sults have to be compared with the properties of pure lipid–protein complexes.

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Phosphorylation of Endogenous Substrates by Erythrocyte Membrane Protein Kinases. I. A Monovalent Cation-Stimulated Reaction†

Joseph Avruch* and Grant Fairbanks

ABSTRACT: Protein kinases in the isolated human erythrocyte membrane catalyze the transfer of ^{32}P from [γ-^{32}P]ATP to endogenous substrates, forming stable phosphopeptides. One of these reactions—phosphorylation of the major 215,000-dalton polypeptide—has several distinguishing features. The reaction is manifested at Mg^{2+} concentrations above 10^{-4} M and is relatively unresponsive to 20 μM cyclic AMP. It is stimulated by Ca^{2+} at 1 mM and by Na^+, K^+, Li^+, and NH_4^+ at 0.05 and 0.2 M. No turnover of this peptide-bound ^{32}P can be detected in the isolated membrane. The 215,000-dalton polypeptide is the only substrate utilized in this reaction. All other endogenous phosphorylations, particularly those strongly stimulated by cyclic AMP, are inhibited by Ca^{2+} and/or by monovalent cations. The specific substrate for the reaction is one of the major components of the elutable polypeptide mixture, "spectrin," and is released from the membrane at low ionic strength. However, the corresponding kinase is not eluted with its substrate: retention of the enzyme in membrane vesicles is demonstrable in reconstitution experiments. These properties are contrasted with those of the cyclic AMP-stimulated kinase activity, which is partially eluted at low ionic strength, while its principal substrates remain tightly bound to vesicles. The phosphorylation of an exogenous substrate, casein, by vesicles lacking "spectrin" is also stimulated by monovalent ions and thus resembles the endogenous reaction. Modes of ionic regulation and possible functional roles of the phosphorylation are discussed.

Erythrocyte membranes require ATP in the performance of a variety of functions. In the active transport of cations, ATP serves directly as a substrate and there is a fixed stoichiometry between ATP hydrolysis and the number of cations translocated. This type of function is well reflected in a specific component of Mg^{2+}-dependent ATP hydrolysis, the Na^+,K^+-stimulated ATPase (Skou, 1965). However, in ion transport, as well as other processes, ATP may participate through other mechanisms. In acting as an allosteric ligand or chelating agent, for example, ATP would not undergo hydrolysis. Likewise, its participation as a phosphate donor in transphosphorylation reactions of glycolysis and in regulatory phosphorylation of proteins need not be well reflected in "ATPase" activities as manifested by release of inorganic phosphate. Under conditions in which phosphorylation is not closely coupled to phosphate release from acceptor molecules, other methods must be used to study the activity and its regulation.

We reported previously that, in association with the Mg^{2+}-dependent, Na^+,K^+-stimulated ATPase reaction of the erythrocyte membrane, a lone membrane polypeptide of

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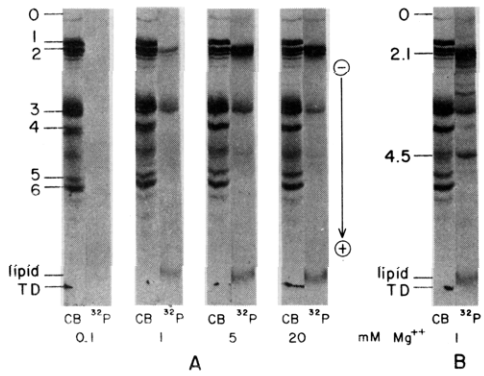

FIGURE 1: Effects of Mg^{2+} and cyclic AMP on phosphorylation of endogenous substrates. Membranes phosphorylated in 10-sec incubations were fractionated by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate at pH 2.4. Dried longitudinal slices from stained gels were radioautographed. For each incubation condition, the Coomassie Blue staining pattern (CB) in a dried slice is pictured alongside a reversed image of the radioactivity distribution (^{32}P) in the corresponding radioautograph. Only the stable phosphopeptides and phospholipid are detected by this technique: (A) phosphorylation in the presence of 0.1, 1, 5, or 20 mM $MgCl_2$, as indicated, without added cyclic AMP; (B) phosphorylation in 1 mM Mg^{2+} with 20 μM cyclic AMP.

molecular weight 105,000 is phosphorylated by ATP (Avruch and Fairbanks, 1972). A portion of the phosphate in this component exhibits properties expected of an intermediate in the transport ATPase reaction, including a very rapid turnover. At low Mg^{2+} concentrations (12 μM), this is the only polypeptide phosphorylation detectable. However, at higher Mg^{2+} concentrations (above 10^{-4} M), we observed, as reported recently by others (Rubin *et al.*, 1972; Guthrow *et al.*, 1972; Roses and Appel, 1973; Rubin and Rosen, 1973), that a separate class of membrane polypeptides can be phosphorylated. These reactions are apparently catalyzed by endogenous membrane-bound protein kinases. We have defined two such protein kinase activities differing in substrate specificity, activation by cyclic AMP, and response to mono- and divalent cations. The properties of the phosphopeptides generated and the rates of the reactions indicate that they contribute minimally to overall "ATPase" activity, though they may nonetheless represent an important expression of ATP involvement in membrane function. In this report we describe the properties of a highly specific salt-stimulated reaction and discuss possible functional roles of the phosphorylation.

Methods

Erythrocyte membranes were prepared and phosphorylated by [γ-^{32}P]ATP (New England Nuclear NEG-002X) as previously described (Avruch and Fairbanks, 1972), with some exceptions as noted. The incubation time was normally 10 sec.

When intact membranes were analyzed for phosphate incorporation without subfractionation, incubations were terminated by sudden addition of cold 0.3 N perchloric acid containing 2.5 mM ATP and 5 mM P_i. In some experiments, phosphorylated membranes were extracted at low ionic strength (Fairbanks *et al.*, 1971). Labeling was terminated by a rapid 50-fold dilution into cold 10 mM Tris-Cl or 5 mM sodium phosphate at pH 8. The membranes were collected by centrifugation, washed twice in the same buffer at 0–4°, and resuspended in 9 vol of warm 1 mM Tris-Cl (pH 8)–1 mM dithiothreitol. After incubation at 37° for 30 min, the suspension was sheared by several passages through a 27 gauge hypodermic needle and subjected to ultracentrifugation to sediment vesicles (2.4×10^6 g_{av}-min). The supernatant fraction was drawn off and the pellet resuspended in Tris-Cl–dithiothreitol. The preparation of vesicles and low ionic strength eluates prior to incubation followed the same procedure; in these experiments the eluates were concentrated approximately tenfold by ultrafiltration (Amicon Diaflo PM-10 membranes).

Electrophoretic fractionation of membrane polypeptides at pH 2.4 in polyacrylamide gels (5.6% monomer) containing 1% sodium dodecyl sulfate was carried out as described previously (Avruch and Fairbanks, 1972). Gel fixation, staining and destaining, densitometry, and molecular weight estimation were carried out as described by Fairbanks *et al.* (1971). Steck's (1972) molecular weight estimates for the two large polypeptides of "spectrin" were assumed as the basis for extending the molecular weight calibration above 130,000 daltons.

Patterns of radioactivity in gels were analyzed by transection and counting in water (Avruch and Fairbanks, 1972) or by radioautography of dried longitudinal slices (Fairbanks *et al.*, 1965).

Protein was assayed according to Lowry *et al.* (1951), with bovine serum albumin as a standard, or by intrinsic fluorescence (Resch *et al.*, 1971).

Results

Substrate specificities of the endogenous membrane phosphorylation reactions can be analyzed conveniently by fractionating the polypeptides electrophoretically in polyacrylamide gels containing sodium dodecyl sulfate. The character of the labeling pattern revealed in this way depends significantly on conditions of sample preparation, electrophoresis, and gel processing for counting. When a phosphorylation reaction is quenched with acid and the precipitated membranes are washed and fractionated electrophoretically without exposing them to elevated temperature or pH, the resulting gel contains multiple distinguishable components. These include unstable phosphopeptide intermediates in cation transport, stable phosphopeptides, phospholipid, and traces of ATP and inorganic phosphate (Avruch and Fairbanks, 1972). The distribution of ^{32}P determined by counting transverse slices from an unstained gel frozen immediately after electrophoresis represents the sum of the distributions of all these labeled constituents. When, however, a gel is sectioned and counted after fixation, staining, and destaining (Fairbanks *et al.*, 1971), only the simpler pattern of the stable phosphate esters—phosphopeptide and phospholipid—is seen. The prolonged soaking at room temperature in acetic acid–isopropyl alcohol solutions appears to leach from gels all ATP and inorganic phosphate and perhaps a portion of the membrane phospholipid. At the same time, the unstable acyl phosphate transport intermediates are discharged, probably by hydrolysis or phosphate transfer to the alcohol.

We have found that the method of transverse sectioning and scintillation counting of gels does not do justice to the inherent high resolution of the electrophoretic technique. Hence, in this study, we used also radioautography of dried longitudinal slices (Fairbanks *et al.*, 1965); this approach (which is probably restricted to analysis of the stable phosphopeptides) does fully reveal details of the ^{32}P distribution in electrophorograms. This is illustrated in Figure 1, which shows relationships between the labeling pattern and the Coomassie Blue staining profile and summarizes data to be discussed in detail below. We have labeled the major bands

TABLE I: Divalent Cation Requirement in Polypeptide 2 Phosphorylation.[a]

Expt	Additions	Incorporation 2(+2.1) (Counts/ 5 min)	Incorporation 3 (Counts/ 5 min)
1	0.1 mM Mg^{2+}	0	88
	1.0	1,006	1084
	5.0	6,891	1838
	20.0	11,459	2156
		Counts/ 4 min	
2	1.0 mM Mg^{2+}	1,029	
	Ca^{2+}	74	
	Co^{2+}	2,077	
	Mn^{2+}	1,356	
	Zn^{2+}	60	
	Fe^{2+}	0	

[a] Membranes were phosphorylated during 10-sec incubations with 2 μM [γ-^{32}P]ATP in 20 mM imidazole chloride (pH 7.4). The membranes were precipitated with perchloric acid, washed, and fractionated electrophoretically. Radioactivity in individual substrates was measured by counting transverse slices from frozen gels.

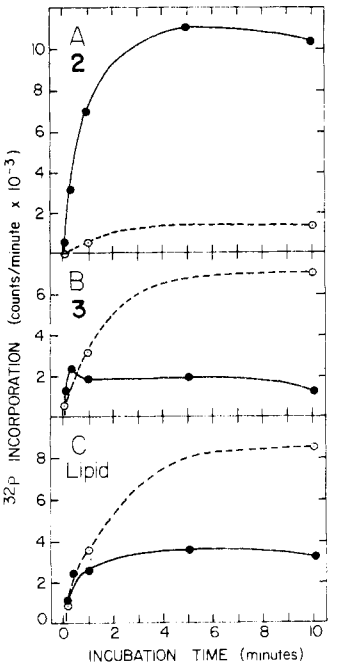

FIGURE 2: Kinetics of endogenous phosphorylation reactions. Isolated membranes were phosphorylated by incubation with [γ-^{32}P]ATP in 20 mM imidazole chloride (pH 7.4)–1 mM Mg^{2+}; sodium chloride was absent (O - - - O) or present at 50 mM (● - - - ●). Reactions were terminated at varying times. The acid-washed membrane precipitates were dissolved in sodium dodecyl sulfate and portions containing 50 μg of protein were fractionated electrophoretically: (A–C) incorporation into components 2, 3, and lipid, respectively, determined by liquid scintillation counting of transverse slices from gels frozen without fixation and staining.

in the staining profile using the decimal notation introduced by Steck (1972). The six major bands described previously (Fairbanks *et al.*, 1971; Fairbanks and Avruch, 1972) are assigned Arabic numerals 1 to 6 and designations for minor bands of interest are interpolated by adding decimal fractions.[1]

In the presence of 0.012 mM Mg^{2+}, minute amounts of radioactivity are transferred from [γ-^{32}P]ATP to phosphopeptides migrating in band 3. Both stable and unstable components can be localized in this zone, which corresponds to an apparent molecular weight of 105,000 (Avruch and Fairbanks, 1972). When the Mg^{2+} concentration is raised above 0.1 mM, the formation of stable phosphopeptides is greatly augmented: incorporation into band 3 is elevated, polypeptide 2 (mol wt 215,000) emerges as a second major phosphopeptide, lipid phosphorylation becomes significant, and slight labeling of several other membrane proteins can be detected (Figure 1A). The incorporation into the high molecular weight zone is increased more than sixfold as the Mg^{2+} concentration is raised from 1 to 5 mM, but less than twofold further as it is raised to 20 mM.[2] Phosphorylation of component 3 is less strongly stimulated by increases in the Mg^{2+} concentration in this range (Table I).

The kinetics of the major phosphorylation reactions at 1 mM Mg^{2+} are shown in Figure 2. In each case, the rate of incorporation declines by 1 min and ceases after several minutes of incubation. In the presence of 50 mM Na^+, incorporation into polypeptide 3 rises to a maximum in less than a few seconds and then falls off rapidly to a lower level. The transient component displays the behavior of the unstable intermediate in the $Na^+ + K^+$ transport ATPase reaction (Blostein, 1968; Avruch and Fairbanks, 1972). Its decline under these labeling conditions is a consequence of the rapid depletion of ATP, which is initially present at 2 μM. At higher ATP concentrations, incorporation into stable phosphopeptides can continue for at least 20 min (Rubin and Rosen, 1973). This indicates that the plateaus reached in reactions prolonged as in Figure 2 reflect exhaustion of the donor ATP, rather than saturation of the endogenous phosphate acceptors. Therefore, in our experiments, we have taken incorporation in a 5- or 10-sec incubation as a measure of the initial rate of a continuing reaction.

The effect of monovalent cations on these phosphorylations allows a sharp distinction to be drawn between the reaction in which component 2 is phosphorylated and reactions in which other polypeptides and lipids are utilized as phosphate acceptors. In the presence of 1 mM Mg^{2+}, the addition of Na^+, K^+, Li^+, or NH_4^+ at 50–200 mM markedly stimulates phosphorylation of 2 while depressing phosphorylation of all other components, among which component 3 and lipid are the most prominent (Figure 3).[3] Al-

[1] Except for the change from Roman to Arabic numerals, this system corresponds to that of Guthrow *et al.* (1972) and Rubin and Rosen (1973); our bands 1, 2, and 3 appear to correspond to bands I, II, and V, respectively, of Roses and Appel (1973).

[2] These measurements include radioactivity incorporated into component 2.1 (see below). The relative contribution of 2.1 declines with increasing concentrations of Mg^{2+} and monovalent ions, and is decreased by 1 mM Ca^{2+} (Fairbanks and Avruch, 1974). Hence, the effects of these ions in stimulating polypeptide 2 phosphorylation (relative to the basal incorporation in 1 mM Mg^{2+}) are underestimated in Table I.

[3] In the case of polypeptide 3, the difference in the extent of phosphorylation supported by Na^+ from the levels observed with K^+, NH_4^+, and Li^+ (Figure 3B) represents formation of the phosphorylated intermediate in the Na^+,K^+ transport ATPase reaction (Avruch and Fairbanks, 1972).

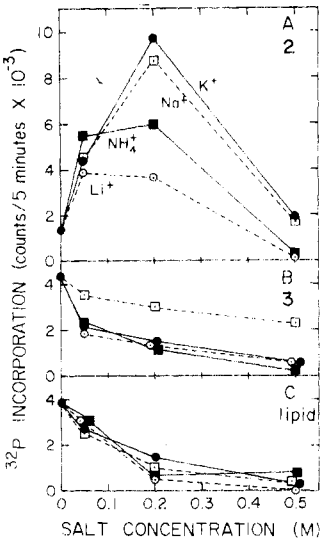

FIGURE 3: Responses of endogenous phosphorylation reactions to monovalent ions. Isolated membranes were phosphorylated during 10-sec incubations with [γ-^{32}P]ATP (89 pmol/10^6 cpm). The incubation conditions were as described under Figure 2 except that Na^+ (□ - - - □), K^+ (● - - - ●), Li^+ (○ - - - ○), and NH_4^+ (■ - - - ■) chlorides were added to varying concentrations. Electrophoretic analysis of the labeled membranes was performed as described under Figure 2: (A–C) incorporation into components 2, 3, and lipid, respectively.

though the degree of stimulation varies for the ions tested, all elicit a similar response pattern—a concentration optimum above 0.1 M with inhibition at 0.5 M. At 10 and 20 mM Mg^{2+}, the degree of stimulation by optimal concentrations of these monovalent ions is attenuated (data not shown).

The addition of 20 μM cyclic AMP results in measurable stimulation of all phosphorylations occurring at 1 mM Mg^{2+}. However, its most marked effect is the activation of incorporation into several minor components. The preferential utilization of these substrates, among which polypeptide 2.1 is most prominent in both the staining and labeling profiles, brings about a profound qualitative change in the pattern of labeling (Figure 1B and Fairbanks and Avruch, 1974). As illustrated in Figures 1–3, the phosphorylation of component 2 is singularly responsive to monovalent ions but not highly stimulated by cyclic AMP. Because of these, and other, distinguishing features, we consider the salt-stimulated phosphorylation of 2 here separately and discuss the salt-inhibited phosphorylations, together with cyclic AMP effects, in the following paper (Fairbanks and Avruch, 1974).

The divalent cation requirement in phosphorylation of 2 can be satisfied by Mn^{2+} and Co^{2+} as well as Mg^{2+}, whereas Ca^{2+}, Fe^{2+}, and Zn^{2+} do not support the reaction (Table I). Phosphorylation by 2 μM [γ-^{32}P]ATP in the presence of 1 mM Mg^{2+} is unaffected by unlabeled GTP, ITP, CTP, AMP, and CMP in tenfold excess, while unlabeled ATP and ADP produce marked reductions in ^{32}P incorporation;[4] unlabeled UTP at the same concentration is slightly inhibitory (Table II). This phosphorylation appears to be unresponsive to ouabain (0.1 mM), colchicine (20 μM), cytochalasin B (10 μg/ml), and prostaglandin E_2 (10^{-8}–10^{-10} M) (data not shown). It is interesting that, although Ca^{2+} will not substitute for Mg^{2+}, its addition at 1 mM to membranes phosphorylated at 1 mM Mg^{2+} stimulates incorporation into 2 by 50–150% (Figure 4 and Fairbanks and Avruch (1974), Figure 3 and Table IV).

TABLE II: Effects of Nucleotides on Endogenous Phosphorylation by [γ-^{32}P]ATP.[a]

Added Nucleotide	Incorporation (Counts/4 min) 2(+2.1)	3
None	958	2050
ATP	358	876
GTP	1007	2060
ITP	927	1968
CTP	1010	2217
UTP	677	1909
ADP	406	924
AMP	926	1878
CMP	889	1868

[a] Phosphorylations and electrophoretic analysis of incorporation were performed as described under Table I. Mg^{2+} was present in all incubation mixtures at 1 mM. Unlabeled nucleotides were present in tenfold excess (20 μM, final concentration) over [γ-^{32}P]ATP.

In contrast to the unstable intermediate in coupled Na^+,K^+ transport (Avruch and Fairbanks, 1972), the phosphorylated form of component 2 does not exhibit turnover in the isolated membrane. After phosphorylation of membranes for 5 sec, the ^{32}P incorporated into 2 neither decays upon addition of EDTA to 10 mM nor can be chased by unlabeled ATP in 100-fold excess (Figure 4).[5]

The identification of the 215,000-dalton phosphorylated component as polypeptide 2 in the staining profile is supported not only by the radioautographic data, but also by its selective elution at low ionic strength. Figure 5 displays results of an experiment in which ghosts were first incubated with [γ-^{32}P]ATP, then extracted under conditions that release "spectrin" (components 1 and 2) with component 5 from the matrix of lipid and tightly bound protein (Marchesi and Steers, 1968; Fairbanks *et al.*, 1971). This experiment demonstrates that the vesicles recovered by centrifugation retain the substrates for the salt-inhibited phosphorylation reaction, while component 2, the substrate in the salt-stimulated reaction, is eluted. The trace of ^{32}P at the zone 2 position in the electrophorogram of the vesicle proteins is consistent with incomplete release of "spectrin" in the one-step fractionation.

Figure 5 also demonstrates differences between the salt-stimulated reaction and phosphorylations dependent on cyclic AMP. A new class of substrates is involved in the latter process, and the most prominent among these are clearly not in the class of elutable proteins. In the presence of cyclic AMP, there is intense labeling of a minor component, 2.1 (mol wt 200,000), that migrates just ahead of 2 (see also

[4] A larger reduction by unlabeled ATP might have been anticipated; however, under these conditions, the effect of dilution is probably moderated by a concomitant stimulation of the reaction rate due to the increased ATP concentration. The action of ADP might involve either competitive inhibition or a reduction in [γ-^{32}P]ATP specific activity produced by the adenylate kinase reaction (Heller and Hanahan, 1972).

[5] Note in Figure 4F that 1 mM Ca^{2+} also stimulates incorporation into the region of polypeptide 3 (mol wt 105,000). This phosphopeptide turns over rapidly (Figure 4G) and has additional properties of an intermediate in the Ca^{2+} transport ATPase reaction (J. Avruch and G. Fairbanks, manuscript in preparation).

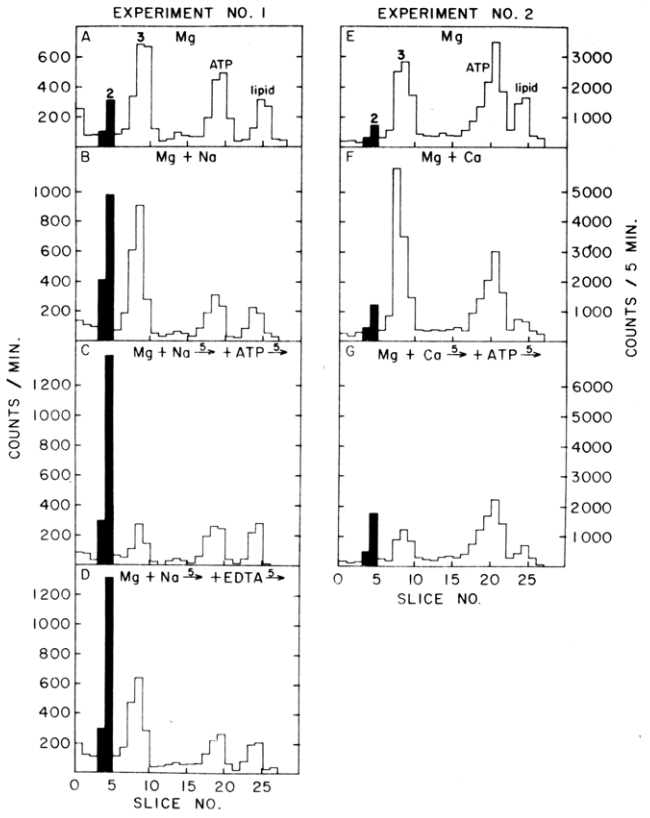

FIGURE 4: Differential turnover of membrane phosphoproteins. Membrane phosphorylation was carried out in presence of 1 mM Mg^{2+}, 2 μM [γ-^{32}P]ATP, and 20 mM imidazole chloride (pH 7.4); 50 mM Na^+ or 1 mM Ca^{2+} was present as indicated. In A, B, E, and F, the incubation time was 5 sec. In C, D, and G, 10 mM EDTA or 0.2 mM unlabeled ATP (final concentrations) was added rapidly after an initial incubation for 5 sec and the reaction was terminated after continued incubation for an additional 5 sec. Electrophoretic analysis of the phosphorylated membranes was performed as described previously (Avruch and Fairbanks, 1972). Radioactivity in component 2 is indicated by blackened areas in the histograms; 10^6 cpm represents about 57 pmol of ^{32}P in experiment 1 and 90 pmol in experiment 2.

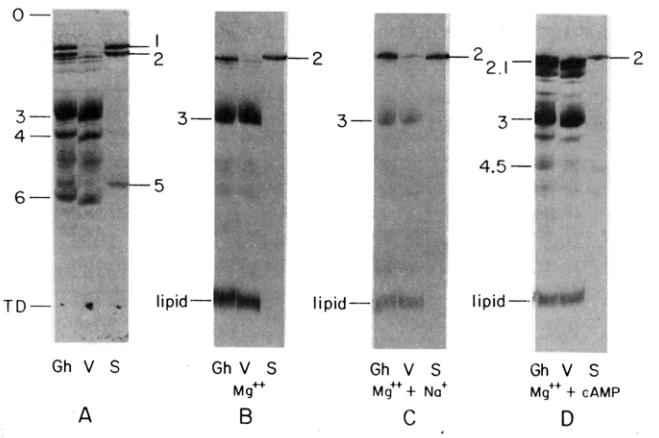

FIGURE 5: Selective elution of membrane phosphoproteins. Membranes were phosphorylated by [γ-^{32}P]ATP during 10-min incubations in the presence of 1 mM Mg^{2+}; 50 mM Na^+ or 20 μM cyclic AMP (final concentrations) were added in C and D, respectively, as indicated. After the incubation, "spectrin" eluates (S) and vesicles (V) were prepared by low ionic strength extraction of a portion of the phosphorylated ghost membranes (Gh). Sample volumes equivalent to about 10 μl of packed ghosts (about 30 μg of total membrane protein) were subjected to electrophoresis. Dried longitudinal slices from the stained gels were radioautographed: (A) Coomassie Blue staining patterns in dried longitudinal slices from electrophorograms of total ghost membrane proteins, vesicles (V), and "spectrin" eluates (S); (B–D) radioautographs showing distributions of ^{32}P in electrophorograms of phosphorylated membranes and membrane subfractions.

Figure 1B). These two bands can be resolved by radioautography if the gels are not overloaded, but are rarely distinguished by transection and counting. With either mode of analysis, however, the failure of 2.1 to elute with 2 establishes an unambiguous distinction between the two phosphate acceptors. Inhibition of the background phosphorylations by 50 mM NaCl is shown in Figure 5C. The reactions stimulated by cyclic AMP are similarly inhibited by monovalent ions (Fairbanks and Avruch, 1974).

Selective elution at low ionic strength can also be used in exploring the physical relationship between the membrane-associated protein kinases and their endogenous substrates. Figure 6 shows results of an experiment in which "spectrin" was eluted from the membranes *before* phosphorylation reactions were carried out. The dilute eluate was concentrated by ultrafiltration, so that the concentration of isolated "spectrin" incubated with [γ-^{32}P]ATP could approximate that in intact ghosts similarly incubated. Under these conditions, self-phosphorylation in the low ionic strength eluate is negligible, even in the presence of 0.2 M Na^+. The "spectrin"-free vesicles, on the other hand, exhibit only slightly impaired phosphorylation of component 3 and lipid; as in the intact membrane, these reactions are salt-inhibited. The vesicles do not themselves manifest an endogenous salt-stimulated phosphorylation reaction, but this activity can be demonstrated by restoring the specific endogenous substrate, polypeptide 2, eluted in "spectrin." This result (Figure 6B) suggests that the enzyme involved is tightly bound to the membrane, while its substrate is not.

Parallel studies using the same protocol have revealed that the addition of cyclic AMP to "spectrin" eluates does elicit significant self-phosphorylation; like cyclic AMP dependent phosphorylation in the intact membrane, these reactions are relatively nonspecific and are strongly inhibited by monovalent ions (Fairbanks and Avruch, 1974).

Although the vesicle enzyme is highly selective in phosphorylating polypeptide 2 in the reconstituted system stimu-

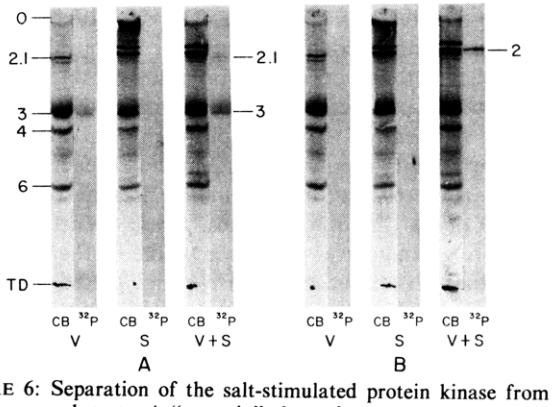

FIGURE 6: Separation of the salt-stimulated protein kinase from its endogenous substrate. A "spectrin" eluate from membranes extracted at low ionic strength was concentrated by ultrafiltration to the same volume as the resuspended vesicles. Vesicles (V), "spectrin" (S), and a 1:1 admixture of the two (V + S) were incubated with [γ-^{32}P]ATP for 10 sec in the presence of 1 mM Mg^{2+} alone (panel A) or Mg^{2+} plus 0.2 M NaCl (panel B). Upon termination of the reactions with perchloric acid, unlabeled vesicles were added to tubes containing "spectrin" alone to serve as carrier in the precipitation and washing steps. The acid-washed material was subjected to polyacrylamide gel electrophoresis. After the gels had been fixed and stained with Coomassie Blue, longitudinal slices were dried and radioautographed. Each dried slice (CB) is pictured aligned with a reversed image of its radioautograph (^{32}P).

TABLE III: Casein Phosphorylation by Membrane Vesicles.[a]

Additions	Total Incorp. (cpm) −Casein	+Casein
None	826	1286
0.05 M NaCl	409	1527
0.2	144	1932
0.5	141	3382
0.05 M KCl		1341
0.2		2051
0.5		3613

[a] All incubation mixtures contained 1 mM $MgCl_2$, 20 mM imidazole chloride (pH 7.4), 2 μM [γ-^{32}P]ATP, and 26 μg/ml of vesicle membrane protein in 1 ml. Casein was added to 4 mg/ml. The vesicles were prepared by incubation at low ionic strength and subjected to ultracentrifugation to separate them from the eluted endogenous substrate. Total incorporation during 15-min incubations was determined by liquid scintillation counting of the material precipitated with perchloric acid and washed according to Miyamoto *et al.* (1969). In this experiment, 10^3 cpm represents 0.93 pmol.

lated with added monovalent cations, the substrate specificity of the reaction is apparently not absolute. Table III shows that casein is phosphorylated when added to vesicles as an exogenous substrate and that this reaction is likewise strongly stimulated by monovalent cations, such as Na^+ or K^+.

Discussion

The results presented above draw attention to the possibility that two or more protein kinases may be involved in the endogenous phosphorylation reactions of the human erythrocyte membrane. One activity is not dependent on cyclic AMP, is stimulated by mono- and divalent cations, and uses as its principal, or sole, substrate the elutable 215,000-dalton polypeptide 2. A second activity is elicited by cyclic nucleotides, is inhibited by calcium and monovalent ions, and phosphorylates several membrane polypeptides, among which certain minor components appear to be preferred substrates. A third activity, exemplified by the salt-inhibited phosphorylation of polypeptide 3 in the absence of cyclic AMP, is less clearly defined and may represent a basal expression of one of the other kinases. These operational distinctions are given greater substance by the further observation that the salt-stimulated kinase is not eluted from the membrane with its specific substrate, whereas the same conditions of low ionic strength elution do cause partial release of kinase activity that is both salt inhibited and cyclic AMP dependent (Fairbanks and Avruch, 1974). It is difficult to reconcile this result with the idea that the endogenous phosphorylations are all functions of a single catalytic moiety. Hence, although the ultimate resolution of this question may require the physical isolation and characterization of the enzymes, we have chosen, on a provisional basis, to consider the mechanism and functional significance of the salt-stimulated phosphorylation independently of cyclic AMP regulation in this system.

In our electrophoretic analysis of the substrate specificities of these reactions, we found perfect coincidence of ^{32}P zones with bands in the Coomassie Blue staining profile.[6] We found also (unpublished observations) that treatment of phosphorylated membranes with pepsin prior to electrophoresis eliminated all slowly migrating components in both the stain and radioactivity profiles, leaving only fragments of low apparent molecular weight. Most of the ^{32}P incorporated into erythrocyte membrane components during long incubations at Mg^{2+} concentrations above 0.1 mM has properties of stable phosphate esters, as distinguished from labile acyl phosphates (Avruch and Fairbanks, 1972; Blostein, 1968). It can be released by alkaline hydrolysis, but is stable to Cl_3CCOOH and hydroxylamine; at least 70% is recoverable as phosphoserine and phosphothreonine after partial acid hydrolysis (Roses and Appel, 1973; Rubin and Rosen, 1973) and much of the remainder is accounted for by labeled phosphoinositides (Guthrow *et al.*, 1972). Although covalently bound phosphorus in individual membrane polypeptides has not yet been determined, the evidence cited supports the conclusion that all the major bands of ^{32}P (other than the lipid zone) in fixed, stained gels represent the formation of phosphopeptides, as opposed to noncovalent binding of ATP, inorganic phosphate, or phospholipid.

The monovalent cation stimulation of polypeptide 2 phosphorylation could be explained in several ways. We envision that the added ions might (a) activate a specific phosphatase; (b) change the state of the substrate; (c) change the state of the enzyme; or (d) alter the physical relationship between the kinase and polypeptide 2. The evidence at hand can be used to argue against the first two possibilities. The activation of a phosphatase with consequent hydrolysis of preexisting phosphoester bonds in polypeptide 2 would stimulate incorporation into that component. However, an effect of this kind would also produce demonstrable turnover of label in 2; this we have been unable to detect in the isolated membrane. It is possible that the monovalent ion stimulation is exerted at the level of the substrate, but, if so, the effect must be nonspecific, because the same ionic conditions augment phosphorylation of an exogenous substrate, casein. The two remaining possibilities are both plausible, and the present data do not support a definitive choice between them. However, the process of polypeptide 2 phosphorylation differs in two respects from the many other, disparate reactions regulated by monovalent cations (Suelter, 1970; Evans and Sorger, 1966). In its response to these ions, phosphorylation by the erythrocyte membrane protein kinase is (a) relatively insensitive, the maximum rate occurring at concentrations above 0.1 M, and (b) relatively nonspecific, with all four ions tested showing a high degree of stimulation.

The fourth mode of regulation, ionic modulation of the interaction between the enzyme and its polypeptide substrate, is of interest because of the likelihood that the two elements are not intimately connected in membranes isolated after hypotonic lysis (see below). It is conceivable that

[6] The incorporation of ^{32}P into phospholipid is an exception. Rubin and Rosen (1973) have suggested that the major erythrocyte membrane sialoglycoprotein, which binds protein dyes very weakly, is also a phosphate acceptor. However, we find only minor differences in the ^{32}P radioactivity profiles produced by electrophoresis at pH 7.4 and 2.4 (unpublished observations). This contrasts with the striking difference in the apparent molecular weights exhibited by the sialoglycoprotein in the two systems (Fairbanks and Avruch, 1972) and makes it unlikely that this protein is a major kinase substrate under the conditions of phosphorylation we have used.

elevation of the ionic strength could reduce repulsive electrostatic forces between the two proteins or that it could produce structural changes in the vicinity of the enzyme, relaxing steric constraints on the interaction. Rubin *et al.* (1972) reported that concentrated salt solutions extracted from erythrocyte membranes material with protein kinase activity against exogenous substrates. We have observed recently that the polypeptide 2 kinase is selectively eluted by 0.5 M NaCl (G. Fairbanks and J. Avruch, unpublished). These findings suggest that solubilization of the enzyme may be one of the factors defining the observed salt optima.

Phosvitin kinases detected in erythrocyte membranes (Judah *et al.*, 1962) and ox brain (Rodnight and Lavin, 1964) resemble in some respects the activity we are studying. These enzymes were stimulated by various monovalent cations and were maximally responsive to both Na^+ and K^+, while Li^+ was ineffective. The addition of Ca^{2+} abolished Mg^{2+}-dependent phosphorylation by the ox brain enzyme and inhibited both the basal and salt-stimulated activities of the erythrocyte membrane enzyme. The failure of the latter to respond to Li^+ does not seriously conflict with our data—the effect of Li^+ is modest and is maximal near 50 mM; it would have been obscured by the relatively high concentrations of Mg^{2+} and buffer ions (5 and 50 mM, respectively) used by Judah *et al.* (1962) in their assays. The Ca^{2+} inhibition of exogenous phosphorylations in these systems contrasts with our finding of Ca^{2+} stimulation of the endogenous reaction in the erythrocyte membrane. The latter result is consistent with a direct action of Ca^{2+} on polypeptide 2 to enhance its rate of phosphorylation.

The physiological role of the polypeptide 2 phosphorylation reaction in the membrane is unknown, and it appears that interpretation of our results at this level hinges on a fuller understanding of the function of the endogenous substrate than is now available. Several lines of evidence have given rise to speculations that "spectrin" interacts with intracellular ATP and divalent cations to define erythrocyte form and deformability. Weed *et al.* (1969) demonstrated that depletion of intracellular ATP produced a marked reduction in cellular deformability that could be reversed by further incubation in the presence of adenosine. Ghosts prepared from depleted cells were likewise more rigid than membranes from normal cells. The introduction of Ca^{2+} into ghosts mimicked the effect of depletion; simultaneous addition of ATP, EDTA, or Mg^{2+} antagonized the Ca^{2+} effect and maintained deformability. Penniston (1972) and Penniston and Green (1968) reported that the addition of ATP to erythrocyte ghosts of several species resulted in extensive endocytosis with concomitant ATP hydrolysis. ATP-dependent conformational changes in the membrane proteins are reportedly augmented by monovalent ions (Graham and Wallach, 1971). "Spectrin" is disposed in a loose fibrillar meshwork localized on the inner surface of isolated membranes (Marchesi and Palade, 1967; Rosenthal *et al.*, 1970; Nicolson *et al.*, 1971). Its release is always accompanied by a dramatic morphological transition leading to breakdown of the membrane into small vesicles (Steck *et al.*, 1970; Kant and Steck, 1972). The large polypeptides of the eluted complex have an amino acid composition resembling that of rabbit myosin (Guidotti, 1972a,b) and the eluted material as a whole manifests a Ca^{2+}-activated ATPase of low activity (Rosenthal *et al.*, 1970; Wolf, 1970; J. Avruch, unpublished observations).

Although we can discern an association between factors regulating polypeptide 2 phosphorylation and some of these variables involved in changes in membrane structure and cellular deformability, an attempt to integrate all of the observations into a single model would be premature. If a relationship between the two processes can be demonstrated, calling for a mechanistic explanation, it will be important to know the stoichiometry of the phosphorylation, inasmuch as this must reflect the organization of "spectrin" components and the interaction of the protein with its kinase in the membrane. Because in our experiments low ATP levels and short incubation times were used, our results do not indicate the acceptor capacity of the endogenous substrates. Rubin and Rosen (1973) reported incorporation of 0.79 pmol of phosphate per pmol of high molecular weight polypeptide, but this figure was obtained after incubation in the presence of cyclic AMP and presumably includes incorporation into polypeptide 2.1 as well as 2. It should be possible to make more precise measurements of stoichiometry by labeling intact cells, in which the protein-bound phosphate is turning over. Palmer and Verpoorte (1971) detected incorporation into a high molecular weight membrane polypeptide in erythrocytes incubated in plasma and added ^{32}P-labeled inorganic phosphate and glucose. We have used the radioautographic technique to demonstrate that several membrane polypeptides are phosphorylated in the intact cell and that phosphopeptide 2 is predominant among these, even in the presence of cyclic AMP (G. Fairbanks and J. Avruch, manuscript in preparation). As we have shown here, the same reaction predominates in isolated membranes when salts are added at physiologic concentrations. The similarity of these results is an encouraging indication that the major phosphorylation reaction in the isolated membrane is a valid expression of enzymatic apparatus active *in vivo*. Both approaches may be useful, not only in defining the stoichiometry, but also in studying modes of regulation *in vivo* and the relationship between membrane protein phosphorylation and erythrocyte function.

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Phosphorylation of Endogenous Substrates by Erythrocyte Membrane Protein Kinases. II. Cyclic Adenosine Monophosphate-Stimulated Reactions†

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ABSTRACT: Cyclic AMP stimulated protein kinase activity in the human erythrocyte membrane is associated with the phosphorylation of endogenous polypeptide substrates. Several features of this reaction distinguish it from the salt-stimulated phosphorylation of the major polypeptide 2 (mol wt 215,000) of "spectrin." The preferred substrates in the cyclic AMP stimulated reaction are minor components, among which polypeptides 2.1 (mol wt 200,000) and 4.5 (mol wt 52,000) are most prominent. These phosphopeptides are tightly held in the membrane and are not eluted at low ionic strength with "spectrin." The response to cyclic AMP is strongly inhibited by monovalent ions at physiologic concentrations and by 1 mM Ca^{2+}. The phosphorylation of polypeptide 3 (mol wt 105,000) is similarly regulated by monovalent ions and Ca^{2+}, and may represent a basal function of the cyclic AMP stimulated enzyme. Relative to polypeptide 2 phosphorylation, these reactions are less strongly stimulated by an increase in the Mg^{2+} concentration from 1 to 20 mM. A portion of the cyclic AMP stimulated activity is eluted with "spectrin." The phosphorylation of endogenous substrates by the free enzyme in such eluates is indiscriminate, but the labeling pattern is normalized when the eluate is admixed with vesicles under the conditions of the reaction.

Protein kinase activity associated with the human erythrocyte membrane (Rubin *et al.,* 1972) has been implicated in the selective phosphorylation of endogenous polypeptide substrates observed both in isolated membranes (Guthrow *et al.,* 1972; Roses and Appel, 1973; Rubin and Rosen, 1973) and intact cells (Palmer and Verpoorte, 1971). In a preceding publication (Avruch and Fairbanks, 1974), we described the reaction in which a single high molecular weight polypeptide, the 215,000-dalton component of "spectrin" (Marchesi and Steers, 1968), is phosphorylated. We drew attention to its high substrate specificity, its stimulation by monovalent cations and Ca^{2+}, and its relative unresponsiveness to cyclic AMP. In this paper we present the contrasting results obtained in the parallel analysis of another set of endogenous phosphorylations—reactions highly stimulated by cyclic AMP, inhibited by monovalent cations and Ca^{2+}, and utilizing multiple polypeptide substrates. These and other features of the reactions suggest that a separate enzyme, or enzyme complex, may be involved.

Methods

The methods of erythrocyte membrane preparation, incubation with [γ-^{32}P]ATP, and electrophoretic analysis of la-

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