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EXTRACELLULAR ATP SHOWS SYNERGISTIC ENHANCEMENT OF DNA SYNTHESIS WHEN COMBINED WITH AGENTS THAT ARE ACTIVE IN WOUND HEALING OR AS NEUROTRANSMITTERS

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The polypeptides PDGF, TGFα, and EGF have previously been shown by others to stimulate proliferation of fibroblasts and keratinocytes in the process of wound healing. Here we demonstrate that extracellular ATP, ADP or AMPPNP caused synergistic enhancement of DNA synthesis in 3T6 mouse fibroblasts and BALB/MK keratinocytes when combined with any of the above polypeptides. TGFβ showed synergistic stimulation with ATP in fibroblasts but it inhibited keratinocytes. ATP acted as a mitogen for NIE-115 neuroblastoma cultures. In 3T6 cells, ATP stimulated thymidine incorporation in combination with carbachol or norepinephrine. The effect of carbachol was sensitive to atropine. We suggest that extracellular ATP and ADP may play a physiological role in wound healing and as a mitogenic neurotransmitter in the nervous system.

In recent years, increasing attention has been devoted to the extracellular roles of ATP and ADP. A significant concentration is found in blood and other extracellular fluids (1–3) and can be increased, as in ischemia (3). In addition, ATP is released from purinergic nerve terminals and from the alpha granules of dense platelets (3). We are interested in possible mitogenic functions for extracellular ATP and ADP in wound healing and in the nervous system.

The initial events in wound repair are platelet deposition and blood coagulation (4). The platelets release PDGF and TGFα, which attract leukocytes and fibroblasts to the wound by chemotaxis (4, 5). Both PDGF and TGFα stimulate fibroblast proliferation and induce synthesis of new extracellular matrix [reviewed in (4, 6)]. Finally, the platelets release ATP and ADP, which we found to be mitogenic for fibroblasts (7). It was therefore reasonable to examine the possibility that one *in vivo* function of ATP and ADP could be synergistic enhancement of DNA synthesis when presented to fibroblasts in combination with polypeptide growth factors that are present in a wound. An important aspect of wound healing is the

Abbreviations: AMPPNP, adenosine 5'-[β,γ-imido]triphosphate; EGF, epidermal growth factor; FBS, fetal bovine serum; PDGF, platelet derived growth factor; TGFα, transforming growth factor α; TGFβ, transforming growth factor β; VIP, vasoactive intestinal peptide; Ado, adenosine; NECA, 5'-N-ethylcarboxamide-adenosine; TPA, phorbol 12-tetradecanoate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium (Gibco); XAC, xanthine amine congener 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl] oxy]phenyl]-1,3-dipropylxanthine.

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proliferation of keratinocytes (8). It has been reported that EGF stimulates growth of primary keratinocytes (9) and the keratinocyte cell line, BALB/MK (10). TGFα (8) and VIP (11) are also active in primary keratinocytes. Extracellular ATP synergizes with EGF (7). In the present communication, we show that ATP, ADP and AMPPNP caused enhancement of DNA synthesis in 3T6 cells when given together with TGFα or TGFβ. Mitogenic effects of ATP and ADP in BALB/MK cells are also described. Thus, it is possible that a variety of the polypeptide growth factors reported in wounds may synergize with ATP and ADP.

Neuropeptides have been shown to be mitogenic. The list includes bombesin, vasopressin, bradykinin and VIP [reviewed in (12)]. In addition, a trophic effect has recently been demonstrated for classical neurotransmitters. Thus, the acetylcholine analogue, carbachol, interacting with muscarinic receptor subtypes that are coupled to inositol lipid metabolism, can elicit DNA synthesis in enriched primary cultures from newborn cerebral cortex (13). With the same preparation, it was found that ATP, acting on P_2–purinergic receptors, causes inositol trisphosphate generation, mobilization of cytosolic Ca^{2+} and prostanoid generation, which are believed to be early signals leading to DNA synthesis (14).

Here we show that extracellular ATP causes synergistic enhancement of DNA synthesis when presented to 3T6 mouse fibroblasts in combination with carbachol and either EGF or insulin. Mitogenic activity was also found for norepinephrine and for VIP, in combination with ATP.

MATERIALS AND METHODS

Swiss 3T6 mouse fibroblasts were maintained, plated and allowed to become quiescent as described (7). The confluent cultures in 35 mm dishes were transferred to DMEM:Waymouth (1:1) (7) and incubated for 24 hrs at 37°C to deplete them of serum growth factors. [^{3}H]thymidine and other additions were made and incorporation into DNA was measured as described (7). For other cell lines, the treatment was similar except that serum starvation was omitted for NIE–115 cells. Other details are shown in the figure legends.

RESULTS

Synergism between ATP and polypeptide growth factors known to be present during wound healing. Fig. 1A demonstrates synergistic enhancement of thymidine incorporation in quiescent 3T6 mouse fibroblasts when serum–free medium was supplemented with 2.5 ng/ml PDGF and increasing levels of extracellular ATP. A similar titration with PDGF in the presence of ATP fixed at 50 μM is shown in Fig. 1B. Not shown is the fact that no enhancement of PDGF effects by adenosine was detected in 3T6 cells. Synergism between ATP and AMPPNP, and either TGFα or TGFβ is documented in Table 1. Adenosine was much less active.

Falco *et al.* demonstrated that EGF is a potent mitogen for the cloned BALB/MK cell lines derived from BALB/c mouse keratinocytes (10). Since proliferation of keratinocytes occurs in wound healing, we examined the effects of ATP in this cell line. Figs. 2A and 2B demonstrate synergism between TGFα and ATP. Figs. 2C and 2D show a large synergistic enhancement of DNA synthesis by ATP in the presence of EGF and insulin compared to a

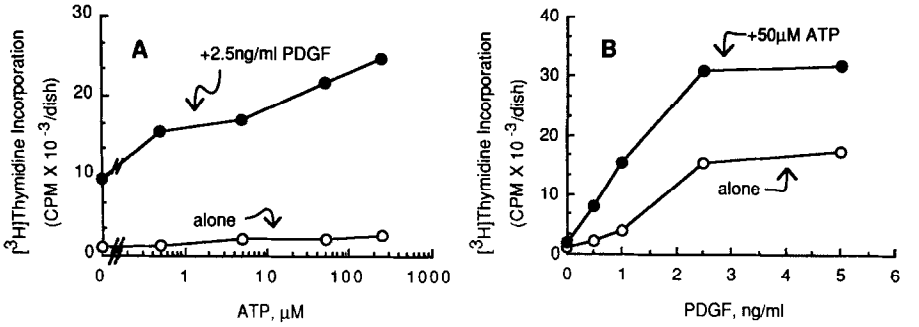

Fig. 1. Synergistic stimulation of DNA synthesis by extracellular ATP and PDGF in 3T6 cells. Quiescent 3T6 cells were serum–starved with a 1:1 mixture of DMEM and Waymouth's medium and then incubated for 24 hr in the same medium containing [^{3}H]thymidine (1 μM, 0.25 μCi/ml, 10^5 cpm/nmole) and the following additions: **Panel A,** variable levels of ATP as shown, in presence or absence of 2.5 ng/ml PDGF. **Panel B,** variable levels of PDGF as shown, in presence or absence of 50 μM ATP. Each dish contained 10^6 cells (0.35 mg protein).

quite modest effect caused by adenosine. In addition, the further stimulation by adenosine in the presence of EGF and insulin was inhibited by XAC and aminophylline, two antagonists of adenosine receptors (18). In contrast, mitogenesis by ATP and ADP was not affected (Table 2).

Synergism between ATP and neuropeptides or neurotransmitters. Extracellular ATP shows synergistic enhancement of DNA synthesis in quiescent 3T6 cells when combined with VIP (Figure 3). This neuropeptide functions as a growth factor by elevating the levels of cAMP in the presence of forskolin or inhibitors of cAMP phosphodiesterase (RO20–1724) (11, 15). Thus, it resembles the action of adenosine, NECA and cholera toxin (16). Extracellular ATP was also found to be synergistic with the neuropeptides bombesin, vasopressin and bradykinin (unpublished). In addition, ATP acted as a mitogen in NIE–115 neuroblastoma cells (Table 3).

Recently, it has been shown that carbachol stimulates DNA synthesis via muscarinic acetylcholine receptors in certain brain–derived astrocytoma and neuroblastoma cell lines and in primary astrocytes derived from perinatal rat brain (13). Fig. 4A demonstrates that

Table 1. Stimulation of DNA synthesis by ATP, AMP-PNP, or adenosine combined with TGF-α or TGF-β in Swiss 3T6 cells

	[^{3}H]thymidine incorporation (cpm/dish)		
Addition	alone	+ TGF–α (10 ng/ml)	+ TGF–β (10 ng/ml)
None	1,080	6,588	4,320
ATP (50 μM)	2,916	24,300	22,788
AMP-PNP (50 μM)	3,132	22,356	26,244
Ado (50 μM)	1,188	14,364	13,284

Experimental conditions were as described in legend to Fig. 1.

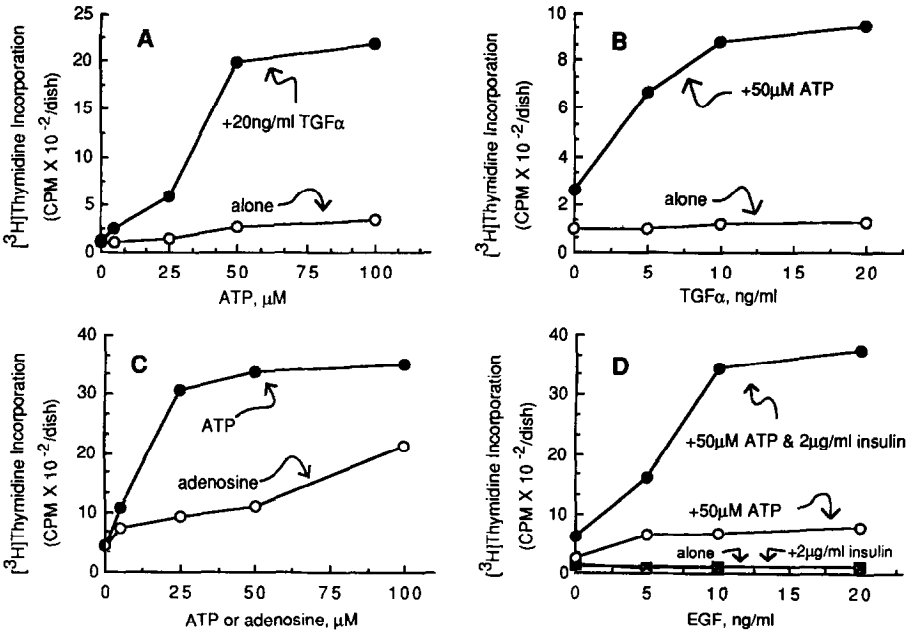

Fig. 2. Synergistic stimulation of DNA synthesis in BALB/MK keratinocytes. Cells were brought to quiescence in MEM plus 10% FBS and 4 ng/ml EGF. They were then incubated for 24 hr in a 1:1 mixture of MEM and Ham's F12 medium, with Ca^{2+} reduced to 0.05 mM, supplemented with 5 μg/ml transferrin, 0.2 mM ethanolamine and 10 nM Na_2SeO_3, followed by 24 hr with [^{3}H]thymidine and the following additions: **Panel A,** various levels of ATP as shown, in presence (●) or absence (○) of 20 ng/ml TGF–α. **Panel B,** various levels of TGFα in presence (●) or absence (○) of 50 μM ATP. **Panel C,** various levels of ATP (●) or adenosine (○) as shown, in the presence of 10 ng/ml EGF plus 2 μg/ml insulin. **Panel D,** various levels of EGF in the presence of 50 μM ATP (○) or 50 μM ATP plus 2 μg/ml insulin (●). Note that EGF plus insulin (□) gave no significant effect compared with EGF alone (X).

Table 2. Effect of XAC and aminophylline on [^{3}H]thymidine incorporation in BALB/MK cells stimulated by various growth factors*

Addition	[^{3}H]thymidine incorporation (cpm/dish)		
	alone	+ XAC (500 nM)	+ aminophylline (0.5 mM)
None	124	125	113
ATP (50 μM)	286	290	300
ADP (50 μM)	161	165	170
Ado (50 μM)	186	170	167
EGF (10 ng/ml) + insulin (2 μg/ml)	533	529	541
ATP + EGF + insulin	2,430	2,542	2,356
ADP + EGF + insulin	2,443	2,728	2,307
Ado + EGF + insulin	1,364	769	558

* Experimental conditions were as described in legend to Fig. 2.

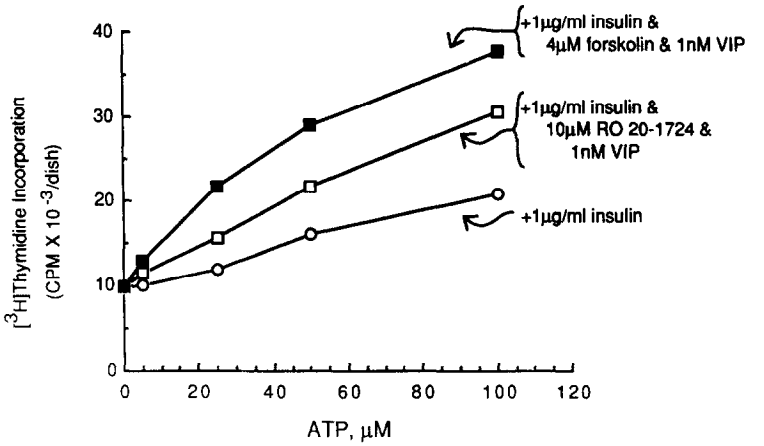

Fig. 3. Synergistic stimulation of DNA synthesis by extracellular ATP and VIP in 3T6 cells. Quiescent 3T6 cells were washed twice with a 1:1 mixture of DMEM and Waymouth's medium and incubated in the same medium containing various concentrations of ATP together with: (○), 1 μg insulin alone; (□), insulin plus 10 μM RO20–1724 plus 1 nM VIP; or (■), insulin plus 4 μM forskolin plus VIP. DNA synthesis was measured after 24 hr of incubation with [^{3}H]thymidine.

extracellular ATP caused further enhancement of DNA synthesis in 3T6 cells in the presence of carbachol plus EGF. By contrast, adenosine had little or no effect (Fig. 4B). Fig. 4C shows the effect on DNA synthesis of increasing levels of carbachol in the presence of fixed levels of EGF and ATP. Similar results (not shown) were obtained in the presence of insulin in place of EGF. Not shown is the fact that the effects of carbachol, but not of EGF, ATP or insulin, were completely inhibited by 10 μM atropine. Finally, ATP (but not adenosine) caused further enhancement of DNA synthesis stimulated by norepinephrine in the presence of insulin (Fig. 4D).

Table 3. ATP acts as a mitogen in NIE–115 neuroblastoma cells*

Addition	[^{3}H]thymidine incorporation (cpm/dish)	
	alone	+ 50 μM ATP
None	993	2,085
EGF (10 ng/ml)	1,390	4,865
TPA (50 ng/ml)	1,092	5,461
Insulin (0.5 μg/ml)	1,489	7,546
Cholera toxin (100 ng/ml)	1,290	6,603
NECA (10 μM)	1,191	5,660
1% FCS	2,979	10,923
10% FCS	12,313	

* Cells (1 x 10^5 per 35 mm dish) were incubated in DMEM plus 5% FBS for 3 days, washed, and incubated for 2 days in 1:1 DMEM and Waymouth medium. Additions were made as indicated and incorporation was assayed after 24 hr further incubation.

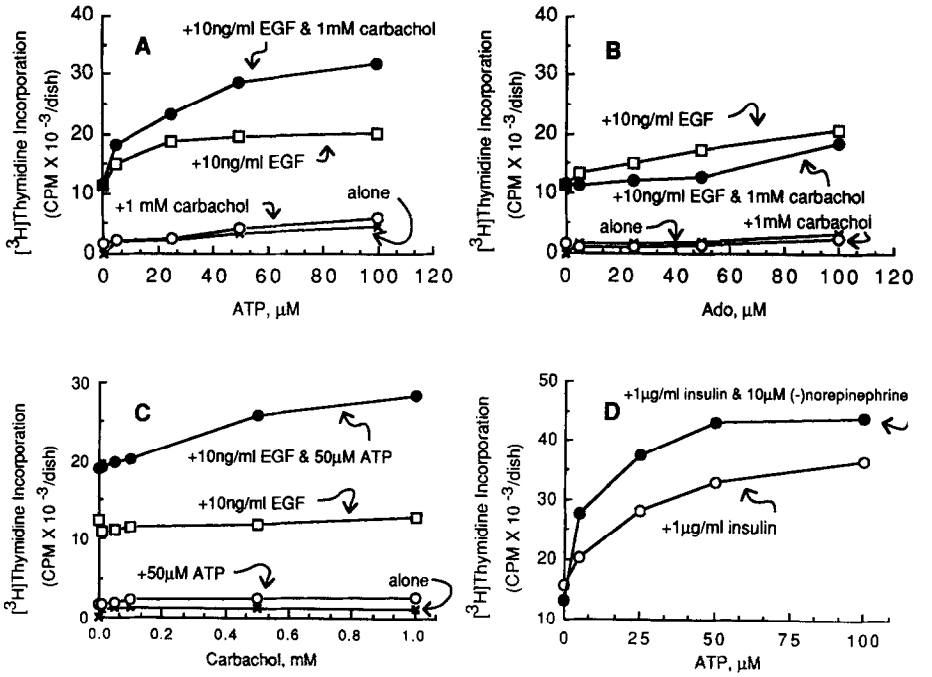

Fig. 4. Synergistic stimulation of DNA synthesis in 3T6 cells involving ATP and either carbachol or (–) norepinephrine. **Panel A,** quiescent 3T6 cells were washed twice with a 1:1 mixture of DMEM and Waymouth's medium and incubated for 40 hr in the same medium containing [^{3}H]thymidine (0.25 μCi/ml, 1 μM) and increasing levels of ATP together with: (X), no further addition; (○), 1 mM carbachol; (□), 10 ng/ml EGF; (●), 10 ng/ml EGF plus carbachol. **Panel B,** same as Panel A except that adenosine replaces ATP. Note that adenosine inhibits carbachol stimulation, whereas ATP caused enhancement. **Panel C,** same as Panel A except that increasing levels of carbachol were mixed with: (X), no further addition; (○), 50 μM ATP; (□), 10 ng/ml EGF; or (●), EGF plus ATP. Note that the maximum stimulation by saturating levels of carbachol plus EGF is enhanced almost 3–fold by 50 μM ATP. **Panel D,** same as Panel A except that increasing levels of ATP were mixed with: (○), 1 μg/ml insulin; or (●), 1 μg/ml insulin plus 10 μM (–) norepinephrine.

DISCUSSION

The present data show that extracellular ATP and ADP caused synergistic enhancement of DNA synthesis when given to quiescent fibroblasts and to the BALB/MK keratinocyte line in combination with polypeptide growth factors known to be released into a wound. Results presented here and previously (7) indicate that these are direct effects of nucleotides and cannot be due to adenosine formation by ectoenzymes. We speculate that ATP and ADP released from aggregating platelets could cause synergistic enhancement of DNA synthesis *in vivo*. Unfortunately, we lack information on the concentrations of ATP and ADP in a wound or on the rates of their hydrolysis. However, we are encouraged by certain considerations. These nucleotides are competence factors and need to be present for as little as 30 min in order to display effective synergism, for example, with EGF (7). Also, as pointed out by Gordon et al. (17), certain kinetic properties make the rate of hydrolysis of ADP slow compared with that of ATP and, at sites of platelet degranulation creates a time lag before the appearance of adenosine. As explained by these authors, the effect is to encourage further platelet aggregation and release and to prolong the lifetime of the mitogenic nucleotides.

With respect to the nervous system, increasing evidence suggests that neurotransmitters themselves have the potential to act as regulators of nerve cell growth and division (12–15). The fact that the neurotransmitter, ATP (19), displays mitogenic activity in combination with the acetylcholine analogue carbachol, as well as with norepinephrine and VIP is interesting in view of their localization. Thus acetylcholine and ATP coexist in cholinergic synaptic vesicles (20). Similarly nonadrenergic nerve endings contain both norepinephrine and ATP. In some cases, ATP is found together with both acetylcholine and norepinephrine. Our studies were carried out with the established cell lines, NIE–115, 3T3 and 3T6. It would clearly be desirable to continue this investigation with a physiologically more relevant preparation. Such studies are under way. Finally, we have noted one other paper on ATP–induced DNA synthesis, in thymocytes (21).

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REFERENCES

1. Rapaport, E. and Fontaine, J. (1989) Proc. Natl. Acad. Sci. USA 86, 1662–1666.
2. Born, G.V.R. and Kratzer, M.A.A. (1984) J. Physiol. 354, 419–429.
3. Gordon, J.L. (1986) Biochem. J. 233, 309–319.
4. Ross, R., Raines, E.W. and Bowen–Pope, D.F. (1986) Cell 46, 155–169.
5. Postlethwaite, A.E., Keski–Oja, J., Moses, H.L. and Kang, A.H. (1987) J. Exp. Med. 165, 251–256.
6. Sporn, M.B., Roberts, A.B., Wakefield, L.M. and de Crombrugghe, B. (1987) J. Cell Biol. 105, 1039–1045.
7. Huang, N., Wang, D. and Heppel, L.A. (1989) Proc. Natl. Acad. Sci. USA 86, 7904–7908.
8. Coffey, R.J. Jr., Derynck, R., Wilcox, J.N., Bringman, T.S., Goustin, A.S., Moses, H.L. and Pittelkow, M.R. (1987) Nature 328, 817–820.
9. Brown, G.L., Curtsinger, L. III, Brightwell, J.R., Ackerman, D.M., Tobin, G.R., Polk, H.C. Jr., George–Nascimento, C., Valenzuela, P. and Schultz, G.S. (1986) J. Exp. Med. 163, 1319–1324.
10. Falco, J.P., Taylor, W.G., DiFiore, P.P., Weissman, B.E. and Aaronson, S.A. (1988) Moll. Cell Biol. 8, 557–563.
11. Haegerstrand, A., Jonzon, B., Dalsgaard, C.–J. and Nilsson, J. (1989) Proc. Natl. Acad. Sci. USA 86, 5993–5996.
12. Zachary, I., Woll, P.J. and Rozengurt, E. (1987) Dev. Biol. 124, 295–308.
13. Ashkenazi, A., Ramachandran, J. and Capon, D.J., (1989) Nature 340, 146–150.
14. Pearce, B., Murphy, S., Jeremy, J., Morrow, C. and Paresh, D. (1989) J. Neurochem. 52, 971–977.
15. Zurier, R.B., Kozma, M., Sinnett–Smith, J. and Rozengurt, E. (1988) Exp. Cell Res. 176, 155–161.
16. Rozengurt, E. (1982) Exp. Cell Res. 139, 71–78.
17. Gordon, E.L., Pearson, J.D. and Slakey, L.L. (1986) J. Biol. Chem. 261, 15496–15504.

18. Jacobson, K.A., Ukena, D., Kirk, K.L. and Daly, J.W. (1986) Proc. Natl. Acad. Sci. USA 83, 4089–4093.
19. Burnstock, G. (1972) Pharmacol. Rev. 24, 500–581.
20. Richardson, P.J. and Brown, S.J. (1987) J. Neurochem. 48, 622–630.
21. El–Moatassim, C., Dornand, J. and Mani, J.–C. (1987) Biochim. Biophys. Acta 927, 437–444.