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Extracellular ATP is a mitogen for 3T3, 3T6, and A431 cells and acts synergistically with other growth factors

(purinoceptor/competence factor/ectoenzymes)

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ABSTRACT **Extracellular ATP in concentrations of 5–50 μM displayed very little mitogenic activity by itself but it caused synergistic stimulation of [^{3}H]thymidine incorporation in the presence of phorbol 12-tetradecanoate 13-acetate, epidermal growth factor, platelet-derived growth factor, insulin, adenosine, or 5′-(*N*-ethyl)carboxamidoadenosine. Cultures of Swiss 3T3, Swiss 3T6, A431, DDT$_1$-MF2, and HFF cells were used. The percent of cell nuclei labeled with [^{3}H]thymidine and cell number were also increased. ADP was equally mitogenic, while UTP and ITP were much less active. The effect of ATP was not due to hydrolysis by ectoenzymes to form adenosine, a known growth factor. Thus, the nonhydrolyzable analogue adenosine 5′-[β,γ-imido]triphosphate was mitogenic. In addition, it was found that ATP showed synergism in 3T6 and 3T3 cells when present for only the first hour of an incorporation assay, during which time no significant hydrolysis occurred. Furthermore, prolonged preincubation of cells with ATP reduced the mitogenic response to ATP but not to adenosine; preincubation with adenosine or N^6-(*R*-phenylisopropyl)adenosine had the reverse effect. Finally, the effect of adenosine, but not of ATP, was inhibited by aminophylline. We conclude that extracellular ATP is a mitogen that interacts with P$_2$ purinoceptors on the plasma membrane.**

Quiescent cultures of Swiss 3T3 mouse fibroblasts and other cell lines have served as useful models for many studies on growth control (1–9). They can be stimulated to reinitiate DNA synthesis and cell division by a variety of growth factors added in serum-free medium. These growth factors activate a number of early signals which vary, depending on the mitogen (9). For example, vasopressin stimulates Ca^{2+} mobilization, monovalent ion fluxes, inositolphospholipid breakdown, and protein kinase C activation (10). On the other hand, agents such as the adenosine analogue 5′-(*N*-ethyl)carboxamidoadenosine (NECA) (11), cholera toxin (12), and cyclic AMP derivatives (13) produce a different pattern of early events; they increase cyclic AMP but do not activate protein kinase C. Specific combinations of growth factors belonging to different classes often promote more than additive effects over a wide range of concentrations, a phenomenon referred to as synergism (9). This has been studied especially in mouse fibroblast lines (9). It is believed that there are parallel, intracellular, signal transduction pathways leading to DNA synthesis and at least some of the effective synergistic combinations involve mitogens that employ different pathways.

We have observed that extracellular ATP stimulates a number of the early events which were found when well-known growth factors were presented to quiescent mammalian cells. These early signals include a transient elevation in cytosolic Ca^{2+} (14, 15), stimulation of Ca^{2+} efflux (16), and increased formation of inositol phosphates (16). In addition, we observed a stimulation of the rate of Na^+, K^+, and uridine entry, and enhanced ornithine decarboxylase activity (unpublished observations). Other workers have reported that extracellular ATP activates similar early signals in hepatocytes (17–20), Ehrlich ascites tumor cells (21–23), mouse macrophages (24), H35 hepatoma cells (25), endothelial cells (26–30), and turkey erythrocytes (31).

These findings raise the possibility that exogenous ATP acts as a growth factor, and this was implied in two recent papers (23, 24), where ATP was compared to "more conventional mitogens." However, it is premature to call ATP a mitogen without more evidence than has been available. One must be careful to rule out the possibility that ATP is converted to adenosine by ubiquitous hydrolytic enzymes on the cell surface (ectoenzymes) (32). It was demonstrated some years ago in Rozengurt's laboratory (11–13) and by others (33) that adenosine is a potent mitogen for Swiss 3T3 cells, and this is also true for certain adenosine analogues such as NECA.

In this paper, we show that exogenous ATP is a mitogen for 3T3, 3T6, A431, HFF (34), and DDT$_1$-MF2 (35) cells. We confirm that adenosine is also mitogenic, but several lines of evidence show that ATP acts independently, in its own right, and not by being first converted to adenosine by ectoenzymes.

MATERIALS AND METHODS

Materials. [^{3}H]Thymidine was purchased from Amersham. Culture media and fetal bovine serum (FBS) were from GIBCO. ATP, adenosine, epidermal growth factor (EGF), adenosine 5′-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*), and all other nucleotides were from Boehringer Mannheim. Bovine insulin, NECA, and phorbol 12-tetradecanoate 13-acetate (PTA) were from Sigma. Platelet-derived growth factor (PDGF) was obtained from Amgen Biologicals. All other chemicals were of the highest purity available.

Cell Culture. Swiss 3T3 and 3T6 mouse fibroblasts, human A431 epidermoid carcinoma cells (14), and DDT$_1$-MF2 cells (35), derived from a vas deferens tumor of the hamster, were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 10 mM Hepes, 44 mM $NaHCO_3$, penicillin (110 units/ml), streptomycin (100 μg/ml), and 0.5% (vol/vol) (3T6), 5% (A431, DDT$_1$-MF2), or 10% (3T3) FBS. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Two days after seeding, the 3T3 cells were given fresh medium supplemented as before and then allowed to become confluent and quiescent for 5 days. The A431, DDT$_1$-MF2, and 3T6 cultures were used 4, 5, or

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Abbreviations: EGF, epidermal growth factor; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; HFF, human foreskin fibroblast; PTA, phorbol 12-tetradecanoate 13-acetate; NECA, 5′-(*N*-ethyl)carboxamidoadenosine; *R*-PIA, N^6-(*R*-phenylisopropyl)adenosine.

6 days after inoculation, respectively. Plating densities were 1×10^5 (3T3, 3T6, DDT_1-MF2) or 4×10^5 (A431) cells per 35-mm dish. Dishes for DDT_1-MF2 cells were pretreated for 5 min with poly(D-lysine) (0.1 mg/ml).

Human foreskin fibroblast (HFF) cells (34) were maintained in DMEM supplemented with 10% FBS and plated at 1×10^5 cells per 35-mm dish, in Ham's F_{12}/DMEM (Irvine Scientific) supplemented with 1% CPSR-2 serum replacement (Sigma). They were used 5 days after plating.

Thymidine Incorporation Assay. DNA synthesis was measured by incubating the cultures in 2 ml of a 1:1 mixture (vol/vol) of DMEM and Waymouth's medium containing 1 μM [^{3}H]thymidine (0.25 μCi/ml; 1 Ci = 37 GBq) under various conditions as indicated. After 24 hr at 37°C, the cells were washed twice with ice-cold phosphate-buffered saline (32), extracted for 30 min with trichloroacetic acid (5%, wt/vol; 4°C) and washed twice with 95% (vol/vol) ethanol. The acid-insoluble material was extracted for 30 min at 23°C with 0.1 M NaOH containing Na_2CO_3 (2 g/100 ml). The radioactivity was measured by liquid scintillation spectrophotometry.

Separation of Adenosine, AMP, ADP, and ATP. The hydrolysis of [^{3}H]ATP was investigated by chromatography on polyethyleneimine-cellulose columns, as described (36).

RESULTS

Synergistic Growth Stimulation by ATP in Combination with Other Growth-Promoting Agents. To determine whether extracellular ATP can stimulate the reinitiation of DNA synthesis in serum-starved 3T6 cells, cultures were incubated with ATP at various concentrations in the presence or absence of EGF at 10 ng/ml. As shown in Fig. 1, as little as 5 μM ATP caused a very large dose-dependent stimulation of [^{3}H]thymidine incorporation into acid-insoluble material when tested in the presence of EGF. Conversely, increasing concentrations of EGF showed a large synergistic effect when assayed in the presence of 50 μM ATP. Similar responses were obtained with the combinations of ATP plus PDGF, ATP plus PTA, and ATP plus insulin (Table 1). These experiments were conducted over a period of 2½ years and some variation in extent of mitogenic response was noted (see Fig. 1 and Table 1). Comparisons made at intervals showed that synergistic combinations involving ATP were as effective in stimulating thymidine incorporation as 10% FBS or EGF plus insulin. Stimulation was also observed when the fraction of [^{3}H]thymidine-labeled nuclei was measured after a 40-hr incubation (Table 1). In addition, we measured the

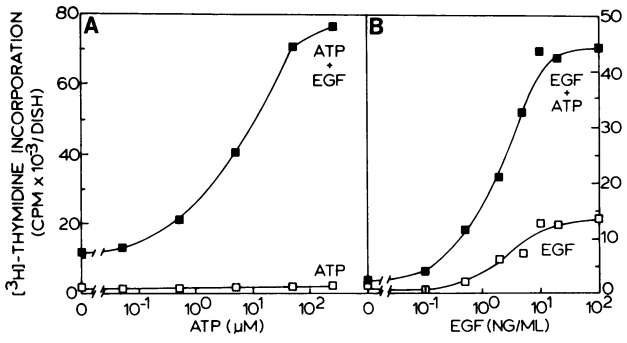

FIG. 1. Synergistic stimulation of DNA synthesis by extracellular ATP and EGF. 3T6 cells were plated and grown for 6 days, and then washed cells were incubated for 20 hr in a 1:1 mixture of DMEM and Waymouth's medium, with the following additions. (*A*) ATP as indicated in the presence (■) or absence (□) of EGF (10 ng/ml). (*B*) EGF as indicated in the presence (■) or absence (□) of ATP (50 μM). The incorporation of [^{3}H]thymidine into DNA was measured as described in the text.

Table 1. Stimulation of DNA synthesis by ATP and other mitogens in quiescent 3T6 cells

Additions	DNA synthesis: [^{3}H]Thymidine incorporation, cpm × 10^{-3}/dish	DNA synthesis: Labeled nuclei, %
None	1.0	0.8
ATP (50 μM)	5.4	2.6
EGF (10 ng/ml)	7.6	5.5
PTA (50 ng/ml)	2.5	2.7
PDGF (3 ng/ml)	11.0	16.0
Insulin (2 μg/ml)	8.8	10.6
Cholera toxin (100 ng/ml)	2.5	2.0
NECA (10 μM)	1.4	1.2
Insulin + cholera toxin	17.1	19.9
Insulin + NECA	10.5	14.2
ATP + EGF	24.0	21.4
ATP + PTA	20.0	31.9
ATP + PDGF	25.7	26.1
ATP + insulin	19.7	20.5
ATP + cholera toxin	9.3	5.5
ATP + NECA	5.3	3.9
ATP + insulin + cholera toxin	27.1	28.0
ATP + insulin + NECA	38.1	26.9

Quiescent cultures of 3T6 cells were washed twice with 1:1 DMEM/Waymouth medium before the indicated additions were made. Labeling with [^{3}H]thymidine (2.5 μCi/ml, 1.0 μM) was carried out for 40 hr. Test substances were present as indicated. The cultures were fixed with 1.5% perchloric acid for 2 hr at room temperature before washing. Autoradiography emulsion (Kodak NTB-3 nuclear track emulsion) was applied to the cultures, and they were incubated in the dark at 4°C for 8 days. After development with D-19 (Kodak), the cultures were stained with Giemsa stain. The cells in at least six separate fields containing about 300 cells each were counted on each culture dish. Data are the mean of four separate experiments.

increase in cell number over a period of several days (Fig. 2). The presence of 50 μM ATP, which had little effect by itself, greatly enhanced the proliferation of 3T6 cells treated with EGF at 10 ng/ml. ADP was equally mitogenic, while UTP and ITP were much less active. Acid-hydrolyzed and ashed ATP were inactive.

Exogenous ATP Exhibits Mitogenic Activity Without First Being Converted to Adenosine. We have confirmed older studies with 3T3 cells (13) and demonstrated that adenosine is mitogenic for 3T3 and 3T6 cells when presented in combination with the phorbol ester PTA (data not shown). The question therefore arises whether ATP was stimulating

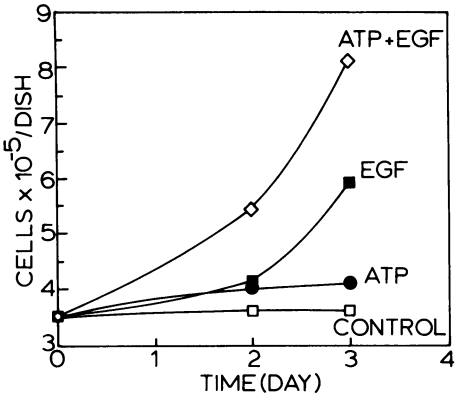

FIG. 2. Synergistic stimulation of cell proliferation. Quiescent 3T6 cells were prepared and then incubated in a 1:1 mixture of DMEM and Waymouth's medium with the following additions: □, none; ●, 50 μM ATP; ■, EGF at 10 ng/ml; ◇, 50 μM ATP plus EGF at 10 ng/ml.

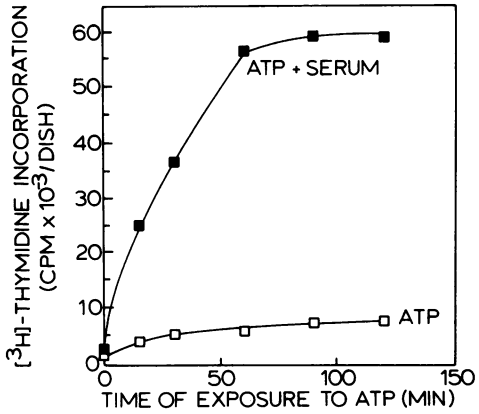

FIG. 3. Extracellular ATP acts as a competence factor for stimulating DNA synthesis in 3T6 cells, in combination with a low concentration of serum. Conditions were as for Fig. 1 with the following changes. □, ATP (50 μM) was present for the indicated times. Then it was removed by repeated washing and incubation was continued in unsupplemented medium. ■, Treatment with 50 μM ATP was followed by medium containing 0.2% FBS. Serum alone did not stimulate DNA synthesis (note *y* axis). Thymidine incorporation was measured after 20 hr of incubation. When both ATP and serum were present for the entire 20 hr, thymidine incorporation was the same as when ATP was present for only the first 60 min.

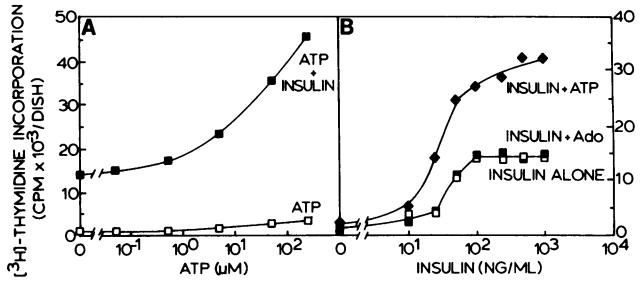

FIG. 4. Synergistic stimulation of DNA synthesis in 3T6 cells occurs with ATP plus insulin, but not with adenosine plus insulin. Conditions were as for Fig. 1. (*A*) ATP was added as indicated in the presence (■) or absence (□) of insulin (1 μg/ml). (*B*) Insulin was added as indicated, together with 50 μM ATP (◆), 50 μM adenosine (■), or no further addition (□).

growth in its own right or whether it was simply being converted to adenosine by ectoenzymes present in the plasma membrane. To answer this question, we first measured the rate of [^{3}H]adenosine formation from [^{3}H]ATP by removing samples of the medium and analyzing them. After 60 min at 37°C, no adenosine could be detected; all of the tritium was recovered in the medium as unchanged ATP except for 10% conversion to ADP. We then established that extracellular ATP need be present for only 60 min to cause synergistic stimulation of DNA synthesis; it could then be washed away and replaced by a second mitogen such as a low concentration of serum (Fig. 3), also insufficient to be mitogenic by itself. With this protocol, [^{3}H]thymidine incorporation was as great as when both agents were present throughout the entire 20-hr assay period.

The following supporting evidence also indicates a direct role for ATP in mitogenesis. (*i*) The ATP analogue Ado*PP*-[NH]*P* was as active as ATP when tested in synergistic combination with serum, EGF, or insulin (data not shown). When Ado*PP*[NH]*P* was incubated with 3T6 cells under conditions used for measurement of [^{3}H]thymidine incorporation, no significant breakdown to adenosine was seen after 4 hr. (*ii*) The mitogenic effects of adenosine were prevented in the presence of aminophylline, which inhibits the binding of adenosine to its receptors. This agent did not interfere with stimulation of DNA synthesis by ATP (data not shown). (*iii*) A surprising result, but obtained repeatedly with 3T6 cells, revealed synergistic stimulation with ATP plus insulin, whereas the combination of adenosine plus insulin was inactive (Fig. 4). The same was true for ATP plus PDGF (synergism). In contrast, no synergism was observed with adenosine plus PDGF (data not shown). These results were not due to metabolic removal of adenosine, since adenosine was as active as ATP when tested in combination with PTA or EGF. (*iv*) Synergism was observed with ATP but not with adenosine in DDT_1-MF2 cells (Table 2). (*v*) In epidermoid carcinoma A431 cells, extracellular ATP and adenosine show synergistic stimulation of [^{3}H]thymidine incorporation (Fig. 5). (*vi*) Under special conditions, in HFF cells, ATP enhanced the mitogenic effect of PDGF, whereas adenosine had little or no effect (Table 3). (*vii*) Prolonged preincubation of A431 and 3T6 cells with ATP reduced the mitogenic response to ATP but not to adenosine, and the reverse was also true. We conclude that ATP acts as a mitogen by interacting with P_2 purinoceptors on the plasma membrane.

DISCUSSION

In the present investigation, we have shown that extracellular ATP is a mitogen and displays synergistic enhancement of activity in combination with other mitogens. It did not act by being first converted to adenosine. For example, in 3T6 cells only 60 min of exposure to ATP was needed, during which no formation of adenosine was detected in the medium (Fig. 3). Then it was washed away and replaced by a low concentration of serum. It is highly likely that receptor-bound ATP was removed by several washes for the following reasons. (*i*) Its affinity is not very great (micromolar concentrations were needed for a response). (*ii*) After 15 min of contact with ATP, the extent of thymidine incorporation was only 40% of that seen after 60 min (Fig. 3). Receptor binding reactions are very rapid, since early signals such as the rise in cytosolic Ca^{2+} and inositol trisphosphate formation require only 10–20 sec. Accordingly, we assume that events following receptor binding require time and make a 60-min contact period necessary for complete development of competence. We conclude that the partial effect seen after 15 min of contact resulted from washing away the ATP. (*iii*) Using digital video fluorescence microscopy and fura-2 (14, 15), we observed that extracellular ATP caused a prolonged elevation in cytosolic Ca^{2+} in indi-

Table 2. Growth stimulation by ATP but not by adenosine in DDT_1-MF2 cells

Addition	[^{3}H]Thymidine incorporation, cpm/dish				
	Alone	+ EGF (10 ng/ml)	+ *R*-PIA (10 μM)	+ NECA (10 μM)	+ Theophylline (0.5 mM)
None	540.0	389.5	335.0	270.0	570.5
ATP (50 μM)	2241.0	6026.5	5708.0	5751.5	5869.0
Adenosine (50 μM)	2025.0	913.5	1453.0	1128.5	1744.0

Cells were grown for 5 days in DMEM plus 5% FBS with no change of medium. Then they were washed and incubated for 22 hr in 1:1 DMEM/Waymouth medium with supplements as indicated. *R*-PIA, N^6-(*R*-phenylisopropyl)adenosine.

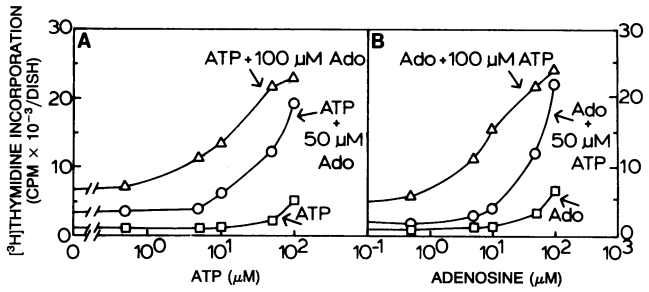

FIG. 5. Synergistic stimulation of DNA synthesis in A431 cells by ATP and adenosine. The cells were plated and grown for 4 days. (*A*) The 1:1 mixture of DMEM and Waymouth's medium was supplemented with different concentrations of ATP (*x* axis) and fixed levels of adenosine (Ado) as indicated. (*B*) The supplements consisted of different concentrations of adenosine and fixed levels of ATP as indicated.

vidual 3T6 cells maintained in the presence of 1 mM external Ca^{2+}. However, when the ATP-containing medium was washed away and replaced with ATP-free medium, the concentration of intracellular Ca^{2+} dropped within seconds, indicating the rapid removal of ATP (unpublished observations).

We found that HFF cells showed an inhibition by adenosine, as well as by ATP, of the PDGF-stimulated thymidine incorporation. After down-regulation of adenosine receptors with *R*-PIA, inhibition no longer occurred. We now observed a substantial enhancement of PDGF-induced stimulation and a small effect of adenosine (Table 3). This requires further investigation, but it appears that the initial inhibition was due to adenosine receptors. While this manuscript was being prepared, a report on the inhibition of PDGF-stimulated thymidine incorporation in HFF cells by forskolin and cholera toxin appeared (37).

There has been increasing interest in the effects of extracellular ATP and their physiological relevance (reviewed in ref. 38). We are particularly interested in a possible role for ATP and ADP in wound repair. After injury, PDGF and transforming growth factor α (TGFα) are released from various sources, including platelets (39). macrophages (40), and keratinocytes (41). It has been reported that topically applied EGF (41) and TGFα (42) promote epidermal regeneration in healing wounds. Significantly, ATP and ADP are also released from platelets after injury, and we have shown synergism between ATP and both EGF and PDGF in fibroblast proliferation. In addition, we have recently found a similar synergistic stimulation of DNA synthesis involving ATP and TGFα (unpublished observations).

The mechanism whereby extracellular ATP stimulates DNA synthesis invites investigation. Interestingly, it did not appear to function by activation of protein kinase C, although ATP-induced changes in the levels of inositol phosphates were noted. The activity of protein kinase C was measured by two independent methods (43). In addition, we observed that down-regulation of protein kinase C by prolonged preincubation of 3T3 cells with phorbol esters did not prevent synergistic stimulation of DNA synthesis by ATP plus insulin.

Table 3. Effect of *R*-PIA on PDGF-stimulated [^{3}H]thymidine incorporation in HFF cells

Additions	[^{3}H]Thymidine incorporation, cpm/dish	
	Control	*R*-PIA pretreated
None	298.5 ± 8	313.5 ± 10
PDGF (5 ng/ml)	6000.0 ± 17	6033.0 ± 21
ATP (50 μM)	235.5 ± 19	237.0 ± 21
Adenosine (50 μM)	301.5 ± 23	235.5 ± 30
ATP + PDGF	2775.0 ± 22	8245.5 ± 25
Adenosine + PDGF	3450.0 ± 19	6634.5 ± 20

Confluent HFF cells were pretreated in the absence or presence of 10 μM *R*-PIA for 40 hr at 37°C and then incubated with test substances for an additional 18 hr. After being washed extensively in DMEM, the cells were incubated in 1 ml of F_{12}/DMEM (34) containing 5% FBS and [^{3}H]thymidine (0.5 μCi/ml) for 4 hr at 37°C. The results are the mean ± SEM of six determinations.

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