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Osteoblast-like cells have a variable mixed population of purino/nucleotide receptors

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Osteoblast-like UMR 106.06 cells respond to extracellular application of nucleotides with a fast intracellular calcium pulse (latency of about 20 s, half-width of about 10 s), as measured with fluo-3 on a confocal laser scanning system. Cross-inhibition experiments at 50 μM show that, on a cell population basis, adenosine triphosphate (ATP) strongly inhibits the effect of uridine triphosphate (UTP) or 2-methylthio-ATP (2-MeSATP) applied within 2 min after the end of the ATP-induced pulse, while prior application of UTP or 2-MeSATP only weakly inhibits the ATP effect, and UTP and 2-MeSATP weakly inhibit each other. Furthermore, there are clear differences in cross-inhibition responses between individual cells. Our measurements provide strong evidence that these cells have at least two types of purino/nucleotide receptors, probably P_{2y} and P_{2u}, with a proportion that varies between individual cells

Osteoblast; ATP; Purinergic receptor; Nucleotide receptor, Intracellular calcium; Cross-inhibition

1. INTRODUCTION

Recently adenosine triphosphate (ATP) receptors in osteoclasts [1] and osteoblastic cells [2–4] have been reported. ATP receptors are classified as P_2 purinergic receptors and have been extensively studied in recent years in other types of cells [5–9]. Studies of P_{2y} purinoceptor mediated Ca^{2+} mobilization found that adenosine diphosphate (ADP) was equipotent to ATP in rat hepatocytes, turkey erythrocytes, and bovine endothelial cells [10–12], while non-adenosine nucleotides were generally inactive or much less potent than ATP. In other cells such as NCB-20 mammalian neuronal cells [9], human neutrophils/monocytes [13], and HL-60 human leukemia cells [14], ADP was much less potent than ATP, while uridine triphosphate (UTP) was equal or more potent than ATP. These data suggested a receptor subtype that some workers called P_{2u} [6,9] and others called the 'nucleotide receptor' [15,16].

UMR 106.06 is an osteosarcoma cell line with many characteristics of the osteoblast, including alkaline phosphatase activity, production of type I collagen, and response to parathyroid hormone with an elevation of intracellular cyclic adenosine monophosphate (cAMP) [17,18] and intracellular free calcium ($[Ca^{2+}]_i$) [19]. Here we report novel cross-inhibition experiments with nucleotides, using a laser scanning imaging system that allows the response of individual cells to be seen. The differences we observe in cross-inhibition between individual cell demonstrate that these cells have a mixed population of purino/nucleotide receptors.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

UMR 106.06 cells were cultured in Dulbecco's modified Eagle's Medium with high glucose (Gibco, Life Technologies Inc Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Flow Laboratories, McLean, VA) and antibiotics (100 μg/ml penicillin G, 50 μg/ml gentamycin, and 250 ng/ml amphotericin B). Experiments were conducted two to three days after subculture, at which time the cells were confluent in 60-mm culture dishes ($\approx 8 \times 10^5$ cells/dish). Cells used are between passages 13–23.

2.2. *Reagents*

All chemicals used were obtained from Sigma except 2-methylthio-ATP (2-MeSATP), which was purchased from Research Biochemicals Inc. (Natick, MA).

2.3. *Confocal measurement of individual cellular $[Ca^{2+}]_i$*

For fluorescence measurements of $[Ca^{2+}]_i$, the cells were loaded with fluo-3/AM (6 μM) at 21°C for about 60 min, and then thoroughly washed, according to the method of Kao et al [20] Measurements were carried out at 37 ± 1°C, in α-minimum essential medium with 25 mM HEPES buffer replacing bicarbonate, with 10 ml of medium per dish [1]. The laser scanner and detectors were attached to a Nikon Optiphot microscope in a Bio-Rad MRC 600 confocal system. Complete images were digitized and collected every 5 s Fluorescence intensity time courses for individual cells or groups of cells were obtained with the Optimas image analysis program.

3. RESULTS

ATP induces a large, transient increase in $[Ca^{2+}]_i$ in UMR 106.06 cells (Fig. 1A). At 25 μM, the time between application of ATP to the bathing medium and

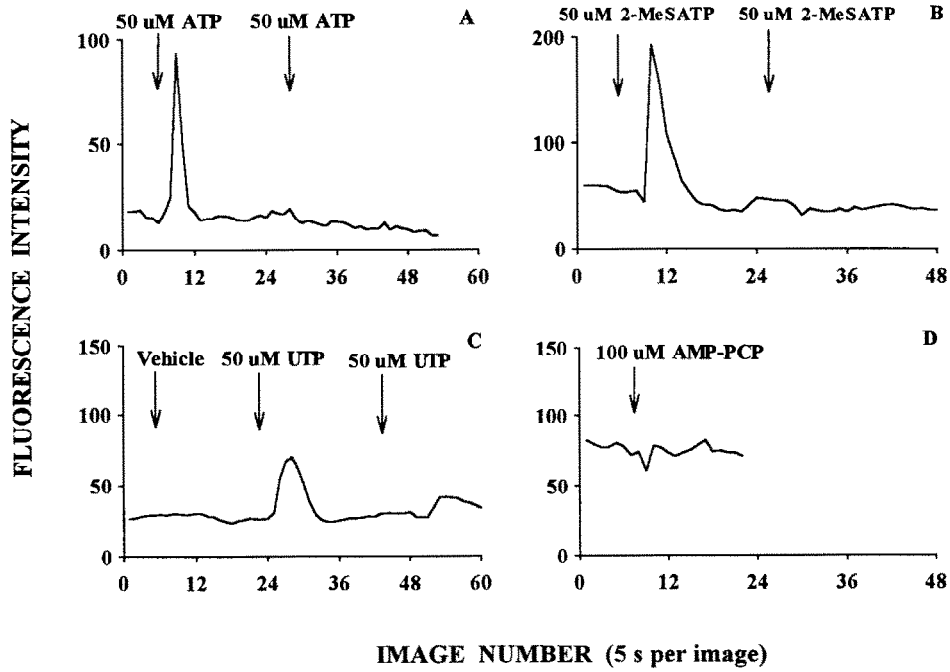

Fig. 1. Time courses of fluorescence intensity averaged over a group of UMR 106 06 cells (about 60 cells), showing typical responses to two applications of (A) ATP, (B) 2-MeSATP, (C) UPT, and to one application of (D) AMP-PCP. The results are representative of three or four experiments (separate dishes) for each nucleotide or ATP analog.

the increase in fluorescence intensity to half maximum ($T_{\frac{1}{2}}$ is 39 ± 7 s (mean ± S.E.M., 6 measurements). The duration of the transient, between attaining the half maximum intensity and declining to the half maximum (T_w), is 16 ± 5 s. The magnitude of the calcium pulse in terms of $\Delta F/F = (F_{peak} - F_{before}/F_{before}$ where F_{peak} is the peak fluorescence intensity after, and F_{before} is the fluorescence intensity just before application of extracellular ATP, is 1.7 ± 0.5. At 50 μM this measure increases to 4.8 ± 0.9 (n = 12; Fig. 1A; Table I). ADP, 2-MeSATP and UTP at 50 μM also produce a transient increase in $[Ca^{2+}]_i$ (Fig. 1B,C; Table I). β,γ-methylene-ATP (AMP-PCP; Fig. 1D) and adenosine produce no discernable $[Ca^{2+}]_i$ increase (100 μM, three or four measurements each). The rank-order at 50 μM in terms of $\Delta F/F$ is ATP > ADP = 2-MeSATP > UTP (Table I). We call this the 'fluorescence intensity order', which defines the 'fluorescence intensity potency'.

At 50 μM, a second dose of the same agonist applied within two minutes after the return of the first pulse to baseline, with no change in bathing medium, produces a negligible effect on $[Ca^{2+}]_i$ with ATP and 2-MeSATP (n = 3 each; Fig. 1A,B), or a smaller pulse in the case of UTP (Fig. 1C; ratio of $\Delta F/F$ values = 0.40 ± 0.20, n = 3). After 10 min, a second application of ATP does produce a small pulse (ratio of $\Delta F/F$ values = 0.35 ± 0.11, n = 4).

In terms of the response of the majority of the cells observed in each experiment, application of 50 μM ATP strongly inhibits the effect of 50 μM UTP or 50 μM 2-MeSATP applied within two minutes after the ATP-induced pulse returns to the baseline, with no change of medium (n = 3 each; Fig. 2). However, prior application of UTP or 2-MeSATP only weakly inhibits the ATP-induced effect (n = 3 each; Fig. 3A,B). Prior application of UTP weakly inhibits the 2-MeSATP effect (n = 4; Fig. 3C) and the inhibitory effect of 2-MeSATP on subsequent application of UTP varies from partial inhibition (n = 5) to very little inhibition (n = 3; Fig. 3D). In many of these cross-inhibition experiments, a number of individual cells can be seen in respond differently from the majority of the cells in the given experiment. In almost all experiments where the majority of cells respond to both the first and second agonist, there are almost always cells that respond to the first agonist but not to the second, and other cells that do not respond

Table I

$[Ca^{2+}]_i$ increase in UMR 106 06 cells induced by extracellular nucleotides or ATP analogs (at 50 μM)

	ATP	ADP	2-MeSATP	UTP
n	12	3	9	7
$T_{\frac{1}{2}}$	21 ± 6 s	25 ± 15 s	13 ± 2 s	17 ± 3 s
T_w	9 ± 2 s	8 ± 3 s	11 ± 4 s	20 ± 5 s
$\Delta F/F$	4.8 ± 0 9	3 9 ± 1.4	3.0 ± 0.5	1.9 ± 0.4

Data expressed as mean ± S E.M.

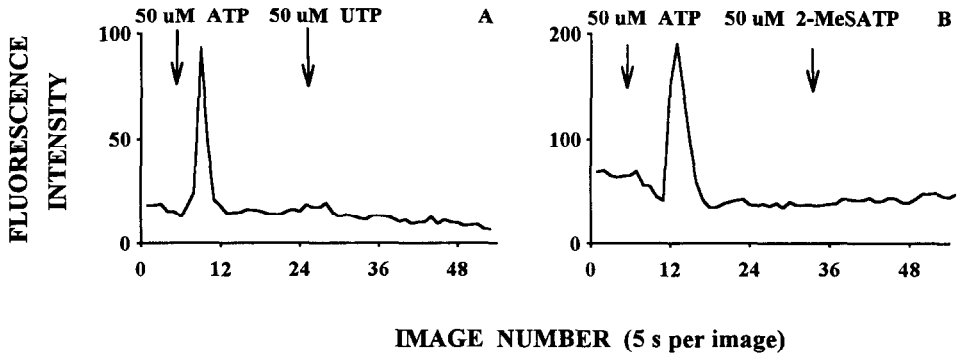

Fig. 2. Time course of fluorescence intensity averaged over a group of cells (about 60 cells), showing that application of ATP virtually abolishes the effect of (A) UTP or (B) 2-MeSATP applied within two min after the ATP-induced pulse returns to the baseline.

to the first agonist but do respond to the second (Fig. 3). In those experiments where the majority of cells show that the effect of the second agonist is inhibited by the first agonist, there are almost always cells that do not show such inhibition, or even cells that do not respond to the first agonist but do respond to the second.

In Ca^{2+}-free bathing medium, the ATP effect is abolished at 25 μM in 3 of 5 measurements, at 50 μM in 2 of 4 measurements, and at 100 μM in 1 of 3 measurements. In the experiments in which ATP induces a calcium pulse in the Ca^{2+}-free medium, the $T_{1/2}$ is longer and the T_w is much shorter than in normal bathing medium at equal ATP concentrations.

4. DISCUSSION

UMR 106.06 cells respond to extracellular ATP with a large, transient increase in $[Ca^{2+}]_i$, showing that this cell line has ATP receptors in the plasma membrane. We do not see two phases of $[Ca^{2+}]_i$ increase as reported by Kumagai et al. [2]. Ca^{2+}-free medium blocks the increase in $[Ca^{2+}]_i$ in response to ATP in some but not all experiments, indicating that internal release of Ca^{2+} is involved, but that the Ca^{2+} influx is important in modulating the internal release. The lack of Ca^{2+} influx in Ca^{2+}-free medium should reduce the steady-state cytosolic $[Ca^{2+}]_i$. Ca^{2+}-free medium also prevents Ca^{2+} influx following ATP binding. Both of these effects should make internal release of Ca^{2+} more difficult.

In contrast to osteoclasts [1], a second application of ATP within two minutes after the first calcium pulse returns to the baseline, with no change in bathing medium, does not produce another calcium pulse. With our volume of bathing medium per cell, and reported rates of hydrolysis of extracellular ATP by osteoblastic cells [3], we estimate that less than 5% of the applied ATP would be hydrolyzed during the 10 min following the first calcium pulse. The partial recovery of the calcium response to the second ADP application after 10 min may thus reflect an adaptation, which is slower than in the osteoclast, of the signaling pathway leading to Ca^{2+} release, or it may reflect a longer period of time needed for refilling of the intracellular Ca^{2+} pools linked to the receptors activated by ATP. Mechanical perturbation experiments with osteoblastic cells found that a second mechanical perturbation required more than a three minute wait to induce a second calcium pulse [21].

Classification of P_2 purinoceptors is largely based on rank-order of potency of the agonists because of the lack of selective, competitive receptor antagonists [6,15]. It has been reported that for P_{2y} receptors, the potency order defined on the basis of receptor-agonist affinity is 2-MeSATP $\gg$ ATP = ADP > AMP-PCP, UTP, while for P_{2x} the affinity order is AMP-PCP $\gg$ ATP = 2-MeSATP > UTP [8,15,16]. Recent reports have shown another receptor subtype, named the P_{2u} or the 'nucleotide receptor' [6,9,15,16] for which agonists elicit an increase in $[Ca^{2+}]_i$ with the affinity order UTP = ATP > ADP > 2-MeSATP > AMP-PCP [15,16]. Since UTP induces a calcium pulse in UMR 106.06 cells, it seems likely that these cells have some P_{2u} nucleotide receptors. However, the receptors can't be just P_{2u}, or else the response to UTP should be greater than the response to ATP at 50 μM [9]. That 2-MeSATP produces a greater fluorescence intensity change than UTP suggests that there are P_{2y} receptors. ATP could produce the greatest fluorescence intensity change among the agonists tested because 2-MeSATP, even though it has a greater affinity for the P_{2y} receptor than does ATP, is only a partial agonist of this receptor [9,16,22,23], and so it would not be expected to produce as large an effect as ATP at 50 μM. Because it is conceivable that UTP could have an affect on P_{2y} receptors, we cannot rule out the possibility of having only P_{2y} receptors on the basis of the fluorescence intensity order.

Another purinergic receptor known to be affected by agonists at these concentrations is the P_{2T}. However, the P_{2T} receptor has been found to have no response to ATP at concentrations up to 1 mM, and no response to UTP up to at least 100 μM [24]. We cannot rule out the

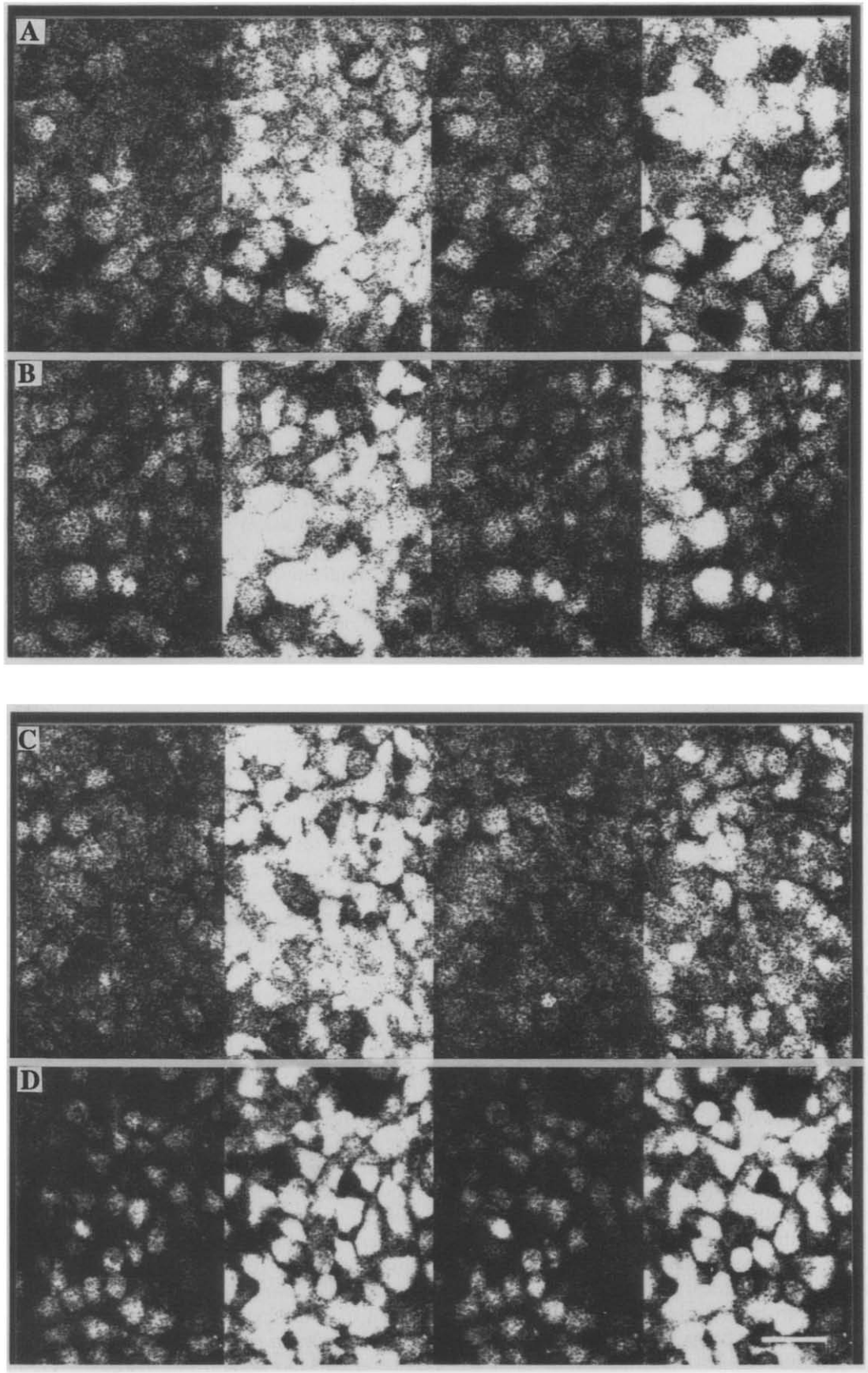
A
B
C
D

Fig. 3. Fluorescence intensity images of UMR 106 06 cells, showing the cross-inhibition effect of (A) UTP (first pair, before and 20 s after application) on ATP (last pair, before and 15 s after application) with an interval of 120 s between applications; (B) 2-MeSATP (first pair, before and 10 s after application) on ATP (last pair, before and 25 s after application) with an interval of 110 s between applications; (C) UTP (first pair, before and 15 s after application) on 2-MeSATP (last pair, before and 10 s after application) with an interval of 150 s between applications; and (D) 2-MeSATP (first pair, before and 50 s after application) on UTP (last pair, before and 20 s after application) with an interval of 160 s between applications. The second agonist is applied within two min after the return to the baseline of the first agonist-induced pulse. These time sequences of images are representative of at least three experiments for each permutation of agonists. Concentrations are all 50 μM. The scale bar is 50 μM. ←

possibility of having P_{2T} receptors, but they would have no role in our measured responses to ATP and UTP.

Our finding, that application of an agonist greatly inhibits the effect of the same agonist applied within two min after the first calcium pulse returns to baseline, suggested to us that, if there is only one receptor type, application of a different agonist at the same concentration with the same or lower fluorescence intensity potency than the first should produce little response. Application of an agonist with a greater fluorescence intensity potency at the same concentration should elicit a response, if the Ca^{2+} pools have not been exhausted. On the other hand, if there is more than one receptor type present, then an agonist should have little inhibitory effect on a second agonist that is lower in the fluorescence intensity order if the second agonist has a greater effect than the first on one of the receptor types, and if the Ca^{2+} pools linked to that receptor type have not been depleted.

For populations of UMR 106.06 cells, our cross-inhibition experiments show that ATP greatly inhibits 2-MeSATP and UTP, but 2-MeSATP weakly inhibits ATP and partially or weakly inhibits UTP, and UTP weakly inhibits ATP and 2-MeSATP. From these cross-inhibition experiments we can establish a cross-inhibition order, in which prior application of an agonist can significantly inhibit the response to another agonist that is lower in the order, but not the response to one that is equal or higher in the order. For our cell populations, we thus have ATP > 2-MeSATP $\approx$ UTP. These data suggest that the receptors cannot be entirely P_{2u}, otherwise UTP should inhibit ATP and 2-MeSATP. These data also suggest that the receptors cannot be entirely P_{2y} either, or else 2-MeSATP at this fairly high concentration should inhibit the effect of UTP and of ATP. However, since 2-MeSATP is known to be only a partial agonist of the P_{2y} receptor, it is conceivable that ATP or perhaps even UTP at this concentration could have an effect on P_{2y} receptors even if the receptors were already saturated with 2-MeSATP.

Our cross-inhibition experiments also show that individual cells can have different responses to the agonists, and thus would have different individual cross-inhibition orders. For example, if one cell has little or no response to the application of ATP, but had a strong response to the prior application of UTP, while another cell in the same experiment has a stronger response to the application of ATP than to the prior application of UTP, then the first cell has a cross-inhibition order with UTP > ATP, while the second cell has a cross-inhibition order with ATP $\geq$ UTP. Our cross-inhibition experiments show that some cells clearly respond more to ATP than to the prior application of UTP, others clearly respond more to UTP than to the subsequent application of ATP (with some not responding at all to ATP), and some respond strongly to both (Fig. 3A). Moreover, some cells respond more to ATP than to the prior application of 2-MeSATP, some respond more to 2-MeSATP than to the subsequent application of ATP, and others respond about equally to both (Fig. 3B). There are also individual cells that respond more to UTP than to the prior application of 2-MeSATP, and others that respond more to the 2-MeSATP (Fig. 3D). These observations show that there are differences in cross-inhibition order between individual cells. The simplest explanation for these differences is that there is more than one type of receptor, with individual cells having a different portion of each receptor type.

In summary, our data show that osteoblast-like UMR 106.06 cells respond to extracellular ATP, ADP, 2-MeSATP and UTP with a $[Ca^{2+}]_i$ pulse. Our cross-inhibition experiments provide strong evidence for a mixed population of purino/nucleotide receptors in these cells, most likely P_{2y} purinoceptors and P_{2u} nucleotide receptors, with differences between individual cells in the proportion of these receptors.

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