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SHORT COMMUNICATION

GLUCOCORTICOID RECEPTOR STABILIZATION: RELATIVE EFFECTS OF MOLYBDATE ION ON INACTIVATION BY ALKALINE PHOSPHATASE AND PHOSPHOLIPASE A_2

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SUMMARY

Glucocorticoid receptors in cytosol preparations from rat liver or mouse L cells are inactivated by phospholipase A_2 or calf intestine alkaline phosphatase. Molybdate ion, an inhibitor of a variety of phosphatase enzymes, does not prevent inactivation of glucocorticoid binding capacity by alkaline phosphatase but it blocks inactivation by phospholipase A_2. In neither case is the enzyme itself inhibited, and the effect of molybdate on phospholipase-mediated inactivation appears to reflect the ability of molybdate to prevent receptor inactivation by the detergent action of lysophosphatides.

INTRODUCTION

The group 6A transition metal oxyanions, like vanadate and molybdate, have recently been shown to affect a variety of biological systems. At low concentrations (in the micromolar range and lower) they have been shown to inhibit ($Na^+ + K^+$)ATPases [1–3] and both acid [4] and alkaline phosphatases [5]. At high concentrations (in the range of 1–10 mM) vanadate and molybdate have been reported to activate adenylate cyclase in plasma membranes [6, 7], to inhibit protein degradation in intact hepatocytes [8], to cause an insulin-like stimulation of glucose oxidation in rat adipocytes [9], to stabilize steroid receptors in cytosol preparations [10, 11], and to inhibit steroid receptor transformation to the DNA-binding state [12, 13]. It has often been assumed that the effects of molybdate and vanadate at high concentrations may reflect inhibition of dephosphorylation or phosphate transfer reactions [6–10]. We report here the unexpected observation that 10 mM molybdate does not prevent loss of glucocorticoid binding capacity caused by calf intestine alkaline phosphatase but it blocks inactivation of receptors by phospholipase A_2.

MATERIALS AND METHODS

Livers were removed from 75–100 g male Sprague-Dawley rats that had been adrenalectomized by the dorsal route and maintained on 0.9% saline for 1–4 days prior to use. Livers were ruptured in 1.5 vol. of 10 mM Hepes buffer (pH 7.4) per g wet weight and cytosol (100,000 *g* supernatant) was prepared as previously described [12]. L929 mouse fibroblasts were grown in monolayer culture in Joklik medium with 10% bovine serum [10]. Cells were harvested in log phase growth, ruptured by Dounce homogenization in Hepes buffer and centrifuged for 1 h at 100,000 *g*.

Cytosol from rat liver was incubated at 20°C with 150 μg/ml of calf intestine alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, 505 units per mg protein at 25°C) and other additions as noted in the legend to Fig. 1. At various times, duplicate 0.4 ml aliquots were removed, incubated for 2 h at 0°C with 50 nM [^{3}H]triamcinolone acetonide* (New England Nuclear, Boston, MA, 5.6 Ci/mmol), and specific binding capacity was assayed as described previously [12]. Calf intestine alkaline phosphatase activity was assayed in 10 mM Hepes buffer, pH 7.4, by a modification of the method of Cox and Griffin[14] using 5 mM *p*-nitrophenyl phosphate as substrate.

Cytosol from mouse L cells was incubated at 20°C with 10 μg/ml of phospholipase A (from *Vipera russelli*, Sigma Chemical Co., 10 units per mg protein at pH 6.5 at 37°C), 100 μg/ml of asolectin, and 10 mM sodium molybdate in the combinations noted in the legend to Fig. 2. All samples contained 0.4 mM $CaCl_2$. At the indicated times, duplicate 0.4 ml samples were removed and specific binding capacity was assayed as above.

The effect of molybdate on phospholipase A activity (Part B of Fig. 2) was determined by incubating 0.1 mg/ml [*N*-methyl ^{14}C]-phosphatidylcholine (New England Nuclear, 50 mCi/mmol) at 20°C with 10 μg/ml phospholipase A, 0.4 mM $CaCl_2$ and L cell cytosol (60% of final incubation volume). After varying times of incubation, 0.15 ml aliquots were removed and added to 0.015 ml 100 mM EGTA to stop the enzyme action. Samples, 0.02 ml, were chromatographed on silicic acid-impregnated paper in chloroform–methanol–7 N ammonium hydroxide (200:25:3, v/v). Phosphatidylcholine and lysophosphatidylcholine were identified with iodine vapor and radioactivity was assayed.

RESULTS AND DISCUSSION

We have previously shown that calf intestine alkaline phosphatase inactivates glucocorticoid receptors in mouse L cell and rat liver cytosol to a state that does not bind steroid [15]. The unbound receptor is sensitive to inactivation whereas the steroid-bound receptor is unaffected. Several observations support the conclusion that the enzyme effect is due to phosphatase action: it is dependent

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* Triamcinolone acetonide is 9α-fluoro-11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione 16,17-acetonide.

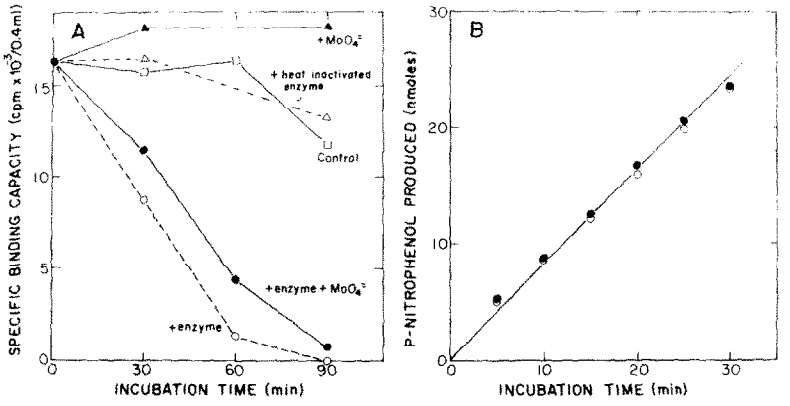

Fig. 1. Inactivation of the glucocorticoid receptor by calf intestine alkaline phosphatase. A, cytosol was incubated at 20°C with calf intestine alkaline phosphatase (○-----○), alkaline phosphatase and 10 mM sodium molybdate (●——●), heat-inactivated alkaline phosphatase (△-----△), sodium molybdate (▲——▲), or 10 mM Hepes buffer alone (□——□). At the indicated times, 0.4 ml aliquots were removed and specific glucocorticoid binding capacity was assayed as described under Methods. B, calf intestine alkaline phosphatase was incubated with 5 mM *p*-nitrophenyl phosphate at 20°C in the presence of 10 mM molybdate (●) or buffer alone (○) and at various times, aliquots were removed and assayed for *p*-nitrophenol.

upon zinc; it is inhibited by arsenate, a competitive inhibitor of the enzyme; and both the dephosphorylating and receptor-inactivating activities of the enzyme preparation copurify. As the transition metal ions have been reported to inhibit both *Escherichia coli* alkaline phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate [5] and protein dephosphorylation by a variety of phosphoprotein phosphatases [16–18], we expected that molybdate would prevent receptor inactivation by calf intestine alkaline phosphatase. As shown in Fig. 1, even at a concentration of 10 mM, molybdate does not prevent receptor inactivation (Panel A), and it does not inhibit dephosphorylation of *p*-nitrophenyl phospate (Panel B) by this enzyme.

Molybdate also has no effect on the ability of phospholipase A to convert phosphatidylcholine to lysophosphatidylcholine (Fig. 2B), but it completely inhibits the inactivation of the L cell receptor by this enzyme at 20°C (Fig. 2A). Phospholipase A has been shown to inactivate glucocorticoid receptors in both mouse L cell [19] and rat liver [20] cytosol. The inactivation is due to the action of lysophosphatides produced by hydrolysis of endogenous phospholipids [20]. As shown in Fig. 2A, addition of both phospholipase A and 100 μg/ml of its substrate asolectin results in greater inactivation than that observed with the enzyme alone and this inactivation is blocked by molybdate. Asolectin alone has no effect on the binding capacity (data not shown) but addition of a lysolecithin, such as L-α-lysophosphatidylcholine from egg yolk, and other types of detergents does inactivate the receptor [12, 19, 20]. We [12] and others [13, 21] have proposed that the transition metal

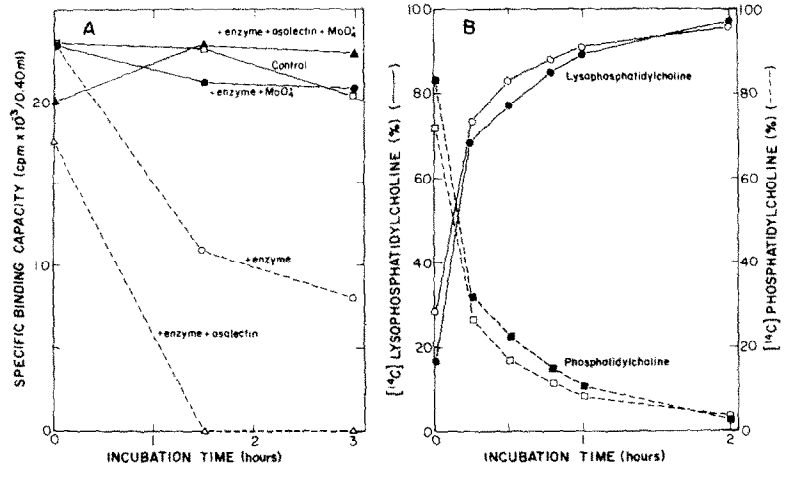

Fig. 2. Inactivation of the glucocorticoid receptor by phospholipase A_2. A, cytosol from mouse L cells was incubated at 20°C with 10 μg/ml of phospholipase A (○-----○); phospholipase A and 10 mM sodium molybdate (●——●); phospholipase A and 100 μg/ml asolectin (△-----△); phospholipase, asolectin and molybdate (▲——▲); or buffer (□). At the indicated times, 0.4 ml aliquots were removed and assayed for their ability to bind triamcinolone acetonide in a specific manner. B, [^{14}C]-phosphatidylcholine was incubated at 20°C with phospholipase A, $CaCl_2$, L cell cytosol and either 10 mM sodium molybdate (closed symbols) or buffer (open symbols). After varying times of incubation, 0.15 ml aliquots were removed and radioactivity in phosphatidylcholine and lysophosphatidylcholine was assayed as described in the Methods. Results are expressed as a percent of the counts recovered as either phosphatidylcholine (■) or lysophosphatidylcholine (●).

oxyanions act through a weak association with the receptor itself and it appears that molybdate in this case is preventing inactivation due to detergent action of lysophosphatides produced by the enzyme.

Although the potent inhibition of several enzymes which hydrolyze phosphate ester bonds by low concentrations of vanadate and molybdate has been studied in some detail, we have no idea how these ions are acting to produce the variety of effects observed at higher concentrations. Van Etten *et al.*[4] have proposed that the early transition metal oxyanions inhibit acid phosphatases at less than μM concentrations because they form complexes with a histidyl residue at the active site of the enzyme. Karlish *et al.*[2] have suggested that vanadate inhibits $(Na^+ + K^+)$ ATPase at low concentrations by blocking the conformational change that follows hydrolysis of the phosphoenzyme and Cantley *et al.*[3] have proposed that vanadate binds to a phosphatase site on the ATPase.

On the basis of the studies on ion effects at low concentration, various investigators have speculated that molybdate and vanadate effects observed at high concentration, such as adenylate cyclase stimulation in plasma membranes [6, 7] and inhibition of protein degradation [8] or stimulation of glucose oxidation [9] in intact cells, may reflect an action of these compounds on phosphate transfer or dephosphorylation reactions. In our original work [10] and in that of Barnett *et al.*[22] it was assumed that molybdate was inhibiting glucocorticoid receptor inactivation and transformation by acting as a phosphatase inhibitor. It should be noted, however, that these compounds can bind to proteins and produce a variety of effects that are not related to inhibition of dephosphorylation or phosphate transfer processes. In the case of steroid receptors, the early transition metal oxoanions can apparently interact in a weak and reversible manner with the receptor itself and inhibit receptor inactivation or transformation produced by low concentrations of salt and precipitation with ammonium sulfate [12, 13, 23]. As shown here, the interaction of molybdate with the receptor does not affect inactivation by alkaline phosphatase but it blocks inactivation by lysophosphatides produced by the action of phospholipase A. Molybdate and vanadate can form complexes with imidazole and sulfhydryl moieties in proteins [24] and probably also with phosphate groups [11]. The biological effects observed at high concentrations of molybdate and vanadate may reflect such weak interactions (e.g. between molybdate and receptor) and are not the same as the more specific effects on phosphatase enzymes observed at much lower concentration.

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