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CALMODULIN-DEPENDENT SPECTRIN KINASE ACTIVITY IN RESEALED HUMAN ERYTHROCYTE GHOSTS

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Membrane protein phosphorylation has been studied in resealed human erythrocyte ghosts by measuring the incorporation of ^{32}P into spectrin and band 3. Norepinephrine- and Ca^{2+}-stimulated phosphate incorporation was diminished in ghosts depleted of calmodulin. Ghosts prepared with endogenous calmodulin showed Ca^{2+}- and norepinephrine-stimulated protein phosphorylation only when the ghosts had been resealed in the presence of [γ-^{32}P]ATP. Ghosts resealed with or without calmodulin in the presence of unlabeled ATP showed no net gain or loss of ^{32}P when exposed to norepinephrine or a Ca^{2+}-specific ionophore. These observations suggest that Ca^{2+} and norepinephrine stimulation of membrane protein phosphorylation is mediated by calmodulin-dependent spectrin kinase activity, and not by increased turnover of spectrin ATPase or by inhibition of phosphospectrin phosphatase.

Introduction

Erythrocyte membrane properties are controlled by a cytoskeleton consisting of spectrin, actin, bands 2.1 and 4.1, and other minor protein components [1,2]. The state of this cytoskeleton affects the shape and deformability of the cell as well as the lateral distribution of membrane proteins [3]. Changes in cell shape and deformability, such as the echinocyte-discocyte transformation of resealed erythrocyte ghosts [4–6], are preceded by phosphorylation of the β-chain of spectrin (band 2 according to Ref. 7). In partially purified cytoskeletal preparations, phosphorylation of spectrin modifies the interaction of spectrin with actin [8–10]; however, in purified preparations the level of spectrin phosphorylation does not affect spectrin-actin association [11], the spectrin tetramer-dimer equilibrium [12], or the binding of spectrin dimers to the erythrocyte membrane [13]. Erythrocyte shape is correlated with spectrin phosphorylation under some but not all conditions [4,5,13,14], suggesting that the phosphorylation of spectrin may be related to the morphological properties of the healthy erythrocyte. How phosphorylation might affect these properties is not understood.

In previous publications, we demonstrated that phosphorylation of spectrin in whole erythrocytes is enhanced by α-adrenergic agents [15] and by increases or decreases in intracellular Ca^{2+} concentration induced by a Ca^{2+}-specific ionophore [16]. Increasing the intracellular Ca^{2+} concentration is known to open a K^+ gate in the membrane [17], leading to K^+ efflux and concomitant cell shrinkage, crenation, and loss of membrane deformability [18]. The observed Ca^{2+}-stimulated increase in spectrin phosphorylation is not the result of either this K^+ flux or the resulting morphological changes [16]. Both norepinephrine and Ca^{2+}

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stimulation of protein phosphorylation can be inhibited by 10 μM trifluoperazine, a phenothiazine derivative that binds to and inhibits the Ca^{2+}-calmodulin complex [19]. On that basis it was suggested that the stimulated ^{32}P incorporation could be calmodulin dependent [16].

Calmodulin, a calcium-dependent regulatory protein, is present in erythrocytes in approximately micromolar concentration [20]. Heretofore the only function ascribed to erythrocyte calmodulin has been activation of the Ca^{2+} transporting $(Ca^{2+} + Mg^{2+})$-ATPase [20]. According to Cheung [19], four criteria must be satisfied in order to demonstrate a calmodulin-dependent reaction: (1) calmodulin must be present in the cell under consideration; (2) the Ca^{2+} concentration-dependence of the reaction studied must be consistent with the Ca^{2+} binding behavior of calmodulin; (3) the reaction must be inhibited by trifluoperazine; and (4) the reaction must be inhibited in the calmodulin-depleted system. Calcium-stimulated spectrin phosphorylation satisfies the first three criteria [16]. To satisfy the fourth criterion, we have studied the incorporation of [^{32}P]phosphate into membrane proteins of resealed ghosts prepared with and without calmodulin. [^{32}P]Phosphate turnover in such ghosts also was studied, in order to discover whether the observed changes in phosphate incorporation arise from increased net phosphorylation (increased kinase or decreased phosphatase activity) or from increased phosphate turnover (ATPase stimulation).

Materials and Methods

Norepinephrine hydrochloride and chromatography resins were obtained from Sigma Chemical Company. The Ca^{2+}-ionophore, A23187, was purchased from Calbiochem. Radioisotopes were the products of New England Nuclear.

Preparation of ^{32}P-labeled erythrocyte membranes. Human erythrocytes were obtained from healthy adult volunteers and washed three times by centrifugation at $3200 \times g$ for 10 min, followed by supernatant aspiration and resuspension in 0.15 M NaCl. The washed cells were suspended in an equal volume of 0.15 M NaCl and left to deplete ATP stores for 36 h at 4°C. Membrane proteins were labelled with ^{32}P by incubating whole erythrocytes for 2 h at 37°C in an equal volume of buffer containing 10 mM $MgSO_4$, 10 mM KH_2PO_4, 10 mM adenosine, 1 mM inosine, 130 mM NaCl, 10 mM D-glucose, 0.01 mM $CaCl_2$, and 0.25 mCi/ml $Na_3{}^{32}PO_4$, adjusted to pH 7.4. Following the incubation the cells were washed three times with three volumes of 0.15 M NaCl, and the membranes were prepared by lysing the cells in three volumes of either 12 mM imidazole, pH 7.4, or 183 mM imidazole, pH 7.4. The membranes were washed three times in five volumes of the lysing buffer.

Preparation and phosphorylation of resealed erythrocyte ghosts. Labeled membranes (4 ml) at 4°C were resealed by addition of an equal volume of a buffer containing 0.252 M KCl, 0.875 mM NaCl, 0.9 mM NaH_2PO_4, and 2 mM Na_4ATP, adjusted to pH 7.4. In some experiments this buffer also contained 500 μCi of tetra(triethylammonium)·[γ-^{32}P]ATP. For calmodulin reconstitution experiments this buffer also contained approx. 3 mg purified erythrocyte calmodulin. After 15 min incubation on ice, the ghosts were pelleted by centrifugation for 10 min at $3200 \times g$ and washed twice in 10 vol. of 0.15 M NaCl. A volume (0.5 ml) of resealed ghosts was added to an equal volume of the buffer used in prelabeling, but containing [^{31}P]ATP and 10 μM $CaCl_2$. Where stated, the suspending buffer also contained 10 μM (*R*)-(−)norepinephrine or 5 μM A23187. The ghosts were incubated at 37°C for 30 min, and the phosphorylation reaction was terminated by addition of 10 vol. of ice-cold 0.15 M NaCl. The ghosts were isolated by centrifugation at $3200 \times g$ for 10 min followed by supernatant aspiration, and lysed by addition of 10 ml distilled water. Membranes were isolated by centrifugation at $9200 \times g$ for 10 min.

Analysis of ^{32}P incorporation into membrane proteins. This was accomplished essentially as described previously [15,16]. Membrane proteins were separated by electrophoresis on sodium dodecyl sulfate polyacrylamide slab gels (7.5% acrylamide) by the method of Ames [21] and Laemmli [22]. Gels were dried onto filter paper and exposed to Kodak NS-5T. X-ray film for three to seven days.

The amount of incorporated radioactivity was determined in two ways. First, the individual bands

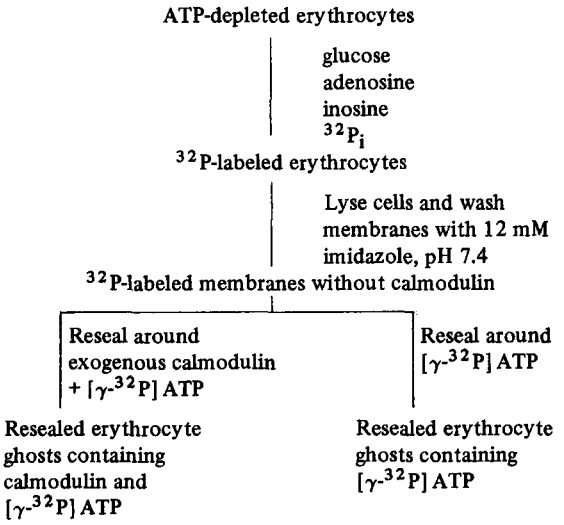
of the dried gels were cut out, dissolved in 0.4 ml of 30% H_2O_2, and analyzed by liquid scintillation counting. Second, individual lanes of the autoradiogram were scanned on a densitometer, and the peaks were integrated by cutting and weighing. Results were normalized to the amount of protein loaded onto the gel as determined by the method of Lowry et al. [23], and then compared with the amount of radioactivity incorporated into proteins in the control samples. Results from individual experiments were discarded if the two radioactivity determinations did not agree to within 15%.

Preparation of human erythrocyte calmodulin. Purified human erythrocyte calmodulin was prepared by a modification of the procedures of Jarrett and Penniston [24] and Jarrett and Kyte [20]. After chromatography, dialysis, and lyophilization as described, the Ca^{2+}-ATPase stimulating activity of the resulting material was found to be within reported ranges [24].

Results

Reconstitution with purified calmodulin

Erythrocyte membranes were isolated under conditions known to remove calmodulin [25] and then resealed in a buffer containing [γ-^{32}P]ATP and, in one sample, approx. 1 μM purified calmodulin (Scheme I). Both samples of ghosts were incubated with norepinephrine or Ca^{2+} ionophore plus 10 μM Ca^{2+} at 37°C for 30 min. Both agents increased the incorporation of ^{32}P into spectrin β-chains and band 3, but the enhancement was two to three fold greater in the ghosts reconstituted with calmodulin (Table I). The gels and autoradiographs of these ghosts showed no evidence of proteolysis, cross-linking, or extraction of membrane proteins during preparation of the ghosts.



Scheme I. Protocol for calmodulin reconstitution experiments.

TABLE I

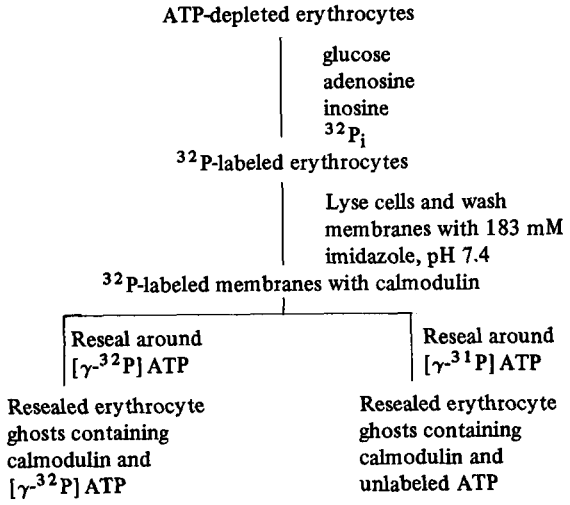
^{32}P INCORPORATION INTO MEMBRANE PROTEINS OF RESEALED ERYTHROCYTE GHOSTS IN THE PRESENCE AND ABSENCE OF CALMODULIN

Erythrocyte membranes were prepared by a technique that removes calmodulin. Some were resealed in the presence of exogenous calmodulin; all were resealed in the presence of [γ-^{32}P]ATP. Resealed ghosts were incubated at 37°C for 30 min in the presence of norepinephrine (10 μM) or A23187 (5 μM). Results are averages of percentage incorporation relative to control (±S.E.).

Agent	Calmodulin	Relative ^{32}P incorporation		
		Spectrin	Band 3	*N*
Control	+	100±4	100±3	4
Norepinephrine	+	128±4	125±4	4
A23187	+	138±1	133±6	4
Control	0	99±1	100±4	4
Norepinephrine	0	110±5	113±3	4
A23187	0	124±7	98±3	4

Mechanism of phosphorylation enhancement: turnover or net incorporation

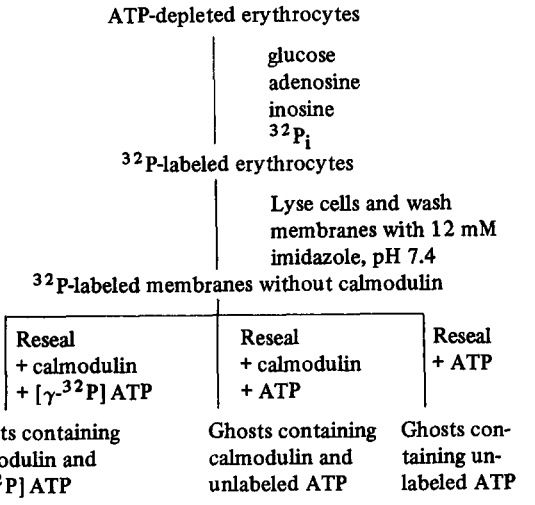
Erythrocyte membranes were prelabeled with ^{32}P by incubating intact cells with $^{32}P_i$, and then isolated under conditions known to leave calmodulin on the membrane [25]. One sample of these labeled membranes was resealed in a buffer containing [γ-^{32}P]ATP, and another was resealed around unlabeled ATP (Scheme II). The specific activity of the added [γ-^{32}P]ATP was adjusted to be the same as that of the $^{32}P_i$ in the prelabeling buffer (10 mCi/mmol). Samples of these ghosts were incubated in the presence of norepinephrine or Ca^{2+} ionophore plus 10 μM $CaCl_2$. Both agents increased the incorporation of ^{32}P into spectrin



Scheme II. Protocol for phosphate turnover vs. net phosphate incorporation experiments.

and band 3 in the ghosts containing [γ-^{32}P]ATP, but neither agent had any effect on the amount of ^{32}P in proteins of ghosts containing only unlabeled ATP (Table II).

One sample of prelabeled erythrocytes was lysed and washed in 12 mM imidazole buffer, a procedure which depletes endogenous calmodulin from the membranes. These membranes were resealed in buffer containing unlabeled ATP and, in one sample, 1 μM purified calmodulin. A second sample contained unlabeled ATP and no calmodulin. A third sample was resealed around 1 μM calmodulin and [γ-^{32}P]ATP (Scheme III). These prepara-



Scheme III. Protocol for kinase stimulation vs. phosphatase inhibition experiments.

TABLE II

^{32}P INCORPORATION INTO MEMBRANE PROTEINS OF RESEALED ERYTHROCYTE GHOSTS IN THE PRESENCE OF ATP

Erythrocyte ghosts were prepared by a method that allows the membranes to retain calmodulin and resealed in the presence of ATP. For half of the membranes, the ATP was enriched in [γ-^{32}P]ATP. The resealed ghosts were incubated at 37°C for 30 min in the presence of norepinephrine (10 μM) or A23187 (5 μM). Results are averages of the percentage incorporated relative to control samples (±S.E.).

Agent	[γ-^{32}P]ATP	Relative ^{32}P incorporation		
		Spectrin	Band 3	*N*
Control	+	99±2	99±2	5
Norepinephrine	+	130±8	112±6	3
A23187	+	130±5	115±3	4
Control	0	100±2	100±2	5
Norepinephrine	0	96±2	94±2	4
A23187	0	107±2	102±2	4

TABLE III

EFFECT OF CALMODULIN ON ^{32}P CONTENT OF ERYTHROCYTE GHOSTS CONTAINING UNLABELED ATP

Erythrocyte membranes were prelabeled with ^{32}P$_i$ and lysed under conditions that remove calmodulin. Ghosts were resealed around (a) 1 μM calmodulin+[γ-^{32}P]ATP, or (b) 1 μM calmodulin+unlabeled ATP, or (c) unlabeled ATP. Resealed ghosts were incubated at 37°C for 30 min in the presence of norepinephrine (10μM) or A23187 (5 μM). Results are averages of the percentage incorporated relative to control samples (±S.E.).

Agent	[γ-^{32}P]ATP	Calmodulin	^{32}P in	
			Spectrin	*N*
Control	+	+	100±4	4
Norepinephrine	+	+	128±4	4
A23187	+	+	138±1	4
Control	0	+	100±4	4
Norepinephrine	0	+	97±6	4
A23187	0	+	115±6	4
Control	0	0	100±6	4
Norepinephrine	0	0	104±5	4
A23187	0	0	100±6	4

tions were incubated in the presence of norepinephrine or Ca^{2+} ionophore for 30 min at 37°C. The presence or absence of calmodulin had no effect on spectrin phosphorylation in ghosts resealed around unlabeled ATP. Ghosts containing exogenous calmodulin and [γ-^{32}P]ATP exhibited the usual enhancements in the presence of norepinephrine or the Ca^{2+} ionophore (Table III).

Discussion

The incorporation of ^{32}P into spectrin and band 3 of whole human erythrocytes is increased by α-adrenergic agents and by 10 μM Ca^{2+} in the presence of a Ca^{2+} ionophore [15,16]. The calcium concentration dependence of the Ca^{2+} + ionophore stimulation is similar to the reported response of calmodulin to Ca^{2+}, and both ionophore- and norepinephrine-stimulated phosphorylation are inhibited by trifluoperazine. These correlations suggested that calmodulin is involved in the mechanism of both responses. Consistent with this suggestion, we find that adrenergic and Ca^{2+}-dependent stimulation of protein phosphorylation are depressed in resealed erythrocyte ghosts depleted of calmodulin (Table I). Ghosts prepared with added calmodulin show greater spectrin phosphorylation in response to both agents. According to the usual criteria [19], this finding supports a calmodulin-dependent mechanism for these phosphorylation responses.

The increased protein phosphorylation induced by these agents could arise by several pathways. Either stimulation of the activity of a spectrin kinase or inhibition of phosphospectrin phosphatase would increase the net phosphate bound to protein, while enhancement of spectrin ATPase activity would produce an increase in the specific activity of the bound phosphate. Either effect would appear as an increase in label bound to the membranes. The parallel enhancement of ^{32}P incorporation into spectrin and band 3 would suggest that kinase stimulation is responsible since both proteins are substrates for spectrin kinase [26]. However, spectrin is reported to show a low-level Ca^{2+}-ATPase activity [27–29] which could give rise to the observed effects by increased phosphate turnover. There is ample precedent for calmodulin-dependent ATPase and kinase activities in a wide range of cells [19].

This question was addressed in experiments on ghosts prelabeled with ^{32}P and then resealed around either ^{32}P or unlabelled ATP. Norepinephrine or calcium stimulation of a spectrin kinase activity or inhibition of a phosphatase in these ghosts should produce an increase in protein-bound ^{32}P in ghosts containing [^{32}P]ATP, while ghosts containing unlabeled ATP should show no response. Stimulation of spectrin ATPase activity should produce a decrease in the label bound to protein in ghosts containing unlabeled ATP, an effect that would be apparent whether the kinase activity is stimulated or not. Such increased turnover should have no effect on the level of ^{32}P label in ghosts containing [^{32}P]ATP, since newly incorporated phosphate would have the same specific activity as that incorporated during preincubation.

The result of this experiment is that expected if norepinephrine or increased intracellular Ca^{2+} increase the net phosphate bound to protein. Both agents increase the relative amount of ^{32}P bound to spectrin and band 3 in ghosts resealed around [γ-^{32}P]ATP, and neither increase nor decrease ^{32}P bound in ghosts resealed around unlabeled ATP.

These increases in net bound phosphate could arise by either of two pathways; by activation of a spectrin kinase or by inhibition of phosphospectrin phosphatase. To distinguish between these possibilities, the experiment described in Fig. 3 and Table III was conducted. Prelabeled, calmodulin-depleted ghosts were resealed around unlabeled ATP in the presence and absence of purified calmodulin. If the calmodulin-dependent response involves activation of spectrin kinase, the presence or absence of calmodulin should have no effect on the amount of ^{32}P bound to spectrin in ghosts containing unlabeled ATP. If calmodulin inhibits a phosphate activity, in contrast, the amount of bound phosphate should decrease in response to the added effectors in cells containing no calmodulin. The former result was found; added calmodulin neither increased nor decreased spectrin phosphorylation in ghosts resealed around unlabeled ATP.

It is concluded that either α-adrenergic stimulation or increases in intracellular Ca^{2+} concentration leads to calmodulin-dependent activation of a

spectrin kinase activity in resealed ghosts. Spectrin is a substrate for at least two distinct red cell kinases that are cyclic AMP-independent and present in both cytoplasmic and membrane-bound forms [30–32]. Ghosts prepared by the methods used here would be expected to retain membrane-bound kinases. The phosphorylation enhancement found in ghosts is only half as great as that observed in intact cells [15,16], suggesting that some of the stimulated kinase activity was lost in lysis and washing. However, two lines of evidence suggest that the casein kinase is not the target of the calmodulin stimulation. First, purified casein kinase from these cells is inhibited by calcium in concentrations greater than 10 nM [33]. It is possible that this direct inhibition could be counteracted by calmodulin in vivo, but preliminary experiments using cell lysate show no stimulation of casein phosphorylation in vitro (Truong, H.T.N., unpublished data). Indeed, direct calcium inhibition of this kinase might be responsible for the stimulation of spectrin phosphorylation observed when cells are depleted of cytosolic calcium by treatment with A23187 in low calcium buffer [16]. Calmodulin itself has been shown to bind the calcium-calmodulin complex in physiological concentration ranges [33]. Experiments are now in progress to examine the effects of this binding on membrane-bound kinase activity.

Prior work showed that calcium has a biphasic effect on the level of spectrin phosphorylation in intact red cells, being minimal at micromolar calcium concentration [16]. The stimulation found at both lower and higher calcium levels may not be due to the same kinase, but the net effect is a kinase system poised at a basal level at normal cytosolic calcium concentration and stimulated by either increases or decreases in that concentration. While intracellular calcium levels are controlled closely by cytoplasmic buffers and the calcium pump, transient changes could arise from membrane perturbations that either displace bound calcium [35], permit influx, or increase binding. The calcium- and calmodulin-dependent spectrin kinase complex could function in reporting such changes in membrane structure to some sort of cytoskeletal control mechanism. It remains to demonstrate a functional role for this intricate and potentially powerful regulatory system.

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