, the data on the phosphorylation of casein and glycogen synthase by casein kinase 1 and casein kinase 2 in

the presence of different concentrations of Mg^{2+} and ATP were plotted as a function of the concentration of bivalent-cation-ATP complex calculated as indicated in the Experimental section (Fig. 3). As can be observed at low concentrations, Mn-ATP served at least as well as Mg-ATP as substrate for both casein kinases. However, inhibition of casein phosphorylation occurred when the concentrations of total ATP and Mn^{2+} were such as to give free Mn^{2+} concentrations above $10\text{ }\mu\text{M}$ with casein kinase 1 and above $50\text{ }\mu\text{M}$ with casein kinase 2.

Stimulation of casein kinases 1 and 2 by free Mg^{2+}

The data plotted in Fig. 3 showed that, at a fixed Mg-ATP concentration, the activity of both casein kinases depended on the amount of free Mg^{2+} present in the assay, this being especially noticeable with casein kinase 2. To confirm that the effect of Mg^{2+} on both casein kinases was not due only to changes in the concentration of the Mg-ATP complexes, experiments were carried out in which the concentration of Mg-ATP complexes was kept constant ($100\text{ }\mu\text{M}$), whereas that of free Mg^{2+} was varied. As shown in Fig. 4, the phosphorylation of casein by both casein kinases was stimulated by free Mg^{2+} , although the effect of the cation was more marked on casein kinase 2 than on casein kinase 1. In addition, free Mg^{2+} also stimulated the phosphorylation of glycogen synthase by both enzymes. However, the optimal concentrations of free Mg^{2+} for the phosphorylation of casein and

Fig. 3. Casein kinase activity as a function of the concentration of bivalent-cation-ATP complexes. The data on the activities of casein kinases 1 (a) and 2 (b) from Figs. 1 and 2 were replotted as a function of the concentration of bivalent-cation-ATP complexes. The molar concentration of the cations, calculated as indicated in the Experimental section, is shown by the scales on the top of the Figure. Symbols are casein phosphorylation in the presence of Mg^{2+} (○) or Mn^{2+} (△), and glycogen synthase phosphorylation in the presence of Mg^{2+} (●) or Mn^{2+} (▲). The activity of both enzymes on casein (□) and glycogen synthase (■) determined in the presence of a fixed amount of MgCl_2 (8 mM) and various amounts of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5–100 μM) is also shown.

Fig. 4. Stimulation of casein kinases 1 and 2 by free Mg^{2+} .

The activities of casein kinases 1 (a) and 2 (b) on casein (○) and glycogen synthase (●) were assayed under conditions similar to those of Fig. 1, except that the concentrations of ATP and MgCl_2 were varied to give a constant (100 μM) concentration of Mg-ATP complex and the indicated amounts of free Mg^{2+} ions, calculated as indicated in the Experimental section.

glycogen synthase by casein kinase 1 were different. Furthermore, 6 mM free Mg^{2+} stimulated by about 4-fold casein phosphorylation by casein kinase 2, but by only 2-fold the phosphorylation of glycogen synthase by this enzyme.

Influence of other bivalent cations on the effect of Mg^{2+}

When assayed in the presence of 0.125 mM-ATP and 1.1 mM- Mg^{2+} , Mn^{2+} inhibited both casein kinases, with 50% inhibition occurring at 44 μM for

casein kinase 1 and at 160 μM for casein kinase 2. However, with glycogen synthase as substrate, the inhibition caused by Mn^{2+} on both enzymes was less marked, with 50% inhibition occurring at 125 μM with casein kinase 1, and 5 mM with casein kinase 2. In addition to Mn^{2+} , Zn^{2+} and Ca^{2+} also inhibited the activity of both enzymes in the presence of Mg^{2+} . Compared with Mn^{2+} , Zn^{2+} was as efficient with casein kinase 2 but less with casein kinase 1, whereas Ca^{2+} was less efficient than Mn^{2+} with both protein kinases with either casein or glycogen synthase as substrate.

Discussion

Casein kinases 1 and 2 were both activated by free Mg^{2+} ions when either casein or glycogen synthase was used as substrate. This fact suggests that both enzymes contain a cation-binding site, in addition to the catalytic site where the cation would bind as a component of the Mg -ATP complex. Direct activation of rabbit reticulocyte casein kinase 2 by Mg^{2+} has been reported (Hathaway & Traugh, 1984b), although that study was restricted to the use of β -casein as substrate. The total Mg^{2+} content in mammalian cells is about 10 mM, of which 0.6–1.3 $\mu mol/g$ wet wt. is present as free Mg^{2+} (Velooso *et al.*, 1973). These data indicate that the effects of free Mg^{2+} on both casein kinases could be operative *in vivo*. Mn^{2+} could substitute for Mg^{2+} in forming the bivalent-cation-ATP complex used as substrate by both casein kinases, but free Mn^{2+} inhibited their activity. Thus Mg^{2+} and Mn^{2+} seem to have a similar role at the level of the catalytic site, but they are antagonistic on the cation-binding site. Nonetheless, total Mn^{2+} concentration in rat liver is about 35 μM , most of which is tightly bound, 13 μM is exchangeable and only 1 μM is free (Ash & Schramm, 1982; Schramm & Ash, 1982). This value is lower than those required to cause inhibition of both casein kinases, either in the absence or in the presence of 1 mM- Mg^{2+} . Thus the possible implication of Mn^{2+} in the physiological control of casein kinases is doubtful, unless some compartmentation exists.

The magnitude of the effects of the cation was influenced by the protein substrate used, which suggests that part of this effect could be due to interactions with the substrate. It is known that casein and phosvitin bind bivalent cations (Panic *et al.*, 1971; Bingham *et al.*, 1972; Taborsky, 1974; Bingham & Farrell, 1974; Osaki *et al.*, 1975; Bingham & Groves, 1979). Binding of Mg^{2+} to glycogen synthase is also indicated by the fact that this cation activates glycogen synthase (Roach & Larner, 1976). As a result of the interaction of the cation with the protein substrate, conformational

changes could occur which may affect the accessibility of the phosphorylation sites to the kinases. Regarding the possibility that Mg^{2+} affected the solubility of the protein substrate, it is known that, although some bivalent cations such as Mn^{2+} and Ca^{2+} precipitate casein at concentrations above 2 mM, this protein remains completely soluble in the presence of 10 mM- Mg^{2+} (Bingham *et al.*, 1972; Bingham & Farrell, 1974; Bingham & Groves, 1979). In consequence, it is unlikely that the differential effects of Mg^{2+} on the phosphorylation of casein by each casein kinase were due to precipitation of the protein substrate. In addition, a different response to Mg^{2+} was also observed with glycogen synthase as substrate, although the effects were less marked than with casein. The data obtained with phosvitin suggest a strong affinity of this protein for bivalent cations: thus the results are difficult to interpret without knowing the binding constants for each of the different cations. Nonetheless they are useful for confirming the different responses of casein kinases 1 and 2 to bivalent cations.

In conclusion, our results demonstrate that, in addition to the effects of Mg^{2+} on the activity of casein kinases 1 and 2 as a component of the bivalent-cation-ATP complex or through interaction with the protein substrate, free Mg^{2+} is also an allosteric effector of both enzymes.

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