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EVIDENCE THAT CALCIUM ACTS AS AN INTRACELLULAR MESSENGER FOR ADRENERGIC RESPONSES IN HUMAN ERYTHROCYTES

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Summary

Adrenergic stimulation of membrane protein phosphorylation has been studied in human erythrocytes. The adrenergic enhancement in phosphorylation of band 2 could be mimicked by the calcium-specific ionophore A23187 in the presence of 10 μm extracellular calcium. Experiments with the potassium ionophore, valinomycin, showed that potassium efflux was not the primary effector of the response. Trifluoperazine, an inhibitor of the Ca^{2+}-dependent regulatory protein, calmodulin, inhibited phosphorylation stimulation by either norepinephrine or the calcium ionophore. The norepinephrine response was observed in the absence of extracellular calcium, implicating Ca^{2+} released from cellular bound pools in mediating the response.

Introduction

Human erythrocytes incubated with micromolar norepinephine exhibit an increase in [^{32}P]phosphate incorporation into the band 2 component of spectrin [1]. This response is effected specifically by α-adrenergic agents, and it does not involve changes in intracellular levels of ATP, cyclic AMP, or cyclic GMP. Many hormonal and neurotransmitter responses in other eucaryotic cells are mediated by changes in the intracellular concentration of calcium achieved by transmembrane fluxes or by release of the cation from membrane-bound stores. In particular, changes in intracellular calcium levels have been invoked as a mechanism for some α-adrenergic responses [2,3]. The work reported here was undertaken to investigate the role of calcium as an intracellular messenger for adrenergic stimulation of human erythrocytes.

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Erythrocytes bear similarities to other cells in which calcium acts as an intracellular messenger. Such cells generally possess a characteristic ATP-driven calcium transport system which maintains an equilibrium calcium concentration below the threshold required to initiate responses [4]. Such a Ca^{2+}-ATPase is a well-characterized component of human erythrocytes [5], where it maintains intracellular calcium at approx. 1 μM. The intracellular calcium concentration can be elevated above micromolar levels in erythrocytes by metabolic depletion [6], calcium-specific ionophores [7], or lysis and resealing [8], but such treatments have profound morphological and mechanical consequences. Concentrations of 100 μM produce decreased cell volume [9], increased osmotic fragility [10], decreased cell deformability [6], and pathological changes in cell shape [7,11]. These effects are associated with calcium-induced potassium efflux [12], and all can be reversed by restoration of normal intracellular calcium concentrations in the presence of ATP [10]. In contrast, millimolar intracellular calcium produces an irreversible loss of deformability that is due to stimulation of an intracellular transglutaminase [13].

Calcium regulation of cellular processes is mediated in many cases by a Ca^{2+}-dependent regulatory protein, calmodulin, which affects a variety of enzymatic process [14]. The most extensively studied calmodulin is derived from bovine brain, but a protein has been isolated from human erythrocytes which is indistinguishable from brain calmodulin in molecular weight and tryptic peptide pattern [15]. Brain and erythrocyte calmodulins bind calcium with similar affinity (K_D = 8 and 1 μM, respectively [14,15]), both activate Ca^{2+}-transport ATPases [15], and the effects of both are antagonized by phenothiazine antipsychotic agents such as trifluoperazine [16—18].

Many of the erythrocyte properties affected by elevated intracellular calcium concentrations are thought to be under control of the spectrin-actin protein network [19]. Phosphorylation of the band 2 component of spectrin in vivo is believed to affect cell deformability by modifying spectrin-actin association [20,21]. In this work we report the effects of calcium concentration of the phosphorylation of band 2 of spectrin, and present evidence concerning the possible role of the regulatory protein, calmodulin, in mediating adrenergic stimulation of the phosphorylation.

Materials and Methods

A23187 was purchased from Calbiochem and used as a 0.2 mM solution in ethanol. Valinomycin was obtained from Boehringer-Mannhein and used as 1.1 mM solution in 50% (v/v) ethanol. Trifluoperazine dihydrochloride (10-(3-(4-methylpiperazin-1-yl)propyl)-2-trifluoromethylphenothiazine dihydrochloride) was the product of Smith, Kline and French. Norepinephrine hydrochloride was purchased from Sigma Chemical Co., and kept frozen. Solutions were prepared immediately before use. ^{32}P was obtained as $Na_3^{32}PO_4$ from New England Nuclear.

Phosphorylation of the membrane proteins of intact human erythrocytes was carried out as described previously [1]. Washed erythrocytes were depleted of ATP by storage in 0.15 M NaCl at 4°C for 2 days. Phosphorylation was initiated by suspending packed cells in an equal volume of buffer solution con-

taining 0.13 M NaCl, 10 mM KH_2PO_4, 10 mM $MgSO_4$, 10 mM adenosine, 1 mM inosine, 10 mM glucose, and 1 mCi/ml $^{32}P_i$, adjusted to pH 7.4. Unless otherwise stated, the buffer also contained 10 μM $CaCl_2$. Where stated, a buffer of the same constituents, except with 0.13 M KCl and 10 mM NaH_2PO_4 (high-potassium buffer) was employed. Effectors were added as concentrated stock solutions. When effectors were added in ethanol, an equal volume of ethanol was added to all other samples and controls. After 2 h at 37°C, the reaction was quenched by addition of 10 vols. ice-cold 0.15 M NaCl. Membranes were prepared by lysis and washing, both in 10 vols. of distilled water. Electrophoresis, autoradiography and densitometric scanning were performed as described [1] to quantitate levels of radioactivity incorporated into proteins. The amount of incorporation was normalized to the control samples using the ratio of total protein, as determined by using the method of Lowry et al. [22] of an aliquot from the same sample loaded onto the gel. In some cases the non-hemoglobin protein was determined by subtracting the amount of hemoglobin in the sample as measured by A_{412} ($\epsilon = 1.25 \cdot 10^5$ based on 16 000 daltons [23]), and that quantity was used in the normalization procedure.

Results

Incorporation of [^{32}P]phosphate into band 2 of spectrin is shown in Fig. 1 as a function of norepinephrine concentration. Phosphorylation is enhanced in the presence of 10 nM norepinephrine, and enhancement increases with increasing norepinephrine concentration up to 10 μM.

The effect of calcium on band 2 phosphorylation is shown in Figs. 2 and 3. In the absence of the calcium ionophore A23187, phosphate incorporation is relatively insensitive to the Ca^{2+} concentration in the suspending buffer. In the presence of 5 μM calcium ionophore, phosphorylation is increased by added

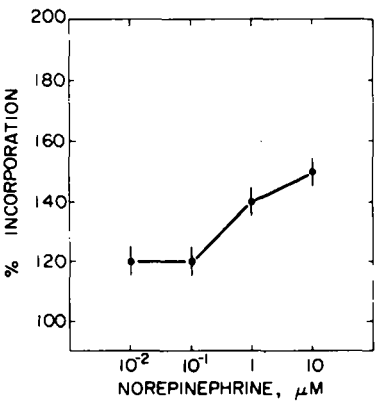

Fig. 1. Norepinephrine-induced stimulation of band 2 incorporation of ^{32}P. Depleted cells incubated with [^{32}P]phosphate were exposed to various concentrations of norepinephrine. The ratio of the amount of ^{32}P found bound to band 2 in the presence and absence of agonist is plotted against norepinephrine concentration. Experimental details are given in the text. Values reported are averages ±S.E. n = 3 for 0.01 and 0.1 μM, 6 for 1 μM and 5 for 10 μM.

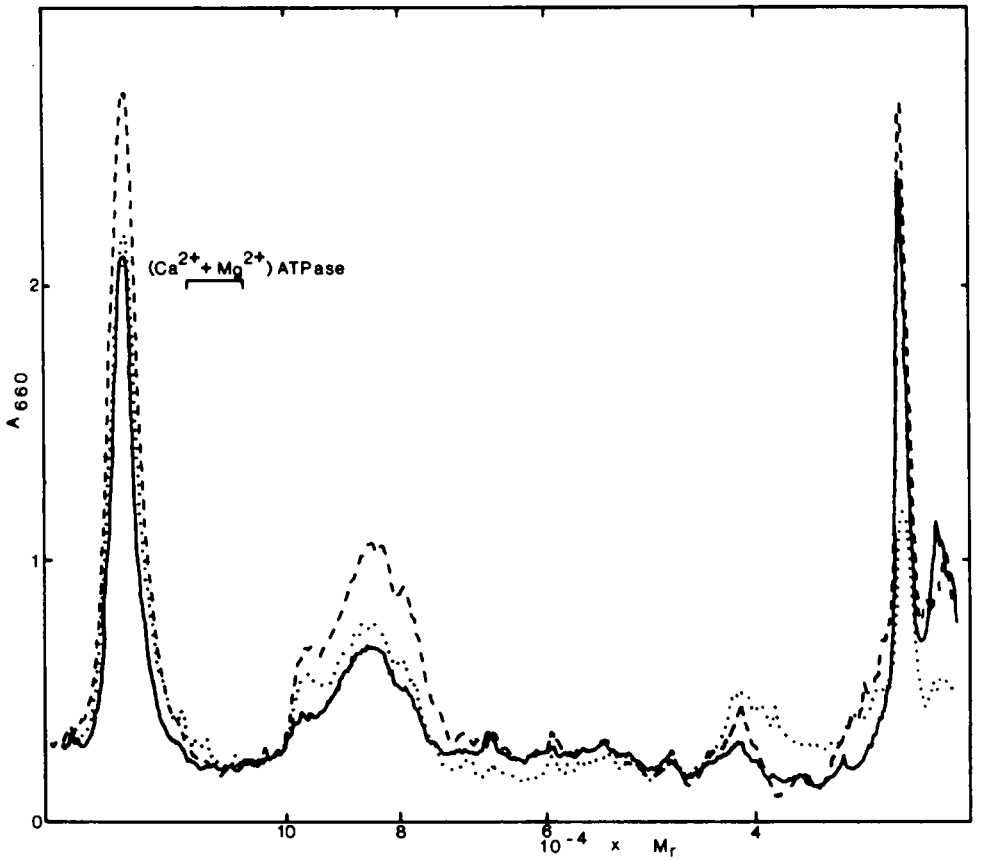

Fig. 2. Incorporation of ^{32}P into membrane proteins. Individual wells of the autoradiogram of the gel were sliced out and scanned at 660 nm using a spectrophotometer tube gel scanner. These traces show the results of a representative experiment. Control sample (———), cells treated with 5 μM A23187 (- - - - -), cells treated with 5 μM A23187 and 10 μM trifluoperazine (· · · · · ·). An approximate molecular weight scale is shown and the region in which the (Ca^{2+} + Mg^{2+})-ATPase is expected (120 000—150 000 daltons) is indicated. The separation between that region and the band 2 peak indicates that the changes in incorporation we observe are not a result of stimulation of that ATPase.

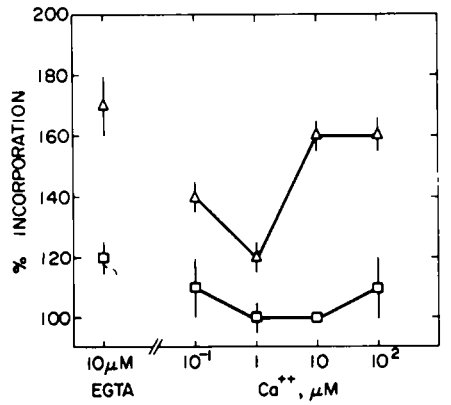

Fig. 3. Ca^{2+} ionophore-induced stimulation of band 2 incorporation of ^{32}P. The relative amount of ^{32}P found bound to band 2 in cells incubated in the presence (△) and absence (□) of 5 mM A23187 is plotted vs. the Ca^{2+} concentration in the external buffer. Values reported are averages ±S.E. $n = 3$ for 10 μM EGTA and 0.1 μM calcium, 4 for 1 and 100 μM calcium, and 8 for 10 μM. The amount of incorporation found with 10 μM Ca^{2+} in the absence of ionophore was defined as 100%.

TABLE I

EFFECT OF VARIOUS AGENTS UPON THE INCORPORATION OF ^{32}P INTO BAND 2

Red cells were incubated as stated in the text in the presence of the listed agents, and relative ^{32}P incorporation into the band 2 component of spectrin (nomenclature of Ref. 30) was measured. 0.13 M Na^+ refers to the normal incubation buffer and 0.13 M K^+ refers to the same buffer in which KCl replaced NaCl, and NaH_2PO_4 replaced KH_2PO_4. Values reported are averages ± S.E. Numbers within parentheses indicate concentration in μM.

Agent	Relative ^{32}P incorporation	n
Control	100	
Valinomycin (1.1) (0.13 M Na^+)	80 ± 2	6
Valinomycin (1.1) (0.13 M K^+)	90 ± 2	4
Trifluoperazine (10)	100 ± 2	7
Norepinephrine (10)	150 ± 5	5
Norepinephrine (10) + trifluoperazine (10)	110 ± 2	7
A23187 (5)	160 ± 5	7
A23187 (5) + trifluoperazine (10)	130 ± 2	6
Norepinephrine (10) + EGTA (10) (no added Ca^{2+})	130 ± 2	4
A23187 (5) (0.13 M K^+)	140 ± 3	5

calcium in the concentration range from 100 nM to 100 μM. The increase is smallest at micromolar calcium, greater at both higher and lower calcium concentrations, and greatest at very low calcium levels (10 μM EGTA and no added $CaCl_2$). The effects of valinomycin (a potassium ionophore) and trifluoperazine (a calmodulin inhibitor) on band 2 phosphorylation are summarized in Table I. Valinomycin decreases band 2 phosphorylation when cells are incubated in the usual high-sodium buffer. When cells are incubated in buffer containing 130 mM KCl and 5 mM NaH_2PO_4 (reversing the concentrations of Na^+ and K^+), valinomycin has less effect on the phosphorylation of band 2. In the same high-potassium buffer, A23187 stimulates the phosphorylation of spectrin to about the same extent as in the normal high-sodium buffer. The calmodulin inhibitor, trifluoperazine [16], antagonizes the stimulatory effects of both norepinephrine and the calcium ionophore.

Erythrocyte proteins other than band 2 are also phosphorylated more extensively in the presence of norepinephrine or the calcium ionophore (data not shown). This effect is most evident for band 3, but is quantitatively less reproducible than that for band 2.

Discussion

Effects of norepinephrine on spectrin phosphorylation

Incubation of whole human erythrocytes with norepinephrine results in an increase in the amount of ^{32}P incorporated into band 2 of spectrin. Enhancement can be detected at norepinephrine concentrations as low as 10 nM, but the response is most apparent for concentrations greater than micromolar (Fig. 1). The concentration of norepinephrine required to initiate neurophysiological responses in systems having α-adrenergic receptors is typically 0.1—1 μM [24]. We have shown previously that the pharmacological characteristics of the

spectrin phosphorylation response and the absence of changes in cyclic AMP or cyclic GMP levels upon adrenergic stimulation of these cells support the suggestion that the erythrocyte response is mediated by an α-adrenergic receptor [1].

Effects of Ca^{2+} on spectrin phosphorylation

Smooth muscle α-adrenergic receptors frequently utilize changes in intracellular calcium concentrations to initiate cellular responses [2]. In human erythrocytes, the adrenergic phosphorylation response can be mimicked by altering intracellular calcium levels using the calcium ionophore A23187. The free intracellular Ca^{2+} concentration in normal erythrocytes is approx. 1 μM [25]. In the presence of 1 μM extracellular calcium, the ionophore has little effect on membrane protein phosphorylation. Increasing extracellular calcium in the presence of the ionophore produces enhanced spectrin phosphorylation. The ionophore also enhances phosphorylation if the added calcium concentration is less than micromolar. Presumably, the effect of the ionophore in the latter case is to deplete the cells of their free Ca^{2+} pool. A spectrin kinase from erythrocytes is inhibited in vitro by free Ca^{2+} in the range from 100 nM to 1 mM [26], so calcium depletion to below micromolar concentration may stimulate this activity in vivo. However, our observation of enhanced phosphorylation at higher calcium concentrations must be accounted for by a separate stimulatory mechanism (vide infra).

Elevation of the internal calcium concentration produces a phosphorylation response similar to that seen with norepinephrine. The norepinephrine response is observed even when the cells are suspended in 10 μM EGTA, forestalling a Ca^{2+} influx from the buffer. Thus, if increases in the Ca^{2+} concentration are the intracellular messenger for norepinephrine, they must arise from release of Ca^{2+} from cellular bound pools rather than from permeability changes in the membrane which admit exogenous ions.

Mechanistic studies of the phosphorylation responses

Several mechanisms might account for the effects of norepinephrine and calcium on band 2 phosphorylation. First, calcium influx is known to be coupled to potassium efflux (the Gárdos effect [7]). There is evidence that this potassium efflux is responsible for calcium-induced morphological changes in erythrocytes [10]. The effects of K^+ efflux on band 2 phosphorylation were investigated by inducing an independent efflux with the potassium-specific ionophore valinomycin. In spite of a possible concomitant calcium influx, the effect of valinomycin was to suppress ^{32}P incorporation, rather than to enhance it. Under conditions which did not result in potassium efflux (suspension of the cells in high-potassium buffer), valinomycin had no effect on ^{32}P incorporation. Spectrin kinase has been shown to require monovalent cations in vitro [27], which may account for these observations. Additionally, the magnitude of the Ca^{2+}-induced enhancement of spectrin phorphorylation is not affected when the A23187-treated erythrocytes are incubated in the high-potassium buffer (conditions which inhibit the Gárdos effect [10]). Therefore, potassium efflux cannot be the mechanism of the calcium-induced phosphorylation response of erythrocytes.

A second mechanism whereby calcium and norepinephrine might enhance

protein phosphorylation is through activation of calmodulin, the calcium-dependent regulatory protein which is known to modulate protein kinase activity in other types of cells [14]. The principle evidence in support of this mechanism is the effect of trifluoperazine, a member of the class of phenothiazine drugs. Phenothiazines have been shown to inhibit a variety of the activities of bovine brain calmodulin [16,17] and the calmodulin-sensitive (Ca^{2+} + Mg^{2+})-ATPase activity of human erythrocytes [18]. In the same concentration range where trifluoperazine inhibits calmodulin, it inhibits both the calcium and norepinephrine stimulation of band 2 phosphorylation (Fig. 2, Table I). Whether this inhibition is related to calmodulin-protein kinase interaction remains to be demonstrated; indeed, erythocryte calmodulin appears not to interact with spectrin kinase in vitro [15]. However, the fact that the calcium and norepinephrine responses are inhibited in the same manner by this agent supports the hypothesis that calcium acts as a messenger for the norepinephrine response, and that both norepinephrine and calcium effects are mediated by calmodulin. We therefore suggest that such a calmodulin stimulation of phosphorylation counteracts the Ca^{2+} inhibition of the kinase observed in presumably calmodulin-free systems [17].

The inverse effects of calcium and potassium depletion on band 2 phosphorylation are consistent with the in vitro cation requirements of spectrin kinase, suggesting that the effects we observe are the results of regulation of a kinase activity. However, the experiments described here do not distinguish between changes in net phosphate incorporation into protein and in the rate of bound phosphate turnover. Stimulation of spectrin kinase could increase bound radioactive phosphate by either mechanism. If accelerated turnover were responsible, increased phosphatase activity also would be implicated. The present data provide no evidence concerning such an increase.

Spectrin phosphorylation and the physiological properties of erythrocytes

It has been suggested [19,20] that spectrin phosphorylation controls spectrin-actin association in erythrocytes, an interaction which is thought to affect the physical properties of the membrane [6,19,28,29]. Calcium is known to affect cell morphology and deformability profoundly in the same concentration range where we observe enhancement in spectrin phosphorylation. Current studies in this laboratory have shown that manipulation of membrane 'fluidity' by independent means may produce countering changes in spectrin phosphorylation (Ferrell, J.E., unpublished results). This raises the intriguing possibility that the spectrin-actin complex may modulate membrane properties in response to a variety of environmental factors. Further work is needed to explore possible mechanistic relationships between spectrin-actin association and the physiological properties of the cell.

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