Modulation of intercellular communication in macrophages: possible interactions between GAP junctions and P2 receptors

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Summary

Gap junctions are connexin-formed channels that play an important role in intercellular communication in most cell types. In the immune system, specifically in macrophages, the expression of connexins and the establishment of functional gap junctions are still controversial issues. Macrophages express $P2X_7$ receptors that, once activated by the binding of extracellular ATP, lead to the opening of transmembrane pores permeable to molecules of up to 900 Da. There is evidence suggesting an interplay between gap junctions and P2 receptors in different cell systems. Thus, we used ATP-sensitive and -insensitive J774.G8 macrophage cell lines to investigate this interplay. To study junctional communication in J774-macrophage-like cells, we assessed cell-to-cell communication by microinjecting Lucifer Yellow. Confluent cultures of ATP-sensitive J774 cells (ATP-s cells) are coupled, whereas ATP-insensitive J774 cells (ATP-i cells), derived by overexposing J774 cells to extracellular ATP until they do not display the phenomenon of ATP-induced permeabilization, are essentially uncoupled. Western-blot and reverse-transcription polymerase chain reaction assays revealed that ATP-s and ATP-i cells express connexin43 (Cx43), whereas only ATP-s cells express the $P2X_7$ receptor. Accordingly, ATP-i cells did not display any detectable ATP-induced current under whole-cell patch-clamp recordings. Using immunofluorescence microscopy, Cx43 reactivity was found at the cell surface and in regions of cell-cell contact of ATP-s cells, whereas, in ATP-i cells, Cx43 immunoreactivity was only present in cytosolic compartments. Using confocal microscopy, it is shown here that, in ATP-s cells as well as in peritoneal macrophages, Cx43 and $P2X_7$ receptors are co-localized to the membrane of ATP-s cells and peritoneal macrophages.

Key words: J774 macrophage-like cells, Connexin43, $P2X_7$ receptor, ATP, Gap junction

Introduction

Gap junctions are clusters of intercellular channels that connect the intracellular milieu of adjacent cells and allow the passage of molecules of up to ~1 kDa (Bennett and Goodenough, 1978). In vertebrates, these channels are formed by a family of proteins termed connexins (Cxs). At present, 19 Cxs have been identified in rodents and named according to their molecular masses as predicted from the cloned DNA sequences (Willecke et al., 2002). Each cell of an adjacent pair contributes with six Cxs to form a hemi-channel or connexon, and the pairing of two connexons forms the gap-junction channel (Makowski et al., 1977; Kumar and Gilula, 1996; Rouach et al., 2002; Evans and Martin, 2002). According to their Cx composition, gap-junction channels show different gating and permeability properties (Dhein, 1998). These channels are regulated by several agents, including voltage, intracellular calcium and pH, adhesion proteins, extracellular matrix, and soluble factors (hormones, cytokines, growth factors and neurotransmitters) (Campos-de-Carvalho, 1988; Mehta et al., 1989; Spray et al., 1982; Spray and Burt, 1990; Rouach et al., 2002; Evans and Martin, 2002).

The contribution of gap junctions to intercellular communication is well established in several tissues and organs (Simon and Goodenough, 1998). Even though reports of junctional communication between lymphocytes were published in the early 1970s (Hulser and Peters, 1972; Oliveira-Castro et al., 1973) and the presence of Cxs has since been demonstrated in various lympho-hematopoietic organs (Alves et al., 2000; Saez et al., 2000), its presence and role are more elusive and remain controversial issues in the immune system.

In macrophages, in which the expression of Cx43 has been demonstrated (Beyer and Steinberg, 1991; Polacek et al., 1993; Jara et al., 1995; Alves et al., 1996), there are conflicting reports regarding the occurrence of functional gap-junction channels or hemi-channels (Polacek et al., 1993; Alves et al., 1996; Porvaznik and MacVittie, 1979; Martin et al., 1998). This might result from different experimental strategies and the

fact that the expression and function of Cxs in these cells seems to be modulated by the inflammatory response. In this regard, Eugenin et al. (Eugenin et al., 2003) have recently described the expression of Cx43 in macrophages stimulated by a combination of lipopolysaccharide (LPS), tissue necrosis factor α (TNF-α) and interferon γ (IFN-γ). Therefore, molecules that modulate the secretion of cytokines might directly or indirectly influence coupling in cells of the immune system in autocrine or paracrine fashion.

In recent years, evidence has been accumulated suggesting that the purinergic receptors can modulate the inflammatory cascade in cells of myeloid and non-myeloid origin. In human macrophages, ATP has been shown to be a necessary co-factor for the secretion of interleukin 1 (IL-1) induced by LPS (Griffiths et al., 1995; Ferrari et al., 1997). These studies showed that $P2X_7$ receptors are involved in the ATP-mediated effect. These receptors are expressed in many cell types and tissues, including cells of the immune system such as lymphocytes, macrophages, phagocytic cells of the thymic reticulum and dendritic cells (North and Surprenant, 2000; Ralevic and Burnstock, 1998; Di Virgilio et al., 2001; Persechini et al., 1998; Coutinho-Silva et al., 1999). In particular, macrophages express $P2X_7$ receptors that mediate ATP-induced permeabilization of the plasma membrane, which is characterized by the opening of transmembrane pores that allow the passage of molecules of up to 900 Da (Steinberg et al., 1987; Dubyak and El-Moatassim, 1993; Surprenant et al., 1996; Persechini et al., 1998).

It has been shown that intercellular calcium-wave propagation in many cell types involves a fine interplay between Cxs and P2-receptor-mediated processes. In astrocytes, in particular, down-modulation of gap-junctional communication using cytokines or gene targeting has resulted in altered expression of P2 receptor subtypes (Scemes et al., 2000). Interplay between P2 receptors and gap junctions in intercellular communication has also been proposed in Hensen's cells (Lagostena et al., 2001) and in airway epithelia (Homolya et al., 2000).

Because high levels of ATP can be released from sources such as platelets, astrocytes, activated leukocytes and dying cells in the course of an inflammatory response, and, in addition, ATP can be released spontaneously by macrophages (Beigi and Dubyak, 2000; Sperlagh et al., 1998; Bodin and Burnstock, 2001; Di Virgilio et al., 2001), a similar interplay might exist in these cells. Therefore, although any direct involvement of Cx43 hemichannels in ATP-induced permeabilization in macrophages (Beyer and Steinberg, 1991) seems unlikely (Alves et al., 1996), there might be a relationship between P2 receptor and Cx expression in macrophages. In this paper, we use the J774.G8 macrophage cell line to examine this question.

Materials and Methods

Cell culture

The mouse macrophage cell line J774.G8 was obtained from the laboratory of W. De Souza (De Carvalho and De Souza, 1986; De Carvalho and De Souza, 1983) and is derived from the original J774.A1 from the American Type Culture Collection (ATCC, Rockville, MD). This cell line was grown in complete RPMI 1640 medium (Sigma Chemical Company, St Louis, MO) supplemented with 10% fetal calf serum (GIBCO-Life Technologies, Rockville, MD), 2 g $l^{-1}NaHCO_3$, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, pH 7.4, on glass cover slips, 35 mm plastic and 25 cm^2 plastic culture flasks (Corning, USA) at 37°C in a 5% CO_2 humidified atmosphere. Because these cells express $P2X_7$ receptors and show ATP-induced permeabilization (Steinberg et al., 1990; Surprenant et al., 1996), they are here called ATP-sensitive J774.G8 cells (ATP-s cells). ATP-insensitive J774.G8 cells (ATP-i cells) were prepared by repeated overexposure of ATP-s cells to increasing doses of ATP using a modification of a protocol originally described by Steinberg and Silverstein (Steinberg and Silverstein, 1987). Briefly, J774.G8 cells were grown for 2-3 days after plating and then exposed to increasing concentrations of ATP (1-10 mM) in Ca^{2+}- and Mg^{2+}-free PBS, pH 7.4, for 30 minutes at 37°C. Floating cells were removed and the surviving cells were allowed to proliferate for another 2-3 days in complete RPMI medium and again exposed to the same ATP concentration until no signs of cell suffering (cell blebbing and detachment) could be observed. ATP concentration was then increased (1 mM, 2 mM, 4 mM, 5 mM, 10 mM) and the process was repeated. Periodically, cells were tested for ATP-induced membrane permeabilization using ethidium-bromide fluorescence as a marker, as described previously (Steinberg et al., 1987; Persechini et al., 1998). Cells usually became ATP insensitive after a period of 6-8 weeks, as judged by the absence of either ATP-induced blebbing or permeabilization. In all experiments herein described, both ATP-s and ATP-i cells were used 48-72 hours after plating at a concentration of 3×10^6 cells ml^{-1}.

Primary cultures of thioglycolate-elicited peritoneal macrophages, used in confocal microscopy, were obtained from the peritoneal cavity of Swiss-Webster mice 4 days after intraperitoneal injection of thioglycolate medium (Difco Laboratories, MI), as previously described (Albuquerque et al., 1993).

Reagents

Unless otherwise specified, all reagents were purchased from Sigma Chemical Company (St Louis, MO).

Isolation of total RNA

Total RNA from 10^6 J774.G8 cells was extracted using Trizol® reagent (Gibco BRL, Grand Island, NY). RNase-free DNase I (1 U μl^{-1}) was used to treat the isolated RNA for 1 hour to eliminate contamination with genomic DNA. The treatment was terminated by extraction with phenol/chloroform/isoamyl-alcohol (PCI) in the proportion 25/24/1 (vol/vol) followed by precipitation with ethanol.

Reverse-transcriptase polymerase chain reaction

In order to prepare first-strand DNA for reverse-transcription polymerase chain reaction (RT-PCR), total RNAs isolated from ATP-s and ATP-i cells (n=4) were reverse transcribed with SuperScript™ (Gibco BRL) at 37°C for 60 minutes, extracted with PCI and precipitated with ethanol. The polymerase chain reaction (PCR) technique was used to amplify the synthesized cDNA. The following solution was used: 0.2 μmol l^{-1} each primer, 0.2 mmol l^{-1} each dNTP, 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-Cl (pH 8.3), 1.5 mmol l^{-1} $MgCl_2$, 2.5 U Amplitaq (Perkin Elmer, New Jersey). 2 μg of each sample was submitted to PCR using specific primers designed for Cx43 (sense, 5′-ATCCAGTGGTACATCTATGG-3′; antisense, 5′-CTGCTGGCTCT-GCTGGAAGG-3′) or the $P2X_7$ receptor (sense, 5′-GGCAGTTC-AGGGAGGAATCATGG-3′; antisense, 5′-AAAGCGCCAGGTGG-CATAGCTC-3′). Two different sets of primers for GAPDH were used: set 1 (sense, 5′-ATCACCATCTTCCAGGAGCG-3′; antisense, 5′-CCTGCTTCACCACCTTCTTG-3′) was used for experiments involving $P2X_7$ receptor; set 2 (sense, 5′-CTTGTCATCAAT-GGGAAG-3′; antisense, 5′-GTCATGGATGACCTTGGCCG-3′) for experiments involving Cx43. This gave rise to 573 bp and 211 bp

products, respectively. Mock reactions consisted of the same reactions with RNA instead of cDNA. Amplification was carried out for 34 cycles of denaturation (94°C, 1 minute), annealing (55°C, 1 minute) and extension (72°C, 1 minute) for Cx43 or 35 cycles of denaturation (94°C for 1 minute), annealing (55°C for 2 minutes), and extension (72°C for 2 minutes) for the P2X$_7$ receptor. The PCR products were resolved in 2% or 1% agarose gels and photographed using the Kodak EDAS 120 System (Eastman Kodak Company, Rochester, NY).

The semiquantitative method of RT-PCR used for Cx43 mRNA expression studies in ATP-s and ATP-i cells was validated in preliminary experiments. First, the optimal PCR conditions that yielded a single band on agarose-gel electrophoresis were determined for Cx43 and GAPDH genes in the same reaction tube. Second, to determine whether the method was semiquantitative, serial quantities of total RNA (62.5 ng, 125 ng, 250 ng, 500 ng, 1000 ng, 2000 ng) extracted from J774.G8 cells were used for RT-PCR amplification for Cx43 and GAPDH genes in the same reaction tube. Third, experiments were performed to determine the optimal number of PCR cycles that yielded PCR products in the linear phase of amplification of both genes in the same reaction. Finally to ensure that the reactions were consistent, PCR reactions were performed at least twice. Only one of these reactions was included for final densitometry analysis, and the selection was arbitrary. For the semiquantitative PCR comparing the ATP-s and ATP-i cells, the optimal total RNA amount was 500 ng and the optimal number of PCR cycles was 34. The identity of the amplification products was confirmed by agarose-gel electrophoresis with 100 bp DNA molecular marker (Gibco BRL). The Cx43 and GAPDH bands from the same sample were submitted to a densitometry analysis using the gel analysis software Sigma Gel (v1.1; Jandel Scientific, USA) and normalized by dividing the Cx43 values by the corresponding GAPDH values.

Immunoblotting

ATP-s and ATP-i cells were plated on 25 cm^2 plastic culture flasks under the same conditions described above. Homogenates were obtained by scraping cells in 1 mM $NaHCO_3$ (pH 8.3) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) diluted in ethanol. The samples were sonicated (Brenson Sonifier 450; Branson Ultrasonics) three times for 30 seconds each. After centrifugation at 10,000 rpm (model Eppendorf 5415C; Eppendorf Netheler-Hinz, Hamburg, Germany) for 10 minutes, the pellet was discarded and the total protein content of the supernatant was determined by the Bradford method (Bradford, 1976) and 100 μg of each sample were loaded in each lane of a 10% polyacrylamide gel and subjected to SDS-PAGE under reducing conditions.

Protein was then transferred onto a nitrocellulose membrane and the membrane was blocked with a PBS solution containing 5% nonfat dry milk and 0.5% Tween 20, and probed with either a polyclonal antibody specific for amino acids 346-360 of the Cx43 sequence (kindly provided by E. Hertzberg, Aecom, New York, NY) (Hertzberg et al., 2000) or a polyclonal antibody specific for amino acids 576-595 of the rat P2X$_7$ protein (Alomone Labs, Jerusalem, Israel). Blots were incubated with primary antibody for Cx43 at 1:1000 or with primary antibody for P2X$_7$ at 1:300 for 2 hours, and with secondary antibody (anti-rabbit IgG 1 mg ml^{-1} conjugated with alkaline phosphatase) (Sigma Chemicals) at 1:1000 dilution for 1 hour. Extracts from murine brain tissue and liver homogenates were processed similarly and used as positive and negative controls for Cx43, respectively. Extracts from elicited peritoneal macrophages were used as positive control for P2X$_7$. Densitometry was performed using the gel analysis software Sigma Gel (v1.1, Jandel Scientific, USA). Results are presented as means±s.d.

Immunocytochemistry

ATP-s and ATP-i cells were plated on glass coverslips and fixed with 70% ethanol for 20 minutes at –20°C. Cx43 was detected as described previously for other cell types (Dermietzel et al., 1984). In short, cells were first incubated for 30 minutes at room temperature with 2% IgG-free bovine serum albumin (BSA) to reduce non-specific binding. This was followed by incubation with the same polyclonal antibody for Cx43 described above (diluted 1:1000) for 1 hour at room temperature. The cells were incubated for 1 hour at room temperature with a goat anti-rabbit secondary antibody (Sigma Chemicals; diluted 1:400) conjugated to FITC. The cover slips were then washed four times for 10 minutes each with PBS and mounted with 10 μl of 5% *p*-phenylenediamine (50 mg dissolved in a solution containing 900 μl glycerol and 100 μl PBS). Fluorescence was observed on a Zeiss Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany).

In another set of experiments, cells (ATP-s and thioglycolate-elicited intraperitoneal macrophages) were double labeled with a mouse anti-Cx43 antibody (1:200; Chemicon International, USA) and with a polyclonal antibody against P2X$_7$ (1:200; Alomone Labs, Jerusalem, Israel). A goat anti-mouse secondary antibody conjugated to FITC (1:200, Sigma Immunochemicals, USA) and a Cy3-conjugated goat anti-rabbit (1:800; Jackson Lab, West Grove, PA) were used to label the primary antibodies. The nuclei of the peritoneal macrophages were labeled with TOPRO3 (1:1000; Molecular Probes, USA). Confocal images and *z*-axis sections were obtained using a LSM 510 Meta Zeiss confocal microscope (Carl Zeiss, Oberkochen, Germany).

The specificity of the immunofluorescent staining was assessed for each experimental condition by performing the reaction in the absence of primary antibodies. No staining was observed under such conditions.

Immunoprecipitation

Total cell lysates of confluent cultures of ATP-s cells and peritoneal macrophages were incubated for 1 hour at 4°C with mouse monoclonal anti-Cx43 antibody (Chemicon International, USA). Appropriate amounts of immobilized protein G were then added to the antigen-antibody complex and incubated overnight with gentle mixing at 4°C. After several washes of the immobilized protein-G-bound complexes, antigen-antibody complexes were eluted from the immobilized protein G with 4% SDS containing dithiothreitol (DTT) and β-mercaptoethanol. Immunoprecipitated samples were electrophoresed and proteins transferred to nitrocellulose membranes as described above. Blots were incubated with primary antibody for P2X$_7$ at 1:300 for 2 hours and with secondary antibody (anti-rabbit IgG 1 mg ml^{-1} conjugated with alkaline phosphatase) (Sigma Chemicals) at 1:1000 dilution for 1 hour.

Dye transfer

Confluent cultures of ATP-s and ATP-i cells plated on 35 mm Petri dishes, were injected with Lucifer Yellow CH (5% in 150 mM LiCl) (457.2 Da), rhodamine dextran (40 kDa) or ethidium bromide (394.3 Da) using glass microelectrodes (resistance between 40-70 MΩ) by the application of short hyperpolarizing current pulses (0.1 nA, 100 milliseconds using a WPI amplifier, model 7060; USA). Fluorescence was observed on an Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany) equipped with appropriate filters (Zeiss BP450-490 / FT510 / LP520) and photographed were taken using Kodak Gold 400 film, 2 minutes after dye injection (Srinivas et al., 1999). In measuring the degree of coupling, a minimum of 150 cells was injected for each cell type in at least five independent experiments. Coupling degree was arbitrarily divided into three classes: no coupling (0); one to three neighbor cells coupled to the injected cell; and four to six neighbors coupled to the injected cell with Lucifer Yellow. Results are presented as mean±s.d.

Electrophysiology

Cells were plated at low density in 35 mm plastic culture dishes in culture medium. Before the experiment the cells were transferred to an extracellular solution containing in mM: 150 NaCl, 5 KCl, 1 $MgCl_2$ and 10 Na-HEPES, pH 7.4. Ionic currents were recorded in whole-cell configuration, using an EPC-7 amplifier (List Electronic, Darmstadt, Germany) according to standard patch-clamping techniques (Hamill et al., 1981). GΩ seals were formed after offset potential compensation, using heat-polished micropipettes of 5-10 MΩ filled with an internal solution (150 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 0.1 mM K-EGTA, 10 K-HEPES, pH 7.2). ATP was applied to cell surface by iontophoresis, using a second micropipette filled with 10 mM ATP (solubilized in deionized distilled water) and connected to a 100 V power supply. Data were collected using pClamp and Fetchex software, and a Digidata 1200 interface (Axon Instruments, USA) and plotted using Origin software (version 4.0; Microcal, USA).

Statistical treatment

One-way analysis of variance followed by Newman-Keuls multiple comparison test was used in Fig. 1B and Fig. 5. Data from Figs 3 and 4 were evaluated by Student's unpaired *t*-test, and considered to be statistically significant when $P<0.05$.

Results

Characterization of $P2X_7$-receptor expression

We first investigated the expression of $P2X_7$ receptors in ATP-s and ATP-i cells (Fig. 1). RT-PCR and western-blot analysis performed in both types of cell showed that $P2X_7$ mRNA and protein could be detected only in ATP-s cells (Fig. 1A,B). To characterize further the state of unresponsiveness to ATP, we compared the whole-cell currents triggered by extracellular ATP in both types of cell (Fig. 1C,D). ATP-s cells respond to ATP application by a cation-selective inward current followed by an outward Ca^{2+}-activated K^+ current (Fig. 1C), as already described for macrophages and other $P2X_7$-expressing cells (Albuquerque et al., 1993; Coutinho-Silva et al., 1996). However, ATP-i cells display no detectable currents even after repeated stimulation (Fig. 1D), in accordance with the RT-PCR and western-blot analysis demonstrating lack of $P2X_7$ molecules in these cells.

Dye coupling

In order to investigate the presence of functional gap-junction communication between cells, we performed dye-injection experiments using Lucifer Yellow (LY) (Figs 2, 3). Confluent cultures of ATP-s cells are well coupled (Fig. 2B), whereas the dye remained restricted to the injected cells in ATP-i cells (Fig. 2D). Octanol, a known gap-junction inhibitor (Johnston and Ramon, 1981), significantly reduced dye coupling in ATP-s cells (Fig. 3). To characterize further dye coupling in ATP-sensitive J774.G8 cells, these cells were simultaneously injected with both LY and rhodamine-dextran, a 40-kDa molecule that does not permeate through gap-junction channels (data not shown). Additionally, we performed injections using ethidium bromide in ATP-i cells and dye remained restricted to the injected cell (data not shown).

Quantitative analyses of LY-injected cells shows that 84% of ATP-s cells were coupled to at least one cell (mean of five coupled cells per injected cell), whereas only 9% of the ATP-i cells were coupled to at least one neighbor (mean of one coupled cell per injected cell). The results shown in Figs 2 and 3 demonstrate that ATP-i cells, obtained after several months of overexposure to ATP, are not dye coupled. To investigate whether short-term exposure to extracellular ATP

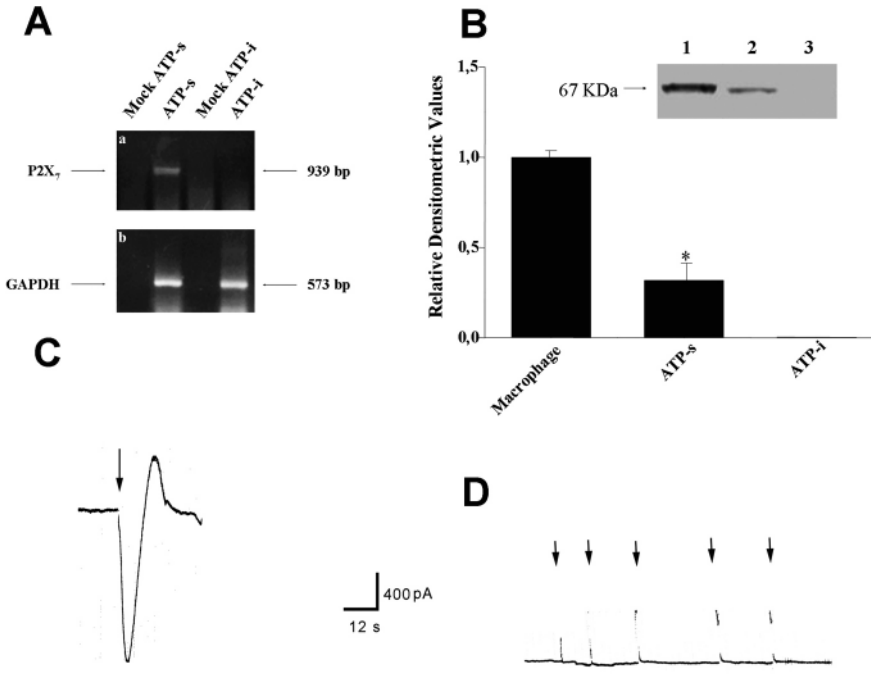

Fig. 1. Expression of $P2X_7$ receptors in macrophages, ATP-sensitive (ATP-s), and ATP-insensitive (ATP-i) J774 cells. (A) ATP-s (second lane) but not ATP-i (fourth lane) cells express $P2X_7$ receptor mRNA. cDNAs obtained from both cells were amplified by PCR using a set of primers specific for either $P2X_7$ (top) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as control of cDNA loading (bottom). RT-PCR product was separated on a 1% agarose gel and stained with ethidium bromide. In mock reactions (first and third lanes), RNA samples obtained from the same preparations were substituted for cDNA as a control for genomic DNA contamination. (B) Western blots showing that peritoneal macrophages (lane 1) and ATP-s cells (lane 2), but not ATP-i cells (lane 3) express $P2X_7$ protein. Each lane was loaded with 100 µg of protein and separated by 10% SDS-PAGE before transfer to nitrocellulose membrane. The bar histograms represent the mean±s.d. (n=5) densitometric values relative to those obtained for macrophages. The asterisk indicates that the intensities of the bands for ATP-s cells are significantly different from those of ATP-i cells ($P<0.05$). Whole-cell patch-clamp recordings show that ATP-s (C) but not ATP-i (D) cells display a biphasic current after iontophoretic application of ATP (arrows). The spikes in (D) are passive currents sensed by the recording electrode during each iontophoretic application of ATP. Holding potential was maintained at –40 mV. Data are representative of at least ten recordings for each cell type.

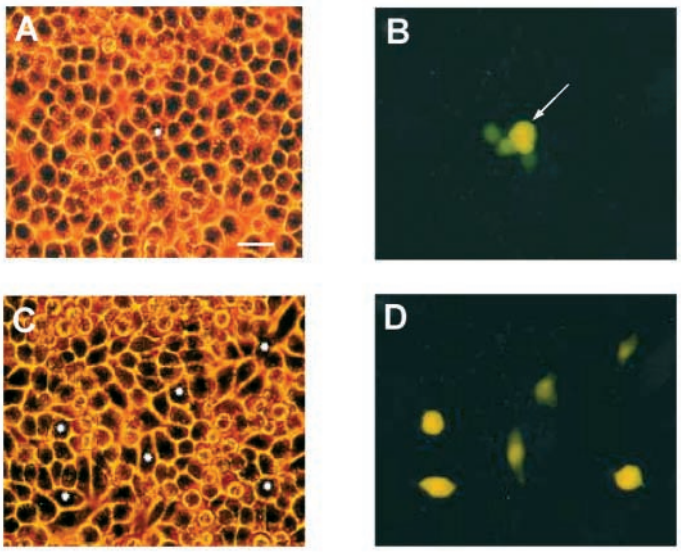

Fig. 2. ATP-s but not ATP-i cells are dye coupled. ATP-s (A,B) and ATP-i (C,D) cells were injected with Lucifer Yellow and observed under phase-contrast (A,C) and fluorescence (B,D) microscopy. In ATP-s cells (B), dye spreads to at least three adjacent cells, whereas, in ATP-i cells (D), dye remained restricted to the injected cells. Arrow and asterisk indicate the injected cells. Bar, 50 μm.

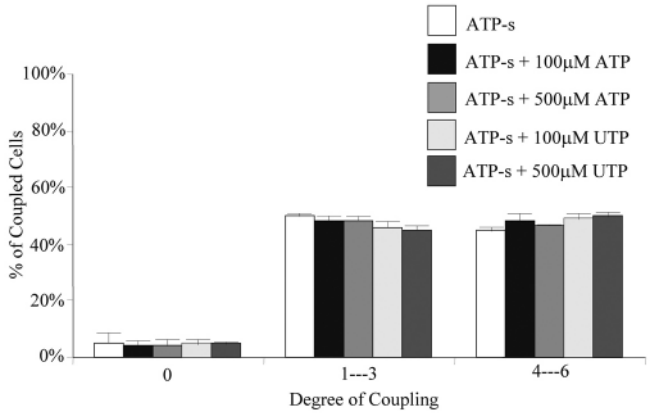

Fig. 4. Short exposures to extracellular ATP or UTP do not interfere with dye coupling. The histogram shows the proportion of dye-coupled ATP-s cells when treated with 100 μM and 500 μM ATP or UTP. The drugs were applied 5 minutes before the experiment and maintained in the culture during the injection procedure. Data represent the mean±s.d. number of injected cells that displayed dye coupling in an individual Petri-dish culture. At least 30 cells per dish were injected with Lucifer Yellow, for a total of 90 cells injected. No difference was observed between ATP-s cells treated or untreated with extracellular ATP and UTP (P>0.05).

or UTP could also change the degree of dye coupling we performed LY injections in ATP-s cells (Fig. 4) exposed to 100-500 μM ATP or UTP. The results (Fig. 4) indicate that, under our experimental conditions, short-term treatment with ATP or UTP did not alter the degree of dye coupling in J774.G8 cells.

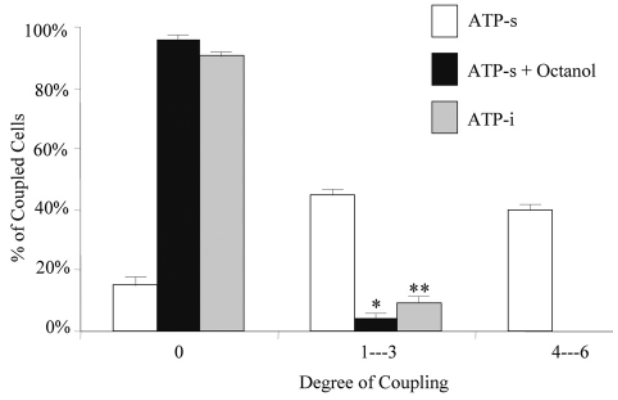

Fig. 3. Quantification of the dye-coupling assays. The bar histograms show the proportions of ATP-s and ATP-i cells that were coupled when in the presence and the absence of octanol. Data represent the mean±s.d. number of injected cells that were coupled to at least one neighboring cell. At least 30 cells were injected with Lucifer Yellow in each culture dish and at least five independent dishes were injected on different days. Degree of coupling: a total of 0 cells coupled to the injected cell; 1-3 cells coupled to the injected cell; or 4-6 cells coupled to the injected cell. The degree of coupling between ATP-i cells and octanol-treated ATP-s cells was significantly decreased compared with that obtained for ATP-s cells to the group of the ATP-s cells. The asterisk and double asterisk indicate P<0.05.

Expression of Cx43

To evaluate whether the difference in the degree of dye coupling observed between ATP-s and ATP-i cells (Figs 2, 3) was related to altered expression levels of Cx43, semiquantitative RT-PCR and western-blot analysis were performed in these two groups of cells. No significant difference in terms of Cx43 mRNA levels was found in the ATP-s and ATP-i cells (Fig. 5A). In accordance with these data, western-blot analysis showed that ATP-i cells that failed to display dye coupling expressed similar amounts of Cx43 to their ATP-s counterparts (Fig. 5B).

Although expressing similar levels of Cx43, immunocytochemical analysis performed on ATP-s and ATP-i cells revealed different patterns of Cx43 distribution (Fig. 6). Whereas ATP-s cells displayed labeling at the membrane (Fig. 6B), ATP-i cells showed Cx43 immunoreactivity distributed around the nucleus and within the cytosol (Fig. 6D). Together, these data suggest that altered Cx43 distribution, rather than Cx43 expression levels, is related to the different degree of dye coupling observed between these two groups of cells.

Colocalization of Cx43 and P2X$_7$

Given that it has been reported that P2X$_7$ receptors are part of a signaling complex involving cytoskeletal elements such as β-actin and α-actinin (Kim et al., 2001), the possibility that the altered cellular distribution of Cx43 was related to the lack of P2X$_7$ receptors in ATP-i cells prompted us to investigate further the localization of these molecules in normal cells. In order to achieve this objective, we labeled ATP-s cells and peritoneal macrophages with both anti-Cx43 and anti-P2X$_7$ antibodies, and analysed the results on a confocal microscope (Figs 7, 8). Both antibodies localized to the cell membrane, including the regions of membrane apposition (Fig. 7A,B, Fig.

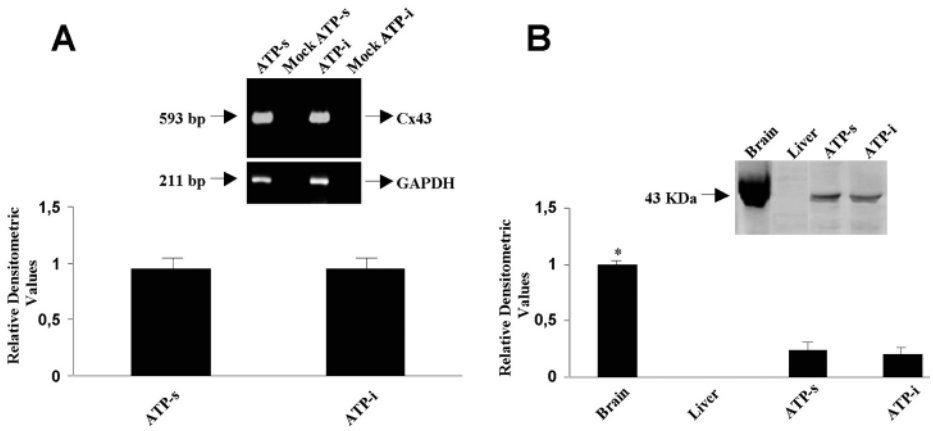

Fig. 5. Expression of Cx43 mRNA (A) and protein (B) in J774 cells. (A) Relative expression of Cx43 mRNA in ATP-s and ATP-i cells, obtained by amplified Cx43 and GAPDH RT-PCR products. The graph represents the mean±s.d. values for the ratio between Cx43 and GAPDH bands (n=5). The insert shows the agarose gel of a semiquantitative RT-PCR experiment. Mock is used as a negative control. There were no significant differences between ATP-s and ATP-i cells. (B) Western blot showing that ATP-s and ATP-i cells express Cx43 protein (43 kDa arrow). Liver and brain homogenates were used as negative and positive controls, respectively. Each lane was loaded with 100 μg of protein and separated by 10% SDS-PAGE before transfer to nitrocellulose membrane. The histogram represents the mean±s.d. (n=5) of relative densitometry values for Cx43 labeling taking the rat-brain homogenate as reference. The intensity of the bands in ATP-s and ATP-i cells are not significantly different from each other. The asterisk indicates that murine brain homogenate expressed significantly more Cx43 than the other cells (P<0.05).

8A,B). Image reconstruction (Fig. 7D, Fig. 8D) showed that extensive colocalization of the two molecules could be observed at areas of cell contact (Fig. 7E,F, arrows, Fig. 8E,F, arrows). Additionally, immunoprecipitation with the anti-Cx43 antibody pulled down $P2X_7$ in both J774.G8 cells (Fig. 7G) and peritoneal macrophages (Fig. 8G).

Discussion

The present work was undertaken to investigate possible interconnections between connexin and nucleotide (P2) receptor expression in a macrophage-derived cell line. Macrophages can be found in practically all tissues, where they mediate several immune-inflammatory reactions. These reactions require intense intercellular communication, and both P2 receptors and gap junctions are among the plethora of membrane proteins that might be involved in the signal-transduction pathways used by these cells to accomplish their task (Hume et al., 2002; Aderem, 2001; Medzhitov and Janeway, 2000; Celada and Nathan, 1994).

The presence of functional gap junctions in macrophages has been a highly controversial issue for years (Kane and Bols, 1980; Dean et al., 1988; Polacek et al., 1993; Alves et al., 1996; Porvaznik and MacVittie, 1979; Martin et al., 1998). Reported differences in homocellular and heterocellular coupling involving macrophages and distinct cell types, such as endothelial cells, fibroblasts and epithelial cells, might be due to diverse experimental conditions leading to the expression of soluble and/or membrane-bound molecules capable of modulating cell coupling. In fact, Eugenin et al. (Eugenin et al., 2003) have recently reported functional gap-junction communication mediated by Cx43 in human macrophages treated with TNF-α plus IFN-γ, and expression of Cx43 has been detected in macrophages at sites of inflammation (Polacek et al., 1993; Afonso et al., 1998).

We have previously reported J774-A1 cells, obtained directly from the ATCC, to be dye and electrically uncoupled (Alves et al., 1996). The cells used in the present study were obtained from the laboratory of W. De Souza, who

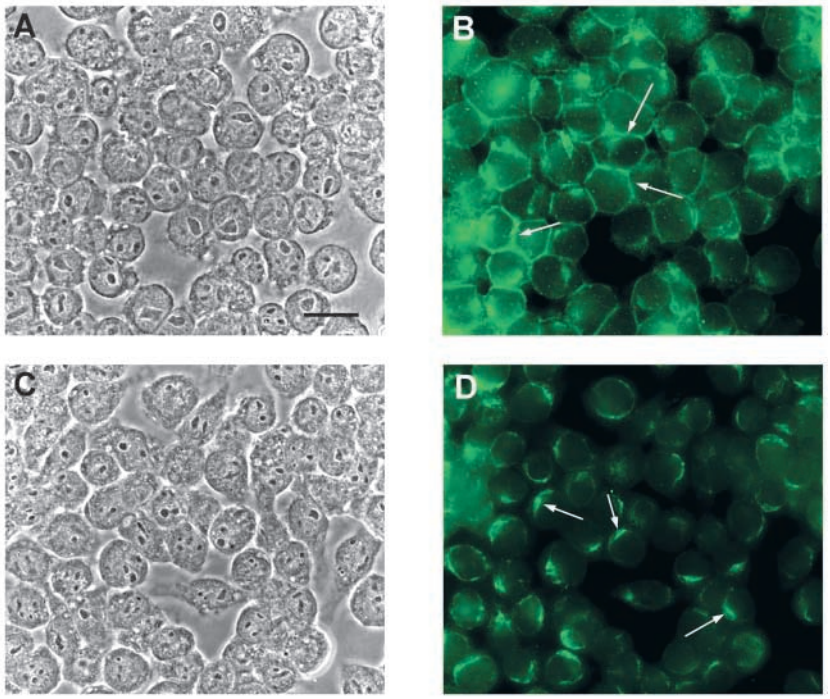

Fig. 6. Spatial distribution of Cx43 in ATP-s and ATP-i cells. Phase-contrast (A,C) and immunofluorescence (B,D) micrographs were taken from ATP-s (A,B) and ATP-i (C,D) cells cultivated on glass cover slips. Notice the Cx43-specific labeling at membrane appositional areas in ATP-s cells (B, arrow). By contrast, labeling is absent from the appositional areas in ATP-i cells and concentrates in the interior of the cells (D, arrow). Bar, 20 μm.

originally acquired them from ATCC as J774-A1 cells. These cells were passaged extensively in De Souza's laboratory over at least 2 years of culturing. This long-term culturing has resulted in a cell line that, although derived from the original ATCC clone, now displays functional coupling mediated by the gap-junction protein Cx43. Given the new properties of the cell line, it was renamed J774-G8 and deposited in the Rio de Janeiro Cell Bank, from where it can be obtained.

We now report that, when the macrophage-like cell line J774-G8 is rendered ATP insensitive by repeated exposures to increasing ATP concentrations, it loses the expression of the P2X$_7$ receptors and the ionic currents associated with their

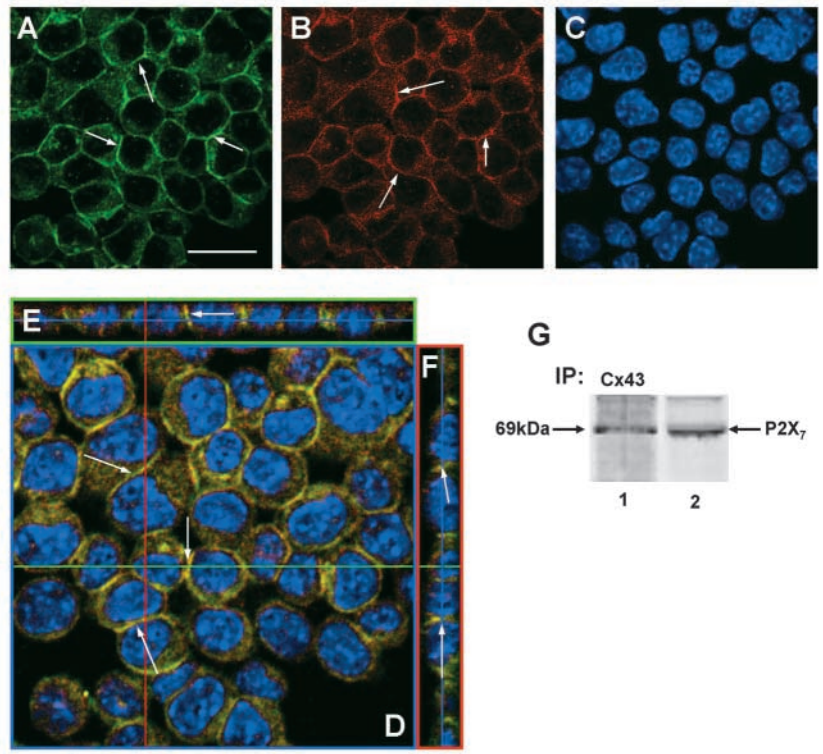

Fig. 7. Co-localization and immunoprecipitation of Cx43 and P2X$_7$ proteins in ATP-s cells. (A) Cx43 immunolabeling, (B) P2X$_7$ immunolabeling and (C) TOPRO3 staining showing the nuclei of the J774 cells. Bar, 20 μm. (D) Reconstruction of 28 optical slices of 0.8 μm thickness. The green line indicates an orthogonal section from (D), projected laterally in (E); the red line indicates an orthogonal section from (D), projected laterally in (F); the blue line indicates the level of the 15th optical slice in (D). Confocal microscopy demonstrates co-localization of the Cx43 and P2X$_7$ proteins (yellow) to the membrane of the ATP-s cells (D-F, arrows). Notice the Cx43 (green) and P2X$_7$ (red) specific labeling located at membrane apposition areas (A,B, arrows). (G) Lane 1 shows a 69 kDa band corresponding to P2X$_7$ receptor obtained after immunoprecipitation of ATP-s cell lysates with Cx43 antibodies, and lane 2 shows the corresponding band obtained from whole ATP-s cell lysate after probing with the P2X$_7$ antibody.

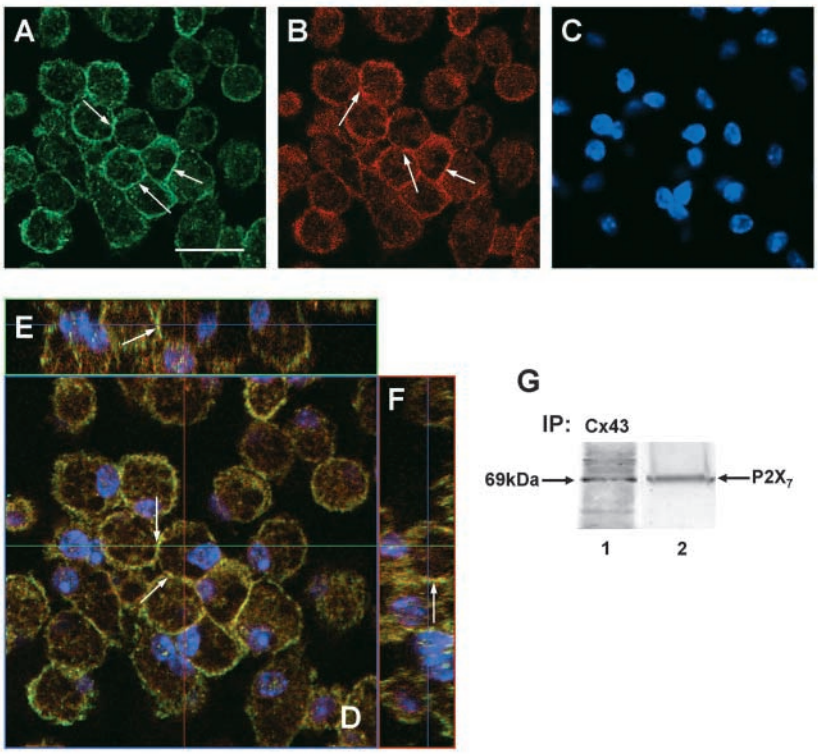

Fig. 8. Co-localization and immunoprecipitation of Cx43 and P2X$_7$ proteins in peritoneal macrophages. (A) Cx43 immunolabeling, (B) P2X$_7$ immunolabeling and (C) TOPRO3 staining showing the nuclei of the J774 cells. Bar, 20 μm. (D) Reconstruction of 49 optical slices of 0.8 μm thickness. The green line indicates an orthogonal section from (D), projected laterally in (E); the red line indicates an orthogonal section from (D), projected laterally in (F); the blue line indicates the level of the 25th optical slice in (D). Confocal microscopy demonstrates co-localization of the Cx43 and P2X$_7$ proteins (yellow) to the membrane of the macrophages (D-F, arrows). Notice the Cx43 (green) and P2X$_7$ (red) specific labeling located at membrane apposition areas (A,B, arrows). (G) Lane 1 shows a 69 kDa band corresponding to P2X$_7$ receptor obtained after immunoprecipitation of peritoneal macrophage lysates with Cx43 antibodies, and lane 2 shows the corresponding band obtained from whole peritoneal macrophage cell lysates probed with the P2X7 antibody.

activation (Fig. 1). Interestingly, although it also loses functional coupling [(assayed by intercellular dye-diffusion experiments (Figs 2, 3)], ATP-insensitive cells still express Cx43 at levels comparable to the ATP-s cells (Fig. 5). Immunolabeling shows that Cx43 has a different pattern of localization in ATP-s and ATP-i cells and seems to be expressed mostly in the cell interior, being absent from the surface membrane and appositional areas in the ATP-i cells, thus explaining the lack of functional coupling. By contrast, ATP-s cells express Cx43 at appositional membrane regions and are functionally coupled (Figs 2, 3, 6).

Loss of gap-junctional communication in ATP-i cells does not seem to result from short-term exposure to ATP, because acute exposure to high nucleotide concentrations had no effect on the degree of coupling of the ATP-s cells (Fig. 4). We therefore speculated that targeting of Cx43 to the membrane might depend on the expression of the $P2X_7$ and/or other P2R subtypes that are expressed in macrophages. In fact, modulation of purinergic receptor activity by gap-junction expression is well established in astrocytes (Scemes et al., 2000; John et al., 1999; Suadicani et al., 2003). In these cells, loss of gap-junction communication in Cx43 knockout mice is compensated by increased purinergic autocrine communication via a switch in P2Y receptor expression (Suadicani et al., 2003). To our knowledge, the present work is the first report on purinergic receptor modulation of gap-junction-mediated intercellular communication.

Protein-protein interactions are known to influence gap-junction-mediated coupling. For Cx43, specific binding sites for SH and PDZ domains have been identified, and ZO-1 and c-Src have been shown to bind to Cx43 at the cardiac nexus (Giepmans and Moolenaar, 1998; Toyofuku et al., 1999). Using confocal microscopy and immunoprecipitation assays, we show here that Cx43 and $P2X_7$ co-localize to the membrane of both ATP-s cells (Fig. 7A,G) and peritoneal macrophages (Fig. 8A,G). These data suggest that surface membrane localization of Cx43 might depend on the presence of a supramolecular complex involving the $P2X_7$ receptor in these cells. In addition, the analysis of several optical slices generated by confocal microscopy suggests that Cx43 and $P2X_7$ can also co-localize to cytoplasmic vesicles. However, owing to the high degree of invagination displayed by macrophage membranes, further experiments are required to clarify this point.

Adhesion molecules are known to influence gap-junction-mediated coupling in different cell types (Jongen et al., 1991; Meyer et al., 1992), and a gap-junction-communication-deficient cell line has been shown to express Cx43 but not E-cadherin (Hernandez-Blazquez et al., 2001). Given that ATP-s cells, which express the $P2X_7$ receptor, undergo spontaneous fusion and ATP-i cells do not (Chiozzi et al., 1997), we propose that the presence of $P2X_7$ receptors at the cell surface facilitates membrane insertion of Cx43 and the formation of gap-junction channels. In addition, the absence of currents upon activation with ATP suggests that ATP-i cells might also lack other P2 receptors. Therefore, we cannot discard the possibility that these other receptors might also be involved in promoting Cx43 insertion into membrane.

Alternatively, soluble factors secreted after activation of the purinergic receptor might be necessary for targeting Cx43 to the plasma membrane. In human macrophages, ATP provides a stimulus for IL-1 secretion after LPS activation, and this response has been shown to involve the $P2X_7$ receptor (Ferrari et al., 1997). Consistent with this idea, mice lacking the $P2X_7$ receptors show altered cytokine production after LPS activation (Solle et al., 2001). It would be interesting to test the ability of macrophages derived from such mice to establish gap-junction-mediated coupling. Such experiments could help to unravel the interplay between Cxs and purinergic receptor expression, and provide important insight into the mechanisms operating during an inflammatory response.

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