REVERSIBLE INHIBITION BY DIAMIDE OF CYCLIC AMP-DEPENDENT PROTEIN KINASES FROM BOVINE THYROID

Michael McClung and Joan Miller

Division of Endocrinology

University of Oregon Health Sciences Center

Portland, Oregon 97201

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SUMMARY: The effects of diamide on protein kinases isolated from bovine thyroid were studied. Cyclic AMP-dependent protein kinase activity was directly, rapidly, and reversibly inhibited by diamide. This inhibition was non-competitive with respect to ATP or histone and could be prevented by thiol-reducing agents. However, a cyclic nucleotide-independent thyroid protein kinase was not affected. Our data indicate that diamide specifically inhibits protein kinases which are cyclic AMP-dependent.

INTRODUCTION: The cyclic AMP-protein kinase cascade has been well studied and is considered to be an important step in the regulation of thyroid function by TSH (1-5). Yet the functional role of this cascade has not been defined. Because of our involvement with thyroid protein kinases, the recent observation by Von Tersch et al. (6) that protein kinase activity from swine kidney was inhibited by diamide was of interest. They subsequently reported that diamide inhibited both protein kinase activity and amino acid and glucose uptake in rat kidney slices (7) and have suggested that diamide might be useful in studies of the physiological role of protein kinases in various systems.

Diamide is an oxidizing agent which has been used to alter intracellular thiol compounds in several tissues (8-10). It has a high affinity for the oxidation of glutathione, but thiol groups of other compounds, including proteins, are also oxidized when intact cells are exposed to diamide (11). In low concentrations, it is non-toxic and its effect can be reversed by the addition of thiol reducing agents.

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Our study was designed to examine the effects of diamide on protein kinases isolated from bovine thyroid glands. These data demonstrate that diamide specifically inhibits the cyclic AMP-dependent protein kinase activity.

MATERIALS AND METHODS: Preparation of protein kinases: Fresh bovine thyroid glands were homogenized and fractionated by the method of Wolff and Jones (12). All procedures were performed at 4° C. The 37,000 X g supernatant was centrifuged at 105,000 X g for 1 h in an IEC Model B-60 ultracentrifuge. This post-microsomal supernatant was dialyzed overnight vs 10 mM Tris·Cl, 1 mM dithiothreitol, pH 7.2 (buffer A), added to a DEAE-cellulose column (2.5 x 40 cm) equilibrated with buffer A, and the protein kinases were eluted with a linear gradient of NaCl, 0-500 mM, in buffer A. Three peaks of protein kinase activity were found. The first two eluted at 50 and 125 mM NaCl, respectively, phosphorylated mixed histones, were stimulated 5-fold by cyclic AMP, possessed cyclic AMP-binding activity, and seemed identical to the two histone kinase peaks previously described in bovine thyroid glands (3,4). The third protein kinase peak eluted at 200 mM NaCl, phosphorylated phosvitin but not histone, and was cyclic AMP-independent. In several respects, this phosvitin kinase has been shown to be distinct from both the cyclic AMP-dependent kinase holoenzymes and their free catalytic subunits (M. McClung, J. Miller, manuscript in preparation). The histone kinase peak which eluted at 125 mM NaCl (peak II) was pooled and precipitated by adding solid ammonium sulfate (32.5 g/100 ml) with constant stirring. The precipitate was resuspended in 50 mM potassium phosphate, pH 7.0, containing 2 mM EDTA (buffer B) and then chromatographed on a 2.5 x 90 cm column of A$_{0.5}$M agarose (BioRad) equilibrated in buffer B. Histone kinase and [^{3}H]cyclic AMP-binding activity eluted as a single peak which was devoid of phosvitin kinase activity. This preparation was used in the following experiments unless otherwise stated.

Protein kinase assays: Standard kinase assays were performed in triplicate in an incubation volume of 0.1 ml containing 50 mM potassium phosphate, pH 7.0, 10 mM magnesium chloride, 0.1 mM [γ^{32}P]-ATP (specific activity 200-400 cpm/pmole), 200 μg histone II-A, and enzyme. When added, cyclic AMP concentration was 2 μM. After incubation for 20 min at 37° in a shaking water bath, reactions were terminated by spotting 50 μl assay mixture onto 2x2 cm squares of Whatman 3MM filter paper. The filter squares were processed as described by Corbin and Reimann (13). Using purified enzyme preparations, kinase activity was linear with respect to time and enzyme concentration. Phosvitin kinase activity was assayed in a similar manner except the $MgCl_2$ concentration was 2 mM and 200 μg phosvitin was used as substrate instead of histone.

Gilman's method of measuring [^{3}H]cyclic AMP-binding to protein kinases was employed using 5 pmoles [^{3}H]cyclic AMP (14). Protein concentration was determined with the method of Lowry (15). Diamide, protein kinase substrates, and thiol reducing agents were obtained from Sigma; [γ^{32}P]ATP and [^{3}H]cyclic AMP were purchased from New England Nuclear.

RESULTS: Diamide markedly inhibited histone kinase in a concentration-dependent manner (Fig. 1). Inhibition was observed in both the presence and absence of cyclic AMP, although the effect on cyclic AMP-stimulated activity was more pronounced. At diamide concentrations of 10 mM and greater, total kinase activity was less than 5% of initial values, and the response to cyclic

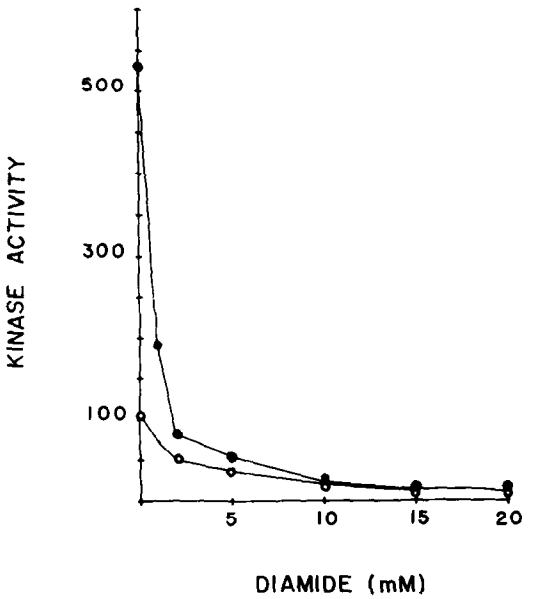

Figure 1. Effect of diamide concentration on thyroid histone kinase activity measured in the presence (●—●) and absence (○—○) of 2 μM cyclic AMP. Kinase activity expressed as pmoles phosphate transferred/mg protein/min (mean ± S.E. of triplicate determinations).

AMP was no longer apparent. The half-maximal inhibitory concentration of diamide was 0.8 mM.

Changes in substrate concentration did not alter the effect of diamide. A reciprocal plot of the data (Fig. 2) revealed that the inhibition was non-competitive with histone. Similar experiments also demonstrated non-competitive inhibition with respect to ATP. The K_m for ATP was 1.9×10^{-5} M in both the presence and absence of 20 mM diamide. Inhibition by diamide was also observed when substrates other than the mixed histone fraction were used. The phosphorylation of protamine, casein, and other histone preparations was decreased approximately 90% by adding 20 mM diamide in the presence of cyclic AMP.

The effect of thiol reducing agents on kinase activity and on its response to diamide was examined. Addition of 10 mM 2-mercaptoethanol, dithiolthreitol,

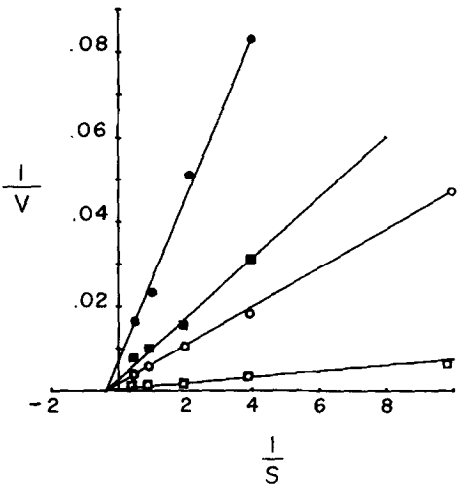

Figure 2. Lineweaver-Burke plot of the relationship between histone concentration and diamide inhibition of thyroid histone kinase activity in the presence and absence of 2 μM cyclic AMP. 1/S = 1/histone concentration (mg/ml). 1/V = 1/pmoles phosphate transferred/mg protein/min. (○—○) No additions, (●—●) 20 mM diamide, (□—□) cyclic AMP, (■—■) cyclic AMP + 20 mM diamide. K_m (histone) = 2.7 mg/ml.

or reduced glutathione increased histone phosphorylation by kinase only slightly (Table I). However, when these agents were added in concentrations sufficient to reduce the diamide in the assay, no significant decrease in kinase activity was observed.

To study the reversibility of kinase inhibition by diamide, purified histone kinase was incubated on ice for 15 minutes in 0.5 ml 50 mM potassium phosphate, 2 mM EDTA, pH 7.0, with and without 10 mM diamide. Each sample was dialyzed overnight vs 50 mM potassium phosphate, 2 mM EDTA, pH 7.0, and then assayed for kinase activity. Even after dialysis, activity was decreased in the sample which had been pre-incubated with diamide (Table II). However, kinase activity in the diamide-treated sample was fully restored by the addition of 18 mM dithiothreitol to the assay medium.

To examine the specificity with which diamide inhibits cyclic AMP-dependent protein kinases, each of the three kinase peaks which were eluted from the DEAE

Table I. Effect of thiol reducing agents on diamide inhibition of protein kinase. Standard histone kinase assay conditions with 2 μM cyclic AMP were used. Kinase activity expressed as pmoles phosphate transferred/mg protein/min ± SE of triplicate determination.

	Protein kinase activity	
Addition	No diamide	5 mM diamide
None	534 ± 27	208 ± 20
Dithiothreitol, 10 mM	641 ± 27	614 ± 44
Glutathione, 10 mM	655 ± 38	626 ± 12
2-mercaptoethanol, 10 mM	680 ± 24	626 ± 59

Table II. Reversibility of diamide inhibition of kinase activity following removal of diamide by dialysis. Assays were performed under standard conditions with 2 μM cyclic AMP and additions as listed. Results shown are the average ± S.E. of triplicate assays expressed as pmoles phosphate transferred/mg protein/min.

	Kinase activity	
Additions to kinase assay	Pre-incubation without diamide	Pre-incubation with 10 mM diamide
None	307 ± 21	42 ± 10
Dithiothreitol, 5 mM	351 ± 18	283 ± 32
Dithiothreitol, 18 mM	317 ± 19	322 ± 24

column were assayed with and without diamide (Table III). Diamide inhibited both peaks of cyclic AMP-dependent protein kinase. However, diamide did not affect the activity of the cyclic nucleotide-independent protein kinase which preferentially phosphorylates phosvitin (peak III). Diamide did not alter [^{3}H]cyclic AMP-binding to either of the histone kinases.

Table III. Specificity of protein kinase inhibition by diamide. Peaks I, II, and III eluted from DEAE-cellulose column at 50, 125, and 200 mM NaCl, respectively, as described under Methods. [^{3}H]cyclic AMP-binding is expressed as pmoles [^{3}H]cyclic AMP bound/mg protein. Histone and phosvitin kinase were assayed under standard conditions, and activity is expressed as pmoles phosphate transferred/mg protein/min. All assays were performed in the presence and absence of 20 mM diamide, and the results listed are means of triplicate determinations.

Fraction and activity assayed	Activity		% inhibition by diamide
	Without diamide	With diamide	
Peak I			
[^{3}H]cyclic AMP binding	2.0	2.0	0
Histone kinase, no cyclic AMP	33.9	11.0	68
Histone kinase, 2 μM cyclic AMP	162.9	14.2	91
Peak II			
[^{3}H]cyclic AMP binding	14.0	14.7	0
Histone kinase, no cyclic AMP	42.9	17.1	60
Histone kinase, 2 μM cyclic AMP	246.1	28.3	89
Peak III			
Phosvitin kinase	224.5	220.1	0

DISCUSSION: The data in this report confirm the observation that protein kinase activity is reversibly inhibited by diamide (6). More importantly, we have shown that this occurs with cyclic AMP-dependent protein kinases. Von Tersch et al. (6) did not convincingly establish that they were studying a cyclic AMP-dependent protein kinase, since addition of cyclic AMP stimulated activity by only 10-16%. As evidenced by the 5-fold increase in enzyme activity in the presence of cyclic AMP and the associated [^{3}H]cyclic AMP-binding activities, the kinases which could be inhibited in our studies were clearly cyclic AMP-dependent.

The effects observed with diamide differ in several important respects from those seen with other protein kinase inhibitors. Kariya and Field recently investigated the inhibition of thyroid protein kinase by adenosine (16). Inhibition was seen only when adenosine or one of its derivatives was present in

DISCUSSION

α_2TPI was separated from α_2-macroglobulin, inter α-trypsin inhibitor and C_1 esterase inhibitor by the difference of their molecular weights, and from α_1-antitrypsin and α_1-antichymotrypsin by their electrophoretic mobilities. The examination of antigenicities further distinguished α_2TPI from another inhibitor, antithrombin III, and from two other serum components (ceruloplasmin and α_2-heat stable glycoprotein) that had very similar physicochemical properties to α_2TPI. Thus, it appears that this inhibitor is a new serum component. α_2TPI inhibited very selectively thiol proteinases of animal and plant origins. The extent of the inhibitory effect was, however, different among several thiol proteinases investigated. Ficin and papain were most sensitive, cathepsin B1 was less sensitive, and fruit and stem bromelains were considerably less sensitive. Such a difference of susceptibility among thiol proteinases may suggest that the inhibition is not due to a simple stoichiometric combination. It is well known that α_2-macroglobulin inhibits not only serine proteinases but also other proteinases like thiol (2, 13) and acidic proteinases (14). In contrast, α_2TPI inhibits selectively thiol proteinases indicating that the mechanism of inhibition by α_2TPI is substantially different from that of α_2-macroglobulin. The biological function of α_2TPI is not yet known. However, one of the probable role of this inhibitor may be in the control of the thiol proteinases released at inflammatory sites. In fact, Beloff (15) and Tokaji (16) have indicated that the prolonged phase of inflammation, which depends on thiol proteinases, is influenced by blood and tissue inhibitors. It is of further interest to determine whether α_2TPI interacts with the proteinases released in the inflammatory loci and by what mechanism this occurs.

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