Adenylate Cyclase Activity in Lymphocyte Subcellular Fractions

CHARACTERIZATION OF NON-NUCLEAR ADENYLATE CYCLASE

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Human peripheral lymphocytes were broken in a Dounce homogenizer and subcellular fractions enriched in plasma membranes or microsomal particles and mitochondria were isolated by centrifugation through a discontinuous sucrose gradient. Various agents that promote cyclic AMP accumulation in intact lymphocytes were compared in their ability to stimulate adenylate cyclase activity in the individual fractions. Plasma-membrane-rich fractions that were essentially free of other subcellular particles as judged by electron microscopy and marker enzyme measurements responded to fluoride, but weakly or not at all to prostaglandin E_1 and other prostaglandins. Microsomal and mitochondrial-rich fractions responded markedly to both prostaglandin E_1 and fluoride. In some, but not all, experiments phytohaemagglutinin produced a modest increase in enzyme activity in plasma-membrane-rich fractions. Catecholamines, histamine, parathyrin, glucagon and corticotropin produced little or no response. In the absence of theophylline, adenosine ($1-10\ \mu\text{M}$) stimulated basal enzyme activity, although at higher concentrations the responses to prostaglandin E_1 and fluoride were inhibited. GTP ($1-100\ \mu\text{M}$) and GMP ($5-1000\ \mu\text{M}$) respectively inhibited or stimulated the response to fluoride, whereas the converse was true with prostaglandin E_1 .

We have previously demonstrated that in intact human peripheral blood lymphocytes an increase in cyclic AMP is obtainable with phytohaemagglutinin, prostaglandin E_1 , isoproterenol and adrenaline (Smith *et al.*, 1970, 1971; Parker, 1974) with 1.5-3.0-fold, 10-30-fold and 6-20-fold increases in cyclic AMP respectively. All three classes of agents stimulate adenylate cyclase activity in crude lymphocyte homogenates, but because of the marked lability of the enzyme and the limited quantities of tissue available, no detailed study characterizing the enzyme has been published. In the present report subcellular fractions of human lymphocytes were prepared and the response of their adenylate cyclase to prostaglandin E_1 , fluoride, phytohaemagglutinin and other stimuli analysed.

Materials and Methods

Materials

L-Adrenaline hydrochloride and DL-isoproterenol hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) were freshly dissolved and diluted in 50 mM-Tris/HCl/5 mM-MgCl₂ buffer, pH 7.5 (Tris/Mg²⁺ buffer), and the pH was re-adjusted to 7.5 with 100 mM-NaOH if necessary. NaF (Sigma) was dissolved in Tris/Mg²⁺ buffer. Purified erythroagglutinating phytohaemagglutinin (MR-68)

(Burroughs Welcome Co., Research Triangle Park, NC, U.S.A.) was dissolved in Tris/Mg²⁺ buffer at concentrations of 200 or 100 $\mu\text{g}/\text{ml}$ and equilibrated with the appropriate buffer by dialysis. Unless otherwise specified the dialysis fluid contained 50 mM-Tris/HCl/1 mM-CaCl₂, pH 7.5 (Tris/Ca²⁺ buffer), and dilutions of phytohaemagglutinin were prepared in the same medium. Ca²⁺ was included in the medium because of reports that phytohaemagglutinin binding to intact cells is not optimal unless Ca²⁺ is present (Kay, 1971; Lindahl-Kiessling, 1972). Stock 3 mM solutions of prostaglandin E_1 and other prostaglandins (obtained through the courtesy of Dr. John Pike, Upjohn Co., Kalamazoo, MI, U.S.A.) were prepared every 7-10 days by dissolving the free acid in 95% (v/v) ethanol/2 mM-Na₂CO₃ (1:9, v/v). Portions of stock solutions were diluted with Tris/Mg²⁺ buffer on the day of the experiment. The solutions were adjusted to pH 7.5 before use. As a control an equimolar solution of acetic acid in 95% ethanol/2 mM-Na₂CO₃ (1:9, v/v) was utilized to exclude non-specific effects of the buffer or the organic solvent on measurements of enzyme activity or cyclic AMP. Prostaglandin E_1 concentrations above 120 μM were not used because of an apparent stimulation of the enzyme at final ethanol concentrations above 0.4% (v/v). Other reagents and their sources were:

bovine serum albumin (Miles Laboratories, Kanakee, IL, U.S.A.); Ficoll (approx. mol.wt. 400000), creatinine phosphate, creatinine kinase, phosphoenolpyruvate, pyruvate kinase, NADH, GTP, GDP, 5'-GMP, 3-phosphoglyceric acid, 3-phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12), bovine corticotropin (adrenocorticotrophic hormone; 150 units/mg), bovine parathyrin (parathyroid hormone; 250 units/mg), glucagon, calf thymus DNA, cytochrome *c*, dextran (mol.wt. 500000) and lactoperoxidase (Sigma); 5'-[³H]AMP (10 Ci/mmol) and cyclic [³H]AMP (24 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.); Palladium Black, EDTA, EGTA and L-histamine hydrochloride (Fisher Scientific, St. Louis, MO, U.S.A.); theophylline (Schwarz/Mann, Orangeburg, NY, U.S.A.); Hypaque (Winthrop Laboratories, New York, NY, U.S.A.).

Preparation of lymphocytes

Blood (500 ml) was obtained by venipuncture from normal healthy human volunteers. The blood was collected in a flask containing heparin at a final concentration of 10 units/ml of blood. Dextran was added to a final concentration of 1.2% (w/v) and the leucocytes were separated from erythrocytes by gravity sedimentation at 37°C for 1 h. Portions (20 ml) of the leucocyte-rich supernatant fraction were layered over 5 ml of a Ficoll/Hypaque mixture [3.53 ml of 9% (w/v) Ficoll in water, 1.00 ml of 50% (w/v) Hypaque and 0.47 ml of water] in a 50 ml conical centrifuge tube. A purified lymphocyte fraction in which 93–98% of the nucleated cells were lymphocytes was obtained by isopycnic centrifugation as described previously (Eisen *et al.*, 1972). The overall recovery of lymphocytes was 50–75%. Platelets were removed by centrifugation at 100g at room temperature for 7 min (preparations contained about one platelet per nucleated cell). In a few experiments lymphocytes were purified additionally by filtration through a nylon-wool column as described previously (Smith *et al.*, 1971). This procedure provides essentially pure lymphocytes, but results in a low overall recovery and a selective loss of bone-marrow-derived (B) lymphocytes.

Disruption of lymphocytes

Cell disruption and all subsequent fractionation steps were carried out at 4°C. The cells were broken by a modification of the procedure of Fisher & Mueller (1971). Preparatory to disruption the cells were washed once with Tris/Mg²⁺ buffer (see above), containing 2 mM-theophylline (Tris/Mg²⁺/theophylline buffer). They were resuspended in the same buffer at 125 × 10⁶ cells/ml and left to equilibrate for 15 min at 0°C to permit cell swelling. Cell disruption was accomplished in a tight-fitting (type B) 7 ml Dounce homogenizer (150 strokes)

(Kontes, Vineland, NJ, U.S.A.). This procedure gave a satisfactory combination of effective cell disruption, as determined by phase-contrast microscopy, and preservation of adenylate cyclase responsiveness to various stimulatory agents.

Isolation of subcellular fractions

Homogenates were fractionated by two methods.

Method A (direct layering). In early experiments a modification of the procedure of Fisher & Mueller (1971) was used in which 2 ml portions of homogenate were layered directly over a discontinuous gradient of 55 and 30% (w/v) sucrose in Tris/Mg²⁺/theophylline buffer (see above). The gradient was prepared by layering 2 ml of 30% sucrose in buffer over 2 ml of 55% sucrose in buffer in a 6 ml cellulose nitrate centrifuge tube. After addition of the homogenate the tubes were centrifuged at 39000 rev./min for 1 h in a Spinco model L centrifuge in the L-50 head (175000g_{max}).

Method B (partial purification before ultracentrifugation). Fractions more highly enriched in plasma membrane or microsomal components were obtained by subjecting the homogenate to two centrifugation steps before addition to the sucrose gradient. Immediately after homogenization the homogenate was centrifuged at 500g_{av} for 2 min. The supernatant solution was obtained and centrifuged at 16500g_{av} for 15 min. The pellet from the 16500g_{av} centrifugation was resuspended in 10 mM-Tris/HCl/5 mM-MgCl₂/2 mM-theophylline, pH 7.5, by two passes of the Dounce homogenizer; the suspension was then mixed with an equal volume of 40% sucrose in the Tris/Mg²⁺/theophylline buffer, followed by two more strokes of the homogenizer. Then 1.5 ml portions of the resulting suspension in 20% sucrose were layered over 1.5 ml each of 55, 40 and 30% sucrose in Tris/Mg²⁺/theophylline buffer and centrifugation was carried out as in the direct layering procedure.

Individual fractions were removed from the interphases of the gradient and the bottom of the tube by aspiration with a capillary pipette. Fractions were pooled and diluted (if necessary) in the buffer used in the sucrose solutions. Unless otherwise specified 0.01 vol. of 1 M-MgCl₂ was added to each recovered fraction. Thus cell-fraction samples used in the adenylate cyclase assay ordinarily contained 30–55% sucrose, 50 mM-Tris/HCl, 15 mM-MgCl₂ and 2 mM-theophylline. In selected experiments one or more components of the final reaction mixture were omitted. For example, when the effect of bivalent cations on adenylate cyclase activity was being evaluated, the final addition of MgCl₂ was not made and Mg²⁺-free buffers were used during homogenization and subsequent tissue processing. The final volume of the individual sucrose-gradient fractions ranged from 0.75 to 2.3 ml (the total yield

of a given fraction from 500 ml of blood). In selected experiments a portion of the original homogenate was stored at 0°C during the ultracentrifugation step and assayed for adenylate cyclase activity together with the gradient fractions. The fractions from direct-layering experiments (method A) are designated: fraction 1 (F-1) from the buffer/30% sucrose interphase; fraction 2 (F-2) from the 30%/55% sucrose interphase; and fraction 3 (F-3), the 55% sucrose pellet. The fractions obtained by method B (partial purification before ultracentrifugation) are designated: purified fraction 1 (PF-1) from the 20%/30% sucrose interphase; purified fraction 1a (PF-1a) from the 30%/40% sucrose interphase; purified fraction 2 (PF-2) from the 40%/55% sucrose interphase; and purified fraction 3 (PF-3) from the 55% sucrose pellet. Because the membrane preparations were highly labile, fractions were used for adenylate cyclase measurements within 30 min after their recovery from the gradient. Dialysis, freezing, or even storage for 1–2 h at 0°C was associated with substantial losses of enzyme activity.

Measurement of adenylate cyclase activity

Unfractionated homogenates and the various density-gradient fractions were assayed in triplicate for adenylate cyclase activity at final protein concentrations of 240–1200 µg/ml; 30 µl of homogenate (12–60 µg of protein) was rapidly added to a mixture of 10 µl of 5 mM-ATP and 10 µl of buffer (containing, when appropriate, the putative adenylate cyclase stimulator under investigation) in a small glass test tube at 4°C (final volume 50 µl). Buffer control tubes contained 10 µl of either Tris/Mg²⁺ buffer (as a control for solutions of prostaglandin E₁, fluoride and adrenaline) or Tris/Ca²⁺ buffer (as a control for solutions of phytohaemagglutinin). Thus in the experiments with phytohaemagglutinin the final Ca²⁺ concentration was 0.2 mM. Unstimulated enzyme activities were similar in the two buffers. Subsequent incubation was carried out for various time-periods at 37°, 30° or 18°C with intermittent gentle shaking; zero-time control tubes were included in each experiment. The reaction was terminated by boiling for 2 min in a heating block. Under these conditions little or no cyclic AMP is formed from ATP over a broad range of bivalent cation and ATP concentrations. An appropriate volume of chilled 0.05 M-acetate buffer, pH 6.2, was added, the tube was agitated in a vortex mixer and centrifuged at 3000 rev./min (model PR-6000, International Equipment Co.) for 10 min at 4°C. The supernatant was removed and frozen at –70°C.

Cyclic AMP was measured by radioimmunoassay in suitable portions of the supernatant. The sensitivity and specificity of antibody used in the cyclic AMP measurement has been described previously (Steiner *et al.*, 1969; Parker, 1971).

The antibody used in the present study had very low cross-reactivity with ATP (1 in 200 000), but since the highest concentration of ATP used did produce small changes in the binding curve for radio-iodinated cyclic AMP, standard curves were performed in the presence and absence of ATP. In a number of early experiments (not shown) there was excellent correspondence between the Krishna assay for adenylate cyclase (Krishna *et al.*, 1968) and the cyclic AMP immunoassay. Cyclic AMP-like immunoreactivity formed during the incubation of tissue with ATP was shown to co-chromatograph with trace quantities of cyclic [³H]AMP in two different chromatographic systems {Dowex 50 and [QAE] diethyl-2-(hydroxypropyl)-aminoethyl-Sephadex}. No cyclic AMP formation was demonstrated in the absence of ATP. In the amounts present in the immunoassay, the various adenylate cyclase-stimulating agents used in the study did not interfere in the measurement of cyclic AMP. Standard curves for cyclic AMP immunoassay were shown to be unaffected by the various sucrose, bivalent-cation and non-cyclic-nucleotide-containing solutions used in the adenylate cyclase experiments. EDTA inhibition in the assay was overcome by adding an excess of Ca²⁺. Protein was determined in each of the cell fractions (see below). Adenylate cyclase activity is expressed in pmol of cyclic AMP formed/10 min per mg of protein.

In early experiments with partially purified subcellular fractions (20–60 µg of total protein), direct ATP measurements indicated that, at the 1 mM-ATP concentration used in the adenylate cyclase reactions, less than 10% of the original ATP was broken down during a 15 min incubation at 37°C. An ATP-regenerating system was not utilized because the quantity of cyclic AMP formed was not increased in its presence. Under the usual conditions of the assay (2 mM-theophylline present), the incubation of 2–20 pmol of cyclic AMP with membrane fractions for 15 min at 37°C was not associated with significant degradation of cyclic AMP, indicating that phosphodiesterase activity, if present, was completely inhibited.

Measurement of protein, DNA, ATP and marker enzyme activities

Protein was measured in 20% (w/v) trichloroacetic acid precipitates of homogenates and individual cell fractions by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. ATP was determined in a coupled enzyme reaction utilizing 3-phosphoglycerate, 3-phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase and NADH (modified from *Sigma Technical Bulletin* 366). ATP produces a stoichiometric decrease in NADH fluorescence (Aminco Bowman Spectrofluorophotometer; activation 340 nm, emis-

sion 470nm) in this system. DNA was determined by the method of Hinegardner (1971).

Cytochrome *c* oxidase activity was determined at 24°C by the method of Smith (1955) by following changes in A_{550} of a freshly reduced 30 μ M solution of cytochrome *c* in 100mM-phosphate, pH7.0. Reduced cytochrome *c* was obtained by catalytic hydrogenation (Palladium Black catalyst); 50–150 μ g samples of membrane protein were used for each determination. NADH oxidoreductase activity was determined at 24°C by a modification of the method of Massey (1966), using 50–200 μ g samples of membrane fraction per determination and following changes in NAD⁺ fluorescence (activation 340nm, emission 470nm) of a solution containing 0.12mM-NAD⁺ and 0.66mM-K₃Fe(CN)₆ in 15mM-Tris/HCl, pH7.4. 5'-Nucleotidase activity was determined at 37°C by a modification of the cyclic AMP phosphodiesterase assay of d'Armiento *et al.* (1972) in which 5'-[³H]AMP is used instead of labelled cyclic AMP; measurements were made in samples containing 10–50 μ g of protein. Incubations were carried out in 50mM-Tris/HCl/5mM-Mg²⁺ with 5mM-5'-AMP. Lactate dehydrogenase activity was determined in a sample containing 10–50 μ g of protein by a modification of the method of Reeves & Fimognari (1966) in the presence of 3.3mM-K₃Fe(CN)₆ at 1.0mM. Bovine serum albumin was omitted from the incubation mixture. Acid phosphatase was measured by a modification of the method of Michell *et al.* (1970), with incubation at room temperature in acetate buffer, pH4.5, rather than 37°C in pH5.0 buffer. All of the enzyme assays were shown to be proportional to enzyme concentrations over the range of concentrations used.

Statistical significance was evaluated by Student's *t* test; *P* values <0.05 were considered to be statistically significant.

Results

Comparison of cell disruption procedures

Initially we compared several cell-disruption techniques, e.g. nitrogen cavitation in Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]/sucrose buffer (Ferber *et al.*, 1972), homogenization in Belco grinding tubes in the same buffer system, or homogenization in a tight-fitting Dounce homogenizer in either Tris/Mg²⁺/theophylline (modified from Fisher & Mueller, 1971), or 1mM-NaHCO₃/0.5mM-CaCl₂ buffer (Marique & Hildebrand, 1973). Dounce homogenization gave the best combination of cell disruption and preservation of responsiveness to hormones and lectin. The cells were more readily disrupted in NaHCO₃/CaCl₂ than in Tris/Mg²⁺/theophylline buffer (only 30 strokes of the Dounce B pestle required for over 95% cell disruption) and the yield of plasma membranes was improved, but the recovery of other subcellular organelles was not as good. Since we wished to compare responses in different subcellular fractions, Dounce homogenization in Tris/Mg²⁺/theophylline buffer was used as our standard preparative method.

Adenylate cyclase activity in partially purified subcellular fractions

Initially the homogenate was layered directly over 30 and 55% sucrose and centrifuged at 39000rev./min (175000*g*_{max}) for 1h (method A). The subcellular composition of the top two ultracentrifugal fractions (F-1 and F-2) was examined by phase-contrast microscopy and enzymic analysis (Table 1). Fraction F-2 had much higher cytochrome oxidase and NADH oxidoreductase activities than fraction F-1, indicating an enrichment of its mitochondrial and microsomal content. Phase-contrast-microscopy studies indicated that fraction

Table 1. *Characterization of lymphocyte subcellular fractions obtained by either method A or method B*
Protein, DNA and enzyme contents are shown of lymphocyte subcellular fractions obtained by either method A or B. Results are mean of two experiments with method B and three experiments with method A, each involving 500ml of blood. The theoretical recovery of the purified ultracentrifugal fractions is about 10% higher (the portion of the homogenate set aside for analysis). A unit of enzyme activity is 1 nmol/min.

	Method A			Method B				
	Original homo- genate	Fraction		Original homo- genate	Fraction			
		F-1	F-2		PF-1	PF-1a	PF-2	PF-3
Total volume of fraction (ml)	4.4	1.5	1.5	4.4	1.8	1.9	1.8	1.7
Protein (μ g/ml)	5040	1170	880	5200	300	300	560	1800
DNA (μ g/mg of protein)	135	42	16	128	38	30	24	270
Acid phosphatase (units/mg of protein)	44	27	46	50	5	48	42	4.8
5'-Nucleotidase (units/mg of protein)	10.5	44	42	12	132	165	10.6	3.7
NADH oxidoreductase (units/mg of protein)	139	30	518	124	<25	62	880	620
Cytochrome <i>c</i> oxidase (units/mg of protein)	32	<8	360	20	<8	<8	162	181
Lactate dehydrogenase (units/mg of protein)	—	—	—	1010	870	512	280	219

EXPLANATION OF PLATE I

Electron microscopy of isolated subcellular fractions

(a) Fraction PF-1a; (b) original homogenate. Sections for electron microscopy were prepared by successive fixation in 2% glutaraldehyde and 2% OsO₄, followed by dehydration with ethanol, embedding in Epon, sectioning (glass knife), and staining with uranyl acetate and lead acetate. The sections were examined in a Philips EM 200 electron microscope. These studies were kindly performed by Dr. Leonard Jarett and Mr. Bob Smith of the Department of Pathology, Washington University School of Medicine. Fraction PF-1 was also shown to be enriched in plasma membranes. The scale bars represent 1 μ m.

F-1 was considerably enriched in fragments with the appearance of plasma membrane. However, the variable NADH oxidoreductase activity in fraction F-1 and the comparable 5'-nucleotidase activities of fractions F-1 and F-2 make the evidence for plasma-membrane enrichment in fraction F-1 less conclusive.

All three partially purified fractions (F-1, F-2 and F-3) had some adenylate cyclase activity. However, this activity was quantitatively and qualitatively quite different among the fractions. Fraction F-1 responded with a statistically significant increase in cyclic AMP when stimulated by phytohaemagglutinin, fluoride and prostaglandin E_1 , although a response to phytohaemagglutinin was observed in only 60% of experiments. Fraction F-2 had a much greater absolute response to fluoride and prostaglandin E_1 than did fraction F-1, whereas the response to phytohaemagglutinin was decreased or absent. Fraction F-3 responded weakly to prostaglandin E_1 and fluoride and not at all to phytohaemagglutinin.

Neither fraction F-1 nor F-2 exhibited an adenylate cyclase response to glucagon ($0.1\text{--}10\mu\text{M}$), histamine ($10\text{--}1000\mu\text{M}$), corticotropin ($0.04\text{--}4\mu\text{M}$) or parathyrin ($0.04\text{--}4\mu\text{M}$). There was a variable, statistically insignificant response ($P>0.10$) to adrenaline ($1\text{--}10\mu\text{M}$) and isoproterenol ($0.1\text{--}10\text{mM}$) in fraction F-3 and in the unfractionated homogenate. Similar results were obtained in lymphocytes additionally purified by filtration through a nylon-wool column indicating that this response is not due to the small numbers of contaminating cells usually present.

Experiments with more highly purified fractions

When lymphocyte homogenates were subjected to centrifugation at $500g$ and $16500g$ before loading on the gradient (method B), a further enrichment in the plasma-membrane or microsomal enzyme content of individual fractions was obtained (Table 1). Fractions PF-1 and PF-1a had 8–40-fold increases in the specific activity of 5'-nucleotidase relative to the original homogenate. Measurements of cytoplasmic, mitochondrial and lysosomal markers indicated that 5'-nucleotidase was the only enzymic activity that was higher in fraction PF-1 than in the original homogenate. As much as 70% of the 5'-nucleotidase activity was lost in the supernatant after the $16500g$ centrifugation step, but increasing the centrifugal force to $40000g$ to give better recovery resulted in higher amounts of NADH oxidoreductase and cytochrome c oxidase activity in fractions PF-1 and PF-1a. Since the $16500g$ supernatant contains small membranous fragments, presumably derived from plasma membrane, much of the 5'-nucleotidase activity may be contained in these structures. Fractions PF-2 and PF-3 had 3–15-fold increases in NADH oxidoreductase, the relative proportions in the two fractions varying somewhat with the preparation, and were

largely devoid of measurable 5'-nucleotidase activity. These fractions also contained increased cytochrome oxidase activity, the mitochondrial enzyme marker.

The results of the enzyme-marker studies were confirmed by electron microscopy (kindly performed by Dr. Leonard Jarrett and Mr. Bob Smith of the Department of Pathology, Washington University School of Medicine). Fractions PF-1 (not shown) and PF-1a (Plate 1) were composed almost entirely of open or vesicular structures with the typical appearance of plasma membranes. It is unlikely that nuclear membranes constitute a significant proportion of the fragments seen, because the nuclei in the original homogenate still had intact perinuclear membranes. Fractions PF-2 and PF-3 (not shown) were a heterogeneous mixture of cellular organelles, including microsomal particles, endoplasmic reticulum, mitochondria and occasional membranous fragments.

(a) *Responses to fluoride.* The responses of sucrose-density-gradient fractions PF-1, PF-1a, PF-2 and PF-3 to fluoride were characterized with regard to dose, time, temperature, bivalent-cation and GTP

Fig. 1. Effect of increasing concentrations of NaF on adenylate cyclase activity in sucrose-density-gradient fractions PF-1 (Δ), PF-1a ($\blacktriangle$), PF-2 ($\circ$) and PF-3 ($\bullet$). Incubations were carried out for 0 or 15 min at 37°C at a final concentration of 1mM -ATP. The 15 min experimental values are shown in the Figure with the control (zero time) cyclic AMP value subtracted. Adenylate cyclase activity is given in pmol of cyclic AMP/15 min per mg of protein.

dependence. Dose-response curves to fluoride (at constant ATP and Mg^{2+} concentrations) are shown in Fig. 1. The curves for fractions PF-1 and PF-1a resembled one another more closely than they resembled the curves for fractions PF-2 and PF-3 with regard to dose of fluoride for half-maximal stimulation, and the shape of the curve at high fluoride concentrations. When the fluoride response was expressed as pmol of cyclic AMP/15 min per mg of protein above the control, fractions PF-1 and PF-1a usually exhibited a higher specific activity of adenylate cyclase than fractions PF-2 and PF-3, although the latter fractions contained more total enzyme activity. The ATP dose-response curve (at 20mM-fluoride) in fraction PF-3 is shown in Fig. 2. By means of a double-reciprocal plot (not shown) the apparent K_m for ATP was calculated to be about 0.2mM. A similar ATP dose-response curve was obtained for fractions F-1 and F-1a, although the K_m values were slightly higher. The effects of temperature, time and various bivalent cations on responses of adenylate cyclase to 20mM-fluoride were similar in the four fractions. There was a linear increase in activity with increasing temperature over the temperature range studied (18–37°C) (results not shown). Linearity was also obtained with

Fig. 2. Effect of different ATP concentrations on basal adenylate cyclase activity and on activity stimulated by fluoride (20mM; $\circ$) and prostaglandin E_1 (60 μ M; $\bullet$). Membrane suspensions (fraction PF-3) were incubated for 10min at 37°C. Results are means of three experiments. Adenylate cyclase activity is given in pmol of cyclic AMP/10min per mg of protein.

Fig. 3. Effect of increasing Mg^{2+} concentrations on the adenylate cyclase response to fluoride (20mM; $\circ$) and prostaglandin E_1 (60 μ M; $\bullet$).

Lymphocytes were homogenized in Tris/theophylline buffer without $MgCl_2$ and ultracentrifuged through 30 and 55% sucrose dissolved in the same buffer. Mg^{2+} was added as $MgCl_2$, to the final concentrations indicated on the abscissa. Membrane suspensions (fraction PF-3) were incubated for 10min at 37°C in the presence of 1mM-ATP. Δ , Control (basal) enzyme activity. Adenylate cyclase activity is given in pmol of cyclic AMP/10min per mg of protein.

increasing protein concentrations (from 250 to 1250 μ g of protein/ml) and time (for at least 15 min). Adenylate cyclase activity increased with increasing Mg^{2+} concentrations until a 10–20mM concentration was reached (shown for fraction PF-3; Fig. 3). At still higher Mg^{2+} concentrations the response was partially inhibited. Mn^{2+} ions were at least as effective as Mg^{2+} in promoting fluoride stimulation (not shown). Inhibition was observed with increasing Ca^{2+} concentrations over the concentration range 10–10000 μ M- Ca^{2+} (Table 2, shown for fraction PF-3). EGTA (0.2–5mM) increased the fluoride response in fractions PF-2 and PF-3 by as much as 35%. GTP (0.1–100 μ M) failed to enhance fluoride stimulation in any of the four fractions. In fact, as a rule, borderline to significant (10–30%) inhibition was seen. In contrast with GTP, 5'-GMP (1–100 μ M) and guanosine (100 μ M) stimulated the fluoride response (not shown). Both agents had only a modest stimulatory effect on enzyme activity in the absence of fluoride.

(b) Responses to prostaglandins. Virtually all of the response to prostaglandin E_1 was in fractions PF-2 and PF-3, supporting the tentative conclusion reached in studies with the partially purified fractions

Table 2. *Effect of Ca^{2+} and EGTA concentrations on stimulated ($60\mu\text{M}$ -prostaglandin E_1 or 20mM - NaF) and unstimulated (basal) adenylate cyclase activity in fraction PF-3 (two separate experiments)*

Purified subcellular fractions were obtained by method B as outlined in the Materials and Methods section. Portions ($30\mu\text{l}$) of fraction PF-3 were incubated for 15 min at 37°C in the absence or presence of stimulator ($60\mu\text{M}$ -prostaglandin E_1 or 20mM - NaF). Enzyme activity is given in pmol of cyclic AMP/15 min per mg of protein. The final Mg^{2+} and ATP concentrations were 5 and 1 mM respectively. The results shown in Tables 2–6 are from one experiment but similar results were obtained from at least two other experiments. The experimental variability among triplicates was almost always less than 10% and usually less than 5% in the individual experiments.

log [Ca^{2+} concn. (μM)]	Unstimulated activity	Stimulated activity	
		60M-Prosta-glandin E_1	20mM- NaF
None	252	897	912
1	225	792	840
2	205	665	829
3	165	361	761
4	107	200	496
log [EGTA concn. (μM)]			
None	150	405	730
1	157	430	752
2	178	477	796
3	232	590	861

F-1 and F-2, that this response originates in microsomal particles or mitochondria (Table 3, GTP not present). In some experiments the NADH oxidoreductase activity was greater in fraction PF-3 than fraction PF-2 and this was associated with an unusually marked prostaglandin E_1 response in fraction PF-3.

The response to prostaglandin E_1 ($60\mu\text{M}$) was optimal between 2 and 10mM - Mn^{2+} (not shown) and 3 and 20mM - Mg^{2+} (Fig. 3). Stimulation was inhibited at 0.1mM - Ca^{2+} and above (Table 2). The three bivalent cations affected responsiveness to prostaglandin E_1 similarly in the various fractions. When responses to prostaglandins E_1 , A_1 and $\text{F}_{2\alpha}$ were compared over a 1000-fold concentration range the order of responsiveness was $\text{E}_1 > \text{A}_1 > \text{F}_{2\alpha}$ (Table 4). The same order of responsiveness is obtained in whole lymphocytes when prostaglandin stimulation of cyclic AMP accumulation is measured (Smith *et al.*, 1971). The apparent K_m for ATP in the prostaglandin E_1 response was about 0.8mM (shown for fraction PF-3 in Fig. 2).

The different order of responsiveness to prostaglandin E_1 and fluoride in fractions PF-1 and PF-1a,

Table 3. *Effect of GTP concentration on prostaglandin-responsive adenylate cyclase activity in purified lymphocyte subcellular fractions*

Fractions were obtained by method B as described in the Materials and Methods section. A $30\mu\text{l}$ portion of each fraction (240 – $1200\mu\text{g}$ of protein/ml) was incubated for 10 min at 37°C with $60\mu\text{M}$ -prostaglandin E_1 or buffer in the presence of 1mM -ATP and various concentrations of GTP. GTP alone (1 and $100\mu\text{M}$) had essentially no effect on measured cyclic AMP values in the various fractions (determined at zero time). Adenylate cyclase activity is given in pmol of cyclic AMP/10 min per mg of protein above buffer control. Results from one experiment done in triplicate. Similar results were obtained in two other experiments.

log [GTP concn. (μM)]	Fraction			
	PF-1	PF-1a	PF-2	PF-3
None	68	161	332	880
-1	82	229	399	906
0	102	312	557	931
1	163	355	766	970
2	181	374	802	985

Table 4. *Stimulation of adenylate cyclase activity in fraction PF-3 by three different prostaglandins*

Experimental conditions are as given in the legend to Table 2 except that the incubation time was 10 min. Adenylate cyclase activity is expressed in pmol of cyclic AMP formed/10 min per mg of protein. The activity in the buffer control was 207pmol of cyclic AMP/10 min per mg of protein.

log [Prostaglandin concn. (μM)]	Prostaglandin		
	E_1	A_1	$\text{F}_{2\alpha}$
-1	341	225	206
0	620	464	225
1	939	713	380
2	996	852	565

as compared with fractions PF-2 and PF-3 (Figs. 1 and 2), suggested that adenylate cyclase activity was contained in multiple subcellular fractions. However, the possibility had to be considered that fractions PF-1 and PF-1a lacked a cofactor necessary for responsiveness to prostaglandin E_1 but not to fluoride, or contained a factor that inhibited the response to prostaglandin E_1 . The response in all fractions was increased by GTP at concentrations in the range 0.1 – $200\mu\text{M}$ with half-maximal stimulation at about 2 – $5\mu\text{M}$ -GTP (Table 3). The effect of GTP on prostaglandin E_1 responsiveness was considerably more marked in fractions PF-1, PF-1a and PF-2 than in fraction PF-3, but even at optimum GTP concentrations the response was still maximal in fraction PF-3. GTP also exerted modest effects on

basal enzyme activity. GDP exhibited less stimulation and 5'-GMP and guanosine either failed to stimulate or were inhibitory (shown for fraction PF-2 in Table 5). (This is especially true if the stimulatory effect of GMP itself on adenylate cyclase activity is considered.) Inhibitory effects of GMP were seen at concentrations as low as 5 μM . GTP has been shown to increase the effect of glucagon and adrenaline in liver (Rodbell *et al.*, 1971; Swislocki *et al.*, 1973) and of prostaglandin E_1 in platelets (Krishna *et al.*, 1972) and pancreatic islets (Johnson *et al.*, 1974) and similar effects are seen in a variety of other tissues. In accord with results with other tissues where GTP is stimulatory, guanylyl-5'-yl imidodiphosphate and ITP also augmented responsiveness to prostaglandin E_1 and basal activity (Table 5).

The possible role of soluble inhibitors of the response to prostaglandin E_1 or accelerated cyclic AMP breakdown in fractions PF-1 and PF-1a was also considered. However, mixing experiments in which fractions PF-1 and PF-1a were recombined with fraction PF-3 failed to reveal consistent inhibitory effects, although modest inhibition was observed in several experiments (results not shown). The response to prostaglandin E_1 was linear with time for at least 15 min in all of the fractions, indicating that loss of substrate or accelerated destruction of cyclic AMP does not explain the greater response in fractions PF-2 and PF-3.

(c) *Responses to adrenaline, isoproterenol and phytohaemagglutinin.* No consistent response to

catecholamines or phytohaemagglutinin was observed in any of the four fractions. In fraction PF-3, adrenaline (10 μM) stimulated a mean increase in adenylate cyclase activity of $20 \pm 7\%$ (S.E.M.; seven experiments, not shown) ($P > 0.10$). In Tris/ Mg^{2+} /theophylline buffer, phytohaemagglutinin produced significant stimulation of adenylate cyclase activity in the plasma-membrane-rich fractions (PF-1 and PF-1a) in only 9 of 26 experiments, with a response in fractions PF-2 and PF-3 in only two of 15 experiments. When the data from the various experiments were pooled, none of the purified fractions responded significantly to phytohaemagglutinin. Somewhat more frequent responses (five of ten experiments) were seen in a 10mM-EDTA/5mM- CaCl_2 buffer system, but even here the degree of stimulation was of borderline significance statistically. The failure to observe a more consistent response to phytohaemagglutinin in purified plasma membranes may indicate that further inactivation of the highly active enzyme is taking place during the more extensive method B purification.

(d) *Effects of adenosine.* High (10–1000 μM) concentrations of adenosine consistently inhibited the responses to both prostaglandin E_1 and fluoride not shown. However, when theophylline was absent from the medium, low concentrations of adenosine stimulated cyclic AMP formation (three of four experiments, not shown).

Discussion

The results of the present study indicate that there is considerable adenylate cyclase activity in human lymphocyte homogenates, with marked responses to fluoride and several of the prostaglandins. Of the various prostaglandins, prostaglandin E_1 was the most effective stimulator, in accord with its greater effectiveness in increasing cyclic AMP in intact human lymphocytes and its greater potency as an inhibitor of DNA synthesis in lymphocytes stimulated by lectins. Since isoproterenol and theophylline also increase cyclic AMP and inhibit DNA synthesis, it seems likely that increases in intracellular cyclic AMP explain the ability of prostaglandins to inhibit mitogenesis.

Adenylate cyclase responsiveness in lymphocytes was inhibited by Ca^{2+} concentrations as low as 10–100 μM and enhanced at comparable concentrations of EGTA. The stimulatory effect of EGTA is presumably due to its ability selectively to chelate the Ca^{2+} present as a contaminant in the salts used to make up adenylate cyclase assay medium. The inhibitory effect of Ca^{2+} is of interest with regard to the changes in cyclic AMP that occur with time in lymphocytes stimulated with mitogenic lectins. After the early increase in cyclic AMP (see the introduction) cyclic AMP decreases over the next

Table 5. *Effect of various nucleotides on the adenylate cyclase activity in response to prostaglandin E_1 in fraction PF-2*

Portions of fraction PF-2 were incubated in the presence (60 μM) and absence of prostaglandin E_1 and nucleoside or base (50 μM) together and separately for 10 min at 37°C. Modest (less than 30%) stimulation of adenylate cyclase activity was obtained with GTP and GMP-PMP in the absence of prostaglandin E_1 (not shown). Adenylate cyclase is expressed in pmol of cyclic AMP/10 min per mg of protein. GMP-PMP, guanylyl-5'-yl imidodiphosphate.

	Adenylate cyclase activity	Ratio of stimulated to basal activity
Basal	162 (± 20)	—
Prostaglandin E_1	430 (± 18)	2.65
Prostaglandin E_1 + GTP	838 (± 25)	5.17
Prostaglandin E_1 + GDP	512 (± 49)	3.16
Prostaglandin E_1 + 5'-GMP	417 (± 4)	2.57
Prostaglandin E_1 + guanosine	417 (± 40)	2.57
Prostaglandin E_1 + guanine	442 (± 54)	2.72
Prostaglandin E_1 + GMP-PMP	912 (± 20)	5.16
Prostaglandin E_1 + ITP	720 (± 33)	4.44

several hours, often to concentrations below those in control cells. Since lectin-activated lymphocytes take up increased amounts of Ca^{2+} it is possible that eventually these cells accumulate enough intracellular Ca^{2+} to inhibit their basal adenylate cyclase activity. Although this could explain the delayed decrease in cyclic AMP it must be kept in mind that lectins produce a wide variety of other changes in lymphocyte transport and metabolism and that the absolute concentrations of intracellular Ca^{2+} at various stages of the activation process are not now known.

In studies of adenylate cyclase in various tissues, responsiveness to hormones but not fluoride has frequently been shown to be stimulated by GTP as well as by metabolically stable GTP analogues such as GMP-PMP (Rodbell *et al.*, 1971; Krishna *et al.*, 1972; Swislocki *et al.*, 1973; Johnson *et al.*, 1974). Our observations in lymphocytes confirm and extend these earlier studies. We have demonstrated that not only do GTP and GMP-PMP enhance responsiveness to prostaglandin E_1 but that the response to fluoride is actually inhibited. Moreover, with GMP the converse effect was observed, with stimulation of the fluoride response and inhibition of the prostaglandin E_1 response. To our knowledge no-one has previously demonstrated an effect of GMP on normal responsiveness, and certainly not one in the opposite direction to that of GTP.

In addition to the effect of guanine nucleotides, both stimulatory and inhibitory effects of adenosine on lymphocyte adenylate cyclase activity were seen with inhibition at high (0.5 mM and higher) concentrations and stimulation at low (1–10 μM) concentrations (in the absence of theophylline). Modulation of adenylate cyclase activity has been observed in studies with adenosine in other tissues. Thus changes in intra- or extra-cellular adenosine concentrations appear to provide another potential control mechanism for modulating the adenylate cyclase response in lymphocytes as well as in other tissues. In this connexion it is noteworthy that 10–100 μM -adenosine increases cyclic AMP in intact human lymphocytes and that lymphocytes from patients with adenosine deaminase deficiency not only respond poorly to mitogenic lectins *in vitro* but appear defective in their functional responses to antigens *in vivo*. The failure of these lymphocytes to respond mitogenically may be due to sustained changes in intracellular cyclic AMP secondary to the deficiency in adenosine breakdown. However, the possibility that high adenosine concentrations are inhibitory because purine and pyrimidine synthesis *de novo* is inhibited has not been excluded.

The earliest known metabolic alteration in lectin-stimulated lymphocytes is an increase in intracellular cyclic AMP. Direct activation of adenylate cyclase by phytohaemagglutinin has been observed previously in studies with crude lympho-

cyte homogenates. However, although some stimulation of the enzyme by phytohaemagglutinin was seen in experiments with enriched plasma-membrane fractions, in contrast with prostaglandin E_1 , which produced easily demonstrable increases in adenylate cyclase activity in every experiment, the response to phytohaemagglutinin was weak and sometimes completely absent; this was particularly true when method B was used to obtain purified membranes. The poor response to phytohaemagglutinin is not particularly surprising considering the relatively modest response to phytohaemagglutinin in intact cells (average increases in cyclic AMP of 1.5–2.0-fold, compared with 15-fold with prostaglandin E_1) and the marked lability of the enzyme preparations. Nonetheless, since the effect of phytohaemagglutinin on adenylate cyclase activity is small, alternative mechanisms for the increase in cyclic AMP in lectin-stimulated lymphocytes (such as inactivation of phosphodiesterase or generation of a cofactor for adenylate cyclase) need to be considered. In this connexion we have recently demonstrated that exogenous arachidonic acid considerably raises intralymphocytic cyclic AMP concentrations and synergistically increases the cyclic AMP response to phytohaemagglutinin. Arachidonic acid might be released by a phospholipase during the early phases of lymphocyte activation.

One of the most interesting questions raised by the present study is with regard to the site of action of prostaglandins in lymphocytes. Adenylate cyclase has usually been assumed to be located in the plasma membrane (Pohl *et al.*, 1969; Forte, 1972), although a nuclear, mitochondrial or microsomal localization has been reported in certain tissues (Davoren & Sutherland, 1963; Rabinowitz *et al.*, 1965; De Robertis *et al.*, 1967). Earlier immunofluorescence studies from our laboratory indicated that cyclic AMP generated in response to prostaglandin E_1 appeared to be localized throughout the cytoplasm, whereas cyclic AMP generated in response to phytohaemagglutinin remained in or near the plasma membrane. In the present study purified sub-cellular fractions enriched in plasma membranes responded considerably less well to prostaglandins than did heavier fractions that were seemingly largely free of plasma membranes as indicated by morphological and enzymic criteria. Control studies indicated that the poor response in purified plasma membranes would not be explained by accelerated cyclic AMP destruction. Moreover, mixing experiments with the heavier, more responsive, fraction gave no indication that the plasma-membrane-rich fractions either contained an inhibitor or lacked a stimulator of adenylate cyclase. All of this suggests that the response is actually taking place in one or more cytoplasmic organelles, probably microsomal particles. Obviously, a site of action of prostaglandins

within the cytoplasm would raise interesting new possibilities with regard to the role of prostaglandins in normal lymphocyte metabolism. Unfortunately, the heavier, more responsive, membrane fractions undoubtedly contain at least some plasma-membrane fragments owing to trapping, binding or the occurrence of a plasma-membrane fraction with an unusually high buoyant density, making it impossible to exclude completely a plasma-membrane site of action for these agents. Absorption experiments using antibodies specific for lymphocyte surface determinants may help to resolve this issue. Additional studies designed to localize and characterize adenylate cyclase activity within lymphocytes seem justified in view of the accumulating evidence that cyclic nucleotides, particularly cyclic AMP, are important in regulating cell growth and differentiation, cell-mediated cytotoxicity and antibody synthesis (Parker, 1974).

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