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REGULAR ARTICLE

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Human melanomas express functional $P2X_7$ receptors

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Abstract Adenosine 5′-triphosphate is known to function as a potent extracellular messenger, producing its effects via a distinct family of cell surface receptors. Different receptor subtypes have been shown to modulate different cellular functions such as proliferation, differentiation and apoptosis. We have investigated the functional expression and apoptotic action of the $P2X_7$ receptor in human malignant melanoma tissue and cells. Incubation of cells with the potent $P2X_7$ receptor agonist 2′–3′-O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate leads to a decrease in cell number, which is dose-dependent and reversible by the antagonist 1-N,O-bis-[5-isoquinoline-sulfonyl]-N-methyl-L-tyrosyl)-4-phenyl-piperazine. Synthesis of the $P2X_7$ receptor by these cells has been established by reverse transcriptase-polymerase chain reaction, immunohistochemistry, immunocytochemistry and cellular accumulation of the fluorescent DNA-binding dye YO-PRO-1. The $P2X_7$ receptors have been shown to mediate apoptotic actions of extracellular nucleotides and represent a novel target for melanoma therapy.

Keywords Purinergic receptors · ATP · $P2X_7$ · Melanoma · Cancer · Human

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Introduction

Malignant melanoma is an aggressive cancer that originates from melanocytes, the pigment-producing cells of the skin. The incidence of melanoma is increasing and the outcome for patients with advanced disease remains poor (Dreiling et al. 1996) because of a high level of therapeutic resistance (Serrone and Hersey 1999).New treatment strategies are therefore needed.

In addition to its key role in cellular metabolism, where it acts as a ubiquitous enzyme co-factor and as the key source of cellular energy unique to phosphate bond formation, the purine nucleotide adenosine 5′-triphosphate (ATP) also functions as a potent extracellular messenger, producing its effects via a distinct family of cell surface receptors (Ralevic and Burnstock 1998). ATP was first shown to be a co-transmitter with noradrenaline in sympathetic nerves in smooth muscle cells of the vas deferens (Sneddon and Burnstock 1984). Sympathetic co-transmission has also been clearly demonstrated in a variety of blood vessels (Burnstock 1998). Co-transmission with acetylcholine in parasympathetic nerves has also been proposed in organs such as the urinary bladder (Burnstock et al. 1972; Hoyle and Burnstock 1993).

A large family of extracellular receptors exists for ATP and related molecules. Purinergic receptors are divided into two groups: P1 purinoceptors are selective for adenosine and P2 purinoceptors are selective for ATP. Four different P1 adenosine receptors have been cloned and are coupled to G proteins (Fredholm et al. 2001). These receptors are widely distributed in the human body regulating the function of virtually every organ and tissue. Two families of P2 purinoceptors are currently recognised: the P2X gated ion channel and the P2Y G protein-coupled receptor family. Seven subtypes of P2X receptors have been described and eight subtypes of P2Y receptors (Abbracchio and Burnstock 1994). The different receptors have been shown to have different functions. For instance, the $P2Y_2$ receptor plays a role in cellular proliferation (Greig et al. 2003b), the $P2X_5$ receptor has been implicated in differ-

entiation (Ryten et al. 2002) and activation of the $P2X_7$ receptor leads to apoptosis (Humphreys et al. 2000).

The $P2X_7$ receptor was first cloned from rat brain and from macrophages in 1996 (Surprenant et al. 1996). $P2X_7$ receptors are formed by the aggregation of homomeric $P2X_7$ receptor subunits (Torres et al. 1999), which combine to form a ligand-gated ion channel that allows the passage of cations when activated. Each subunit consists of two transmembrane domains, which are separated by an extensive N-glycosylated extracellular loop (always containing ten cysteine residues), and intracellular amino (N) and carboxy (C) termini.

$P2X_7$ receptors are non-selective cation channels. Under normal physiological conditions, the activation of $P2X_7$ receptors will result in Na^+ and Ca^{2+} influx and K^+ efflux across the cell membrane leading to depolarisation of the plasma membrane and an increase in intracellular Na^+ and Ca^{2+} concentrations. Membrane depolarisation can in turn activate voltage-gated channels, causing the firing of action potentials. The $P2X_7$ receptor functions like other P2X receptors when briefly activated but, when exposed to ligands such as ATP at high concentrations or for long periods, the cation channel can also be converted to a large non-selective transmembrane pore that allows the passage of not only cations, but also small molecules up to the size of 900 Da (Surprenant et al. 1996). This effect is associated with the mediation of a number of physiological processes, including apoptosis via the caspase enzyme system (Humphreys et al. 2000), release of cytokines such as interleukin-1β, interferon-γ and tumour necrosis factor-α (Ferrari et al. 2000; Humphreys and Dubyak 1996; Mehta et al. 2001), vesicle release (MacKenzie et al. 2001) and cell fusion (Chiozzi et al. 1997). $P2X_7$ receptors have been described in a wide range of human tissues and cells including lymphocytes (Wiley et al. 1998), macrophages (Chiozzi et al. 1997), dendritic cells (Coutinho-Silva et al. 1999) and keratinocytes (Greig et al. 2003b).

Raised extracellular ATP has been shown to inhibit tumour growth in vitro and in vivo (Greig et al. 2003a; Janssens and Boeynaems 2001; Rapaport 1983; Rapaport and Fontaine 1989). Several clinical trials have been carried out with respect to the beneficial use of ATP against cancer (Agteresch et al. 2000; Haskell et al. 1996). Attempts have been made to identify the mechanism of action of ATP on cancer cells and the purinergic receptor subtypes involved. Decreases in cancer cell number may be attributable to an inhibition of cell proliferation mediated via P2Y receptors (Dubyak and De Young 1985; Lin and Chuang 1993; Maaser et al. 2002), the stimulation of differentiation with the subsequent inhibition of proliferation via $P2X_5$ receptors (Cowen et al. 1991; Popper and Batra 1993) or the induction of cell death (apoptosis) via $P2X_7$ receptors (Chueh and Kao 1993).

P1 receptors have been described in the human melanoma A375 cell line (Merighi et al. 2001), with evidence for all four subtypes of receptor being present, and have been postulated as a possible target for the treatment of this cancer (Merighi et al. 2002). Recently, evidence for the presence of $P2X_7$ receptors in human melanomas has been demonstrated in specimens of superficial spreading melanoma by immunohistochemistry (Slater et al. 2003).

In this study, we confirm the presence of $P2X_7$ receptors in human melanoma tissue and show that the A375 melanoma cell line expresses functional $P2X_7$ receptors at the cellular and molecular level. We also demonstrate that the activation of this receptor causes apoptosis of melanoma cells in a dose-dependent manner.

Materials and methods

Cell culture

The melanoma cell line A375 (Giard et al. 1973) was obtained from the Wellcome Trust Functional Genomics Cell Bank (St. Georges Hospital Medical School, London, UK). Melanoma cells were grown in 90% Dulbecco's modified Eagle's medium and 10% heat inactivated fetal calf serum supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) in 75-cm^2 tissue culture flasks (Corning, New York, USA). All other cell culture reagents were obtained from Sigma (Poole, UK). Cells were incubated at 37°C in 5% CO_2/95% air and were sub-cultured at 70% confluence. Cell viability was determined by using the trypan blue exclusion method.

Reverse transcriptase-polymerase chain reaction

The synthesis of mRNA encoding the seven human P2X receptor subtypes in A375 melanoma cells was determined by using reverse transcriptase (RT) and the polymerase chain reaction (PCR). Total RNA was isolated from A375 cells grown in culture, to confluence, by using the SV Total RNA Isolation System (Promega, Wis., USA). RT-PCRs were carried out with Ready-To-Go RT-PCR beads (Amersham Biosciences, N.J., USA). RT was carried out on 1 μg RNA for 30 min at 42°C. PCR cDNA samples were initially denatured for 5 min at 95°C prior to the addition of the primer sequences. The human P2X primer sequences used were: $P2X_1$ 5′-gac aac tcc ttc gtg gtc at-3′ and 5′-ccg tac gtg cca gtc cag gt-3′, product size 510 base pairs; $P2X_2$ 5′-gca tcg gag tgc aac ccc aa-3′ and 5′-tca cag gcc agc tac ct gag-3′, product size 355 base pairs; $P2X_3$ 5′ atc aac cga gta gtt cag c-3′ and 5′-gat gca ctg gtc cca gg-3′, product size 695 base pairs; $P2X_4$ 5′-gag att cca gat gcg acc-3′ and 5′-gac ttg agg taa gta gtg g-3′, product size 295 base pairs; $P2X_5$ 5′-tcg act aca aga cc gaga ag-3′ and 5′-ctt gac gtc cat cac att g-3′, product size 595 base pairs; $P2X_6$ 5′-aaa aac agg cca gtg tgt ggt gtt c-3′ and 5′-tgc ctg ccc ggt gac gag gat gtc ga-3′, product size 520 base pairs; $P2X_7$ 5′-aac atc act tgt acc ttc c-3′ and 5′-tgt gaa gtc cat cgc agg-3′, product size 674 base pairs (Adrian et al. 2000; Janssens and Boeynaems 2001; Nakamura et al. 2000). All primer sequences were obtained from Invitrogen (Paisley, UK). The PCR cDNA samples were run for 35 cycles (30 s at

95°C, 1 min at 54-60°C and 1 min at 72°C) with a final extension at 72°C for 5 min in a DNA thermal cycler (Hybaid, UK). The size of the PCR products was determined by agarose gel electrophoresis (Gibco, Paisley, UK) and compared with a DNA ladder (Sigma).

Immunohistochemistry of paraffin-embedded specimens

A total of 14 specimens of melanoma of differing histological subtype were examined. Paraffin blocks were sectioned at a thickness of 4 μm on a Reichert-Jung Microtome. Sections were mounted on Snowcoat Extra slides (Surgipath, Cambridgeshire, UK), dried in an oven for 2 hours at 60°C, dewaxed and rehydrated through xylene and graded concentrations of ethanol. Antigen retrieval was performed by microwaving the slides for 10 min in a solution of 1 mM ethylenediamine tetraacetic acid (Tris EDTA) at pH 9.0. Endogenous alkaline phosphatase was blocked by a 20-min incubation in 20% acetic acid. Sections were washed and then incubated with avidin D blocking solution, biotin blocking solution and 1:5 normal swine serum (Vector laboratories).

$P2X_7$ receptor antibody (100 μl, diluted 1:100) was applied to the sections for 12 h at 4°C. The immunogen used for the production of the polyclonal $P2X_7$ antibody was a synthetic peptide corresponding to 15 receptor-type-specific amino acids (peptide sequence TWRFGSQDMA DFAIL) in the intracellular C-termini of the cloned human $P2X_7$ receptor, obtained by a similar method to that previously described (Giard et al. 1973; Oglesby et al. 1999). The polyclonal antibody was raised by multiple monthly injections of New Zealand rabbits with the peptide.

Biotinylated anti-rabbit antibody (DAKO E0353; 100 μl, diluted 1:200 in DAKO ChemMate dilutent) was applied for 30 min followed by 100 μl streptavidin alkaline phosphatase (Vector SA5100; diluted 1:200 in DAKO ChemMate) for 30 min. Vector Red substrate (Vector alkaline phosphatase substrate, SK5100) prepared in 200 mM TRIS-HCl (pH 8.2) was then applied for 10 min. Positive $P2X_7$ staining appeared bright pink, whereas nuclei were counterstained purple with haematoxylin. All sections were subsequently dehydrated, cleared and mounted.

Negative controls were performed by either omission of the primary antibody or preabsorption of the primary antibody with the corresponding peptide sequence.

Immunocytochemistry of cells grown in culture

A375 cells were grown in culture on chamber slides (Nunc, Ill., USA). They were fixed in 4% formaldehyde in 0.1 M phosphate buffer for 2 min. Non-specific binding sites were blocked by a 20-min preincubation with 10% normal horse serum (NHS) in 0.1 M phosphate buffer containing 0.05% merthiolate, followed by incubation with primary $P2X_7$ antibody (diluted 1:100), with 0.2% Triton X-100, for 12 h at 4°C. Subsequently, the slides were incubated with donkey anti-rabbit Cy3 (Jackson Immunoresearch, Pa., USA; diluted 1:300) with 1% NHS in phosphate buffer. Slides were then mounted with Eukitt (BDH laboratories, Dorset, UK) and examined. Control experiments were carried out with the primary antibody being omitted from the staining procedure or the primary antibody being preabsorbed with the corresponding peptide. All other reagents were obtained from Sigma.

YO-PRO-1 fluorescent imaging of $P2X_7$ receptor mediated pore formation

A375 human melanoma cells were grown in 96-well plates (BD Falcon, N.J., USA) to confluence. For the measurement of $P2X_7$ receptor-mediated pore formation, cells were incubated in an extracellular bath solution consisting of 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $CaCl_2$ and 10 mM D-glucose (pH adjusted to 7.4 with 1 M NaOH), containing 1 μM YO-PRO-1 (Molecular Probes, Eugene, Ore., USA). The plates were placed into a Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices, Calif., USA) in which they were incubated at 37°C prior to preincubation with the $P2X_7$ selective antagonist 1-N,O-bis-[5-isoquinoline-sulfonyl]-N-methyl-L-tyrosyl)-4-phenyl-piperazine (KN-62) for 20 min and the addition of the $P2X_7$ selective agonist 2′– 3′ -O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate (BzATP). Both compounds were obtained from Sigma. The cells were excited by an argon-ion laser source at a wavelength of 488 nm and emission was measured at 520 nm at 30-s intervals for 60 min. The change in fluorescence measured was plotted in arbitrary units as a change from baseline fluorescence prior to the addition of BzATP.

Cell proliferation assay

A375 human melanoma cells were seeded onto a 96-well plate at a density of 250 cells per well (2,500 cells/ml with 100 μl cell suspension/well). This seeding density gave the best growth curve over a 96-h period. The medium was aspirated 24 h after seeding and fresh medium (control) or medium containing $P2X_7$ receptor agonist BzATP and/or $P2X_7$ antagonist KN-62 was added to the well plates. Prior to addition to the cells, the pH of the drug solutions prepared in the culture medium was adjusted to 7.36–7.44 with 1 M NaOH.

Changes in cell number were quantified via a colorimetric assay by using crystal violet (Gillies et al. 1986) and read by using a spectrophotometric plate reader (Labtech, East Sussex, UK) 48 h after the addition of the purinergic agents.

For the colorimetric assay, a solution of 0.5 g crystal violet, 0.85 g NaCl, 5 ml 10% formal saline, 50 ml absolute ethanol and 45 ml distilled water was used. Medium was aspirated from the wells and 100 μl colorimetric assay

mixture was added to each well and incubated at room temperature for 10 min. This mixture allowed the simultaneous fixation of the cells and the penetration of the crystal violet dye into the cells. After three washes with phosphate-buffered saline, 33% acetic acid was used to elute colour from the cells and the optical density was read at a wavelength of 570 nm by using a spectrophotometric plate reader (Labtech). To confirm that the optical density of the wells correlated with cell number, a control assay was performed for each experiment: known numbers of cells were seeded in ascending seeding densities and the plate read as soon as the cells had attached. Cell number versus optical density was plotted. The R^2 value of the trend line was always greater than 0.98.

Caspase 3/7 apoptosis assay

A375 melanoma cells were incubated with 100 μl medium containing 30–300 μM BzATP for 6 h in a 96-well white-walled luminometer plate. Caspase-Glo 3/7 reagent (100 μl; Promega) was added to each well to be measured, including blank baseline and untreated control wells. The contents were gently mixed at 300 rpm for 30 s and then incubated at room temperature for 1 h. Luminescence was measured with a plate reading luminometer (Labtech). The blank baseline signal was subtracted from the signal produced by the treated and untreated cells.

Results

$P2X_7$ receptor mRNA is present in A375 melanoma cells

RT-PCR analysis of P2X receptor subtypes demonstrated a positive band at the expected size for the $P2X_7$ receptor (Fig. 1). No mRNA for the $P2X_{1–6}$ receptor subtypes was detected.

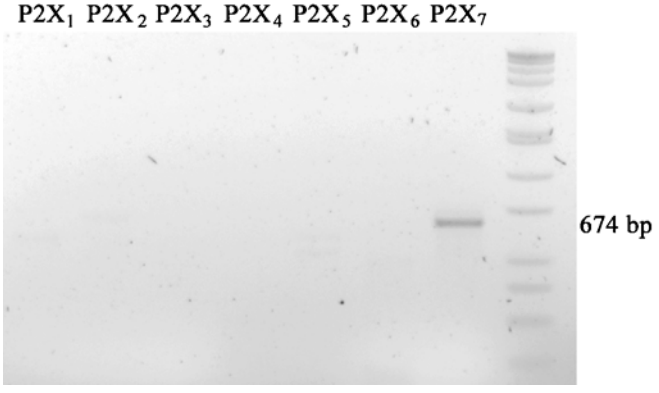

Fig. 1 $P2X_7$ receptor mRNA is present in melanoma cells. RT-PCR analysis of total RNA extracted from A375 melanoma cells generated a fragment of the expected size of 674 bp for the $P2X_7$ receptor. No mRNA for the other P2X receptor subtypes was demonstrated. A size ladder is shown *right*

$P2X_7$ receptors are present in paraffin-embedded sections of melanoma

Positive labelling for $P2X_7$ receptors was found in all specimens of examined melanoma (Fig. 2a). Over 50% of the cells in each specimen were positive for $P2X_7$ receptors and, in the majority of specimens, over 75% of the cells stained positive. Between the different histological subtypes, no differences were seen in the number of cells in each specimen with positive staining for $P2X_7$ receptors. As an internal positive control, $P2X_7$ receptors were also appropriately stained in the stratum corneum of the epidermis (Fig. 2b) as previously described (Greig et al. 2003b).

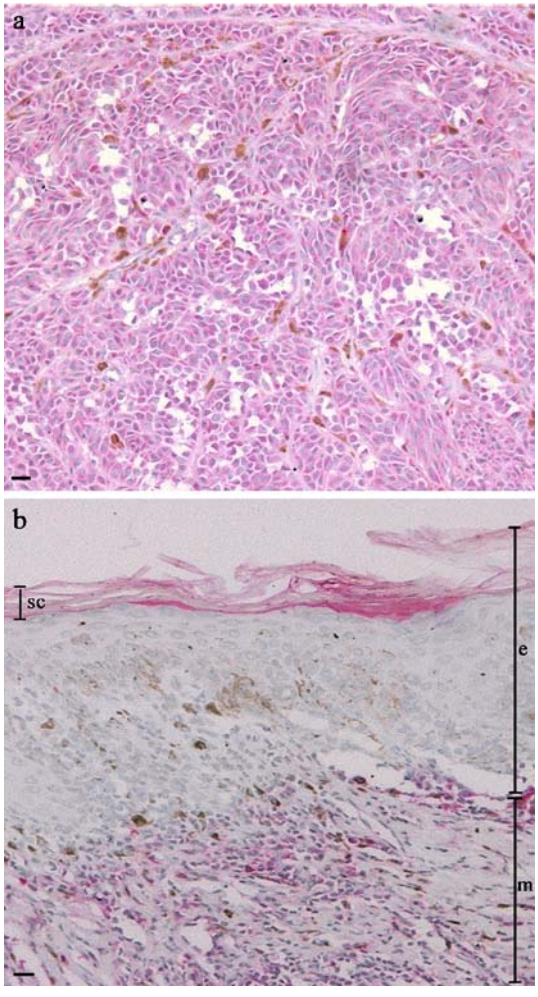

Fig. 2 $P2X_7$ receptors are present in sections of melanoma. **a** Streptavidin alkaline phosphatase immunostaining of $P2X_7$ receptors (*pink*) with haematoxylin counterstain (*purple*) in a formalin-fixed paraffin-embedded melanoma. *Bar* 250 μm. **b** Staining of melanoma cells infiltrating the dermis (*m*) and the stratum corneum (*sc*) of the epidermis in the same section as an internal positive control. Note no positive staining in the other layers of the epidermis (*e*). *Bar* 500 μm

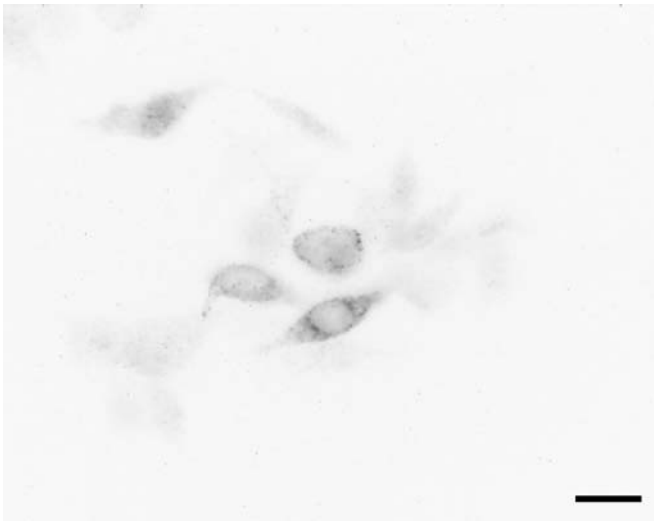

Fig. 3 $P2X_7$ receptors are present in A375 human melanoma cells grown in culture. Fluorescent extracellular staining for the $P2X_7$ receptor in human melanoma A375 cells grown in culture (reverse image). *Bar* 25 μM

$P2X_7$ receptors are expressed in A375 human melanoma cells grown in culture

A375 melanoma cells grown in culture synthesised $P2X_7$ receptors (Fig. 3). Both preabsorption with the corresponding peptide and omission of the primary antibody were performed as controls; minimal immunoreaction was seen under these conditions.

$P2X_7$ receptor agonist mediates YO-PRO-1 uptake

$P2X_7$ receptor-mediated pore formation was assessed by imaging the uptake of YO-PRO-1, a 629-Da propidium diiodide dye that fluoresces when bound to nucleic acids. Activation of $P2X_7$ receptors results in the opening of non-specific pores permeable to molecules smaller than 900 Da, such as YO-PRO-1 (Surprenant et al. 1996). A time-dependent increase in fluorescence intensity occurred in response to the application of the $P2X_7$ receptor agonist BzATP. This was also dose-dependent. The effect of 10 μM BzATP was completely blocked by preincubation with 100 nM KN-62, the $P2X_7$ receptor-specific antagonist. Figure 4 shows the response to 10 μM or 100 μM BzATP alone and after preincubation with the antagonist KN-62. These results demonstrate the presence of functional $P2X_7$ receptors in human A375 melanoma cells.

Decrease in melanoma cell number caused by treatment with $P2X_7$ receptor agonist is reversed by competitive antagonist

The proliferative effects of a sustained application of the $P2X_7$ receptor agonist BzATP was studied in human melanoma cells by using a crystal violet colorimetric assay. BzATP was found to cause a dose-dependent decrease in cell number, which was partially reversed by addition of the competitive antagonist KN-62 (Fig. 5). At a dose of 100 μM BzATP or higher, a significant ($P<0.001$) reduction in cell number was seen after 48 h. A maximum decrease in cell number of 29% was obtained with 300 μM BzATP. This decrease in cell number was partially reversed in the presence of 100 nM KN-62, with only a 21% decrease being measured when cells were treated with 300 μM BzATP. The decrease in cell number caused by 100 μM BzATP was also partially reversed by 100 nM KN-62 but this result was not statistically significant. The antagonist KN-62, which is a potent calmodulin kinase inhibitor that has been shown to induce cell death in other cell types, had a minimal affect on A375 cell number in isolation.

$P2X_7$ receptor activation results in apoptosis

To ascertain whether the anti-proliferative action of $P2X_7$ receptor agonist BzATP was attributable to an induction of

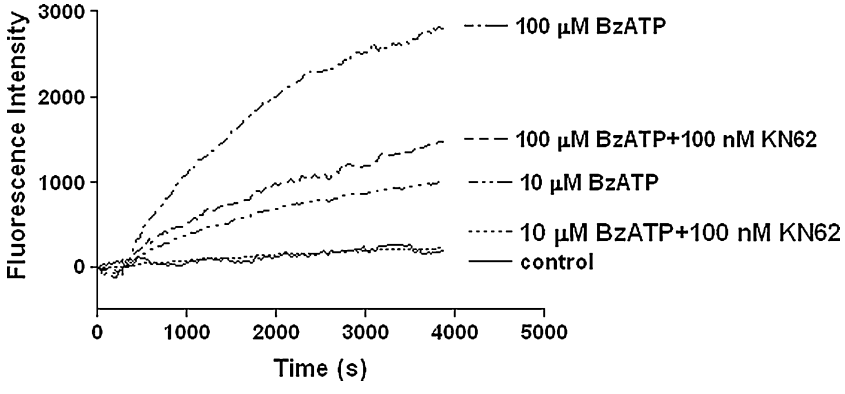

Fig. 4 Demonstration of functional $P2X_7$ receptors in human A375 melanoma cells. The $P2X_7$ receptor agonist BzATP mediates uptake of the fluorescent DNA-binding dye YO-PRO-1. Fluorescence intensity increases in a time-dependent manner in response to application of the $P2X_7$ receptor agonist BzATP up to timepoint 300 s. This response is also dose-dependent. Preincubation with 100 nM KN-62, the $P2X_7$ receptor-specific antagonist, completely blocks the effect of 10 μM BzATP and reduces the effect of 100 μM BzATP

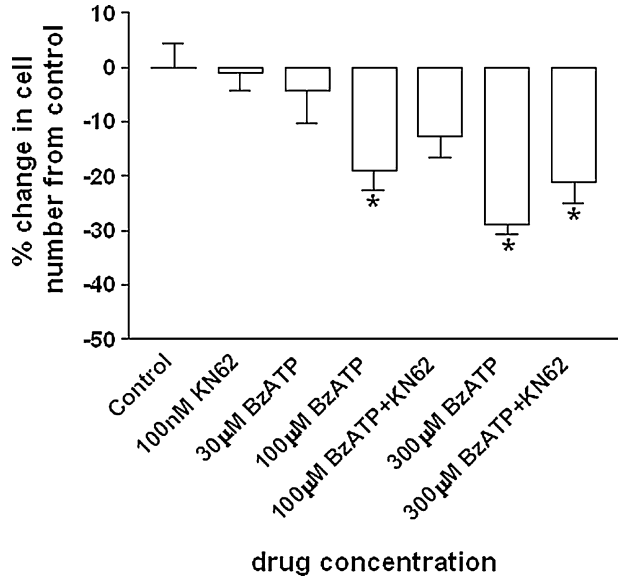

Fig. 5 Cell proliferation assay following sustained treatment of human melanoma cells with the $P2X_7$ receptor agonist BzATP; a crystal violet colorimetric assay. BzATP caused a dose-dependent decrease in cell number, which was partially reversed by addition of the competitive antagonist KN-62. At 100 μM BzATP and higher, a significant reduction in cell number occurred after 48 h when compared with the control. This decrease in cell number was partially reversed in the presence of 100 nM KN-62 (300 μM BzATP compared with 300 μM BzATP+100 nM KN-62). Means± SEM (n=9, *P<0.001)

programmed cell death, the activation of caspase-3 and caspase-7, key enzymes in the process of apoptosis, was investigated in A375 melanoma cells. After a 6-h incubation, BzATP dose-dependently induced an increase in caspase activity. At 300 μM BzATP, caspase activity was increased by 75% when compared with the untreated control (Fig. 6).

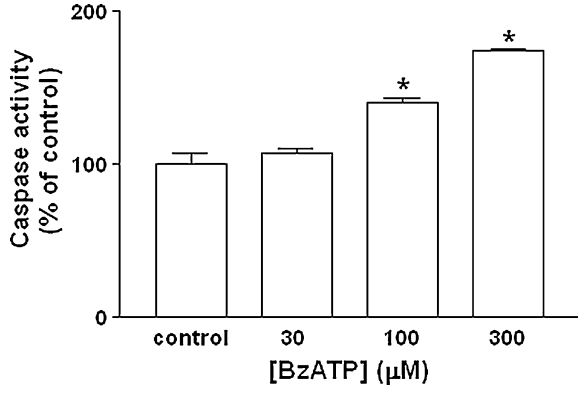

Fig. 6 Application of the $P2X_7$ receptor agonist BzATP causes an increase in caspase 3/7 activity in the A375 melanoma cell line. After incubation with BzATP, caspase-3/7 activity increased in a dose-dependent manner. At 300 μM BzATP, caspase-3/7 activity was increased by 75% compared with the untreated control. Means ±SEM (n=9, *P<0.001)

Discussion

Malignant melanoma is the most important cutaneous malignancy, since it accounts for over 95% of all deaths from skin disease, its incidence is on the increase and it is resistant to current treatments (Dreiling et al. 1996; Osborne 2002). ATP is known to inhibit cancer cell growth in a variety of models and through a number of mechanisms (Abraham et al. 2003). One of these is thought to be through the activation of the apoptotic $P2X_7$ receptor (Chueh and Kao 1993). $P2X_7$ receptors have been previously described in several cancer types, including neuroblastomas (Per et al. 2002), osteosarcomas (Gartland et al. 2001), squamous cell carcinoma of the skin (Greig et al. 2003a) and prostate cancer (Calvert et al. 2004). Recently, $P2X_7$ receptors were described in human melanomas for the first time by immunohistochemical localisation of receptors in specimens of superficial spreading melanoma (Slater et al. 2003). In this paper, we provide evidence of functional $P2X_7$ receptors in the human melanoma A375 cell line at both the cellular and molecular levels and demonstrate that the activation of this receptor causes apoptosis of melanoma cells in a dose-dependent and reversible manner.

We have shown the synthesis of both $P2X_7$ receptor mRNA and protein in the human A375 melanoma cell line. To validate this cell line further as a model of purinergic receptor expression in melanoma, we have used the same antibody to $P2X_7$ receptors to confirm the presence of this purinoreceptor subtype in formalin-fixed paraffin-embedded specimens of melanoma excised from patients. $P2X_7$ receptors are consistently present in these specimens across differing histogenetic subtypes; this correlates with previous observations.

Currently, no selective agonists exist for all the purinoceptors (Ralevic and Burnstock 1998) and therefore pharmacological identification relies on the effects of a combination of compounds. ATP itself is active over the entire range of metabotropic P2Y and ionotropic P2X receptors. BzATP is a potent agonist active at the $P2X_7$ receptor, although it has also been shown to have effects on other P2 receptors (Khakh et al. 2000; King et al. 2000). KN-62 is a potent selective inhibitor of $P2X_7$-mediated responses in human cells (Gargett and Wiley 1997). The inhibition of BzATP-induced YO-PRO-1 entry into A375 melanoma cells by KN-62 implies that the effect of BzATP is mediated through $P2X_7$ receptors.

We have shown that the effect of the incubation of A375 melanoma cells in the presence of BzATP is a statistically significant (P<0.001) decrease in cell number at doses of 100 μM or higher. This is dose-dependent and partially reversed by KN-62 and is to be expected if BzATP acts on an extracellular receptor linked to cell death. Consistent with these findings is the increase in the activity of caspase-3 and caspase-7 enzymes after the treatment of cultured melanoma cells with BzATP. Both these enzymes are involved in intracellular pathways mediating apoptosis (Nicholson and Thornberry 1997) and their up-regulation

is indicative of an apoptotic mechanism, such as the activation of $P2X_7$ receptors.

In summary, we have shown, for the first time, that functional $P2X_7$ receptors are present in human melanomas and that their activation causes a decrease in cell number by apoptosis. Purinergic receptors are distributed widely throughout normal and diseased tissue and further subtypes of either P2X or P2Y receptors will probably be found in malignant melanoma. The targeting of $P2X_7$ receptors may provide a novel treatment for this disease.

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