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Effects of extracellular nucleotides on single cells and populations of human osteoblasts: contribution of cell heterogeneity to relative potencies

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1 Human osteoblasts responded to the application of extracellular nucleotides, acting at P_2-receptors, with increases in cytosolic free calcium concentration ($[Ca^{2+}]_i$).

2 In populations of human osteoblasts, adenosine 5′-diphosphate (ADP) evoked a rise in $[Ca^{2+}]_i$ with less than 40% of the amplitude of that induced by adenosine 5′-triphosphate (ATP).

3 ATP and uridine 5′-triphosphate (UTP) were applied to single human osteoblasts and induced $[Ca^{2+}]_i$ rises of comparable amplitude in every cell tested.

4 However, from the results of single cell studies with ADP (and 2-methylthioATP (2-meSATP)) two groups of cells were delineated: one group responded to ADP (or 2-meSATP) with a rise in $[Ca^{2+}]_i$ indistinguishable from that evoked by ATP; whereas the second group failed completely to respond to ADP (or 2-meSATP).

5 Therefore heterogeneity of receptor expression exists within this population of human osteoblasts. The limited distribution of the ADP-responsive receptor underlies the small response to ADP, compared with ATP, recorded in populations of human osteoblasts. This heterogeneity may reflect differences in the differentiation status of individual cells.

Keywords: P_2-receptors; cytosolic free calcium concentration; human osteoblasts; heterogeneity; 2-meSATP; ADP; ATP; UTP

Introduction

Osteoblasts are the cells that synthesize and secrete the organic matrix of bone. Expression of P_{2Y}-receptors has been demonstrated in human osteoblasts and osteosarcoma cell lines of rat and human origin, which retain osteoblastic characteristics (Kumagai *et al*., 1989; 1991; Schofl *et al*., 1992; Reimer & Dixon, 1992; Gallinaro *et al*., 1995; Bowler *et al*., 1995). These G-protein-coupled receptors mediate the effects of extracellular nucleotides and, in most cases, are coupled to hydrolysis of phosphatidylinositol 4,5-bisphosphate and hence Ca^{2+} mobilization from intracellular stores (Boarder *et al*., 1995; Harden *et al*., 1995). Calcium is an important and well characterized intracellular regulator of a diverse array of physiological functions (Exton, 1987). In human osteoblasts (Bowler *et al*., 1996), as in isolated cardiomyocytes (Zheng *et al*., 1994), nucleotide-induced increases in cytosolic free calcium concentration ($[Ca^{2+}]_i$) have been shown to induce expression of the proto-oncogene, *c-fos*. This immediate early gene has been implicated in the processes that govern cell growth, differentiation and, more specifically, skeletal tissue remodelling (Johnson *et al*., 1992).

In the continued absence of specific antagonists for G-protein-coupled P_2-receptors, studies to identify the receptors expressed by a particular cell type have relied upon the rank order of potency series for adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and synthetic nucleotide analogues (O'Connor *et al*., 1991; Abbracchio & Burnstock, 1994). Such studies have been performed on cell populations which have associated problems, including the hydrolysis of nucleotides by extracellular enzymes. Indeed the influence of ecto-ATPases on rank order of potency series has received much attention recently; different orders of potency have been recorded in some preparations in the presence or absence of an ecto-ATPase inhibitor (Crack *et al*., 1995; Kennedy & Leff, 1995).

Here, we show that single cell studies can reveal information about the receptors present on cells which is hidden in studies on larger numbers of cells. Thus, the response of groups of human osteoblasts to ATP and ADP indicates that ADP is a poor agonist in these cells. However, when individual cells are studied it becomes clear that this is the result of heterogeneity of the receptors expressed within the population, rather than weak agonist characteristics of ADP.

Methods

Cell culture

Human osteoblasts were isolated from bone as previously described (Gallagher *et al*., 1996). Briefly, specimens of human bone obtained at surgery were finely minced into Dulbecco's modified Eagle's medium (DMEM) and then washed vigorously in DMEM to remove adherent marrow cells. Fragments of bone were then transferred to 90 mm diameter petri dishes containing DMEM supplemented with 10% foetal calf serum, 100 μg ml^{-1} streptomycin, 100 u ml^{-1} penicillin and 2 mM L-glutamine, and incubated at 37°C in a humidified atmosphere of 93% air and 7% CO_2. After 3–6 weeks a confluent monolayer of cells had grown out which was passaged onto 22 mm diameter glass cover slips. All experiments were performed on cultures at first passage, and cells were all derived from the same donor. The human bone cell populations obtained have previously been shown to possess osteoblastic characteristics, including responsiveness to parathyroid hormone and to 1,25-dihydroxyvitamin D_3, and synthesis of type I collagen and the bone-specific protein, osteocalcin (Gallagher *et al*., 1996).

Measurement of $[Ca^{2+}]_i$

Cells were serum-starved for 2 h before being loaded with fura-2 by incubation with fura-2 acetoxymethyl ester (5 mM) (Molecular Probes) for 20 min at 37°C in HEPES buffer

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(composition in mM: HEPES 10, NaCl 121, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 5, and glucose 10; pH 7.2) containing 2% bovine serum albumin (BSA). Cells were subseqently washed 3 times in buffer of the same composition but containing 0.2% BSA.

Experiments were carried out with a photon-counting spectrophotometer (Cairn Research Limited) on a Nikon TM Diaphot microscope with a 40x oil immersion lens. The cell-coated coverslip was attached with silicone grease to form the base of a stage-mounted, thermostatically-regulated chamber maintained at 37°C. Recordings were made either from the complete field of view, encompassing a group of 6–8 cells (a population), or from a window surrounding a single cell. Cells were illuminated with excitation light (340 nm and 380 nm) at a rate of 32 times per second, and the emission measurements (at 510 nm) were integrated into 1 second averages, then stored to memory. Addition of agonists, in HEPES buffer with 0.2% BSA, was performed manually by Pasteur pipette. Agonists were added for short periods (60–120 s) and recovery periods of at least 10 min were allowed between additions. The fluorescence ratio values under Ca^{2+}-free and saturating conditions, R_{min}, and R_{max}, and autofluorescence values were obtained *in situ* with ionomycin, as described by Thomas & Delaville (1991). $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence at the two excitation wavelengths, after subtraction of autofluorescence (Grynkiewicz *et al*., 1985). The increases in $[Ca^{2+}]_i$ are expressed as a percentage of the response to a maximal concentration of ATP (10 μM) in the same cell(s). ADP, ATP and uridine 5′-triphosphate (UTP) were obtained from Sigma, and 2-methylthioATP (2-meSATP) from ICN Biomedicals.

Results

Effects of ATP and ADP on populations of human osteoblasts

Application of 10 μM ATP to populations of fura-2-loaded human osteoblasts, consisting of 6–8 cells, induced a rise in $[Ca^{2+}]_i$ in 7/7 preparations. As depicted in Figure 1, increasing the concentration of ATP to 100 μM did not result in a $[Ca^{2+}]_i$ rise with increased amplitude in 4/5 preparations; 100 μM ATP induced a rise in $[Ca^{2+}]_i$ which was 102.1±5.0% (mean± s.e.mean) of that recorded in response to 10 μM ATP. The remaining group of human osteoblasts showed an increase in the amplitude of the response to 100 μM ATP compared with 10 μM ATP (157.5%).

In the majority of cell populations (5/7), ADP was a much less effective agonist than ATP, as illustrated in Figure 1;

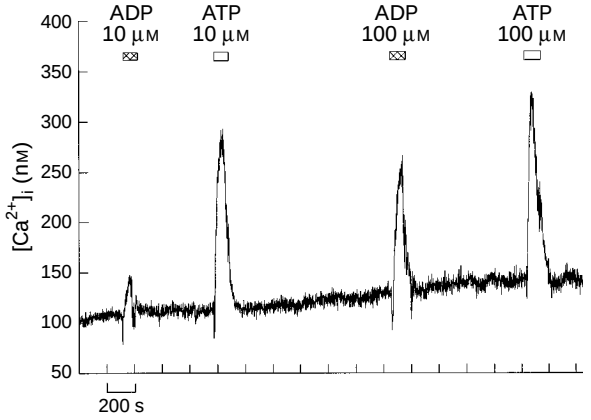

Figure 1 Groups of 6–8 fura-2-loaded human osteoblasts were stimulated with ADP (10 μM and 100 μM) and ATP (10 μM and 100 μM) for the periods indicated, and responded with rises in $[Ca^{2+}]_i$. Stimulation with 100 μM ADP evoked a smaller amplitude rise in $[Ca^{2+}]_i$ than that induced by 10 μM ATP in the same cells.

10 μM ADP evoked a rise in $[Ca^{2+}]_i$ with an amplitude only 38.1±5.4% of that recorded in response to 10 μM ATP in the same cells. (The remaining 2 groups of human osteoblasts responded to 10 μM ADP with a rise in $[Ca^{2+}]_i$ with similar amplitude to that induced by 10 μM ATP (98.0±6.5%)). The smaller amplitude of the responses to ADP compared with ATP cannot be accounted for by decreased sensitivity of the cells, as the application of ADP was always before stimulation with ATP, as illustrated in Figure 1. When the ADP concentration was raised from 10 μM to 100 μM, the amplitude of the rise in $[Ca^{2+}]_i$ increased, but still represented only 61.1±11.0% ($n=6$) of the response to 10 μM ATP (see Figure 1).

Effects of ATP, ADP and 2-meSATP on human single osteoblasts

All single cells responded to application of 10 μM ATP with a rise in $[Ca^{2+}]_i$ ($n=11$). However, when stimulated with ADP, 2 sub-populations of cells were delineated: those responsive to ADP; and those which were non-responsive to this nucleotide. In the first sub-population (3/8 cells), 10 μM ADP evoked a rise in $[Ca^{2+}]_i$ indistinguishable from that induced by 10 μM ATP (101.8±3.3%), as illustrated in Figure 2. Two of these cells were tested for responsiveness to the P_{2Y1}-receptor agonist, 2-meSATP, which also led to a rise in $[Ca^{2+}]_i$ similar in amplitude to that evoked by 10 μM ATP (92.1±3.5%; Figure 2). The second sub-population, comprising the remaining 5 cells, and represented in Figure 3, was unresponsive to 10 μM or 100 μM ADP (0.4±3.6% and 2.8±1.6% of the response to 10 μM ATP, respectively). Two of these 5 cells were subsequently stimulated with 10 μM 2-meSATP; both cells failed also to respond to this nucleotide (2.2±4.0% of the $[Ca^{2+}]_i$ rise induced by 10 μM ATP), as shown in Figure 3.

Effect of UTP on human single osteoblasts

The P_{2Y2}-receptor agonist, UTP (10 μM), was applied to 6 single osteoblasts, and elicited a $[Ca^{2+}]_i$ response comparable in amplitude to that evoked by 10 μM ATP in the same cells (90.5%±8.6%; Figure 3). Five of these 6 cells were tested for responsiveness to ADP (10 μM), 3 were responsive to ADP, whereas the remaining 2 cells, one of which is illustrated in Figure 3, were unresponsive to ADP.

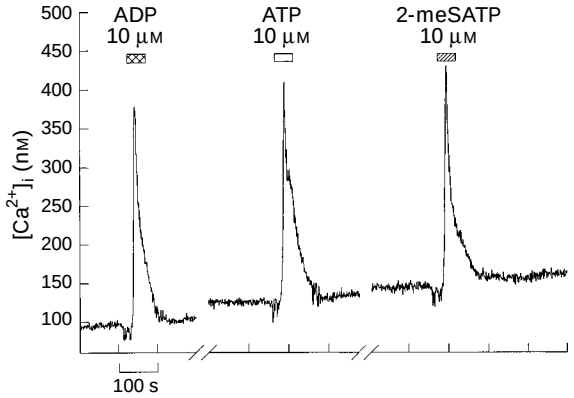

Figure 2 $[Ca^{2+}]_i$ increases in a single fura-2-loaded human osteoblast induced by ADP, ATP and 2-meSATP. When the effects of extracellular ADP were studied in human single osteoblasts, two groups of cells were delineated: one group was unresponsive to this nucleotide; whereas the other group, represented here, responded to 10 μM ADP with an increase in $[Ca^{2+}]_i$ which was indistinguishable from that evoked by 10 μM ATP. This result is typical of the response recorded in 3/8 cells. Two of these 3 cells were tested for responsiveness to 2-meSATP; it evoked a response similar in amplitude to that evoked by the same concentration of ADP or ATP.

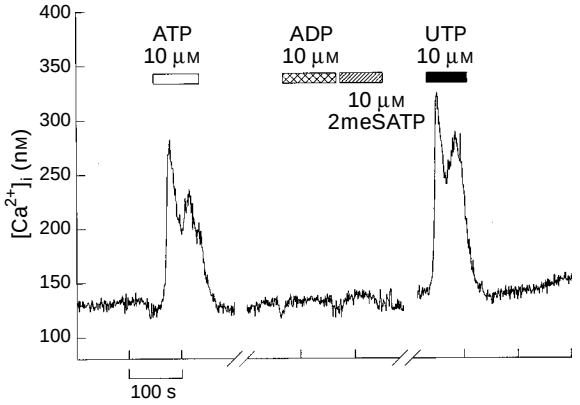

Figure 3 $[Ca^{2+}]_i$ increases evoked by UTP and ATP in a single fura-2-loaded human osteoblast which failed to respond to ADP or 2-meSATP. Five out of 8 single cells failed to respond to ADP, and two of these cells were further stimulated with 2-meSATP, which similarly was ineffective. All single cells tested responded to the application of UTP with a rise in $[Ca^{2+}]_i$ similar to that induced by ATP.

Discussion

The studies described here highlight the need for caution when interpreting data aimed at identifying the purinoceptors present on populations of cells. Considering, in isolation, the data from groups of human osteoblasts, it appears that ADP is a poor agonist in these cells in comparison with ATP; 10 μM ADP evoked a rise in $[Ca^{2+}]_i$ with less than 40% of the amplitude of that recorded in response to 10 μM ATP. Although the effects of UTP were not tested on populations of cells, this nucleotide was as effective an agonist as ATP in all single cells tested. Human osteoblasts are known to express P_{2Y2}-receptors (Bowler *et al*., 1995). This receptor is activated equipotently by ATP and UTP, whilst ADP has been described as a partial agonist (Erb *et al*., 1995). The responses recorded in populations of human osteoblasts could therefore be explained by the sole expression of a P_{2Y2}-receptor. As all cells tested responded to UTP, this receptor appears to have a ubiquitous distribution throughout the population of human osteoblasts employed here.

However, single cell studies reveal a situation which would not be predicted from a consideration of population studies alone. Such studies uncover the existence of 2 sub-populations of cells with distinct profiles. One group of cells is completely unresponsive to ADP and to 2-meSATP, and the effects of nucleotides are consistent with these cells expressing only the P_{2Y2}-receptor. In the second sub-population of human osteoblasts, the response to 10 μM ADP or 2-meSATP was indistinguishable from that evoked by 10 μM ATP or UTP. In addition to the P_{2Y2}-receptor expressed by all cells, this sub-population expresses a second receptor responsive to ADP and 2-meSATP (possibly the P_{2Y1}-receptor subtype). When measured in a population of cells, therefore, the response to ADP will be smaller than that to ATP, reflecting the limited distribution of this receptor.

UMR-106 cells, osteosarcoma cells of rat origin which retain osteoblastic characteristics, have been shown to express 2 Ca^{2+}-mobilizing purinoceptors, probably the P_{2Y1} and P_{2Y2} subtypes (Reimer & Dixon, 1992; Yu & Ferrier, 1993; Gallinaro *et al*., 1995). The results of cross-desensitization experiments led Yu & Ferrier (1993) to conclude that individual UMR-106 cells expressed the two receptors in differing proportions. Some cells did not respond to ATP, UTP or 2-meSATP, indicating that both purinoceptors were not expressed by all individual cells. These results demonstrate that heterogeneity in terms of the receptors expressed exists within populations of cells from a clonal cell line, which may be expected to be homogeneous. Heterogeneity in $[Ca^{2+}]_i$ responses of single hepatocytes to extracellular nucleotides has been demonstrated previously (Dixon *et al*., 1990; Keppens *et al*., 1993). It is therefore not unreasonable to assume that similar heterogeneity exists amongst individual cells in many populations. If so, this has important consequences for characterization studies based on rank order series in cell populations.

Dubyak *et al*. (1996) found that differential expression of ATP receptor subtypes was associated with distinct stages of differentiation during the development of human mononuclear phagocytes. Thus, the P_{2Y1}-receptor was found to be expressed in early stages of differentiation, whilst the P_{2Y2}-receptor was expressed at later stages. Osteoblasts are derived from local mesenchymal stem cells which, when exposed to the correct extracellular signals, will proliferate and differentiate first into pre- and then into mature osteoblasts. The population of cells studied here may be expected to contain osteoblasts at different stages of maturity and this may underlie the heterogeneity in responses to extracellular nucleotides. Interestingly, SaOS-2 cells, osteosarcoma cells of human origin with an immature osteoblastic-phenotype, respond to 2-meSATP but not to UTP, consistent with these cells expressing P_{2Y1}-, but not P_{2Y2}-receptors (unpublished observations). The pattern of receptor expression during the development of osteoblasts may therefore prove to be analogous to that seen during differentiation of mononuclear phagocytes, where the P_{2Y1}-receptor is expressed in the early stages of maturation, and the P_{2Y2}-receptor at later stages of development (Dubyak *et al*., 1996). The osteoblast population studied here would then appear to contain cells at an intermediate stage of development and at different stages of differentiation. Studies designed to correlate receptor expression with stages of osteoblast maturation, characterized by markers of differentiation, are under way.

The current study emphasizes the value of single cell studies, which yield information that is masked in populations. Single cell studies can, as demonstrated here, provide a clearer understanding of the purinoceptors present on a given cell type, particularly if heterogeneity exists within the cell population. Such heterogeneity may prove to be widespread, and may, in part, account for the great diversity of rank order of potency series obtained. We believe that the differences in receptor expression between cells may reflect the differentiation status of the individual cells.

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