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ADRENERGIC STIMULATION OF MEMBRANE PROTEIN PHOSPHORYLATION IN HUMAN ERYTHROCYTES

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Summary

Adrenergic modification of membrane protein phosphorylation was studied in intact human erythrocytes. Micromolar norepinephrine increased ^{32}P incorporation into Band 2 by 70%, and into Band 3 by 40%. Phosphorylation levels observed with a series of specific agonists and antagonists suggest that an α-adrenergic receptor is involved in this effect. The mechanism of linkage between this receptor and protein phosphorylation does not appear to involve modulation of intracellular concentrations of ATP, cyclic AMP, or cyclic GMP.

Adrenergic agents have been shown to alter the flow properties and osmotic fragility of human erythrocyte suspensions, presumably by affecting cell deformability [1, 2]. These agents also produce subtle changes in the fluidity of the cell membranes which can be detected as increases in the order parameter of a spin-labelled lipid [3]. Since these membrane fluidity changes can be inhibited by ATP depletion or calcium depletion, or by cytochalasin B [4] at concentrations which abolish actin-myosin association, it has been suggested that membrane fluidity might reflect the state of the spectrin-actin complex of the erythrocyte [3]. These proteins, which resemble actin-myosin complexes of other cells in several biochemical and immunological respects [5, 6], also have been implicated in control of the mechanical properties of the cell [7].

The interaction of spectrin and actin is thought to be regulated by the extent of spectrin phosphorylation [8], which in turn is controlled by a cyclic AMP-independent spectrin kinase [9, 10] and a phosphospectrin phosphatase [10, 11]. The activities of these enzymes are modulated in vitro by

a variety of ions and metabolic intermediates, but their regulation by exogenous agents has not been demonstrated. We therefore have examined the phosphorylation of spectrin and other membrane proteins in intact cells under adrenergic stimulation.

Erythrocytes were incubated with adrenergic agonists and/or antagonists in the presence of $^{32}P_i$. Fig. 1 shows polyacrylamide slab gels of the membranes isolated from these cells; the Coomassie Blue-stained gel is shown on the left and the autoradiogram of the same gel on the right. The greatest incorporation of ^{32}P was found in bands 2 (spectrin) and 3, although some phosphorylation was observed in virtually every band, as reported previously [12]. As is shown in Fig. 2 and Table I, 1 μM norepinephrine enhanced ^{32}P incorporation into bands 2 and 3. Changes in phosphorylation of other bands could not be quantitated with confidence. A similar pattern of enhancement was induced by 10 μM phenylephrine (a specific α-adrenergic agonist) but not by isoproterenol (a β-adrenergic agonist). The norepinephrine-induced enhancement could be inhibited partially by phenoxybenzamine (an α-adrenergic antagonist), but not by propranolol (a β-adrenergic antagonist). Other workers have shown that the β-agonist isoproterenol does not increase the phosphorylation of the membrane protein glycophorin under similar conditions [13]. These results suggest that the observed phosphorylation enhancement is mediated by an α-adrenergic receptor.

TABLE I

RELATIVE ^{32}P INCORPORATION AND ATP AND CYCLIC NUCLEOTIDE CONCENTRATIONS IN HUMAN ERYTHROCYTES UNDER ADRENERGIC STIMULATION

^{32}P incorporation into gels was quantitated from densitometer scans of autoradiograms by cutting and weighing the peaks. These values were normalized according to the total protein loaded onto the gel (vide infra). ATP concentrations were determined by the luciferin-luciferase assay, for cells incubated for two hours at 37°C in NaCl/P_i/glucose buffer [21]. Cyclic nucleotide concentrations were measured by radioimmunoassay [22]. Washed cells were incubated in NaCl/P_i/glucose buffer in the presence of 0, 1, or 10 μM norepinephrine, or 1 μM norepinephrine, Data shown in the table represent average relative nucleotide levels in aliquots taken after 5, 15, 30 and 60 min of incubation (1 μM norepinephrine or 1 μM isoproterenol); 0.25, 0.50, 1, 2, 5, 15, and 60 min of incubation (10 μM norepinephrine); or after 5 and 15 min of incubation (0 and 1 μM norepinephrine or 1 μM isoproterenol, after a 20-min preincubation with 200 μM 3-isobutyl-1-methyl xanthine). Cyclic nucleotide concentrations did not vary significantly from these averages at any time point. Values are % control ± S.D.

Agent	^{32}P Incorporation		Concentration of		
	Band 2	Band 3	ATP	Cyclic AMP	Cyclic GMP
None	100	100	100	100	100
Norepinephrine (1 μM)	170 ± 15	140 ± 15	100 ± 10	100 ± 30	110 ± 30
(10 μM)	—	—	—	110 ± 20	100 ± 20
Phenoxybenzamine (1 μM)	80 ± 15	80 ± 15	100 ± 10	—	—
Propranolol (1 μM)	120 ± 15	110 ± 15	110 ± 10	—	—
Norepinephrine (1 μM) + phenoxybenzamine (1 μM)	150 ± 15	100 ± 15	100 ± 10	—	—
Norepinephrine (1 μM) + propranolol (1 μM)	200 ± 15	120 ± 15	100 ± 10	—	—
Phenylephrine (10 μM)	170 ± 15	120 ± 15	—	—	—
Isoproterenol (1 μM)	—	—	—	100 ± 40	90 ± 50
(10 μM)	110 ± 15	90 ± 15	—	—	—
3-Isobutyl-1-methylxanthine (200 μM)	—	—	—	170 ± 30	110 ± 20
3 Norepinephrine (1 μM) + 3-isobutyl-1-methylxanthine (200 μM)	—	—	—	160 ± 40	110 ± 40
Isoproterenol (1 μM) + 3-isobutyl-1-methylxanthine (200 μM)	—	—	—	190 ± 40	130 ± 30

Since protein phosphorylation can be influenced by ATP concentration [14], the effect of catecholamines on intracellular ATP levels was determined. As shown in Table I, no significant difference in ATP concentration was detected in the presence and absence of 1 μM norepinephrine. Thus, it does not appear that the phosphorylation enhancement is caused by a general metabolic stimulation which might provide additional substrate for a protein kinase.

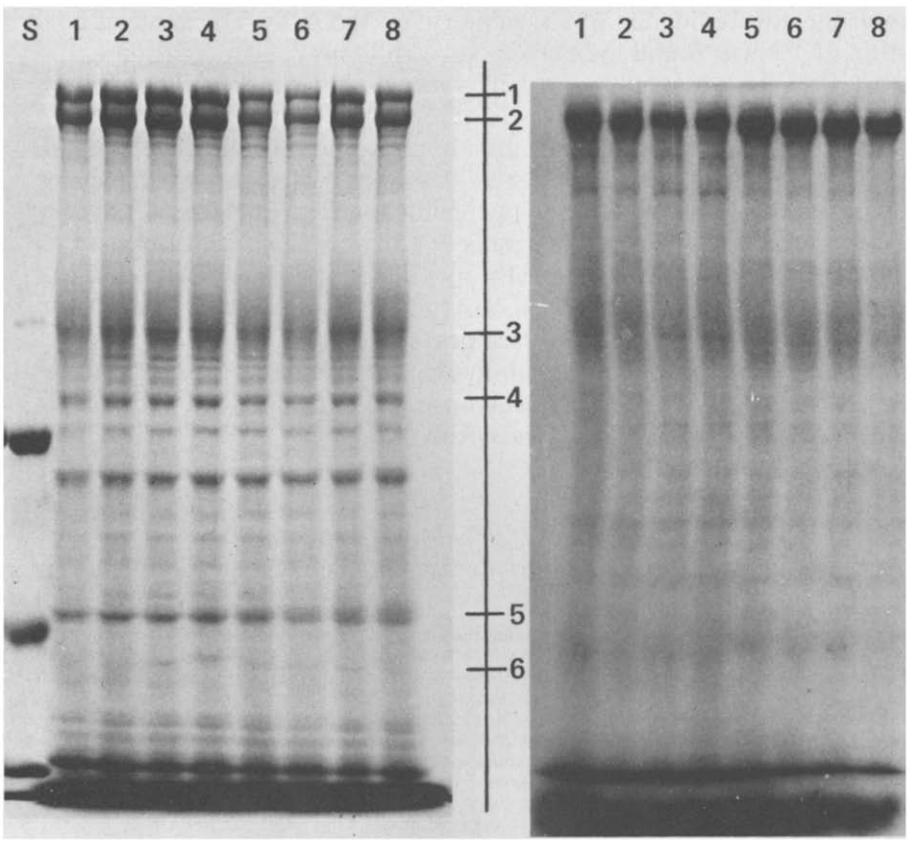

Fig. 1. ^{32}P incorporation into whole erythrocyte ghosts. Erythrocytes were obtained from adult volunteers and washed four times in 0.15 M NaCl, suspended in an equal volume of 0.15 M NaCl and left overnight at 4° C. These ATP depleted cells (0.5 ml) were suspended in 0.5 ml of a solution containing 10 mM KH_2PO_4, 10 mM $MgSO_4$, 10 mM adenosine, 1 mM inosine, 10 mM glucose, 130 mM NaCl, 10 μM $CaCl_2$, and 1 mCi/ml $^{32}P_i$ adjusted to pH 7.4 with 1 M NaOH. The appropriate agonists and/or antagonists were added as concentrates, and the cells incubated at 37° C for 2 h. To terminate the incubation, 10 ml of ice-cold 0.15 M NaCl was added to each sample and they were centrifuged. The supernatants were removed, and the cells were lysed with 10 ml distilled water. The membranes were pelleted and washed once with 10 ml distilled water. Polyacrylamide slab-gel electrophoresis was performed [23, 24] using a 4.5% acrylamide stacking gel and a 7.5% acrylamide separating gel. Samples were prepared by heating a suspension of 25% (v/v) pelleted membranes in 50 mM Tris, pH 6.8, 140 mM glycine, 4% (w/v) sodium dodecylsulfate, and 1% (v/v) 2-mercaptoethanol with a trace of bromophenol blue, at 90° C for 2 min. Total protein in electrophoresis samples was determined by Lowry assay [25]. Electrophoresis was performed at 27 mA over 3 h. The slab was stained overnight with 0.2% (w/v) Coomassie blue in 20% (v/v) methanol, 10% (v/v) acetic acid in water, and destained in 12.5% (v/v) methanol, 12.5% (v/v) acetic acid in water. After photography it was dried under vacuum with gentle warming onto Whatman No. 1 filter paper, and exposed to Kodak NS-5T film for 48 h. Sample wells are labelled: S, standard protein sample; 1, control; 2, + 1 μM norepinephrine; 3, + 1 μM phenoxybenzamine; 4, + 1 μM propranolol; 5, + 1 μM norepinephrine and 1 μM phenoxybenzamine; 6, + 1 μM norepinephrine and 1 μM propranolol; 7, + 10 μM phenylephrine; 8, + 10 μM isoproterenol. Numbers on the vertical axis label the membrane protein bands according to Ref. 14.

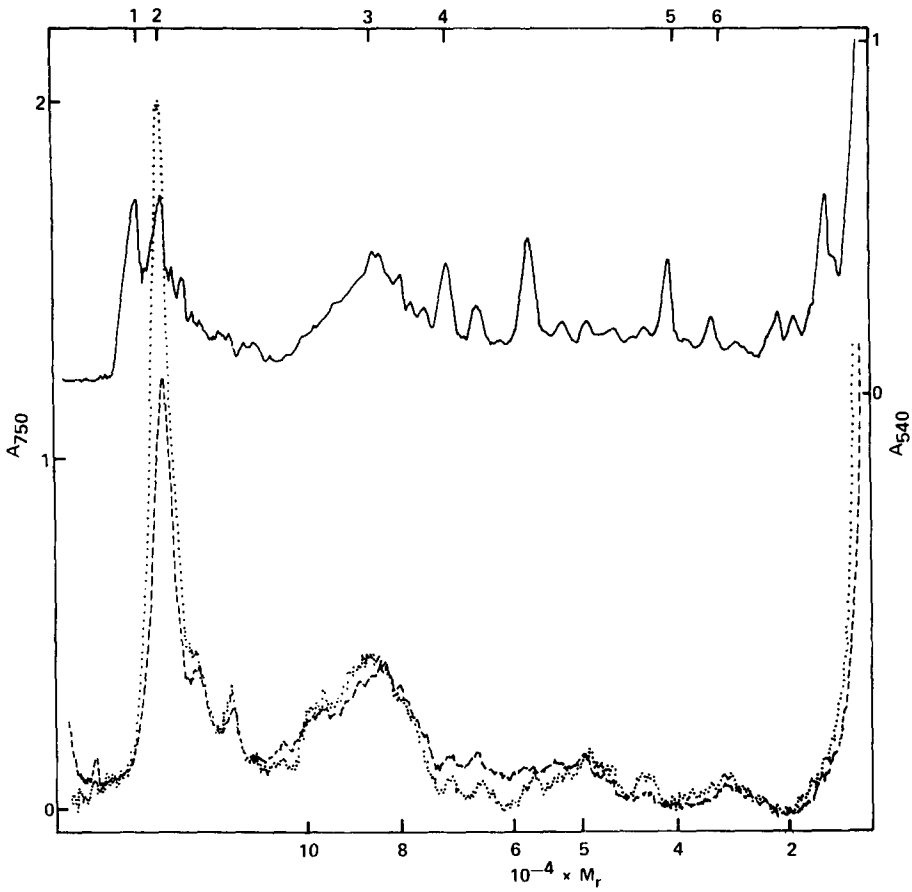

Fig. 2. Enhancement of ^{32}P incorporation into membrane proteins. Individual wells of the autoradiogram were sliced out and scanned at 750 nm using a spectrophotometer tube-gel scanner. A representative well of the Coomassie blue-stained gel was scanned at 540 nm for comparison. Bands are labelled according to the nomenclature of Ref. 14. For display purposes, the vertical scale of the scans of the autoradiogram wells has been adjusted to reflect the normalization of the amount of protein loaded onto the gel as determined by Lowry assay. Coomassie blue (——), autoradiogram of the control sample (- - - -), autoradiogram of the norepinephrine-treated sample (. . . . .). Qualitatively similar results were obtained by slicing and counting tube gels (four trials, data not shown).

Another possible link between hormone-receptor interaction and the enhanced protein phosphorylation is modulation of cyclic nucleotide concentrations. Human erythrocytes possess a low activity catecholamine-stimulated adenylate cyclase [15] and cyclic AMP or cyclic GMP-dependent protein kinases [14, 16]. Furthermore, many of the catecholamine-induced changes in erythrocytes can be mimicked by cyclic AMP or cyclic GMP [2, 17, 18]. However, two lines of reasoning argue against cyclic AMP or cyclic GMP mediation of these catecholamine effects. First, work on erythrocyte ghosts has shown that a kinase which requires cyclic AMP or cyclic GMP phosphorylates bands 2.1 and 4.5, whereas cyclic nucleotide-independent kinases phosphorylate bands 2 and 3. The principle enhancement in phosphorylation noted after α-adrenergic stimulation corresponds to the pattern for cyclic nucleotide-independent kinases, although we cannot exclude some enhance-

ment in the cyclic nucleotide-dependent kinase activity. Second, no significant changes in cyclic AMP or cyclic GMP levels were detected in response to 1μM or 10μM norepinephrine or 1μM isoproterenol, even in the presence of 3-isobutyl-1-methylxanthine, a cyclic nucleotide-phosphodiesterase inhibitor [19]. These findings are consistent with a previous report [18] that 1nM epinephrine has no effect on cyclic AMP levels in human erythrocytes.

Other workers have reported that a protein kinase activity in permeable erythrocyte ghosts is sensitive to β-adrenergic agonists [20]. Moreover, erythrocyte flow properties and osmotic fragility are reported to respond to β-adrenergic control, although no receptor-adenylate cyclase coupling was detected [2]. Our studies with intact erythrocytes provide evidence for α-adrenergic responses.

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