SPECIFIC INHIBITION OF PROTEIN KINASE BY DIAZENEDICARBOXYLIC ACID BIS-(N,N-DIMETHYLAMIDE)

Francis J. Von Tersch and Joseph Mendicino

Department of Biochemistry
University of Georgia
Athens, Georgia 30601

and

Dennis J. Pillion and Fredrick H. Leibach

Department of Cell and Molecular Biology
Medical College of Georgia
Augusta, Georgia 30902

Received March 7,1975

SUMMARY: Diazenedicarboxylic acid bis (N,N-dimethylamide), commonly called diamide, inhibits purified protein kinase isolated from swine kidney. The extent of inhibition is increased in the presence of cysteine, reduced glutathione and dithiothreitol. The inhibition of protein kinase is reversible and greater than 80% of the activity of an inhibited preparation can be recovered by dialysis. Diamide appears to be specific for the inhibition of protein kinase. The activities of various other kinases acting on low molecular weight substrates were unaffected by diamide. Diamide is the first known low molecular weight inhibitor of protein kinase and may prove to be an extremely useful agent in elucidating the physiological role of this enzyme.

INTRODUCTION: Diazenedicarboxylic acid bis (N,N-dimethylamide) is an oxidizing agent with specificity toward reduced glutathione (1). It has been demonstrated that the reaction of diamide with reduced glutathione is reversible and that other thiol compounds are much less reactive (2). There have been reports (3,4) that diamide causes oxidation of NADPH and NADH, but it appears likely (2,3) that this is a secondary effect caused by elevated levels of GSSG. While diamide has been shown to react with some protein thiols (5) there is very little evidence to indicate that it affects enzyme activities (2,6). Siliprandi et.al.have recently reported that diamide stimulates the ATPase activity of rat liver mitochondria (7) although there is no indication that this is the result of

a direct interaction of diamide with the enzyme. The biological effects of diamide have been investigated in a number of systems in order to help define the physiological role of GSH. Kosower (8) reported that diamide inhibited the growth of bacteria, inhibited protein and RNA synthesis, and affected red cell membrane permeability. Epstein and Kinoshita reported that diamide inhibited cation transport in the lens (9). In previous reports from our laboratory we have presented evidence that diamide inhibits amino acid and sugar transport in slices of kidney cortex (10,11), and that this effect could be reversed by glutathione.

cAMP, which acts by stimulating various protein kinases (12), has been shown to influence many of the processes which are inhibited by diamide. cAMP stimulates amino acid transport (13), protein synthesis (14), and the transport of various metabolites as well as being involved in the regulation of glycogen metabolism (12).

As a result of information obtained in our transport studies, we suggested that diamide interferred with membrane proteins or enzymes involved with transport processes. We now present evidence that diamide reacts specifically with kidney protein kinase.

MATERIALS AND METHODS: Protein kinase was isolated from swine kidney according to the procedure of Abou-Issa et al (15). The activity of the enzyme was assayed as described previously (15) and the standard reaction mixture was incubated for 10 min at 30° and contained in 0.35 ml: 30 mM 2(N-Morpholino) ethanesulfonic acid (MES), pH 6.5; 12 mM $MgCl_2$, 0.12M sucrose, 0.03 mM 3',5' cyclic AMP, 9mM cysteine, 1 mg calf thymus histone and enzyme. Preliminary incubation was for 30 min at 30° and the reaction was initiated by the addition of 0.12 µmole of (γ) ^{32}P-ATP (specific activity 2 x 10^7 cpm/µmole). Other kinases were assayed by standard procedures. Diamide, reduced glutathione, dithiothreitol and cysteine were obtained from Sigma.

RESULTS: The activity of protein kinase decreases in the presence of diamide as shown in Fig. 1. Addition of higher concentrations of diamide 24mM, resulted in greater than 90% inhibition of the activity of the enzyme. The experiments summarized in Fig. 2 demonstrate that the extent of inhibition is proportional to the concentration of enzyme. The addition of sulfhydryl compounds increase the affinity of the enzyme for diamide, as seen in Fig. 3. Under these conditions 50% inhibition of protein kinase, in the absence of sulfhydryl compounds, required 12.7 mM diamide. In the presence of 9 mM dithiothreitol, reduced glutathione, or cysteine, the 50% inhibition was observed at 9mM, 5.4mM, or 4.2mM diamide respectively.

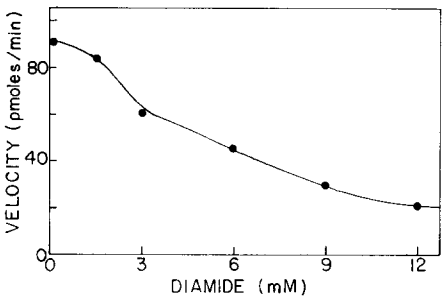

Fig. 1. Influence of the concentration of diamide on the activity of swine kidney protein kinase. The standard assay conditions described in the text with the concentrations of diamide shown were used.

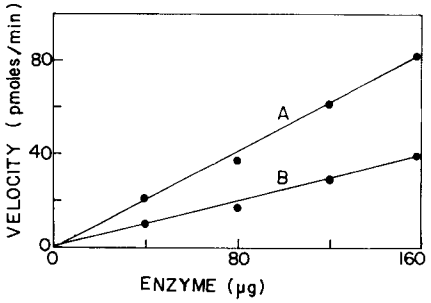

Fig. 2. Influence of the concentration of enzyme on the inhibition of protein kinase by diamide. Conditions are the same as those in Fig. 1. Curve A was obtained in the absence of diamide and curve B with 6 mM diamide.

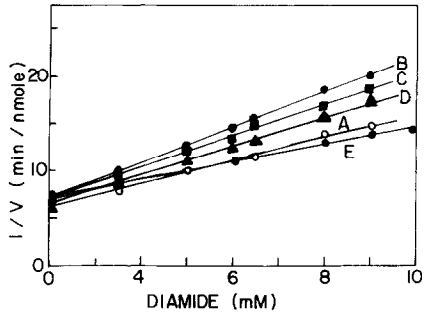

Fig. 3. Influence of cysteine, reduced glutathione, oxidized glutathione and dithiothreitol on the inhibition of protein kinase by diamide. Curve A (O—O) was obtained with no additions; Curve B (●—●) with 9 mM dithiothreitol; Curve C (■—■) with 9 mM reduced glutathione; Curve D (▲—▲) with 9 mM oxidized glutathione, and curve E (●—●) with 9 mM cysteine. The enzyme was idalyzed against 0.02 M Tris, pH 7.0 and assayed under the standard conditions described in the text, except that cysteine was omitted as shown in the figure.

Experiments to determine whether the inhibition of protein kinase by diamide was reversible were carried out by treating the enzyme (initial activity, 60 pmoles/min/0.1mg) with 12mM diamide. Under these conditions, the activity decreased to 6 pmoles/min/0.1mg. The sample was then dialyzed against 4 liters of 12mM reduced glutathione for 4 hours. The dialyzed sample had an activity of 64 pmoles/min/0.1mg. These results demonstrate that the inhibition by diamide can be reversed by dialysis and that all of the activity can be recovered after removal of the inhibitor. Addition of reduced glutathione in amounts sufficient to oxidize all of the diamide present was also effective in reversing the inhibition of protein kinase.

The inhibitor appears to be specific for the protein kinase. The data summarized in Table I shows that diamide had little or no effect on the activity of pyruate kinase, hexokinase, phophofructokinase and creatin kinase. The activity of the enzyme was inhibited to the same degree when histone, protamine or casein were used as substrates, as shown in Table I. The rate of phosphorylation of glycogen synthetase was much less

TABLE I

Specificity of Inhibition of Protein Kinase and the Effect of Different Protein Substrates.

The standard assay systems described in the text were used. The concentration of diamide was 6 mM.

Enzyme or Protein Substrate Tested	Activity Minus diamide (pmoles/min)	Activity Plus diamide (pmoles/min)	% Inhibition
Protein kinase	90	50	44
Pyruvate kinase	2.5×10^4	2.4×10^4	2
Hexokinase	4.3×10^4	4.2×10^4	2
Phosphofructokinase	1.8×10^4	1.7×10^4	5
Creatin Kinase	1.8×10	1.8×10^4	0
Histone, 1 mg	109	46.6	57
Protamine, 1 mg	143	46.5	67
Glycogen Synthetase, 2 mg	42	34	19
Casein, 1 mg	13.5	6.3	53

sensitive to inhibition by diamide. The higher molecular weight of this substrate (380,000) may hinder the accesibility of the inhibitor.

The % inhibition of the enzyme by 6mM diamide was not significantly influenced by the concentration of histone, $MgCl_2$, 3',5' cyclic AMP or ATP added to the reaction mixture.

DISCUSSION: The use of diamide as a thiol oxidizing agent has increased in recent years, because of the ease with which its effects can be reversed in biological systems. Our earlier investigations had demonstrated the effectiveness of diamide as an inhibitor of amino acid and sugar transport in slices of rat and rabbit kidney cortex (10,11). Even though the reaction of diamide with GSH is well documented and represents the initial event caused by diamide in a biological system, it seemed to us worthwhile to look for other effects of diamide on membrane enzymes or transport processes.

TABLE II

Influence of the concentration of components of the reaction mixture on the inhibition of protein kinase by diamide.

The standard reaction conditions with addition as noted were used. The concentration of diamide was 6 mM except for experiment.

Additions	Activity		%
	Minus diamide pmoles/min	Plus diamide pmoles/min	Inhibition
Histone, 0.5 mg	64	31	52
1.0 mg	137	28	77
2.0 mg	145	35	76
$MgCl_2$, 6mM	82	34	59
12mM	125	33	74
3',5'-cyclic AMP, none	50	18	64
1×10^{-6}M	58	20	66
2×10^{-6}M	55	16	71
ATP, 0.24 mM	98	15	85
0.48 mM	96	17	83

Diamide can reversibly inhibit isolated kidney protein kinase as shown in Figure 1. It would appear that diamide is not inhibiting protein kinase by means of a random oxidation of protein thiol groups. The various other kinases shown in Table I, as well as the protein kinase, all contain reactive sulfhydryl groups and are capable of being inhibited by sulfhydryl reagents such as mercurials (16). However, diamide has no effect on the activity of these other kinases, just as it had no effect on various glycolytic and hexose monophosphate shunt enzymes studied previously (6).

The inhibition of protein kinase cause by diamide was more pronounced in the presence of thiol (Fig. 3). The reasons for this effect are not immediately apparent. However, disturbances of the GSH/GSSG ratio are known to have noticeable effects on a number of biological reactions (17). It is also possible that the presence of thiol stimulates mixed disulfide

formation between the added thiol and the enzyme. Further investigation of this area is currently in progress.

The data presented in this report demonstrate that diamide is the first specific low molecular weight inhibitor of protein kinase. There are numerous reports in the literature of a protein which is capable of inhibiting protein kinase; this factor is not well characterized. Other investigators have reported the stimulatory effects of cyclic AMP and dibutyryl cyclic AMP on protein phosphorylation (18). Weis et al (19) reported that cAMP and dibutyryl cAMP increase the uptake of amino acids in slices of renal cortex. Segal et al (20) have shown that dibutyryl cyclic AMP enhances the accumulation of α-methyl-D-glucoside in kidney cortex slices, which suggests that cAMP is involved in the process of active transport. Our earlier demonstration of the inhibition by diamide of amino acid and sugar transport along with the current finding that diamide inhibits protein kinase would indicate that diamide exerts its inhibitory effect on transport and other processes by reacting with protein kinase.

ACKNOWLEDGEMENTS: This investigation was supported by USPHS Grant AM 13150 from the National Institute of Arthritis, Metabolism and Digestive Diseases, and USPHS-NIH Grant FR 5365.

REFERENCES

1. Kosower, N.S., Kosower, E.M. and Wertheim, B. (1969) Biochem. Biophys. Res. Commun. 37, 593-596.

2. Kosower, E.M., Correa, W., Kinon, B.J., and Kosower, N.S. (1972) Biochim. Biophys. Acta 264, 39-44.

3. Brown, J.S. (1971) Biochem. J. 124, 665-667.

4. Biaglow, J.E. and Nygaard, O.F. (1973) Biochem. Biophys. Res. Commun. 54, 874-881.

5. Harris, T.W. and Biaglow, J.E. (1972) Biochem. Biophys. Res. Commun. 46, 1743-1749.

6. Srivastava, S.K., Awasthi, Y.C., and Beutler, E. (1974) Biochem. J. 139, 289-295.

7. Siliprandi, D., Scutari, G., Zoccarato, F., and Siliprandi, N. (1974) FEBS Letters 42, 197-199.

8. Kosower, E.M. and Kosower, N.S. (1969) Nature 224, 117-120.

9. Epstein, D.L. and Kinoshita, J.H. (1970) Invest. Ophthalmol. 9, 629-638.

10. Hewitt, J., Pillion, D. and Leibach, F.H. (1974) Biochim. Biophys. Acta 363, 267-276.

11. Pillion, D. and Leibach, F.H. (1975) Biochim Biophys Acta, in press.

12. Brostrom, M.A., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) Adv. Enzyme Regul. 8, 191-203.

13. Naokaju, N. and Rasmussen, H. (1970) Proc. Nat. Acad. Sci., 65, 368-374.

14. Adiga, P.R., Murthy, P.V.N. and McKenzie, J.M., (1971) Biochemistry 10, 711-715.

15. Abou Issa, H., Kratowich, N. and Mendicino, J., (1974) Eur. J. Biochem., 42, 461-473.

16. Morrison, J.F. and Heyde, E. (1972) Ann. Rev. Biochem. 44-46.

17. Eggleston, L.V. and Krebs, H.A. (1974) Biochem. J. 138, 425-435.

18. Dousa, P.T., Sands, H. and Hechter, O. (1972) Endocrinol. 91, 757-763.

19. Weiss, I.W., Morgan, K. and Phang, J. (1972) J. Biol. Chem. 247, 760-764.

20. Rea, C. and Segal, S. (1973) Biochim. Biophys. Acta 311, 615-624.