Signaling in Human Osteoblasts by Extracelluar Nucleotides

THEIR WEAK INDUCTION OF THE c-fos PROTO-ONCOGENE VIA Ca^2+ MOBILIZATION IS STRONGLY POTENTIATED BY A PARATHYROID HORMONE/cAMP-DEPENDENT PROTEIN KINASE PATHWAY INDEPENDENTLY OF MITOGEN-ACTIVATED PROTEIN KINASE*

(Received for publication, October 15, 1998, and in revised form, January 27, 1999)

Wayne B. Bowler‡‡, Catherine J. Dixon¶, Christine Halleux**, Rainer Maier**, Graeme Bilbe**, William D. Fraser‡‡, James A. Gallagher‡, and Robert A. Hipskind¶¶

From the ‡Human Bone Cell Research Group, ‡Department of Human Anatomy and Cell Biology, and ¶¶Department of Clinical Chemistry, University of Liverpool, Liverpool L69 3OE, United Kingdom; **Novartis Pharma AG, CH-4002, Basel, Switzerland, and ¶¶Institut de Genetique Moleculaire de Montpellier, UMR 5535, CNRS, 1319 Route de Mende, 34293 Montpellier cedex 5, France

Extracellular nucleotides acting through specific P2 receptors activate intracellular signaling cascades. Consistent with the expression of G protein-coupled P2Y receptors in skeletal tissue, the human osteosarcoma cell line SaOS-2 and primary osteoblasts express P2Y_1 and P2Y_2 receptors, respectively. Their activation by nucleotide agonists (ADP and ATP for P2Y_1; ATP and UTP for P2Y_2) elevates [Ca^2+]_i and moderately induces expression of the c-fos proto-oncogene. A synergistic effect on c-fos induction is observed by combining ATP and parathyroid hormone, a key bone cell regulator. Parathyroid hormone elevates intracellular cAMP levels and correspondingly activates a stably integrated reporter gene driven by the Ca^2+/cAMP-responsive element of the human c-fos promoter. Nucleotides have little effect on either cAMP levels or this reporter, instead activating luciferase controlled by the full c-fos promoter. This induction is reproduced by a stably integrated serum response element reporter independently of mitogen-activated protein kinase activation and ternary complex factor phosphorylation. This novel example of synergy between the cAMP-dependent protein kinase/CaCRE signaling module and a non-mitogen-activated protein kinase/ternary complex factor pathway that targets the serum response element shows that extracellular ATP, via P2Y receptors, can potentiate strong responses to ubiquitous growth and differentiative factors.

Extracellular nucleotides act by inducing c-fos proto-oncogene expression, which downstream targets the serum response element (SRE), at position 300 in the promoter, mediates induction by many extracellular signals via a ternary complex composed of a dimer of serum response factor (SRF) together with one molecule of the TCF family of Ets proteins (Elk-1, SAP-1a, or ERK/ERK21–26). TCFs, particularly Elk-1 and SAP-1a, are important nuclear targets of various MAPK cascades (11), while SRF is apparently sufficient for activation by certain Ca^2+ signals and signals emanating from the Rho/Rac/CDC42 family of small GTPases (12–14). Upstream of the SRE is the v-sis-inducible element, the binding site for homo- and heterodimers of signal transducer and activator of transcription 1 and 3 upon their cytoplasmic activation by cytokines and certain growth factors (15, 16). While transient transfections have proven useful in attributing a role to each element, the results from mice containing c-fos transgenes (17) and more recent data in vitro indicate that multiple elements are necessary for a strong response (18), thereby implying that they are targeted simultaneously by intracellular signals.

The importance of c-fos induction in vivo in driving immortalized fibroblasts to enter the cell cycle and plays an important role in vivo in the skeleton. Mice lacking the c-fos gene fail to develop osteoclasts and thus show an osteopetrotic phenotype in which the dynamic process of bone remodeling has been shifted toward bone accumulation (19, 20). Conversely, constitutive overexpression of c-fos in the bone environment of transgenic mice leads to the development of

---

1 The abbreviations used are: CaCRE, calcium/cAMP-responsive element; SRE, serum response element; PCR, polymerase chain reaction; RT-PCR, reverse transcript PCR; MAPK, mitogen-activated protein kinase; CaMK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; ATPyS, adenosine 5’-O-(3-thiotriphosphate); 2-meSATP, 2-methylthioadenosine 5’-triphosphate; PTH, parathyroid hormone; BSA, bovine serum albumin; FCS, fetal calf serum; CREB, cAMP response element-binding protein; SRF, serum response factor; TCF, ternary complex factor; HBBDC, human bone-derived cells; SSC, saline-sodium citrate; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2 M. Bebien, C. Becamel, V. Richard, and R. A. Hipskind, manuscript in preparation; R. A. Hipskind, C. Halleux, S. Decker, M. Bebien, D. B. Evans, and G. Bilbe, manuscript in preparation.
ostesarcomas (21, 22). Accordingly, a number of proteins characteristic of differentiating bone cells have regulatory activator protein 1 sites in their promoters, and a variety of extracellular factors documented to stimulate bone cell growth and differentiation activate c-fos transcription in cultured bone cells in vitro (2, 22–25).

Parathyroid hormone (PTH) is essential for the modeling and remodeling of the skeleton. In bone-derived cell lines as well as in primary osteoblasts in culture, PTH strongly induces transcription of the c-fos gene (23, 24). This occurs via the cAMP/cAMP-dependent protein kinase/CREB pathway in the osteosarcoma cell line SaOS-2 (25) but may involve protein kinase C and the ERK pathway in other cells (2). Because PTH is a systemic factor and the process of bone remodeling is essentially a focal phenomenon, cellular responsiveness to PTH in vivo is likely to be modulated by other factors. We wondered whether ATP might play such a modulatory role, since ATP can be released from osteoblasts into the local bone microenvironment via a nonlytic mechanism (26) and since ATP synergizes with mitogens to enhance DNA synthesis in a variety of cells (27–29).

Extracellular nucleotides, such as ATP, exert stimulatory effects on cells at micromolar concentrations through the P2 family of membrane-bound receptors (30–33). Two major classes of P2 receptors have been delineated: P2X receptors, which are ligand-gated ion channels, and P2Y receptors, which are coupled to G proteins (32). More pertinent to our hypothesis is, P2Y receptors are expressed in osteoblastic cells of rat (34, 35) and human origin (36, 37). Two major subtypes of P2Y receptor, P2Y1, and P2Y2, are coupled through G1 to phosphatidylinositol 4,5-bisphosphate hydrolysis and hence Ca2+-mobilization from intracellular stores (32).

Here we show that both the osteosarcoma cell line SaOS-2 and primary cells in culture express P2Y receptors that functionally couple to c-fos activation. This involves increased intracellular Ca2+ and a signaling pathway that can activate an SRE-driven reporter gene independently of the predominant ERK/TCF signaling module. Co-activation of this pathway and that induced by PTH increases c-fos mRNA levels well above those induced by either stimulus alone, thereby providing a novel example of synergy between a cAMP-dependent protein kinase and a Ca2+-triggered signaling system not involving MAPK. These data demonstrate that extracellular nucleotides can strongly potentiate the response of bone cells to systemic factors and suggest that this may be a common mechanism to generate strong localized responses to systemic growth and differentiation factors.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Dulbecco’s modified Eagle’s medium, α-modified Eagle’s medium, Ham’s F-12, and RPMI 1640 were obtained from Flow Laboratories (United Kingdom). Fetal calf serum (FCS) was purchased from Life Technologies Ltd. and fura-2 acetoxyethyl ester was from Molecular Probes, Inc. (Eugene, OR). dNTPs, oligo(dT), RNase inhibitor, and some restriction enzymes were from Roche Molecular Biochemicals, while Taq DNA polymerase and Superscript II reverse transcriptase were from GIBCO BRL (Gaithersburg, MD). Nucleotides, bovine serum albumin (BSA), EGF, and peroxidase-coupled goat anti-rabbit antibodies were obtained from Sigma. Luciferase reagent according to the protocols of the manufacturer, and the cells were maintained in RPMI/FCS for h. Stably transduced cell pools were selected for resistance to G418 following standard protocols (39). UMR-106 cells were cultured in Ham’s F-12 and Dulbecco’s modified Eagle’s medium (1:1) supplemented with 10% FCS at 37°C in a humidified atmosphere containing 95% air and 5% CO2. Stable pools of UMR-106 cells containing the SRE-luciferase reporter were described above.

**Luciferase Reporter Gene Assays**—For reporter gene assays, the cells were seeded into 96-well plates at a density of 96,000 cells/well. At near confluency the medium was replaced with one containing 0.5% FCS for 16 h, followed by serum-free RPMI containing 0.1% BSA for 24 h. Agents were added to cells as a 10x stock solution prepared in the same medium to the final concentrations indicated in the figures. After a 4-h incubation, cells were washed twice in cold PBS and lysed in luciferase cell culture lysis reagent for 15 min at room temperature (25°C/96-well plate). The plates were subsequently centrifuged at 3000 rpm for 2 min. After addition of luciferase reagent, the plates were transferred to a 4°C humidified atmosphere containing 95% air and 5% CO2. Stable pools of UMR-106 cells containing the SRE-luciferase reporter were described above.

**Measurement of [Ca2+]i**—SaOS-2 cells and HBDC were grown to confluence on 22-mm diameter glass coverslips. [Ca2+]i, was measured after 2 h of serum deprivation. Cells were loaded with fura-2 by incubation with fura-2 acetoxyethyl ester (5 μM) for 20 min at 37°C in HEPES buffer (10 mM HEPES, 121 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 μM CaCl2, 5 μM NaHCO3, 10 mM glucose, pH 7.2) containing 2% BSA. Cells were subsequently washed three times in the same buffer containing 0.2% BSA. Measurements were performed with a photon-counting spectrophotometer on a Nikon TM Diaphot microscope with a ×40 oil immersion objective. The cell-coverage slides were attached with silicone grease to form the base of a stage-mounted, thermoregulated chamber maintained at 37°C. Groups of 30 cells were illuminated with excitation light (340–380 nm) at a rate of 32 times/s, and emission maxima at 510 nm were integrated into 1-s averages and stored. Agent was, in HEPES buffer with 0.2% BSA, were added for 60–120 s, followed by at least 10 min of recovery prior to further stimulation. Rmax, Rmin, and autofluorescence values were obtained in situ using ionomycin, as described previously (40). [Ca2+]i, was calculated from the ratio of fluorescence at the two excitation wavelengths after subtraction of autofluorescence.
**Synergistic Effect of ATP/[Ca^{2+}], and PTH on c-fos**

(41). The results were evaluated statistically using the Student's *t* test, assuming a significance of *p* < 0.05.

**RNA Isolation and cDNA Synthesis**—Total RNA was extracted from control and stimulated cells with 4 M guanidine thiocyanate, 0.5% sarkosyl, 0.1 M mercaptoethanol, 25 mM sodium citrate, pH 7.0, followed by phenol-chloroform extraction (9). RNA extraction was as described by Evans et al. (42). The primer sequences were as follows: P2Y1 sense, TGTGGTGTACCCCCTCAAGT; P2Y2 sense, TCCCTCAAGTGGTGTACCCCCTCAAGT; GAPDH sense, GGTCATGAGTCCTTCCACGAT; and GAPDH antisense, GAAGCTTATCCACACTGTCTT. RNA was used as template for first strand cDNA synthesis in a 50-μl reaction containing 0.5 mM dNTPs, 1.25 mM of oligo(dT), 20 units of RNase inhibitor, 10 mM dithiothreitol, 6 mM MgCl₂, 40 mM KCl, 50 mM Tris·HCl (pH 8.3), 1000 units of RNase-free DNAse, 10 mM Tris·HCl, and 10 mM EDTA. The primer extension reactions were as described previously (42). At the appropriate time point, plates were washed for 30 min at 65 °C, and the complexes were visualized by autoradiography using enhanced chemiluminescence. The antisera used were anti-pan-ERK, diluted 1:5000; anti-phospho-Thr202/Tyr204 ERK, anti-phospho Thr183/Tyr185 SAPK, anti-phospho Thr389/Tyr422 p38, anti-SAPK, and anti-p38, diluted 1:1000.

**RESULTS**

**Differential Expression of P2Y₁ and P2Y₂ Receptors by SaOS-2 and Primary Human Bone-derived Cells**—Since osteoblasts have been described to express different purinergic receptors of the P2Y family, we used RT-PCR to analyze which subtypes are expressed in the human osteosarcoma cell line SaOS-2. In addition, we tested two populations of primary osteoblastic cells derived from explants of human bone in *vitro* culture, termed HBDC1 and HBDC2. RT-PCR on cDNA from SaOS-2 cells gave rise to an intense signal for P2Y₁ receptor transcripts but only a weak signal for P2Y₂ receptor (Fig. 1). In contrast, only P2Y₂ receptor transcripts were visualized in the HBDC1 cDNA, while amplification of HBDC2-derived cDNA showed similar levels of amplification for both P2Y₁ and P2Y₂. The signal with primers specific for GAPDH was similar in all reactions (Fig. 1), confirming the integrity and amount of cDNA in each sample.

**Elevation of [Ca^{2+}]i in SaOS-2 Cells and Primary Human Osteoblasts following P2 Receptor Stimulation**—P2Y receptors are coupled to heteromeric G proteins intracellularly, and have been reported to activate, via G₄q, phosphatidylinositol 4,5-bisphosphate hydrolysis and Ca²⁺ mobilization from intracellular stores (32). Since SaOS-2 cells and HBDC1 express predominantly the P2Y₁ and P2Y₂ receptors, respectively, we tested whether different P2Y₁ and P2Y₂ receptor agonists could mobilize intracellular Ca²⁺, measured using fluorescence increases in groups of 6–8 fura-2-loaded cells. ATP consistently induced a rise in [Ca²⁺], in both cell types (Table II and I). In SaOS-2 cells, ATP was effective at concentrations ranging from 1 to 100 μM (Fig. 2A). In these cells, the P2Y₂ agonist UTP evoked only a minor increase in [Ca²⁺], at 10 μM and a more significant increase at 100 μM (Fig. 2B) that nevertheless was smaller than that induced by 1 μM ATP (Fig. 2, compare A and B). This difference in responsiveness was also observed upon the sequential addition of UTP and ATP, thus indicating that it did not reflect decreased sensitivity of the cells. More importantly, UTP induced the same increase as ATP in HBDC1 (Fig. 3 and Table I), clearly showing that the difference is due to the P2Y₂ receptor subtype expressed by the cells.

The effects of other agonists confirmed this differential responsiveness between the primary cells and the established cell line. In SaOS-2 cells, the ATP analogues ATP-γS and 2-me-SATP, as well as ADP (all 10 μM), induced [Ca²⁺], increases comparable with ATP (Table I). The primary cells did not show the same behavior, since these P2Y₂ agonists did not elevate [Ca²⁺], in HBDC1 (Fig. 3). These differences in the functional response to extracellular nucleotides are consistent with and thereby confirm the receptor profiles obtained by RT-PCR (Fig. 1).

**Induction of c-fos mRNA in SaOS-2 Cells following P2 Receptor Stimulation**—Since nucleotide addition elevated intracellular calcium levels, we tested whether this second messenger pathway activated nuclear signaling, as measured by the induction of the proto-oncogene c-fos. This event is particularly...
relevant in bone-derived cells, since c-fos has been implicated in many of the processes that govern skeletal tissue remodeling (21, 22).

ATP_gS stimulation of quiescent SaOS-2 cells led to a dose-dependent induction of c-fos mRNA, measured by Northern blotting (Fig. 4A), which was maximal at 10 μM ATP_gS. This represented a typical transient activation in which fos mRNA levels peaked 45 min after stimulation and then rapidly decayed (Fig. 4B). In addition, we tested the same range of P2Y receptor agonists used to analyze Ca^{2+} mobilization. 10 μM ATP_gS, ATP, and ADP stimulated c-fos expression, whereas UTP and 2-meSATP did not (Fig. 4C). While this generally reflects the activation of the P2Y<sub>1</sub> receptor, this correlation is not universal, as shown by the lack of induction by 2-meSATP and the decrease between 10 and 100 μM ATP_gS. This was not due to differing levels of RNA, since rehybridization of the blots with a GAPDH probe and/or ethidium bromide staining confirmed equal loading of mRNA in all lanes. This apparent discrepancy between increased [Ca^{2+}]_i and c-fos induction will be discussed in more detail below.

Synergistic Induction of c-fos by the Combination of P2 Re-

![Fig. 1. Differential P2 receptor expression by SaOS-2 and primary human bone-derived cells. PCR amplification of cDNAs with P2Y<sub>1</sub>, P2Y<sub>2</sub>, or GAPDH primer pairs. cDNAs were synthesized from RNA templates prepared from SaOS-2 cells, two different primary osteoblast populations (HBDC1 and -2), and an osteoclastoma tumor. The tumor expresses both receptor subtypes and thus serves as the positive control (37). As indicated on the left, the bands of 259 and 362 base pairs (bp) correspond to amplification products specific for P2Y<sub>1</sub> and P2Y<sub>2</sub> receptor cDNAs, respectively, and GAPDH yields a larger 519-base pair band. The control reaction contained H<sub>2</sub>O instead of template cDNA, and the unmarked lanes contain molecular size markers.

![Fig. 2. Nucleotide-induced increases in [Ca^{2+}]_i in SaOS-2 cells. Groups of fura-2-loaded SaOS-2 cells (6–8 per point) were sequentially stimulated with increasing concentrations of ATP (A) and UTP (B) for the times indicated on the x axis scale. The curves plot intracellular calcium concentration in nM, as indicated on the y axis. The values are representative of the following numbers of measurements. A, 1 μM, n = 4; 10 μM, n = 16; 100 μM, n = 3. B, n = 3.

| Nucleotide | [Ca^{2+}]_i (nM) SaOS-2 | [Ca^{2+}]_i (nM) HBDC1 |
|------------|-------------------------|-------------------------|
| ATP_gS     | 47 ± 5^a (n = 3)         | 47 ± 5^a (n = 3)         |
| UTP        | 93 ± 5 (n = 6)           | 93 ± 5 (n = 6)           |
| 2-meSATP   | -1 ± 1^a (n = 3)         | -1 ± 1^a (n = 3)         |
| ADP        | 1 ± 1^a (n = 10)         | 1 ± 1^a (n = 10)         |

^a A response significantly different from that induced by ATP at p < 0.05.
Synergistic Effect of ATP/[Ca\(^{2+}\)]\(_i\) and PTH on c-fos

---

**Fig. 3.** Nucleotide-induced increase in [Ca\(^{2+}\)]\(_i\) in primary human bone-derived cells. Primary cultures of human osteoblasts (HBDC) cells, 6–8 per point) were loaded with fura-2 and stimulated sequentially with the different P2Y agonists (10 \(\mu\)M) indicated at the top. Table I presents the statistics concerning these measurements.

---

**Receptor Agonists and PTH in SaOS-2 Cells**—While P2Y\(_1\) receptor agonists induced c-fos, the level of expression was low relative to serum (Fig. 4C). Since nucleotides enhance the proliferative response to mitogens in other cell types (27, 28), we tested whether they might enhance c-fos induction in SaOS-2 cells by PTH, a potent stimulator of bone growth in vivo. Quiescent SaOS-2 cells were treated with different nucleotides alone or in combination with PTH and mRNA levels analyzed by Northern blotting. Cotreatment with PTH and either ATP or ADP resulted in a synergistic induction of c-fos mRNA relative to either inducer alone (Fig. 5A). In contrast, UTP did not augment PTH-induced c-fos activation in SaOS-2 cells, which is again consistent with its inability to stimulate the P2Y\(_1\) receptor.

To confirm that this synergy arose from direct transcriptional activation and facilitate further characterization of this effect, we transfected SaOS-2 cells with a reporter gene in which the full c-fos promoter, spanning positions −711 to −1, was linked to the firefly luciferase gene. To avoid the problems inherent in assaying signaling by transient transfection of non-physiological quantities of DNA, we selected pools of stably transfected cells using the neomycin resistance gene present in physiological quantities of DNA, we selected pools of stably transfected cells using the neomycin resistance gene present in the full-length reporter construct. Co-stimulation of the stable transfected cells using the neomycin resistance gene present in the same construct. Both serum and PTH induced a robust response of this reporter construct, elevating luciferase activity 15–20-fold in the stable transfectants (Fig. 5B). ATP showed only a weak effect on its own, consistently several fold above the background level. However, the combination of ATP and PTH cooperatively induced the c-fos reporter, which is in direct contrast to the failure of UTP to significantly augment luciferase activity in combination with PTH (Fig. 5B). Thus, we are able to reproduce the synergy between ATP and PTH using the c-fos promoter in stable transfection assays, which indicates that this effect arises primarily from increased transcriptional activation and not from stabilization of the fos mRNA. Furthermore, it suggests that ATP and PTH activate intracellular signals that target regulatory elements in the c-fos promoter.

**Synergistic Induction of c-fos by the Combination of P2 Receptor Agonists and PTH in HBDC**—To determine whether extracellular nucleotides might also potentiate c-fos activation by PTH in primary osteoblasts, we treated HBDC with PTH and a range of P2Y receptor agonists. As above, ATP cooperated with PTH to strongly enhance c-fos mRNA levels above those induced by either factor alone (Fig. 6). In these cells, the P2Y\(_2\) receptor agonist also synergized with PTH, whereas the P2Y\(_1\) agonist ADP was inactive (Fig. 6). Thus, the primary cell population shows a response consistent with their receptor subtype and that contrasts with the response of the P2Y\(_1\)/SaOS-2 combination to the same nucleotides.

**PTH and ATP Utilize Distinct Second Messengers in Human Osteoblasts**—In clonal rat osteoblastic cells, ATP increases PTH-induced [Ca\(^{2+}\)]\(_i\) responses (32); therefore, we tested if the synergy between ATP and PTH resulted from enhanced Ca\(^{2+}\) mobilization. In neither SaOS-2 cells nor the primary osteoblasts did PTH, at 20–500 ng/ml, generate a significant calcium response, nor did we ever observe that PTH enhanced the effect of either ATP or UTP on [Ca\(^{2+}\)]\(_i\). In SaOS-2 cells, PTH-(1–34) induces c-fos expression through cAMP-dependent phosphorylation of CREB independently of protein kinase C activation, TCF phosphorylation, or signal transducer and activator of transcription induction (25). Therefore, we tested if the synergy on c-fos expression following nucleotide/PTH stimulation might reflect modulation of cAMP levels. A radioimmunoassay was used to assess the effects of nucleotide stimulation, alone and in combination with PTH, on cAMP accumulation in SaOS-2 cells. Forskolin, a potent activator of adenyl cyclase at 10 \(\mu\)g/ml, elevated intracellular cAMP to 170 pmol/ml above the basal level. On the other hand, the addition of 10 \(\mu\)M ATP, ATP\(_7S,\) UTP, or ADP had no significant effect on cAMP levels either alone (Fig. 7A) or in combination with PTH (Fig. 7B).

**The CaCRE in the c-fos Promoter Is Insufficient for a Synergistic Response**—The CaCRE in the c-fos promoter, which is situated immediately upstream of the TATA box, can mediate activation of reporter genes by certain Ca\(^{2+}\) signaling pathways (see Introduction). We used stably transfected SaOS-2 cell lines to test the possibility that the ATP/Ca\(^{2+}\) and PTH/cAMP pathways converge on this site synergistically inducing c-fos. A reporter driven by a truncated promoter, containing only the CaCRE linked to luciferase, was compared with the full-length reporter construct. Co-stimulation of the stable pools containing the truncated reporter with PTH and nucleotides resulted in luciferase expression levels slightly above those observed with PTH alone (Table II). Notably, this increase reflected an additive effect of ATP and PTH rather than the multiplicative effect seen on the endogenous gene and the full promoter-driven reporter. This strongly suggested that

---

\(^3\) C. J. Dixon and W. B. Bowler, unpublished observations.
synergistic activation did not arise by the convergence of these two signaling systems on the CaCRE alone.

ATP Activates the c-fos SRE Independently of ERK and TCF—These data implied that the ATP/Ca\(^{2+}\) signaling pathway targeted another site in the c-fos promoter. The SRE seemed the most likely candidate, since it can also mediate activation by various calcium-dependent pathways. We again chose the stable transfection approach. Unfortunately, SaOS-2 cells have proven refractory to stable transfection with SRE-driven reporter constructs, so we resorted to another bone-derived cell line, UMR-106, that shows the same responses as SaOS-2 cells to ATP and PTH. 4 Pools of UMR-106 cells were generated that contain a reporter construct in which the luciferase gene is controlled by three c-fos SREs cloned in front of the c-fos TATA box. These cells responded strongly to EGF and slightly less so to serum, which increased luciferase activity 12- and 6-fold, respectively (Table II). Treatment with PTH did not

Synergistic Effect of ATP/[Ca\(^{2+}\)]\(_i\) and PTH on c-fos

![Fig. 4. Induction of c-fos mRNA in SaOS-2 cells following nucleotide stimulation. A, SaOS-2 cells were induced for 45 min with ATP as indicated. RNAs were isolated and purified using the guanidine one-step procedure and visualized by hybridizing Northern blots containing 10 \(\mu\)g of total RNA/lane with a \(^{32}\)P-labeled c-fos probe labeled by random priming (top). Equal RNA loading and integrity were confirmed by ethidium bromide staining (bottom). Numerical values for the c-fos mRNA:28 S ribosomal RNA ratio are as follows: 10\(^{-1}\) M ATP, 0.195; 10\(^{-6}\) M ATP, 2.50; 10\(^{-5}\) M ATP, 11.59; 10\(^{-4}\) M ATP, 3.10. B, kinetics of c-fos mRNA induction in response to 10 \(\mu\)M ATP. Northern blots were hybridized as above, and the maximal induction level, which occurred 45 min postinduction, was taken as 100%. Numerical values for the c-fos mRNA:28 S ribosomal RNA ratio are as follows: 30 min, 0.23; 45 min, 0.41; 60 min, 0.23. C, SaOS-2 cells were stimulated for 45 min with a range of nucleotide agonists at 10 \(\mu\)M concentration, and c-fos mRNA induction was analyzed as described above (top). The membrane was stripped and rehybridized with a GAPDH probe \(^{32}\)P-labeled by random priming to confirm equal loading and mRNA integrity (bottom). Numerical values for the c-fos:GAPDH mRNA ratio are as follows: 10% FCS, 4.58; ADP, 0.19; ATP, 0.134. Medium, UTP, and 2-meSATP did not induce c-fos expression.

SaOS-2 cells to ATP and PTH. 5 Pools of UMR-106 cells were generated that contain a reporter construct in which the luciferase gene is controlled by three c-fos SREs cloned in front of the c-fos TATA box. These cells responded strongly to EGF and slightly less so to serum, which increased luciferase activity 12- and 6-fold, respectively (Table II). Treatment with PTH did not

4 C. Halleux and G. Bilbe, unpublished observations.

![Fig. 5. Nucleotides synergize with PTH to induce c-fos expression in SaOS-2 cells. A, SaOS-2 cells were stimulated with PTH (100 ng/ml) and nucleotide agonists (10 \(\mu\)M) for 45 min. RNAs were analyzed by Northern blotting (top) and ethidium staining (bottom) as described in the legend to Fig. 4. The c-fos signal in each lane was standardized to 28 S ribosomal RNA, and the level of induction relative to control was expressed numerically. B, SaOS-2 cells stably transfected with the fos (-711/-1) promoter-luciferase reporter were serum-starved and then induced for 4 h with PTH (100 ng/ml), ATP (10 \(\mu\)M), UTP (10 \(\mu\)M), or the combination of PTH and NTP. Luciferase activity was measured as described under “Experimental Procedures.” The data represent the average of three measurements. Error bars indicate the S.D.

![Fig. 6. Synergy between nucleotides and PTH in c-fos induction in primary human osteoblasts. Populations of primary human bone-derived cells were serum-starved and then stimulated for 45 min with 10 \(\mu\)M ATP, ADP, UTP, and/or 100 ng/ml PTH. 5 \(\mu\)g of total RNA were analyzed by Northern blotting and hybridization with c-fos, and GAPDH riboprobes mixed 15:1. The c-fos signal in each lane was standardized to the GAPDH internal control, and the level of induction relative to control was expressed numerically.

[^4]: C. Halleux and G. Bilbe, unpublished observations.
stimulation of pools of SaOS-2 or UMR-106 cells stably transfected with phosphorylation of TCF, a major nuclear target for the MAPKs particularly responsive to activated MAPK cascades via the promoter elements. Values represent -fold increase in luciferase expression in SaOS-2 cells.

The SRE integrates signals from many pathways but is targeted by the ATP/Ca\(^{2+}\) pathway in bone cells. ERK, SAPK/c-Jun N-terminal kinase, and p38 MAPK (2). We wondered whether this synergy reflected the activation of one of these cascades by nucleotides, especially since other bone growth factors can induce ERK activity (45–47). To test for MAPK activation, we used antisera directed against the activated kinases, which are highly specific for the molecules phosphorylated on Thr and Tyr in their Thr-Xaa-Tyr activation motif. Western blots of SaOS-2 whole cell extracts, probed with anti-phospho-Thr\(^{202}\)/Tyr\(^{204}\)ERK1/2, anti-phospho-Thr\(^{183}\)/Tyr\(^{185}\)SAPK, and anti-phospho-Thr\(^{187}\)/Tyr\(^{188}\)p38, followed by peroxidase-coupled antirabbit antisera and detection using enhanced chemiluminescence. The blots were stripped and reprobed with anti-pan-ERK, anti-SAPK, and anti-p38 MAPK antisera to confirm the presence of similar levels of the kinases in each lane.

ERK, SAPK/c-Jun N-terminal kinase, and p38 MAPK (2). We used the same strategy to test whether ATP activated the stress-responsive MAPKs, namely SAPK/c-Jun N-terminal kinase and p38 MAPK, in these cells. Neither kinase was induced by ATP (Fig. 8), whereas they were weakly activated by EGF (Fig. 8). UMR-106 cells show similar behavior (49), which may represent another example of antagonism between cAMP-dependent protein kinase and the ERK cascade apparent in some cell contexts (49–52). Thus, the synergy between ATP and PTH does not involve the ERK pathway.

It remained possible that ATP activated a non-MAPK pathway that could nevertheless target TCF, as has been documented in a mouse macrophage cell line (53). TCF can be readily seen in the complexes formed by the EGF-treated whole cell extract (Fig. 9). Antibody supershift experiments show that complex I\(\text{I}\) contains the TCF Elk-1 and that I\(\text{I}\) represents another example of antagonism between cAMP-dependent protein kinase and the ERK cascade apparent in some cell contexts (49–52). Thus, the synergy between ATP and PTH does not involve the ERK pathway.

The SYNGenic Effect of ATP/\([\text{Ca}^{2+}]\), and PTH on c-fos

The increase in luciferase expression was assessed following agonist stimulation of pools of SaOS-2 or UMR-106 cells stably transfected with truncated c-fos reporter constructs containing the CaCRE or SRE promoter elements. Values represent -fold increase in luciferase expression relative to serum-deprived cells and are presented as the mean ± S.D. of at least three separate experiments.

| Treatment | Reporter constructs |
|-----------|---------------------|
|           | CRE                 | SRE             |
| 0% FCS    | 1.00 ± 0.21         | 1.00 ± 0.04     |
| 10% FCS   | 4.90 ± 0.31         | 5.73 ± 0.26     |
| EGF, 10 ng/ml | ND\(^a\)          | 12.98 ± 0.24    |
| ATP, 10 \(\mu\) | 2.20 ± 0.08       | 4.60 ± 0.29     |
| PTH(1–34), 100 ng/ml | 10.39 ± 1.61   | 1.01 ± 0.03     |
| ATP + PTH | 11.34 ± 0.44        | ND              |

\(^a\) ND, not determined.
consistent with expression of the P2Y<sub>1</sub> receptor subtype. Only marginal levels of P2Y<sub>2</sub> mRNA could be detected, and accordingly the P2Y<sub>2</sub> receptor agonist UTP was only weakly effective at elevating [Ca<sup>2+</sup>]<sub>i</sub>. In contrast, UTP and ATP strongly elevated [Ca<sup>2+</sup>]<sub>i</sub> in primary osteoblast population HBDC1, which correlated with high levels of P2Y<sub>2</sub> receptor mRNAs. Consistent with the very low levels of P2Y<sub>2</sub> mRNA, these cells showed no response to either ADP or 2-meSATP, while intermediate levels of P2Y<sub>1</sub> receptor expression by HBDC2 were consistent with the ability of ADP/2-meSATP to mobilize intracellular calcium in a subpopulation of these cells as previously reported (55). As indicated above, we attribute this heterogeneity of receptor expression to the well characterized differentiation-dependent heterogeneity of osteoblast phenotype within primary populations cultured from explants of bone. Notably, nucleotide treatment did not lead to increased [cAMP], in either cell type, thus eliminating cAMP as an intracellular second messenger for P2Y receptors in these osteoblasts.

To assess the biological activity of NTPs in osteoblasts, we have analyzed the activation of the c-fos proto-oncogene, which is strongly implicated in controlling the proliferation and differentiation of bone cells (see Introduction). We find that P2 receptor agonists induce c-fos expression in correspondence with the elevation of [Ca<sup>2+</sup>]<sub>i</sub>, via specific receptor subtypes (discussed above). This correlation is upheld in the primary population of HBDC1: UTP and ATP induced c-fos, while ADP and 2-meSATP were ineffective. Similarly, in SaOS-2 cells ADP and ATP also led to increased c-fos mRNA levels, while UTP did not. However, while 2-meSATP effectively increased [Ca<sup>2+</sup>]<sub>i</sub> in SaOS-2 cells, this agonist failed to induce c-fos gene expression. This indicates that increased [Ca<sup>2+</sup>]<sub>i</sub>, alone is not sufficient for induction of the c-fos gene in SaOS-2 cells. Many reports have demonstrated that single G protein-coupled receptor species can be linked to multiple effector systems (55, 56). Occupation of the P2Y<sub>1</sub> receptor by 2-meSATP could induce a receptor conformation that activates G protein-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate, but not the G protein(s) that trigger other effector pathways leading to c-fos induction. Consistent with this notion, others have documented agonist-dependent coupling of G protein-coupled receptors to various intracellular effectors (57, 58).

The relatively modest increase in c-fos mRNA levels induced by nucleotides contrasted with the robust increase generated by PTH treatment. Even more striking was the synergistic effect on c-fos by the combination of NTPs and PTH, which resulted from the activation of two distinct signaling pathways. While NTPs lead to increased [Ca<sup>2+</sup>]<sub>i</sub>, without affecting cAMP, PTH does the opposite, namely elevating the levels of cAMP, cAMP-dependent protein kinase activity, and CREB phosphorylation independently of increased Ca<sup>2+</sup>. CREB phosphorylation alone might suffice to explain the synergistic induction of c-fos, since its major binding site in the fos promoter, the CaCRE, can integrate both cAMP and Ca<sup>2+</sup> signals in transfection assays (61). However, the assays using stably transfected reporter genes suggest that the NTP-Ca<sup>2+</sup> and PTH-cAMP pathways do not converge on the same promoter element. The CaCRE reporter was strongly induced by PTH. This induction was not significantly increased by including promoter sequences out to position −711, ruling out the contribution of other cryptic cAMP response elements active in transient transfection analyses (3, 4). On the other hand, the full promoter mediated the synergy between NTPs and PTH. NTPs alone consistently gave a severalfold induction of the fos −711-linked reporter gene and background levels with the CaCRE. There are several possible explanations for this observation. One is that the NTP-Ca<sup>2+</sup> pathway weakly targets the
CREB/activating transcription factor complexes bound to cAMP response elements located elsewhere in the c-fos promoter. This seemed unlikely, since NTPs activated neither the CaCRE reporter gene nor one driven by an array of six cAMP response elements. The other is that synergism arises from the effects of the NTP-Ca\(^{2+}\) pathway targeting another promoter element, either the SRE or the v-sis-inducible element. Ca\(^{2+}\) signals can selectively target the SRE in neuronal (62, 63), mesangial (64), and T cells (65). Accordingly, we observed that NTPs induced luciferase expression from a SRE-driven reporter gene stably transfected into bone cells.

The SRE is bound and activated in vitro by a ternary complex, containing SRF and TCF, that reproduces the pattern observed in genomic footprints (6, 7, 11). Transactivation by the SRF complex, containing SRF and TCF, that reproduces the pattern activation of any MAPK pathway or to detectable levels of TCF phosphorylation, thus ruling out a contribution by this signal.
Synergistic Effect of ATP/\([Ca^{2+}]_i\) and PTH on c-fos

(1993) Science 262, 1065–1069
53. Hipskind, R. A., Buscher, D., Nordheim, A., and Baccarini, M. (1994) Genes Dev. 8, 1803–1816
54. Rodan, S. B., Imai, Y., Thiede, M. A., Wesolowski, G., Thompson, D., Bar-Shavit, Z., Shull, S., Mann, K., and Rodan, G. A. (1987) Cancer Res. 47, 4961–4966
55. Dixon, C. J., Bowler, W. B., Walsh, C. A., and Gallagher, J. A. (1997) Br. J. Pharmacol. 120, 777–780
56. Gudermann, T., Schoenberg, T., and Schultz, G. (1997) Annu. Rev. Neurosci. 20, 399–427
57. Robb, S., Cheek, T. R., Hannan, F. L., Hall, L. M., Midgely, J. M., and Evans, P. D. (1994) EMBO J. 13, 1325–1330
58. Post, S. R., Rump, L. C., Zambon, A., Hughes, R. J., Buda, M. D., Jacobson, J. P., Kao, C. C., and Insel, P. A. (1998) J. Biol. Chem. 273, 23093–23097
59. Schoff, C., Cuthbertson, K. S., Gallagher, J. A., Pennington, S. R., Cobbold, P. H., Brahant, G., Hesch, R. D., and Muhlen, A. V. S. (1991) J. Biochem. (Tokyo) 274, 15–20
60. Harden, T. K., Boyer, J. L., and Nicholas, R. A. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 541–579
61. Ginty, D. D. (1997) Neuron 18, 183–186
62. Bading, H., Ginty, D. D., and Greenberg, M. E. (1993) Science 260, 181–186
63. Xia, Z., Dudek, H., Miranti, C. K., and Greenberg, M. E. (1996) J. Neurosci. 16, 5425–5436
64. Wang, W., and Simonson, M. S. (1996) Mol. Cell. Biol. 16, 5915–5923
65. Lee, G., and Gilman, M. (1994) Mol. Cell. Biol. 14, 4579–4587
66. Patel, V., Brown, C., Goodwin, A., Wilkie, N., and Boarder, M. R. (1996) Biochem. J. 320, 221–226
67. Yu, S. M., Chen, S. F., Lau, Y. T., Yang, C. M., and Chen, J. C. (1996) Mol. Pharmacol. 50, 1000–1009
68. Neary, J. T., McCarthy, M., Kang, Y., and Zuniga, S. (1998) Neurosci. Lett. 242, 159–162
69. Soltoff, S. P., Avraham, H., Avraham, S., and Cantley, L. C. (1998) J. Biol. Chem. 273, 2653–2660
70. Rosen, L. B., Ginty, D. D., Weber, M. J., and Greenberg, M. E. (1994) Neuron 12, 1207–1221
71. Collart, M. A., Tourkine, N., Belin, D., Vassalli, P., Jeanteur, P., and Blanchard, J.-M. (1991) Mol. Cell. Biol. 11, 2828–2831
72. MacDonald, B. R., Gallagher, J. A., and Russell, R. G. G. (1986) Endocrinology 118, 2445–2449
73. Raisz, L. G. (1965) Nature 197, 1015–1017