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Osteoclast ATP receptor activation leads to a transient decrease in intracellular pH

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SUMMARY

Application of extracellular adenosine triphosphate (ATP) induces a pulsed decrease in osteoclast intracellular pH (pH_i), as measured with seminaphthofluorescein (SNAFL)-calcein on a laser scanning confocal microscope. Adenosine diphosphate also produces a pH_i decrease, but adenosine monophosphate, uridine triphosphate, 2-methylthio-ATP, and β,γ-methylene-ATP have little effect on pH_i. The ATP-induced pH_i decrease is largely inhibited by suramin, a P_2 purinergic receptor blocker. Clamping intracellular free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) with BAPTA/AM does not affect the ATP-induced pH_i change, showing that this pH_i decrease is not caused by the increased intracellular $[Ca^{2+}]_i$ that is produced by activation of osteoclast purinergic receptors. We show that an increase in $[Ca^{2+}]_i$ by itself will produce a pH_i increase. The ATP effect is not blocked by inhibition of Na^+/H^+ exchange by either Na^+-free bathing medium or amiloride. Two inhibitors of the osteoclast cell membrane proton pump, *N*-ethylmaleimide and vanadate, produce partial inhibition of the ATP-induced pH_i decrease. Two other proton pump inhibitors, bafilomycin and *N,N′*-dicyclohexylcarbodiimide, have no influence on the ATP effect. None of the proton pump inhibitors but vanadate has a direct effect on pH_i. Vanadate produces a transient pH_i increase upon application to the bathing medium, possibly as a result of its known effect of stimulating the Na^+/H^+ exchanger. Inhibition of Cl^-/HCO_3^- exchange by decreasing extracellular Cl^- gives a pronounced long-term pH_i increase, supporting the hypothesis that this exchange has an important role in osteoclast pH_i homeostasis. In Cl^--free extracellular medium, there is a greatly reduced effect of extracellular ATP on pH_i. The ATP effect is partially inhibited by diisothiocyanatostilbene sulfonic acid, an inhibitor of the Cl^-/HCO_3^- exchanger. These data provide evidence that ATP binding to a P_2 purinergic receptor results in a transient enhancement of Cl^-/HCO_3^- exchange across the osteoclast cell membrane.

Key words: purinergic receptor, chloride/bicarbonate exchanger, proton pump, SNAFL-calcein, intracellular pH, osteoclast

INTRODUCTION

Regulation of intracellular pH (pH_i) in osteoclasts has three aspects: the normal metabolic homeostasis that is important in all cells, an augmented pH_i homeostasis requirement during bone resorption when there is a large flux of proton-equivalents through the cell into the resorption lacuna on the bone surface, and the possibility of a signal-related alteration of pH_i in response to the binding of extracellular signaling molecules to cell membrane receptors. The homeostasis of osteoclast pH_i, in both the resorbing and the non-resorbing state, has been shown to require Cl^-/HCO_3^- exchange across the cell membrane (Teti et al., 1989; Hall and Chambers, 1989; Schlesinger et al., 1994). The Na^+/H^+ exchanger also has some importance during osteoclastic bone resorption (Hall and Chambers, 1990), and there is considerable evidence that proton pumping across the cell membrane is necessary for bone resorption (Blair et al., 1989; Väänänen et al., 1990; Sundquist et al., 1990; Schlesinger et al., 1994). Much of the experimental evidence indicates that during resorption a cell membrane H^+-ATPase extrudes protons into the extracellular bone resorption compartment between the osteoclast and the mineralized surface, while a Cl^-/HCO_3^- exchanger is responsible for an efflux of HCO_3^- across the opposite cell membrane (Hall and Chambers, 1989; Schlesinger et al., 1994). Both H^+ and HCO_3^- are derived from metabolically produced CO_2 via the enzyme carbonic anhydrase (Hunter et al., 1991; Schlesinger et al., 1994). The cell membrane proton pump in chicken osteoclasts has been shown to be sensitive to inhibitors of the V-type H^+-ATPase, such as bafilomycin and *N*-ethylmaleimide (NEM), but also to vanadate, usually used as an inhibitor of the P-type H^+-ATPase (Chaterjee et al., 1992, 1993). Little is known about changes in osteoclast pH_i in response to extracellular signaling molecules, but this is thought to be a potentially important signaling pathway in other cell types (e.g. Ganz and Boron, 1994; Dällenbach et al., 1994; LaPointe and Battle, 1994; Baltz, 1993).

Osteoclasts have been shown to have P_2 purinergic receptors which mediate an intracellular free [Ca^{2+}] ([Ca^{2+}]$_i$) increase in response to an increase in extracellular [ATP] (Yu and Ferrier, 1993, 1994). Purinergic receptors have been studied in a number of other cell types, in which they are thought to be involved in important cellular signaling systems (Abbracchio et al., 1993; Burnstock, 1993; Dubyak and El-Moatassim, 1993; El-Moatassim et al., 1992; Garritsen et al., 1992; O'Conner, 1992; Pirotton et al., 1993). The physiological role of such receptors in osteoclasts is not yet understood, but it is likely that signal transduction systems other than those involving [Ca^{2+}]$_i$ may be linked to P_2 receptors in osteoclasts, as in other cell types. In particular, ATP induced pH_i changes have been observed, in endothelial cells and ascites tumor cells (El-Moatassim et al., 1992).

A number of P_2 receptor types have been distinguished in other cell types on the basis of their affinity for the various nucleotides and synthetic analogues, as well as their molecular properties and the nature of the intracellular signaling pathways they are linked to. For example, the P_{2y} receptor has a high affinity for both adenosine triphosphate (ATP) and adenosine diphosphate (ADP), with little affinity for uridine triphosphate (UTP), while the P_{2u} receptor has a high affinity for both UTP and ATP, with less affinity for ADP. Both the P_{2y} and P_{2u} are thought to be of the 'serpentine' class of receptor, with seven membrane spanning segments, which interact with G-proteins leading to activation of phospholipase C, production of inositol trisphosphate and intracellular release of Ca^{2+}, or in some cases to inhibition of adenylyl cyclase (e.g. Dubyak and El-Moatassim, 1993; Pirotton et al., 1993; Abbracchio and Burnstock, 1994). Recently, cDNA clones for these receptors have been prepared and functionally expressed (Filtz et al., 1994; Parr et al., 1994). In contrast, the P_{2x} receptor and the P_{2z} receptor, which have a high affinity for ATP, are thought to have an intrinsic ion channel that would allow Ca^{2+} and Na^+ influx across the cell membrane (Dubyak and El-Moatassim, 1993; Abbracchio and Burnstock, 1994). The P_{2t} receptor, which has a high affinity for ADP, was thought to have intrinsic channel properties (Dubyak and El-Moatassim, 1993), but is now thought to be a G-protein linked receptor (Abbracchio and Burnstock, 1994).

In this paper we report an investigation into the effect of extracellular ATP on osteoclast pH_i. We have carried out studies on the relation of ATP-induced changes in pH_i to ATP-induced changes in [Ca^{2+}]$_i$ and on the mechanisms by which pH_i change is produced. We use an intracellular fluorescent pH indicator, seminaphthofluorescein (SNAFL)-calcein, which has largely separate excitation spectra for its protonated and unprotonated forms, and also largely separate emission spectra for these two forms (Haugland, 1992; Zhou et al., 1995). This allows simultaneous excitation of the two forms and simultaneous measurement of the emission from the two forms. The main advantage of this technique over the widely used BCECF (bis-carboxyethyl-carboxyfluorescein) technique is that it does not require the use of chopped dual excitation illumination in order to use a ratio method (e.g. Muallem et al., 1992) as opposed to the less accurate single excitation wavelength method (e.g. Redhead, 1988; Green et al., 1988). This allows a ratiometric measurement to be made using the laser scanning confocal microscope.

MATERIALS AND METHODS

Culture of osteoclasts

Osteoclasts were isolated from one-day-old New Zealand white rabbits (Yu and Ferrier, 1993). The rabbits were decapitated and the femora and tibiae were dissected out. The bone shafts were cut longitudinally. Cells were removed from trabecular bone by curetting and scraping and released by pipetting. The supernatant was centrifuged for 10 minutes at 1,200 rpm, and the cells were resuspended and distributed to 60 mm culture dishes (about 16 dishes per rabbit). The cells were placed in a 5% CO_2 incubator at 37°C. After one hour, 5 ml fresh α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS) and antibiotics was added into each dish. The next day, the dishes were gently washed and the medium changed. Measurements were done on the following two days.

Reagents and media

We obtained α-MEM from Gibco and FBS from Flow Laboratories. ATP, ADP, UTP, 2-methylthio-ATP (2-MeSATP), β,γ-methylene-ATP (AMP-PCP), amiloride, BAPTA/AM, *N*-ethylmaleimide (NEM), and dicyclohexylcarbodiimide (DCCD) were purchased from Sigma. Vanadate was obtained from Fisher Scientific. Seminaphthofluorescein (SNAFL)-calcein acetoxymethyl (AM), fluo-3/AM, valinomycin, and nigericin were purchased from Molecular Probes (Eugene, OR). Suramin was provided by Miles Canada Inc. (Etobikoke, Ontario).

Fluorescence measurements were done in α-MEM (10 ml/dish) with 25 mM HEPES buffer replacing bicarbonate (medium A). Calibration of intracellular pH (pH_i) was done in medium B (in mM): NaCl 15, KCl 130, $CaCl_2$ 1.8, $MgSO_4$ 0.81, glucose 10, HEPES 25; pH was adjusted with NaOH. Sodium-free medium had the same components as medium A except that NaCl was isotonically replaced by choline chloride, and the pH was adjusted to pH 7.4 with Trisma-base (Sigma). Chloride-free medium was prepared by substituting sodium gluconate in medium A for NaCl, potassium gluconate for KCl, hemicalcium gluconate for $CaCl_2$ isotonically, and pH was adjusted to pH 7.4 with NaOH.

Intracellular pH measurement

Cells were exposed to the acetyoxymethyl form of the pH sensitive fluorescent indicator SNAFL-calcein (SNAFL-calcein/AM; 6 μM) in medium A at 21°C for 60 minutes. Measurements were carried out at 37±1°C in medium A. The laser scanner and detectors were attached to a Nikon Optiphot microscope in a Bio-Rad MRC 600 argon/krypton laser scanning system, using dual excitation (488 nm and 568 nm) laser lines. The system was equipped with a FITC/Texas Red filter set, which allows simultaneous recording of an image from fluorescence between 520 and 540 nm and a second image from fluorescence beyond 600 nm. The protonated form of SNAFL-calcein absorbs strongly between 450 and 490 nm with emission largely from 500 to 575 nm, with a peak near 525 nm, while the unprotonated SNAFL-calcein absorbs strongly from 550 to 580 nm with emission largely at wavelengths greater than 600 nm, with a peak near 615 nm (Haugland, 1992; Zhou et al., 1995). Fluorescence images at 525 nm and 615 nm were collected at a rate of 5 seconds per image pair. Average cellular fluorescence intensities were obtained from each image using the Optimas image analysis program or the Cfocal image analysis program. Ratios were obtained by dividing the average cellular fluorescence intensity at 615 nm by the average cellular fluorescence intensity at 525 nm.

pH_i calibration

For calibration, cells loaded with SNAFL-calcein were incubated in medium B with nigericin (10 μg/ml) and valinomycin (5 μg/ml) for more than 10 minutes at a given extracellular pH. This should result in pH_i becoming equal to the extracellular pH. Some 615 nm/525 nm

fluorescence intensity ratios as a function of pH are shown in Fig. 1A. Baseline pH_i values in medium A and agonist-stimulated changes in pH_i were determined by interpolation from these calibration curves.

Intracellular calcium measurements

Fluo-3 was used to measure intracellular calcium with the laser scanning confocal microscope (Yu and Ferrier, 1993, 1994). Loading conditions for the fluo-3/AM were the same as for the SNAFL-calcein/AM. Excitation of the fluo-3 was at 488 nm, and emission fluorescence was collected at wavelengths ≥515 nm.

Statistical methods

The effect of ADP, AMP, UTP, 2-MeSATP and AMP-PCP on pH_i was compared to the effect of ATP on pH_i by using a two-tail *t*-test without assuming equal variances. The effect of various changes in extracellular medium (either exchanges of medium or application of inhibitors to the medium) on baseline pH_i was compared to the appropriate control (either exchanging normal medium A for normal medium A or applying vehicle solution to the medium) by using a one-tail *t*-test without assuming equal variances, as well as by an F test for the entire group of medium changes (1-way ANOVA). The effect of ATP in the presence of the various inhibiting agents was compared to the effect of ATP without inhibitors by using a one-tail *t*-test without assuming equal variances, as well as by an F test for the entire group of inhibitor measurements (1-way ANOVA). Our results throughout the paper are given as mean ± standard error of the mean (s.e.m.).

RESULTS

ATP and ADP induce a decrease in pH_i

Application of ATP (100 μM) induced a transient decrease in fluorescence intensity at 615 nm and a transient increase in fluorescence intensity at 525 nm. The peak change in the 615 nm/525 nm ratio was reached 10 to 25 seconds after application of ATP, and the baseline ratio was restored 20 seconds to 2 minutes after the peak (n=23). The peak ATP-stimulated change in the 615 nm/525 nm fluorescence ratio was –0.63±0.05 (mean ± s.e.m.; n=23). The duration of the ratio change, taken as the time from initiation of the change until the ratio returns to the pre-stimulation baseline value, was 66±6 seconds (n=23).

A complete calibration was done twice, on different days, with three measurements of the ATP effect carried out immediately after each calibration. In these measurements, the peak change in the 615 nm/525 nm ratio induced by ATP was –0.63±0.09 (n=6; Fig. 1B), representing a peak change in pH_i of –0.72±0.23 pH units.

To determine if the ATP induced pH_i decrease was mediated by a purinergic receptor, we incubated osteoclasts in 500 μM suramin, a blocker of some of the P_2 purinergic receptor subtypes (Abbracchio and Burnstock, 1994), for 2 minutes. The ATP induced change in the 615 nm/525 nm ratio was then –0.09±0.04 (n=8). This is significantly different from the value under control conditions with $P<10^{-8}$.

Among the known P_2 receptor subtypes, ATP is known to be an agonist of the P_{2y} and P_{2u} receptors. We further tested the involvement of a P_2 receptor by applying other nucleotides. We found that 100 μM ADP, an agonist of both the P_{2y} and P_{2t} receptors, induced a 615 nm/525 nm ratio change of –0.56±0.03 (n=6), which is quite close to the effect of ATP, while AMP induced a change of only –0.15±0.02 (n=7). The latter change is significantly different from the ATP effect at $P<10^{-9}$. These results are further evidence that the pH_i decrease is brought about via a P_2 receptor. We also found that 100 μM UTP, a main agonist of the P_{2u} receptor, had little effect on the 615 nm/525 nm fluorescence ratio (–0.10±0.04, n=6; which is significantly different from the ATP induced change, with $P<10^{-6}$). A chief agonist of the P_{2y} receptor, 2-MeSATP, had a small effect at 100 μM (–0.23±0.05, n=4; significantly different from the ATP induced effect at $P<0.001$), while AMP-PCP, an agonist of the P_{2x} receptor, had an effect of only –0.12±0.07 (n=5; significantly different from the ATP effect at $P<0.001$).

The ATP-induced pH_i decrease is not produced by an increase in $[Ca^{2+}]$

To prevent an intracellular $[Ca^{2+}]$ increase, osteoclasts were incubated with a cell permeable Ca^{2+} chelator, the acetoxymethyl form of BAPTA (BAPTA/AM; 100 μM) in Ca^{2+}-free medium (medium A without Ca^{2+}). A series of measurements was carried out with the fluorescent Ca^{2+} indicator fluo-3. Following two minutes of incubation with BAPTA/AM, the fluo-3 fluorescence declined steadily with time, and there was no response to 100 μM ATP (n=4). The ATP-induced pH_i response was measured following five to

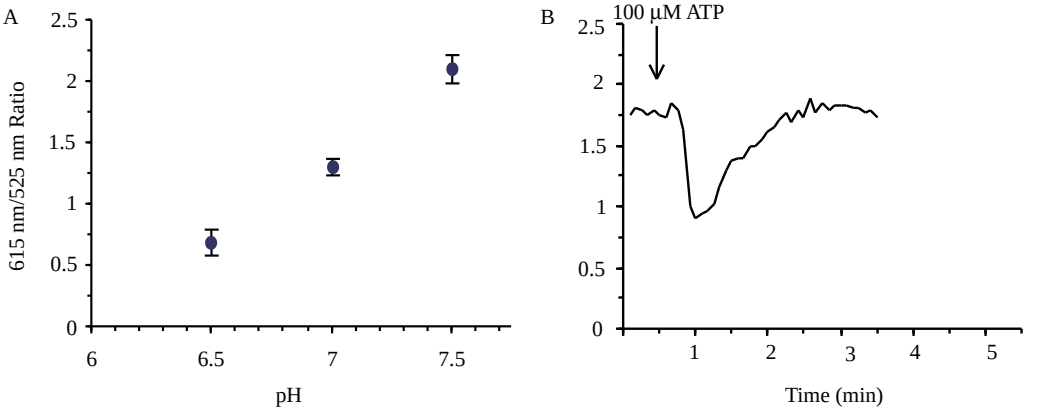

Fig. 1. (A) The 615 nm/525 nm fluorescence intensity ratio as a function of pH. Cells loaded with SNAFL-calcein were incubated in medium B with freshly prepared nigericin (10 μg/ml) and valinomycin (5 μg/ml) for more than 10 minutes at a given extracellular pH. Fluorescence from the unprotonated form of the SNAFL-calcein, which peaks near 615 nm, and from the protonated form, which peaks near 525 nm, were separately measured and averaged over the entire cell body of an osteoclast. Ratios were taken of these averages. The number of separate cells in separate dishes measured for each point was: pH 6.5, n=6; pH 7.0, n=4; pH 7.5, n=4. (B) Time course of the measured 615 nm/525 nm fluorescence ratio averaged over the cell body of an osteoclast, following application of 100 μM ATP to the extracellular medium. This result is representative of measurements on twenty-three cells.

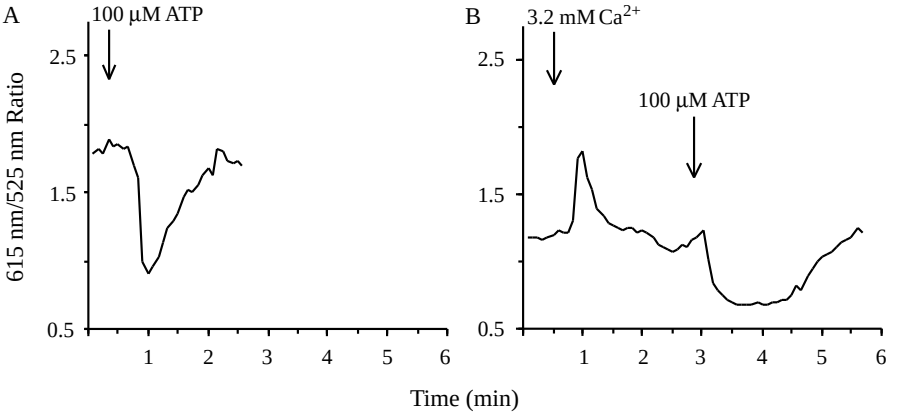

Fig. 2. (A) Time course of the 615 nm/525 nm fluorescence ratio averaged over the cell body of an osteoclast, following application of ATP to the extracellular medium. The extracellular medium was Ca^{2+}-free, containing a cell permeable Ca^{2+} chelator, BAPTA/AM (100 μM). The cell was incubated in the medium 4.5 minutes before image collection began. This result is representative of measurements on eight cells. (B) Time course of the 615 nm/525 nm fluorescence ratio averaged over a cell, showing that an increase in extracellular [Ca^{2+}] induces an increase in the 615 nm/525 nm fluorescence ratio, while ATP induces a decrease in the 615 nm/525 nm fluorescence ratio.

eleven minutes of incubation with BAPTA/AM. This response was not blocked, with ATP inducing a change in the SNAFL-calcein 615 nm/525 nm fluorescence ratio of −0.87±0.14 (n=8, Fig. 2A). An increase of 3.2 mM extracellular [Ca^{2+}] by application of calcium into normal medium A, which has been shown to induce a large transient increase in $[Ca^{2+}]_i$ in rabbit osteoclasts (Yu and Ferrier, 1993), induced an increase in the ratio of 615 nm/525 nm fluorescence of 0.58±0.07 (n=11; Fig. 2B), representing a pH_i increase of 0.67±0.10 units.

The ATP-induced pH_i decrease does not involve Na^+/H^+ exchange

To investigate the involvement of Na^+/H^+ exchange in the ATP effect, we measured the ATP-induced pH_i change in Na^+-free extracellular medium. Changing the extracellular medium from normal medium A to Na^+-free medium produced a long lasting increase in the 615 nm/525 nm fluorescence ratio of +0.21±0.07 (n=6), while in control experiments, changing from normal medium A to normal medium A produced a change of +0.08±0.03 (n=6). This is not a significant difference. In Na^+-free medium, the ATP-induced change in the 615 nm/525 nm ratio was −0.72±0.09 (n=10), which is quite close to the response to ATP in normal medium. In another series of experiments, the Na^+/H^+ exchange blocker amiloride (1 mM) produced a very small decrease in the 615 nm/525 nm ratio of −0.07±0.03 three minutes after application to the extracellular medium (n=3). The ATP-induced change in the 615 nm/525 nm ratio in the presence of 1 mM amiloride was −0.50±0.08 (n=5). Neither Na^+-free medium nor amiloride produced a statistically significant block of the pH_i response to ATP. These results are summarized in the bar graphs in Figs 3 and 4.

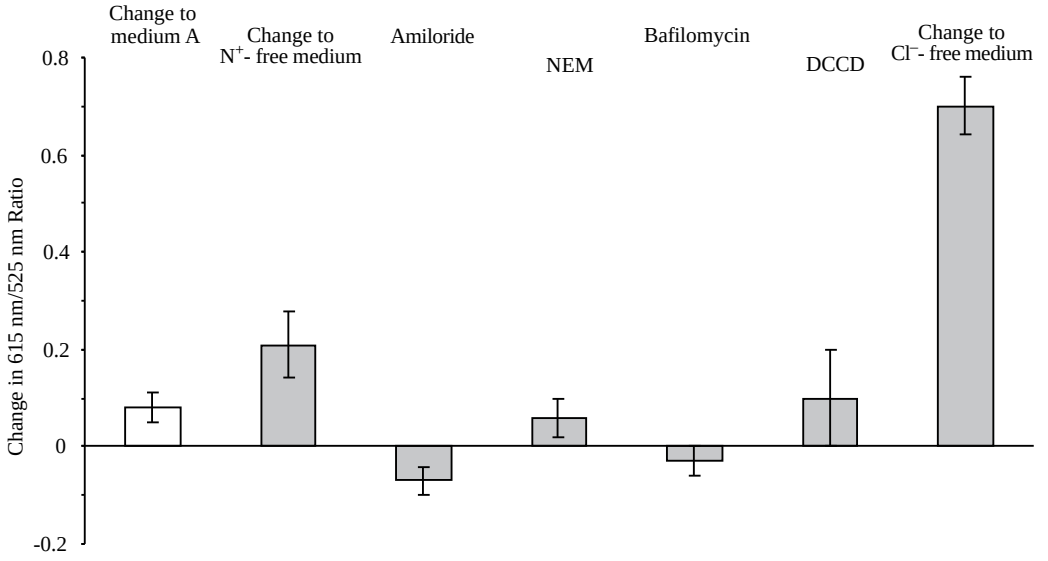

Fig. 3. Effect on the baseline of changes in the extracellular medium. The bars are mean values of the change in the 615 nm/525 nm fluorescence ratio, three to four minutes after changing the medium or adding a reagent. The error bars are s.e.m. Changes in extracellular media and numbers of measurements (separate dishes) are: changing from medium A to medium A, n=6; changing from medium A to Na^+-free medium, n=6; application of 1 mM amiloride into medium A, n=3; application of 1 mM NEM into medium A, n=7; application of 200 nM bafilomycin into medium A, n=6; application of 0.2 mM DCCD into medium A, n=2; changing from medium A to Cl^--free medium, n=13. The latter produced a significant effect with $P<10^{-7}$. None of the other changes in extracellular medium produced a statistically significant change in baseline. A one way analysis of variance (ANOVA) over all groups here showed significant effects at $P<10^{-12}$.

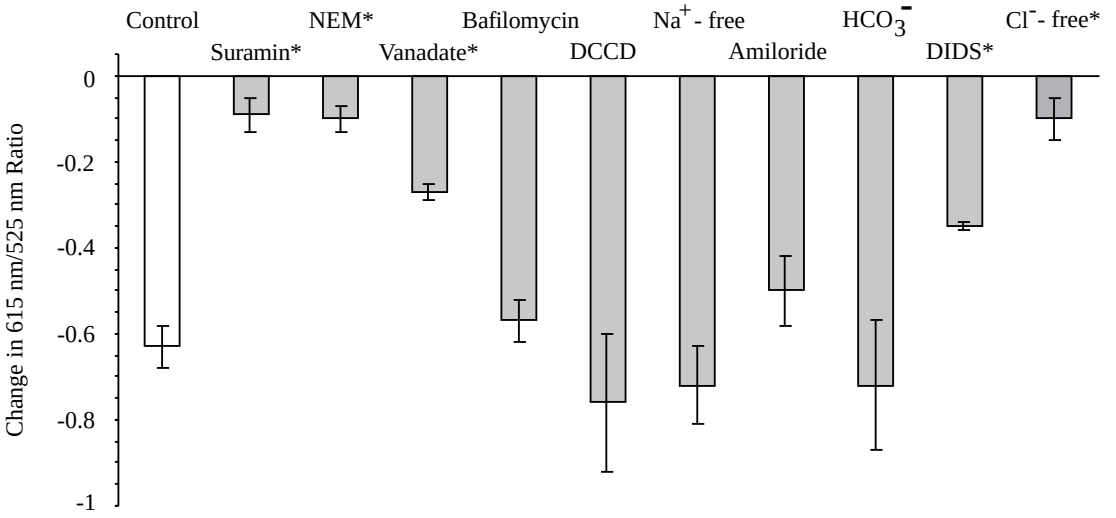

Fig. 4. Amplitude of the effect of 100 μM ATP under various conditions. The bars are mean values of the peak change in the 615 nm/525 nm ratio, for cells in normal medium A (control) and in extracellular medium containing various reagents or in Na^+-free or Cl^--free medium. The error bars are s.e.m. Conditions and numbers of measurements (separate dishes) are: control, n=23; suramin, n=8; 1 mM NEM, n=13; 0.2 mM vanadate, n=7; 200 nM bafilomycin, n=8; 0.2 mM DCCD, n=5; Na^+-free medium, n=10; amiloride, n=5; 17-25 mM HCO_3^-, n=3; 1 mM DIDS, n=3; Cl^--free medium, n=19. Results marked with (*) are significantly different from the control, with at least P<0.05, using a one-tail t-test. A one way analysis of variance (ANOVA) over all groups showed significant effects at $P<10^{-15}$.

The ATP-induced pH_i decrease is affected by some proton pump inhibitors

Application of 1 mM *N*-ethylmaleimide (NEM), an inhibitor of the V-type H^+-ATPase, into the bathing medium, produced little change in the baseline 615 nm/525 nm ratio over a period of four minutes (+0.06±0.04, n=7). In the presence of NEM, the peak change induced by ATP was diminished to –0.10±0.03 (n=13; significantly different from the effect of ATP without inhibitor at $P<10^{-10}$). Following application of 200 μM vanadate, an inhibitor of the P-type H^+-ATPase, there was a transient increase in the 615 nm/525 nm ratio, with a peak change of +0.57±0.11, and a duration of 71±11 seconds, followed by a decline in the 615 nm/525 nm ratio at a rate of about 0.1 ratio units per minute (n=7). The ATP effect was also significantly reduced by vanadate: the peak of the ATP-induced change in the 615 nm/525 nm ratio with vanadate in the bathing medium was –0.27±0.05 (n=7; significantly different from the effect of ATP without inhibitor at $P<10^{-4}$). However, 200 nM bafilomycin, a V-type H^+-ATPase inhibitor, had no effect on the 615 nm/525 nm ratio after four minutes (–0.03±0.04, n=6), and no effect on the response to ATP. The ATP effect on the 615 nm/525 nm ratio in the presence of bafilomycin was –0.57±0.05 (n=8), which is quite close to the effect of ATP in normal medium. Another proton pump inhibitor, N,N′-dicyclohexylcarbodiimide (DCCD; 200 μM) had no effect on the baseline (+0.1± 0.1, n=2), and no influence on the ATP effect, which was –0.77±0.13 (n=5). These results are summarized in the bar graphs in Figs 3 and 4.

The ATP-induced pH_i decrease and Cl^-/HCO_3^- exchange

Changing the extracellular medium from normal medium A to Cl^--free medium produced a large long lasting increase in the 615 nm/525 nm fluorescence ratio (mean change, +0.70±0.06, n=13, which is significantly different from the change induced by changing from normal to normal medium, with $P<10^{-7}$; Fig. 3). The effect of ATP on pH_i was significantly inhibited in Cl^--free medium, with all cells showing a significantly reduced pH_i transient. In eleven cells there was no measurable ATP effect, in three cells there was a small reversal of the normal ATP effect (Fig. 5), and in five cells there was a small decrease in the 615 nm/525 nm ratio (mean change = –0.10±0.05, n=19; this is significantly different from the effect of ATP in normal medium, with $P<10^{-7}$). The ATP effect was reduced in a dose-

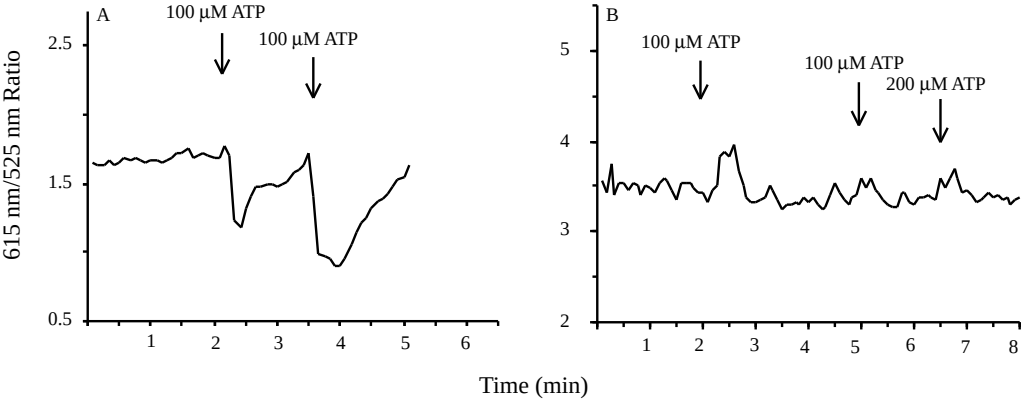

Fig. 5. Time courses of the 615 nm/525 nm fluorescence ratio averaged over an osteoclast, showing that Cl^--free medium blocks the ATP-induced decrease in pH_i. (A) Response to 100 μM ATP in normal medium. (B) Response to 100 μM ATP in Cl^--free medium. The baseline 615 nm/525 nm ratio was increased after changing to Cl^--free medium from normal medium. This measurement was replicated in nineteen cells.

dependent manner by 4,4′-diisothiocyanatostilbene sulfonic acid (DIDS), an inhibitor of the Cl^-/HCO_3^- exchanger. The DIDS concentrations used and the corresponding ATP-induced changes in the 615 nm/525 nm ratio are: 0.2 mM, −0.54±0.06 (n=5); 0.4 mM, −0.38±0.12 (n=3); 1 mM, −0.36±0.05 (n=3; P<0.05; Fig. 4).

Application of 17 or 25 mM HCO_3^- into medium A resulted in a large transient increase of the 615 nm/525 nm ratio (peak value: +2.01±0.48, n=8; the duration was 136±7 seconds, from initiation to returning to baseline). Application of ATP into extracellular medium containing 17 or 25 mM HCO_3^- induced a decrease in the 615 nm/525 nm ratio of −0.70±0.15 (n=3; Fig. 4).

DISCUSSION

Our data indicate that a transient decrease in osteoclast pH_i is induced by activation of a P_2 purinergic receptor. The main evidence for this is that the decrease in pH_i is induced by ATP and by ADP, but not by AMP. The blocking effect of suramin is additional evidence that the pH_i decrease is mediated by a P_2 receptor. In further characterizing the receptor, we find that it does not fit any of the defined subtypes. ATP is an agonist of the P_{2y} and P_{2u} receptors, and ADP is an agonist of the P_{2y} and P_{2t} receptors. However, 2-MeSATP, an agonist of the P_{2y} receptor, and UTP, an agonist of the P_{2u} receptor, have only small effects on pH_i. Furthermore, ATP is known to be an antagonist of the P_{2t} receptor, indicating that this receptor is not involved. Also, we find little effect on pH_i of AMP-PCP, an agonist of the P_{2x} receptor.

These results are similar to our previously reported measurements of $[Ca^{2+}]_i$ responses to nucleotides. We found that ATP and ADP had good responses (with ADP having less response than ATP), with AMP, UTP, 2-MeSATP, and AMP-PCP providing little or no response (Yu and Ferrier, 1993, 1994). This suggests that the same receptors are involved, and that the agonist potency for a pH_i response is similar to that for a $[Ca^{2+}]_i$ response.

On the other hand, our results clearly show that the mechanisms linking the P_2 receptors and a $[Ca^{2+}]_i$ change are different from those linking the P_2 receptors and a pH_i change. Our experiments reported here based on reducing and clamping intracellular free calcium with BAPTA/AM (Fig. 2A) demonstrate that the pH_i decrease induced by ATP is not dependent on the ATP stimulated $[Ca^{2+}]_i$ increase. Furthermore, our results show that a transient increase in $[Ca^{2+}]_i$, which has been shown to occur when the extracellular $[Ca^{2+}]$ is increased (Yu and Ferrier, 1993), by itself induces a pulsed pH_i increase (Fig. 2B). This means that, although osteoclast pH_i and $[Ca^{2+}]_i$ changes may affect each other, the osteoclast P_2 receptors are linked to two distinct intracellular signaling pathways, one involving $[Ca^{2+}]_i$ changes and one involving pH_i changes. We have previously presented evidence that the ATP-stimulated signaling pathway that involves $[Ca^{2+}]_i$ has two separate branches, one that produces a calcium influx, and one that leads to an internal release of calcium that does not depend on influx (Yu and Ferrier, 1994). Combined with these results, our present data indicate that the signaling pathways leading from the activated purinergic receptors in osteoclasts have at least three branches.

Since stimulation of sodium/proton exchange has been found to be possibly involved in transmembrane signal transduction in other cell types (Ganz and Boron, 1994; Dällenbach et al., 1994; LaPointe and Batlle, 1994), as well as in homeostasis of pH_i in osteoclasts (Hall and Chambers, 1990), we investigated whether the ATP-stimulated decrease in pH_i is through inhibition of the Na^+/H^+ exchanger. Our results show that the ATP-stimulated pH_i decrease in rabbit osteoclast is not a result of slowing down Na^+/H^+ exchange, since the use of Na^+-free extracellular medium, or of amiloride, does not significantly affect the ability of ATP to induce a decrease in pH_i (Fig. 4). Moreover, our data indicate that this exchanger is relatively inactive, since there is only a small effect on baseline pH_i of amiloride or Na^+-free medium (Fig. 3).

A cell membrane proton pump (H^+-ATPase) plays an important role in secreting H^+ into the bone resorption compartment between osteoclasts and bone (Blair et al., 1989; Sundquist et al., 1990; Chatterjee et al., 1993). If it is active, a decreased pumping rate for this H^+-ATPase should lead to a decrease in pH_i. That is, although the osteoclasts are on a plastic substrate and are not resorbing bone, the carbonic anhydrase could still be active, producing H^+ and HCO_3^- from metabolic CO_2, which would require active cell membrane H^+-pumps and Cl^-/HCO_3^- exchangers to maintain intracellular pH at the normal level.

The properties of the osteoclast cell membrane proton pump are still not completely clear. The available evidence shows that it is inhibited by agents that are known to inhibit the V-type proton pump, such as bafilomycin (Sundquist et al., 1990) and NEM (Väänänen et al., 1990; Bekker and Gay, 1990), as well as by vanadate, which is usually found to be an inhibitor of the P-type proton pump (Chaterjee et al., 1992, 1993), although this latter result has been questioned (Hall and Schaueblin, 1994). Our data show that 1 mM NEM and 200 μM vanadate partially inhibit the ATP-induced decrease in pH_i, implying that the osteoclast cell membrane proton pump may be involved in the ATP effect. However, our data also show that 200 nM bafilomycin, and 200 μM DCCD (a nonspecific inhibitor of proton pumps; Moriyama and Nelson, 1988), have no influence on the ATP effect (Fig. 4). This could mean that NEM and vanadate have effects on the osteoclast beyond that of inhibiting the cell membrane proton pumps. NEM can change the properties of proteins by alkylating thiol groups (Stavros et al., 1993; Sargianos et al., 1994), and has been shown to have many kinds of effects on cell membrane signal transduction systems. For example, NEM has been found to partially inactivate various cell membrane receptors, including H_3 receptors (Luo et al., 1994), acetylcholine receptors (Misle et al., 1994), human growth hormone receptors (Frank et al., 1994), and endothelin receptors (Stavros et al., 1993). Inhibition of amino acid transport has also been reported (Novak et al., 1994; Deves et al., 1993), as has activation of K^+ channels by NEM (Kennedy, 1994). Vanadate, a potent inhibitor of protein tyrosine phosphatases (Swarup et al., 1982), has been shown to have many effects on cell membrane transport systems, including stimulation of Na^+/Ca^{2+} exchange (DiPolo and Beauge, 1994) and stimulation of Na^+/H^+ exchange (Macara et al., 1986). The latter effect could explain the transient increase in pH_i that we see upon application of vanadate to the bathing medium.

Furthermore, neither NEM, bafilomycin, nor DCCD have a

large effect on the baseline pH_i (Fig. 3). These data would indicate that the osteoclast cell membrane proton pumps are not active with the osteoclast on a plastic substrate. However, it is possible that the proton pump is active under our normal conditions of measurement, but that there is a fast compensatory increase in Na^+/H^+ exchange, or a decrease in Cl^-/HCO_3^- exchange, as pH_i begins to decrease following proton pump inhibition.

Our results support the hypothesis that the Cl^-/HCO_3^- exchanger plays an important role in pH_i homeostasis in osteoclasts, as proposed by Teti et al. (1989) and Hall and Chambers (1989). Decreasing extracellular $[Cl^-]$ should decrease the Cl^-/HCO_3^- exchange rate because of a decreased thermodynamic driving force on the exchange, leading to an increase in $[HCO_3^-]$ within the cell, thus producing a decrease in $[H^+]$. Our measurements show that changing to Cl^--free medium does dramatically increase pH_i. This change in pH_i is long lasting, demonstrating that there is no other process that can compensate for a lack of Cl^-/HCO_3^- exchange in osteoclast pH_i regulation. Increasing extracellular $[HCO_3^-]$ should also slow down the exchange of Cl^- and HCO_3^- because of a reduced driving force, and thus increase pH_i. In our experiments, adding HCO_3^- to normal medium A does produce a rapid but transient increase in pH_i. That this change is transient may be because there is a feedback mechanism that will lead to restoration of the original Cl^-/HCO_3^- exchange rate. This would be expected, since Cl^-/HCO_3^- exchange is thought to be an important mechanism for preventing excessive cytoplasmic alkalinization (Alper, 1991). Another possibility is that there is a long-term reduction in Cl^-/HCO_3^- exchange rate that is compensated by a reduction in proton efflux through the pumps, if they are active.

The ATP effect on the 615 nm/525 nm fluorescence ratio was inhibited in Cl^--free extracellular medium (Fig. 5). This is evidence that the ATP-stimulated decrease in pH_i in normal medium A involves an increase in the Cl^-/HCO_3^- exchange rate, resulting in enhancement of the efflux of HCO_3^-, a basic equivalent. Furthermore, the ATP effect was reduced by DIDS, an inhibitor of the Cl^-/HCO_3^- exchanger. Our data show that the ATP effect is largely unaffected by having a physiological concentration of HCO_3^- in the bathing medium (Fig. 4). This is not inconsistent with having an enhanced rate of Cl^-/HCO_3^- exchange as a basis for the reduced pH_i, since the outside to inside concentration ratio for Cl^- should still be higher than that for HCO_3^-, providing a thermodynamic driving force for HCO_3^- extrusion.

Our data further suggest that an ATP-induced increase in Cl^-/HCO_3^- exchange rate is transient, and that the return of the Cl^-/HCO_3^- exchanger to the pre-ATP activation rate is by itself enough to return pH_i to the pre-activation level. An alternative explanation is that the proton pumps, if they are active, may initially have a reduced velocity as a result of the ATP-induced signal, but that they then speed up, finally having a pump rate higher than the original one, thus compensating for a long term increased rate of Cl^-/HCO_3^- exchange. If reduction of proton pump velocity is involved in the ATP effect, the result that Cl^--free extracellular medium produces a substantial reduction in the ATP effect would mean that the low cytosolic $[H^+]$ produced by Cl^--free medium is sufficient to largely prevent proton pumping. This would be in agreement with the finding in macrophages that proton pumps are not active at higher pH_i (Swallow et al., 1993). However, our data showing that bafilomycin has no effect on the ATP-induced pH_i change, and that neither bafilomycin nor NEM have an effect on the pH_i baseline, are evidence that the osteoclast cell membrane proton pumps are not active under our conditions of measurement. This implies that the ATP-induced transient decrease in pH_i results solely from a transient enhancement of the Cl^-/HCO_3^- exchange rate.

In summary, the experimental results presented in this paper show that in osteoclasts there is a signaling pathway linked to purinergic receptors that leads to a pulsed increase in $[H^+]_i$. Our data demonstrate that this pathway is distinct from the one that links osteoclast purinergic receptors to a pulsed $[Ca^{2+}]_i$ increase. Our results also indicate that activation of osteoclast purinergic receptors leads to a transient increase in Cl^-/HCO_3^- exchange across the cell membrane.

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