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INHIBITION OF PROTEIN KINASE ACTIVITY AND AMINO ACID AND α-METHYL-D-GLUCOSIDE TRANSPORT BY DIAMIDE

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SUMMARY

Dizene dicarboxylic acid bis-(*N*,*N*-dimethylamide), commonly called diamide, is known to oxidize stoichiometrically intracellular pools of reduced glutathione and inhibit the accumulation of sugars and amino acids by rat kidney slices. Incubation of rat renal cortical slices in diamide also leads to a significant decrease in the level of endogenous protein kinase activity. The inhibition of sugar and amino acid transport and protein kinase activity by diamide is partially reversible by the addition of exogenous glutathione or other thiols. A comparison of protein kinase activity with amino acid and sugar transport at various concentrations of diamide indicates that there is a high degree of correlation between these two processes.

INTRODUCTION

The accumulation of amino acids and sugars by slices of mammalian renal cortex is a process which has been studied extensively in recent years [1–10]. At present, the concept of a membrane-bound carrier protein which serves to bind stereospecifically the transport substrate is generally accepted. The means by which translocation occurs, the method of physiological control over the process and the source of energy which drives the reaction are all topics of considerable debate. It is extremely difficult to resolve these questions because the carrier protein, by virtue of its close association with the lipid membrane, cannot be readily isolated in a form which retains its in vivo activity.

The finding that the addition of exogenous cyclic AMP leads to an increase in the accumulation of sugars and amino acids [6, 8, 11] has led to speculation that protein kinases are involved in the regulation of this process. Recent investigations in this laboratory have sought to establish evidence consistent with this hypothesis. It has been shown that the mammalian kidney contains endogenous protein kinase, phosphoprotein phosphatase, and a protein which can serve as a substrate for phosphory-

Abbreviation: Diamide, diazene dicarboxylic acid bis-(*N*,*N*-dimethylamide).

lation [12]. More recently it was discovered that diazene dicarboxylic acid bis-(*N*,*N*-dimethylamide) (diamide) is a specific inhibitor of an isolated porcine renal protein kinase [13]. Previous studies with diamide have shown that this GSH-oxidizing agent is also a reversible, non-toxic inhibitor of sugar and amino acid transport [9, 10].

This study presents data that show a high degree of correlation between the magnitude of protein kinase inhibition and transport inhibition at various concentrations of diamide. Since diamide does not inhibit the activity of other enzymes with which it has been tested [13, 14], these results suggest that diamide inhibits amino acid and sugar transport by inhibiting a protein kinase which is required for the transport process.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats were fed Purina rat chow and water ad libitum until killed by cervical dislocation. Kidneys were quickly removed and used to make cortical slices; tissue preparation and the techniques used in transport studies have all been described previously [9, 10]. Briefly, three cortical slices per sample are placed in 3.0 ml Krebs-Ringer bicarbonate buffer plus or minus 30 mM diamide for 15 min on ice (Incubation 1). After rinsing in saline, the slices are next placed in 3.0 ml buffer plus or minus 20 mM GSH for 15 min at room temperature (Incubation 2). Finally the samples are rinsed in saline and placed in 2.0 ml buffer which contains labelled and unlabelled amino acids or sugars. After various lengths of incubation at 37 °C, the tissue slices are rinsed in saline, weighed and placed in centrifuge tubes containing 2.0 ml water. These centrifuge tubes are placed in a boiling-water bath for 10 min and then centrifuged at 5000 rev./min for 15 min. An aliquot of the supernatant is counted in a Packard Tricarb liquid scintillation spectrometer to determine tissue levels of radioactivity, while an aliquot of the incubation media is also counted in order to determine the amount of radioactivity contained in the extracellular fluid of the tissue. After correcting for extracellular radioactivity (inulin space), the distribution ratio (cpm/ml intracellular fluid)/(cpm/ml extracellular fluid) was determined [1]. Distribution ratios greater than one are characteristic of active uptake.

The level of protein kinase activity found in renal cortical slices was determined as follows. Kidney slices were prepared and manipulated in the same manner as those used in transport studies except that no substrate was added during the final 37 °C incubation. At various times throughout this procedure, samples were taken for protein kinase determination. Slices were rinsed, blotted and weighed, and a 5 % (w/v) homogenate was made in a tight-fitting glass homogenizer with Tris · HCl (0.01 M, pH 7.8); these samples were then frozen in a solid CO_2/acetone bath and stored at −20 °C until assayed. Samples used in this study were never stored more than 1 week and protein kinase activity remained essentially constant over this time period.

The reaction mixture (0.35 ml) contained the following reagents: 30 mM 2(*N*-morpholino)-ethanesulfonic acid, pH 6.5; 12 mM $MgCl_2$, 120 mM sucrose, 0.03 mM 3′,5′-cyclic AMP, 9 mM cysteine, 5 mM NaF, 1 mg calf thymus histone and 0.10 ml of the tissue homogenate. Preliminary incubation was for 3 min at 30 °C and the reaction was initiated by the addition of 0.12 μmol of [γ-^{32}P]ATP (specific activity $2 \cdot 10^7$ cpm/μmol). The reaction was stopped by the addition of 3 ml of 10 %

trichloroacetic acid, followed by an additional mg of histone. The sample was centrifuged at 5000 rev./min for 10 min, the supernatant discarded, and the pellet resuspended in 0.3 ml of 1 M NaOH. This was followed by the addition of 0.7 ml H_2O and 3.0 ml 10 % trichloroacetic acid. The sample was spun down again and the washing procedure repeated. Finally, the pellet was resuspended in 0.2 ml 1 M NaOH, followed by the addition of 0.5 ml H_2O. The sample was then poured into scintillation vials which contained 0.3 ml of 1 M HCl plus 10 ml of a Triton/Toluene cocktail (0.4 % PPO, 0.01 % POPOP, toluene plus Triton X-100 in a ratio of 2 : 1). Activity was expressed as pmol ^{32}P incorporated/min.

Diamide and dithiothreitol were purchased from Nutritional Biochemicals Corp., mercaptoethanol from Eastman-Kodak Co. and 2,3-dimercapto-1-propanol (BAL) from Pfaltz and Bauer Chemicals. Labelled sugar was obtained from Calbiochem. and labelled amino acids were obtained from New England Nuclear, while labelled ATP was prepared according to Glynn and Chappell [15].

RESULTS

Slices of rat renal cortex were analyzed for protein kinase activity at various times during uptake studies, just as GSH levels had been determined at various times in a previous report [9]. Arbitrarily, the beginning of the final incubation at 37 °C was assigned a value of 0 min as a time reference; since it takes approximately 60 min to reach this point after sacrifice of the animal, fresh tissue is referred to as −60 min and the first incubation starts at −30 min. The data shown in Fig. 1 demonstrate that fresh

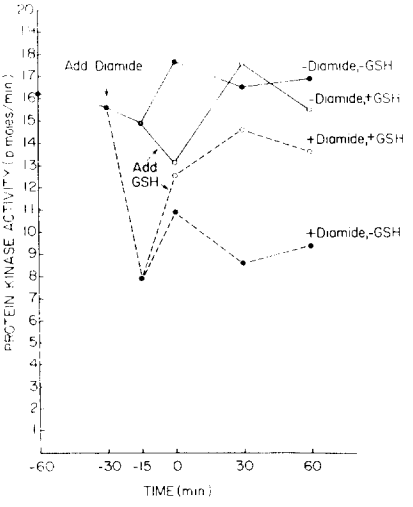

Fig. 1. The effect of diamide and GSH on the protein kinase activity of rat renal cortical slices. Slices were prepared and placed in ice-cold buffer with or without the addition of 30 mM diamide at −30 min (Incubation 1). At −15 min, the slices were transferred to flasks at 25 °C which contained buffer plus and minus 20 mM GSH (Incubation 2). The slices were then placed in buffer at 37 °C at 0 min and incubated for 60 min at 37 °C. At various times throughout this procedure, samples were removed and assayed for protein kinase activity. The data shown represent the average of triplicate or quadruplicate determinations, and the S.E.M. for these values never exceeded 1.27 pmol/min.

tissue (−60 min) contains a considerable amount of protein kinase activity. It is apparent that in the untreated tissue (−diamide, −GSH) the level of enzymatic activity does not decrease continually during the time course of these experiments, although large fluctuations are evident from one time point to the next. In contrast, the level of GSH in renal cortical slices studied under identical conditions decreased steadily with time [9]. These results would argue against the existence of a direct correlation between the concentration of GSH and the level of protein kinase activity found in kidney slices.

The addition of 30 mM diamide to the slices (Fig. 1) led to a significant decrease in kinase activity ($p < 0.005$ for all four time periods tested). This finding is consistent with an earlier report which stated that diamide is capable of inhibiting protein kinase isolated from pig kidney [13]. Once again it is apparent that changes in protein kinase activity do not parallel changes in the concentration of reduced glutathione. Protein kinase activity fell to a value approximately 40 % below that of untreated tissue, and the enzymatic activity remained relatively close to this level after the removal of the slices from diamide (Fig. 1). In contrast, GSH values fell over 95 % after diamide treatment and then showed a considerable regeneration at subsequent times [9]. Thus, it would appear that the regeneration of endogenous GSH is not capable of reversing the effects of diamide on protein kinase activity.

Finally, it was necessary to test whether or not exogenous GSH could reverse the effects of diamide on protein kinase activity. 20 mM GSH was added to untreated tissue slices as a control to determine whether or not this tripeptide was capable of stimulating protein kinase activity. The results shown in Fig. 1 demonstrate that reduced glutathione does not significantly increase the level of protein kinase activity in rat kidney slices. In view of the finding that the level of protein kinase activity in diamide-treated slices remained low throughout the course of this experiment, despite the regeneration of endogenous GSH, it seemed unlikely that the addition of exogenous GSH would lead to a restoration of enzyme activity. The results depicted in Fig. 1 demonstrate that the addition of GSH to diamide-treated samples did partially reverse the inhibition of protein kinase activity. While enzymatic activity never reached the level found in the untreated tissue, it was significantly improved over samples which were treated with diamide alone ($p < 0.01$ for all three time periods tested). Comparison of these findings with our previously published results on amino acid and sugar transport [9, 10] reveals that GSH was capable of reversing inhibition of transport in a similar manner.

Further experiments were undertaken to determine the thiol specificity of reversing protein kinase inhibition by diamide. The results presented in Table I show the effectiveness of various thiol compounds in reversing this inhibition as well as the effect of treatment with oxidized glutathione (GSSG). One possible explanation of the inhibitory effect of diamide on the protein kinase of rat renal cortical slices is that diamide treatment causes an increase in tissue GSSG levels and GSSG may inhibit the enzyme. The data presented here indicate that GSSG is not an inhibitor of protein kinase.

As shown in Fig. 1, the addition of GSH to diamide-treated tissue causes a reversal of the protein kinase inhibition. The data in Table I show that cysteine and mercaptoethanol are equally effective in reversing the effects of diamide, while 2,3-dimercapto-1-propanol is not. Dithiothreitol appears to be an even more potent agent,

TABLE I

REVERSIBILITY OF PROTEIN KINASE INHIBITION

Slices of rat kidney cortex were incubated as described in Fig. 1. Diamide and GSSG (30 mM) or various thiols (20 mM) were added to the incubation medium where indicated. The uptake period lasted 60 min, after which slices were assayed for protein kinase activity. Values represent the average of triplicate determinations ± S.E.M.

Incubation 1	Incubation 2	pmol/min	%
None	None	33.09±2.61	100.00
Diamide	None	21.29±0.45	64.34
GSSG	None	34.85±3.13	105.32
Diamide	GSH	28.46±1.47	86.01
Diamide	Dithiothreitol	35.77±1.67	108.10
Diamide	Cysteine	26.69±1.75	80.66
Diamide	2,3-Dimercapto-1-propanol	20.33±1.43	61.44
Diamide	Mercaptoethanol	28.72±1.70	86.79

and complete reversibility of inhibition by diamide can be achieved with this reagent. While it is not clear why dithiothreitol is more effective than the other thiol compounds, the complete reversal of inhibition shows that the effect of diamide is not due to irreversible tissue damage.

In order to elucidate the relationship between transport and protein kinase activity, rat renal cortical slices were prepared in an identical fashion and assayed for each parameter. The data shown in Table II illustrate the direct relationship between the uptake of sugars and amino acids and the level of protein kinase activity. The addition of 30 mM diamide to renal cortical slices, as demonstrated previously [9, 10], leads to a consistent reduction in transport of approximately 40 % for a wide variety of amino acids, as well as the nonmetabolized sugar, α-methyl-D-glucoside.

TABLE II

THE EFFECT OF DIAMIDE ON DISTRIBUTION RATIOS AND ON PROTEIN KINASE ACTIVITY

Slices of rat kidney cortex were incubated as described in Fig. 1, with or without the addition of 30 mM diamide during Incubation 1. The uptake period lasted 60 min and distribution ratios for the various transport substrates were calculated as described in the text. Protein kinase activity was determined in samples which contained no transport substrate but which were treated in an identical fashion. The values shown represent mean ±S.E.M. with the number of determinations given in parentheses.

Substrate	- Diamide	+ Diamide	% Inhibition	*P* values
Arginine	3.13±0.19 (8)	1.98±0.17 (8)	36.7	0.005
Lysine	3.20±0.29 (6)	1.89±0.03 (6)	41.3	0.005
Leucine	2.90±0.46 (7)	1.64±0.12 (7)	43.4	0.0125
Glycine	6.57±0.23 (4)	4.13±0.19 (4)	37.1	0.005
Methionine	3.38±0.13 (7)	2.17±0.09 (7)	35.8	0.005
α-Methyl-D-glucoside	3.80±0.12 (8)	2.09±0.26 (7)	38.7	0.005
Protein kinase activity*	16.12±1.02 (5)	9.70±0.94 (5)	39.8	0.005

* Values expressed as pmol ^{32}P incorporated/min.

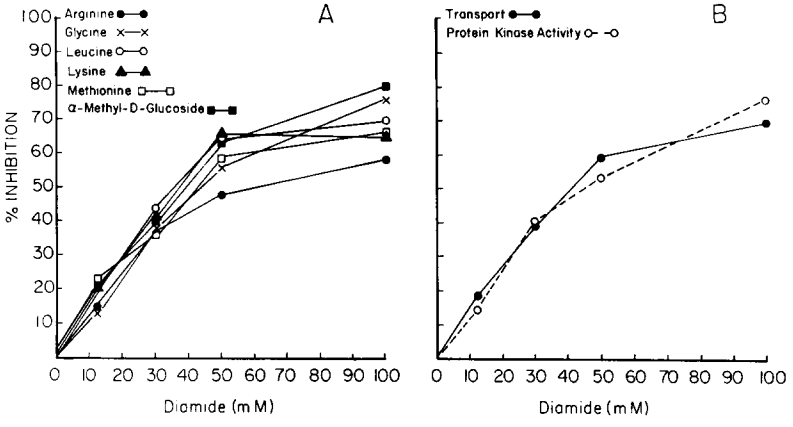

Fig. 2. The effect of the concentration of diamide on transport activity and on protein kinase activity. Slices of rat kidney cortex were prepared and incubated as described in Fig. 1. Various concentrations of diamide were used during Incubation 1, while Incubation 2 was performed with buffer only. The uptake period lasted 60 min, after which the distribution ratio of each sample was determined. Samples treated in an identical fashion, but which contained no transport substrate, were analyzed for protein kinase activity. Each point represents the average of at least quadruplicate determinations. The transport line in B represents the average of all the points in A.

The distribution ratios for each of these substrates varies considerably, but the percent decrease found after treatment with diamide is quite consistent in magnitude. It is extremely interesting to note that the addition of the same amount of diamide to the slices leads to a reduction in protein kinase activity which parallels the decreases seen in the distribution ratios. This finding is consistent with the theory that renal protein kinase functions at a rate-limiting step in amino acid and sugar transport.

In order to rule out the possibility that the observed correlation between the inhibition of transport and the inhibition of protein kinase activity was a chance occurrence at one particular concentration of diamide, a study was undertaken using various amounts of diamide. The data shown in Fig. 2A are derived from earlier studies on the uptake of amino acids and α-methyl-D-glucoside [9, 10]. In these experiments, renal cortical slices were incubated with increasing amounts of diamide and then distribution ratios were calculated for each of the substrates. To facilitate comparison, these values were converted to percentages, with distribution ratios obtained in the absence of diamide serving as controls. Clearly there is a direct correlation between the percent inhibition of transport activity and the concentration of diamide used for all the substrates studied. This indicates that diamide and, presumably, protein kinase are not interacting with only one particular carrier system [18]. Fig. 2B depcits the percent inhibition of transport, which is simply the average value for the data in Fig. 2A. The percent inhibition of protein kinase activity, derived from tissues treated in an identical fashion, is also shown in Fig. 2B. Inspection of the data reveals that the two lines are virtually identical; this again suggests that the activity of protein kinase may be directly related to sugar and amino acid transport, since both the amount of sugar and amino acid transported and the level of protein kinase activity showed the same dose-response curve with diamide.

DISCUSSION

The data presented in this report establish for the first time that diamide is a reversible inhibitor of the protein kinase activity of rat kidney slices. This finding has important ramifications for investigators of active transport and any other processes regulated by cyclic AMP and protein kinase. The involvement of a cyclic AMP-dependent protein kinase in the regulation of membrane transport might be simplistically stated as follows: a membranous carrier protein, which is capable of binding a transport substrate, exists in the renal brush border in two forms, phosphorylated or unphosphorylated. Physiological stimuli regulate the level of cyclic AMP, which in turn activates a protein kinase found in the renal brush border membrane. This protein kinase phosphorylates the carrier protein which induces a change in its conformation and thus mediates the transfer of the substrate from one side of the membrane to the other.

Such a model does little to elucidate the proposed conformational changes in the membranous carrier protein. Nonetheless, this model allows a much clearer look at those processes which might serve to regulate active transport in vivo. Several investigators have shown that cyclic AMP can stimulate the active accumulation of amino acids and sugars [6, 8, 11]. Since cyclic AMP is known to mediate its effects through protein kinases, these findings would suggest a role for protein kinase in the transport process [16–19]. More recent work in our laboratory has demonstrated that the renal brush border does indeed contain an endogenous protein kinase [12]. In addition, we have found that the brush border also contains a protein which is capable of being phosphorylated and dephosphorylated. Thus it is now known that kidney cortex contains all of the functional components required for a protein kinase-regulated transport system.

When our current findings with diamide are viewed in light of these previous reports, the evidence for protein kinase involvement in transport seems quite formidable. The direct correlation between distribution ratios and protein kinase activity levels over a wide range of diamide concentrations suggests that protein kinase may act at the rate-limiting step of the transport process. Epstein and Kinoshito have shown that diamide can also inhibit cation transport in the lens [20]. Such a finding is particularly significant in view of the proposal that Na^+ is cotransported with amino acids and sugars [21]. Further work is currently underway in this laboratory to determine the effects of diamide on the Na^+ gradient in renal cortical slices (Moree, L. H., Pillion, D. J., Pashley, D. H. and Leibach, F. H., unpublished observations).

Diamide was introduced by Kosower et al. as a highly specific GSH oxidant [22]. Our studies employed diamide to inhibit protein kinase and active transport. Certainly there is no diamide in vivo to inhibit these processes, and there is no indication that oxidized glutathione is capable of such inhibition. The question must be raised, then, as to the role of GSH in the process of protein kinase-dependent active transport. The data presented in this report show that there is no obvious correlation between GSH levels and the amount of protein kinase activity found in renal cortical slices. Several thiols were as effective as GSH, or even more so, in reversing the inhibition of protein kinase activity by diamide. Nonetheless, in renal tissue GSH serves as the major source of non-protein thiol and it is likely that GSH, or a protein thiol which is maintained in the reduced state by GSH, is required for enzymatic activity.

Considerations such as these must remain speculative until it is possible to construct artificial systems which contain both the carrier protein and the protein kinase in an active form. In such a system it should be possible to demonstrate amino acid or sugar binding to, as well as phosphorylation of, the carrier protein; these processes should be stimulated by cyclic AMP and inhibited by diamide. Hopefully such a system will be available in the near future to test this hypothesis.

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