Human primary fibroblasts in vitro express a purinergic P2X7 receptor coupled to ion fluxes, microvesicle formation and IL-6 release

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SUMMARY

We have investigated responses to extracellular ATP in human fibroblasts obtained by skin biopsies. Our data show that these cells express a P2X7 purinergic receptor, as judged by (1) RT-PCR with specific primers, (2) reactivity with a specific anti-P2X7 antiserum, (3) activation by the selective P2X agonist benzoylbenzoylATP and (4) stimulation of transmembrane ion fluxes. Stimulation with benzoylbenzoylATP, and to a lesser extent with ATP, also caused striking morphological changes and increased formation of cytoplasmic microvesicles. These changes were fully reversible upon nucleotide removal. Two known blockers of P2X receptors, oxidised ATP and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, inhibited the morphological changes fully and the ion fluxes partially. The residual rise in intracellular Ca2+ levels and membrane depolarization observed in the presence of the inhibitors were dependent upon activation of a P2Y-type receptor exhibiting a peculiar pharmacological profile, in that CTP was the preferred agonist. ATP stimulation triggered release of the pro-inflammatory cytokine IL-6 in fibroblasts pre-treated with PMA and bacterial endotoxin. These observations reveal a novel pathway for fibroblast activation and for their recruitment in the inflammatory response.

Key words: Cytoplasmic calcium, Cytokine, Extracellular nucleotide, Inflammation, Golgi apparatus, Fibroblast

INTRODUCTION

Many different cell types express purinergic receptors of the P2X and P2Y subtypes (Harden et al., 1995; Brake and Julius, 1996; North and Barnard, 1997). While a wealth of data has been collected from neuronal cells (Buell et al., 1996; Chen et al., 1995; Lewis et al., 1995; Soto et al., 1996) or leukocytes (Di Virgilio et al., 1989, 1996; Filippini et al., 1990; Humphreys and Dubyak, 1996), comparatively little information is available about human primary fibroblasts, apart from a few anecdotal reports (Fine et al., 1989; Okada et al., 1984). Human skin fibroblasts are an interesting model as they share several features with smooth and striated muscle cells and are directly involved in chronic degenerative diseases such as atherosclerosis and diabetic angiopathy (Shats et al., 1997; Nasrin et al., 1997; Sigari et al., 1997). Furthermore, they can be obtained by a very easy isolation procedure (skin biopsies) from well controlled healthy or diseased subjects.

Most data on the effect of extracellular nucleotides on fibroblasts have been obtained in mouse cells, where ATP has a mitogenic effect, either alone or in combination with more classical growth factors, possibly due to stimulation of Ca2+-dependent pathways or inhibition of arachidonate metabolism (Gonzales et al., 1989; Huang et al., 1993). Besides these responses, which are mediated by P2Y receptors, some fibroblast lines also undergo changes that are suggestive of expression of P2X receptors, as they are susceptible to the typical ATP-dependent plasma membrane permeabilization diagnostic of P2X7 receptors, while others exhibit transmembrane ion fluxes that are suggestive of the presence of smaller members of the P2X family (Pizzo et al., 1992). However, a molecular identification of the P2X receptor responsible for these responses has not yet been attempted in human fibroblasts.

Fibroblasts are non-excitable cells, thus it is unlikely that external nucleotides serve the function of fast mediators of intercellular communication as in the nervous system. Rather they might be involved in the modulation of fibroblast responses to the variety of different microenvironmental situations to which these cells are exposed in the vessel wall or in the interstitial tissue. There is increasing evidence that ATP can accumulate in the external milieu as a consequence of different events: release by secretory exocytosis (e.g. platelets or nerve cells), transport across the plasma membrane via as yet unknown carriers (e.g. endothelial cells), passive release from damaged cells (see Hoyle and Burnstock, 1996). It is well known that cells in the vessel wall are exposed to intense shear stress forces or actual damage that may release cellular ATP;
furthermore, during thrombus formation fibroblasts in the vessel wall are also exposed to ATP released from platelets.

In the present work we show that human skin fibroblasts express purinergic receptors of the P2X7 subtype and that activation of these receptors is coupled to induction of ion fluxes and extensive cytoplasmic microvesiculation. Furthermore, in fibroblasts pre-treated with PMA and LPS, ATP causes release of the pro-inflammatory cytokine IL-6.

MATERIALS AND METHODS

Cells and solutions

Human skin fibroblasts were grown in DMEM medium supplemented with 10% heat-inactivated fetal calf serum. Mouse microglial cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Both culture media contained penicillin (100 units/ml) and streptomycin (100 μg/ml). Short-term experiments were performed in saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM Na2HPO4, 5.5 mM glucose, 5 mM NaHCO3 and 20 mM Hepes (pH 7.4). We will refer to this saline solution as standard saline. In some experiments, 1 mM CaCl2 was also added (Ca2+-containing standard saline).

Cytoplasmic free Ca2+ concentration measurements

Changes in cytoplasmic free Ca2+ concentration ([Ca2+]i) were measured with the fluorescent indicator fura-2/AM, as described previously (Falzoni et al., 1995). Briefly, cells were loaded for 15 minutes with 2 μM fura-2/AM and incubated at 37°C in a thermostat-controlled and magnetically stirred fluorescence cuvette (LS50; Perkin Elmer Ltd., Beaconsfield, UK) at a concentration of 106/ml in the presence of 250 μM sulfinpyrazone. Intracellular Ca2+ concentration was determined with the 340/380 excitation ratio at an emission wavelength of 505 nm.

Semiquantitative measurement of plasma membrane potential

Changes in plasma membrane potential were measured with the fluorescent dye bis[1,3-diethyliothiacarbocyanine] trimethineoxonal (bisoxonol; Molecular Probes, Inc., Eugene, OR, USA) at the wavelength pair 540-580 nm (Falzoni et al., 1995). Experiments were performed in a spectrofluorometer (LS50; Perkin Elmer) at 37°C at a concentration of 2.5x105 cells/ml.

Changes in plasma membrane permeability

ATP-dependent increases in plasma membrane permeability were measured with the extracellular fluorescent tracers YO-PRO and Lucifer Yellow (Molecular Probes, Inc.). Cell monolayers or suspensions were incubated for 15 minutes at 37°C in standard saline containing 250 μM sulfinpyrazone and 10 μM YO-PRO or 1 mg/ml Lucifer Yellow, and stimulated with 3 mM ATP. After several washings to remove the extracellular dye, cells were analysed with an inverted fluorescence microscope (Olympus IMT-2; Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a 40× objective and a fluorescein filter.

Measurement of enzymatic activity

Lactate dehydrogenase activity was measured according to standard methods (Bergmeyer, 1983).

Western blotting

Cells were lysed in lysis buffer containing 300 mM sucrose, 1 mM K2HPO4, 1 mM MgSO4, 5.5 mM glucose, 20 mM Hepes, pH 7.4, 1 mM benzamidine, 1 mM PMSF, 0.2 μg of DNase and 0.2 μg of RNase by repeated freeze/thawing (three cycles). Proteins were separated on 7.5% SDS-polyacrylamide gels according to Laemmli (1970) and blotted overnight on nitrocellulose paper (Schleicher and Schuell Italia Srl, Legnano, Italy). The rabbit polyclonal anti-P2Z/P2X7 serum was raised against the synthetic peptide corresponding to the last 20 amino acids of the P2X7 protein (KIRKEFPKTQGQYSFGKYPY), and was kindly provided by Dr Gary Buell (Glaxo-Wellcome, Geneva) (Surprenant et al., 1996). The primary antibody was used at a dilution of 1:100 in TBS buffer (10 mM Tris-Cl, 150 mM NaCl, pH 8.0). The secondary antibody was a goat anti-rabbit antibody conjugated to alkaline phosphatase.

Immunofluorescence

Fibroblasts were seeded on glass slides, washed with PBS, and fixed with paraformaldehyde (2% in PBS). After 2 hours, fixed cells were permeabilized with Triton X-100 (1% in PBS) and incubated for 1 hour with the anti-AP-1 monoclonal antibody (1:40 dilution in PBS). Cells were then rinsed three times with PBS and incubated for 30 minutes with an anti-mouse Ig FITC-labelled antibody (1:50 dilution in PBS). At the end of this incubation, slides were rinsed 3 times and analyzed with the fluorescence microscope.

RT-PCR

Total cytoplasmic RNA was extracted from fibroblast cells by the acid guanidium thiocyanate-phenol-chloroform method, as described by Chomczynski and Sacchi (1987).

RT-PCR was performed as described by Rappolee et al. (1989). Amplification primers and probe were chosen on the basis of the published sequence of human P2X7 receptor (Rassendren et al., 1997). The 5’ primer was 5′-AGATCGTGGAATGGAGTG-3′ and the 3’ primer, 5′-TTCTCGTGTTGATTGTTG-3′; the probe was 5′-TCATGCACACACCTTTCC-3′. Amplification primers for β-actin were: 5′ primer, 5′-TGACGGGGGTCACCCACTGTC-3′; 3′ primer, 5′-ATGTCATAGTCGCCCTAGAAC-3′. Oligonucleotides were synthesized by M-Medical Genencio-Life Science (Firenze, Italy). Labelling of the probe and blotting were carried out as described in Dig-labelling and detection protocols (Boehringer Mannheim, Germany). Briefly, RT-PCR products were separated in 1.2% agarose gel and transferred to a positively charged nylon membrane (ICN Biomedicals Inc. Aurora, Ohio, USA) by a vacuum blotter system (BioRad Laboratories, Hercules, CA, USA) for 2 hours. After hybridization, the digoxigenin-labelled P2X7-specific internal oligoprobe was visualized by chemiluminescence detection after incubation with a dilution of anti-digoxigenin Fab conjugated to alkaline phosphatase.

IL-6 determination

IL-6 was measured with the BioSource Cytoscreen Immunoassay kit (BioSource International Inc., Camarillo, CA, USA).

Reagents

Nucleotides were HPLC-purified. All other chemicals were reagent grade.

![Fig. 1. Human fibroblasts express the P2X7 receptor mRNA. Total RNA was extracted as described in Materials and methods and used for RT-PCR. 10 μl of PCR product were loaded in each lane. Detection of the P2X7 fragment was performed by transfer to nylon membrane and hybridization with a specific internal probe. Lane 1, human macrophages; lane 2, human fibroblasts.](image-url)

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RESULTS

Expression of P2X\textsubscript{7} receptor has so far only been reported in immune cells, but very recent evidence suggests that it may also be present in other cell types (for example, see Cario-Toumaniantz et al., 1998). Fig. 1 shows that P2X\textsubscript{7} mRNA is present in human fibroblasts, although to a much lower level than in macrophages, the prototypical cell in which this receptor was originally characterized. P2X\textsubscript{7} expression by fibroblasts is also confirmed by using an anti-P2X\textsubscript{7} polyclonal antibody (Collo et al., 1997). Fig. 2, lane 1, shows a western blot of human fibroblasts stained with a serum raised against the COOH tail (residues 576-595) of the rat P2X\textsubscript{7} receptor. Immunoreactivity of mouse microglial cells, a cell type well known for expressing the P2X\textsubscript{7} receptor, is shown in lane 2. In lane 3 we also show lower reactivity to the anti-P2X\textsubscript{7} antiserum of a microglial cell clone selected for hypo-expression of P2X\textsubscript{7}.

One of the earliest changes associated to P2X\textsubscript{7} receptor activation is plasma membrane depolarization, due to a fast transmembrane cation flux. Fig. 3A, left panel, shows that this response is also present in ATP-stimulated fibroblasts. Surprisingly, while the larger depolarization in response to BzATP was anticipated, given the higher affinity of this ATP analogue for the P2X\textsubscript{7} receptor, the fact that CTP was also active was unexpected, as no other nucleotides or nucleosides besides ATP, some ATP analogues and, to a lesser extent, ADP, are active at P2X\textsubscript{7}. Interestingly, depolarization triggered by CTP was preceded by a small transient hyperpolarization. Experiments performed in the virtual absence of extracellular Ca\textsuperscript{2+} helped to solve this paradox, as they showed (Fig. 3A, right panel) that while responses to ATP...
and benzoylbenzoylATP (BzATP) were potentiated as expected, CTP-dependent depolarization was totally abrogated, thus indicating that CTP caused depolarization not by directly activating a depolarizing flux, but rather by causing an increase in the intracellular Ca$^{2+}$ concentration. The pharmacological sequence for plasma membrane depolarization in Ca$^{2+}$-containing standard saline was: BzATP>CTP>>ATP=A TP$_{\text{g}}$=2 methylthio ATP.

Fig. 3B shows ATP, BzATP and CTP dose-dependency for plasma membrane depolarization in Ca$^{2+}$-containing standard saline and challenged with increasing concentrations (a$_1$,b$_1$,c$_1$ = 0.1 mM; a$_2$,b$_2$,c$_2$ = 0.5 mM; a$_3$,b$_3$,c$_3$ = 1 mM) of the three nucleotides.

Fig. 4 shows Ca$^{2+}$ changes triggered by increasing concentrations of CTP (a), ATP (b) and BzATP (c) in fibroblasts. Fibroblasts were incubated in Ca$^{2+}$-containing standard saline and challenged with increasing concentrations (a$_1$,b$_1$,c$_1$ = 0.1 mM; a$_2$,b$_2$,c$_2$ = 0.5 mM; a$_3$,b$_3$,c$_3$ = 1 mM) of the three nucleotides.

respectively. Inhibition of plasma membrane depolarization by oATP or PPADS was evaluated using BzATP as a stimulus, as this ATP analogue is a better depolarizing agent than ATP. On the contrary, inhibition of [Ca$^{2+}$] increases was evaluated with respect to the maximal increase caused by ATP, as BzATP caused a very small increase in [Ca$^{2+}$]. Confirming previous observations performed in our laboratory (O. R. Baricordi and F. Di Virgilio, unpublished), oATP was a much better inhibitor than PPADS on a purely P2X$^7$-mediated response, such as depolarization, while both oATP and PPADS partially blocked (about 50%) the [Ca$^{2+}$] increase, a response that was dependent on activation of both P2Y and P2X$^7$ receptors.

The hallmark of P2X$^7$ receptor activation is an ATP-dependent reversible permeabilization of the plasma membrane to solutes of molecular mass <900 Da (Steinberg et al., 1987; Di Virgilio, 1995). To this end, different fluorescent dyes such as Lucifer Yellow, YO-PRO or Ethidium Bromide can be used. Rather surprisingly, in human fibroblasts we were unable to document early changes in plasma membrane permeability due to stimulation with ATP or BzATP. Only after a few hours (2 hours in the experiment shown in Fig. 6), could some dye uptake be observed, in the presence of ATP (Fig. 6B) or BzATP (Fig. 6C). Most of the fibroblasts were positive for YO-PRO uptake at this late time point and, as expected, fluorescence was preferentially localized in the nucleus, YO-PRO being a DNA-binding dye, and was more intense in BzATP-stimulated cells (cf. Fig. 6B,C). Even after this prolonged incubation, fibroblasts did not show the usual alterations observed in other ATP-challenged cells, i.e. rounding, swelling, blebbing, detachment from the substrate (see Fig. 6D-F). In addition, release of lactic dehydrogenase, an index of cell injury, was minimal, as only at exceedingly high BzATP concentrations (>2 mM) was there a significant level of cell death (not shown). ATP and CTP had no cytotoxic activity at any concentrations tested. However, although overt signs of ATP-dependent cytotoxicity were lacking, it was difficult to rule out the possibility that YO-PRO uptake was the consequence of a non-specific increase in plasma membrane permeability as a result of the prolonged alteration of intracellular ion homeostasis.

One of the main drawbacks in the study of P2 receptors is the lack of selective antagonists. However, two compounds that over the last few years have turned out to be useful are the dialdehyde ATP derivative, oxidized ATP (oATP) (Murgia et al., 1993), and the aldehyde compound pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (Lambrecht et al., 1992). These reagents react with lysine residues in the vicinity of plasma membrane ATP-binding sites and have been shown to inhibit P2X$^7$-dependent responses. Fig. 5 shows that these two compounds also inhibit depolarization and [Ca$^{2+}$] increases triggered in fibroblasts by BzATP and ATP,

![Fig. 4. Changes in [Ca$^{2+}$] triggered by CTP (a), ATP (b) and BzATP (c). Fibroblasts were incubated in Ca$^{2+}$-containing standard saline and challenged with increasing concentrations (a$_1$,b$_1$,c$_1$ = 0.1 mM; a$_2$,b$_2$,c$_2$ = 0.5 mM; a$_3$,b$_3$,c$_3$ = 1 mM) of the three nucleotides.](image)

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![Fig. 5. Inhibition of depolarization and [Ca$^{2+}$] increase by oATP and PPADS. Fibroblasts were incubated in Ca$^{2+}$-containing standard saline solution for 2 hours at 37°C in the absence (controls) or presence of 300 μM PPADS or oATP, rinsed, and challenged with 1 mM BzATP (depolarization; left) or ATP ([Ca$^{2+}$] increases; right). Data are means ± s.d. of triplicate determinations from one representative experiment repeated three times with similar results.](image)
While on the one hand P2X₇ receptor stimulation did not induce overt signs of cell injury, on the other it caused a large increase in cytoplasmic microvesicle formation (Fig. 7D). This effect was clearly visible with BzATP, even after relatively short incubations, while was only occasionally observed with ATP (Fig. 7B), and never with CTP. CTP, on the contrary, stimulated an intriguing rearrangement of the monolayer, where the fibroblasts assumed a spindle-like morphology and generated intersecting cellular cords (Fig. 7C). Within 15-30 minutes of BzATP removal, microvesicles disappeared, and fibroblasts resumed a normal morphology (not shown). The ability of extracellular nucleotides to trigger cytoplasmic vesicle formation is not a new phenomenon, as this is one of the most extensively documented early changes observed upon stimulation of macrophages with extracellular nucleotides (Cohn and Parks, 1967; Di Virgilio and Steinberg, 1993). However, in fibroblasts this phenomenon was rather peculiar as vesicles were of small size and had a perinuclear location. Thus we further investigated the nature of these vesicles. Extracellular nucleotides are known to increase the rate of pinocytosis; however, we found that upon BzATP stimulation uptake of Texas Red-labelled ovalbumin was only marginally increased (about 10% with respect to unstimulated controls). Since the perinuclear location of the vesicles suggests an expansion of the Golgi apparatus, we labelled BzATP-stimulated fibroblasts with a monoclonal antibody directed against a specific Golgi marker (γ-adaptin, AP-1) (Ahle et al., 1988). As shown in Fig. 8, fibroblasts incubated in the presence

Fig. 6. P2X₇ activation causes delayed YO-PRO uptake. Fibroblasts were incubated for 2 hours at 37°C in Ca²⁺-containing standard saline supplemented with 10 μM YO-PRO, in the absence of added nucleotides (A and D, controls), or in the presence of 1 mM ATP (B,E) or 1 mM BzATP (C,F). A-C, fluorescence microscopy; D-E, phase-contrast microscopy. Bars, 25 μm.
of BzATP show an increased staining of the Golgi apparatus (cf. Fig. 8A,B), suggesting that microvesicles originate, at least in part, from the Golgi.

Although the physiological function of the P2X7 is as yet unknown, in mononuclear phagocytes this receptor has been associated with cytokine release (Perregaux and Gabel, 1994; Ferrari et al., 1996). Fibroblasts release several cytokines and pro-inflammatory factors, including IL-6 (Baumann and Kushner, 1998), thus we investigated whether extracellular ATP could promote secretion of this cytokine. Extracellular nucleotides by themselves were unable to stimulate IL-6 secretion (not shown); however, addition of ATP or BzATP, but not CTP, to fibroblasts pre-treated with bacterial endotoxin and PMA almost doubled IL-6 release (Fig. 9), a behaviour reminiscent of the effect of ATP on IL-1β release from human and mouse macrophages and microglial cells (Perregaux and Gabel, 1994; Ferrari et al., 1996).

DISCUSSION

Purinergic P2X receptors are becoming one of the most interesting families of ligand-gated ion channels. Seven P2X receptor subunits have been cloned and sequenced so far, and their amino acid sequence suggests a rather unusual membrane topology in that these molecules are understood to span the membrane twice, with both the N and C termini on the cytoplasmic side of the plasma membrane (Harden et al., 1995; Brake and Julius, 1996; North and Barnard, 1997; Buell et al., 1996). The seven members of the P2X subfamily show a tissue- and differentiation-dependent expression, suggesting an involvement in specialized cell functions such as neurotransmission, cell proliferation, cell-to-cell communication, cytokine release and cytotoxic reactions (Buell et al., 1996; Chen et al., 1995; Lewis et al., 1995; Soto et al., 1996; Di Virgilio et al., 1996). In this study we show that human skin-derived fibroblasts express the P2X7 receptor, although its permeability properties appear to be slightly different with respect to the better characterized macrophage P2X7 receptor.

Stimulation of fibroblast P2X7 triggered a fast plasma membrane depolarization and a large Ca²⁺ influx. However, the increase in plasma membrane permeability, a typical response consequent to P2X7 activation, was very small and delayed. We were able to detect some uptake of an extracellular fluorescent dye only 2 hours after addition of BzATP. Although even after such a long incubation fibroblasts did not show any overt sign of cytotoxicity, nor released lactic dehydrogenase, it is difficult to link this delayed dye uptake unambiguously to activation of P2X7. Interpretation of fibroblast responses to nucleotides is further complicated by expression of a previously undescribed receptor responsive to CTP that also causes a fast depolarization of the plasma membrane, albeit with a mechanism different from ATP, as in the case of the CTP receptor depolarization is secondary to the increase in cytoplasmic Ca²⁺ levels. It is not completely unusual for CTP to be an agonist at P2 receptors, as activatory reponses have been described in mouse macrophages and in oocytes injected.

Fig. 7. P2X7 receptor activation causes cytoplasmic microvesiculation. Fibroblasts were plated as described in Fig. 6 and stimulated with 1 mM ATP (B) or 1 mM CTP (C) for 6 hours, or with 0.5 mM BzATP for 2 hours (D). (A) Controls. Phase-contrast microscopy. Bars, 25 µm.
with a P2Y receptor cloned from *Xenopus* (Greenberg et al., 1988; Burnstock, 1997), and a member of the P2X subfamily, P2X4, is also weakly responsive to CTP (Garcia-Guzman et al., 1997). Nonetheless it is surprising that CTP appeared to be by far the most potent among the nucleotides tested in our study.

While in other cells expressing P2X7 sustained stimulation with ATP causes swelling, blebbing and eventually death, in human fibroblasts the most striking morphological alteration consisted in an expansion of the cytoplasmic vesicular compartment. There is a precedent for such an alteration in mouse macrophages, in which ATP causes a dramatic but reversible increase in the content of large cytoplasmic phase-lucent vesicles (Cohn and Parks, 1967; Di Virgilio and Steinberg, 1993). In fibroblasts microvesiculation occurs, rather than macrovesiculation. These microvesicles do not arise from stimulation of pinocytosis, but rather from an expansion of the Golgi compartment, although further experiments are clearly needed to fully characterize this process.

For fibroblasts, as for other cell types, the question arises as to the physiological significance of ATP-gated ion channels. Until a few years ago it was doubtful that significant amounts of ATP were ever released in the extracellular milieu, but now it is clear that non-lytic ATP release is an event far more common than previously believed (von Kugelgen and Starke, 1991; Pearson and Gordon, 1979; Meyers et al., 1982; Reisin

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**Fig. 8.** Staining of BzATP-treated fibroblasts with a monoclonal antibody directed against an antigen selectively expressed in the Golgi. Fibroblasts were plated on glass coverslips (5×10^4/coverslip) and further incubated at 37°C. (A,D) Controls; no BzATP was present. (B,C,E,F) 0.5 mM BzATP was added for 1 hour. For immunofluorescence, samples were processed as described in Materials and methods. In C and F only the secondary FITC-labelled antibody, and not the primary anti-AP-1, was added. A-C, fluorescence microscopy; D-F, phase-contrast microscopy. Bars, 25 μm.
et al., 1994), not forgetting that every injured cell will also discard its cytoplasmic ATP content, usually in the 5-10 mM range. Thus, it has to be expected that cells are continuously exposed to varying extracellular ATP concentrations that, by acting at membrane receptors with widely different affinities, will elicit complex cellular responses. In this way P2 receptors may endow fibroblasts with an array of very sensitive sensors capable of detecting minute alterations in the metabolic activity or integrity of neighbouring tissues.

Fibroblasts are well known not only for being the major producers of extracellular matrix, but also a cell type actively involved in synthesis of inflammatory mediators and tissue repair; thus we consider the observation that ATP triggers IL-6 release may suggest the observation that the fibroblasts of the subendothelial layer. Nucleotides, in synergy with other factors released from platelets and blood leukocytes (growth factors, cytokines, prostaglandins), may enhance stimulation of fibroblasts, thus leading to a second wave of release of pro-inflammatory molecules such as IL-6. Incidentally, among the various NF-κB isoforms involved in IL-6 gene expression, the most active is the unusual p65 homodimer, and it is therefore intriguing that Ferrari et al. (1997) have recently shown that in mouse microglial cells the p65 homodimer is the NF-κB isoform selectively induced by P2X7 receptor activation (Ferrari et al., 1997). Ability of fibroblast P2X7 receptor to trigger IL-6 release may suggest additional targets for pharmacological intervention in a number of diseases in which fibroblasts are implicated, such as atherosclerosis, degenerative joint diseases and diabetic angiopathy.

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