ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells

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It is well known that intracellular ATP plays fundamental roles in nucleic acid synthesis, ion channel modulation, energy metabolism, and enzyme regulation. Accumulating evidence has shown that extracellular ATP also acts as an extracellular signaling molecule mediating cell-cell communication in nonneuronal cells (31, 45) and as a neurotransmitter or neuromodulator in the peripheral and central nervous system (10, 11). Extracellular ATP is known to exert its effects via a family of specific receptors termed P2 purinoceptors. The P2 purinoceptors are divided into the P2X receptors, which are ligand-gated ion channels, and the P2Y receptors, which are G protein-coupled receptors (29). Up to seven P2X subtypes (P2X1–P2X7) (5) and several types of P2Y receptors (P2Y1, P2Y2, P2Y4, and P2Y6) have been cloned, and a wide range of tissue expression of P2X and P2Y receptors has been reported (29).

Fracture healing of bone occurs through a complex sequence of cellular processes, and proliferation and differentiation of osteoblasts have pivotal roles in bone formation (2). Platelet-derived growth factor (PDGF) or insulin-like growth factor I (IGF-I) has been suggested to modulate the healing of bone fractures, in part by promoting proliferation and differentiation of osteoblasts, possibly in an autocrine/paracrine manner (1, 2).

It has been reported that at sites of tissue injury and inflammation, nucleotides, including ATP, are released from damaged cells (3) or from activated platelets or leukocytes (31), and they reach concentrations sufficient to activate purinoceptors (14). Thus ATP is considered to function as an autocrine/paracrine mediator to regulate osteoblast activity in fracture healing and inflammation. Recently, ATP has been shown to be released by mechanical stresses, such as stretch, compression, or shear stress, in a variety of cells, including osteoblasts (16, 19). In bone tissues, much attention has been paid to how mechanical stimuli transduce the signal to the osteoblasts or osteocytes (28). Although extracellular ATP is considered a possible candidate involved in the mechanical responses of the bone (19),...
it is not certain whether ATP mediates cellular responses and what are the signal transduction pathways of the ATP-induced cellular events in osteoblasts. P2 purinoreceptors are expressed in several species of osteoblasts, and extracellular ATP causes an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) in osteoblasts and osteoblast-like cells (19, 22, 34). In osteoblasts, a recent study has shown that extracellular ATP increased [Ca\(^{2+}\)], as well as DNA synthesis and cell proliferation in MC3T3-E1 mouse osteoblast-like cells (36). In addition, ATP synergistically potentiated the PDGF-induced DNA synthesis in the cells (36). However, no direct proof was presented regarding the involvement of [Ca\(^{2+}\)], in the effect of ATP. It is not clear how extracellular ATP mediates cell proliferation and which types of ATP receptors are involved in the ATP-induced cellular responses in osteoblasts.

In the present study we focused on the characterization of ATP receptor subtypes and intracellular signal transduction pathways responsible for the ATP-induced proliferative effects in osteoblasts. We also investigated how ATP synergistically activates the growth factor-induced cell proliferation. To this end, we employed human osteoblast-like MG-63 cells, which have been widely used to investigate the signaling pathway of osteoblasts (8, 27). We showed that extracellular ATP causes DNA synthesis via activation of P2X, but not P2Y, receptors in a mitogen-activated protein (MAP) kinase-dependent manner.

**MATERIALS AND METHODS**

**Materials.** MG-63 human osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). Culture plates or dishes were obtained from Falcon (Meylan Cedex, France). ATP was obtained from Kohjin (Tokyo, Japan); UTP, UDP, adenosine 5’-O-(3-thiotriophosphate) (ATP\(_7\)S), fura 2-AM, and herbimycin A from Calbiochem (La Jolla, CA); 1,2-bis(2-aminophenoxy)ethane-N\(_2\),N\(_2\),N\(_2\),N\(_2\)-tetraacetic acid (BAPTA)-AM from Dojindo (Kumamoto, Japan); ATP-(2R,3S,4R,5R)-1,2-bis(2-aminophenoxy)ethane-N\(_2\),N\(_2\),N\(_2\),N\(_2\)-tetraacetic acid (BAPTA)-AM from Dojindo (Kumamoto, Japan); pyridoxal phosphate-6-azophenyl-2’-4-disulfonic acid (PPADS) and reactive blue 2 from Tocris Cookson (Bristol, UK); human PDGF-BB from Pepro Tech (Rocky Hill, NJ); human IGF-I from Sumitomo Pharmacy (Tokyo, Japan); H-7 and KN-93 from Seikagaku Kogyo (Tokyo, Japan); staurosporine from Kyowa Hakko (Tokyo, Japan); dibutyryl-cAMP (db-cAMP), myelin basic protein (MBP), and IBMX from Sigma Chemical (St. Louis, MO); MEM from Nissui (Tokyo, Japan); fetal bovine serum (FBS) from Biocell (Rancho Dominguez, CA); [\(^{3}H\)]thymidine (5.0 Ci/mmol) and p42/44 MAP kinase assay kit from Amersham Japan (Tokyo, Japan); and activated form of p42/44 MAP kinases from Stratagene (La Jolla, CA). The Abacus cell proliferation kit was obtained from Clontech (Palo Alto, CA). Other chemicals were analytic grade and were obtained from Nacalai Tesque (Kyoto, Japan).

**Cell culture.** MG-63 cells were cultured at 37°C in MEM (pH 7.2–7.4) containing 10% FBS, 2 mM L-glutamine, 0.1 U/ml penicillin, and 0.1 mg/ml streptomycin under a humidified atmosphere of 95% air-5% CO\(_2\). The cells were detached by exposure to 0.05% trypsin, washed twice with PBS, and seeded in 24- or 96-well 35- or 60-mm dishes at a density of 1.75 × 10\(^4\) cells/cm\(^2\). Quiescence was induced by incubating cells for 24 h in MEM containing 10% FBS and then for 48 h in MEM containing 0.1% FBS. The medium was changed to serum-free MEM just before experiments (36).

**DNA synthesis.** Cultured cells were seeded in 24-well plates (3.5 × 10\(^4\) cells/well, 1.75 × 10\(^4\) cells/cm\(^2\)) in 96-well plates, rendered quiescent, and treated with various test compounds in serum-free MEM for 24 h. [\(^{3}H\)]thymidine (37 kBq) was added to the serum-free MEM during the last 2 h of the 24-h incubation. Antagonists were added 1 h before ATP stimulation and maintained throughout the experiment. Cells were washed twice with ice-cold PBS, once with 5% (wt/vol) TCA, and once with ethyl alcohol-diethyl ether (3:1, vol/vol) and then harvested with 0.3 M NaOH. After neutralization with 0.6 M HCl, the suspension was passed through a cellulose acetate filter, and the retained radioactivity was determined with a liquid scintillation spectrometer (model LS7000, Beckman, Fullerton, CA).

**Cell proliferation.** Cells were seeded at a density of 4.5 × 10\(^3\) cells/well (1.75 × 10\(^4\) cells/cm\(^2\)) in 96-well plates, rendered quiescent, and treated with ATP or ATP-S. The cells were rendered quiescent and then incubated in CO\(_2\)-saturated HEPES-buffered solution (HBS) containing (in mM) 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose (pH adjusted to 7.4 with NaOH); with the amount of 5 μM fura 2-AM for 1 h. The perfusion fluid was HBS warmed to 37°C, and cells were continuously perfused at a constant flow rate of 1.5 ml/min. Ca\(^{2+}\)-free solution was modified HBS containing 2 mM EGTA without CaCl\(_2\) and MgCl\(_2\). Fluorescence was measured from fura 2-loaded cells in the perfusion chamber, which had a glass coverslip bottom and was positioned on the stage of an inverted microscope (model TMD-300, Nikon, Tokyo, Japan), with use of a Ca\(^{2+}\)-imaging system equipped with an intensified charge-coupled device camera (Quanticell700, JEOL, Tokyo, Japan). Fluorescence intensities at 510 nm with excitation at 340 and 380 nm were recorded at an interval of 5 s. [Ca\(^{2+}\)], in individual cells was calculated from the ratio of fluorescence images measured with excitation at 340 nm to those with excitation at 380 nm by use of the equation of Grynkiewicz et al. (15). Autofluorescence in MG-63 cells was negligible compared with the fluorescence in the fura 2-loaded cells.

**RT-PCR of P2X receptor mRNA.** Total RNA was extracted from quiescent MG-63 cells (60-mm dish) with a Midi kit (Qiagen, Hilden, Germany), and RT-PCR was performed with a thermal cycler (Perkin-Elmer, Norwalk, CT) with use of an RT-PCR kit (Toyobo, Osaka, Japan). Seven independent forward and reverse primers specific for P2X1–P2X7 receptors were designed on the basis of the cloned human P2X receptors of GenBank (Table 1). The sizes of PCR products expected on the basis of each primer pair are also shown in Table 1. Reverse transcription was performed in a final volume of 20 μl by use of random primers and an RT supplied with the RT-PCR kit. PCR was performed in a final volume of 50 μl containing 1 μl primers, 1 μl each deoxynucleoside triphosphate, 2.5 U of recombinant Taq DNA polymerase, 10 U of RNase inhibitor, and the RT-PCR buffer supplied with
Table 1. Individual P2X receptor primers for RT-PCR

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Sources represent GenBank accession numbers. Sequences are 5' to 3'. S, sense; AS, antisense.

RESULTS

Extracellular ATP increases [³H]thymidine incorporation and cell proliferation in MG-63 cells. Quiescent MG-63 cells were stimulated with ATP. Treatment of the cells with ATP at 100 µM for 24 h increased [³H]thymidine incorporation to 183.2 ± 6.8% of the control value (P < 0.05), and the effect was concentration dependent (EC₅₀ = 4.2 µM; Fig. 1A). We also measured cell number with a cell proliferation assay kit. Treatment of the cells with 100 µM ATP or the hydrolysis-resistant analog of ATP ATPγS (100 µM) for 48 h significantly increased the cell number to 113.0 ± 4.4 and 136.0 ± 2.2% of the control value, respectively (Fig. 1B).

ATP synergistically activates polypeptide growth factor-induced [³H]thymidine incorporation. Quiescent cells were stimulated with ATP and/or several polypeptide growth factors. IGF-I or PDGF, each given at 10 ng/ml for 24 h, also increased [³H]thymidine incorporation to 183.2 ± 6.8% of the control value, respectively (Fig. 2A). When ATP was added with IGF-I or PDGF, the nucleotide caused synergistic increases in [³H]thymidine incorporation to 986.8 ± 197.2 or 763.4 ± 55.4% of the control values, respectively (Fig. 2A). This synergistic action was observed in other growth factors, such as basic fibroblast growth factor, but not hepatocyte growth factor (data not shown). ATP augmented the IGF-I (10 ng/ml)-induced [³H]thymidine incorporation in a concentration-dependent manner (Fig. 2B). The EC₅₀ of ATP with IGF-I (EC₅₀ = 5.0 µM) was essentially similar to the EC₅₀ of ATP.

Effects of various purinergic agonists or antagonists on [³H]thymidine incorporation. To determine whether the mitogenic effects of extracellular ATP are mediated by activation of P2 purinoceptors, selective agonists or antagonists for P2 purinoceptors were tested. The hydrolysis-resistant analogs of ATP, ATPγS, and AMP-
P2X receptors (18), caused a complete inhibition of the ATP-induced \( [3H] \) thymidine incorporation at 100 \( \mu M \), suggesting that the effects of ATP were not due to P1 purinoceptor activation by adenosine or other hydrolyzed metabolites of ATP (Fig. 3A). \( \alpha \beta \)-mATP (100 \( \mu M \)), a selective agonist for P2X \(_1\) and P2X \(_2\) receptors, failed to mimic the ATP response. UTP or its hydrolyzed form UDP (100 \( \mu M \) each), which stimulates a family of P2Y receptors, except P2Y \(_1\) receptors, inhibited the \( [3H] \) thymidine incorporation by \(-30\%\) (Fig. 3A).

Suramin, a nonselective antagonist for P2X and P2Y receptors (18), caused a complete inhibition of the ATP-induced \( [3H] \) thymidine incorporation at 10 \( \mu M \) (Fig. 3B). PPADS, an antagonist for the P2X receptor (12), also completely inhibited the ATP-induced \( [3H] \) thymidine incorporation. On the other hand, reactive blue 2, an antagonist for P2Y \(_1\) receptors (7), failed to inhibit the ATP-induced \( [3H] \) thymidine incorporation even at 100 \( \mu M \), which is reported to inhibit the P2Y \(_1\) receptor-mediated cellular responses in other types of cells (44) (Fig. 3B).

RT-PCR analysis of the P2X receptor subtypes in MG-63 cells. RT-PCR analysis showed that MG-63 cells expressed mRNAs for the P2X\(_4\), P2X\(_5\), P2X\(_6\), and P2X\(_7\), but not the P2X\(_1\), P2X\(_2\), and P2X\(_3\) receptors (Fig. 4). The bands for P2X\(_4\), P2X\(_5\), P2X\(_6\), and P2X\(_7\) receptors were sequenced and found to be identical to the reported human P2X receptors. When we used mRNAs as templates prepared from rat vas deferens, which expresses the P2X\(_1\) receptor (45), or rat pheochromocytoma PC-12 cells, which express P2X\(_2\) and P2X\(_3\) receptors (25), a fragment for P2X\(_1\), P2X\(_2\), and P2X\(_3\) receptors was amplified with the corresponding primer pairs for each rat P2 purinoceptor (data not shown).

Ca\(^{2+}\) imaging of MG-63 cells stimulated with nucleotides or growth factors. \( [\text{Ca}^{2+}]_i \), responses to extracellular nucleotides and/or growth factors were analyzed in fura 2-loaded MG-63 cells. ATP (100 \( \mu M \)) increased \( [\text{Ca}^{2+}]_i \) in MG-63 cells (Fig. 5A), and the peak increase in \( [\text{Ca}^{2+}]_i \) from the baseline \( (\Delta[\text{Ca}^{2+}]_i) \) was 866.0 ± 55.6 nM \((n = 65; \text{Fig. 5B})\). UTP (100 \( \mu M \)) also increased \( [\text{Ca}^{2+}]_i \) \((n = 43)\), with \( \Delta[\text{Ca}^{2+}]_i \) of 781.2 ± 73.4 nM. PDGF (10 ng/ml) increased \( [\text{Ca}^{2+}]_i \), with \( \Delta[\text{Ca}^{2+}]_i \) of 561.7 ± 81.8 nM \((n = 12)\). In contrast, IGF-I did not increase \( [\text{Ca}^{2+}]_i \) \((n = 45; \text{Fig. 5, A and B})\), even when IGF-I was applied for a longer period (up to 5 min; data not shown). The selective agonist \( \alpha \beta\)-mATP did not elicit any increase in \( [\text{Ca}^{2+}]_i \) (data not shown).
addition, ATP and UTP increased \([\text{Ca}^{2+}]_i\), even when cells were perfused with \(\text{Ca}^{2+}\)-free buffer, and the amplitude of the \([\text{Ca}^{2+}]_i\) increase was similar to that obtained with normal, \(\text{Ca}^{2+}\)-containing buffer (data not shown). ATP did not further increase \([\text{Ca}^{2+}]_i\) when added with PDGF or IGF-I compared with the increase in \([\text{Ca}^{2+}]_i\) caused by ATP alone (Fig. 5B).

Effects of inhibitors for various protein kinases on ATP-induced \[^{3}H\]thymidine incorporation. The protein kinase C inhibitors H-7 (10 \(\mu M\)) and staurosporine (3 \(nM\)) or the \(\text{Ca}^{2+}/\text{calmodulin-dependent protein kinase II inhibitor KN-93 (0.3 \(\mu M\)}\) had little effect on ATP-induced \[^{3}H\]thymidine incorporation at concentrations reported to sufficiently inhibit each kinase activity in several types of cells (30) (Fig. 6). On the other hand, herbimycin A (3 \(\mu M\)), an inhibitor for Src-related intracellular protein tyrosine kinases (37), completely inhibited the ATP-induced \[^{3}H\]thymidine incorporation (Fig. 6).

Involvement of the cAMP pathway in the ATP- or growth factor-induced \[^{3}H\]thymidine incorporation. To examine whether the ATP-induced mitogenic effect is mediated through activation of a MAP kinase or a phosphatidylinositol-3 kinase (PI-3 kinase) pathway, a selective inhibitor for each kinase was employed. PD-98059, a highly selective inhibitor of the MAP kinase pathway, and also measured cAMP levels in response to ATP, PDGF, or IGF-I. The cAMP-elevating agent forskolin and the membrane-permeable cAMP analog db-cAMP significantly inhibited the control level of \[^{3}H\]thymidine incorporation by up to 40% (100 ± 7, 160 ± 10, 51 ± 8, and 61 ± 9% for control, 100 \(\mu M\) ATP, 50 \(\mu M\) forskolin, and 1 mM db-cAMP, respectively, \(n = 6\) each). cAMP assay showed that although forskolin elevated cAMP (12.0 ± 1.2 and 280.0 ± 19.7 pmol/well for control and forskolin, respectively, \(n = 6\), \(P < 0.05\)), neither ATP and UTP (100 \(\mu M\) each) nor the growth factors (10 ng/ml each) changed the cAMP levels (14.0 ± 2.2, 12.0 ± 1.1, 10.5 ± 1.1, and 12.0 ± 2.1 pmol/well for ATP, UTP, IGF-I, and PDGF, respectively, \(n = 6\) each). ATP, when added with each growth factor, did not alter the cAMP level (11.5 ± 1.1 and 12.1 ± 2.1 pmol/well for IGF-I + ATP and PDGF + ATP, respectively, \(n = 6\) each).

Involvement of a MAP kinase or a phosphatidylinositol-3 kinase pathway in ATP-induced \[^{3}H\]thymidine incorporation. To identify the P2X receptor mRNAs in MG-63 cells, total RNA from MG-63 cells was reverse transcribed (+) or not (−) and amplified by PCR with each primer pair described in Table 1. Primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as internal standard and generated a 450-bp fragment. Amplification products were electrophoresed on 2% agarose gel and visualized by GelStar staining. Lane M, DNA marker.
for MAP kinase kinase (9), inhibited ATP-induced [3H]thymidine incorporation in a concentration-dependent manner (Fig. 7A). By contrast, wortmannin, a selective inhibitor for PI-3 kinase (33), did not have significant inhibitory effects on ATP-induced [3H]thymidine incorporation (Fig. 7B).

**Effects of ATP and growth factors on MAP kinase activity.** ATP (100 μM) increased MAP kinase activity, which reached a peak within 5 min and then slightly decreased at 10 min (Fig. 8A). In-gel protein kinase assay showed that ATP (100 μM), IGF-I (10 ng/ml), or PDGF (10 ng/ml) increased MAP kinase activity, as evidenced by the ability of cell lysates to phosphorylate MBP (Fig. 8B). When ATP was added with the growth factors, the nucleotide caused synergistic increases in MAP kinase activity (Fig. 8B). The ATP-induced phosphorylation of MAP kinase was completely inhibited by PD-98059 (data not shown). ATP also activated MAP kinase activity, even in the Ca2+ -free, BAPTA-AM-containing KR-HEPES buffer (Fig. 8B). In fura 2-loaded MG-63 cells treated with the same Ca2+ -free buffer, neither ATP nor UTP (each 100 μM) increased [Ca2+](i) (n = 26; data not shown).

**DISCUSSION**

**ATP receptors responsible for cell proliferation in MG-63 cells.** We have shown that the nonhydrolyzable ATP analogs ATPγS and AMP-PNP, as well as ATP, activated DNA synthesis or cell proliferation, and the P2 purinoceptor antagonist suramin abolished the ATP-induced DNA synthesis, suggesting that ATP-induced DNA synthesis was mediated by P2-type, but not P1-type, purinoceptors. We also showed that UTP or UDP did not increase DNA synthesis and that PPADS, but not reactive blue 2, inhibited the ATP-induced DNA synthesis, indicating that P2X, but not P2Y, receptors are involved in the ATP-induced DNA synthesis in MG-63 cells. RT-PCR analysis revealed that mRNAs for receptors of P2X4, P2X5, P2X6, and P2X7, but not those of P2X1, P2X2, and P2X3, were expressed in MG-63 cells. Our results that the selective agonist for P2X1 and P2X3 receptor αβ-mATP neither activated the DNA synthesis nor increased [Ca2+](i), also support the RT-PCR results. To our knowledge, this is the first evidence demonstrating that P2X receptors are expressed in osteoblasts.

P2X4, P2X6, and P2X7, among the P2X receptor subtypes, are reported to be insensitive to suramin and PPADS in expression studies with each of the cloned
receptors (6, 29). Our results showed that suramin and PPADS almost completely inhibited the ATP-induced DNA synthesis, suggesting that P2X4, P2X6, and P2X7 receptors expressed in MG-63 cells may not be involved in the proliferative effects of ATP. From these results, P2X5 receptors could be responsible for the ATP-induced DNA synthesis. P2X5 receptors were expressed in rat heart, and an electrophysiological study with cells heterologously expressed in rat P2X5 receptors showed that the currents induced by ATP were completely inhibited by PPADS and suramin, and the effects of ATP were not mimicked by αβ-mATP (13). Furthermore, in rat pituitary gonadotrophs, ATP caused gonadotropin release through P2X2 and/or P2X5 receptors, where the ATP-induced events were inhibited by suramin but not mimicked by UTP or αβ-mATP (42). Our data, however, do not exclude the possibility of the involvement of recently reported heterooligomeric P2X receptors composed of different subtypes of P2X receptors [i.e., P2X2/3 (25) and P2X1/5 (23) or other combinations (43)] in ATP-induced cellular responses in MG-63 cells.

Intracellular signaling pathways involved in ATP-induced DNA synthesis. A previous study in osteoblasts showed that ATP increased [3H]thymidine incorporation in parallel with the increment of [Ca2+]i (36). Our results also showed that ATP increased [3H]thymidine incorporation and [Ca2+]i. However, we showed that not only ATP, but also UTP, which did not increase [3H]thymidine incorporation, increased [Ca2+]i with similar amplitudes. In addition, ATP and UTP increased [Ca2+]i, even in a Ca2+-free buffer, suggest-

Fig. 7. Effects of PD-98059 or wortmannin on ATP-induced [3H]thymidine incorporation. Quiescent cells were pretreated with or without PD-98059 (A) or wortmannin (B) for 1 h before stimulation with ATP for 24 h. Each inhibitor was present throughout the incubation with ATP, and then [3H]thymidine incorporation was determined. Values (means ± SE, n = 6) are expressed as a percentage of values obtained in the absence of ATP and each inhibitor (control). *P < 0.05 vs. ATP(-).

Fig. 8. In vitro (A) and in-gel (B) MAP kinase assay. A: quiescent MG-63 cells were treated with ATP (100 μM). After reaction, the cell lysates were prepared, and MAP kinase activity was measured with a MAP kinase assay kit. Values (means ± SE, n = 6) are expressed as a percentage of the control value. *P < 0.05 vs. control. B: quiescent cells were treated with ATP and growth factors for 5 min, and then cell lysates were prepared. The cell lysates were then subjected to SDS-PAGE including myelin basic protein as a substrate. Autoradiography (bottom) shows ATP- and/or growth factor-induced MAP kinase activity (left) and Ca2+ dependency of the ATP-induced MAP kinase activity (right). Quantification of each band (top) represents the activity of p44MAPK (left, n = 3; right, n = 8). Values (means ± SE) are expressed as a percentage of values obtained in the absence of ATP and growth factors (control, None). *P < 0.05 vs. ATP(-). *P < 0.05 vs. None.
ing that the ATP-induced increase in [Ca\(^{2+}\)]\(_i\) is due to Ca\(^{2+}\) release from intracellular stores via P2Y receptors. Collectively, our results indicate that an increase in [Ca\(^{2+}\)]\(_i\) is not necessary for the ATP-induced DNA synthesis and that there is a [Ca\(^{2+}\)]\(_i\) increase-independent cell proliferation mechanism in MG-63 cells. The increment of [Ca\(^{2+}\)]\(_i\) via the P2Y receptors observed in our study could be associated with other cellular functions such as cell differentiation.

We further demonstrated that several Ca\(^{2+}\)-dependent protein kinases, such as protein kinase C and Ca\(^{2+}\)/calmodulin-dependent protein kinase II, are not involved in ATP-induced DNA synthesis. These results support the belief that [Ca\(^{2+}\)]\(_i\)-independent signaling pathways underlie the proliferative effects induced by ATP in MG-63 cells. On the other hand, herbimycin A completely inhibited ATP-induced DNA synthesis, suggesting that non-receptor-associated protein tyrosine kinase pathways are downstream of ATP receptors.

Accumulating evidence has shown that stimulation of tyrosine kinase leads to the activation of several protein kinases such as MAP kinases, which are well known to be involved in cell proliferation (26). Our results showed that ATP increased MAP kinase activity, and the activation of MAP kinase and P2X receptor-mediated DNA synthesis were abolished by PD-98059, indicating that a MAP kinase pathway is involved in the P2X receptor-mediated DNA synthesis, which is inconsistent with the previous study in rat pheochromocytoma PC-12 cells (39). However, in contrast to the previous study in PC-12 cells (39), we demonstrated that ATP increased MAP kinase activity in a Ca\(^{2+}\)-independent manner. The exact mechanism of the P2X receptors involved in the activation of the MAP kinase pathway remains to be determined; however, P2X receptors might transduce their signal to MAP kinases in a manner independent of ion influx. In fact, it has been recently reported that ionotropic glutamate receptors transduce the signal to the MAP kinase pathway in a tyrosine kinase Lyn-dependent, but a Ca\(^{2+}\) and Na\(^{+}\) influx-independent, manner (17).

Several growth factors, including PDGF, promote DNA synthesis by activating a PI-3 kinase pathway in MG-63 cells (40); however, we showed that the PI-3 kinase inhibitor wortmannin failed to inhibit the ATP-induced DNA synthesis. Taken together, the ATP-induced cell proliferation may be mediated through several protein kinase pathways, including the tyrosine kinases and the MAP kinases, but not the Ca\(^{2+}\)-dependent protein kinases or the PI-3 kinase pathways.

**Mechanisms by which ATP causes synergistic effects of DNA synthesis induced by the growth factors.** The mechanism by which ATP accelerates the DNA synthesis induced by the growth factors in osteoblasts has not been well clarified. In the osteoblast cell line UMR-106, ATP was shown to augment the parathyroid hormone-induced [Ca\(^{2+}\)]\(_i\) increases, which may lead to modulation of the parathyroid hormone-induced cellular functions (20, 24). These results suggest that enhancement of [Ca\(^{2+}\)]\(_i\) increase results in the synergistic effects of cellular responses. Our results, however, showed that ATP administered with IGF-I or PDGF caused an increase in [Ca\(^{2+}\)]\(_i\) similar to that caused by ATP alone, suggesting that augmentation of the increase in [Ca\(^{2+}\)]\(_i\) does not account for the ATP-induced synergism of the growth factor-induced DNA synthesis in MG-63 cells.

The cAMP signaling pathways are involved in cell proliferation of a variety of the cells (32). Although we showed that an increase in cAMP levels inhibits the DNA synthesis in MG-63 cells, ATP did not change the levels regardless of the presence of the growth factors. These results suggest that cAMP-dependent pathways are not involved in the synergistic proliferative effects of ATP in MG-63 cells.

We showed that ATP augmented the MAP kinase activity induced by PDGF or IGF-I, and we hypothesized that ATP-mediated augmentation of growth factor-induced MAP kinase activity contributes to the synergistic proliferative effects of ATP. Previous reports have shown that, in human erythroid colony-forming cells, stem cell factor synergistically activated both DNA synthesis and MAP kinase activity induced by erythropoietin, and these effects were inhibited by PD-98059 (38). In Swiss 3T3 cells, the polypeptide bombesin synergistically activated the mitogenic effects of insulin by enhancing the p42 MAP kinase activity (21).

**Physiological significance of the ATP-induced cellular responses in osteoblasts.** ATP and related nucleotides are widely distributed in a variety of tissues, inasmuch as ATP is stored in cytosol or in secretory vesicles in most of the cells (14). A recent study showed that, in rat aortic smooth muscle cells, ATP released by mechanical stretch from the cells activates Jun NH\(_2\)-terminal kinase/stress-activated protein kinase and MAP kinase pathways via activation of P2Y receptors in an autocrine/paracrine manner (16). On the basis of these results and our present results, it is possible to speculate that extracellular ATP released by mechanical stimulation or from damaged cells in bone fracture may contribute to the bone formation and remodeling.

In summary, we have demonstrated that extracellular ATP increased the DNA synthesis and also enhanced the proliferative effects induced by IGF-I or PDGF through P2X receptors, presumably of the P2X\(_5\) subclass, expressed in MG-63 cells and that ATP-induced DNA synthesis was mediated through a MAP kinase-dependent pathway.

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**REFERENCES**

MITOGENIC EFFECTS OF ATP IN OSTEOBLAST-LIKE CELLS


32. Tomic M, Jobin RM, Vergara LA, and Stoilkovic SS. Expression of purinergic receptor channels and their role in-cal...
