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Hormone and adenosine 3′, 5′ cyclic monophosphate stimulated phosphorylation of human erythrocyte membranes

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ADENOSINE 3′, 5′ cyclic monophosphate (cyclic AMP) has now been implicated in a large number of control mechanisms in the cell.[1] The widespread distribution of cyclic AMP-dependent protein kinases led Kuo and Greengard[2] to postulate that the primary function of cyclic AMP was to stimulate phosphorylation of specific proteins. *In vitro* a variety of proteins serve as substrate for cyclic AMP-dependent protein kinases: histones,[3] phosphorylase kinase,[4] ribosomal proteins[5] and troponin.[6] However with the exception of phosphorylase kinase[4] the cyclic AMP stimulated phosphorylation has not been associated with any specific modulation of biological activity.

We therefore decided to study the cyclic AMP-stimulated phosphorylation of the erythrocyte membrane and its possible role in the regulation of cation transport in the intact cell.

MATERIALS AND METHODS

ATP and cyclic AMP were purchased from Sigma (London) Chemicals Co. Ltd, London, S.W.6, U.K. and N^6, O^2-dibutyryl cyclic AMP from Boehringer Corp., London Ltd, London, W.5, U.K. Acetyl choline and noradrenaline were supplied by Koch-Light Laboratories Ltd, Colnbrook, Bucks, U.K. and theophylline by BDH (Chemicals) Ltd, Poole, Dorset, U.K. The sodium salt of (γ-^{32}P) ATP (2200–3000 mCi/m-mole) and [^{32}P] Pi orthophosphate (30 Ci/g phosphorus) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Prostaglandin E_1 and E_2 were kindly supplied by Dr. J. E. Pike, The Upjohn Co., Kalamazoo, Michigan 49001, U.S.A. and persantin, RA 233 and RA 433 were a generous gift from Boehringer Ingelheim Ltd, Isleworth House, Great West Road, Isleworth, Middlesex, U.K.

The erythrocytes were prepared from recently expired acid–citrate–dextrose blood supplied by the Clinical Pathology Department, Manchester Royal Infirmary. Fresh human blood was obtained from local volunteers by venepuncture and fresh rat blood by cardiac puncture. Citrate was the anti-coagulant when fresh blood was taken.

Erythrocyte membranes were prepared by washing the ghosts six times with hypothonic sodium phosphate buffer as previously described by Duffy and Schwarz[7] except no EDTA was included in the washing buffers. Phosphorylation of the isolated membranes was carried out by the method of Duffy and Schwarz.[8] For the phosphorylation of intact erythrocyte membranes, red blood cells were allowed to synthesize their own [γ-^{32}P] ATP from [^{32}P] Pi (1 μCl/ml whole blood) added to fresh whole blood. The incubation was carried out at 37° for 45 min in a shaking water bath. The erythrocytes were freed of extracellular [^{32}P] Pi, plasma and white cells by washing four times with ice cold isotonic choline chloride. They were then suspended in an isotonic solution of the following composition: NaCl 135 mM, KCl 5·0 mM, $MgCl_2$ 1·0 mM, $CaCl_2$ 1mM, Na_2HPO_4 2·5 mM and glucose 11 mM. The dibutyryl derivative of cyclic AMP (1 mM) was added and incubation was carried out at 37° for 45 min. The reaction was terminated by the addition of 60 mOsm ice cold sodium phosphate buffer (pH 7·2). The membranes were then prepared as described[7] except that ice cold washing buffers were used and 1 mM carrier ATP was added to the last two washes. Protein was measured by the method of Lowry *et al.*,[9] using crystalline bovine serum albumin as standard.

RESULTS AND DISCUSSION

Cyclic AMP stimulated phosphorylation by 166 per cent in the isolated membranes and its dibutyryl derivative by 49 per cent in membranes of intact cells (Table 1). Separation of the lipid and protein fractions in the isolated membranes by the method of Hajra *et al.*,[10] showed the stimulatory effect of cyclic AMP was confined to protein (Table 1). Cyclic AMP had no effect on the dephosphorylation of the membrane as has recently been reported for toad bladder plasma membranes.[11]

TABLE 1. EFFECT OF CYCLIC AMP AND DIBUTYRYL CYCLIC AMP ON PHOSPHORYLATION OF ERYTHROCYTE MEMBRANES

Substrate	^{32}P Incorporation into membrane (cpm/mg membrane protein)		Stimulation by cyclic AMP (%)
	minus C-AMP	plus C-AMP	
Isolated membranes	3056 ± 102·7 (6)	8134 ± 379·4 (6)	166·2
Intact cell membranes	103 ± 2·1 (4)	154 ± 3·3 (4)	49·4
Membrane protein	2556 ± 133·4 (4)	7592 ± 482·1 (4)	197·0
Membrane lipid	463 ± 37·6 (4)	476 ± 44·5 (4)	—

Values are means ± S.E.M. with number of observations given in parenthesis.

TABLE 2. EFFECT OF HORMONES ON THE PHOSPHORYLATION OF MEMBRANES IN INTACT ERYTHROCYTES

Hormone	Human erythrocytes ^{32}P Incorporation (cpm/mg membrane protein)	Stimulation (%)	Rat erythrocytes ^{32}P Incorporation (cpm/mg membrane protein)	Stimulation (%)
Control	106·0 ± 2·8	—	87·8 ± 1·5	
Noradrenaline (10^{-4} M)	141·3 ± 3·5	33·3	126·7 ± 2·8	44·3
Prostaglandin E_2 (10^{-6} M)	124·4 ± 3·9	17·4	118·1 ± 1·3	34·5
Prostaglandin E_1 (10^{-6} M)	—		121·2 ± 1·7	38·0

Values are means of four experiments ± S.E.M.

Noradrenaline and prostaglandins which increase adenyl cyclase in rat erythrocytes[12] stimulated phosphorylation of the intact erythrocyte membrane from humans and rats (Table 2). Acetyl choline (10^{-6} M) which has been shown to stimulate ^{32}P incorporation into phospholipids[13] had no effect in these experiments. It is probable that noradrenaline and the prostaglandins stimulated phosphorylation via the endogenous adenyl cyclase–cyclic AMP–protein kinase system.

The dibutyryl cyclic AMP stimulated phosphorylation in the intact cell was increased in the presence of phosphodiesterase inhibitors. The control value of 50 per cent stimulation was increased to 71·5 per cent by theophylline (5 mM), 64·8 per cent by persantin* (0·25 mM) 66·8 per cent by RA 233† (0·25 mM) and 68·5 per cent by RA 433‡ (0·25 mM). These compounds had no effect on phosphorylation in the absence of dibutyryl cyclic AMP.

* 2,6-*bis*-(Diethanolamino)-4,8-dipiperidino-pyrimido-(5,4-*d*)pyrimidine.
† 2,6-*bis*-(Diethanolamino)-4-piperidino-pyrimido-(5,4-*d*)pyrimidine.
‡ 2,4,6-Trimorpholinopyrimido(5,4-*d*)pyrimidine.

Since cyclic AMP is widely believed to stimulate active transport of Na^+ in the toad bladder[14] and may control passive Na^+ permeability in the frog photoreceptor[15] attempts were made to correlate the cyclic AMP stimulated phosphorylation with both active and passive transport of Na^+ in the erythrocyte. However neither cyclic AMP nor its dibutyryl derivative had any effect on Na^+ transport in the present investigation. The failure of cyclic AMP to stimulate active Na^+ efflux is in agreement with the finding that the nucleotide did not enhance that Na^+–K^+–ATP ase activity in isolated erythrocyte membranes or the phosphorylation of microsomal Na^+–K^+–ATP ase from pig brain (M. J. Duffy and V. Schwarz, unpublished). It is also of interest to note here that in erythrocytes from cystic fibrosis patients where a defect in the active transport of Na^+ exists,[16] cyclic AMP-stimulated protein kinase is normal.[8] These findings seem to eliminate the possibility that cyclic AMP controls active Na^+ transport via phosphorylation in the erythrocyte.

The results presented in this paper show that the erythrocyte can be used as a useful model system to study cyclic AMP and hormone stimuated phosphorylation of membranes. However it was not a useful system to examine the effects of these compounds on Na^+ transport.

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