Adenylate Cyclase Activity in Lymphocyte Subcellular Fractions

CHARACTERIZATION OF A NUCLEAR ADENYLATE CYCLASE

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Nuclei from purified human peripheral lymphocytes were prepared by incubations with Triton X-100 to disrupt the cells, followed by sucrose-density gradient centrifugation. The nuclei were pure as judged by phase-contrast microscopy and had low contents of non-nuclear marker enzymes. In addition, nuclei prepared from lymphocytes surface-labelled with ^{125}I had only 2–7% of the radioactivity bound to intact lymphocytes. At 3.3 mM- Ca^{2+} and 100 μM -ATP a fluoride-sensitive adenylate cyclase was demonstrated in nuclei prepared in 0.2% Triton X-100 or 0.33% Triton X-100. There was linear accumulation of cyclic AMP for 10 min in both preparations. The apparent K_m for ATP was 90 μM . Adenylate cyclase activity was augmented by 1.0 mM- Mn^{2+} and inhibited at higher concentrations. Ca^{2+} showed two peaks of stimulation, at 1.0–2.5 mM- and above 10 mM- Ca^{2+} . Mg^{2+} was inhibitory at all concentrations. EDTA or EGTA only slightly decreased adenylate cyclase activity, suggesting that another metal ion may be necessary for activity. Adenylate cyclase activity was stimulated by 10 mM-isoproterenol and 10 μM -adrenaline in the presence of a phosphodiesterase inhibitor. Phytohaemagglutinin and prostaglandin E_1 alone or in combination with isoproterenol had no effect on nuclear adenylate cyclase activity in either nuclei preparation. These results indicate that human lymphocyte nuclei contain one or several adenylate cyclases which differ from adenylate cyclases found in other subcellular fractions of these cells with regard to their bivalent-cation requirements and responsiveness to pharmacological agents.

Fluoride-sensitive adenylate cyclase activity has been reported in rat ventral prostate nuclei (Liao *et al.*, 1971), and glucagon- and fluoride-sensitive adenylate cyclases have been demonstrated in hepatic nuclei (Soifer & Hechter, 1971). Although rigorous control experiments to exclude the existence of cytoplasmic or plasma-membrane contamination of the nuclear preparations were not presented, these observations are consistent with other evidence indicating that eukaryotic nuclei contain cyclic AMP-dependent protein kinases (Langan, 1973; Castagna *et al.*, 1975), raising the possibility that nuclear adenylate cyclases may be involved in the modulation of nuclear function. By using an immunocyto-technical technique for the localization of cyclic AMP (Wedner *et al.*, 1972), we have demonstrated that isoproterenol and adrenaline produce local increases in cyclic AMP in the nucleus of intact human peripheral blood lymphocytes (Wedner *et al.*, 1975a), suggesting that the enzymic machinery for generating cyclic AMP is present in the nuclei of these cells as well. We now report the results of studies of adenylate cyclase in isolated nuclei from these cells. Evidence strongly supporting the existence

of a highly active adenylate cyclase in human lymphocyte nuclei is presented.

Materials and Methods

Materials

Reagents and their sources were as follows: Triton X-100, bovine lactoperoxidase, L-adrenaline, and DL-noradrenaline (Sigma Chemical Co., St. Louis, MO, U.S.A.); carrier-free ^{125}I (Industrial Nuclear Co., St. Louis, MO, U.S.A.); foetal calf serum and Gey's solution (Grand Island Biologicals, Grand Island, NY, U.S.A.); AG1-X2 resin (Bio-Rad, Richmond, CA, U.S.A.); Instagel (Packard, Downers Grove, IL, U.S.A.); L-propranolol (Aldrich, Milwaukee, WI, U.S.A.). Other reagents and their sources are given in the preceding paper (Snider & Parker, 1977).

Bivalent cations, propranolol, noradrenaline hydrochloride and theophylline were dissolved directly in 0.25 M-sucrose/3.3 M- CaCl_2 (sucrose/ Ca^{2+} medium). Isoproterenol and erythroagglutinating phytohaemagglutinin were dissolved in 0.1 M- NaCl . Adrenaline was dissolved at 10 mM in 0.1 M-HCl and diluted further in 0.1 M- NaCl . Prostaglandin E_1 was

dissolved at 10mg/ml in 95% (v/v) ethanol and diluted to 1 mg/ml in 0.1% Na_2CO_3 . Further dilutions were made in 0.1 M-NaCl. NaF, EDTA and EGTA were dissolved in water.

Preparation of nuclei

Peripheral lymphocytes from normal human volunteers were prepared by dextran sedimentation and isopycnic centrifugation on a Ficoll/Hypaque gradient as described in the preceding paper (Snider & Parker, 1977). After two washes with 0.15 M-NaCl/0.01 M-phosphate, pH 7.35 (NaCl/phosphate buffer) the lymphocytes were suspended in sucrose/ Ca^{2+} medium and chilled to 4°C. All further purification steps were carried out at this temperature. The nuclei were isolated by a modification of the methods of Darzynkiewicz & Jacobson (1971) and Blobel & Potter (1966). The cells were suspended in sucrose/ Ca^{2+} medium at a density of $100 \times 10^6/\text{ml}$, and an equal volume of sucrose/ Ca^{2+} medium containing various concentrations of Triton X-100 (usually 0.3 or 0.5%) was added. The suspension was agitated in a Vortex mixer for 30 s and rapidly diluted with 7 vol. of sucrose/ Ca^{2+} medium. Nuclei and incompletely disrupted cells were sedimented at $100g_{\text{max}}$ for 4 min. The pellets were resuspended in the original volume of sucrose/ Ca^{2+} medium. Then 2 vol. of 0.3 or 0.5% Triton X-100 in sucrose/ Ca^{2+} medium were added and the agitation and washing were repeated. After a final wash in sucrose/ Ca^{2+} buffer, the nuclei were resuspended in sucrose/ Ca^{2+} at 10×10^6 – $20 \times 10^6/\text{ml}$. Then 4 ml of the nuclear suspension was underlaid with 2 ml of 2.25 M-sucrose containing 3.3 mM- Ca^{2+} in 6 ml nitrocellulose tubes. The mixture was centrifuged for 30 min at $105\,000g_{\text{max}}$ in a Beckman L5-65B ultracentrifuge. The pellet was resuspended in 30 ml of sucrose/ Ca^{2+} medium and washed once with sucrose/ Ca^{2+} medium. The nuclei were counted in a haemocytometer under phase-contrast illumination. The yield of nuclei was 70–90% of the starting number of purified lymphocytes.

Enzyme and protein measurements

Protein, cytochrome oxidase, 5'-nucleotidase and NADH oxidoreductase were measured as described in the preceding paper (Snider & Parker, 1977). Appropriate controls were carried out to exclude effects of residual Ca^{2+} and sucrose in the enzyme assays. Lymphocyte subcellular fractions prepared as described in the preceding paper (Snider & Parkers, 1977) were used as enzyme standards.

Radioiodination of intact cells

As an aid in evaluating the degree of contamination of the nuclear preparations by plasma

membrane, nuclei were prepared from cells that had been enzymically radioiodinated by the technique of Marchalonis *et al.* (1971) before addition of Triton X-100. Because of possible non-specific lymphocyte damage during iodination and covalent labelling with ^{125}I inside cells, procedures leading to either light or intermediate degrees of iodination were used. The extent of iodination was controlled by varying the quantity of ^{125}I and H_2O_2 and the incubation time. For light iodination, 40×10^6 lymphocytes in 2 ml of NaCl/phosphate buffer were mixed with 80 μl of lactoperoxidase solution (0.3 mg/ml in water) and 80 μl of ^{125}I solution (approx. 6×10^8 c.p.m./ml) in plastic tubes at room temperature. Four 50 μl additions of 3 mM- H_2O_2 were made at 70 s intervals. For heavier iodination a sixfold higher amount of ^{125}I was used and eight additions of 3 mM- H_2O_2 were made at 30 s intervals. Then 1 min after the final addition of H_2O_2 the reactions were stopped by the addition of 10 ml of cold Gey's solution containing 5% foetal calf serum. All subsequent steps were performed at 4°C. The cells were harvested by centrifugation at 1000g for 5 min. The cell pellet was resuspended in 1 ml of Gey's solution containing 5% (v/v) foetal calf serum and 5 ml of cell suspension was layered over 10 ml of 100% foetal calf serum and tubes were centrifuged at 1000g for 10 min. This procedure was repeated a second time. Finally the cells were washed once in Gey's solution and once in sucrose/ Ca^{2+} medium. A sample was removed for determination of ^{125}I bound to the cells. The nuclei were isolated from the remaining cells as described above and the nuclear preparations were examined for residual ^{125}I . ^{125}I was determined in a Nuclear-Chicago auto-gamma counter.

Incubation conditions for nuclei

Washed nuclear preparations were suspended in sucrose/ Ca^{2+} medium at a density of 5×10^6 or 10×10^6 nuclei/ml and maintained at 4°C. Solutions of stimulatory agents, ATP, bivalent cations, chelating agents and buffer control solutions were added to chilled glass tubes (10 mm $\times$ 75 mm) in a final volume of 0.2 ml or less followed by the addition of 0.5 ml of nuclear suspension (at 4°C). This sequence of addition was particularly important with solutions containing EDTA, EGTA or Mg^{2+} , which cause nuclear clumping, making it impossible to dispense the nuclei accurately into tubes once mixing has occurred. The reaction mixtures were incubated at 37°C for various periods of time. The reaction was terminated by centrifuging the nuclei at 2200g for 2 min, decanting the supernatant and freezing the pellet rapidly in ethanol cooled with solid CO_2 . In selected experiments the supernatant also was frozen and analysed. All incubations were

done in duplicate or triplicate unless otherwise noted. The results presented are the means of three or more experiments. Intra-experimental variability was usually less than 10% and the standard deviation for three experiments was 15% or less.

Assay of cyclic AMP

Frozen nuclear pellets or supernatants were stored at -90°C until assay. Samples were diluted with 50mM-sodium acetate buffer, pH 6.10, boiled, sonicated (10s at 35% of output, Bronson Biosonik III, Bronwill Scientific, Rochester, NY, U.S.A.), and evaluated by radioimmunoassay as described in the preceding paper (Snider & Parker, 1977). In experiments in which EDTA or EGTA was used, the acetate buffer contained 5mM- CaCl_2 . In the absence of excess of Ca^{2+} , these two agents inhibited the binding of cyclic AMP and iodinated cyclic AMP marker to the anti-(cyclic AMP) antibody. This could be completely reversed by the addition of Ca^{2+} to the assay mixture. None of the other agents interfered with the radioimmunoassay of cyclic AMP. The immunoassay was validated by adding known amounts of cyclic AMP to nuclear pellets or extracts, by incubation of samples with cyclic nucleotide phosphodiesterase and column chromatography in the presence of tracer amounts of cyclic $[^3\text{H}]\text{AMP}$ (Wedner *et al.*, 1975b).

Results

Effect of Triton X-100 concentrations

Essentially pure nuclear preparations with adenylate cyclase activity were obtained by successive treatments with 0.15 and 0.2% Triton X-100 (0.2% Tx nuclei) or with 0.25 and 0.33% Triton X-100 (0.33% Tx nuclei) followed by sucrose-density-gradient centrifugation. These two preparations had comparable resting cyclic AMP concentrations (6–12pmol/ 10^7 nuclei), but their responses to fluoride and catecholamines differed markedly. The 0.33% Tx nuclei had a threefold greater response to fluoride but a considerably decreased (or absent) response to catecholamines. For this reason most of the subsequent experiments with fluoride were

carried out with 0.33% Tx nuclei, whereas with catecholamines 0.2% Tx nuclei were used. Nuclei obtained at final Triton X-100 concentrations above 0.33% had greatly decreased or absent adenylate cyclase activity. The effect of Triton X-100 on responsiveness to fluoride and catecholamine in lymphocyte nuclei is similar to that observed with detergents in other tissues, where the typical result is an increase in fluoride responsiveness and a decrease in hormonal responsiveness (Birnbaumer *et al.*, 1971).

Nuclear purity

After purification as described above, no 5'-nucleotidase, NADH oxidoreductase or cytochrome *c* oxidase activity was found in sonicated or intact purified nuclei (enzyme activities of less than 1% of those present in whole lymphocyte sonicates when expressed per cell or per nucleus). However, it was possible that marker-enzyme inactivation might have occurred during the exposure of the lymphocytes to detergent, or because of failure to remove residual Triton X-100, giving a falsely low estimate of nuclear contamination. This proved not to be the case. Plasma-membrane and microsomal-rich fractions were obtained by homogenization and density-gradient centrifugation (method B) as described in the preceding paper (Snider & Parker, 1977). When fractions enriched in plasma membranes were combined and exposed to 0.33% Triton X-100 in sucrose/ Ca^{2+} medium the 5'-nucleotidase activity increased from 180nmol/min per mg to 310nmol/min per mg in membranes with 0.33% Triton X-100 in sucrose/ Ca^{2+} medium. When fractions PF-2 and PF-3 (enriched in microsomal particles) were combined and studied in the same way for changes in NADH oxidoreductase activity, no effect of the detergent was observed.

As another control for plasma-membrane contamination, cells were radioiodinated by the lactoperoxidase technique before disruption and nuclear purification. Since this procedure selectively iodinated the cell surface, any radioactivity associated with isolated nuclei would presumably be of plasma-membrane origin. The results of two experiments are shown in Table 1. When cells are heavily iodinated

Table 1. Iodination of human lymphocytes and residual iodine present on washed lymphocyte nuclei

Purified human peripheral lymphocytes were iodinated by the technique of Marchalonis *et al.* (1971) as described in the text. Nuclei were then isolated from the iodinated lymphocytes using 0.2% Triton X-100. Expt. 1, intermediate iodination, 1.84% residual iodine counts; Expt. 2, light iodination, 7.14% residual iodine counts.

	Expt. 1	Expt. 2
Radioactivity added (c.p.m./ 10^7 cells)	29.5×10^6	5.0×10^6
Radioactivity bound to washed cells (c.p.m./ 10^7 cells)	3.8×10^5	2.1×10^4
Radioactivity bound to washed 0.2% Tx nuclei (c.p.m./ 10^7 nuclei)	7×10^3	1.5×10^3

Table 2. *Cyclic AMP accumulation in washed lymphocyte nuclei from iodinated human lymphocytes*

Nuclei from Expt. 2, Table 1, containing 7.14% residual ^{125}I counts, were incubated with 10mM-NaCl, 10mM-isoproterenol or 1mM-isoproterenol as described and cyclic AMP accumulation was determined as described in the text.

	Cyclic AMP (pmol/ 10^7 nuclei)
10mM-NaCl (control)	13.7
10mM-Isoproterenol	104.0
1mM-Isoproterenol	52.0

Table 3. *Effect of fluoride and ATP on cyclic AMP response in human lymphocyte nuclei*

The indicated agents or 10mM-NaCl (control) were incubated with 5.0×10^6 0.2% Tx nuclei for 15min. Cyclic AMP accumulation was measured as described in the text. Results are means $\pm$ S.E.M. for three experiments.

Stimulating agent	Cyclic AMP (pmol/ 10^7 nuclei)
Control	9.6 ± 2.0
Fluoride (10mM)	13.1 ± 2.3
ATP (1mM)	23.0 ± 5.0
Fluoride (10mM)+ATP (1mM)	115.5 ± 23.1

(Expt. 1) only 2% of the radioactivity bound to intact lymphocytes was found in the final washed nuclear preparation. When the cells were lightly iodinated 7% of the radioactivity was associated with the nuclei. The nuclei from this experiment were stimulated with 10mM-isoproterenol in the absence of ATP (see below). The intranuclear concentration of cyclic AMP rose from 13.7 to 104pmol/ 10^7 nuclei (Table 2), which is equivalent to the degree of stimulation of cyclic AMP accumulation by isoproterenol in the same number of intact cells (Parker & Smith, 1973).

Fluoride stimulation

In nuclei prepared in medium with final concentrations of either 0.2 or 0.33% Triton X-100, and incubated with fluoride and ATP, there was a time-related increase in intranuclear cyclic AMP. In the absence of ATP no increase in cyclic AMP occurred (Fig. 1 and Table 3). The response to fluoride was linear with time for at least 10min (15min with 0.2% Tx nuclei). Intranuclear cyclic AMP increased to a lesser extent in nuclei not incubated at 37°C presumably owing to cyclic AMP formation during the 2min centrifugation step at room temperature. Almost all of the generated cyclic AMP remained associated with the nuclei, as indicated by a virtual absence of cyclic AMP from the medium. The

Fig. 1. *Time-course for cyclic AMP accumulation in fluoride-stimulated nuclei*

Incubations were carried out with 5×10^6 0.2% Tx nuclei in the presence of 10mM-NaCl (control, □), 10mM-NaF (■) or 10mM-NaF+1mM-ATP (○). Reactions were terminated as described in the text.

0.33% Tx nuclei had a much higher cyclic AMP accumulation in response to fluoride than the 0.2% Tx nuclei [276.6 (S.E.M. ± 33.6) and 115.5 (S.E.M. ± 23.1) pmol/15 min per 10^7 nuclei respectively].

The kinetics of cyclic AMP accumulation were studied in 0.33% Tx nuclei by using an incubation time of only 3min to assure linearity of the reaction. In the standard sucrose/ Ca^{2+} medium the apparent K_m for ATP was $90 \mu\text{M}$. An identical K_m value was noted in nuclei incubated with 2mM-EDTA and 10mM- Ca^{2+} (see below). For this reason subsequent experiments were done at ATP concentrations of $100 \mu\text{M}$ or greater.

The importance of bivalent cations in adenylate cyclase activity has been demonstrated in a variety of cellular systems. In most of these Mg^{2+} seems to be necessary for optimum activity, but stimulatory effects of Mn^{2+} and Ca^{2+} have also been reported. The Ca^{2+} -containing solution used in the isolation of nuclei was studied initially because it provided the most homogeneous preparation of nuclei and could be used without nuclear clumping. When bivalent cations were added to nuclei suspended in sucrose/ Ca^{2+} medium (Fig. 2), Mn^{2+} produced the most marked increase in fluoride responsiveness, with maximum stimulation at 1mM- Mn^{2+} concentrations. Higher concentrations of Mn^{2+} (above

Fig. 2. Effect of bivalent-cation concentration on cyclic AMP accumulation in fluoride-stimulated nuclei

Incubations were performed with 2.5×10^6 0.33% Tx nuclei in the presence of 10mM-NaF, 100 μ M-ATP and various concentrations of Ca^{2+} ($\bullet$), Mn^{2+} ($\square$) and Mg^{2+} ($\circ$). The incubations were for 10min at 37°C and the reactions were terminated and cyclic AMP assayed as described in the text. Results are the means of three experiments.

2.5mM) were inhibitory. When Ca^{2+} was increased above the 3.3mM- Ca^{2+} concentrations already present in sucrose/ Ca^{2+} medium, a more complicated pattern emerged. Two peaks of stimulation were observed at total Ca^{2+} concentrations of 5mM and above 12mM. Mg^{2+} was inhibitory at all concentrations tested, with maximum inhibition at concentrations in the 1–10mM range. The effects of Mg^{2+} were not due to changes in ionic strength, since equivalent quantities of NaCl had no effect on adenylate cyclase activity (not shown).

Similar results were seen in the presence of EDTA (Fig. 3). EDTA decreased the adenylate cyclase activity (compare Figs. 2 and 3), but the shape of the curves with added Mn^{2+} , Ca^{2+} and Mg^{2+} were similar. In this case Mn^{2+} was clearly the best stimulator at low concentrations. EGTA also caused an overall decrease in adenylate cyclase activity without altering the shape of the bivalent-cation curves.

The biphasic response seen with Ca^{2+} and the rise in activity seen with Mn^{2+} might logically be attri-

Fig. 3. Effect of EDTA and bivalent cations on cyclic AMP accumulation in fluoride-stimulated nuclei

Incubations were performed with 2.5×10^6 0.33% Tx nuclei in the presence of 2mM-EDTA, 10mM-NaF, 100 μ M-ATP and various concentrations of Ca^{2+} ($\bullet$), Mn^{2+} ($\square$) or Mg^{2+} ($\circ$). The incubations were for 10min at 37°C, and the reactions were terminated and cyclic AMP was measured as described in the text. Results shown are the means of three experiments.

buted to two separate enzymes with different bivalent-cation requirements. However, the fluoride dose-response curve in sucrose/ Ca^{2+} medium was the same in 2.0mM-EDTA or 2.0mM-EDTA/5mM- Mg^{2+} (Fig. 4). Similarly studies of ATP-concentration-dependence of the fluoride response at 3.3 and 10mM- Ca^{2+} revealed a difference in the $V_{\max}$ value but not in the K_m value for stimulation by ATP. Thus, by the above two criteria, no conclusive evidence for two separate enzymes was obtained.

Catecholamine stimulation

With 10mM-isoproterenol, there was a marked and consistent increase in cyclic AMP over a 10min time-period, with essentially no change in the control (Table 3). The increase in cyclic AMP was linear with time for approx. 10min, after which the curve paralleled the slow rise in cyclic AMP seen in control nuclei. In contrast with the response to fluoride, the stimulation of cyclic AMP accumulation by isoproterenol did not require exogenous ATP. The addition of ATP had no effect whatsoever on the magnitude or duration of the response, even when the ATP was added after 15min, the time at which specific isoproterenol stimulation seems to stop.

Fig. 4. Fluoride dose-response curve

Incubations were performed with 2.5×10^6 0.33% Tx nuclei in the presence of various concentrations of NaF and 3.3mM- Ca^{2+} (●), 3.3mM- Ca^{2+} +2mM-EDTA (○) or 3.3mM- Ca^{2+} +2mM-EDTA+5mM- Mg^{2+} (△). Incubations were for 10min at 37°C and the reaction was terminated and cyclic AMP measured as described in the text. Results shown are the means of three experiments.

Thus what we presume to be a catecholamine-responsive adenylate cyclase does not behave in the same manner as the fluoride-stimulated enzyme, at least as far as ATP-dependence is concerned, although the effects of bivalent cations and chelating agents are similar (results not shown).

The dose-response curve of nuclei to isoproterenol indicated that in the absence of theophylline definite stimulation was obtainable only at concentrations of 1mM and higher (Fig. 5). Noteworthy, the shape of the isoproterenol dose-response curve in isolated nuclei was similar to that obtained in intact lymphocytes (Parker & Smith, 1973), although the latter have a 50% greater average increase in cyclic AMP on a per-cell or per-nucleus basis. Nuclei preincubated with 5mM-theophylline for 30min accumulated increased amounts of cyclic AMP at isoproterenol and adrenaline concentrations as low as 1μM. Theophylline alone produced a less marked cyclic AMP response, providing presumptive evidence for cyclic AMP phosphodiesterase activity in lymphocyte nuclei, again in accord with the observations in intact lymphocytes. Less marked stimulation was seen with 10mM-noradrenaline.

Because relatively high doses of isoproterenol and adrenaline were necessary for maximum stimulation of nuclear cyclic AMP accumulation and the response did not require the addition of ATP

Fig. 5. Isoproterenol dose-response curve

Incubations were performed with 5.0×10^6 0.2% Tx nuclei in the presence of various concentrations of isoproterenol. The incubation was for 10min at 37°C. The reaction was terminated and cyclic AMP assayed as described in the text. Results shown are the means of three experiments.

to the medium, the possibility was considered that the increase in cyclic AMP might be a manifestation of non-specific phosphodiesterase inhibition, as described by Goren & Rosen (1972). In apparent support of this possibility, stereospecificity of the stimulation by adrenaline was limited in that L-adrenaline was only a slightly better stimulator than D-adrenaline (results not shown). This is also true of the stimulation of cyclic AMP accumulation by adrenaline in intact lymphocytes (C. W. Parker, unpublished work). However, studies with adrenaline in the presence and absence of a β-adrenergic blocking agent cast considerable doubt on the non-specific phosphodiesterase inhibition hypothesis. In the presence of 5mM-theophylline an increase of 23.5 (S.E.M. ± 1.7) pmol/10⁷ nuclei was seen with 10μM-L-adrenaline. The response to adrenaline was partially inhibited by 100μM-propranolol (an increase of 10.0 ± 1.2 pmol/10⁷ nuclei) and to a lesser extent by 10μM-propranolol. The non-specific phosphodiesterase inhibition described by Goren & Rosen (1972) was seen only at high catecholamine concentrations and was not blocked by β-adrenergic inhibitors.

Effect of phytohaemagglutinin and prostaglandin E₁

Under conditions in which isoproterenol was effective in increasing intranuclear cyclic AMP, prostaglandin E₁ and phytohaemagglutinin had no effect whatsoever (Table 4). In contrast with the additive effects of prostaglandin E₁, phytohaemagglutinin and isoproterenol in intact lymphocytes (Wedner *et al.*, 1975a), prostaglandin E₁ and phytohaemagglutinin did not augment the isoproterenol

Table 4. *Effect of isoproterenol, prostaglandin E₁ and phytohaemagglutinin alone or in combination on the cyclic AMP response in human lymphocyte nuclei*

The agents indicated were incubated with 5.0×10^6 0.2% Tx nuclei for 15 min and the cyclic AMP was measured as described in the text. Results are means $\pm$ S.E.M. for three experiments.

Stimulating agent	Cyclic AMP (pmol/ 10^7 nuclei)
Control	10.1 $\pm$ 0.9
Isoproterenol (10mM)	32.5 $\pm$ 4.9
Prostaglandin E ₁ (3.3mM)	9.0 $\pm$ 0.8
Phytohaemagglutinin (20 μ g/ml)	9.2 $\pm$ 0.8
Isoproterenol (10mM)+prostaglandin E ₁ (1mM)	32.0 $\pm$ 4.8
Isoproterenol (10mM)+phytohaemagglutinin (20 μ g/ml)	28.5 $\pm$ 5.3
Prostaglandin E ₁ (3.3mM)+phytohaemagglutinin (20 μ g/ml)	9.7 $\pm$ 1.7

response in isolated nuclei. The addition of GTP, which markedly enhances the response of mitochondrial plus microsomal-rich fractions of lymphocytes to prostaglandin E₁ (Snider & Parker, 1977), had no effect on the failure to respond to prostaglandin E₁. This failure did not appear to be due to inactivation by detergents. In microsomal-rich fractions obtained by homogenization and density-gradient centrifugation (a combination of fractions PF-2 and PF-3; see above) at least 80% of the response to prostaglandin E₁ and GTP was retained after exposure to 0.33% Triton X in sucrose/Ca²⁺ medium. In addition, 10mM-Mg²⁺ augmented the response to fluoride in detergent-treated fractions PF-2 and PF-3, in accord with the effect of Mg²⁺ when these fractions were free of detergent (see Snider & Parker, 1977). Thus, even if marker enzymes derived from fractions PF-2 and PF-3 had been present in the nuclear preparations, the failure of Mg²⁺ to enhance the response would be difficult to explain.

Discussion

Although classically, adenylate cyclase has been considered to be primarily a plasma-membrane enzyme, there have been a number of reports of adenylate cyclase activity in intracellular organelles, including mitochondria (Rabinowitz *et al.*, 1965), microsomal fractions (De Robertis *et al.*, 1967) and nuclei (Soifer & Hechter, 1971; Liao *et al.*, 1971). However, because of difficulties in excluding contamination of subcellular organelles by plasma membranes some scepticism has remained. A number of lines of evidence indicate that the nuclear cyclic AMP responses seen in this study are produced by one or more adenylate cyclases intimately related to the cell nucleus and are not the result of contamination of the nuclear preparation by plasma membranes or other intracellular organelles. (1) In nuclei obtained at low Triton X-100 concentrations, total fluoride-stimulatable adenylate cyclase activity averages about 50% of that found in whole

lymphocyte homogenates, or 30% of that found in homogenates exposed to 0.33% Triton X-100. By contrast, experiments with lymphocytes labelled with ¹²⁵I indicate that purified nuclei contain only 2–7% of the radioactivity present on their cell surfaces. Since low concentrations of iodination reagents presumably get inside cells during labelling, this is likely to be a maximum value for plasma-membrane contamination. Thus there appears to be too little plasma membrane present to explain the marked response to fluoride. (2) The bivalent-cation-dependence for responsiveness to fluoride in purified nuclei is entirely different from that in microsomal plus mitochondrial-rich fractions and less strikingly different from that in purified plasma membranes. (3) Experiments in which known quantities of Triton X-100 were added to purified subcellular fractions of lymphocytes obtained by homogenization and density-gradient centrifugation (Snider & Parker, 1977) failed to provide adenylate cyclase reactivity patterns that resembled those in isolated nuclei. (4) Catecholamine responsiveness is observed in nuclei in the absence of added ATP. Although it is conceivable that the catecholamine is acting to prevent cyclic AMP breakdown (see below), it seems more likely that adenylate cyclase is indeed being stimulated and that endogenous ATP present inside the lymphocyte nucleus (McEwen *et al.*, 1963a,b,c) is serving as the substrate for cyclic AMP formation. This would not be predicted if plasma-membrane tags were responsible for the catecholamine response. (5) No stimulation of cyclic AMP accumulation was demonstrated in the nuclear preparation by phytohaemagglutinin or prostaglandin E₁, as might have been expected if the nuclear preparation was contaminated with plasma-membrane or microsomal fractions. Moreover, efforts to demonstrate stimulation of cyclic AMP accumulation by use of prostaglandin E₁ and GTP in combination also were unsuccessful. (6) When cyclic AMP generation was stimulated by fluoride and catecholamines much or all of the cyclic AMP formed was present in the nucleus, presumably bound to the

regulatory subunit of a nuclear cyclic AMP-dependent protein kinase. On the other hand, when various amounts of exogenous cyclic AMP are added to suspended nuclei and an incubation is carried out for 15 min under the normal conditions of the cyclase assay very little of the cyclic AMP is associated with the nucleus confirming that most of the cyclic AMP is being made inside rather than outside the nucleus.

The bivalent-cation requirements of the nuclear adenylate cyclase, with stimulation by Ca^{2+} and Mn^{2+} and inhibition by Mg^{2+} , are unusual but not by any means unique. Although most mammalian adenylate cyclases are stimulated by Mg^{2+} and Mn^{2+} and inhibited by Ca^{2+} (Drummond & Duncan, 1970; Robison *et al.*, 1971), a pattern similar to that found in lymphocyte nuclei has been reported in a number of tissues (for example, Bradham *et al.*, 1970; Flawiá & Torres, 1972; Weinryb & Michel, 1974). It may be that one or more enzymes capable of responding to either Ca^{2+} or Mn^{2+} are present. An alternative explanation comes from the work of Johnson & Sutherland (1973), who noted that EDTA (but not EGTA) did not inhibit the activity of detergent-solubilized rat brain adenylate cyclase and suggested that the enzyme required a second metal ion in addition to a bivalent cation.

Whether or not changes in intracytoplasmic or intranuclear bivalent-cation concentrations modulate adenylate cyclase activity in the nuclei of intact lymphocytes and contribute to nuclear activation by mitogenic agents remains to be established. Lymphocytes stimulated with a variety of mitogenic agents take up increased amounts of Ca^{2+} during the early phases of lymphocyte activation (Parker, 1974), and changes in the concentration of other bivalent cations are not excluded. On the other hand, the Ca^{2+} concentrations used in the present experiments were high. Unfortunately, problems of nuclear clumping make it difficult to examine the response in isolated lymphocyte nuclei at lower, more physiological, Ca^{2+} concentrations.

As predicted from the immunohistochemical localization of cyclic AMP (Wedner *et al.*, 1975a), isoproterenol and adrenaline promote the accumulation of cyclic AMP in isolated nuclei. Although it seems probable that an intranuclear adenylate cyclase is in fact being stimulated, since the catecholamine stimulation exhibits very little stereospecificity, the catecholamine concentrations involved are relatively high (1–10000 μM) and exogenous ATP is not required, alternative explanations need to be considered. Of these the most attractive would be a direct inhibitory effect on cyclic AMP phosphodiesterase at a time when a catecholamine-insensitive adenylate cyclase is continuing to produce cyclic AMP. Whatever the explanation there is a close similarity between the response seen in the intact cells and in isolated nuclei with regard to time-

course, catecholamine-concentration-dependence, stereospecificity, propranolol inhibition, and even to a lesser extent magnitude, suggesting that very similar or identical processes are involved. In view of the high concentrations of catecholamines required, the physiological significance of this response is uncertain. The significance of a fluoride-sensitive adenylate cyclase on the nuclear membrane or within the nucleus of human lymphocytes is equally unclear, but in view of the presence of cyclic AMP-dependent protein kinases in the nucleus (Castagna *et al.*, 1975) a role for cyclic AMP in the modulation of nuclear function seems likely.

The evidence for a nuclear adenylate cyclase in lymphocytes, taken with the demonstration of adenylate cyclase activity on both the external plasma membrane and microsomal fractions of these cells (Snider & Parker, 1977), provides further evidence for the concept of compartmentalization of cyclic AMP within the lymphocyte (Wedner *et al.*, 1975a). Thus cyclic AMP may be generated and act locally within the cell without affecting other cellular functions. This may provide for finer control by cyclic AMP of a variety of lymphocyte functions.

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