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INHIBITION OF PROTEIN PHOSPHORYLATION AND INDUCTION OF PROTEIN CROSS-LINKING IN ERYTHROCYTE MEMBRANES BY DIAMIDE

M. MARLENE HOSEY, DAVID A. PLUT and MARIANO TAO

Department of Biological Chemistry, University of Illinois at the Medical Center, 835 S. Wolcott Avenue, Chicago, Ill. 60612 (U.S.A.)

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Summary

This report presents studies on the effect of diamide on protein phosphorylation in erythrocyte membranes. Diamide, a thiol-oxidizing reagent, nonspecifically inhibits cyclic AMP-dependent and -independent autophosphorylation of red cell membranes, but not the activity of the solubilized membrane cyclic AMP-independent protein kinases. Analysis of diamide-treated membranes by gel electrophoresis indicates that diamide is capable of inducing cross-linking of membrane proteins. The action of diamide, both in the inhibition of membrane autophosphorylation and in the cross-linking of membrane proteins, is very similar to that of Cu^{2+} · *o*-phenanthroline complex. Our data indicate that diamide inhibits erythrocyte membrane autophosphorylation by perturbing the protein substrates.

Introduction

Diamide, diazene dicarboxylic acid bis (*N,N*-dimethylamide), was originally introduced by Kosower et al. [1] as a specific oxidizing agent for glutathione in human erythrocytes. Except for the oxidation of glutathione, they showed that diamide did not interfer with normal cellular functions [1,2]. However, a subsequent report indicated that diamide could also oxidize protein-SH groups [3], suggesting that the oxidizing agent might be relatively nonspecific and might cause other cellular modifications. Recently, diamide was found to inhibit amino acid and sugar transport in rat kidney cortex [4,5] and the activity of cyclic AMP-dependent protein kinases of swine kidney [6]. The inhibition of transport processes was subsequently attributed to the inhibitory action of diamide on the cyclic AMP-dependent protein kinases [7].

Our laboratory has been interested in the study of the role of protein phos-

phorylation in the regulation of membrane functions. We have previously reported that human and rabbit erythrocyte membranes contain multiple protein kinases and phosphoryl acceptors [8—10]. In view of the reported action of diamide on cyclic AMP-dependent protein kinases and in order to further characterize the erythrocyte membrane autophosphorylation systems, we have undertaken studies to determine whether diamide could be used to selectively inhibit membrane-bound kinases. Our data suggest that diamide inhibits nonspecifically cyclic AMP-dependent and -independent protein phosphorylation in erythrocyte membranes but not the phosphorylation of casein by the solubilized membrane cyclic AMP-independent protein kinases. In addition, low concentrations of diamide produced cross-linking of membrane proteins in a manner analogous to the Cu^{2+} · *o*-phenanthroline complex, another thiol-oxidizing agent [11,12]. Based on our data, it is concluded that the inhibition of membrane phosphorylation in erythrocytes by diamide results from the perturbation of membrane structure due to the formation of membrane protein cross-links.

Experimental Procedures

Hemoglobin-free ghosts of human and rabbit erythrocytes were prepared from freshly drawn blood according to the procedure of Dodge et al. [13] as previously described [14]. Cyclic AMP-independent protein kinases of rabbit erythrocyte membranes were extracted and partially purified according to Hosey and Tao [10]. Phosphorylation of membrane proteins was performed as previously described [8,9] and assayed by trichloroacetic acid precipitation [15] or analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis [16] followed by radioautography [8,9]. The reaction mixture (in a final volume of 50 or 100 μl) contained: 0.1 M buffer (either Tris/acetate, pH 6.0; Tris · HCl, pH 7.5; or glycine/NaOH, pH 8.5, as specified); 10 mM $MgCl_2$; 0.2 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP; ±2 μM cyclic AMP; 30—60 μg membrane proteins. Phosphorylation of casein by the solubilized protein kinases was measured as described by Hosey and Tao [10]. The reaction mixture was as above but contained 2 mg/ml of casein instead of the membrane proteins, 0.1 M KCl, and the solubilized enzymes. Diamide or Cu^{2+} · *o*-phenanthroline treatment was performed by incubating equal volumes of membranes and the oxidizing agents at 0°C for 10 min. In most experiments, the phosphorylation reaction components were added directly to the treated membranes. However, in some experiments, the oxidized membranes were washed once in 20 vols. of 20 mM Tris · HCl, pH 7.5, and then phosphorylated. In the experiments where phosphorylation preceded thiol-oxidation, the phosphorylated membranes were washed once with 20 volumes of 150 mM KCl, 10 mM Tris · HCl, pH 7.5, and 1 mM EDTA (KCl-stopping solution) and once with 20 vols. of 20 mM Tris · HCl, pH 7.5, prior to the addition of the thiol-oxidizing agents.

Materials. [γ-^{32}P]ATP and [γ-^{32}P]GTP were purchased from New England Nuclear or Amersham/Searle; ATP and GTP from P-L Biochemicals; cyclic AMP, diamide, and 1,10-*o*-phenanthroline from Sigma Chemical Co; casein from Mann Research. All supplies for gel electrophoresis were obtained from Bio-Rad. The X-ray film was Kodak No-Screen NS-2T. Radioautograms were scanned in a Zeineh Soft-Laser densitometer.

Results

Table I shows the effect of diamide on the autophosphorylation of rabbit erythrocyte membranes and on the phosphorylation of casein by the solubilized cyclic AMP-independent rabbit erythrocyte membrane protein kinases, I ($M_r \approx 100\,000$) and II ($M_r \sim 30\,000$) [10]. Although diamide produced a significant inhibition of membrane autophosphorylation, it did not inhibit the activity of the solubilized membrane protein kinases towards casein. The results suggest that inhibition of membrane phosphorylation does not occur as a result of the specific inhibition of the membrane protein kinases. That the inhibition of membrane phosphorylation was more dramatic in the presence of GTP than with ATP cannot be explained adequately at this time.

Fig. 1 shows the effects of varying concentrations of diamide on autophosphorylation in rabbit and human erythrocyte membranes. In both species, the concentration of diamide necessary for near maximal inhibition in the presence of ATP or GTP is 5 mM or less. Again, the effects of diamide on autophosphorylation in the presence of GTP occurred to a greater extent than in the presence of ATP. In the human erythrocyte membranes, which contain a cyclic AMP-dependent protein kinase (the rabbit does not), diamide promoted the same degree of inhibition of phosphorylation in the presence or absence of cyclic AMP.

Fig. 2 shows the radioautograms depicting the effect of diamide on the phosphorylation of membrane polypeptides of human erythrocytes in the presence of ATP or ATP plus cyclic AMP. The results indicate that the inhibition of cyclic AMP-dependent and -independent phosphorylation in the human erythrocyte membranes is nonspecific. That is, there is decreased labelling in all the phosphoprotein species, and the decreases appear to be quantitatively similar.

Fig. 3 consists of densitometric tracings of a radioautogram depicting the effect of diamide on rabbit erythrocyte membrane autophosphorylation in the presence of ATP. The results differ from those presented for the human ery-

TABLE I

EFFECT OF 30 mM DIAMIDE ON MEMBRANE-BOUND AND SOLUBILIZED RABBIT ERYTHROPROTEIN KINASES

The phosphorylation reaction was conducted at pH 6.0 with [γ-^{32}P]GTP. The specific activity of the radioisotopes was about 24 cpm/pmol. The amounts of ^{32}P incorporated into the membrane proteins or casein were measured by the trichloroacetic acid precipitation method as described in the text.

Incubation	Treatment	nmoles ^{32}P incorporated per 10 min per mg protein		% of control	
		ATP	GTP	ATP	GTP
Membranes	none	8.5	4.6		
	diamide	4.9	0.1	57	3
Kinase I + casein	none	18.8	7.7		
	diamide	14.9	6.9	79	90
Kinase II + casein	none	111			
	diamide	85		77	

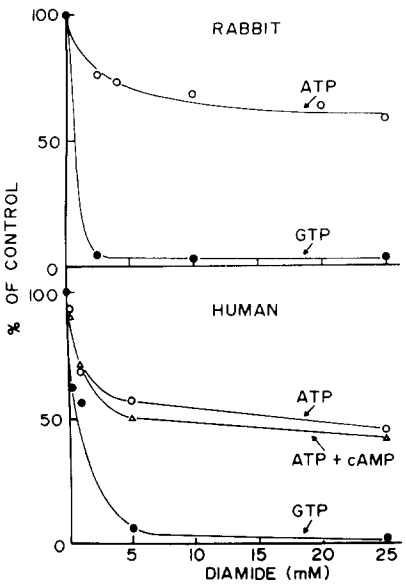

Fig. 1. Effects of varying concentrations of diamide on the autophosphorylation of human and rabbit erythrocyte membranes. Rabbit erythrocyte membranes were autophosphorylated for 10 min at pH 6.0 with [γ-^{32}P]ATP (20 cpm/pmol) or at pH 8.5 with [γ-^{32}P]GTP (20 cpm/pmol). Human erythrocyte membranes were autophosphorylated at pH 7.5 with [γ-^{32}P]ATP (50 cpm/pmol) ± 2 μM cyclic AMP for 10 min or with [γ-^{32}P]GTP (50 cpm/pmol) for 30 min.

throcyte membranes (Fig. 2) in that not all the phosphorylation reactions occurring in the rabbit erythrocyte membranes appear to be inhibited by diamide. As seen in Fig. 3, the phosphorylation of bands 2—2.1 and of a minor protein migrating between 2—2.1 and 2.9—3 is not decreased by treatment with 1—25 mM of diamide. Furthermore, those proteins whose phosphorylation is inhibited by diamide do not appear to be equally sensitive to diamide. The phosphorylation of bands 2.9—3 is inhibited to the same degree at 1 and 25 mM diamide, whereas that of 4.1 is greater at 25 mM than at 1 mM diamide.

Fig. 4 illustrates the effect of diamide on rabbit erythrocyte membrane phosphorylation in the presence of GTP. In this case, the inhibition is nonselective and phosphorylation is decreased overall. The profiles of human erythrocyte membranes autophosphorylated in the presence of GTP and diamide are identical to those illustrated in Fig. 4 and thus are not shown. That spectrin band 2 phosphorylation in the rabbit erythrocyte membranes is inhibited by diamide in the presence of GTP but not ATP is puzzling and suggests an inherent difference in these two reactions.

Inhibition of erythrocyte membrane phosphorylation by diamide appeared to occur nonspecifically and did not seem to be due to a direct inhibition of the membrane kinases (Table I). These observations, together with the knowledge that thiol-oxidizing agents are capable of cross-linking membrane proteins [11,12] suggest that the inhibition of phosphorylation may be a secondary effect resulting from a change in membrane structure. This led us to examine the effects of varying concentrations of diamide on the protein profiles of rabbit and human erythrocyte membranes as visualized in Coomassie Blue

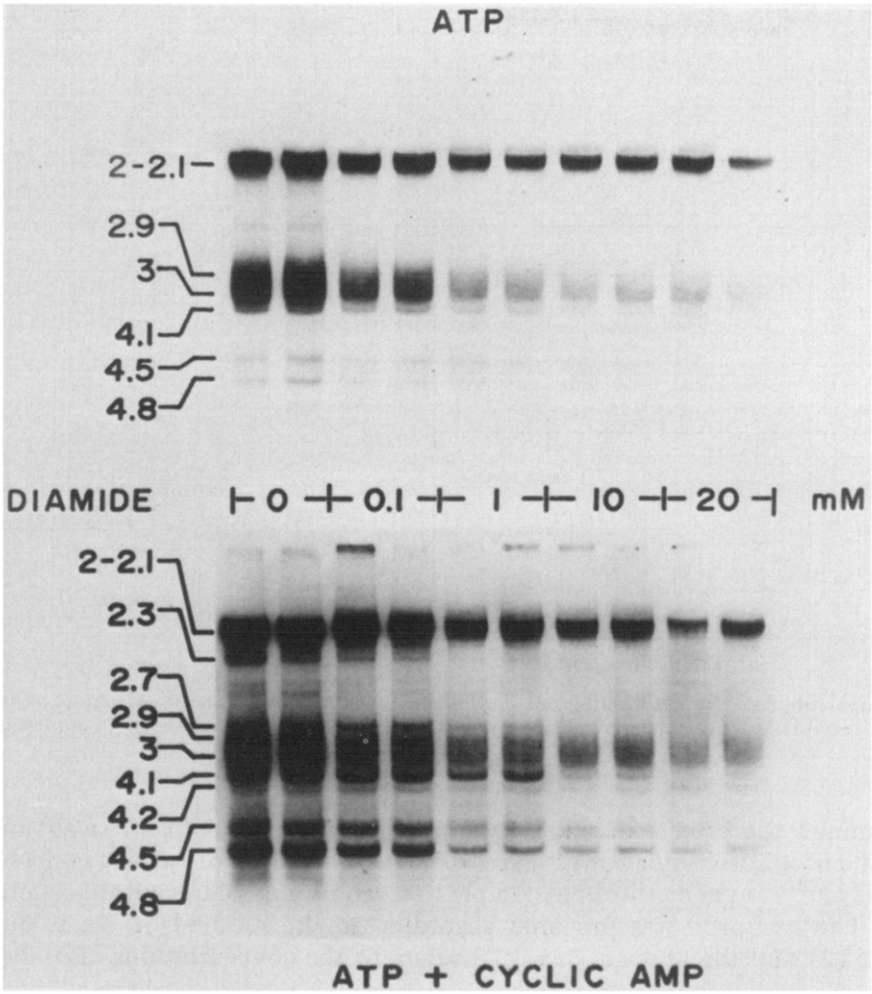

Fig. 2. Radioautogram depicting the inhibition of autophosphorylation in human erythrocyte membranes by varying concentrations of diamide. Membranes were phosphorylated at pH 7.5 for 10 min in the presence of [γ-^{32}P]ATP (300 cpm/pmol) without (top) or with (bottom) 2 μM cyclic AMP. The phosphorylated membranes were dissolved in a SDS diluent containing dithiothreitol [8] and electrophoresed in a slab gel containing 4.5% acrylamide. The radioautogram was exposed for 3 days.

stained SDS polyacrylamide gels which had been electrophoresed in the absence of dithiothreitol. The results are shown in Fig. 5. Cross-linking of membrane proteins is apparent at the lowest concentrations of diamide studied. With higher concentrations, large aggregates can be found at the origins of the gel. These aggregates do not enter 3.2% gels. Data from preliminary experiments utilizing two-dimensional gel electrophoresis in which the disulfide groups were reduced in the second dimension [12] suggests that the aggregated protein which remains at the origin may be composed of most, if not all, of the membrane proteins (data not shown). Such drastic perturbations of membrane structure might explain the inhibition of membrane autophosphorylation.

In order to substantiate the hypothesis concerning the action of diamide, we

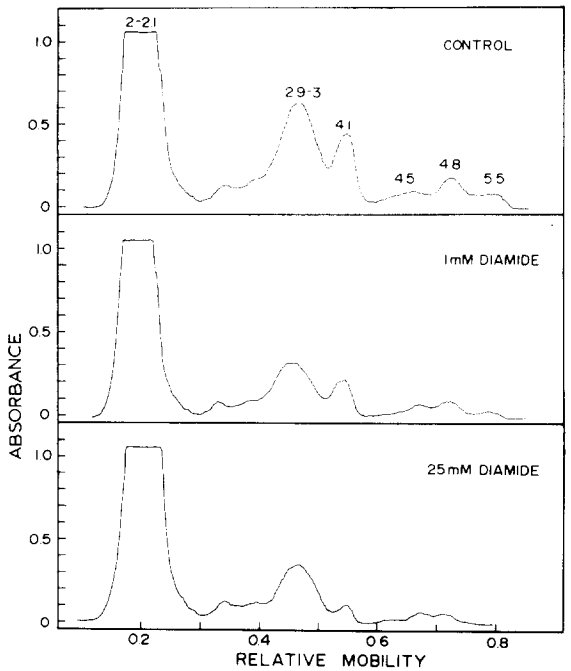

Fig. 3. Densitometric tracings of radioautograms showing the effect of diamide on the autophosphorylation of rabbit erythrocyte membranes in the presence of ATP. Phosphorylation was carried out at pH 6.0. Other conditions were as in Fig. 2.

have examined the effects of another thiol-oxidizing cross-linking reagent on membrane autophosphorylation. Figure 6 shows the effects of varying concentrations of Cu^{2+} · *o*-phenanthroline complex on rabbit membrane autophosphorylation. The complex was prepared according to the method of Wang and Richards [12] and the abscissa in Fig. 6 refers to the concentrations of *o*-phe-

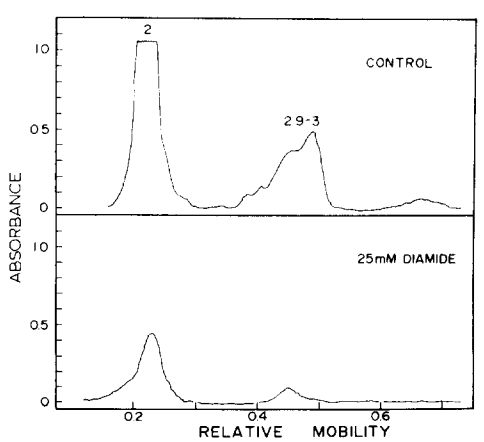

Fig. 4. Densitometric tracings of radioautograms depicting the effect of diamide on the autophosphorylation of rabbit erythrocyte membranes in the presence of GTP. Membranes were phosphorylated at pH 8.5 for 30 min in the presence of [γ-^{32}P]GTP (147 cpm/pmol). The radioautogram was exposed for 5 days.

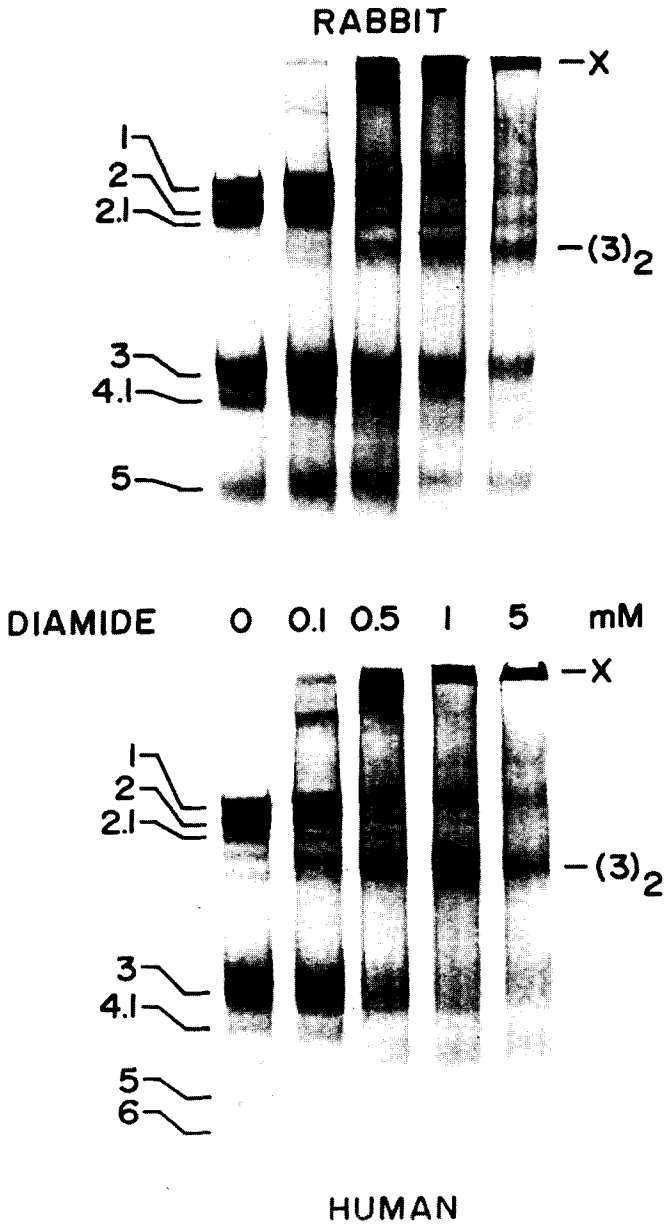

Fig. 5. Cross-linking of erythrocyte membrane proteins by diamide. The electrophoresis was carried out in the absence of dithiothreitol. Other experimental conditions were as described in the text.

nanthroline. The effects are strikingly similar to those illustrated for diamide in Fig. 1.

Fig. 7 compares radioautograms depicting the autophosphorylation of human erythrocyte membrane proteins before and after cross-linking with 140 μM/70 μM of Cu^{2+} · *o*-phenanthroline or 20 mM of diamide. The radioautograms were prepared from 4% gels electrophoresed in the absence of dithio-

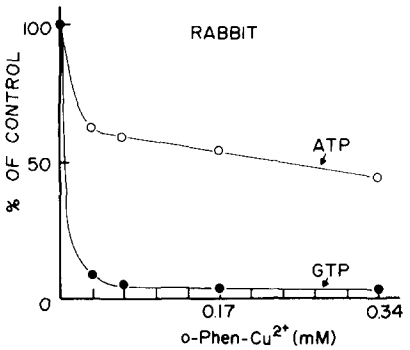

Fig. 6. Effect of Cu^{2+} · *o*-phenanthroline on autophosphorylation in rabbit erythrocyte membranes. Conditions were as described in Fig. 1.

threitol in order to the preserve the cross-links. The phosphorylation reactions were performed in the presence of ATP (A), ATP plus cyclic AMP (B), or GTP (C). The control samples (panel 1) represent the normal patterns of autophosphorylation. Fig. 7 shows that the phosphoproteins can be cross-linked by diamide (panel 3) or Cu^{2+} · *o*-phenanthroline (panel 2) into high molecular weight complexes. The cross-linking patterns of the two thiol-oxidizing reagents are qualitatively similar. The most prominent cross-linked products are the high molecular weight aggregates (X) which remain at the origin and the dimer of band 3, designated as $(3)_2$. Based on preliminary two-dimensional gel electrophoresis studies (data not shown) we have tentatively identified other

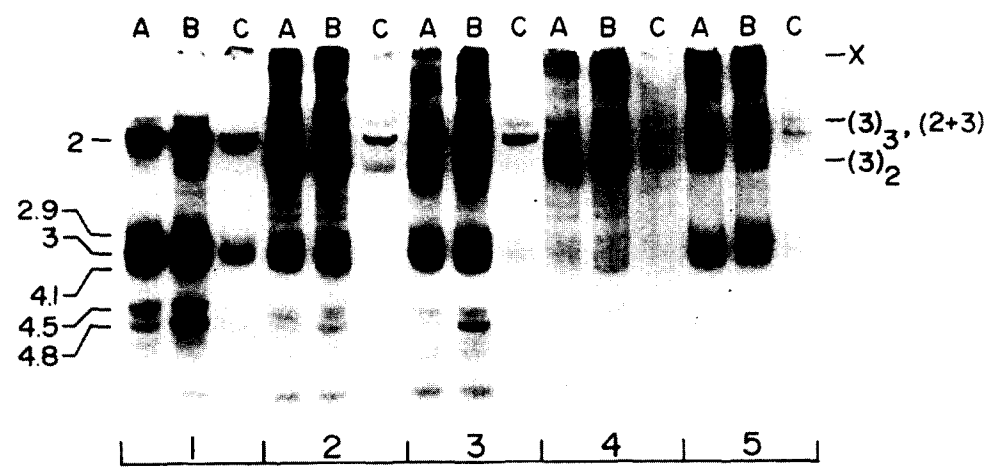

Fig. 7. Radioautograms depicting the autophosphorylation of human erythrocyte membranes before and after treatment with Cu^{2+} · *o*-phenanthroline or diamide. Membranes were phosphorylated at pH 8.5 for 10 min with [γ-^{32}P]ATP (100 cpm/pmol) without (A) or with (B) cyclic AMP or with [γ-^{32}P]GTP (115 cpm/pmol) (C). Panel 1, control. Panel 2, membranes were phosphorylated and then cross-linked with Cu^{2+} · *o*-phenanthroline. Panel 3, membranes were phosphorylated and then cross-linked with diamide. Panel 4, membranes were cross-linked with Cu^{2+} · *o*-phenanthroline and then phosphorylated. Panel 5, membranes were cross-linked with diamide and then phosphorylated. Details are in the text. The membranes were electrophoresed in 4% gels in the absence of dithiothreitol. The radioautograms were exposed for 6 days.

cross-linked products as the trimer of band 3, designated as $(3)_3$, and the complex of bands 2 and 3, (2 + 3). These observations need to be further corroborated. The fate of the cyclic AMP-dependent protein kinase substrates, bands 4.5 and 4.8, is not apparent. However, studies conducted using the two-dimensional gel electrophoresis procedure suggest that band 4.8 may be cross-linked into a complex which migrates slightly slower that spectrin (data not shown).

The phosphorylation patterns after cross-linking (with diamide in panel 5 and $Cu^{2+} \cdot o$-phenanthroline in panel 4) are quite different from those obtained before cross-linking. The major phosphoproteins observed are bands 2 and 3 and their tentatively identified cross-linked products and also the high molecular weight cross-links (X) which remain at the origin of the gels. The data suggest that some phosphorylation can occur in these components in spite of their cross-linking.

Discussion

The results presented in this communication show that low concentrations of diamide inhibit cyclic AMP-dependent and -independent membrane autophosphorylation. In contrast to the observations of Von Tersch et al. [6] and McClung and Miller [19] that diamide directly inhibits cyclic AMP-dependent protein kinases, we have found that diamide does not inhibit directly the activities of two cyclic AMP-independent protein kinases which have been solubilized from erythrocyte membranes. Our studies suggest that the inhibition of membrane autophosphorylation results from a perturbation of membrane structure. This conclusion is supported by several lines of evidence. First, high concentrations of diamide do not inhibit the solubilized membrane cyclic AMP-independent protein kinases. Second, the inhibitory effect of diamide is mimicked by another thiol-oxidizing agent, the $Cu^{2+} \cdot o$-phenanthroline complex. Third, low concentrations of both diamide and the $Cu^{2+} \cdot o$-phenanthroline complex produce cross-links of membrane proteins. Finally, the phosphorylation of the different membrane polypeptides is not inhibited to the same extent by diamide or the $Cu^{2+} \cdot o$-phenanthroline. This latter observation can be attributed, in part, to differences in susceptibility of the membrane proteins to oxidation, and to whether thiol-oxidation or cross-linking causes a drastic structural alteration in certain membrane proteins such that they can no longer serve as phosphoryl acceptors. It is interesting to note, however, that the cross-linked products of some membrane proteins can still serve as substrates for phosphorylation.

Based on our observations, we hope that other investigators will recognize that diamide is a very reactive compound capable of perturbing protein structures. Perhaps these observations should be kept in mind in evaluating new and previous studies of the effect of diamide on transport processes [2,5,7], neurotransmitter release [17,18] and cyclic AMP-dependent protein kinase activities [6,7,19].

Acknowledgements

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