ATP-induced $P2X_7$-associated uptake of large molecules involves distinct mechanisms for cations and anions in macrophages

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Summary

Macrophages express the $P2X_7$ receptor and other nucleotide (P2) receptors, and display the phenomenon of extracellular ATP (ATP_e)-induced $P2X_7$-dependent membrane permeabilization, which occurs through a poorly understood mechanism. We used patch-clamp recordings, cytoplasmic Ca^{2+} measurements and fluorescent dye uptake assays to compare $P2X_7$-associated transport phenomena of macrophages and HEK-293 cells transfected with $P2X_7$ receptors (HEK-$P2X_7$ cells). Both cell types showed inward currents, increase of free cytoplasmic Ca^{2+} concentration and the uptake of cationic dyes upon exposure to ATP_e, as previously described. However, in contrast to the macrophages, HEK-$P2X_7$ cells did not take up anionic dyes and did not display the 440 pS channels (Z pores) under cell-attached patch-clamping conditions. In addition, the transport mechanism of anionic dyes displayed by macrophages was also able to support dye efflux and, once activated at 37°C, it remained active at 4°C, whereas uptake of cationic dyes was temperature-dependent and unidirectional. Our results indicate that the mechanism of ATP_e-induced dye uptake, usually called a 'permeabilization phenomenon' and associated with a 'permeabilization pore' can be ascribed to at least two distinct mechanisms in macrophages: a diffusional pathway, possibly associated with the 440 pS Z pores, and a cation uptake mechanism that is not diffusional and should be ascribed to an, as yet, unidentified transport mechanism.

Key words: ATP, P2 receptor, $P2X_7$, Permeabilization, Macrophage, Cation, Anion, Pore

Introduction

Extracellular nucleotides and adenosine have been included among the immunomodulators during immune response/inflammation (Di Virgilio et al., 2001; Ferrari et al., 2006; Kornbluth and Stone, 2006). Their activities are mediated to a large extent by nucleotide (P2) and adenosine (P1) receptors (Ralevic and Burnstock, 1998; North, 2002), and modulated by ecto-nucleotidases such as CD39 and CD73, expressed in macrophages, dendritic and endothelial cells, as well as by other cells that participate in the immune response (Dwyer et al., 2007).

The $P2X_7$ receptor is an ATP-gated cation-selective channel permeable to Na^+, K^+ and Ca^{2+} that has also been associated with the opening of a non-selective pore that allows the passage of large organic ions (Steinberg et al., 1987a; Persechini et al., 1998; North, 2002; Erb et al., 2006). Its activation can induce cell death by apoptosis and necrosis, cytokine release, killing of intracellular pathogens, and membrane blebbing (Di Virgilio et al., 2001; Coutinho-Silva et al., 2003; Ferrari et al., 2006). In addition, $P2X_7$ receptors are upregulated by interferon-γ (IFN-γ) plus LPS (Ferrari et al., 2006) and cooperate with toll-like receptors (TLRs) and NOD-like receptors (NLRs) in the process of pathogen recognition, activation of inflammasome and maturation and secretion of interleukin-1β (IL-1β) and IL-18 (Mariathasan and Monack, 2007).

Although different signaling pathways are known to be triggered by $P2X_7$ and other P2 receptors in macrophages, the specific pathways that lead to each of the $P2X_7$-dependent responses are still poorly understood (Persechini et al., 1998; Humphreys et al., 2000; Kim et al., 2001; North, 2002; Mackenzie et al., 2005; Monteiro-da-Cruz et al., 2006; Erb et al., 2006; Monteiro-da-Cruz et al., 2007; Pfeiffer et al., 2007). In particular, although the elucidation of the pathway or pathways that link extracellular ATP (ATP_e) and $P2X_7$ to membrane permeabilization still await further clarification, the requirement for $P2X_7$ receptors has been firmly established by pharmacological tools and experiments with knock-out mice (North, 2002; Coutinho-Silva et al., 2003; Adinolfi et al., 2005; Garcia-Marcos et al., 2006). The permeabilization phenomenon is itself poorly characterized. It is generally accepted that ATP_e can induce the opening of permeabilization pores that allow the passage of molecules of up to approximately 900 Da in macrophages and below 400 Da in lymphocytes (North, 2002; Adinolfi et al., 2005). However, few molecules have in fact been studied. In macrophages, the DNA-binding cationic dyes of different M_r (here we use values corresponding to the ionized form in solution) such as ethidium (314 Da), propidium (414 Da), and YO-PRO-1 (375 Da) and the anionic dyes carboxyfluorescein (CF; 376 Da), Lucifer Yellow (LY; 443 Da) and Fura-2 (637 Da) are taken up on ATP or BzATP stimulation (Steinberg et al., 1987a; Virginio et al., 1999). Anions of larger M_r such as Evans Blue (869 Da) and Trypan Blue (869 Da) are not taken up (Steinberg et al., 1987a). In lymphocytes, ATP_e induces the uptake of ethidium but not of propidium (Wiley et al., 1993).

LY and DNA-binding dyes have been widely used to investigate the permeabilization phenomenon. Based on the evidence that LY concentration in the cytoplasm reaches a value close to the

extracellular concentration of the dye (Steinberg et al., 1987a), it has been proposed that the mechanism underlying the dye uptake phenomena is free diffusion through a permeabilization pore, and it has been assumed that cations and anions uses the same pathway. However, evidence from several different sources indicate the involvement of distinct mechanisms (North, 2002; Egan et al., 2006). Whole-cell patch-clamp recordings performed on cells transfected with $P2X_7$ receptors indicate a permeability shift from low to high M_r molecules in the first seconds after stimulation by ATP_e, while keeping the selectivity for cations (Virginio et al., 1999). In addition, although ATP_e induces the uptake of both cationic and anionic dyes in macrophages (Steinberg et al., 1987a), the uptake of anions has not been reported either in HEK-293 cells or astrocytomas transfected with $P2X_7$, neither has it been reported in lymphocytes (Wiley et al., 1993; Surprenant et al., 1996; Rassendren et al., 1997; Chessell et al., 1998; Ferrari et al., 2000; Paukert et al., 2002; Duan et al., 2003; Suadicani et al., 2006), suggesting that in these experimental situations, ATP_e might not induce membrane permeabilization to anions.

Based on cell-attached patch-clamping experiments demonstrating the opening of large cation- and anion-permeant channels (Z pores) induced by ATP_e in macrophages, we have hypothesized that the permeabilization pore of macrophages could be distinct from the receptor itself, but coupled to $P2X_7$ through an unidentified signaling mechanism (Coutinho-Silva and Persechini, 1997; Persechini et al., 1998). This possibility has recently been corroborated by data showing that pannexin-1, a protein that forms large non-selective transmembrane channels (Barbe et al., 2006), is involved in the phenomenon of $P2X_7$-associated, ATP_e-induced membrane permeabilization (Pelegrin and Surprenant, 2006; Locovei et al., 2007). However, there have been no reports of any large and non-selective unitary channels similar to the Z pores in cells transfected with $P2X_7$ receptors under conditions of ATP_e-induced dye uptake. Moreover, pannexin-1 channels are expected to be permeable to both cations and anions (Bao et al., 2004; Locovei et al., 2006; Locovei et al., 2007), whereas there are no reports of either whole cell currents or dye uptake of anions in cells transfected with $P2X_7$ alone (Rassendren et al., 1997; Virginio et al., 1997; Virginio et al., 1999; Hibell et al., 2001). The lack of data regarding the downstream events associated with $P2X_7$ receptors prevents us from fully understanding their role in macrophage physiology and in the immune system.

To further dissect the events involved in ATP_e-induced $P2X_7$-associated phenomena in macrophages we compared macrophages with HEK-293 cells transfected with $P2X_7$ receptors (HEK-$P2X_7$ cells) using fluorescent dye uptake, cytoplasmic Ca^{2+} measurement with Fura-2, and patch-clamp recordings. We concluded that the $P2X_7$-triggered phenomena are more diverse than previously thought and the so called 'permeabilization phenomenon' usually identified with a 'permeabilization pore', can be ascribed to at least two distinct mechanisms: one for cations and another for anions. Our results imply that previous data obtained using only one approach to study $P2X_7$-associated phenomena (e.g. whole cell current, LY uptake, YO-PRO-1 uptake, cytoplasmic Ca^{2+} measurement) should be re-addressed and the uptake of anions and cations should be treated separately.

Results

Functional expression of $P2X_7$ receptors in HEK-293 cells

We stably transfected HEK-293 cells with $P2X_7$ cDNA and performed whole-cell current recordings, measurement of free cytoplasmic Ca^{2+} concentration, and dye uptake experiments in order to ascertain the functionality of the transfected receptors. Macrophages were used as control wild-type $P2X_7$-expressing cells. HEK-$P2X_7$ cells expressed the $P2X_7$ receptor protein (Fig. 1H) and displayed typical ATP_e-induced inward currents (Fig. 1C) similar to what was observed in macrophages (Fig. 1A), as previously described (Surprenant et al., 1996). In macrophages, in addition to the inward current, ATP_e also triggered an outward current (Fig. 1A) previously shown to be ascribed to a Ca^{2+}-dependent K^+ channel (Albuquerque et al., 1993) that was not present in HEK cells (Fig. 1B,C). HEK-$P2X_7$ cells and macrophages also displayed a sustained ATP_e-induced increase in the free cytoplasmic Ca^{2+} concentration not present in wild untransfected HEK-293 cells (Fig. 1D-G). A small Ca^{2+} signal also observed in untransfected HEK-293 cells (Fig. 1E) was possibly due to the presence of other P2 receptors in these cells. The EC_{50} obtained for the Ca^{2+} signal in HEK-$P2X_7$ cells was 0.8 mM ATP corresponding to 86 μM ATP^{4-}; a value

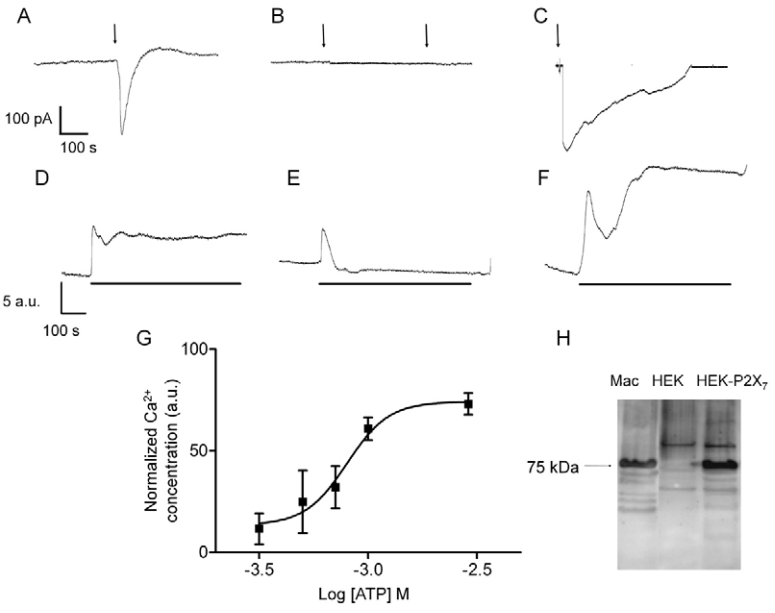

Fig. 1. ATP_e-induced inward currents and Ca^{2+} signaling in HEK-$P2X_7$ cells and macrophages. (A-C) Whole-cell voltage clamp recordings of macrophages (A), HEK-293 cells (B), and HEK-$P2X_7$ cells (C). Cells were kept at room temperature and ATP (1 mM) was applied (arrows) pneumatically. (D-F) Changes in free cytoplasmic Ca^{2+} concentration of macrophages (D), HEK-293 cells (E), and HEK-$P2X_7$ cells (F) loaded with Fura-2-AM. Cells were kept at 37°C and 5 mM ATP (final concentration) was applied as indicated by the horizontal bars. Ca^{2+} concentration was measured for 20-30 cells in the same microscope field using arbitrary fluorescence ratio units (a.u.). (G) Dose-effect curve of ATP_e in HEK-$P2X_7$ cells. Cells were exposed to different ATP_e concentrations in the same conditions as in F and the fluorescence signal was normalized taking the maximum fluorescence obtained after the addition of 0.1% saponin. (H) Anti-$P2X_7$ western blotting of macrophages (lane 1) HEK-293 cells (lane 2), and HEK-$P2X_7$ cells (lane 3). Each result is representative of at least three independent experiments. In G, the bars represent the mean ± s.d. of at least three measurements per data point.

consistent with previously published results for the EC_{50} of the inward current of the cloned rat P2X$_7$ receptor in the absence of divalent cations (Surprenant et al., 1996).

We next showed that macrophages (Fig. 2A,B) and HEK-P2X$_7$ cells (Fig. 2E,F), but not untransfected HEK-293 cells (Fig. 2C,D) could take up of the cationic DNA-binding dyes ethidium (Fig. 2) and YO-PRO-1 (data not shown), using ATP_e, as previously described (Virginio et al., 1999). In addition, the uptake of ethidium by HEK-P2X$_7$ cells was blocked by periodate-oxidized ATP (oxATP; Fig. 2G) as it is in macrophages [(Murgia et al., 1993); data not shown], another hallmark of the P2X$_7$-associated phenomena.

Large ATP_e-induced channels (Z pores) are observed in macrophages but not in HEK-P2X$_7$ cells

The above results confirm data in the literature and ascertain that the P2X$_7$ receptors expressed in transfected cells are functional, establishing the ground for further investigation of other P2X$_7$-associated phenomena.

We have previously identified an ATP_e-activated P2X$_7$-associated non-selective channel activated in macrophages (Coutinho-Silva and Persechini, 1997). These channels were called Z pores and could only be recorded under cell-attached patch-clamping conditions. Because they carry currents of large cations (Tris, 121 Da and N-methyl-D-glucamine (NMDG), 195 Da) and anions (glutamate, 146 Da) we have proposed that they could be a pathway for dye uptake (Coutinho-Silva and Persechini, 1997; Persechini et al., 1998). We therefore investigated whether a similar large conductance non-selective channel could be detected in HEK-P2X$_7$ cells under cell-attached patch-clamping conditions. We observed that, whereas Z pores were readily detected in 35 out of 42 macrophages during the first 4 minutes after ATP_e application (Fig. 3A, upper trace), no large conductance channels were observed under similar conditions in HEK-P2X$_7$ cells (n=10; Fig. 3B) for up to 30 minutes after ATP_e application. In all experiments, the holding potential was kept initially at –40 mV inside the pipette, but no channels were observed by varying pipette potential from –80 to +40 mV, which corresponds to a patch transmembrane potential ranging from –40 to +80 mV considering that the cellular transmembrane potential should be 0 mV in the presence of ATP, because of the opening of the non-desensitizing P2X$_7$ cation currents, as previously described (Coutinho-Silva and Persechini, 1997).

In addition, in some experiments we also added 10 mM ATP to the pipette solution but no pores were observed either (n=13). The Z pores of macrophages were found to have a conductance state of 440±16 pS (n=13) and of 260±49 pS (n=9). They displayed a strong rectification, being detected only at negative pipette potentials (Fig. 3C). The reversal pipette potential was –2.7 mV, a value consistent with a reversal membrane potential of 0 mV.

To further investigate the relationship between the Z pores and the phenomenon of dye uptake by macrophages, we also performed cell-attached recordings and uptake assays using both ethidium and LY in the absence of divalent cations, a condition known to interfere in the activation of P2X$_7$ receptors and in the uptake of dyes (Steinberg et al., 1987a) (Fig. 3A, recording b; Table 1). In the divalent-free solution, the unitary events were rarer, leading to a significantly decreased open probability, while the mean open time of each event increased. These data suggest that any Z-pore-associated permeability would decrease in the absence of divalent cations. In keeping with this possibility, the uptake of LY by macrophages also decreased significantly under the same

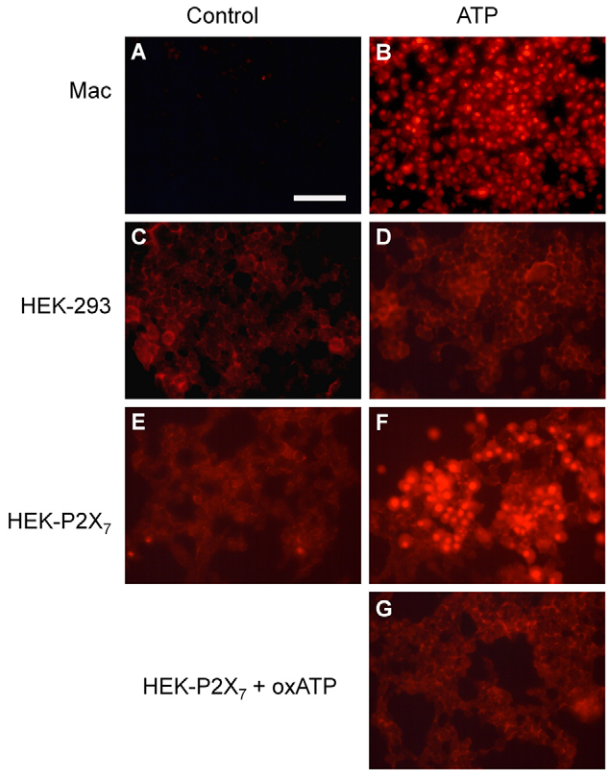

Fig. 2. ATP_e-induced ethidium uptake in HEK-P2X$_7$ cells and in macrophages. Fluorescence images of cells incubated for 15 minutes at 37°C in the presence (B,D,F,G) or not (A,C,E) of 5 mM ATP. Ethidium bromide (10 μM) was added in all preparations. (A,B) Macrophages; (C,D) HEK-293 cells; (E,F) HEK-P2X$_7$ cells. (G) HEK-P2X$_7$ cells pre-incubated for 2 hours with 300 μM oxATP before the addition of ATP. Each result is representative of at least three independent experiments. Bar, 100 μm.

experimental conditions, whereas the uptake ethidium did not (Fig. 3D). Similar results were obtained when macrophages were pre-loaded with BAPTA-AM and kept in the same divalent-free solution (data not shown), a condition that completely abrogates ATP_e-induced Ca^{2+} signaling in macrophages (Monteiro-da-Cruz et al., 2006). These results indicate that the Z pores are associated with the uptake of LY (an anion) but not ethidium (a cation). In accordance with this hypothesis, the uptake of ethidium HEK-P2X$_7$ cells, a phenomenon that does not seem to involve Z pores, was not significantly changed in divalent-free solution regardless of pre-loading with BAPTA-AM (data not shown). Therefore, the changes

Table 1. Open probability and open time of Z pores

Solution	Open probability†	Open time‡ (seconds)
Normal	0.110±0.02	1.87±0.01
Divalent-free	0.070±0.04*	3.7±1

Cell-attached recordings of macrophages were performed using the same solution in the pipette and in the extracellular medium at 37°C in the presence of 1 mM ATP at a holding potential of –40 mV.

Values are mean ± s.d. (n=5 independent experiments).

*P<0.05.

†Time opened/total time of recording.

‡Mean time of a single channel in the open state.

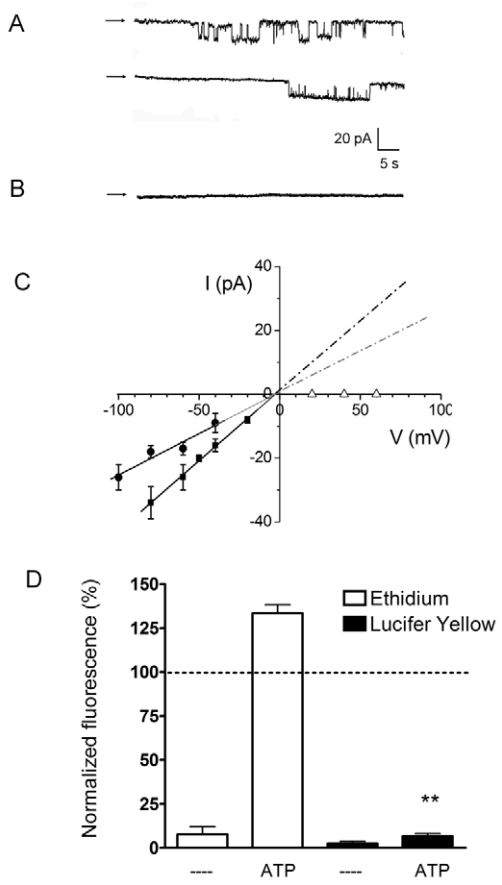

Fig. 3. Large non-selective channels observed in macrophages but not in HEK-P2X$_7$ cells correlates with ATP$_e$-induced anion uptake. Cell-attached recordings (A-C) and dye uptake assays (D) were made in macrophages (A,C,D) and in HEK-P2X$_7$ cells (B). For patch-clamping experiments, cells were kept at 37°C and recordings were performed with a holding potential of –40 mV. ATP was applied manually with a micropipette (1 mM final concentration). Results are representative of at least ten independent experiments. (A) Cell-attached recordings of macrophages. Upper trace: normal extracellular solution. Lower trace: divalent-free extracellular solution. (B) Cell-attached recording of a HEK-P2X$_7$ cell. (C) I-V curves of the macrophage channels obtained in the same conditions as in the upper trace of A. (D) ATP$_e$-induced uptake of ethidium and LY in macrophages using the same solutions as in A. Data in D was obtained by fluorescence microscopy as described in Materials and Methods. The values were normalized by taking the specific fluorescence, obtained in normal solution in the presence of ATP, as 100%. Data points and bars represent the mean ± s.d. of at least four independent experiments.

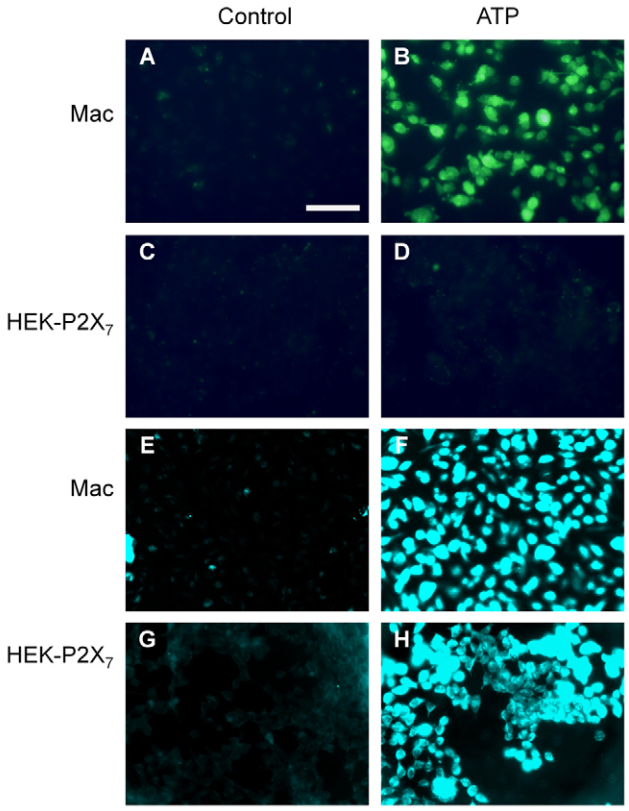

Fig. 4. ATP$_e$ induces the uptake of anionic dyes in macrophages but not in HEK-P2X$_7$ cells. Fluorescence microscopy of cells incubated for 10 minutes at 37°C in the absence (A,C,E,G) or in the presence (B,D,F,H) of 5 mM ATP. LY (3 mM) or MQAE (5 mM) were used as fluorescent dyes. (A,B) Macrophages in the presence of LY; (C,D) HEK-P2X$_7$ cells in the presence of LY; (E,F) Macrophages in the presence of MQAE. (G,H) HEK-P2X$_7$ cells in the presence of MQAE. Each result is representative of at least three independent experiments. Bar, 100 μm.

in LY uptake require Ca^{2+} signaling and correlate well with the changes in the open probability of the Z pores, whereas the uptake of ethidium requires neither the presence of Z pores nor cytoplasmic Ca^{2+} signaling.

ATP$_e$ induces the uptake of anionic dyes in macrophages but not in HEK-P2X$_7$ cells

The above results suggest that anions and cations are taken up by distinct pathways and prompted us to further investigate possible differences between the ATP$_e$-induced dye uptake phenomena of these two cell types. We began by using different anionic and cationic dyes. The anion LY (443 Da) is promptly taken up by macrophages (Fig. 4A,B) but not by HEK-P2X$_7$ cells (Fig. 4C,D). Similar results were obtained with CF, another anion, of a smaller M_r (376 Da; data not shown). We next performed dye-uptake experiments with MQAE, a 246 Da cationic dye used as a Cl^- detector and that does not bind to DNA, being directly visible in the cytoplasmic compartment of the cells (Munkonge et al., 2004). MQAE can be loaded into both macrophages (Fig. 4E,F) and HEK-P2X$_7$ cells (Fig. 4G,H) in a regular ATP$_e$-induced dye uptake assay. These results showed that the uptake of cations is not an exclusive property of DNA-binding dyes and is present in both macrophages and HEK-P2X$_7$ cells, whereas the uptake of anions occurs only in macrophages.

Uptake of anions and not cations through a diffusional pathway

The differences in electrophysiological and dye uptake properties of ATP$_e$-stimulated cells described above suggest that at least two distinct transport mechanisms are activated by ATP$_e$ in P2X$_7$-bearing cells. To further distinguish between these two pathways we decided to investigate the temperature dependence of the dye uptake phenomenon. First, as the effect of ATP$_e$ has been reported to be temperature-dependent (Steinberg et al., 1987a; Nuttle and Dubyak, 1994; Coutinho-Silva and Persechini, 1997; Persechini et al., 1998; Gibbons et al., 2001) we stimulated macrophages and HEK-P2X$_7$ cells with 5 mM ATP for 10 minutes in the presence of either LY or ethidium, at 0, 10, 20, 25, 30 and 37°C. The results showed that

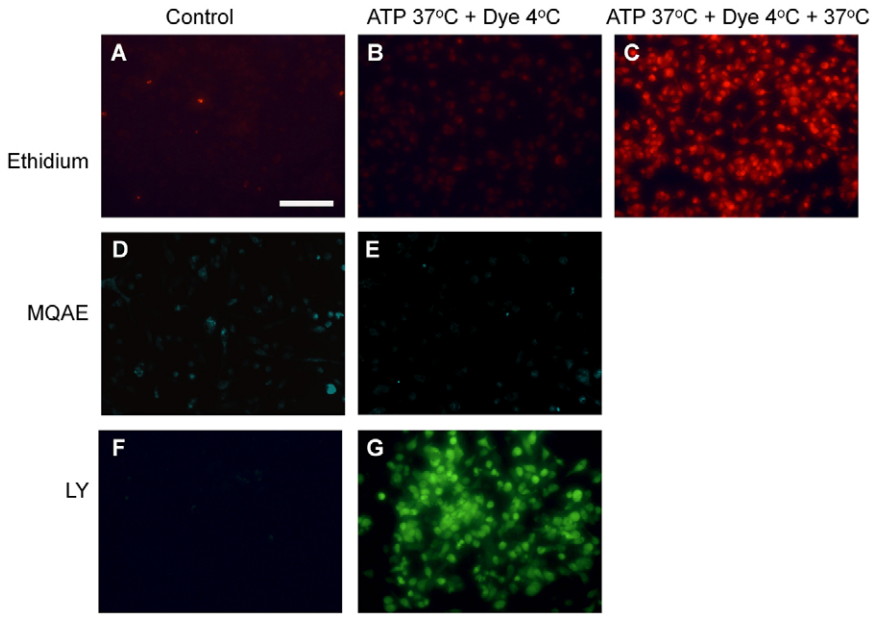

Fig. 5. Uptake of anions but not cations occurs through a diffusion process. Fluorescence microscopy of cells incubated for 10 minutes at 37°C in the absence (A,D,F) or in the presence (B,C,E,G) of 5 mM ATP and cooled on ice in the absence of any dyes. Ethidium bromide (A,B), MQAE (D,E), or LY (F,G) was then added for 15 minutes and the cells were immediately transferred to the stage of the microscope. Temperature was kept all the time below 4°C. In C, cells treated as in B were heated up to 37°C and kept in the presence of ATP_e and ethidium bromide for 10 minutes. Experiments using MQAE were performed in a low chloride solution obtained by substituting Cl^- for glutamate in the normal extracellular solution. Each image is representative of the results of at least three independent experiments performed in duplicates. Bar, 100 μm.

both the uptake of LY or ethidium by macrophages and the uptake of ethidium by HEK-P2X$_7$ cells, decrease in the range 25–20°C and are not observed below 20°C (data not shown). Therefore, anion and cation uptake in both cell types are temperature-dependent, suggesting the involvement of an active mechanism such as an active transport or signaling pathway. However, it is not clear yet whether a single mechanism regulates the uptake of anions and cations.

We next investigated the possibility that, once opened at 37°C, either or both uptake pathways could remain opened if the cells were cooled down in the presence of ATP_e. This feature was uncovered and shown in a recent report where J774 macrophages were loaded with trehalose (a sugar used to protect cells during dehydration) under similar experimental conditions (Elliott et al., 2006). We stimulated macrophages with ATP_e at 37°C for 10 minutes, cooled them down to 4°C on ice, and only then added the dye to the culture plate. Cells were then washed gently with cold medium containing ATP and moved to the stage of the microscope. Under these conditions, any dye uptake taking place at a low temperature would allow us to distinguish between active and passive transport. We observed that ethidium (Fig. 5A,B) and MQAE (Fig. 5D,E) were not taken up by macrophages, whereas LY (Fig. 5F-G) and CF (not shown) were taken up. The result obtained with MQAE indicates that the blockade of ethidium uptake is not an exclusive characteristic of DNA-binding dyes that could also be influenced by any putative cytoplasm-nucleus communication mechanisms. In addition, we also showed that the blockade of cation uptake at low temperature is reversible, since the typical staining was observed after heating the cells to 37°C, again in the continuous presence of ATP_e (Fig. 5C).

The persistence of CF and LY uptake at a low temperature supports that the pathway used by anions to permeate macrophage membranes is by diffusion and is consistent with the presence of a pore that, once activated at 37°C, opens at a low temperature. The lack of permeation of ethidium and MQAE at a low temperature provides additional evidence in support of the hypothesis that the uptake of cations follows a different pathway.

To further differentiate the mechanisms of cation and anion transport we next investigated the efflux of cytoplasmic dyes using LY as an anion (Fig. 6A-D) and sulforhodamine B (SR-B; 559 Da) as a cation (Fig. 6E-H). Cells were initially loaded by the addition of ATP_e (Fig. 6B,F) washed free of the dye and ATP and, after a 10-minute resting period in the absence of any dyes or agonist, re-exposed (Fig. 6D,H) or not (Fig. 6C,G) to ATP_e for an additional period of 10 minutes. We observed that whereas LY leaked out from the cells (Fig. 6D), SR-B remained trapped in the cytoplasm (Fig. 6H). No significant decrease in the fluorescence emitted by SR-B was observed even after 30 minutes in the presence of ATP_e (data not shown) but it could be readily observed after the permeabilization of plasma membranes with 0.01% saponin (data not shown), indicating that it was not retained inside the cell as a result of unspecific binding. Ethidium could not be used in these efflux experiments because of its DNA-binding properties which means that it remains in the nuclei even after addition of saponin (data not shown). Interestingly, the efflux of LY could only be observed in the presence of probenecid, a blocker of anion transporters, required to avoid the uptake of LY into intracellular compartments, as previously described (Steinberg et al., 1987b) and was confirmed by us under our experimental conditions (data not shown).

Besides the differences shown above, the uptake of both cations and anions require the same high doses of the agonist (3 mM ATP_e) to reach 50% of the maximum uptake under our experimental conditions (Fig. 6I).

Uptake of cations in the presence of pannexin-related reagents

We next investigated the effects of two gap-junction inhibitors, mefloquine (MFQ) and carbenoxolone (CBX), as well as ^{10}Pnx1, a pannexin-1 peptide, which have been reported to diminish pannexin-1-associated currents and the P2X$_7$-associated uptake of cationic dyes (Bruzzone et al., 2005; Suadicani et al., 2006; Pelegrin and Surprenant, 2006; Locovei et al., 2007). In these experiments, in addition to the fluorescence microscopy (not shown) we also used a more precise fluorimetric method to quantify the total amount of SR-B taken up by the cells (Fig. 7). CBX was used in the range 10-100 μM, MFQ from 10 to100 nM and the peptide from 100 to 200 μM. Pre-incubation periods varied from 0 to 30 minutes. In none of these conditions did we observe

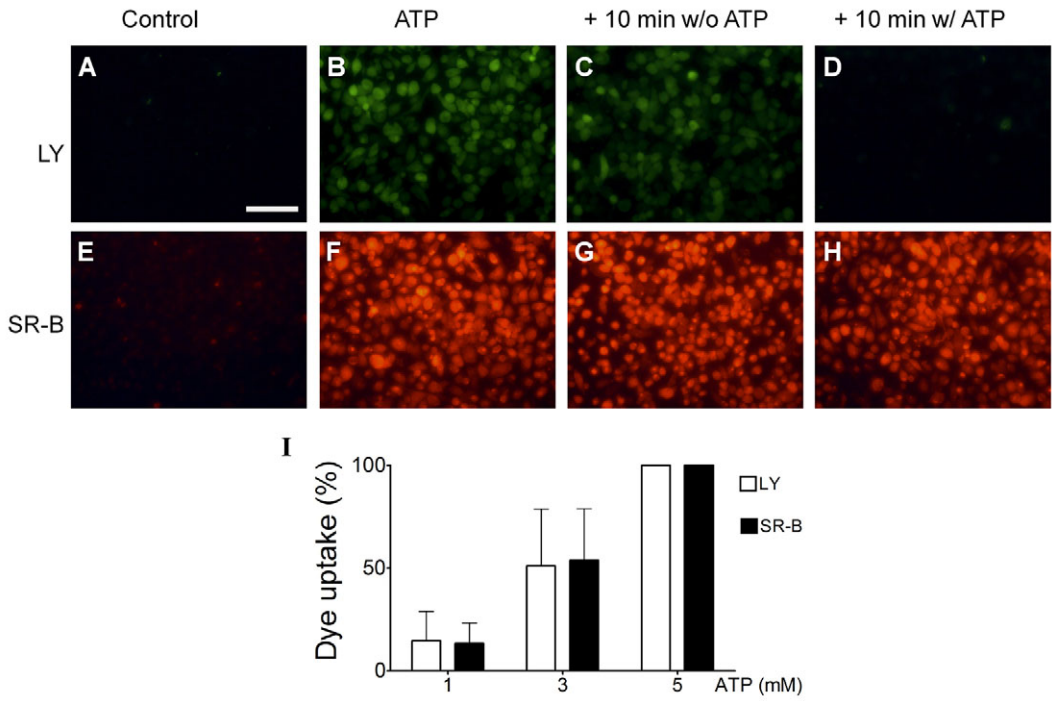

Fig. 6. ATP_e induces the efflux of anions but not cations from macrophages. Fluorescence microscopy of macrophages incubated for 10 minutes at 37°C in the absence (A and E) or in the presence (B-D and F-H) of 5 mM ATP. LY (A-D) or SR-B mM (E-H) was added at 37°C and cells were kept at this temperature until transferred to the stage of the microscope. In B and F, cells were loaded with the dye by incubation for 10 minutes in the presence of one of the dyes and ATP. In C and G cells were prepared as in B and F, respectively, washed, and kept in normal solution without the dye or ATP for an additional period of 10 minutes. In D and H cells were prepared as in B and F, respectively, washed, and kept in normal solution in the presence of 5 mM ATP and without the dye for an additional period of 10 minutes. In the LY experiments (A-D), macrophages were pre-incubated for 30 minutes and kept in the presence of probenecid (5 mM) during all incubations. Images in A-H is representative of the results of at least three independent experiments. Bar, 100 μm. (I) A quantitative comparison of the uptake of LY and SR-B by macrophages. Cells were incubated at 37°C with LY or SR-B for 10 minutes in the presence of 0, 1, 3 and 5 mM ATP and the amount of dye taken up by the cells was measured by spectrofluorimetry as described in the Materials and Methods. Data were normalized for a better comparison of the different data sets. Typical absolute values at 0 and 5 mM ATP were 1.2 and 28 mg of SR-B/mg protein and 0.2 and 1.0 mg of LY/mg protein. The bars represent the means of three independent experiments.

significant inhibition of the uptake of SR-B by any of these substances. Similar results were obtained with HEK-$P2X_7$ cells (not shown).

Discussion

In this work we investigated the uptake of different cationic and anionic dyes and performed patch clamping and intracellular Ca^{2+} measurements to compare ATP_e-induced $P2X_7$-associated phenomena in murine macrophages and HEK-$P2X_7$ cells. ATP_e-induced inward currents and a rise in free cytoplasmic Ca^{2+} concentration were present in both cell types, consistent with previously reported data for $P2X_7$-associated phenomena (Rassendren et al., 1997; Persechini et al., 1998). In addition, HEK-$P2X_7$ cells showed ATP_e-induced dye uptake when probed with cationic dyes such as ethidium and YO-PRO-1, usually referred to as membrane permeabilization or pore formation (Rassendren et al., 1997; Virginio et al., 1997; Virginio et al., 1999).

Besides these similarities, there were also important differences between macrophages and HEK-$P2X_7$ cells. Although the uptake of cationic dyes occurred in both cells, the uptake of anionic dyes took place only in macrophages. Moreover, in cell-attached patch-clamp experiments, only macrophages displayed an ATP_e-induced 440 pS channel (Z pores), previously shown to be associated with $P2X_7$ (Coutinho-Silva and Persechini, 1997; Faria et al., 2004). The ability to take up both cations and anions is not the sole property of murine macrophages since rat peritoneal macrophages (data not shown), rat astrocytes (Duan et al., 2003) and human macrophages (Suh et al., 2001) are also able to do this. Therefore the differences between the uptake capabilities induced by native $P2X_7$ expressed in macrophages and $P2X_7$ transfected into HEK cells are more likely due to differences in the signal transduction cascade and/or in the transport mechanisms.

As a result of these observations we investigated the possibility that the pathway for uptake of anionic dyes involves the opening of Z pores, whereas the uptake of cationic dyes does not. In keeping with this possibility we showed that when dyes were added to ATP_e-activated cells after cooling them down on ice, the uptake of cationic dyes was inhibited in both cell types, whereas anionic dyes still permeated through macrophage membranes. These results support the view that the mechanisms for uptake of anionic dyes are present only in macrophages and involve a diffusional pathway, whereas the uptake of cationic dyes requires a different mechanism. The use of several different dyes (CF, LY, ethidium, YO-PRO-1, MQAE and SR-B), two different methods to assess fluorescence intensity (microscopy and quantitative fluorimetry) as well as efflux experiments, make it difficult to ascribe these results to a different sensitivity of the detection systems for the fluorescence of anionic and cationic dyes. The diffusional nature of the anion pathway was further confirmed by showing that anions, but not cations, can be released from the cells after re-exposure to ATP_e.

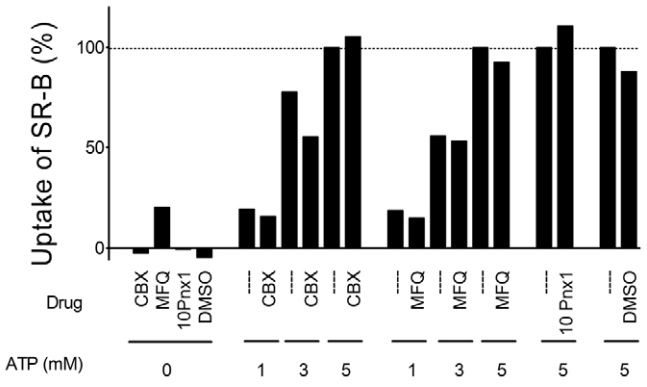

Fig. 7. Uptake of SR-B in the presence of carbenoxolone, mefloquine and ^{10}Pnx1. Representative experiments showing the uptake of SR-B by macrophages in different conditions. Cells were pre-incubated at 37°C with 50 μM carbenoxolone for 5 minutes (CBX), 100 nM mefloquine (MFQ) for 30 minutes, 100 μM ^{10}Pnx1 for 30 minutes, or vehicle (DMSO) in the case of mefloquine and ^{10}Pnx1. ATP (0, 1, 3, and 5 mM) and SR-B were then added for an additional period of 10 minutes and the amount of dye taken up by the cells was measured by spectrofluorimetry as described in the Materials and Methods. Data were normalized for a better comparison of the different data sets. Each result is representative of at least three independent experiments.

These results confirm the original work from Silverstein's group that used anionic dyes to show that ATP$_e$-induced dye uptake is a diffusional process (Steinberg et al., 1987a). However, although this concept has been frequently generalized to all dyes, limited only to their M_r, we could find no similar reports regarding cationic dyes. In keeping with this possibility, we have also shown that both the uptake of anionic dyes and the open probability of Z pores decrease in the absence of divalent cations. It should be emphasized that even though divalent-free extracellular solutions increase the effect of extracellular ATP, possibly by increasing the concentration of free ATP^{4-} ions (Steinberg et al., 1987a), the inhibition observed by us requires the chelation of these divalent ion with 10 mM EDTA; a condition that reduces the concentration of free extracellular Mg^{2+} in our normal extracellular solution from 1 mM to 0.5 μM and the concentration of free Ca^{2+} to 0.5 nM or even less (assuming a maximum of 100 μM residual concentration of this ion). In accordance with this view, it has been shown that the flow of LY from the patch pipette into the cell through attached membrane patches is coincident with the opening of Z pores (Faria et al., 2004). The blockade of the uptake of LY after chelating extracellular Ca^{2+} with EGTA has also been previously reported (Alves et al., 1996), indicating that Ca^{2+} rather than Mg^{2+} is the critical divalent cation. However, the uptake of ethidium was resistant to the chelation of divalent cations, even in cells loaded with BAPTA-AM, a condition that completely abrogates the intracellular Ca^{2+} signaling (Monteiro-da-Cruz et al., 2006). Taken together these results also imply that it is unlikely that Z pores are involved in the uptake of large cationic dyes.

We therefore propose that, besides the small (8-9 pS) and non-selective cation channels directly associated with the P2X$_7$ receptors (Persechini et al., 1998; Riedel et al., 2006), at least two additional transport mechanisms are activated by ATP$_e$ in macrophages: the anion uptake mechanism, which is associated with Z pores and allows the free diffusional flow of anionic dyes through the plasma membrane, and the mechanism for the non diffusional uptake of cations, which is yet to be elucidated. It is interesting to notice that besides the differences, both transport mechanisms require similar high concentrations of ATP$_e$ in the mM range to be activated (Fig. 6I), as previously shown for the activation of Z pores (Coutinho-Silva and Persechini, 1997). These data are consistent with the interpretation that both transport mechanisms and the Z pores are activated by the same low-affinity interaction, a know characteristic of P2X$_7$ receptors.

The molecular nature of these two transport mechanisms remains to be elucidated. Recently, the involvement of pannexin-1 has been suggested, based on immunoprecipitation of P2X$_7$ by anti-pannexin-1 antibodies in HEK-P2X$_7$ cells and by the use of iRNA technique (Pelegrin and Surprenant, 2006; Locovei et al., 2007). Pannexin-1 can form large non-selective transmembrane channels that have some biophysical properties that resemble the Z pores, such as a large unitary conductance (475 pS), a reversal potential of 0 mV, multiple conductance states, and a voltage-dependent gating that favors the opening at positive membrane potentials (Bao et al., 2004; Shestopalov and Panchin, 2007). Moreover, pannexin-1 channels expressed in oocytes can also carry currents for large anions such as ATP^{3-} (504 Da) in inside-out recording patches (Bao et al., 2004), which is consistent with the flux of LY (443 Da). However, we found no single channel studies for the permeability of pannexin-1 unitary channels for large cations. On the other hand, we have previously shown that under cell-attached conditions, the reversal potential of the Z pores of macrophages did not change significantly in solutions where Tris (121 Da) or NMDG (195 Da) were substituted for Na^+, glutamate (146 Da) was substituted for Cl^- or mannitol was partially substituted for NaCl (Coutinho-Silva and Persechini, 1997). These results are consistent with both cation and anion permeability but more experiments are needed to examine the selectivity for larger molecules, the opening probability and other properties of these pores. Taken together, our data are consistent with the possibility that the Z pores of macrophages are involved in the uptake of anionic dyes but are difficult to reconcile with the possibility that the same pores mediate the uptake of cationic dyes.

Our experiments were not conclusive regarding the role of pannexins, since we did not observe any significant inhibition of the uptake of cations when cells were treated with CBX, MFQ or ^{10}Pnx, three reagents that have been reported to diminish pannexin-1-associated currents and the P2X$_7$-associated uptake of cationic dyes (Bruzzone et al., 2005; Suadicani et al., 2006; Pelegrin and Surprenant, 2006; Locovei et al., 2007). However, it should be noticed that important differences have also been reported regarding the action of these drugs on P2X$_7$-associated phenomena. MFQ had no effect on the uptake of YO-PRO-1 in HEK cells and 1321-N1 cells transfected with P2X$_7$ (Pelegrin and Surprenant, 2006) and CBX did not inhibit the uptake of ethidium macrophages (Faria et al., 2004). In addition, even in studies where CBX inhibited the uptake of YO-PRO-1, one group reported the inhibition of Ca^{2+} transients in 1321-N1 cells (Suadicani et al., 2006), whereas another group observed no inhibition of either the Ca^{2+} transients or the P2X$_7$-specific whole cell currents (Pelegrin and Surprenant, 2006). Moreover, the mechanism of action and the specificity of connexin- and pannexin- mimetic peptides have been questioned (Wang et al., 2007). The different experimental conditions that might generate all these differences are not clear at the moment.

Therefore, although we do not exclude the involvement of pannexins in the P2X$_7$-coupled uptake of anionic and cationic dyes, more studies are necessary to understand the precise role of these newly described channel-forming proteins. Our data show that the transport mechanisms associated with P2X$_7$ are more complex than

previously thought, and the possibility that this receptor might couple to other transport mechanisms and channel-forming proteins such as connexins (Fortes et al., 2004; Saez et al., 2005; Iacobas et al., 2007) also deserves further experimental analysis.

Irrespective of the molecular identity of Z pores and the role they play in the phenomena of ATP_e-induced dye transport, its role in $P2X_7$-associated phenomena and in macrophage physiology also deserves further investigation. Their large and non-selective conductance together with their strong voltage dependence make them potential regulators of membrane potential, osmotic balance and transport of small molecules.

Our results highlight the need in any studies aimed to elucidate $P2X_7$-associated phenomena and their roles in macrophage physiology, that the transport of large cationic and anionic molecules, as well as Z pores and the small cation channels should be independently analyzed. It would also be interesting to investigate to what extent these observations could be applied to other $P2X_7$-expressing cells such as lymphocytes, astrocytes, mastocytes and dendritic cells.

These results open new possibilities in the planning of P2-based strategies to intervene in the immune system, as each one of these phenomena could have different consequences in different situations, such as intracellular infection by pathogenic agents, differentiation, cell death, drug transport through cell membranes, cytokine secretion and the activation of the inflammasome.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin and streptomycin were obtained from Gibco-BRL (São Paulo, SP, Brazil). Thioglycollate medium was from Difco (Detroit, MI, USA). NaCl, $MgCl_2$, $CaCl_2$, KCl, methanol, Trizma-base, glycine and glycerol were from Reagen (Rio de Janeiro, RJ, Brazil). ATP, EGTA, EDTA, Tween 20, sodium dodecyl sulfate (SDS), periodate-oxidized ATP (oxATP), ethidium bromide, sucrose, poly-L-lysine, Hepes, dimethyl sulfoxide (DMSO), sulforhodamine B (SR-B), Lucifer Yellow (lithium salt), mefloquine (MFQ) and cabenoxolone (CBX) were purchased from Sigma-Aldrich (St Louis, MO, USA). BAPTA-AM, YO-PRO-1, Fura-2-AM, MQAE (*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide) and probenecid were from Molecular Probes (Eugene, OR, USA). Lipofectamine-2000, pcDNA6/V5-His A and blasticidin S HCl were from Invitrogen (São Paulo, SP, Brazil). Bovine serum albumin was from USB Corporation (Cleveland, Ohio, USA), and 5(6)-carboxyfluorescein from Eastman Kodak (Rochester, NY, USA). Polyvinylidene difluoride (PVDF) membranes, horseradish peroxidase-conjugated anti-mouse-IgG antibody, Bradford reagent and an enhanced chemiluminescence (ECL-Plus) kit were from Amersham Pharmacia Biosciences (São Paulo, SP, Brazil). Nonfat dry milk was obtained from Molico (Rio de Janeiro, RJ, Brazil). Anti-$P2X_7$ antibody was purchased from Alomone Labs (Jerusalem, Israel). Phenylmethylsulfonyl fluoride, leupeptin and aprotinin were from Biochemica Boehringer Mannheim (Mannheim, Germany). Precision Plus Protein Standards, Kaleidoscope, was from Bio-Rad (São Paulo, SP, Brazil). The pannexin-1 peptides, ^{10}Pnx1 (WRQAAFVDSY) (Pelegrin and Surprenant, 2006) was synthesized by GenScript (GenScript Corporation, Piscataway, NJ, USA).

Animals

Swiss-Webster mice were obtained from the animal facilities of the Instituto de Microbiologia Paulo de Goes and from the Transgenic Animal Facility of the Instituto de Biofísica Carlos Chagas Filho of the Federal University of Rio de Janeiro. All animals were 8- to 12-weeks old, weighed approximately 16-30 g and were handled according to the guidelines for animal use in scientific experiments of the Instituto de Biofísica Carlos Chagas Filho of the Federal University of Rio de Janeiro.

Macrophage isolation and culture

Thioglycollate-elicited macrophages were obtained from the intra-peritoneal cavity of mice, collected 4 days after thioglycollate injection, as previously described (Coutinho-Silva and Persechini, 1997). In brief, cells were washed in DMEM and kept on ice at a concentration of 10^6 cells/ml. For permeabilization assays and electrophysiological experiments, cells were plated on 35 mm culture dishes at a concentration of 2×10^5 cells/dish in 2 ml DMEM supplemented with 10% fetal bovine serum, 2 g/l sodium bicarbonate, 0.3 mg/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were then removed after 1 hour and the macrophages were kept for 4 days under the same culture conditions. For calcium measurements, cells were plated on glass coverslips at a concentration of 5×10^5 cells/dish.

Stable expression of $P2X_7$ in HEK-293 cells

The rat $P2X_7$ cDNA was a generous gift from Dr Alan North (Surprenant et al., 1996) and was subsequently sub cloned into pcDNA6/V5-His A. For transfection, HEK-293 cells were maintained in DMEM containing 10% fetal bovine serum and plated at 70-80% confluence. About 30 μg of linearized plasmid was used to transfect cells with Lipofectamine 2000, following the manufacturer's instructions. The next day the medium was replaced and 5 μg/ml blasticidin S HCl was introduced to select for transfected cells. One week after transfection, with the medium being replenished every 2 days, a few clones were picked with a sterile tip and placed individually in a well of a 12-well plate. After expansion of the clones they were assayed for permeabilization with ATP and ethidium bromide and the best responses selected.

Culture of HEK-293 cells

Cells were plated in poly-L-lysine-coated 35 mm culture dishes in 2 ml DMEM complete medium at 37°C in a humidified atmosphere containing 5% CO_2. The HEK-$P2X_7$ cell medium was supplemented with 5 μg/ml blasticidin S HCl.

Cellular extracts

Cells were lysed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 250 mM sucrose, 17 μg/ml phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupeptin and 25 μg/ml aprotinin) by 30 passages through a 28 gauge needle, and kept on ice for a few minutes until used as described below.

Anti-$P2X_7$ western blots

Cell extracts prepared as above, were mixed with a gel loading solution (250 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.02% bromophenol, 10% β-mercaptoethanol), boiled and 10 μg of total protein per lane were separated by electrophoresis using 10% SDS-PAGE and transferred to PVDF membranes using standard protocols. The protein concentration in the extracts was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as standard. Membranes were then incubated with a blocking buffer consisting of TTBS (50 mM Tris, 200 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% nonfat dry milk for 2 hours at room temperature and then incubated overnight with anti-$P2X_7$ C-terminal antibody (diluted 1:200) at 4°C. After three rinses in TTBS for 5 minutes each, membranes were incubated for 1 hour with horseradish peroxidase-conjugated goat anti-IgG (1:2000) diluted in blocking buffer. Membranes were then washed three times for 10 minutes in TTBS, and bound antibodies were detected by ECL-plus reaction according to the manufacturer's instructions.

Dye uptake and efflux assays

Unless otherwise specified, cells were kept at 37°C for 5 minutes, in a solution containing (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, and 10 Na-Hepes, pH 7.4 (normal extracellular solution). Ethidium bromide (10 μM final concentration) or YO-PRO-1 (25 μM), carboxyfluorescein (5 mM), SR-B (3 mM), or LY (3 mM) and ATP (5 mM) were then added and the cells were kept under the same conditions for an additional period of 10 minutes (macrophages) or 15 minutes (HEK cells). In some experiments, cells were pre-incubated with the indicated drugs for a period of 0-30 minutes, as specified in the figure legends. The drugs were prepared in 100-fold-concentrated stock solutions prepared in the normal extracellular solution (carbenoxolone) or in DMSO (mefloquine and ^{10}Pnx1) and kept frozen until use. In assays requiring BAPTA-AM-loaded cells, macrophages were first prepared as described below for 'Intracellular calcium measurements'. In experiments using the Cl^- indicator MQAE, glutamate was substituted for Cl^- in the bathing solution. The dye uptake was then determined either by fluorescence microscopy or by quantitative spectrofluorimetry as described below.

Fluorescence microscopy was performed using an Axiovert 100 microscope (Karl Zeiss, Oberkochen, Germany) equipped with an HBO lamp, an Olympus digital camera (Olympus American Inc., PA, USA) and Image-pro plus v 6.2 software (Media Cybernetics, Inc. Bethesda, MD, USA). In the experiments with MQAE, we used an Axiovert 200 microscope equipped with an HBO lamp, an Axio Cam MRn digital camera and Axiovision v 4.1 software (Karl Zeiss, Oberkochen, Germany). In all fluorescence microscopy observations, cells were also observed with clear field illumination (not shown in the figures) and at least 50 macrophages were present in each microscope field studied.

Quantitative spectrofluorimetry (Figs 6 and 7) was performed in experiments with LY and SR-B using an FLX-800 plate reader (BioTek Instruments Inc., Winooski, VT, USA) according to the following protocol. The cells were gently washed five times with PBS, lysed by the addition of 100 μl PBS containing 0.01% BSA and 0.05% Triton X-100, scraped off the plate, and used for fluorescence determination using the following excitation and emission wavelengths ranges (nm): 420-450 and 528-520 for LY, and 516-520 and 620-640 for SR-B. Protein concentrations were determined by the Bradford method and the results were expressed as mg of dye/mg protein.

Electrophysiology

Macrophages and HEK-293 cells were plated in 35 mm plastic culture dishes for 3-5 days as described above. Before the experiment, the culture medium was exchanged for one of the salt solutions described below: unless otherwise specified, experiments were performed in normal solution (composition defined above) at –40 mV holding potential. In some experiments, Tris-HCl was used in place of NaCl (low Na^+ solution) or 10 mM EDTA was added to the normal solution (divalent-free solution), as specified in the text and legends. Ionic currents were recorded in whole cell or cell-attached configurations, using an EPC-7 amplifier (List Electronic, Darmstadt, Germany) according to standard patch-clamping techniques (Hamill et al., 1981). Giga-ohm seals were formed after offset potential compensation, using heat-polished micropipettes of 5–10 MΩ filled with a solution of (in mM): 135 KCl, 5 NaCl, 2 $MgCl_2$, 0.1 K-EGTA, and 10 K-Hepes, pH 7.4, in the whole cell experiments, and with the same extracellular solution in cell-attached experiments. ATP was applied to the cell surface by pneumatic injection, using a second micropipette filled with ATP, at the indicated concentration, dissolved in extracellular solution and connected to a PPM-2 pneumatic pump (NeuroPhore BH-2 system, List). Data were collected using pClamp and Fetchex software, version 6.0, and a Digidata 1200 interface (Axon Instruments, USA) and plotted using Origin software (Microcal Inc, USA, version 4.0).

Intracellular calcium measurements

Macrophages were plated on glass coverslips for 3-5 days and loaded with 5 μM Fura-2 AM with or without 10 μg/ml BAPTA-AM for 30 minutes at room temperature in Hepes-buffered culture medium containing 2.5 mM probenecid. The cells were then washed twice and placed in a three-compartment superfusion chamber the base of which was formed by the coverslip containing the cells. The central chamber containing the cells had a volume of 200 μl, and was perfused at a rate of 1 ml/minute. The perfusion solution contained (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 10 Na-Hepes, pH 7.4; and either 1 mM $CaCl_2$ or 1 mM EGTA. This solution was pre-heated to reach 37°C at the perfusion chamber. Cytoplasmic calcium concentrations of groups of 20–40 cells were monitored continuously at 37°C with the use of a fluorescence photometer (Photon Technology, Princeton, NJ, USA). Fura-2 was excited alternately at 340 and 380 nm, and the emission at 510 nm was measured. The ratio measurement, which is proportional to the cytoplasmic calcium concentration, was determined every 100 ms. ATP application was via continuous perfusion of the same solution containing the indicated concentrations of the drug (Bisaggio et al., 2001).

Data analysis

Differences between experimental groups were evaluated by the two-tailed unpaired Student's *t*-test. Each experiment was performed at least three times in duplicate. Data were analyzed using GraphPad InsTat software (GraphPad Software Inc., version 4.0). Values are mean ± s.d. The quantification of the fluorescence intensity was performed using the ImageJ 1.38X program (National Institute of Health, USA). The calculation of the concentration of ATP^{4-} was performed using the Sliders v2.00 program (Chris Patton, Stanford University, CA, USA).

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