THE ROLE OF CYCLIC AMP IN THE PHOSPHORYLATION OF PROTEINS IN HUMAN ERYTHROCYTE MEMBRANES *

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SUMMARY: Three protein components of human erythrocyte membranes served as substrates for membrane-bound protein kinase. Cyclic AMP stimulated the rates of phosphorylation of two polypeptides, but the phosphorylation of the principal phosphate-acceptor was not enhanced by the cyclic nucleotide. These observations demonstrate the presence of cyclic AMP-dependent protein kinase and its homologous substrates in erythrocyte membranes and suggest that cyclic AMP could mediate alterations in the properties of membranes.

A considerable body of evidence has been accumulated (1-8) in support of the hypothesis (2) that cytoplasmic protein kinases are the molecular receptors of cyclic AMP and mediators of cellular responses to catecholamines and polypeptide hormones. Cyclic AMP may also participate in the regulation of such membrane functions as aggregation and differentiation in the slime mold (9), growth and contact inhibition in mammalian cells (10,11), secretory processes (12), cellular permeability (13) and synaptic transmission (14,15). It is possible that some of these phenomena are controlled by cyclic AMP-dependent protein kinases which are components of the cell membrane since membrane-associated protein kinases and endogenous protein substrates have been observed in synaptic plasma membranes, synaptic vesicles (15-17), and adenohypophyseal granules (12).

Recently, we described and characterized a membrane-associated protein kinase derived from human erythrocytes which catalyzes the phosphorylation of exogenous and endogenous proteins (18). The erythrocyte membrane provides an

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excellent model system for studying the relationship between protein kinase activity and possible alterations in membrane structure and function because purified plasma membranes may be obtained in large quantities (19) and some of the structural and enzymic components of these membranes have been characterized (20-22). Since modifications in membrane properties effected by protein kinase will depend on the nature of the endogenous substrates, we have attempted to characterize these substrates and determine if the rate of phosphorylation of any of these proteins is controlled by cyclic AMP.

Materials and Methods

$[\gamma\text{-}^{32}P]$ ATP (15 Ci/mmole) was purchased from Amersham-Searle and its purity determined by ion-exchange chromatography (23). Erythrocyte membranes were prepared according to the method of Dodge et al. (19).

Erythrocyte membranes were solubilized, subjected to electrophoresis in 1% sodium dodecyl sulfate (SDS) - 5.6% acrylamide gels. Gels were stained for protein and carbohydrate by the methods of Fairbanks et al. (21). The radioactivity incorporated into membrane proteins was determined by slicing the gels into 1 mm segments, placing the segments in vials with 5 ml H_2O and determining the Cerenkov radiation in a liquid scintillation spectrometer (24). Counting efficiency was 33%. All data obtained from electrophoresis experiments were corrected for recovery (approximately 90%) and the decay of ^{32}P. Radioactivity in the various protein bands was determined in unstained gels and in parallel gels which had been fixed, stained with Coomassie blue and destained according to the procedure of Fairbanks et al. (21). The latter treatment did not lead to a significant loss (<6%) of incorporated ^{32}P. Quantitative densitometry of the stained gels was carried out according to Fairbanks et al. (21).

Results

Figure 1 depicts the time course of phosphorylation of endogenous substrates by membrane-associated protein kinase. The amount of phosphate incorporated into membrane proteins was not a linear function of time and a

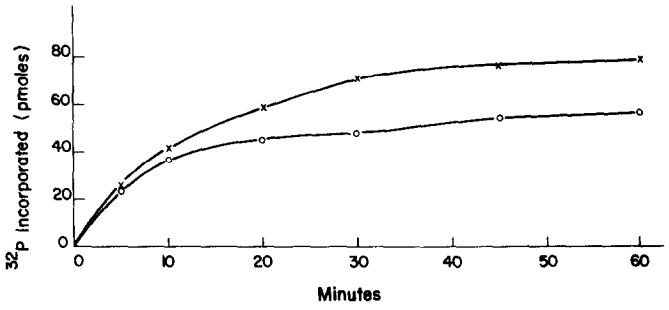

Figure 1. Phosphorylation of proteins in human erythrocyte membranes. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 15 mM $MgSO_4$, 6 mM dithiothreitol, 66 μM $[\gamma\text{-}^{32}P]$ ATP (41 CPM/pmole), 3μM cyclic AMP (when included), and 840 μg membrane protein in a final volume of 1.6 ml. Incubations were carried out at 35°. Aliquots (200 μl) were withdrawn at the indicated times and the incorporation of ^{32}P into membrane proteins was assayed as previously reported (18). A correction for incorporation of ^{32}P into phospholipids and proteolipids was made by subtracting the amount of radioactivity which was extracted into chloroform-methanol (2:1) in duplicate samples (approximately 30%). x–x–x, cyclic AMP present, o–o–o, cyclic AMP absent.

maximal level of phosphorylation was reached in 45 min. Protein kinase activity was slightly, but consistently, stimulated about 35% by 3μM cyclic AMP. This degree of stimulation suggested that the rate of phosphorylation of only one or a few of the possible substrates of protein kinase was subject to control by cyclic AMP. For this reason, erythrocyte membranes were phosphorylated with $[\gamma\text{-}^{32}P]$ ATP in the presence or absence of cyclic AMP and then dissociated into their component protein chains by treating them with 3% SDS - 2% mercaptoethanol. The individual polypeptides were then resolved by electrophoresis (Fig. 2A). The pattern of protein bands closely resembled that obtained by Fairbanks et al. (21) and identical patterns were observed for untreated membranes and membranes incubated with ATP in the presence or absence of cyclic AMP (Fig. 2a). Using the protein band and molecular weight (MW) assignments of Fairbanks et al. (21), proteins II (MW = 215,000), III (MW = 88,000)[1] and IVc (MW = 50,000) were found to be substrates for membrane-

1 A major erythrocyte glycoprotein migrates with a mobility which is indistinguishable from that of protein III under the standard conditions of electrophoresis (See Methods). Although preliminary experiments suggest that the incorporated ^{32}P migrates with the glycoprotein on 8% gels, we have temporarily assigned this radioactivity to protein III pending more conclusive studies.

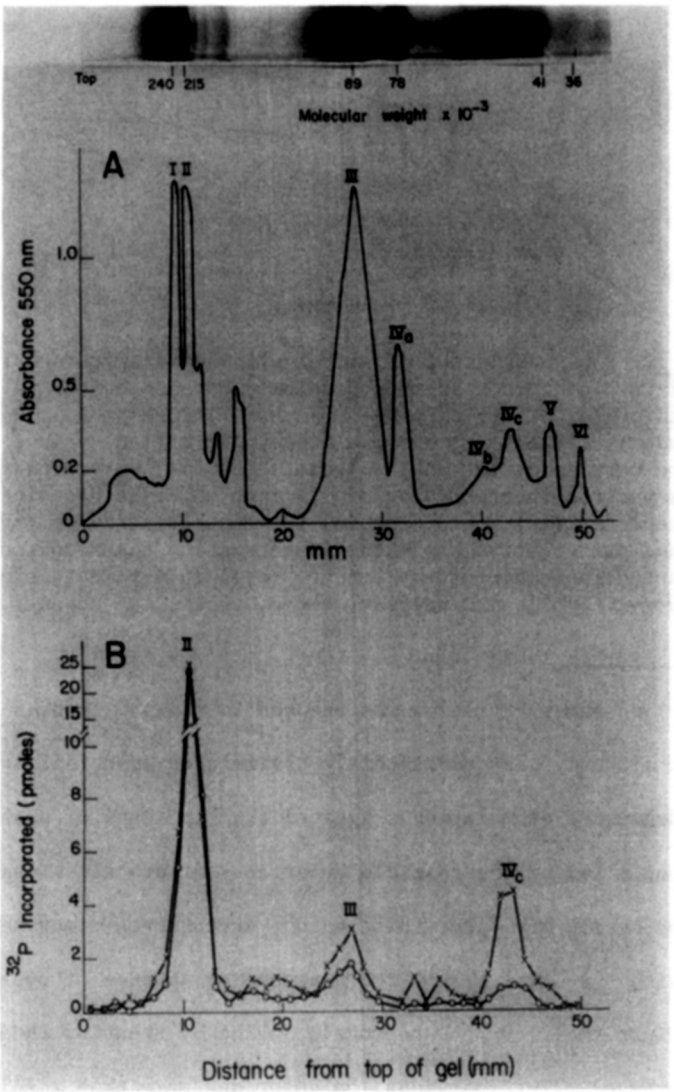

Figure 2. Resolution of the endogenous substrates of membrane-associated protein kinase. Fig. 2A shows the electrophoretic pattern given by membranes following SDS-acrylamide electrophoresis and staining with Coomassie blue (see Methods). The numbering system corresponds to that of Fairbanks et al. (21) except subscripts have been introduced to indicate bands IVb and IVc. An optical density scan of a stained gel which was loaded with 35μg of membrane protein is also shown.

Fig. 2B shows the distribution of ^{32}P in the polypeptide chains derived from aliquots containing 80 μg of membrane protein which had been incubated in the presence of cyclic AMP. Incubations were carried out as described in the legend to Fig. 1 except that the specific activity of [γ -^{32}P] ATP was (338 CPM/pmole). o—o—o cyclic AMP absent, x—x—x cyclic AMP present.

associated protein kinase (Fig. 2B) with protein II accounting for approximately 60% of the phosphate incorporated in the presence of cyclic AMP.

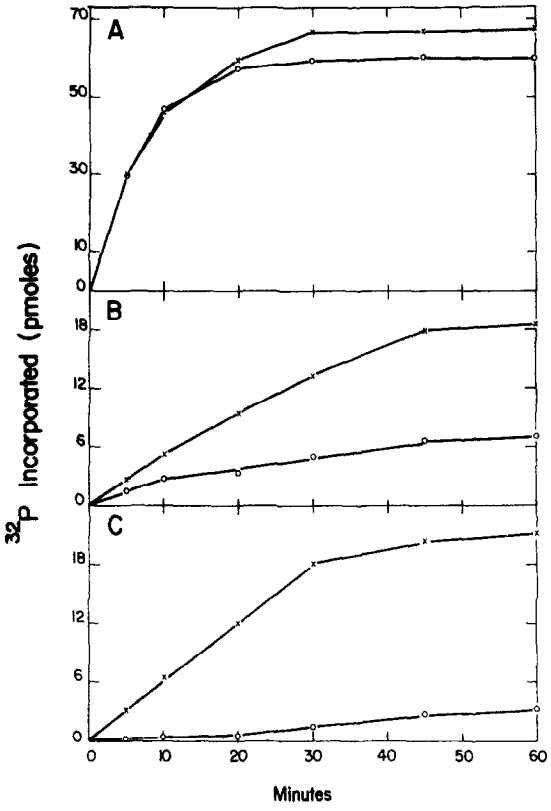

Figure 3. Kinetics of ^{32}P incorporation into proteins II, III and IVc. Erythrocyte membranes were incubated as described in Fig. 1 except that the specific activity of [γ-^{32}P] ATP was 319 CPM/pmole. Aliquots containing 110 µg protein were removed at the indicated times and the reaction terminated by the addition of 3% SDS. The samples were subjected to electrophoresis and the radioactivity in the substrates determined as described in Methods. The data for proteins II, III and IVc are plotted separately in 3A, 3B and 3C, respectively. o—o—o cyclic AMP absent, x—x—x cyclic AMP present.

The kinetics of phosphate incorporation were then ascertained for each substrate protein in the presence and absence of cyclic AMP (Fig. 3). Protein II was rapidly phosphorylated but no rate enhancement was observed in the presence of cyclic AMP. Proteins IVc and III were phosphorylated at constant rates for 30 min. and the addition of cyclic AMP caused a 5-fold stimulation of the velocity of phosphorylation of IVc and a 2-fold increase in III (Fig. 3).

An estimate of the stoichiometry of phosphorylation (Table 1) showed that large fractions of the polypeptide chains comprising proteins II and IVc

were available as substrates for membrane-associated protein kinase (assuming 1 site for phosphorylation per polypeptide chain), while only a minor portion of protein III was phosphorylated.

When the phosphorylated erythrocyte membranes were treated with hot trichloroacetic acid or hydroxylamine prior to electrophoresis no loss of incorporated ^{32}P was observed. All the incorporated radioactivity was released when the membranes were treated with 1N NaOH at 95° for 5 min. These observations indicated that the phosphorylated proteins contained phosphoester bonds rather than acyl phosphate moieties (25,26) and this was confirmed by the finding that 70% of the total ^{32}P incorporated into the membranes could be identified as phosphoserine (59%) and phosphothreonine (11%) after partial acid hydrolysis (27).

Discussion

Three components of erythrocyte membranes were substrates for the membrane-associated protein kinase. Protein II was phosphorylated most extensively (Table 1), but its rate of phosphorylation was not affected by cyclic AMP (Fig. 3A). This protein accounts for approximately 10-15% of the total erythrocyte membrane protein (21) and is probably a component of spectrin (28) and analagous to tektin α found in bovine erythrocyte membranes (29). Its function is not known.

Protein III is also a substrate for membrane-associated protein kinase and its rate of phosphorylation is stimulated 2-fold by cyclic AMP (Fig. 3B). It is difficult to evaluate the significance of the cyclic AMP-dependent phosphorylation of protein III because of the ambiguity resulting from the co-migration of two or more components during electrophoresis (See footnote 1 and (21)). Recently, Avruch and Fairbanks (24) tentatively identified a component of band III as an acyl phosphate intermediate of Na^+,K^+ - ATPase. It is unlikely that the phosphate incorporation we have observed in protein III is the consequence of acyl phosphate formation since the incorporated ^{32}P is stable to treatment with hydroxylamine (26) and to electrophoresis at 25° at pH 7.4 (24).

Table 1

Stoichiometry of Phosphorylation

Protein	pmole ^{32}P incorporated	moles phosphate/mole protein
II	46.1	0.79
III	7.9	0.024[2]
IVc	9.5	0.17

Erythrocyte membranes (93μg) were incubated for 60 min. under the conditions described in Fig. 1. The reaction mixture contained cyclic AMP and [γ-^{32}P] ATP (229 CPM/pmole). After termination of the reaction the protein components of the membranes were resolved by electrophoresis, stained and assayed for radioactivity. The relative amounts of protein in the bands were estimated by densitometry (21). Each value is the average of four determinations.

2 This value is significantly underestimated if the glycoprotein component migrating with protein III is the actual substrate (See Footnote 1).

Protein IVc is a relatively minor component (2-4%) of the membrane protein and is phosphorylated *in vitro* to the extent of 0.17 moles P/mole protein (Table 1). The rate of phosphorylation of this polypeptide is greatly enhanced by the presence of cyclic AMP (Fig. 3C) and 5-12-fold stimulations have been observed. No information is currently available on its structure or function.

The role of membrane-associated cyclic AMP-dependent protein kinase in mammalian erythrocytes is unknown. Our membrane preparations contained adenylate cyclase and experiments using intact cells have demonstrated cyclic AMP synthesis and intracellular concentrations of cyclic AMP sufficient to activate protein kinase.[3]

When intact membranes were studied, the cyclic AMP-stimulated phosphorylation of proteins III and IVc was obscured by the high level of cyclic AMP-independent phosphorylation of component II (Fig. 1). The low levels of cyclic AMP-stimulation reported in the endogenous phosphorylation of synaptic plasma membranes (16,17) and adenohypophyseal granules (12) may be the result

3 Rubin, C. S., and Rosen, O. M., unpublished observation.

of a similar phenomenon. In fact, Johnson et al. (30) have recently reported the qualitative observation, that one minor component of synaptic membranes was subject to cyclic AMP-stimulated phosphorylation. The observations in erythrocyte and synaptic membranes show that simultaneous cyclic AMP-dependent and independent phosphorylations of endogenous substrates occur in membranes. This necessitates the resolution and characterization of the phosphorylated polypeptide chains as a first step towards understanding the roles of cyclic AMP and protein kinase in membrane function.

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