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Problems Caused by High Concentration of ATP on Activation of the $P2X_7$ Receptor in Bone Marrow Cells Loaded with the Ca^{2+} Fluorophore fura-2

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Fura-2 is one of the most used fluorophore for measuring intracellular calcium concentration ($[Ca^{2+}]i$). In mouse bone marrow cell suspensions ATP produces a biphasic effect: till 1 mM, ATP produces increases in $[Ca^{2+}]i$; from 1 mM on an increase is observed, that is followed by the decrease in the 340/380 nm ratio (R340/380). At high ATP (4 mM) concentration fura-2 leaked from loaded bone marrow cell suspensions. We observed that ATP decreases fluorescence in the absorption and excitation spectra of fura-2, consequently the emitted one is decreased including the isobestic point (360 nm). ATP analogs: BzATP, ATPγS and UTP, but not $\alpha\beta$ATP, ADP or AMP, promote decrease of fluorescence in the isobestic point of fura-2. The physical/chemical process that reduces the absorption and excitation of fura-2 by ATP is unknown. The $P2X_7$ inhibitors, Mg^{2+} (5 mM), OxATP (300 μM) and Brilliant Blue (100 nM), blocked the efflux of fura-2 and ATP-induced R340/380 decrease. The J774 cell line and mononuclear cells with a higher expression of $P2X_7$ receptors show the same decrease in R340/380 as that induced by ATP. In the HL-60 cell line, myeloid cells and erythroblasts extracted from bone marrow, such effect does not occur. It is concluded that the use of the fluorescent Ca^{2+} indicator fura-2 does not allow the correct measurement of $[Ca^{2+}]i$ in these cells in the presence of a higher concentration of ATP which activated the $P2X_7$ receptor.

KEY WORDS: ATP; $P2X_7$ receptor; fura-2; bone marrow.

INTRODUCTION

The Ca^{2+} indicator fura-2, has been widely used to measure the dynamic changes in cytosolic Ca^{2+} in various cells types. Fura-2 is derived from the EGTA molecule, a well-known Ca^{2+} chelator. Upon Ca^{2+} binding, fura-2 increases the amplitude of the excitation spectrum at 340 nm at the expense of that at 380 nm, with little change in the peak emission spectrum between 505 nm and 520 nm [1]. Based on these properties a ratiometric measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) can be obtained through the 340/380 nm ratio (R340/380), by calibrating the system with maximal and minimal ratio values (R_{max} and R_{min}), and the Ca^{2+}-dissociation constant (Kd) of fura-2 (near 224 nM in physiological intracellular medium). Such ratioing largely cancels out the effects of cell thickness, dye content or instrumental efficiency allowing more accurate measurements of $[Ca^{2+}]i$ [1,2].

Fura-2 dye is a powerful tool for the pharmacological characterization of many receptors since when activated it may induce the increase in $[Ca^{2+}]i$ as it occurs with P2 receptors. These P2 receptors are divided into P2Y and P2X subtypes [3]. The activation of these subtypes by a wide range of concentrations of purinergic agonists (picomolar to millimolar), increases $[Ca^{2+}]i$. The P2Y receptors belong to the superfamily of 7-transmembrane domain G-protein coupled receptors and are activated by

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ATP, ADP, UTP and 2meSATP [4–7]. The P2X receptors are membrane ligand-gated ionic selective cation channels (Na^+, K^+, Ca^{2+}), constitute a family of seven proteins ($P2X_{1-7}$) and are activated by ATP, $\alpha\beta$ATP, $\beta\gamma$ATP and BzATP [8–11].

Steinberg *et al*. [12] reported, in murine macrophag es, the action of millimolar concentration of ATP, which increased plasma membrane permeability by the formation of a non-selective pore, permeable to molecular mass up to 900 Da, lately termed P2Z receptor. Nuttle & Dubyak [13], in electrophysiological studies on *Xenopus* oocytes injected with mRNA from the murine BAC12F5 macrophage cell line, characterized two properties of the P2Z receptor: it is a ligand-gated channel and non-selective pore. Later, Surprenant *et al*. [11] cloned from rat brain the last known P2X receptor termed $P2X_7$; this receptor expressed the same pharmacological properties of the P2Z receptor. The activation of the $P2X_7$ receptor is selectively antagonized by oxidized ATP (OxATP) [14], Brilliant Blue [15], and by high divalent cation concentrations (Mg^{2+}, Ca^{2+} and others) [16]. The $P2X_7$ receptor is mainly expressed in cells of the lymphohematopoietic system [12,17–22]. The cellular pathways to produce openings of this non-selective pore and their physiological role are unknown, but appear to be related to necrosis and apoptosis [23–25].

In this study, we describe the particular fluorescence problems using high concentrations of ATP that activate the $P2X_7$ receptor, and open non-selective pores, promoting both fura-2 release and absorbance decrease of this Ca^{2+} fluorophore, in bone marrow cells. These studies are important for the analysis of fluorescence results in order to not attribute the response only to cellular changes of $[Ca^{2+}]i$.

MATERIAL AND METHODS

Chemicals

ATP (adenosine 5′-triphosphate), UTP (uridine 5′-triphosphate), $\alpha\beta$ATP (α, β-methyleneadenosine 5′-trip hosphate), ATPγS (adenosine 5′-0-(3-thiotriphosphate), BzATP (2′-& 3′-O-[4-Benzoyl-Benzoyl] adenosine 5′-triphospahate), OxATP (adenosine 5′-triphosphate), Coomassie Brilliant Blue G, digitonin, ethylene glycol-bis(beta-aminoethyl-ether)-N,N,N′,N′tetraacetic acid (E GTA) and Hystopaque solutions (1119 and 1077) were purchased from Sigma Chemicals Co, (St. Louis, MO, USA). Fura-2 acetoxymethyl ester (fura-2/AM), fura-2 salt and pluronic acid were obtained from Molecular Probes (Eugene, OR, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium and fetal calf serum were purchased from Cultilab (Campinas, SP, Brazil). All other chemicals were obtained from Merck (Rio de Janeiro, RJ, Brazil).

Bone Marrow Cell Isolation and Culture

Bone marrow from femurs of three-month-old female C57BL/10 mice was used. The animals were killed by rapid cervical dislocation. Bone marrow cells were flushed out of two femurs with 3 mL Tyrode solution by using a syringe fitted to a 21-gauge needle. The experiments were approved by the Ethics in Research Committee of the Federal University of São Paulo (CEP 1247/01).

Separation of bone marrow cell types was performed by gradient centrifugation methods using different density solutions at the same volumes. A pool of bone marrow suspensions from 6 mice (6 mL), Hystopaque 1119 (3 mL) and 1077 (3 mL) was centrifuged at 700 × *g* for 30 min separating the cell types into easily differentiated interfaces [26].

After separation, contaminants such as erythroblasts, in mononuclear cell and myeloblast fractions, were lysed by the same volumes of cool hypotonic NaCl (0.2%) for 20 s and hypertonic NaCl (1.3%). Mononuclear contaminants of erythroblasts and myeloblasts were eliminated by three cycles of centrifugation at 200 × *g* for 10 min [28]. The purity of the cell suspension was higher than 90%.

The mouse J774 macrophage cell line and human HL-60 myeloid cell line were obtained from Rio de Janeiro Cell Bank. The J774 cell line was grown in DMEM and the HL-60 cell line in RPMI 1640 media, both supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin and cultured at 37°C in a humidified atmosphere (5% CO_2-95% air).

Cell Loading and Measurement of Intracellular Calcium

Intracellular Ca^{2+} concentration was measured in 5×10^6 cells per mL, suspended in 2.5 mL Tyrode solution (mM concentration: 137 NaCl; 2.68 KCl; 1.36 $CaCl_2{\cdot}2H_2O$, 0.49 $MgCl_2{\cdot}6H_2O$, 12 $NaHCO_3$, 0.36 NaH_2PO_4, 5.5 D-glucose) and loaded at room temperature with the fluorescent Ca^{2+} indicator fura-2/AM (2 μM) and pluronic acid (0.01%) under constant stirring. The Perkin Elmer Live Science Spectrofluorometer (LS 5B, Buckinghamshire, UK) was used to monitor the fluorescence spectrum of fura-2 in the excitation range of 300–400 nm with emission collected at 520 nm. In the esterified form, its maximal fluorescence was observed at 390 nm. As the indicator fura-2/AM was converted to the acid form, the fluorescence peak shifted to 350 nm within an average

period of 1 h, thus indicating the maximal incorporation of the dye into the cell suspension. At that time the cell suspension was washed with 15 mL Tyrode and centrifuged at 400 × *g* for 4 min and the supernatant was discarded, to remove the excess of fluorescent indicator from the extracellular medium. The pellet was suspended in 2.5 mL Tyrode solution and transferred to a quartz cuvette of the SPEX FluoroLog-2 fluorometer (AR-CM System). The variation of $[Ca^{2+}]i$, expressed as fluorescence, was measured in photons/S(CPS) using the SPEX fluorometer set at two excitation wavelengths, 340 nm ultraviolet and 380 nm (UV) and the emission collected at 505 nm, under constant magnetic stirring at 37°C. The Spex AR-CM system is equipped with the optional front-face/right-angle feature. Front-face mode is typically selected for very dense suspensions such as bone marrow cells. Data were processed to calculate the R340/380 fluorescence. Maximum fluorescence ratio (R_{max}) was determined after disruption of the cells with 50 μM digitonin while the minimum (R_{min}) was obtained by the use of 2 mM $MnCl_2$ followed by addition of EGTA (10 mM) in alkaline medium. The $[Ca^{2+}]i$ was calculated by the equation of Grynkiewicz *et al*. [1]. For inhibition of $P2X_7$ receptors the bone marrow cells were preincubated for 3 h in the presence of OxATP (300 μM) and left for another 40 min for loading with fura-2.

Determination of the fura-2 Indicator Efflux

The efflux of fura-2 indicator was measured after observing the decrease in fluorescence intensity at the excitation wavelengths of 340 nm and 380 nm monitored in the SPEX system by addition of ATP (4 mM). The cell suspension was centrifuged and the pellet resuspended in 2.5 mL Tyrode solution, preserving the supernatant. Resuspended cell and supernatant fluorescence was measured using the Perkin Elmer spectrofluorometer in the excitation range of 300–400 nm, the emission being collected at 520 nm.

Monitoring the Fluorescence of fura-2 at the Isobestic Point

After diluting fura-2 salt (2.2 μM) in milli-Q water the fluorescence decrease by the addition of ATP and analogs was measured using the Perkin Elmer spectrofluorometer, at excitation wavelength of 360 nm which is the isobestic point of the fura-2 spectrum (independent of Ca^{2+} concentration) with emission collected at 520 nm. The baseline level before adding ATP at different concentrations was taken as the maximum value (100%).

Absorption Measurements

The absorption spectra of ATP (4 mM) and fura-2 (330 μM) in milli-Q water were obtained using a Hitachi U-2000 absorption spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Quartz cuvettes with a path length of 1 cm were used. Background samples without the fluorophore were used for baseline corrections.

RESULTS

Low Concentration of ATP Induces 340/380 nm Ratio Increase and High Concentration Produces Decreased Ratio in Bone Marrow Cells

The presence of P2 receptors in different types of bone marrow cells was described previously [11,12,15,29–32]. In the suspension of bone marrow cells, the addition of ATP produces $[Ca^{2+}]i$ increase in a concentration-dependent manner (0,01 mM, 36 ± 2 nM, $n = 3$; 0,1 mM, 30 ± 4 nM, $n = 3$; 1 mM, 57 ± 7 nM, $n = 8$) (Fig. 1A). Concentration of ATP up to 1 mM produces a biphasic response, an initial transient increase in $[Ca^{2+}]i$ followed by a decrease in the fluorescence ratio (Fig. 1B). Figure 1C–D shows a representative record of the changes in the fluorescence ratio (R340/380) induced by cumulative addition of ATP. The second phase is characterized by a simultaneous decrease in fluorescence excitation intensities at 340 nm and 380 nm (Fig. 1D). The simultaneous decrease in both fluorescence intensities, when excited at 340 nm and 380 nm, is typical of the efflux of the fluorescent indicator [33].

ATP Causes Ca^{2+} Indicator (fura-2) Efflux in Bone Marrow Cel Suspension

The efflux of the Ca^{2+} indicator fura-2 from the cytosolic medium to the supernatant was determined after centrifugation of the sample for 4 min at 700 × *g*. The excitation spectrum of bone marrow cells loaded with fura-2 was monitored from 300 nm to 400 nm, and emission collected at 520 nm. A control of fura-2 fluorescence spectrum is shown in Fig. 2A[i] obtained before ATP addition. After the stimulus with ATP (4 mM), the sample was centrifuged and the cell pellet was resuspended in Tyrode solution (Fig. 2A[ii]). In the resuspended cells less fluorescence than in the supernatant was observed as shown by the excitation spectrum (Fig. 2A[iii]).

Addition of digitonin (50 μM) to bone marrow cell suspension causes maximal R340/380 fluorescence by permeabilization of the cell membrane (Fig. 2B). High concentration of digitonin (200 μM) causes complete

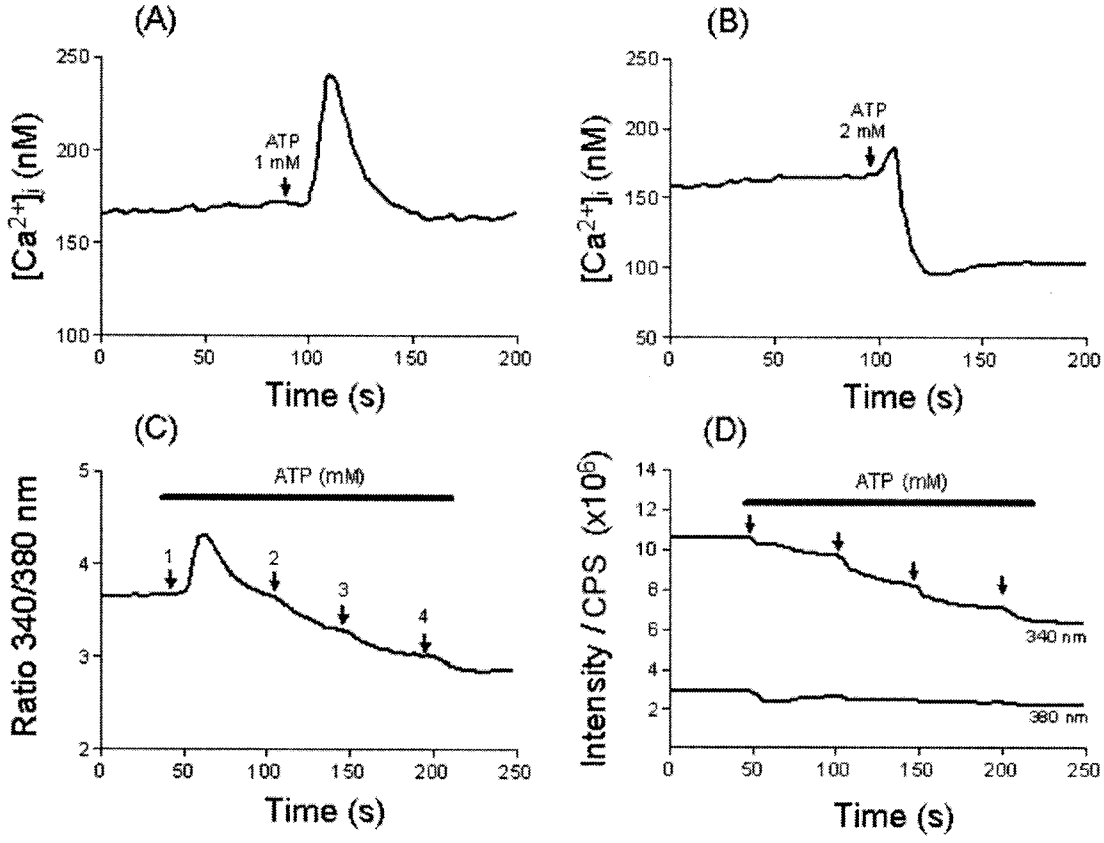

Fig. 1. Effects of ATP on murine bone marrow cell suspensions loaded with fura-2. (A) 1 mM of ATP induced a transient response of [Ca2+]*i*- (B) 2 mM ATP has a biphasic effect: a rapid increase in [Ca2+]*i* followed by its decrease. (C) Typical cumulative concentration-response curve to ATP, showing that R340/380 increases till 1 mM; and the decrease of ratio is observed with the cumulative concentration of ATP (2, 3 and 4 mM). (D) The corresponding profile of fluorescence intensities to alternated excitation, at 340 and 380 nm, with emission collected at 505 nm, showing the reduction in intensity to the additions of ATP up to 1 mM. All data are representative responses of at least four experiments.

membrane lysis allowing the efflux of the fura-2 indicator but not promoting the decrease in fluorescence ratio, but the subsequent cumulative addition of ATP (1, 2, 3 and 4 mM) produces a rapid decrease in R340/380. This means that the efflux of the indicator is not sufficient to decrease R340/380 and a possible quenching of fura-2 fluorescence by ATP must be checked.

ATP and Analogs Decrease Fluorescence Properties of fura-2

The absorption peak of fura-2 in physiological saline with Ca^{2+} (1.36 mM) is 340 nm and in Ca^{2+}-free solution it is 380 nm, approximately; the emission peaks of the 340 nm and 380 nm excitation wavelengths are between 505 nm and 520 nm. The excitation wavelength of fura-2, independent of Ca^{2+} concentration, is 360 nm and corresponds to the isobestic point.

In Fig. 3A[i], the typical excitation spectrum of an aqueous fura-2 (2.2 μM) is shown, where peak excitation is near 340 nm. The addition of the Ca^{2+}chelator EGTA (1, 2, 3 and 4 mM) promotes the decrease in fluorescence at 340 nm and fluorescence near 380 nm can be observed. In Fig. 3A[ii] the effect of the addition of $CaCl_2$ (10^{-2} M) is shown: the peak excitation changes from 380 nm to 340 nm. The addition of ATP (Fig. 3A[iii]) causes a different change in the spectrum of fura-2 related to EGTA: the fluorescence decrease promoted by ATP occurs in the whole excitation spectrum.

ATP absorbs in the wavelength interval of 200–300 nm and thus there is no overlap between fura-2 absorbance and emission spectra (Fig. 3B). However the absorbance of fura-2 (330 μM) in the interval of 300–400 nm is reduced by increasing concentrations of ATP, the reduction in peak absorbance at 340 nm was of 60% for 10^{-2} M of ATP (Fig. 3C).

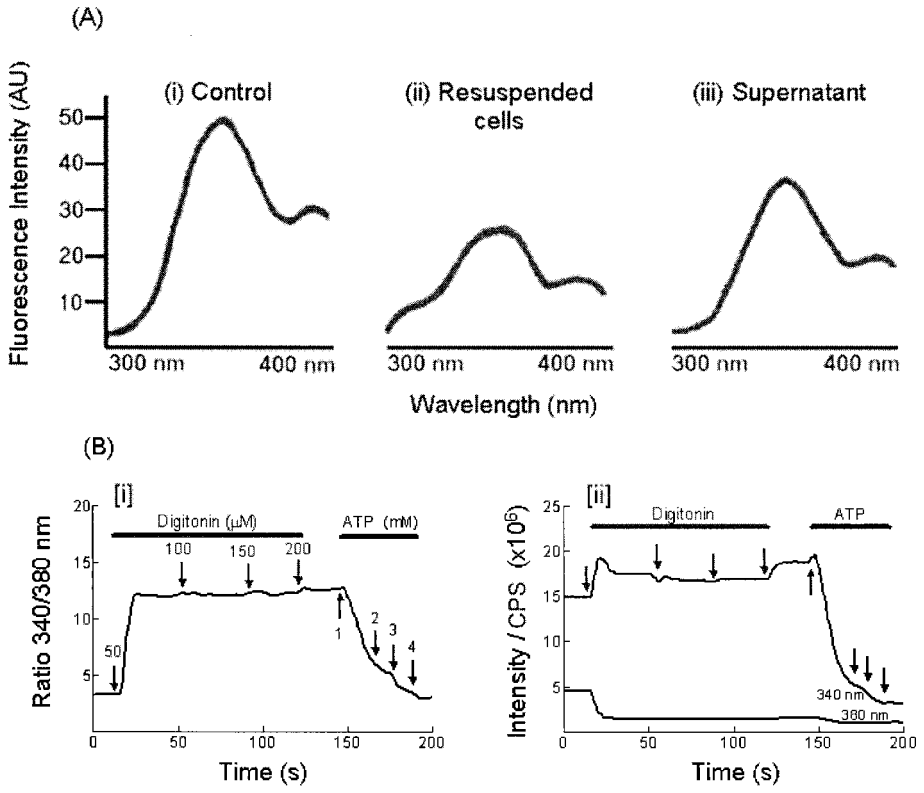

Fig. 2. ATP causes leakage of fura-2 incorporated in the murine bone marrow cell suspensions and decreases the measured fluorescence intensity. (A) Excitation spectra from 300 nm to 400 nm in bone marrow cell suspension loaded with fura-2 indicator; the emission was collected at 520 nm; [i] control excitation spectrum of bone marrow cell suspension before stimulation with ATP (4 mM). [ii] After the addition of ATP (4 mM) the sample was centrifuged and the cell pellet resuspended in 2.5 mL Tyrode solution and its excitation spectrum recorded. [iii] The excitation spectrum of the supernatant is more fluorescent than in resuspensed cells suggesting that efflux of fura-2 dye occurred. (B) The addition of digitonin permeabilizes the cell membrane increasing R340/380 fluorescence to maximal values, since subsequent cumulative digitonin addition promotes complete cell lysis releasing fura-2 but does not decrease fluorescence intensities. When ATP is added to the solution, a rapid decrease in fluorescence is observed at both excitation wavelengths (340 nm and 380 nm). On the other hand, only the addition of ATP (1 mM) after permeabilization of the membrane causes decrease in fluorescence. All data are representative responses of at least three experiments.

The consequence of decrease in absorbance of fura-2 in the presence of high concentrations of ATP is the decrease in fluorescence emission. ATP and analogs decrease fluorescence intensity at the isobestic point of fura-2 salt (2.2 μM) diluted in milli-Q water (Fig. 4A). The $\alpha\beta$ATP, ADP and AMP analogs do not produce reduction in fura-2 fluorescence, but BzATP, ATPγS and UTP decrease fluorescence intensity by 20% at the isobestic point of the fura-2 spectrum (Fig. 4B). Concentrations of BzATP, ATPγS and UTP less than 1 mM decrease fluorescence intensity at the isobestic point of fura-2 by less than 7% (data not shown).

Despite the fact that ATP produces fluorescence intensity decrease at the isobestic point of fura-2 salt, it also increases $[Ca^{2+}]i$ in bone marrow cell suspensions at up to 1 mM concentration. One possibility of ATP to decrease fluorescence of fura-2, is the absorption of photons emitted by fura-2, but the absorption of ATP near the 500–550 nm emission band was not observed. In order to determine if the simple presence of ATP in the extracellular medium promotes the decrease in R340/380 by absorption of light excitation, or if the presence of ATP and fura-2 in the same compartment, through permeabilization of the cell membrane allowing indicator efflux is necessary, we divided the quartz cuvette into two spaces with a coverslip (Fig. 5A). In both compartments [i] and [ii] 1 mL Tyrode solution was placed. To space [ii] fura-2 salt (2.2 μM) was added. The additions of ATP (1, 2, 3 and 4 mM) to space [i] do not produce fluorescence intensity decrease in

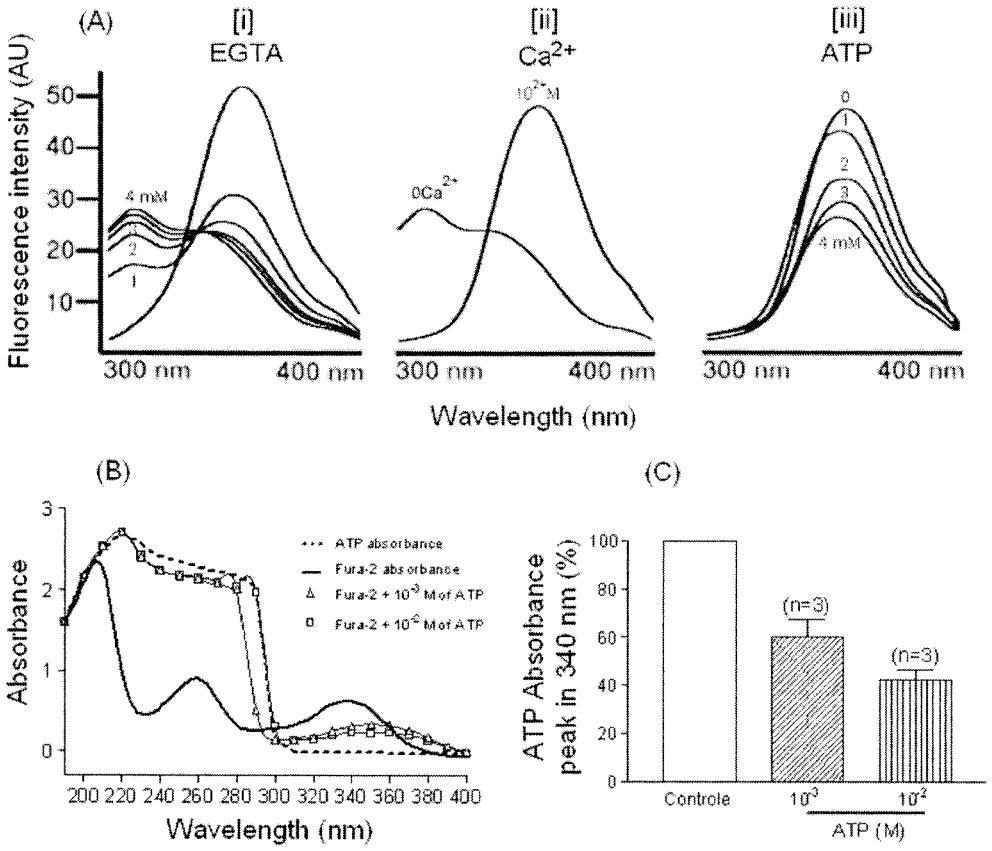

Fig. 3. ATP decreases fluorescence intensity in the excitation and absorption fura-2 spectra. (A) Typical excitation spectrum of fura-2 in aqueous solution (2.2 μM). [i] The addition of EGTA (1, 2, 3 and 4 mM) alters the excitation peak at 340–380 nm. [ii] The addition of Ca^{2+} (10^{-2} M) changes the excitation peak at 380–340 nm. [iii] The addition of ATP (1, 2, 3 and 4 mM) decreases fluorescence intensity of fura-2 excitation spectrum, suggesting that its action is not by chelating Ca^{2+}. (B) Absorption spectra of ATP and fura-2 in water solution. Fura-2 (330 μM) absorbs between 300 nm to 400 nm (solid line) and ATP absorbs between 200 nm to 300 nm (dashed line). The presence of high concentration of ATP decreases absorption of fura-2. (C) Maximal decrease in fura-2 absorbance at 340 nm was 60% for 10^{-2} M ATP.

R340/380 (Fig. 5B). This corroborated the idea that ATP does not absorb photons emitted by fura-2. However, the addition of ATP (1, 2, 3 and 4 mM) to space [ii] immediately decreases fluorescence (Fig. 5C); this result suggests that the presence of both fura-2 and ATP is necessary in the same compartment to decrease fluorescence and that this is triggered when ATP permeates the cell membrane of bone marrow cells.

The $P2X_7$ Inhibitors, OxATP, Coomassie Blue and Mg^{2+} and Ca^{2+} Ions Blocked the Decrease in 340/380 nm Fluorescence Ratio

Among the P2X receptors, the last described member is the $P2X_7$ receptor. This receptor is an ionic channel activated by high ATP concentrations of the millimolar order, forming a non-selective pore permeable to molecules up to 1000 Da [11,12]. This receptor is antagonized by divalent ions, such as Mg^{2+} and Ca^{2+} [16,17] or specifically by OxATP and Brilliant Blue [14,15]. Pretreatment of the cells with OxATP (300 μM) or Brilliant Blue (100 nM), and the presence of Mg^{2+} or Ca^{2+} (5 mM), blocked the decrease in R340/380 fluorescence ratio induced by ATP and only the increase in fluorescence ratio is observed (Figs. 6A–D and 7A–B) (Ca^{2+} inhibition not shown). In Ca^{2+}-free Tyrode solution the ATP-induced $[Ca^{2+}]i$ increase was totally inhibited but the decrease in R340/380 by ATP persists with the cumulative additions of ATP, from 10 μM to 4500 μM (Fig. 7C–D). The use of OxATP also blocked the ATP-induced efflux of fura-2, which is shown

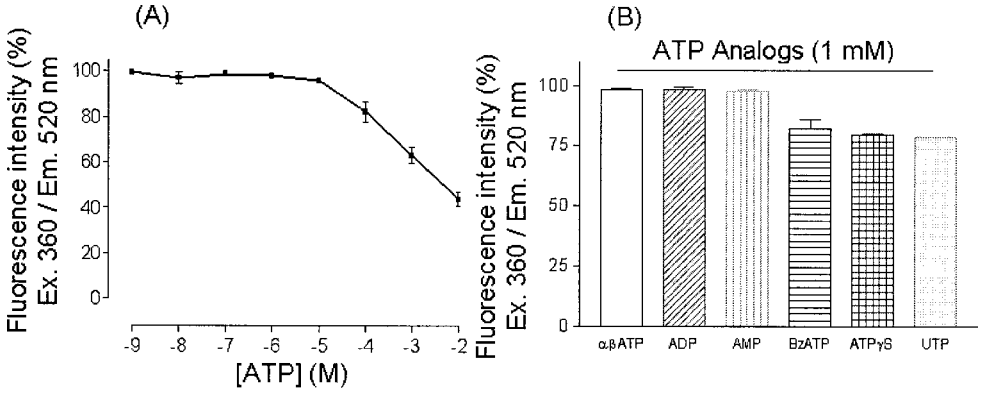

Fig. 4. Fluorescence intensity of fura-2 (2.2 μM) decreases at the isobestic point (360 nm, not Ca^{2+}-dependent) by addition of ATP and analogs. The emission was collected at 520 nm. (A) Decreased fluorescence intensity by ATP was concentration-dependent and reached 50% with 10^{-2} M ATP. (B) The addition of the ATP analogs, $\alpha\beta$ATP, ADP and AMP, did not decrease fluorescence intensities at this isobestic point, but BzATP (1 mM), ATPγS (1 mM) and UTP (1 mM) reduced fura-2 fluorescence by 20%. Each point and histogram represents the mean $\pm$ SEM of three independent experiments.

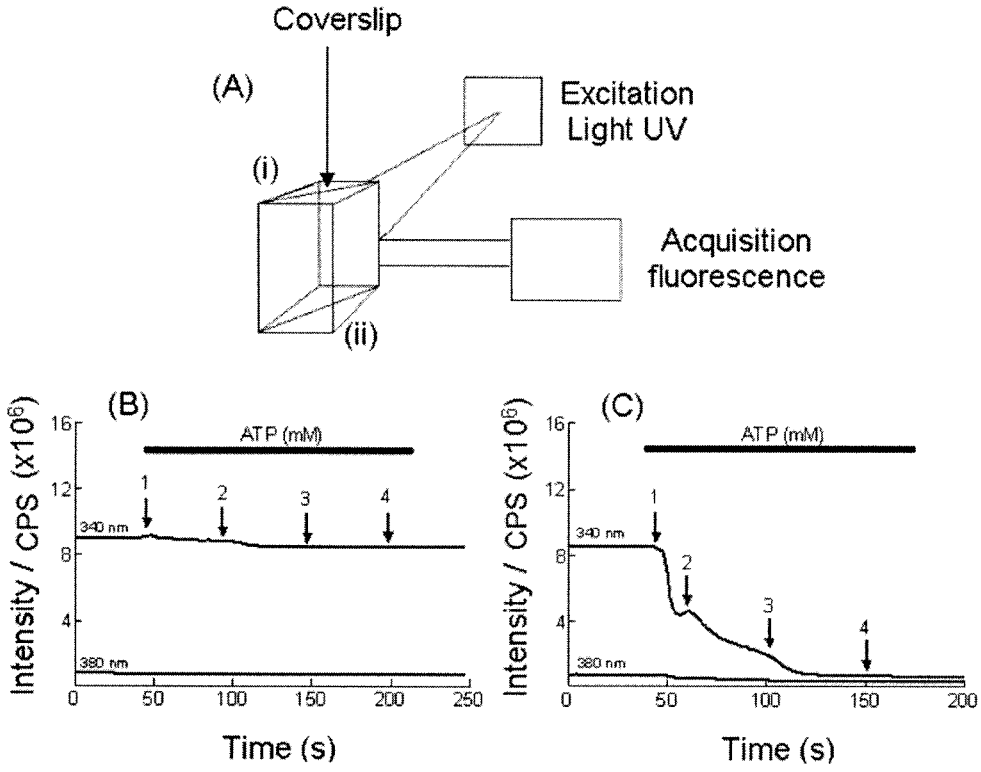

Fig. 5. ATP does not absorb the fluorescence by excitation in the Spex system and the presence of ATP and fura-2 is necessary in the same compartment for the decrease of fluorescence intensity to occur. (A) Scheme of the system used to detect decrease in 340/380 ratio by ATP: the quartz cuvette was divided into two spaces. Both spaces [i] and [ii] have 1 mL Tyrode solution and to the second space [ii] fura-2 salt (2.2 μM) was added. (B and C) Records of fluorescence intensity of fura-2 alternately excited at 340 nm and 380 nm, with emission collected at 505 nm. (B) The record of fluorescence in space [i] when ATP (4 mM) was added showing no alteration of fluorescence. (C) The record of fura-2 fluorescence in space [ii] when ATP (4 mM) was added showing the decrease in dye fluorescence till self-fluorescence values, proving that ATP does not absorb the fluorescence and the presence of ATP and fura-2 in the same space is necessary to observe fluorescence decrease.

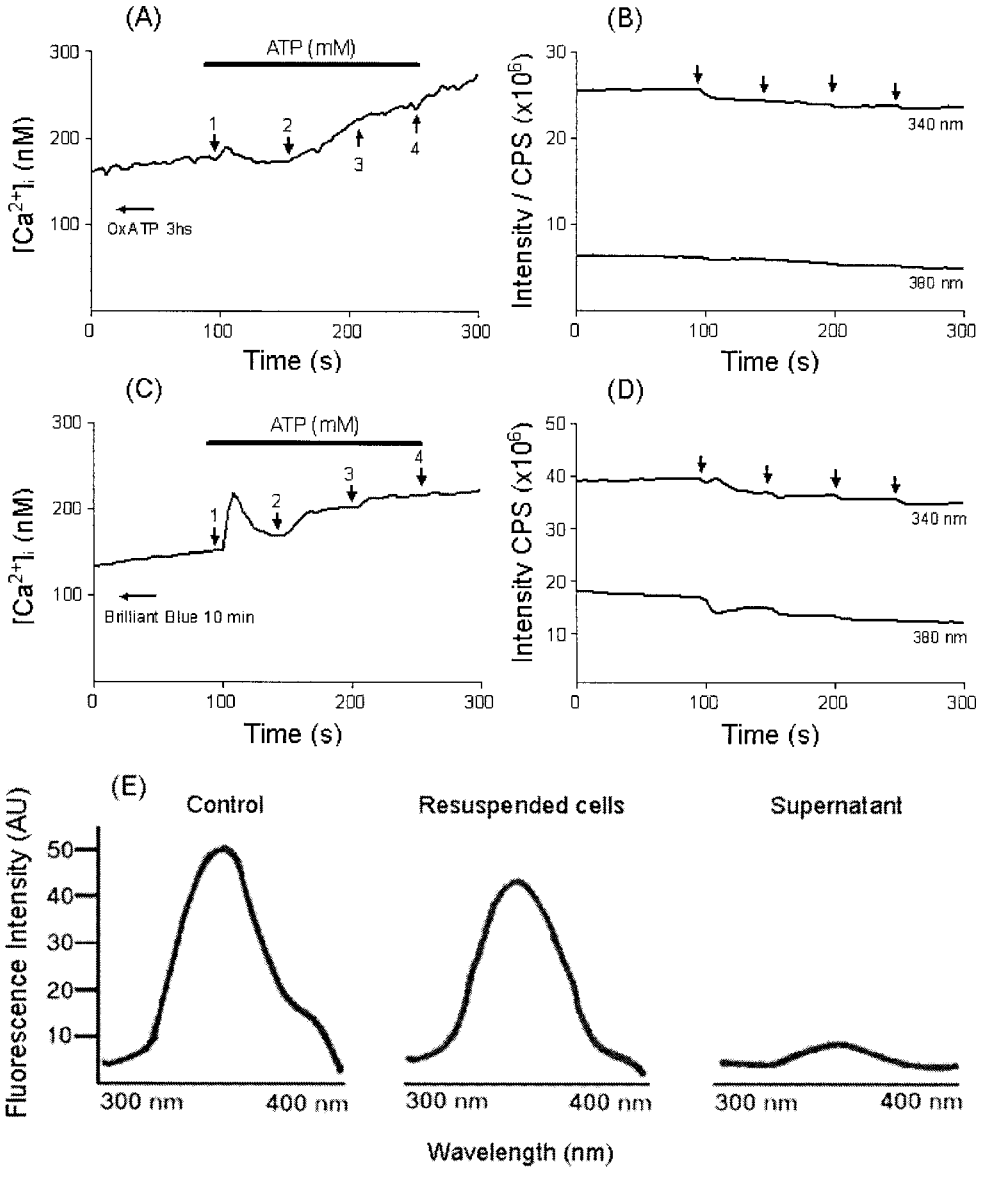

Fig. 6. Effect of $P2X_7$ inhibitors on the decrease in ATP-dependent R340/380 fluorescence of fura-2 in murine bone marrow cell suspension. $[Ca^{2+}]_i$ and fluorescence intensity profiles obtained with the alternated excitation of the samples at 340 nm and 380 nm, with emission collected at 505 nm are shown. (A–B) The pretreatment with OxATP (300 μM) for 3 h and (C–D) with Brilliant Blue (100 nM) for 10 min blocked the ATP-dependent decrease in R340/380. (E) The cell suspension and its supernatant spectrum show that $P2X_7$ is blocked by OxATP (300 μM). The addition of ATP (4 mM) to the cell suspension pretreated with OxATP produces minimal release of fura-2, confirming previous results. All data are representative responses of at least five experiments.

in Fig. 6E where a small fluorescence in the supernatant is observed after the stimulus with ATP (4 mM).

Effects of ATP on Different Cell Types Extracted from Bone Marrow

Since bone marrow is a tissue with different cell population mixtures, we utilized the murine J774 macrophage cell line as positive control cells that are known to strongly express the $P2X_7$ receptor [20], the undifferentiated human HL-60 myeloid cell line and different mouse bone marrow cell populations, after performing the isolations, to discriminate P2 receptor activation. The ATP-induced effects vary depending on the different cell populations. The J774 cell line (Fig. 8A) and mononuclear cell fraction (Fig. 8B) showed similar responses when compared to

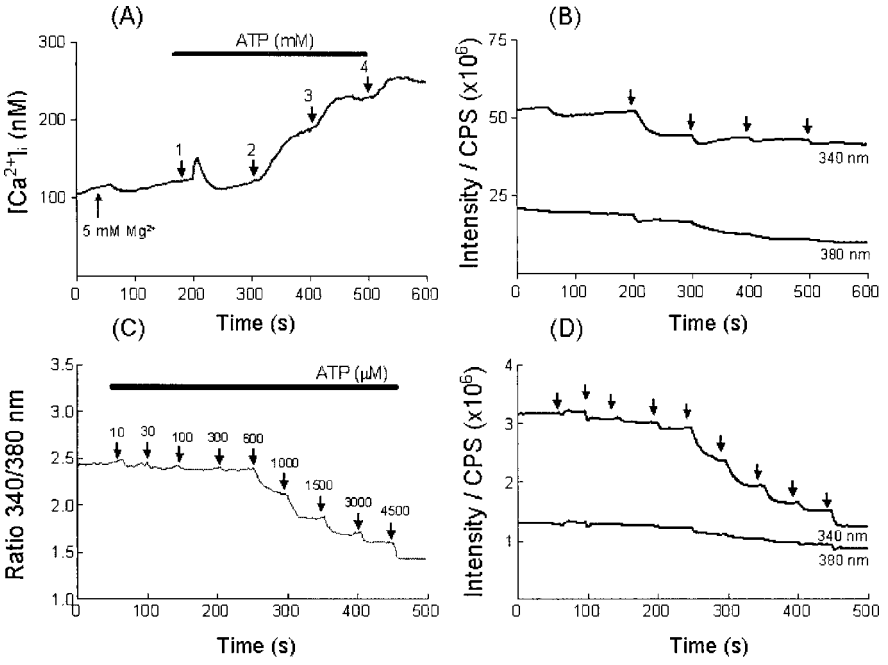

Fig. 7. Effect of divalent cations (Mg^{2+} and Ca^{2+}) on fura-2 leakage in loaded murine bone marrow cell suspensions. $[Ca^{2+}]_i$, in nanomolar units and the profile corresponding to the fluorescence intensities obtained with the alternated excitation of the samples at 340 nm and 380 nm, with emission collected at 505 nm are shown. (A–B) The presence of Mg^{2+} (5 mM) blocked the decrease in R340/380. (C–D) In the case of suspensions in Ca^{2+}-free Tyrode solution the cumulative concentration-response curve of ATP shows decrease in R340/380 as well as decrease in the corresponding 340 nm and 380 signal profiles.

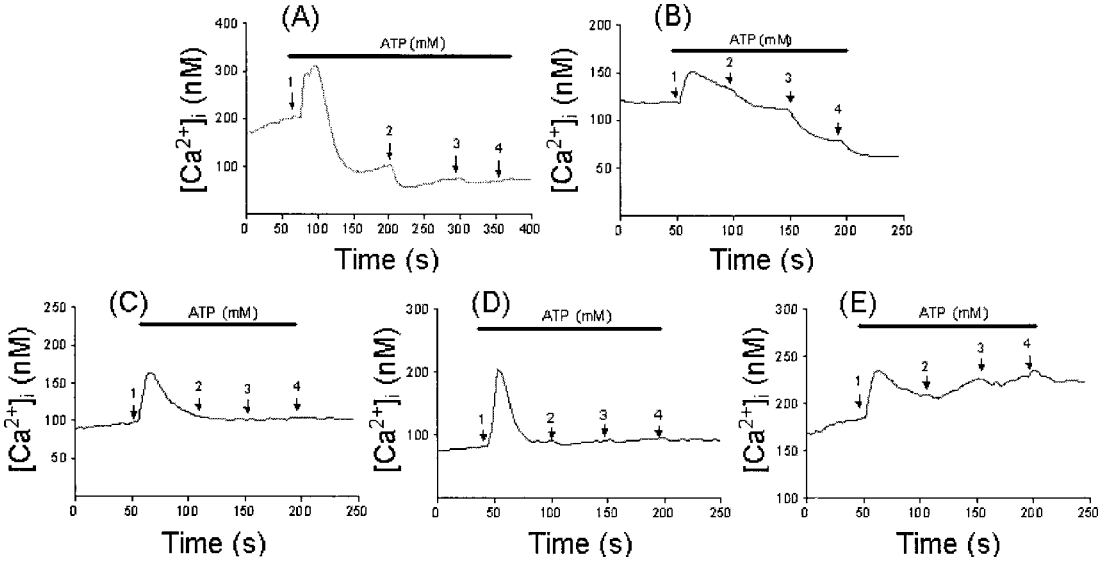

Fig. 8. Effects of ATP on different cell lines extracted from bone marrow and loaded with fura-2. (A) J774 macrophage cell line and (B) mononuclear cell fractions have a similar response to that of bone marrow cells: 1 mM of ATP produced transient $[Ca^{2+}]_i$ increase and concentrations up to 1 mM produce decreased R340/380. (C) Regarding undifferentiated myeloid HL-60 cell line and (D) myelocyte suspensions, ATP produced transient $[Ca^{2+}]_i$ increase and with concentrations up to 1 mM it had no effect on $[Ca^{2+}]_i$. In erythroblasts (E) ATP produced a biphasic response: first $[Ca^{2+}]_i$ increases and then it is followed by a sustained increase. All data are representative responses of at least four experiments.

total bone marrow cell suspensions. The mononuclear cell fraction contains monocytes and lymphocytes that express the $P2X_7$ receptor [19,34]. The undifferentiated HL-60 myeloid cell line (Fig. 8C) and murine myeloid cells (Fig. 8D) showed transient $[Ca^{2+}]i$ increases when stimulated with ATP (1 mM); concentrations of ATP up to 1 mM did not produce new increases or decreases in R340/380 fluorescence ratio in both cell suspensions. The erythroblast suspension showed a biphasic response: initially a transient $[Ca^{2+}]i$ increase followed by a sustained increase (Fig. 8E).

DISCUSSION

The presence of P2 receptors in many tissues and systems, including bone marrow-derived cells where ATP activates both P2X and P2Y receptor subtypes, is known. All known P2 receptors cause $[Ca^{2+}]i$ increases [32,35–37], For this reason, the most utilized approach for the characterization of P2 receptors, through the use of fluorimetric methodologies, is the fluorophore fura-2, which allows monitoring intracellular Ca^{2+} changes which are associated with desensitization protocols [38–41].

Here, we report the problems of the use of the fluorescent Ca^{2+} indicator fura-2 in the presence of higher concentrations of ATP in bone marrow cell suspensions. ATP produces a concentration-dependent biphasic effect: initially it increases R340/380 (or $[Ca^{2+}]i$) (up to 1 mM) which is followed by a decrease in fluorescence by ATP concentrations up to 1 mM of ATP (Fig. 1).

The R340/380 decrease is due to the simultaneous reduction in fluorescence intensity at both 340 nm and 380 nm wavelength excitations, and which is characterized by the leakage of indicator from the cells or by quenching of the fluorescence [33]. The leakage of the Ca^{2+} indicator fura-2 was observed by the 300–400 nm fluorescence excitation spectrum of the supernatant after sample centrifugation (Fig. 2).

One possibility of fluorescence decrease is the chelator effect of ATP^{4-} complexes with Ca^{2+}. In Fig. 3A the typical decrease of Ca^{2+} by the known Ca^{2+} chelator EGTA is shown. The decrease of Ca^{2+} promotes a change in the excitation peak of fura-2. However, ATP does not produce a change in this excitation peak, but it decreases the fluorescence in all excitation spectra, although we do not exclude the possibility that part of decrease would be due to the decrease in Ca^{2+} concentration. Another possibility to explain the decrease in the collected fluorescence would be the alteration of the emission of fura-2, but no change in emission peak was observed, only a decrease of intensities occurs (data not shown). Analogs of ATP, such as BzATP, ATPγS and UTP, in also cause decrease in R340/380 (Fig. 4) at 1 mM concentration. We measured the pH of the Tyrode solution (pH 7.4), in the presence and absence of cells, after additions of the agonists (ATP and analogues), but its highest variation was near, 0.5–1, thus it did not elucidate the reason for the decrease in fluorescence of fura-2 (data not shown).

The quenching for ATP, by absorption of emitted photons, was also hypothesized. Absorption of ATP at a wavelength near band emission of fura-2 (500–550 nm) was not observed. The maximal absorbance of ATP is between 200 nm and 300 nm (Fig. 3B), although the presence of ATP decreases the absorbance of fura-2 (Figs. 3B and C).

On using a similar methodology as that shown in Fig. 5, but placing ATP (4 nM) in space [i] and fura-2 (2,2 μM) in space [ii] no alteration of fluorescence collected in the Spex system was observed, corroborating the records of absorption of ATP. If ATP absorbed photons emitted by fura-2, we would observe fluorescence decrease in both spaces when the sample was excited at 340 nm and 380 nm, but ATP in space [i] did not absorb the emitted light and only when ATP is in the same space as fura-2 (space [ii]) a decrease in R340/380 is observed (Fig. 5C). This result corroborates the experiment shown in Figs. 2 and 3, suggesting the need for the permeabilization of the cell membrane to allow contact of fura-2 with ATP. What interaction occurs between fura-2 and ATP is unknown, and it is difficult to suppose such an interaction, because both molecules are strongly negative.

Based on the present results we can summarize: ATP does not absorb between 500 nm and 550 nm, and no overlap between absorbance or emission spectra of fura-2 and ATP occurs. The possibility that the decrease in fluorescence is caused by the emission inner filter effect is excluded because ATP did not absorb photons emitted by fura-2. Increases in concentration can promote excitation inner filter effect in many systems, but the front-face illumination mode which was used is much less sensitive to the excitation inner effect. We conclude from these experiments that ATP promotes the decrease in fura-2 absorption by decrease in concentration of fura-2 or by an unknown process. The reduction in emission of fura-2 (fluorescence) is just an unexplained side effect.

Among the P2 receptors, the $P2X_7$ receptor was the last member known; it differs strikingly from the other members in that it is activated by a high concentration of ATP (above 1 mM), forming a non-selective pore, permeable to molecules up to 900 Da, including fluorescent dyes, such as the fura-2 indicator [11,12]. We antagonized the release of fura-2 and the decrease at R340/380 with Mg^{2+}, OxATP and Brilliant Blue (Fig. 6), that are specific

inhibitors of the $P2X_7$ receptor [14–17], suggesting the presence of this receptor in our samples. Similar results (decreased R340/380) were obtained with high concentration of BzATP (1 mM), a selective $P2X_7$ receptor agonist, but the initial $[Ca^{2+}]i$ increase was not observed, in other words, the biphasic effect was absent. Lower concentrations were used but without interfering in those of $[Ca^{2+}]i$ or in the R340/380 decrease. The activation of the non-selective pore is not fully understood. Several authors described the initial activation of an ionic channel followed by pore formation as separate phenomena [11,13], and the intracellular pathways that induce the activation of the large permeabilization pore are unknown. The inhibition of ATP-dependent decreases in fluorescence by OxATP and Mg^{2+} is a pharmacological effect. In experiments similar to those shown in Fig. 4, where the isobestic point of fura-2 was monitored, in the presence of Mg^{2+} (5 mM) and OxATP (300 μM), the cumulative addition of ATP decreased fura-2 fluorescence in a concentration-dependent way and this is a proof of the pharmacological effect of these inhibitors in suspended bone marrow cells loaded with fura-2 (data not shown).

The activation of the $P2X_7$ receptor would allow the efflux of the fluorescent indicator but this explanation is not sufficient to justify the decrease in R340/380 since the presence of ATP in the bathing solution is necessary to alter the properties of the Ca^{2+} indicator fura-2 (Fig. 2).

In this study the intracellular pathways triggered by ATP in bone marrow cell suspension were not described and because this tissue consists of different cell populations that express different P2 receptor subtypes they were analyzed separately. The mononuclear cell fractions and J774 macrophage cell line, that have a higher expression of the $P2X_7$ receptor [20,34], also showed R340/380 decrease on addition of up to 1 mM ATP. Mouse bone marrow myeloid cells and the myeloid HL-60 lineage showed rapid transient increases in $[Ca^{2+}]i$. The presence of the P2 receptor in macrophages and myelocytes, mainly the P2Y subtype, is known [30–32,40]. In erythroblasts ATP induced a biphasic effect: an initial transient increase of $[Ca^{2+}]i$ followed by a sustained increase. In such cells the P2 receptor subtypes are poorly characterized.

Schilling *et al*. [43,44] described, in fibroblast cell suspension, the increase in plasma membrane permeability to fura-2 and other fluorophores with maximal fluorescence response of these cells by activation of the $P2X_7$ receptor induced by BzATP (100 μM), but no higher concentration of BzATP or ATP was used. The concentration used by these authors is lower than that used in this study and, as shown in Fig. 4B, 1 mM of BzATP decreased the fluorescence of fura-2 by 20% at the isobestic point. These results suggest that a concentration of up to 1 mM ATP, BzATP, ATPγS and UTP would interfere in the measurements of fura-2 fluorescence.

Summarizing, we observed that some problems appeared when the fluorescent dye fura-2 is used to monitor changes in intracellular Ca^{2+} induced by high concentrations of ATP and its analogues in some types of cell suspensions. The decrease in the absorption of fura-2 occurs when the cells are stimulated by a concentration of ATP higher than 1 mM activating the $P2X_7$ receptor, which promotes the leakage of fura-2, and the interaction of the Ca^{2+} fluorophore with ATP, by an unknown process, results in the reduction in R340/380. So it must be kept in mind that ATP and analogs, used for the pharmacological characterization of the P2 receptor, can interfere in their cell $[Ca^{2+}]i$ measurements when fura-2 is used as Ca^{2+} fluorophore in the fluorimetric methods. Our studies contribute to avoid such inaccurate interpretation of $[Ca^{2+}]i$ values in the ATP-dependent cell signaling.

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