ERRATUM

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Extracellular ATP Triggers Cyclic AMP-Dependent Differentiation of HL-60 Cells

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Extracellular ATP and ATPγS (1–1000 μM) stimulated cyclic AMP (cAMP) production in undifferentiated HL-60 cells. The potency order for adenine nucleotides and adenosine was ATPγS > ATP $\gg$ ADP $\geq$ AMP = Adenosine. Indomethacin (50 μM) had no effect on ATP-induced cAMP production. ATP and ATPγS also suppressed cell growth and induced differentiation as revealed by fMLP-stimulated β-glucuronidase release 48 h after exposure. The potency order for the induction of fMLP-stimulated β-glucuronidase release by adenine nucleotides and adenosine was ATPγS $\geq$ ATP > ADP > AMP = Adenosine $\approx$ 0. The protein kinase A inhibitor Rp-8-Br-cAMPS (10–200 μM) suppressed ATP-induced differentiation but had no effect on ATP-dependent growth suppression. UTP which, like ATP, activates P_{2U} receptors on HL-60 cells, had no effect on cAMP production, cell growth, or differentiation. The data suggest the existence of a novel receptor for ATP on undifferentiated HL-60 cells that is coupled to the activation of adenylate cyclase and cAMP-dependent differentiation.

Human promyelocytic HL-60 leukemia cells can be induced to differentiate to neutrophil-like cells by various agents including 3′, 5′ cyclic AMP. Agents that elevate intracellular cAMP levels by activating adenylate cyclase e.g., PGE_2 (1), histamine (2) and epinephrine (3) are all recognised differentiating agents. Cell-permeable forms of cAMP such as N^6, 2′-O-dibutyryl cAMP (4) and 8-Cl-cAMP (5) also induce HL-60 cell differentiation.

Extracellular ATP has been reported to induce the differentiation of HL-60 cells (1, 6) but the mechanism of action remains unclear. ATP might act after its hydrolysis to adenosine (which induces HL-60 cell apoptosis; see Refs 7, 8), by the activation of an ecto-protein kinase, or via surface receptors. Two forms of ATP receptor have been described on HL-60 cells: (i) P_{2U} receptors present on both undifferentiated and differentiated HL-60 cells (9) which induce Ca^{2+} mobilization in response to extracellular ATP or UTP and (ii) P_{2X1} receptors which are ATP-gated Ca^{2+}-permeable ion channels present on differentiated cells (10). Recently, extracellular ATP has been reported to elevate intracellular cAMP levels in several different cell types e.g., bovine aortic endothelial cells (11), NG108-15 cells (12) and MDCK-D1 cells (13). Given that cAMP is a recognised differentiation agent for HL-60 cells, we examined whether the differentiating effect of extracellular ATP was dependent upon the generation of cAMP. Our data suggest the presence of a novel ATP receptor on HL-60 cells that is coupled to the generation of cAMP which, in turn, triggers cellular differentiation.

Abbreviations: ATPγS, adenosine 5′-[γ-thio]triphosphate; dbcAMP, N^6, 2′-O-dibutyryl-adenosine 3′, 5′-cyclic monophosphate; fMLP, fMet-Leu-Phe; IBMX, 3-isobutyl-1-methyl xanthine; PK-A, protein kinase A; Rp-8-Br-cAMPS, Rp isomer of adenosine 3′,5′-(cyclic)-phosphoro[thioate].

MATERIALS AND METHODS

HL-60 cell culture and proliferation assay. HL-60 cells were grown in suspension culture in Hepes-buffered RPMI-1640 (pH 7.4, Sigma, St Louis MO, USA) supplemented with fetal bovine serum (FBS, 10%, Cytosystems, Australia) and gentamicin (50 μg/ml; Sigma) at 37°C. Cell density was maintained within the range 1–10 $\times$ 10^5 cells/ml which falls in the log phase of HL-60 cell growth curve. Cell counts were performed routinely using a Coulter Counter (Model Zf, Dunstable, Bedfordshire, UK).

Doubling times of undifferentiated HL-60 cells were approximately 30-36 h. For proliferation studies, HL-60 cells were cultured in either 10 ml flasks (Greiner, Australia) or in multi-well plates (Linbro, ICN Biomedicals, Ohio, USA; 1-2 ml) with a starting density of 10^5 cells/ml. Nucleotides or other chemicals were added as required in a single dose at the beginning of each experiment.

cAMP assay. HL-60 cells were harvested in log phase by centrifugation and resuspended in fresh RPMI-1640 medium supplemented with 10% FBS at a density of 10^6 cells/ml. The cells (in 0.2 ml aliquots) were stimulated with nucleotides at various concentrations

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in the presence or absence of IBMX (2 mM) at 37°C. Reactions were stopped by the addition of absolute ethanol (0.5 ml). Supernatants were evaporated by vacuum centrifugation and the precipitates were re-dissolved in 0.2 ml of cAMP assay buffer which contained Na acetate (0.05 M), $CaCl_2$ (0.025 M) and bovine serum albumin (0.1%), pH 6.2. The radioimmune assay for cAMP was performed as described previously (14).

β-Glucuronidase secretion assay. Assays of β-glucuronidase release were performed on cytochalasin B (Sigma)-treated cells as described previously using 4-methylumbelliferyl β-D-glucuronide (Calbiochem, CA, USA) as the fluorescent substrate (15, 16).

Ion-pair HPLC. Ion-pair reverse-phase chromatography was used to separate adenosine and adenine nucleotides using a C-18 endcapped column (Regis, Morton Grove, IL USA; 250 mm ×4 mm) attached to a Pharmacia/LKB HPLC system. A binary gradient based on increasing acetonitrile (Rhone-Poulenc, Melbourne, Victoria, Australia) concentration was used. Buffer A had the following composition: TBA (tetrabutyl ammonium chloride; Sigma) 5 mM, KH_2PO_4 80 mM, pH 5.0. Buffer B was composed of equal volumes of Buffer A and acetonitrile. The gradient profile was as follows: 0-3 min, 24-50% B, 3-14 min, 50% B, 14-15 min, 50-24% B, 15-30 min, 24% B. The flow rate was 1 ml/min. Nucleotides were detected by absorbance at 254 nm. The retention times (min) were as follows (means ± SEM, n=13): adenosine (4.29±0.01), AMP (5.19±0.01), ADP (8.72±0.01), ATP (10.82±0.01) and ATPγS (11.49±0.01).

Data analysis. The data are reported as means ± SEM. Student's unpaired and paired *t*-tests were used to test the statistical significance of the differences observed between experimental means. Curve fitting for concentration-response curves using the Hill equation was performed using DeltaGraph Professional software to perform non-linear regression analysis. EC_{50}s (means ± SEM) were determined using MacCurveFit software.

RESULTS

Extracellular ATPγS and ATP stimulated cAMP production in HL-60 cells. ATP (100 μM) induced a rapid increase in cellular cAMP levels that reached a maximum (66.3 ± 8.5 pmol/10^6 cells; n = 3) after 5-10 min of incubation (Fig. 1); cAMP levels returned to baseline after 1–2 h (not shown). ATPγS (100 μM) induced a similar maximal response (88.7 ± 16.2 pmol/10^6 cells; n = 3). In the presence of the cyclic nucleotide phosphodiesterase inhibitor IBMX (2 mM), the ATP- and ATPγS-induced cAMP responses were similar but cAMP levels returned to baseline levels more slowly (not shown). Extracellular UTP (1-1000 μM) which activates Ca^{2+}-mobilizing P_{2U} receptors on HL-60 cells (9, 17) had no effect on cAMP levels.

The concentration dependence of various nucleotides on cAMP generation were obtained after 5 min of incubation in the presence of IBMX (2 mM; Fig. 2). The potency order for the production of cAMP by adenine nucleotides and adenosine was: ATPγS > ATP >> ADP ⩾ AMP = Adenosine ≈ 0. Similar data were obtained in the absence of IBMX (not shown). The EC_{50} values for ATPγS and ATP were 17.7 ± 2.7 μM and 158 ± 15.6 μM respectively. Aristolochic acid (200 μM), an inhibitor of phospholipase A_2 and indomethacin (50 μM), an inhibitor of cyclo-oxygenase, had no effect on ATP-induced cAMP production.

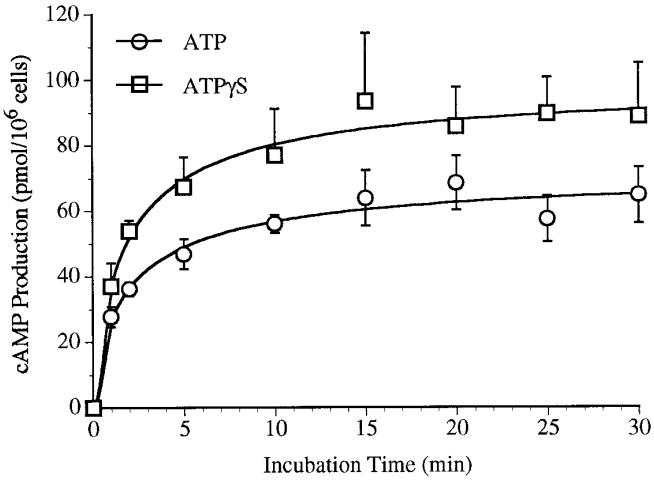

FIG. 1. The effect of ATP and ATPγS on cAMP production in undifferentiated HL-60 cells. HL-60 cells were incubated in RPMI-1640 medium containing FBS (10%) for various times at 37°C in the presence of ATP or ATPγS (100 μM). cAMP was determined by radioimmunoassay of aqueous samples prepared from cell pellets by ethanol extraction.

The greater apparent potency of ATPγS might have arisen as a result of the hydrolysis of ATP by ectonucleotidases. However, the half-life of ATP under these conditions was found to be greater than 1 h and only about 5% of ATP was hydrolysed under the conditions of the assay. Therefore, the data support the conclusion that ATPγS is a more potent activator of cAMP production than ATP.

Extracellular ATP induced the differentiation of HL-60 cells as determined by fMLP-stimulated β-glucuronidase secretion. Control undifferentiated HL-60 cells did not release β-glucuronidase when exposed to the chemotactic peptide fMLP (1 μM). HL-60 cells that had been exposed to a single dose of ATP or ATPγS (1-1000 μM) and cultured for 48 h, released β-glucuronidase in response to fMLP (1 μM; Fig. 3). The potency order for adenine nucleotides and adenosine was: ATPγS ⩾ ATP > ADP > AMP = Adenosine ≈ 0 (Fig. 3B). UTP was without effect. After 48 h exposure to ATP at an initial concentration of 1 mM, fMLP released 16.4 ± 2.8% (n = 5) of their β-glucuronidase content. By way of comparison, cells exposed to dbcAMP (500 μM) for 48 h and then exposed to fMLP (1 μM) released 23 ± 1.6 % (n = 3) of the total cell content of β-glucuronidase. Unlike ATP, cells treated with high concentrations of ATPγS (0.3 mM and 1.0 mM) exhibited suppressed fMLP-induced β-glucuronidase release (Fig. 3A). These cells lost viability as determined by phase contrast microscopy and trypan blue exclusion. Less than 40% of cells exposed to ATPγS (1 mM) on day zero excluded trypan blue after 48 h (0.04%, 20-25°C, 5 min).

Growth suppression of HL-60 cells by extracellular ATP and ATPγS. Single doses of ATP or ATPγS (10-

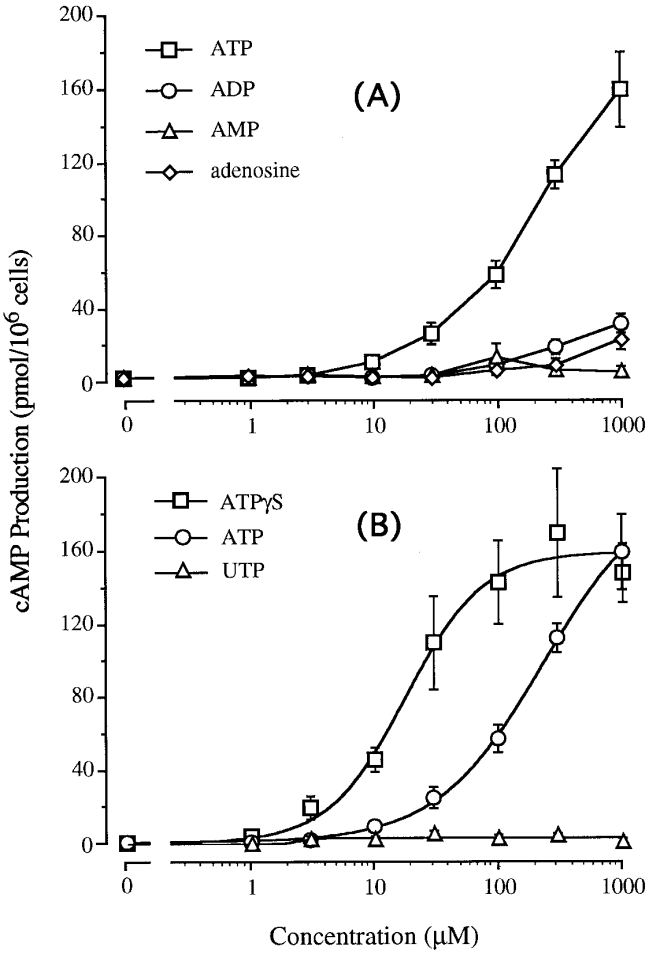

FIG. 2. The concentration dependence of nucleotide-induced cAMP production. HL-60 cells were incubated in RPMI-1640 medium containing FBS (10%) for 5 min at 37°C. (A) The effects of ATP and its hydrolysis products on cAMP production. (B) The effects of ATP, ATPγS, and UTP on cAMP production.

1000 μM), but not UTP, inhibited the growth of HL-60 cells in FBS-supplemented RPMI-1640 medium over the succeeding 7 days of culture (Fig. 4A). Adenosine (100 μM) had no effect on cell growth and ATP was more effective than its products of hydrolysis (Fig. 4B).

Effects of the cell-permeable protein kinase A inhibitor Rp-8-Br-cAMPS. Rp-8-Br-cAMPS (10 – 200 μM) suppressed the differentiation effect of extracellular ATP (Fig. 5). After 48 h exposure to ATP in the presence of Rp-8-Br-cAMPS (200 μM), HL-60 cells failed to release β-glucuronidase in response to fMLP (1 μM). The effect of Rp-8-Br-cAMPS was concentration dependent with an apparent IC_{50} of about 10 μM. Rp-8-Br-cAMPS (200 μM), however, failed to reverse the growth inhibitory effect of ATP (not shown).

DISCUSSION

Extracellular ATP and its more stable analog ATPγS, induced cAMP generation, cell differentiation and growth suppression in HL-60 cells. HL-60 cell differentiation by ATP has been noted previously (1, 6) however, the mechanism responsible has been unclear. Olsson et al. (1) suggested that the effect was due to ecto-protein kinase activity. On the other hand, Cowen et al. (6) suggested that the differentiation effect depended upon P_{2U} receptor activation. Our data are not compatible with the idea that P_{2U} receptors mediate the differentiation effect of ATP because UTP, which is an equipotent agonist at P_{2U} receptors, had no effect. In fact, our results suggest that extracellular ATP induces cell differentiation via the stimulation of cAMP

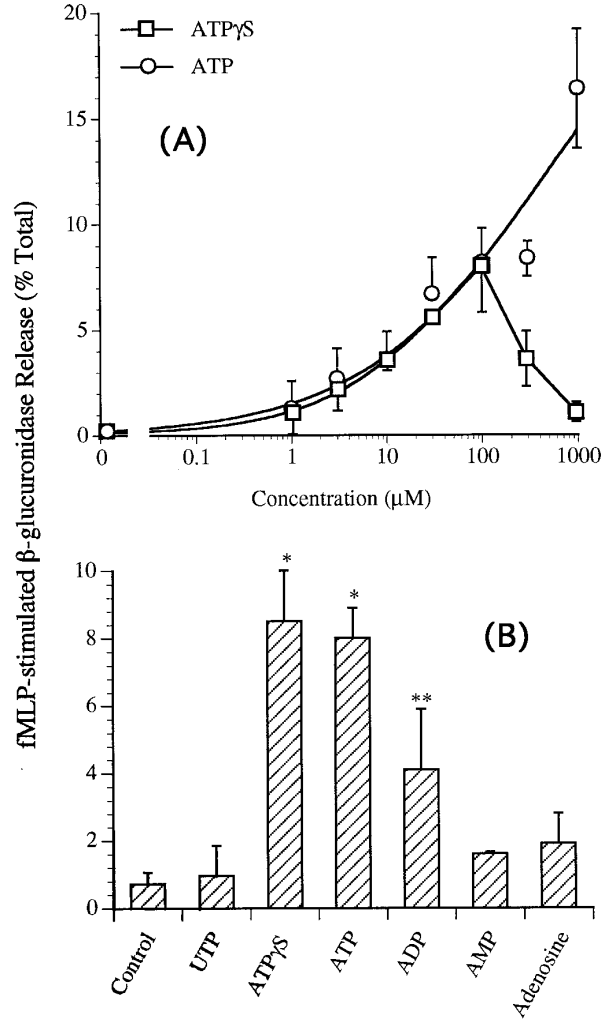

FIG. 3. The effect of exposure of undifferentiated HL-60 cells to extracellular nucleotides on fMLP-induced β-glucuronidase release. HL-60 cells were cultured in RPMI-1640 medium containing FBS (10%) and exposed to nucleotides or control solution on day zero. After 48 h the cells were harvested and assayed for fMLP-induced β-glucuronidase release. (A) The dependence of ATP and ATPγS-induced fMLP-stimulated β-glucuronidase release on initial nucleotide concentration. (B) The effect of various nucleotides (all 100 μM; compared to control: *p = 0.0001 for ATP and ATPγS; **p = 0.007 for ADP).

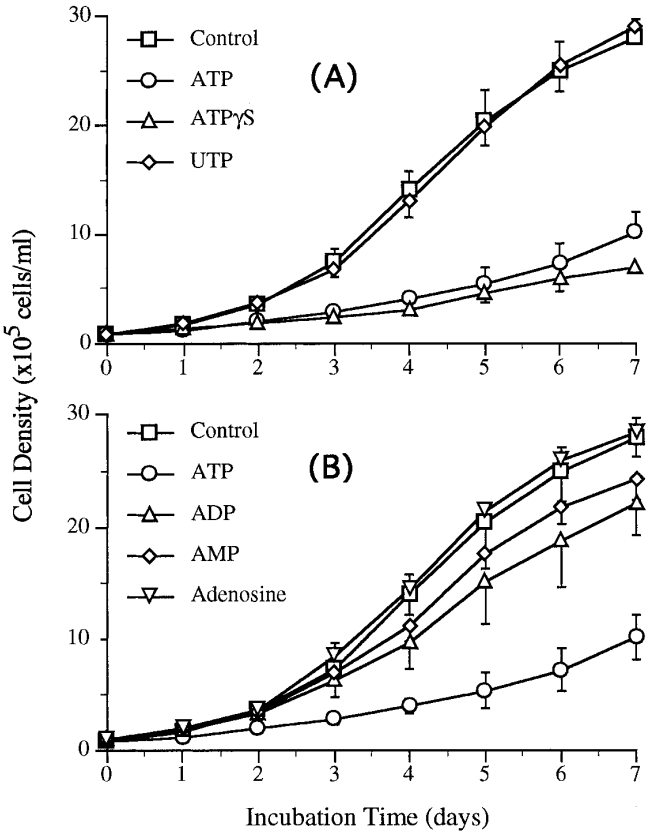

FIG. 4. The effect of extracellular nucleotides on HL-60 cell proliferation. HL-60 cells were cultured in RPMI-1640 (FBS 10%) and exposed to nucleotides (initial concentration 100 μM) on day zero. (A) The effects of ATP, ATPγS, and UTP on cell proliferation over the succeeding 7 days of culture. (B) The effects of ATP and its products of hydrolysis, ADP, AMP, and adenosine, on HL-60 cell proliferation.

production and activation of protein kinase A. Consistent with this, ATP and ATPγS promptly elevated cAMP levels in a concentration-dependent fashion (Figs. 1 and 2), ATP induced HL-60 cell differentiation (Fig. 3) and the protein kinase A inhibitor Rp-8-Br-cAMPS (10-200 μM) inhibited ATP-dependent differentiation (Fig. 5).

ATP is broken down by surface ectonucleotidases to ADP, AMP and adenosine. This raises the possibility that the stimulation of cAMP production and differentiation, and the suppression of growth were due to the generation of one or other of these breakdown products. However, we obtained potency orders for all three effects which took the form: ATPγS $\geqslant$ ATP $>$ ADP $\geqslant$ AMP = Adenosine $\approx$ 0. This potency order for cAMP generation, in particular, excludes the involvement of adenylate cyclase-activating adenosine receptors.

ATPγS (EC_{50} 17.7 $\pm$ 2.7 μM) was found to be a more potent agonist than ATP (EC_{50} 158 $\pm$ 15.6 μM) for cAMP production from undifferentiated HL-60 cells. This difference might have arisen from the greater stability of ATPγS. However, HPLC analysis of ATP (initial concentration 100 μM) and its breakdown products under the conditions of these experiments demonstrated that only about 5% of ATP was broken down. Therefore, ATPγS appears to be a selective agonist suggesting the presence of a novel P_2 receptor. The effects of ATPγS on cell differentiation and growth inhibition, however, were complex because at the highest concentrations (0.3 and 1.0 mM) it induced cell death. This effect may have arisen from ATPγS-derived thiophosphate. We have shown previously that thiophosphate (10-1000 μM) induces apoptosis in HL-60 cells (18).

ATP receptors that activate adenylate cyclase have been reported previously. In some cases ATP-activated cAMP production is sensitive to the cyclo-oxygenase inhibitor indomethacin which blocks the generation of the autocrine activator PGE_2 (13, 19, 20). In MDCK-D1 cells, activation of PLA_2-coupled P_{2U} receptors by ATP or UTP induces cAMP production via this mechanism (13). In other cells, however, ATP-activated cAMP production is insensitive to indomethacin (11, 21) and UTP is ineffective despite the presence of P_{2U} receptors e.g., NG108-15 cells (12). The results of our study indicate that HL-60 cells fall into this latter group: indomethacin (50 μM) did not block ATP-activated cAMP production and UTP was ineffective despite the presence of P_{2U} receptors (9, 17, 22) A sub-group of ATP receptors that stimulate cAMP production are inhibited by xanthines (11, 12, 23). The receptor responsible for cAMP production in HL-60 cells does not appear to belong to this group because IBMX (2 mM) did not inhibit cAMP production. Instead, IBMX prolonged the

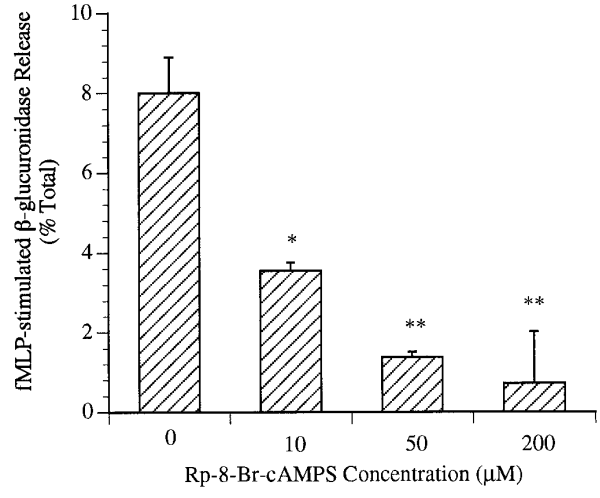

FIG. 5. The effect of Rp-8-Br-cAMPS on ATP-induced HL-60 cell differentiation. HL-60 cells were exposed to Rp-8-Br-cAMPS at the concentrations shown for 20 min prior to exposure to ATP (100 μM). After 48 h culture in RPMI-1640 (FBS 10%) the cells were harvested and assayed for fMLP-induced β-glucuronidase release and total β-glucuronidase content (compared to 100 μM ATP alone: *p = 0.016; **p $<$ 0.005).

ATP-induced elevation in cAMP levels as predicted for an inhibitor of cAMP phosphodiesterase.

The conclusion that ATP induces the differentiation of HL-60 cells via the production of cAMP is consistent with a large body of evidence that links elevated intracellular cAMP levels to HL-60 cell differentiation. This effect of intracellular cAMP has been observed in cells exposed to cell-permeable forms of cAMP e.g., dbcAMP (4) or 8-Cl-cAMP (5) as well as in cells exposed to activators of adenylate cyclase e.g., PGE_2 (1), epinephrine (3) and histamine (2, 24). cAMP is a recognised differentiation agent in a variety of cell systems. In addition to its differentiation effects on HL-60 cells, cAMP also induces the differentiation, for example, of certain neurons (25), PC12 pheochromocytoma cells (26), thyrocytes (27) and LNCaP and PC-3-M prostate cancer cells (28). Depending on the distribution of adenylate cyclase coupled ATP receptors, extracellular ATP may act as a more general signal for cellular differentiation.

ATP-activated cAMP production has been linked to increased DNA synthesis and mitogenesis (19, 20, 29). In HL-60 cells, however, we observed ATP-dependent suppression of cell proliferation. The mechanism is unclear. The cell-permeable inhibitor of PK-A, Rp-8-Br-cAMPS (200 μM) which suppressed ATP-dependent differentiation, failed to reverse the inhibitory effect of ATP on cell growth. The growth suppressing effect of ATP may depend upon an alternative mechanism e.g., the activation of an ecto-protein kinase (30). However, intracellular cAMP has been shown to suppress the growth of a variety of cell-types in culture (31) and cell-permeable forms of cAMP, dbcAMP and 8-Cl-cAMP, as well as the adenylate cyclase activators PGE_1 and PGE_2, suppress the growth of HL-60 cells (4, 5). Rp-8-Br-cAMPS is a more potent inhibitor of the PK-AI isoenzyme than PK-AII (32). It is possible that ATP induces differentiation via the activation of PK-AI, whereas ATP suppresses cell growth via the activation of PK-AII.

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