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Effects of extracellular nucleotides and nucleosides on prostate carcinoma cells

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1 The purpose of this work was to characterize the receptors involved in the action of nucleotides on the human prostate carcinoma cell lines LNCaP, PC-3 and DU145.

2 Northern blotting revealed the presence of $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ messengers in the three cell lines. $P2Y_1$ mRNA was only observed in the DU145 cells. In both PC-3 and DU145 cells, ATP and UTP stimulated inositol phosphate accumulation in an equipotent, equiactive and non-additive way, suggesting the involvement of $P2Y_2$ receptors. ATP also increased cyclic AMP, but this effect is likely to result from degradation into adenosine and activation of A_2 receptor. A_2 receptor activation led to a synergistic enhancement of prostate-specific antigen secretion induced by vasoactive intestinal peptide.

3 RT–PCR experiments detected the expression of the $P2X_4$ and $P2X_5$ receptors in the DU145 cells and the $P2X_4$, $P2X_5$ and $P2X_7$ receptors in the PC-3 cells. The calcium influx induced by BzATP confirmed the functional expression of P2X receptors.

4 ATP inhibited the growth of PC-3 and DU145 cells. This effect was mimicked neither by UTP nor by adenosine, indicating that it does not result from phospholipase C or adenylyl cyclase activation. On the contrary, in PC-3 cells, BzATP reproduced the effect of ATP, which was associated to a moderate decrease of proliferation and an increase of apoptosis. In DU145 cells, ATP was more potent than BzATP and growth inhibition was mainly associated with necrosis. We suggest that P2X receptors might be involved in the inhibition by nucleotides of prostate carcinoma cell growth.

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Keywords: P2Y receptors; P2X receptors; adenosine receptors; LNCaP; PC-3; DU145; PSA; cell growth; apoptosis; necrosis

Abbreviations: AC, adenylyl cyclase; AS, antisense; ATPγS, adenosine 5′-O(3-γ-thio)triphosphate; bp, base pair; BzATP, 2′,3′-O-(4-benzoyl-benzoyl)adenosine 5′-triphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; FBS, fœtal bovine serum; FK, forskolin; $InsP_3$, inositol trisphosphates; kb, kilobase; KRH, Krebs-Ringer-Hepes; α,βmeATP, α,β-methylene adenosine 5′-triphosphate; MEM, Eagle's modified essential medium; 2-MeSADP, 2-methylthio-adenosine 5′-diphosphate; 2-MeSATP, 2-methylthio-adenosine 5′-triphosphate; pA RNA, polyA RNA; PBS, phosphate buffer saline; PLC, phospholipase C; PSA, Prostate Specific Antigen; 8-PST, 8-p-sulphophenyltheophyllin; RT–PCR, reverse transcription-polymerase chain reaction; S, sense; s.d., standard deviation; SDS, sodium dodecyl sulphate; s.e.mean, standard error of the mean; SSC, standard saline citrate; VIP, vasoactive intestinal peptide

Introduction

Extracellular adenine nucleosides and nucleotides as well as uracil nucleotides exert many effects on a variety of tissues and cell lines. These effects are mediated respectively by the P1 and P2 receptors. The P1 family includes four subtypes of adenosine receptors, A_1, A_{2A}, A_{2B} and A_3, mainly involved in the modulation of the adenylyl cyclase (AC) activity (Fredholm *et al*., 1994). The effects of nucleotides are mediated by the P2 receptors that are segregated in two subfamilies according to their structure: the P2X and the P2Y receptors (Abbrachio & Burnstock, 1994). The former are ligand-gated ion channels possessing two transmembrane segments: seven different members have been cloned (Valera *et al*., 1994; Brake *et al*., 1994; Chen *et al*., 1995; Buell *et al*., 1996; Collo *et al*., 1996; Surprenant *et al*., 1996). The later are G-protein-coupled receptors and five different mammalian subtypes have been cloned and proven to be responsive to nucleotides: $P2Y_1$ (Webb *et al*., 1993), $P2Y_2$ (Lustig *et al*., 1993), $P2Y_4$ (Communi *et al*., 1995a), $P2Y_6$ (Chang *et al*., 1995) and $P2Y_{11}$ (Communi *et al*., 1997) receptors. All these receptors are coupled to the phospholipase C pathway (PLC); moreover the $P2Y_{11}$ receptor is also positively coupled to the AC pathway. Other subtypes ($P2Y_5$, $P2Y_7$, $P2Y_9$ and $P2Y_{10}$) have been mistakenly included in the P2Y family on the basis of structural homology, but in the absence of a functional response to nucleotides (Janssens *et al*., 1997; Li *et al*., 1997; Yokomizo *et al*., 1997).

Three human prostate carcinoma cell lines have been established and well characterized: DU145 and PC-3, both androgen-independent, and LNCaP, an androgen-sensitive cell line. Effects of nucleotides on these cell lines have been

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investigated previously, but the receptors involved in these actions were not reliably identified. ATP and UTP were shown to increase cytosolic calcium in PC-3 and DU145 cell lines (Fang *et al*., 1992; Wasilenko *et al*., 1997). ATP (UTP was not tested) strongly inhibited the growth of PC-3 and DU145 cells (Fang *et al*., 1992): the authors speculated that this inhibition resulted from the activation of phospholipase C and rise in $[Ca^{2+}]_i$ observed in these cells. Similarly, ATP increased the inositol phosphate level and inhibited growth of rat prostate epithelial cells (Guijarro *et al*., 1996). The inhibition of prostate carcinoma cell proliferation by ATP is potentially interesting, since prostate cancer is a major cause of cancer death in male subjects. While there is initially a high rate of response to hormonal therapy, most treated patients relapse with androgen-independent tumor, refractory to further hormonal therapy and traditional chemotherapy: this underscores the need of new therapeutic approaches (Isaacs *et al*., 1987).

In this study, we have expanded these previous observations with the purpose of identifying the receptors involved in the action of nucleotides, by a combination of pharmacological and molecular biology experiments.

Methods

Cells

The human prostatic carcinoma cell lines LNCaP, PC-3 and DU145 were a gift of Dr R. Kiss, Medical Faculty of the Université Libre de Bruxelles. LNCaP were grown in RPMI 1640 medium with 10% foetal bovine serum (FBS); PC-3 cells in Ham's F-12 medium with 8% FBS and DU145 in Eagle's modified essential medium (MEM) plus 8% FBS. All media were supplemented with 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 300 μg ml^{-1} glutamine and 2.5 μg ml^{-1} amphotericin B.

Inositol phosphates measurements

Thirty thousand prostatic cells were labelled for 48 h with 1-*myo*-D-2[^{3}H]-inositol (10 μCi ml^{-1}) in inositol-free Ham's F-12 (PC-3) or inositol-free MEM (DU145), containing 5% FBS, antibiotics, amphotericin B and glutamine. The cells were preincubated 20 min in a Krebs-Ringer-HEPES medium (KRH) (in mM): NaCl 124; KCl 5; $MgSO_4$ 1.25; $CaCl_2$ 1.45; KH_2PO_4 1.25; HEPES 25 and glucose 8) containing LiCl 10 mM, and then stimulated for various times with the agonist in a KRH medium supplemented with LiCl. Incubations were terminated by the addition of ice-cold 3% perchloric acid solution. Extraction and isolation of inositol trisphophates ($InsP_3$) on Dowex AG1-X8 were done as described (Communi *et al*., 1995b). Experiments were performed at least three times, each with triplicate samples. The EC_{50} values (mean $\pm$ s.e.mean) were calculated on basis of three independent experiments using SigmaPlot v2.0 Curve Fit. Student's *t*-tests were performed with Microsoft Excel 97 software.

Calcium measurements

Intracellular calcium ($[Ca^{2+}]_i$) was measured in PC-3 cells using the fluorescent Ca^{2+} indicator fura 2-AM (Molecular Probes). Practically, the cells were concentrated at 10^7 ml^{-1} and incubated at 25°C for 45 min in a loading medium (in mM): glucose 11; NaCl 96; KCl 6; $MgCl_2$ 1; NaH_2PO_4 2.5; HEPES 24.5; BSA 0.5%; $CaCl_2$ 0.25) containing 2 μM of fura 2-AM and 0.005% of pluronic acid. After loading, the cells were quickly washed and diluted at the level of 10^6 cells ml^{-1} in a medium containing (in mM): glucose 11; NaCl 96; KCl 6; $MgCl_2$ 1; NaH_2PO_4 2.5; HEPES 24.5 and $CaCl_2$ 1. Two ml of the cells suspension were poured in a quartz cuvette and allowed to equilibrate at 25°C for 10 min. Measurements were then performed with the Luminescence Spectrometer LS50B (Perkin Elmer) at the same temperature. Fura 2-loaded cells were maintained under agitation and were alternatively excited at 340 and 380 nm. Maximal and minimal fluorescence values were respectively measured after the addition of solutions of digitonin (0.5 mM) and EGTA (40 mM). The emission light (510 nm) was collected and analysed through the FL WinLab software. The ratio between the fluorescence intensities recorded after excitation at 340 and 380 nm is related to the $[Ca^{2+}]_i$, which was calculated according to Grynkiewicz *et al*. (1985).

Cyclic AMP measurements

Cells were seeded on Petri dishes (200,000 cells) in complete medium and cultured overnight (as described in the 'Cells' section of Methods). The cells were first preincubated 30 min with 25 μM rolipram, a phosphodiesterase inhibitor, and then stimulated for 15 min with the nucleotides in presence or absence of forskolin (FK; 1 or 3 μM, according to the experiment and cell line), in a KRH medium supplemented with rolipram (25 μM). Alternatively, for the AC inhibition experiments, a 15 min preincubation with FK was performed, before the addition of the nucleotide. The incubation was stopped with HCl 0.1 M. After evaporation to dryness, the samples were diluted as required. Cyclic AMP was quantified by radioimmunoassay after acetylation (Brooker *et al*., 1979). Experiments were performed at least three times with triplicate samples.

Prostate-specific antigen measurements

Quantitative analysis of prostate-specific antigen (PSA) secreted by LNCaP cells was performed using a MEIA (Microparticles Enzyme ImmunoAssay) procedure on the AXSYM analyser (Abbott). Briefly, 300,000 cells cultured in complete medium were washed twice with serum-free RPMI 1640 medium and incubated in the same medium containing the agonist for 2 h. After the incubation, the medium was removed, centrifuged to remove any suspended cells and assayed.

Cell growth

At day 0, the PC-3 and DU145 cells were seeded at the density of 50,000 cells per 3.5 cm diameter Petri dish, respectively in a complete Ham's F-12 or MEM medium containing the agents; the seeding density was 100,000 cells for the LNCaP cell line. Addition of tested agents was repeated every day and the medium was renewed at day 2. The cell number at day 4 was determined by haemocytometer counting of trypan blue excluding cells. Serum concentration (FBS) were 10, 8 and 4% respectively for the LNCaP, PC-3 and DU145 cells.

Laddering experiments

At day 0, 400,000 PC-3 or DU145 cells were seeded in 10 cm diameter Petri dishes and cultured respectively in complete Ham's F-12 or MEM medium without or with nucleotides (ATP or 2′,3′-O-(4-benzoyl-benzoyl)adenosine 5′-triphosphate (BzATP) at 100 μM). Supernatant was taken every day from the second day and replaced by fresh medium and agonist. The supernatant, which contains cell fragments or dead cells, was centrifuged. The pellet was resuspended in 50 μl of phosphate buffered saline (PBS) and dissolved in the AL lysis buffer of the QIAamp DNA mini kit (Qiagen) supplemented with proteinase K, incubated 10 min at 56°C and stored at 4°C. At day 4, the remaining adherent cells in the Petri dish were detached, lysed, pooled together with the other lysates and centrifuged 30 min at $15{,}000 \times g$ at 4°C. Supernatant was then applied on QIAamp column, and DNA was eluted, separated by electrophoresis on a 2% agarose gel (8 μg DNA/lane) and visualized by ethidium bromide staining.

Cell proliferation assay

Fraction of cells entering into DNA synthesis was measured by the frequency of ^{3}H-thymidine-labelled nuclei, as estimated by autoradiography. At day 0, 50,000 PC-3 or DU145 cells were seeded in 3.5 cm diameter Petri dishes and cultured respectively in complete Ham's F-12 or MEM medium containing the agonist (ATP, BzATP) at 100 μM. At day 1 or 3, cells were incubated in presence of 10 μM [^{3}H]-thymidine, 10 μM D-cytidine and 3 μM cold thymidine for 12 h in 4% serum complete MEM medium for DU145 cells and 18 h in 8% serum complete Ham's F-12 medium for PC-3 cells. The cells were then fixed with methanol and washed with water. Photographic emulsion (Kodak) was poured on the dishes, which were stored for one week at 4°C before development and counting (at least 1000 cells per dish).

Northern blotting (P2Y receptors)

Total RNA was extracted with the RNeasy mini kit (Qiagen) and polyA RNA with the polyATtract, mRNA isolation system IV kit (Promega). Northern blots containing 10 μg of total RNA and 4 μg of polyA RNA of the three cell lines were prehybridized at least 4 h at 42°C and hybridized overnight at 42°C with a ^{32}P-labelled probe specific for the studied P2Y receptor. The membranes were washed 2×15 min at 42°C in a solution of standard saline citrate 2× (SSC) and 0.1% sodium dodecyl sulphate (SDS) and then 4×15 min at 65°C in a solution of $0.2 \times$ SSC and 0.1% SDS. The blots were exposed to a X-ray autoradiography film in presence of intensifying screen at −80°C for 2 to 7 days according to the probe.

RT–PCR (P2X receptors)

Total RNA of PC-3 and DU145 cells was submitted to DNAse treatment using the deoxyribonuclease I, amplification grade (GIBCO BRL) and reverse transcribed with the Superscript preamplification system (GIBCO BRL). Oligonucleotides specific for the seven different P2X receptors were used in PCR reaction using *Taq* DNA polymerase (93°C 3 min; then 93°C 1 min, 50–60°C 2 min and 72°C 3 min; 35 cycles; 72°C 6 min). Amplification products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. For each receptor, two different pairs (each pair = sense (S) and antisense (AS)) of oligonucleotides were designed on basis of the known sequences of cloned human P2X receptors (GenBank) and tested in PCR reactions. Oligonucleotide sequences and expected amplification product size (ps) in bp are given hereunder. First pair: $P2X_1S$: GACAACTCCTTCGTGGTCAT, $P2X_1AS$: CCGTACGTGCCAGTCCAGGT, ps = 510; $P2X_2S$: GACTACGAGACGCCCAAGGT, $P2X_2AS$: CTCGTGGAACGTGCAGCGCT, ps = 611; $P2X_3S$: AGTCGGTGGTTGTGAAGAGC, $P2X_3AS$: GCTGTAGTTCACGCAGCGGC, ps = 391; $P2X_4S$: ACTGCTCATCCTGGCCTACG, $P2X_4AS$: AAGCAGGTTGTGGCACGTGT, ps = 424; $P2X_5S$: GCCAGATATTACCGAGACGC, $P2X_5AS$: GTGCTCCTGTGGGGCTCCAG, ps = 373; $P2X_6S$ ($=P2X_MS$): CACAGGACCTGTGAGATCTG, $P2X_6AS$ ($=P2X_MAS$): AAGGTGACCACGCCCAGCCA, ps = 556; $P2X_7S$: GCAGAGGTGAAAGAGGAGAT, $P2X_7AS$: CTGTGGATTCTGAGTCTTGT, ps = 470. Second pair: $P2X_1S'$: CACGGCGGTTCCAGG, $P2X_1AS'$: CTCAGCCAGGGTGCT, ps = 740; $P2X_2S'$: ATCGTGGTGAGGAAC, $P2X_2AS'$: TGCACTCCGATGCAG, ps = 838; $P2X_3S'$: CTGCATATCCGACTT, $P2X_3AS'$: GCAGGATGATGTCAC, ps = 1025; $P2X_4S'$: ACGACACGCCGCGCA, $P2X_4AS'$: GGAAGGATATTCCTC, ps = 575; $P2X_5S'$: TCTGTCTCCTCCGGG; $P2X_5AS'$: CAGGGCCTGAACGTA, ps = 434; $P2X_6S'$ ($=P2X_MS'$): CAGGCCAGTGTGTGG, $P2X_6AS'$ ($=P2X_MAS'$): GAGTTGGCGGTTGCT, ps = 670; $P2X_7S'$: GGTGAGTGACAAGCT, $P2X_7AS'$: CTAGTCGGAAAATGG, ps = 560. RT–PCR experiments were performed three times on two different batches of RNA with the first set of oligonucleotides and once with the second set of oligonucleotides.

Results

Northern blotting study of P2Y receptors expression in PC-3, DU145 and LNCaP cells

Blots prepared with both total and polyA RNA of the different cell lines were hybridized with probes corresponding to the coding sequence of the different cloned human P2Y receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ (data not shown). The hybridization with the $P2Y_1$ probe revealed a characteristic doublet at high molecular weights (6.7 and 5.2 kb; Janssens *et al*., 1996) in the DU145 polyA RNA lane. The $P2Y_2$ messenger was detected in the three cell lines, but only after a 2 day exposure. A double band could be observed in PC-3 and DU145 lanes at 2.2 and 2.4 kb, while in LNCaP cells lanes, only the lowest size messenger was detectable. No $P2Y_4$ messenger was observed. The $P2Y_6$ receptor hybridization revealed a unique messenger of 2 kb in the androgen-insensitive cell lines, especially DU145. The faint band observed in the LNCaP polyA RNA lane had a slightly higher size (2.2 kb). Finally, the signal observed after the $P2Y_{11}$ probe hybridization was the most complex and revealed multiple messengers: signals were observed at 9, 5.0 and 4.2 kb (only 9 and 4.2 kb for the LNCaP polyA RNA

lane). A less intense and more diffuse signal could also be observed around 2.7 kb.

Pharmacological characterization of the P2Y receptors of PC-3, DU145 and LNCaP cells

In order to correlate the Northern blotting data with functional expression, we characterized the pharmacological response of the three cell lines to nucleotides: $InsP_3$ as well as cyclic AMP measurements were performed.

$InsP_3$ measurements We first established a time-course of $InsP_3$ accumulation induced by ATP (100 μM) in the PC-3 and DU145 cells, in presence of LiCl at 10 mM. The accumulation of $InsP_3$ was faster in the PC-3 cells: it was observed after 30 s of stimulation (65% of maximal response) and reached a plateau after 5 min. The accumulation of $InsP_3$ in the DU145 cells was slower and was observed only after 5 min (70% of maximal response), raising to a plateau at 15 min. No $InsP_3$ accumulation could be detected in the LNCaP cells when stimulated by ATP, ATPγS, ADP, UTP, UDP or carbachol in different experiments; the lack of effect of nucleotides on PLC stimulation was probably due to the poor incorporation of [^{3}H]-inositol into membrane lipids (data not shown). Full concentration-responses curves were obtained after a 5 min (PC-3 cells) or 15 min (DU145 cells) incubation with UTP and ATP in presence of LiCl 10 mM. For both cell lines, the results were similar: UTP and ATP raised almost equipotently and to the same extent the level of $InsP_3$ (Figure 1). The EC_{50} values (mean $\pm$ s.e.mean) were as follows: EC_{50} PC-3 cells (UTP $= 0.7 \pm 0.1$ μM; ATP $= 1.3 \pm 0.2$ μM), EC_{50} DU145 cells (UTP $= 0.9 \pm 0.2$ μM; ATP $= 1.2 \pm 0.2$ μM). Responses to 2-MeSADP, BzATP and UDP were also tested (concentrations ranged from 10–100 μM). The first two did not stimulate the PLC of either androgen-insensitive cells, whereas UDP increased the level of $InsP_3$ when incubated for 5 min or more in the PC-3 and DU145 cells. Increases of [^{3}H]-$InsP_3$ generated by ATP, UTP or UDP were not additive, whereas the histamine + ATP and histamine + UTP combinations produced additive effects (data not shown).

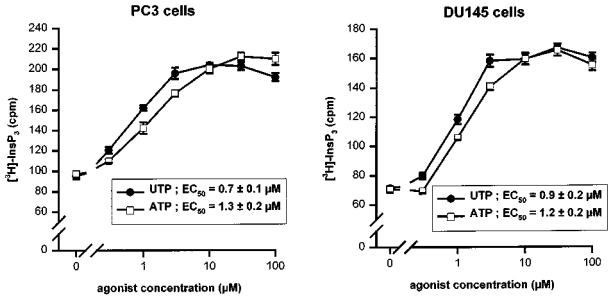

Figure 1 Concentration-action curves of UTP and ATP on the $InsP_3$ accumulation in PC-3 or DU145 cells. ^{3}H-inositol-labelled cells, preincubated 20 min with 10 mM LiCl, were incubated in the presence of various concentrations of nucleotides for 5 min (PC-3 cells) or 15 min (DU145 cells). The data are mean $\pm$ s.e.mean of three independent experiments with triplicate experimental points.

Cyclic AMP measurements In view of the expression of $P2Y_{11}$ transcripts, we tested the effects of the most potent agonists of that receptor (ATPγS > BzATP > ATP: Communi *et al*., 1999). They all increased intracellular cyclic AMP in PC-3 cells (Figure 2A). However, the BzATP stimulation was clearly less pronounced than the effect of ATP and ATPγS, and ADP, which is a weak agonist of $P2Y_{11}$ receptor, strongly raised the cyclic AMP concentration. Adenosine mimicked the effect of ATP and the stimulation generated by adenosine as well as ATP was abolished in presence of the adenosine receptors antagonist, 8-p-sulphophenyltheophylline (8-PST) at 300 μM (a 80% inhibition was observed at 100 μM, data not shown) (Figure 2A). These data demonstrate that the effect of ATP on cyclic AMP in the PC-3 cell line is mediated by its degradation into adenosine and does not involve $P2Y_{11}$ receptors. In DU145 cells, as represented in Figure 2B, ATP, ATPγS (100 μM) or adenosine (1 μM) produced only a slight increase of the basal cyclic AMP level. However, ATP and adenosine strongly potentiated the stimulatory effect of forskolin (FK; 1 μM), ATPγS being less effective. This potentiation was abolished by 8-PST (100 μM), again suggesting that the effect of ATP is mediated by its degradation into adenosine (Figure 2B). In the LNCaP cells (Figure 2C), only adenosine had an effect: *per se* it weakly increased the basal cyclic AMP level, but it strongly enhanced the stimulation by FK (3 μM). No increase of the cyclic AMP level (as compared to FK alone) was observed after a 15 min challenge with ATP or ATPγS. We also tried to detect a potential inhibition of AC by nucleotides. The LNCaP cells, preincubated 15 min with FK 3 μM, were exposed to different nucleotides (ADP, ATP, ATPγS and BzATP at 100 μM), either in presence or in absence of 8-PST (100 μM). No decrease of the cyclic AMP level could be detected (data not shown).

PSA release assays

The effects of adenosine and nucleotides on the release of the prostate-specific antigen (PSA) were studied in LNCaP cells only, since PC-3 and DU145 cells do not secrete PSA (data not shown). The cells were incubated for 2 h in serum-free medium with several nucleotides (ATP, BzATP, UTP, and UDP at 100 μM) or adenosine (10 μM), but no increased release could be measured as compared to the control condition. On the contrary, the Vasoactive Intestinal Peptide (VIP; 100 nM), which strongly activates AC in these cells (Gkonos *et al*., 1996), increased PSA secretion by about 50% (Figure 3). The secretory effect of VIP at a low concentration (1 nM) was enhanced in a synergistic way by adenosine (10 μM; Figure 3) and ATP (100 μM), but not by UTP (data not shown). Similarly, adenosine enhanced the cyclic AMP accumulation induced by VIP (Figure 3).

Cell growth

PC-3, DU145 and LNCaP cells were seeded at low density and cultured for 4 days in serum-containing medium, with or without different nucleotides, which were readded every day. As represented in Figure 4, ATP (100 μM) completely prevented cell growth, whereas UTP (100 μM) had no detectable effect. The ATP effect was less pronounced in

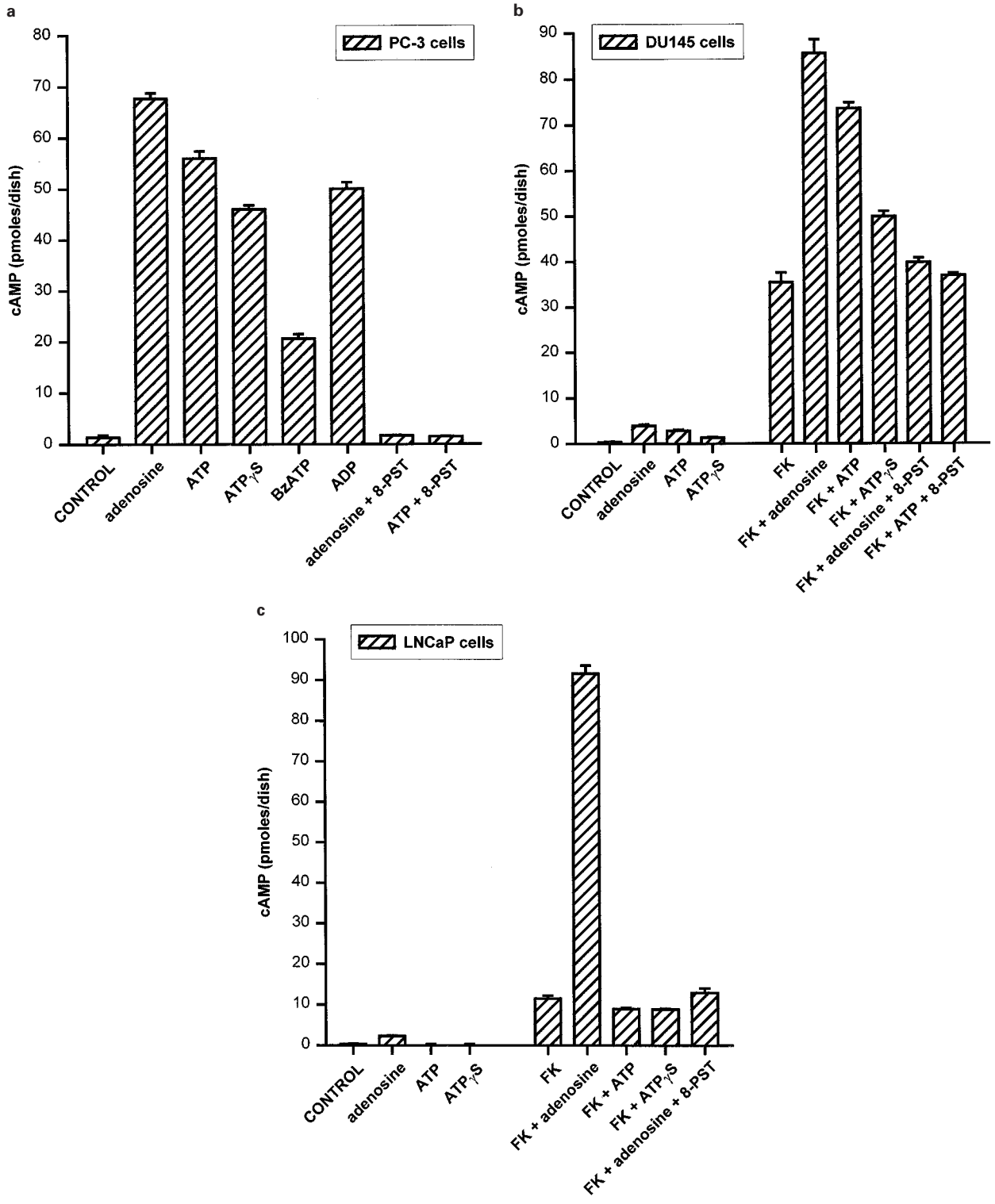

Figure 2 Effects of various nucleotides and adenosine on the cAMP production in prostatic carcinoma cells. (a) The PC-3 cells, preincubated 30 min with rolipram 25 μM, were stimulated for 15 min with adenosine (1 μM), ATP (100 μM), ATPγS (100 μM), BzATP (100 μM), ADP (100 μM), adenosine + 8-PST (300 μM), ATP + 8-PST or without agonist (CONTROL) in a medium containing rolipram 25 μM. The data represent the mean $\pm$ s.e.mean of three independent experiments with triplicate experimental points. (b) The DU145 cells, preincubated 30 min with rolipram 25 μM, were stimulated for 15 min with adenosine (1 μM), ATP (100 μM), ATPγS (100 μM), FK (1 μM), FK + adenosine, FK + ATP, FK + ATPγS, FK + adenosine + 8-PST (100 μM) or FK + ATP + 8-PST in a medium containing rolipram 25 μM. The data represent the mean $\pm$ s.e.mean of three independent experiments with triplicate experimental points. (c) The LNCaP cells, preincubated 30 min with rolipram 25 μM, were stimulated for 15 min with adenosine (1 μM), ATP (100 μM), ATPγS (100 μM), FK (3 μM), FK + adenosine, FK + ATP, FK + ATPγS or FK + adenosine + 8-PST in a medium containing rolipram 25 μM. The data represent the mean $\pm$ s.e.mean of three independent experiments with triplicate experimental points.

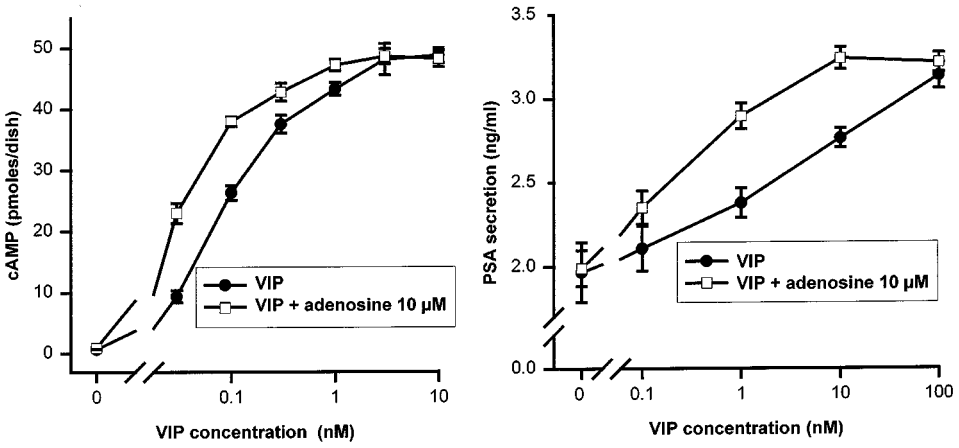

Figure 3 Concentration-action curves of VIP or VIP + adenosine (10 μM) on the cyclic AMP accumulation and PSA secretion in LNCaP cells. 300,000 LNCaP cells were incubated for 2 h in a serum free RPMI 1640 medium containing the agonist or not (control). Extracellular medium was removed and PSA assayed using a MEIA procedure on an AXSYM device. Measurements of cyclic AMP were performed as described under Methods. Data are the mean ± s.e.mean of three different experiments performed with triplicate experimental points.

the LNCaP cells. As detailed in Figure 5, the inhibitory effect of ATP on the growth of PC-3 cells could not be mimicked by UTP (100 μM), adenosine (100 μM), FK (10 μM) or the combinations UTP + adenosine or UTP + FK. Moreover, the ATP effect was not abolished by the addition of the adenosine receptors antagonist, 8-PST (up to 300 μM). Similar results were observed with the DU145 cells, but the serum concentration had to be decreased to 4% in order to obtain an amplitude of inhibition comparable to that found in PC-3 cells (Figure 5). In contrast to PC-3 cells, the growth of LNCaP cells was inhibited by the FK + UTP or FK + ATP combinations, and the combinations were more effective than ATP alone (Figure 5). Figure 6 shows the relationship between adenine nucleotide (BzATP, ATP, ATPγS, 2-MeSATP and α,βmeATP) concentration and the number of cells after 4 days in culture. The rank order of growth-inhibiting activity was: ATPγS $\approx$ ATP > 2-MeSATP > α,β meATP. BzATP had a different behavior on the two cell lines: it produced more growth inhibition than ATP in PC-3 cells and less in DU145.

The effect of ATP or BzATP (100 μM) on DNA synthesis was evaluated by measuring the percentage of ^{3}H-thymidine-labelled nuclei. As shown in Table 1, no significant difference between control and nucleotide conditions was observed after 2 days of treatment in both PC-3 and DU145 cell lines. However, after 4 days of treatment, significant differences were observed in the PC-3 cells: a decrease of labelled nuclei of about 20 and 35% was observed respectively when cells were treated with ATP or BzATP. Proliferation rate was not affected by nucleotides in DU145 cells. Most of the nonproliferative cells presented a normal aspect.

Detection of apoptosis

Since the decrease in cell number in response to nucleotides was more impressive than the decrease in proliferation rate, we investigated whether nucleotides induce the death of prostatic cells. In order to detect the internucleosomal DNA degradation that occurs in many apoptotic cells (Wyllie, 1980), the genomic DNA of PC-3 and DU145 cells challenged or not by ATP or BzATP (100 μM) for 4 days, was submitted to electrophoresis and stained with ethidium bromide. As observed in Figure 7, the lanes corresponding to PC-3 cells incubated with BzATP or ATP presented the characteristic laddering of apoptotic cells, whereas the control cells did not present any specific degradation. On the contrary, the lane of DU145 cells incubated with ATP presented a smear, characteristic of a random DNA degradation on which one may however distinguish some bands of weak intensity.

Expression of P2X receptors

The lack of UTP and adenosine effect suggested that the inhibition of PC-3 and DU145 cell growth by ATP is unrelated to either inositol phosphate-linked P2Y receptors or AC-linked adenosine receptors. Nucleotide-induced cell death, and apoptosis in particular, has been linked to the activation of P2X receptors (Di Virgilio *et al*., 1989; Coutinho-Silva *et al*., 1999; Ferrari *et al*., 1999). RT–PCR experiments were performed on PC-3 and DU145 cells cDNA in order to detect the expression of P2X transcripts. PCR experiments were performed with pairs of oligonucleotides (sense and antisense) specific of each P2X receptor, synthesized on basis of the sequence submitted to Gen-Bank/EMBL databases. Three different P2X receptors ($P2X_4$, $P2X_5$ and $P2X_7$) were detected in the PC-3 cDNA, whereas only the $P2X_4$ and $P2X_5$ receptors were amplified in the DU145 cDNA; the amplification products had the expected size ($P2X_4$: 424 pb; $P2X_5$: 373 pb and $P2X_7$: 470 pb) (Figure 8). These results were confirmed with a new pair of specific oligonucleotides (second set, as described under the Methods section). Negative controls were performed (PCR on RNA of PC-3 or DU145 cells; PCR without cDNA) and no contamination was observed (data not shown). The presence of P2X receptors on prostatic cells was confirmed by intracellular calcium measurements. As shown in Figure 9, BzATP (100 μM) induced a transient, although still appreciable after 15 min, increase of cytosolic calcium which was almost abolished (80% inhibition of the maximal response) in

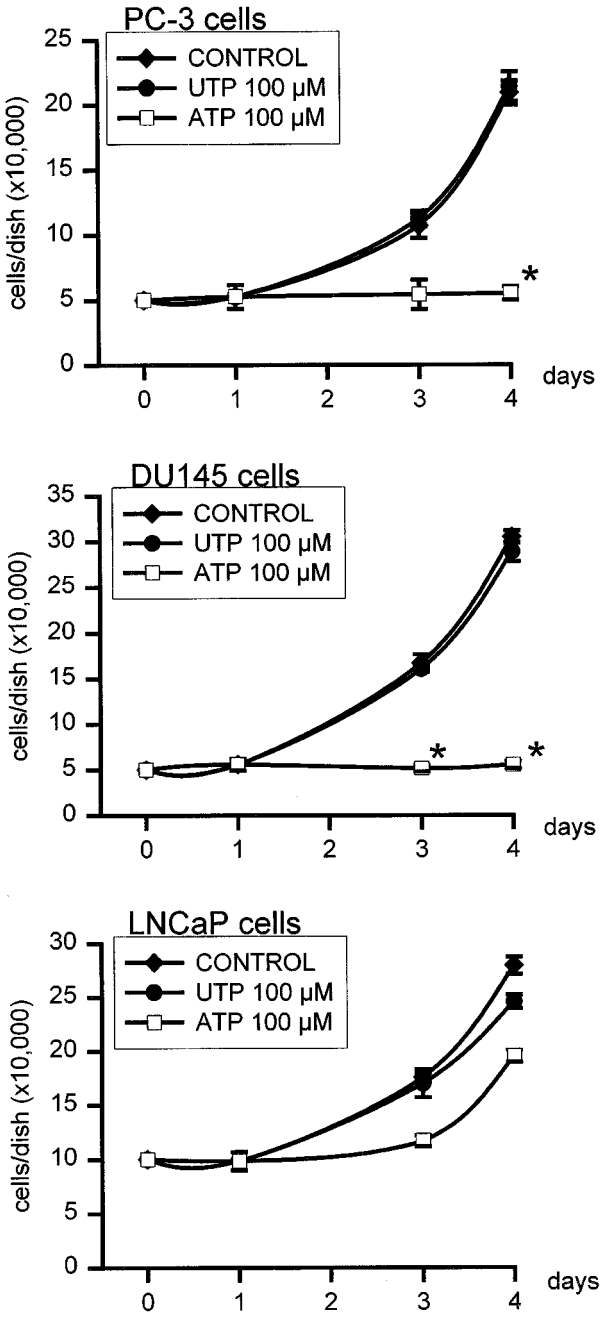

Figure 4 Effects of ATP or UTP on growth of prostatic carcinoma cells. PC-3 cells, at a density of 50,000 cells/3.5 cm dishes, were seeded at day 0 in a complete Ham's F-12 medium containing 8% FBS, with or without the agonist (100 μM). Nucleotides were renewed each day, and medium changed at day 2. The number of adherent living cells that excluded trypan blue was determined at days 1, 3 and 4 using a haemocytometer. DU145 cells were seeded at a density of 50,000 cells/3.5 cm dishes in a complete Eagle's modified essential medium containing 4% FBS. LnCAP cells were seeded at a density of 100,000 cells/3.5 cm dishes in complete RPMI 1640 medium containing 10% FBS. Treatment and counting of DU145 and LNCaP are similar to those described for PC-3 cells. The data represent the mean$\pm$s.e.mean of three independent experiments with quadruplicate experimental points. *Indicates that the level of significance (P) between control and ATP conditions is $P<0.005$.

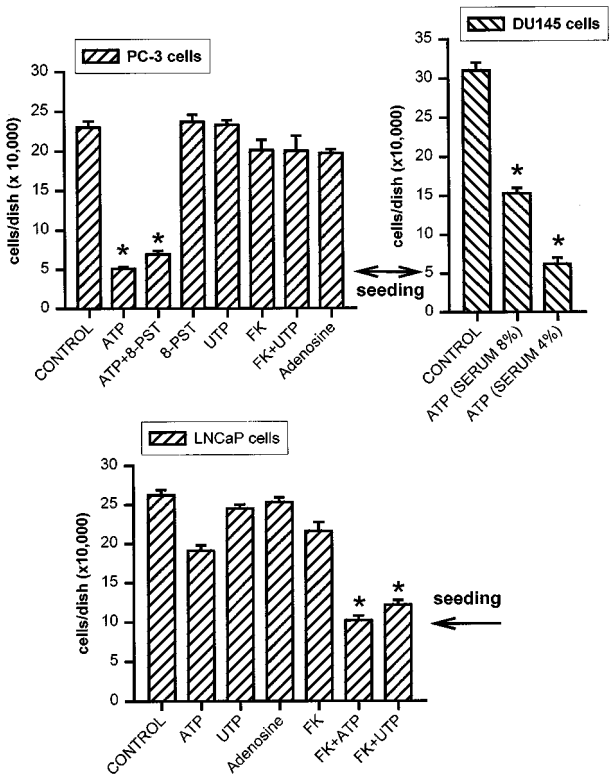

Figure 5 Effects of nucleotides, adenosine and/or FK on growth of prostatic carcinoma cells. Culture conditions are similar to those described in Figure 4. Cells were incubated in the presence or absence of nucleotides for 4 days and the number of living cells that excluded trypan blue was determined using a haemocytometer. Stimulations were performed with ATP (100 μM), UTP (100 μM), adenosine (100 μM), FK (10 μM) or 8-PST (300 μM for PC-3 cells and 100 μM for other cell lines alone or in combination). The data represent the mean$\pm$s.e.mean of three independent experiments with quadruplicate experimental points. *Indicates that the level of significance (P) between control and stimulated conditions is $P<0.005$.

presence of EGTA, an extracellular calcium chelator. In contrast, the response to ATP was about 2 fold greater, but in presence of EGTA, it was reduced by only 50%. Measurements of fluorescent dyes (ethidium bromide and lucifer yellow) influx as described by Chaïb *et al.* (2000) were also performed in the PC-3 cells following the addition of BzATP 100 μM for 15 min. However, no specific increase of intracellular fluorescence was detected (data not shown).

Discussion

In this paper, we have investigated the effects of nucleosides and nucleotides on three different prostate carcinoma cell lines: LNCaP, PC-3 and DU145 cells. The growth of the first one is androgen-dependent, whereas the PC-3 and DU145 cells are androgen-insensitive. Little is known about the effects of nucleosides and nucleotides on the prostate or prostatic cell lines. In particular, no effect of adenosine has been described. $P2Y_1$ receptor mRNA was strongly expressed in the normal human prostate (Janssens *et al.*, 1996), as well as $P2X_1$ receptor messengers (Longhurst *et al.*, 1996). ATP and UTP elicited an increase of cytoplasmic calcium in the androgen-insensitive cell lines and, interestingly, ATP (UTP was not tested) inhibited the growth of rat epithelial prostatic cells and of the PC-3 and DU145 cell lines, but the

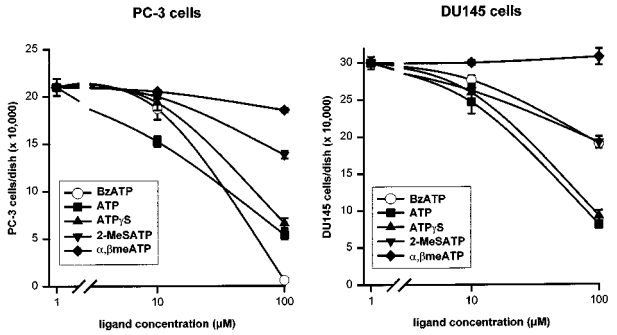

Figure 6 Concentration-action curves of effects of various adenine nucleotides on androgen-independent prostatic cell growth. PC-3 and DU145 cells were cultured and counted in the same conditions as described in Figure 4. Cells were submitted or not to stimulation with BzATP, ATP, ATPγS, 2-MeSATP or α,βmeATP (all at 100 μM). After 4 days, the number of living cells that excluded trypan blue was determined using a haemocytometer. The data represent the mean $\pm$ s.e.mean of three independent experiments with quadruplicate experimental points.

Table 1 Effects of ATP or BzATP on proliferation of androgen-independent cells

Treatment time	*Control*	*ATP*	*BzATP*
PC-3 cells			
2 days	91.1 $\pm$ 2.2	90.0 $\pm$ 3.1	87.0 $\pm$ 1.9
4 days	75.6 $\pm$ 1.5	57.4 $\pm$ 2.8	41.9 $\pm$ 3.8
DU145 cells			
2 days	96.4 $\pm$ 3.2	95.1 $\pm$ 2.6	97.8 $\pm$ 3.9
4 days	86.2 $\pm$ 2.9	80.9 $\pm$ 2.2	88.8 $\pm$ 2.5

Cells, at a density of 50,000/3.5 cm dishes, were stimulated or not by agonist (100 μM) in culture conditions described in the Methods section. At the end of day 1 or 3, cells were labelled with ^{3}H-thymidine for 18 h (PC-3 cells) or 12 h (DU145 cells). The percentage of labelled nuclei was established by counting (at least 1,000 cells/dish). Data are mean $\pm$ range of two independent experiments with triplicate points.

receptor(s) involved has(ve) not been identified (Fang *et al*., 1992; Guijarro *et al*., 1996; Wasilenko *et al*., 1997). Growth-inhibitory effects of nucleotides have been observed in other cell types. For instance, activation of $P2Y_2$ receptors, decreases of proliferation of human colorectal HT29 cells, (Hopfner *et al*., 1998), human endometrial cancer HEC-1A and Ishikawa cells (Katzur *et al*., 1999). On the other hand the ATP-induced decrease in the proliferation of HL-60 (Seetulsingh-Goorah & Stewart, 1998) and NK (Miller *et al*., 1999) cells is associated with a rise in cyclic AMP presumably mediated by the $P2Y_{11}$ receptor.

Our approach was first to detect P2Y receptors transcripts by Northern blotting in the three cell lines and to compare these data with pharmacological assays. PC-3 cells express mainly $P2Y_2$ receptors and this can be correlated with an equipotent stimulation of $InsP_3$ accumulation by ATP and UTP, which is a landmark of that receptor (Lazarowski *et al*, 1995). Although a significant expression of $P2Y_1$, $P2Y_6$ and $P2Y_{11}$ mRNA was detectable in DU145 cells, in addition to $P2Y_2$ mRNA, $InsP_3$ regulation by nucleotides was similar to the PC-3 cells, suggesting the predominant involvement of $P2Y_2$ receptors. This is supported by the lack of additivity of the ATP and UTP effects. The lack of stimulation by 2-MeSADP, a potent agonist of $P2Y_1$ receptors (Leon *et al*., 1997), or BzATP, a $P2Y_{11}$ agonist (Communi *et al*, 1999), suggests that these subtypes are not functionally expressed in these cells. A response to UDP was detectable but its time course suggests that it might result from an interconversion into UTP and an activation of $P2Y_2$ rather than $P2Y_6$ receptors (Nicholas *et al*., 1996). This hypothesis was confirmed by the lack of additivity of the UDP and ATP or UTP effects. Finally, the stimulation of cyclic AMP accumulation and potentiation of FK action by ATP cannot be explained by an action on the $P2Y_{11}$ receptor. Indeed, BzATP and ATPγS, which are more potent than ATP on the recombinant $P2Y_{11}$ receptor (Communi *et al*., 1999), produced a weaker effect than ATP itself, and the action of ATP was mimicked by adenosine and abolished by 8-PST, suggesting that it results entirely from ATP degradation into

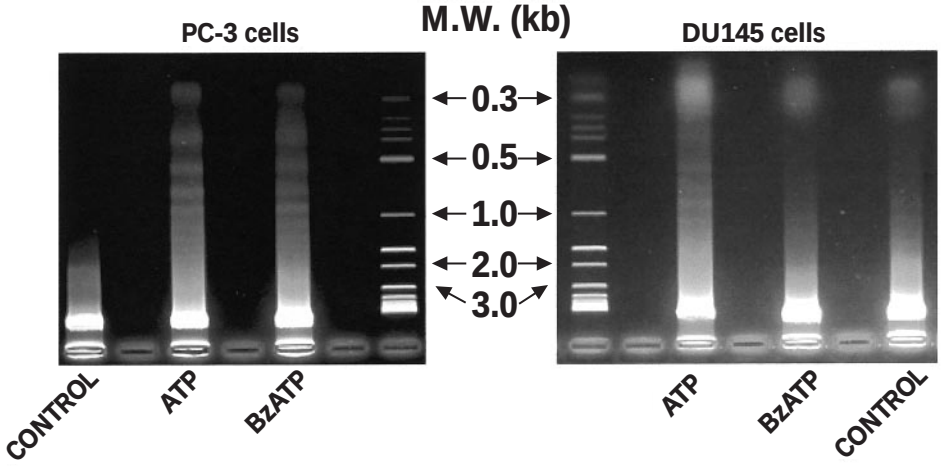

Figure 7 Qualitative detection of apoptosis among androgen-independent PC-3 and DU145 cells. PC-3 cells, at a density of 400,000/10 cm dishes, were cultured in complete Ham's F-12 containing 8% FBS and stimulated or not by BzATP or ATP (100 μM). DU145 cells, 400,000/10 cm dishes, were cultured in a complete Eagle's modified essential medium containing 4% FBS. Experimental procedures are described under Methods. DNA (8 μg/lane) was separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

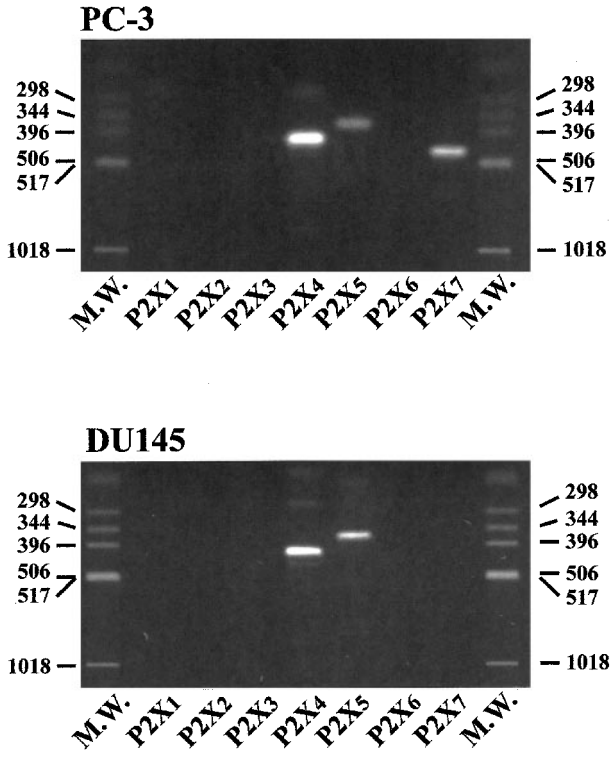

Figure 8 Detection of P2X receptors mRNA in human androgen-independent prostatic carcinoma cells by RT–PCR experiments. The experimental procedure as well as oligonucleotide sequences are described under Methods. PCR products ($P2X_4$: 424 pb; $P2X_5$: 373 pb; $P2X_7$: 470 pb) are visualized after electrophoresis, and ethidium bromide staining of a 2% agarose gel. These data are representative of three different RT–PCR experiments, performed on two different batches of RNA.

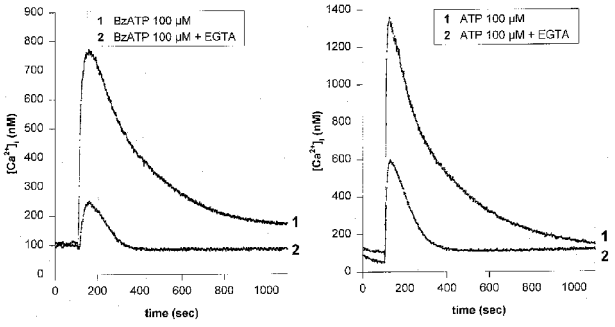

Figure 9 Measurements of intracellular calcium using the fluorescent marker fura 2-AM. Effects of BzATP (A) and ATP (B) at 100 μM on the PC-3 cells, in a medium containing or not EGTA (1 mM) added just prior to the ligand stimulation. The experimental procedure is described under Methods. The data represent the mean of three independent experiments (s.e.means were below 10%).

adenosine and an action on A_2 receptors. In LNCaP cells, no effect of nucleotides on either $InsP_3$ or cyclic AMP was detectable, despite a significant expression of $P2Y_2$ and $P2Y_{11}$ transcripts. In summary, our data show that the $InsP_3$ response to nucleotides in PC-3 and DU145 cells is mediated by $P2Y_2$ receptors, that the three cell lines express adenosine receptors coupled to AC stimulation, presumably an A_2 subtype, and that there are discrepancies between Northern blotting showing the expression of $P2Y_1$, $P2Y_6$ and $P2Y_{11}$ receptor mRNA in prostatic cells and the lack of functional expression of these receptors.

We have also investigated the influence of nucleotides and adenosine on PSA secretion. PSA is commonly measured in serum and is an important tool in the diagnosis and monitoring of prostate cancers (Stamey *et al*., 1987). The synthesis and secretion of PSA is under control of androgen hormones (Lee *et al*., 1995). However, secretion of PSA is also acutely increased by stimuli that activate AC, such as VIP (Gkonos *et al*., 1996). Among the three cell lines, only the LNCaP cells synthesise PSA (Takahashi *et al*., 1999). Adenosine *per se* did not stimulate PSA release, but it enhanced the secretory effect of low concentrations of VIP. This enhancement is likely to result from a synergistic stimulation of cyclic AMP. ATP also potentiated the PSA secretory effect of VIP: the mechanism of this potentiation remains unknown since we could not detect effects of ATP on cyclic AMP level in LNCaP cells.

The last aspect of this paper is the study of the action of nucleotides and nucleosides on the growth of prostatic carcinoma cells. First, we reproduced the observation made by Fang *et al*. (1992) that ATP inhibits the growth of PC-3 and DU145 cells. In addition, we have shown that adenine nucleotides induce the death of these cells, presumably by apoptosis in PC-3 cells and mainly by necrosis in DU145 cells. Fang *et al*. had concluded that growth inhibition by nucleotides was a consequence of phospholipase C activation and rise in cytoplasmic Ca^{2+}. However, unlike these authors, we also tested the effect of UTP which had no effect on growth, whereas it was equipotent and equiactive to ATP on $InsP_3$. Therefore, the growth inhibitory effect of ATP cannot be explained by the activation of $P2Y_2$ receptors coupled to phospholipase C. It cannot be explained either by an accumulation of cyclic AMP, since the effect of ATP was neither mimicked by adenosine nor reduced by 8-PST. P2X receptors constitute an alternative mechanism and this hypothesis is consistent with the report by Fang *et al*. (1992) that as much as 50% of the Ca^{2+} rise observed in response to ATP is due to an influx of extracellular calcium. Indeed, RT–PCR experiments performed on PC-3 and DU145 cDNA amplified the $P2X_4$, $P2X_5$ and $P2X_7$ sequences in the PC-3 cells and the first two of them in the DU145 cells. In PC-3 cells, we confirmed the presence of functional P2X receptors by measuring the cytosolic calcium concentration: BzATP, which is more potent than ATP on several P2X subtypes, induced a prolonged increase of cytosolic calcium (over 15 min), which was almost suppressed in presence of EGTA, indicating that it is mostly due to extracellular calcium influx. The residual part (about 20 % of the maximal response) would involve calcium release from the intracellular pools, although BzATP did not stimulate InsP3 accumulation in these cells. ATP also induced extracellular calcium influx in a proportion (about 50 % of the maximal response) compatible with the study of Fang *et al*. (1992) in the same cells. In fluorescent dye experiments, BzATP application did not result in entry of ethidium bromide suggesting the lack of $P2X_7$ receptor-related pores in the PC-3 cells. This could be due to the lack of a significant expression of the $P2X_7$ protein or of other factors needed for pore formation, as demonstrated in *Xenopus* oocytes (Petrou *et al*., 1997). Our data broaden the number of epithelia in which P2X receptors are

expressed: they have been previously described in primary cultures or cell lines derived from airways (especially $P2X_4$ and $P2X_5$ subtypes), intestine, pancreas, liver and salivary glands (Tenneti *et al*., 1998; Taylor *et al*., 1999; Luo *et al*., 1999).

The main conclusion of our work are as follows. $InsP_3$ accumulation in response to nucleotides in prostate carcinoma cells involves the $P2Y_2$ receptor, whereas their stimulation of cyclic AMP accumulation is mediated by degradation into adenosine and activation of A_2 receptors. One consequence of the adenosine-induced cyclic AMP accumulation is the potentiation of PSA secretion. We have confirmed the observation by Fang *et al*. (1992) that ATP inhibits the growth of PC-3 and DU145 cells, but we have shown that this effect is unrelated to $InsP_3$ or cyclic AMP accumulation. P2X receptors activation with a resulting calcium influx and cell death might constitute an alternative explanation.

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References

ABBRACHIO, M.P. & BURNSTOCK, G. (1994). Purinoceptors: are there families of P_{2X} and P_{2Y} purinoceptors? *Pharmacol. Ther.*, **64,** 445–475.

BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotrophic ATP receptor. *Nature,* **371,** 519–523.

BROOKER, G., HARPER, J.F., TERASEKI, W.L. & MOYLAN, R.D. (1979). Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv. Cyclic Nucleotides Res.,* **10,** 1–33.

BUELL, G., LEWIS, C., COLLO, G., NORTH, R.A. & SURPRENANT, A. (1996). An antagonist insensitive P2X receptor expressed in epithelia and brain. *EMBO J.,* **15,** 55–62.

CHAIB, N., KABRE, E., ALZOLA, E., POCHET, S. & DEHAYE, J.P. (2000). Bromoenol lactone enhances the permeabilization of rat submandibular acinar cells by $P2X_7$ agonists. *Br. J. Pharmacol.,* **129,** 703–708.

CHANG, K., HANAOKA, K., KUMADA, M. & TAKUWA, Y. (1995). Molecular cloning and functional analysis of a novel P2 nucleotide receptor. *J. Biol. Chem.,* **270,** 26152–26158.

CHEN, C., AKOPIAN, A.N., SIVILOTTI, L., COLQUHOUN, D., BURNSTOCK, G. & WOOD, J.N. (1995). A P2X purinoceptor expressed in a subset of sensory neurons. *Nature,* **377,** 428–431.

COLLO, G., NORTH, R.A., KAWASHIMA, E., MERLO-PICH, E., NEIDHART, S., SURPRENANT, A. & BUELL, G. (1996). Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and the properties of an extended family of ATP-gated ion channels. *J. Neurosci.,* **16,** 2495–2507.

COMMUNI, D., GOVAERTS, C., PARMENTIER, M. & BOEYNAEMS, J.M. (1997). Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.,* **272,** 31969–31973.

COMMUNI, D., PIROTTON, S., PARMENTIER, M. & BOEYNAEMS, J.M. (1995a). Molecular cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.,* **270,** 30849–30852.

COMMUNI, D., RASPE, E., PIROTTON, S. & BOEYNAEMS, J.M. (1995b). Coexpression of P_{2Y} and P_{2U} receptors on aortic endothelial cells. *Circ. Res.,* **76,** 191–198.

COMMUNI, D., ROBAYE, B. & BOEYNAEMS, J.M. (1999). Pharmacological characterization of the human $P2Y_{11}$ receptor. *Br. J. Pharmacol.,* **128,** 1199–1206.

COUTINHO-SILVA, R., PERSECHINI, P.M., BISAGGIO, R.D., PERFETTINI, J.L., NETO, A.C. & KANELLOPOULOS, J.M. (1999). P_{2Z}/$P2X_7$ receptor-dependent apoptosis of dendritic cells. *Am. J. Physiol.,* **276,** C1139–C1147.

DI VIRGILIO, F., BRONTE, V., COLLAVO, D. & ZANOVELLO, P. (1989). Responses of mouse lymphocytes to extracellular adenosine 5′-triphosphate (ATP). Lymphocytes with cytotoxic activity are resistant to the permeabilizing effects of ATP. *J. Immunol.,* **143,** 1955–1960.

FANG, W.G., PIRNIA, F., BANG, Y.J., MYERS, C.E. & TREPEL, J.B. (1992). P2-purinergic receptor agonists inhibit the growth of androgen-independent prostate carcinoma cells. *J. Clin. Invest.,* **89,** 191–196.

FERRARI, D., LOS, M., BAUER, M.K., VANDENABEELE, P., WESSELBORG, S. & SCHULZE-OSTHOFF, K. (1999). P_{2Z} purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. *FEBS Lett.,* **447,** 71–75.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DALY, J.W., HARDEN, T.K., JACOBSON, K.A., LEFF, P. & WILLIAMS, M. (1994). Nomenclature and classification of purinoceptors. *Pharmacol. Rev.,* **46,** 143–156.

GKONOS, P.J., ASHBY, M.H. & ANDRADE, A.A. (1996). Vasoactive intestinal peptide stimulates prostate-specific antigen secretion by LNCaP prostate cancer cells. *Regul. Pept.,* **65,** 153–157.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

GUIJARRO, L.G., JUARRANZ, M.G., MARINERO, M.J., PAJUELO, L., CARMENA, M.J. & PRIETO, J.C. (1996). Modulation of cyclic AMP and inositol phosphate production in rat prostatic cultures by VIP/PACAP, ATP, and carbachol: role in prostatic proliferation. *Ann. N.Y. Acad. Sci.,* **805,** 723–728.

HOPFNER, M., LEMMER, K., JANSEN, A., HANSKI, C., RIECKEN, E.O., GAVISH, M., MANN, B., BUHR, H., GLASSMEIER, G. & SCHERUBL, H. (1998). Expression of functional P2-purinergic receptors in primary cultures of human colorectal carcinoma cells. *Biochem. Biophys. Res. Commun.,* **251,** 811–817.

ISAACS, J.T., SCHULZE, H. & COFFEY, D.S. (1987). Development of androgen resistance in prostatic cancer. *Prog. Clin. Biol. Res.,* **243A,** 21–31.

JANSSENS, R., BOEYNAEMS, J.M., GODART, M. & COMMUNI, D. (1997). Cloning of a human heptahelical receptor closely related to the $P2Y_5$ receptor. *Biochem. Biophys. Res. Commun.,* **236,** 106–112.

JANSSENS, R., COMMUNI, D., PIROTTON, S., SAMSON, M., PARMENTIER, M. & BOEYNAEMS, J.M. (1996). Cloning and tissue distribution of the human $P2Y_1$ receptor. *Biochem. Biophys. Res. Commun.,* **218,** 783–788.

KATZUR, A.C., KOSHIMIZU, T., TOMIC, M., SCHULTZE-MOSGAU, A., ORTMANN, O. & STOJILKOVIC, S.S. (1999). Expression and responsiveness of $P2Y_2$ receptors in human endometrial cancer cell lines. *J. Clin. Endocrinol. Metab.,* **84,** 4085–4091.

LAZAROWSKI, E.R., WATT, W.C., STUTTS, M.J., BOUCHER, R.C. & HARDEN, T.K. (1995). Pharmacological selectivity of the cloned human P2U-purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.,* **116,** 1619–1627.

LEE, C., SUTKOWSKI, D.M., SENSIBAR, J.A., ZELNER, D., KIM, I., AMSEL, I., SHAW, N., PRINS, G.S. & KOZLOWSKI, J.M. (1995). Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. *Endocrinology,* **136,** 796 – 803.

LEON, C., HECHLER, B., VIAL, C., LERAY, C., CAZENAVE, J.P. & GACHET, C. (1997). The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett.,* **403,** 26 – 30.

LI, Q., SCHACHTER, J.B., HARDEN, T.K. & NICHOLAS, R.A. (1997). The 6H1 orphan receptor, claimed to be the $P2Y_5$ receptor, does not mediate nucleotide-promoted second messenger responses. *Biochem. Biophys. Res. Commun.,* **236,** 455 – 460.

LONGHURST, P.A., SCHWEGEL, T., FOLANDER, K. & SWANSON, R. (1996). The human $P2X_1$ receptor: molecular cloning, tissue distribution, and localization to chromosome 17. *Biochim. Biophys. Acta.,* **308,** 185 – 188.

LUO, X., ZHENG, W., YAN, M., LEE, M.G. & MUALLEM, S. (1999). Multiple functional P2X and P2Y receptors in the luminal and basolateral membranes of pancreatic duct cells. *Am. J. Physiol.,* **277,** C205 – C215.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 5113 – 5117.

MILLER, J.S., CERVENKA, T., LUND, J., OKAZAKI, I.J. & MOSS, J. (1999). Purine metabolites suppress proliferation of human NK cells through a lineage-specific purine receptor. *J. Immunol.,* **162,** 7376 – 7382.

NICHOLAS, R.A., WATT, W.C., LAZAROWSKI, E.R., LI, Q. & HARDEN, K. (1996). Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol. Pharmacol.,* **50,** 224 – 229.

PETROU, S., UGUR, M., DRUMMOND, R.M., SINGER, J.J. & WALSH, J.V. (1997). $P2X_7$ purinoceptor expression in Xenopus oocytes is not sufficient to produce a pore-forming P2Z-like phenotype. *FEBS Lett.,* **411,** 339 – 345.

SEETULSINGH-GOORAH, S.P. & STEWART, B.W. (1998). Growth inhibition of HL-60 cells by extracellular ATP: concentration-dependent involvement of a P2 receptor and adenosine generation. *Biochem. Biophys. Res. Commun.,* **250,** 390 – 396.

STAMEY, T.A., YANG, N., HAY, A.R., MCNEAL, J.E., FREIHA, F.S. & REDWINE, E. (1987). Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N. Engl. J. Med.,* **317,** 909 – 916.

SURPRENANT, A., RASSENDREN, F., KAWASHIMA, E., NORTH, R.A. & BUELL, G. (1996). The cytolytic P_{2Z} receptor for extracellular ATP identified as a P2X receptor $P2X_7$. *Science,* **272,** 735 – 738.

TAKAHASHI, T., HOSHI, S., SATOH, M., KANEDA, T., SUZUKI, K.I., NAKAGAWARA, K.I. & ORIKASA, S. (1999). The study of PSA gene expression on urogenital cell lines. *Int. J. Urol.,* **6,** 526 – 531.

TAYLOR, A.L., SCHWIEBERT, L.M., SMITH, J.J., KING, C., JONES, J.R., SORSCHER, E.J. & SCHWIEBERT, E.M. (1999). Epithelial P2X purinergic receptor channel expression and function. *J. Clin. Invest.,* **104,** 875 – 884.

TENNETI, L., GIBBONS, S.J. & TALAMO, B.R. (1998). Expression and trans-synaptic regulation of $P2X_4$ and P_{2Z} receptors for extracellular ATP in parotid acinar cells. Effects of parasympathetic denervation. *J. Biol. Chem.,* **273,** 26799 – 26808.

VALERA, S., HUSSY, N., EVANS, R.J., ADAMI, N., NORTH, R.A., SURPRENANT, A. & BUELL, G. (1994). A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature,* **371,** 516 – 519.

WASILENKO, W.J., COOPER, J., PALAD, A.J., SOMERS, K.D., BLACKMORE, P.F., RHIM, J.S., WRIGHT, JR, G.L. & SCHELLHAMMER, P.F. (1997). Calcium signaling in prostate cancer cells: evidence for multiple receptors and enhanced sensitivity to bombesin/GRP. *Prostate,* **30,** 167 – 173.

WEBB, T.E., SIMON, J., KRISHEK, B.J., BATESON, A.N., SMART, T.G., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1993). Cloning and functional expression of a brain G protein-coupled ATP receptor. *FEBS Lett.,* **324,** 26 – 30.

WYLLIE, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature,* **284,** 555 – 556.

YOKOMIZO, T., IZUMI, T., CHANG, K., TAKUWA, Y. & SHIMIZU, T. (1997). A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature,* **387,** 620 – 624.

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