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ATP stimulates the proliferation of MCF-7 cells through the PI3K/Akt signaling pathway

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ABSTRACT

We studied the modulation of the PI3K/Akt signaling pathway by ATP in MCF-7 cells. Western blot analysis showed that ATP stimulated the phosphorylation of Akt in a dose- and time-dependent manner. Akt phosphorylation in response to nucleotides followed the potency order ATP = UTP = ATPγS ≫ ADP = UDP > ADPβS = adenosine, suggesting participation of $P2Y_{2/4}$ receptors. Inhibitors of PI3K, PLC, PKC and Src or Src antisense oligonucleotides prevented ATP-induced phosphorylation of Akt. Incubation of cells with 2-APB or in a nominally Ca^{2+}-free medium plus EGTA showed that Akt phosphorylation by ATP depends on intracellular calcium release but is independent of calcium influx. The PI3K inhibitor was not effective in reducing MAPKs phosphorylation by ATP. ATP and UTP stimulated MCF-7 cell proliferation, effect that was inhibited by PI3K, PLC, PKC, Src and MAPKs inhibitors. These findings suggest that ATP modulation of $P2Y_{2/4}$ receptors increases MCF-7 cell proliferation by activation of the PI3K/Akt signaling pathway through PLC/IP_3/Ca^{2+}, PKC and Src.

Introduction

Purinergic and pyrimidinergic nucleotides have widespread and specific extracellular signaling actions in the regulation of a variety of functions including development, proliferation and differentiation in many tissues [1]. They bind to cell surface receptors designated P2 purinergic receptors, which can be divided into two families: metabotropic, P2Y receptors (G-protein-coupled receptors) and ionotropic, P2X receptors (ligand-gated ion channels) [2]. Currently, seven P2X ($P2X_{1-7}$) and eight P2Y ($P2Y_{1,2,4,6,11-14}$) receptor subtypes have been cloned and characterized [3]. P2Y receptors couple to multiple G proteins and, so, its activation by extracellular nucleotides can lead to the activation of multiple signaling transduction pathways [4], including the mitogen activated protein kinases (MAPKs)[1] [5–7] and the Src family of tyrosine kinases [8]. The Src family of non-receptor protein tyrosine kinases interacts with a diverse array of molecules involved in cell division, motility, adhesion, angiogenesis and survival [9]. Despite the ubiquitous presence of Src, Fyn and Yes, it is Src that is mostly associated with tumor progression. Compared with normal tissues, elevated Src expression and/or activity has been reported in a wide range of tumor types, including breast and, in many of these tissues, an increase in Src activity correlates with disease stage or malignant potential [10]. These data support the importance of studying the role of c-Src in the activation of PI3K/Akt signaling pathway in breast cancer cells.

PI3Ks are heterodimeric enzymes that consist of a catalytic subunit and a regulatory subunit [11]. Upon activation, the catalytic subunit phosphorylates phosphoinositides (PI) at the 3-position of the inositol ring to generate phosphatidyl-inositol triphosphate (PIP_3) and other lipid products. These cofactors, together with the PH domain-dependent membrane translocation step, followed by phosphorylation of two key regulatory sites at Thr 308 and Ser 473, are important for Akt activation [12,13]. The Akt serine/threonine kinase (also called protein kinase B) has emerged as a critical signaling molecule within eukaryotic cells. This kinase has a role in a range of diverse cellular functions that are important physiologically and pathophysiologically, such as cell growth and survival, angiogenesis, glycogen synthesis, protein synthesis and transcription [14]. The PI3K/Akt signaling pathway has an important role in the development of breast cancer [15,16]. Akt is over-expressed in human breast cancer cells and plays a crucial role during the development and progression of breast malignancy. In normal breast epithelial cells, Akt controls proliferation and apoptosis; and functions as a survival factor during breast tumorigenesis [17]. Recent studies have demonstrated that extracellular ATP can stimulate the PI3K/Akt signaling pathway in some cell types [18–20]. However, the modulation of the PI3K/Akt signaling pathway by extracellular nucleotides in breast cancer cells has not been studied yet.

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[1] *Abbreviations used:* MAPKs, mitogen activated protein kinases; PI, phosphorylates phosphoinositides; PIP_3, phosphatidyl-inositol triphosphate; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; PLC, phospholipase C.

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In this study we show that $P2Y_2$ and/or $P2Y_4$ receptor stimulation by extracellular ATP induces the activation of PI3K/Akt through a mechanism involving phospholipase C, IP_3-sensitive-intracellular calcium release, protein kinase C and Src in MCF-7 cells. In addition, PI3K/Akt signaling induced by ATP is involved in MCF-7 cell proliferation.

Materials and methods

Materials

ATP, UTP, ADP, ATPγs, ADPβS, UDP, adenosine, RPMI-1640 medium, neomycin sulfate, compound U73122, diphenylboric acid 2-aminoethyl ester (2-APB) and calphostin C were from Sigma–Aldrich Co. (St. Louis, MO, USA). Caffeine was from J.T. Baker (Phillipsburg, NJ, Canada). Ro318220, Ly294002 and wortmannin were from EMD Chemicals, Inc. (San Diego, CA, USA). SB203580, PD98059, SP600125 and PP2 were from Tocris Bioscience (Ellisville, MO, USA). Lipofectamine 2000 transfection reagent was provided by Invitrogen Corp. (Carlsbad, CA, USA). Src antisense oligonucleotides (antisense 5′-CTC TTG TTG CTA CCC ATG GTCC-3′ and mismatch 5′-AGC AAC GAC AAG TCA AGC TG-3′) were from Ruralex (Buenos Aires, Argentina). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was from Promega (Madison, WI, USA). Monoclonal antibodies recognizing dually phosphorylated ERK1/2 (Thr 202/Tyr 204) and JNK1/2 (Thr 183/Tyr 185); phosphorylated Src (Tyr 416) and phosphorylated Akt (Ser 473) were from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies recognizing dually phosphorylated p38 (Thr 180/Tyr 182), polyclonal anti p38α, polyclonal anti Akt1/2/3, monoclonal anti c-Src, polyclonal goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein size markers were from Amersham Biosciences (Piscataway, NJ, USA), Immobilon P (polyvinylidene difluoride) membranes and Amersham ECL chemiluminescence detection kit were from GE Healthcare (Little Chalfont, Buckinghamshire, England). All other reagents used were analytical grade.

Cell culture

The human breast cancer epithelial cell line MCF-7 (American Type Culture Collection; Manassas, VA, USA) was seeded at a density of 20,000 cells per cm^2 and cultured at 37 °C in RPMI-1640 medium containing 10% FBS under humidified air (5.5% CO_2). After 48 h, the medium was replaced by RPMI-1640 without FBS and cells were starved for 24 h before agonist stimulation. Controls with vehicle (water) were used.

SDS–PAGE and immunoblotting

As indicated in the figure legends, MCF-7 cells were treated with ATP in the presence or absence of the inhibitors Ly294002, wortmannin, neomycin, U73122, Ro318220, calphostin C and PP2. Cells were then washed with PBS buffer plus 25 mM NaF and 1 mM Na_3VO_4, and lysed in buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% Tween-20, 1% Nonidet P-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF and 1 mM Na_3VO_4. The lysates were incubated on ice for 10 min, vortexed for 45 s and maintained on ice for another 10 min. After centrifugation at 14,000g and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [21]. Lysate proteins dissolved in 6X Laemmli sample buffer [22] were separated (30 μg/lane) using SDS–polyacrylamide gels (10% acrylamide) and electrotransferred to PVDF membranes. After blocking with 5% non-fat milk in TBST buffer (50 mM Tris pH 7.2–7.4, 200 mM NaCl, 0.1% Tween-20), the membranes were incubated 90 min with the appropriate dilution of primary antibody in TBST plus 1% non-fat milk. After washing, the membranes were incubated with the appropriate dilution of horse radish peroxidase-conjugated secondary antibody in TBST plus 1% non-fat milk. Finally, the blots were developed by ECL with the use of Kodak Bio-Max Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA).

Stripping and re-probing of membranes

The complete removal of primary and secondary antibodies from the membranes was achieved by incubating the membranes in stripping buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS and 50 mM β-mercaptoethanol) at 55 °C for 30 min with agitation. Membranes were then washed for 10 min in TBST (1% Tween-20) and blocked, as indicated above, for 1 h at room temperature. Thereafter, membranes were ready to re-probe with the corresponding antibodies.

Cell treatment with c-Src antisense and mismatch oligonucleotides

Cells were pre-treated with c-Src antisense oligonucleotides as follows. MCF-7 cells were seeded in plates at a density of 20,000 cells per cm^2 and cultured at 37 °C in RPMI-1640 medium containing 10% FBS under humidified air (5.5% CO_2). When cells reached 50–60% confluence, they were washed twice with PBS solution. Then 4 ml of serum-free RPMI-1640 medium were added to each plate. Transfections were performed using Lipofectamine 2000 reagent according to the manufacturer's instructions. Antisense or mismatch oligonucleotides (antisense: 5′-CTC TTG TTG CTA CCC ATG GTCC-3′; mismatch: 5′-AGC AAC GAC AAG TCA AGC TG-3′) [23] were used at a final concentration of 20 μM. Cells were incubated with the oligonucleotides/liposome mixture at a final volume of 5 ml for 24 h at 37 °C in a CO_2 incubator. After 24 h, medium was changed to serum-free RPMI for 1 h and then followed by Western blot analysis of cell extracts.

Proliferation assays

The number of cells was determined performing cell counts in a Neubauer chamber. Cells were seeded in 12-well plates at 75,000 cells/well. After 48 h, the medium was replaced by RPMI-1640 with 0.5% FBS, and cells were starved for 24 h. Cell treatment was performed in triplicate and started by adding the indicated inhibitors/vehicle and then agonists/vehicle in a medium containing 0.5% FBS. The cells were harvested, stained with trypan blue (to exclude non viable cells) and counted in a Neubauer chamber 72 h after.

Alternatively, the number of viable cells in proliferation was determined employing the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit. MCF-7 cells were plated into 96-multiwell plates (2400 cells/well) and after 48 h, the medium was replaced by RPMI-1640 without FBS and the cells were starved for 24 h. Cell treatment was performed in quadruplicate and started by adding the indicated inhibitors/vehicle, and then agonists/vehicle in a medium containing 0.5% FBS. To finish cell treatment, the medium was replaced by one containing MTS (20 μl MTS/100 μl RPMI-1640). Cells were incubated in the dark during 120 min at 37 °C (5.5% CO_2). Then, 10% SDS (25 μl/well) was added to stop the color reaction and subsequently the optical density (O.D.) was measured at 490 nm. Controls (without cells) containing the same volumes of culture medium and MTS solution as in the experimental conditions were used to substract background O.D.

from O.D. values of the samples. The results obtained were expressed as O.D. values ± standard deviation.

Statistical analysis

Data are shown as means ± standard deviation (S.D.). Statistical differences between groups were calculated by a two-tailed *t*-test. $P < 0.01$ (**) and <0.05 (*) were considered highly statistically significant and statistically significant, respectively.

Data from cell growth were first analyzed with a one-way ANOVA test; this analysis was followed by application of the multiple comparison Bonferroni test to compare means.

Results

Akt activation is a multistep process involving the phosphorylation of Ser 473 and Thr 308 residues, and the phosphorylation of these sites closely correlates with the activity of Akt [24]. To determine whether this serine/threonine kinase is modulated by ATP in MCF-7 cells, we performed dose–response (1–100 μM ATP) and time–course (1–60 min) studies. As shown in Fig. 1A, ATP (5 min) stimulated the phosphorylation of Akt, at the serine 473 residue, in a concentration-dependent manner. A significant rate of phosphorylation was already observed at 1 μM ATP, the response still increasing within the concentration range of 5–100 μM ATP. The EC50 value of ATP-induced Akt phosphorylation was 2.5 μM. Fig. 1B shows that Akt was activated in a time-dependent fashion reaching the maximum within 3–5 min of ATP exposure.

To obtain information on the possible purinergic receptor subtype/s involved in the phosphorylation of Akt, several nucleotide receptor agonists were tested. UTP was as effective as ATPγS in activating Akt (Fig. 2A and B). The EC50 values for UTP- and ATPγS-induced phosphorylation of Akt were near 2.5 μM. ADP and UDP significantly increased Akt (Ser 473) phosphorylation (Fig. 2C and E, respectively) in a similar manner as ATP and UTP but only at higher doses (50 and 100 μM; EC50 values = 30 μM); the response to ADPβS, non-hydrolysable analog of ADP, was weaker (EC50 value = 35 μM) (Fig. 2D).

Since ATP and other nucleotides can be hydrolyzed by extracellular ectoenzymes, the effects observed upon ATP addition might

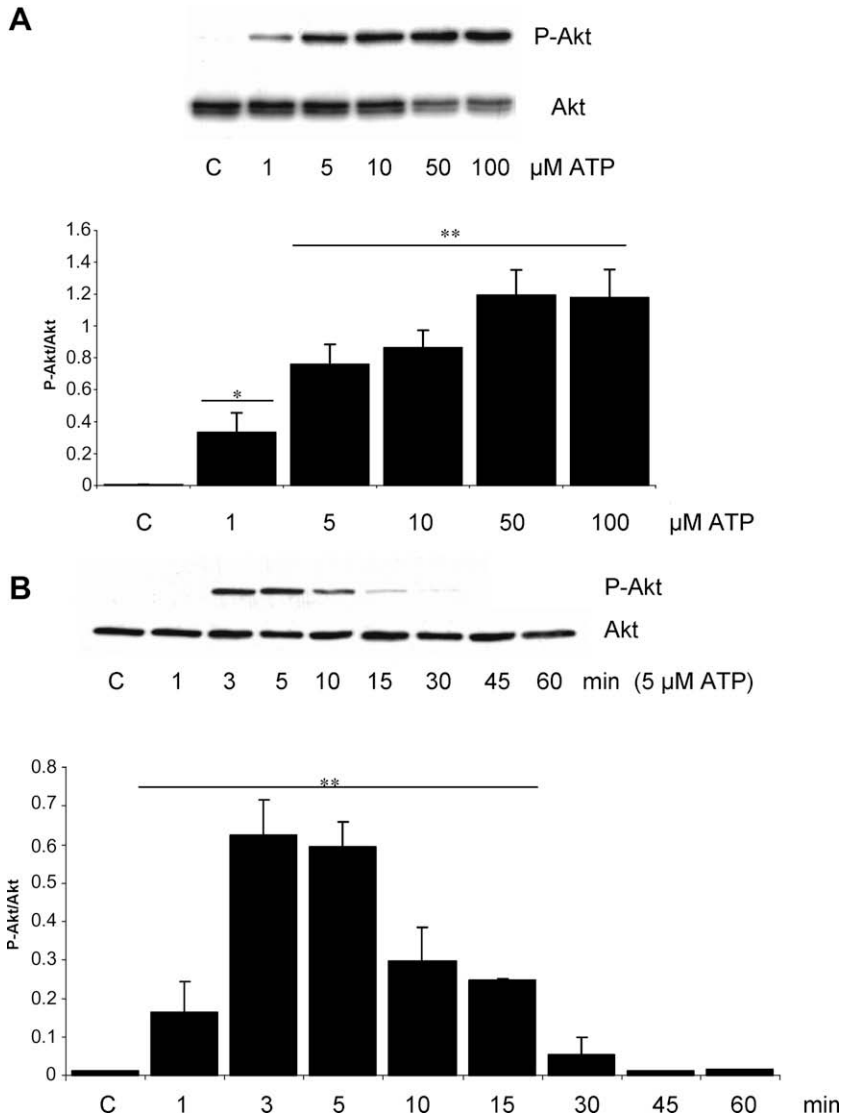

Fig. 1. Time- and dose–response studies of ATP-dependent phosphorylation of the serine/threonine kinase Akt in MCF-7 cells. Cells were treated with 1–100 μM ATP for 5 min (A) or 5 μM ATP (T) or vehicle (C) during 1–60 min (B). After cell lysis, comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting with anti-pAkt (Ser 473) antibody and then the membrane was stripped and re-probed with anti-Akt antibody as loading control. Representative immunoblots and quantifications by scanning volumetric densitometry of blots from three independent experiments are shown. Bars show the quantification of the blots expressed as P-Akt/Akt ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$; where *p* values refer to differences in phosphorylation of Akt between cells in the presence or absence of ATP.

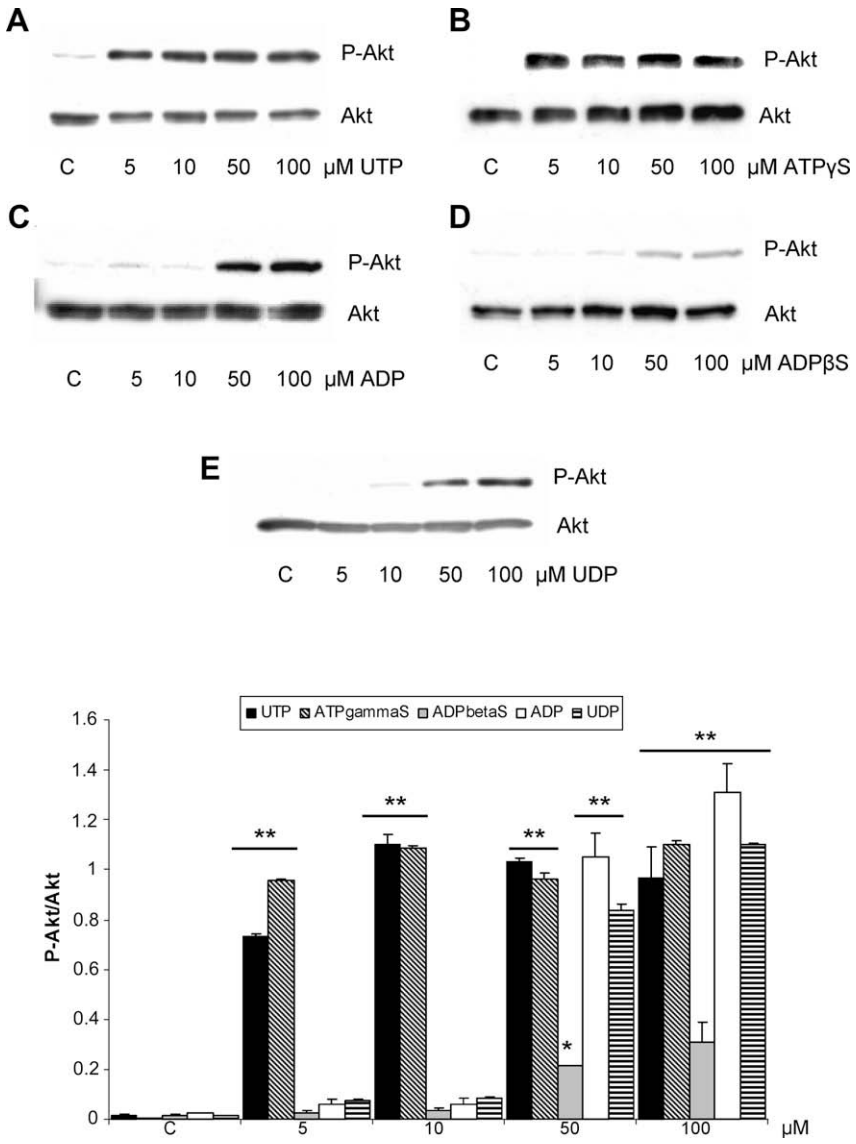

Fig. 2. Dose–response studies on the phosphorylation of Akt by different extracellular nucleotides. MCF-7 cells were treated with 5–100 μM of UTP (A), ATPγS (B), ADP (C), ADPβS (D), UDP (E) or vehicle (C) for 5 min. To compare the response elicited by several nucleotide agonists, cell lysate proteins were immunoblotted with anti-phospho Akt (Ser 473) antibody, and then the membranes were stripped and re-probed with anti-Akt as loading control. The immunoblots shown are representative of at least three independent experiments. Representative immunoblots and quantification by scanning volumetric densitometry of blots from three independent experiments are shown. Means ± SD are given. $^{*}p < 0.05$, $^{**}p < 0.01$, where p values refer to differences in phosphorylation of Akt between cells in the presence or absence of ATP.

result also from the activation of adenosine receptors [25]. To evaluate the contribution of these receptors to the phosphorylation of Akt we performed dose–response studies using adenosine. As seen in Fig. 3A, adenosine is a weaker inducer of Akt phosphorylation when compared to the effect exerted by ATP and ATPγS. To further confirm this, we tested the effect of caffeine, an antagonist of adenosine receptors, on the phosphorylation of Akt by ATP. Fig. 3B shows that although caffeine suppressed the phosphorylation of Akt by adenosine, it did not appreciably affect the activation of Akt by ATP. These results indicate that the contribution of adenosine receptors activation to Akt phosphorylation by ATP is not significant.

Fig. 4 summarizes the effects of several purinergic agonists (5 μM) on the phosphorylation of Akt. ATP, ATPγS and UTP induced a significant rate of Akt phosphorylation; whereas ADP, ADPβS, UDP and adenosine affected it to a lesser extent suggesting that the phosphorylation of Akt mainly occurs through $P2Y_2/P2Y_4$ receptors activation.

The phosphorylation of Akt can occur through a PI3K-dependent or -independent mechanism [26–30]. PI3K specific inhibitors, Ly294002 and wortmannin, were effective in reducing the phosphorylation of Akt at Ser 473 by ATP (Fig. 5A and B), suggesting that PI3K is a component of the Akt signaling pathway in MCF-7 cells.

To study the pathway upstream Akt, we treated MCF-7 cells with specific inhibitors prior to the addition of ATP. The main signal transduction pathway of $P2Y_{2/4}$ receptors (responsive to ATP and UTP) activation involves phospholipase C (PLC), which leads to generation of IP_3 and a transient rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [5,6]. Therefore, we tested the effect of two PLC inhibitors on the phosphorylation of Akt by ATP: neomycin and U73122. Fig. 6A and B show that both compounds reduced ATP-induced phosphorylation of Akt. Then, we determined the involvement of calcium in the activation of Akt by ATP. The use of a nominally Ca^{2+} free buffer containing EGTA had no effect on the phosphorylation of Akt by the nucleotide (Fig. 7A). To study the participation of intracellular Ca^{2+} release in the phosphorylation of Akt, we employed 2-APB, an IP_3 receptor antagonist [31]. The concentration used (150 μM) was that previously shown to

Fig. 3. Role of adenosine receptors in the phosphorylation of Akt in MCF-7 cells. (A) Dose–response study of the phosphorylation of Akt by adenosine. Cells were treated with 1–100 μM adenosine, 5 μM ATP, 5 μM ATPγS or vehicle (C) for 5 min. (B) Effect of caffeine on the phosphorylation of Akt by ATP. Cells were pre-incubated for 60 min with 10 μM caffeine; then, cells were treated with 5 μM ATP or adenosine (Ado) or vehicle (C) for 5 min. After cell lysis, comparable aliquots of proteins were separated by SDS–PAGE followed by Western blotting with anti-pAkt (Ser 473) antibody. Then, the membrane was stripped and re-probed with anti-Akt antibody as loading control. Representative immunoblots from three independent experiments are shown.

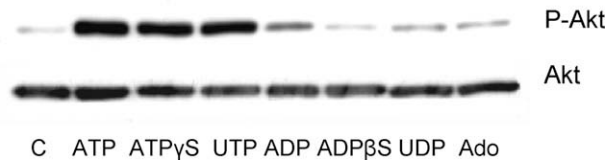

Fig. 4. The phosphorylation of Akt (Ser 473) depends on $P2Y_2$ and/or $P2Y_4$ receptor activation. MCF-7 cells were treated with 5 μM ATP, ATPγS, UTP, ADP, ADPβS, UDP, adenosine (Ado) or vehicle (C) for 5 min. The cells were then lysed, comparable protein aliquots were separated by SDS–PAGE followed by Western blotting using anti-phospho Akt (Ser 473) and anti-Akt (loading control) specific antibodies. Representative immunoblots of at least three independent experiments are shown.

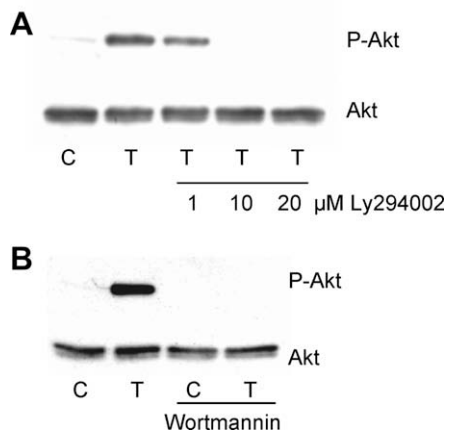

Fig. 5. The phosphorylation of Akt (Ser 473) by ATP depends on PI3K. MCF-7 cells were pre-incubated for 30 min with 1–20 μM Ly294002 (A) or 100 nM wortmannin (B), and afterwards treated with 5 μM ATP (T) or vehicle (C) for 5 min. The cells were then lysed and comparable protein aliquots were separated by SDS–PAGE followed by Western blotting using anti-phospho Akt (Ser 473) and anti-Akt (loading control) specific antibodies. Representative immunoblots of at least three independent experiments are shown.

block ATP-induced Ca^{2+} release in MCF-7 cells [6]. As seen in Fig. 7B, 2-APB reduced ATP-induced phosphorylation of Akt. Together, these results suggest that the phosphorylation of Akt at Ser 473 by ATP is linked to PLC activation and to intracellular Ca^{2+} release.

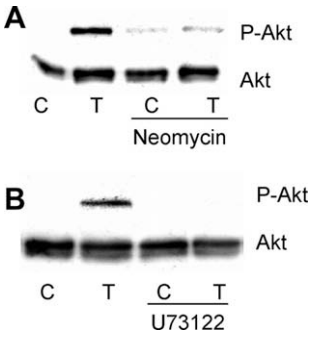

Fig. 6. PLC is involved in the phosphorylation of Akt at Ser 473 by ATP in MCF-7 cells. Cells were pre-incubated in RPMI medium in the presence and absence of 1 mM neomycin (A) or 5 μM U73122 (B) for 15 min, followed by the addition of either vehicle (C) or 5 μM ATP (T) for 5 min. Cell lysate proteins were immunoblotted with anti-phospho Akt (Ser 473) antibody; then the membrane was stripped and re-probed with anti-Akt antibody as loading control. A representative immunoblot of three independent experiments is shown.

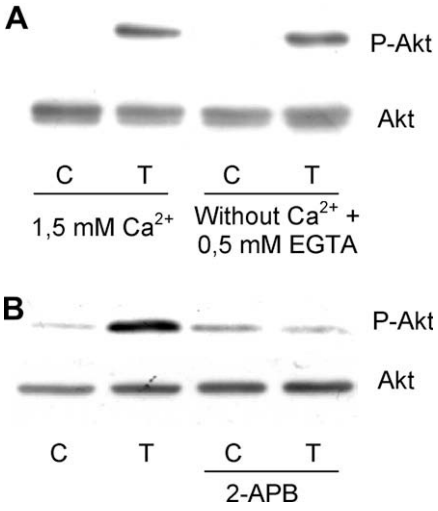

Fig. 7. The phosphorylation of Akt at Ser 473 induced by ATP depends on intracellular calcium release but is independent of extracellular calcium influx in MCF-7 cells. Cells were pre-incubated for 15 min in 1.5 mM Ca^{2+} or in a free-calcium medium containing 0.5 mM EGTA (A); or for 7 min in a calcium-free medium containing 0.5 mM EGTA in the absence and presence of 150 μM 2-APB (B). Then, cells were treated with 5 μM ATP (T) or vehicle (C) for 5 min and Western blot analysis was performed using anti-phospho Akt (Ser 473) phosphospecific antibody and anti-Akt as a loading control. Representative immunoblots of at least three independent experiments are shown.

Increases in $[Ca^{2+}]_i$ can activate some isoforms of protein kinase C, and this kinase has been shown to lie upstream of Akt in some cells [32,33]. To evaluate the participation of PKC on the phosphorylation of Akt by ATP, we tested two PKC inhibitors: Ro318220, an inhibitor of all PKC isoforms, and calphostin C, an inhibitor of conventional (cPKCs) and novel (nPKCs) PKC isoforms [34]. Although Ro318220 increased the basal phosphorylation of Akt; ATP was ineffective in inducing the phosphorylation of Akt over that control (Fig. 8A). In addition, ATP-induced Akt phosphorylation was inhibited by 0.5 μM calphostin C (Fig. 8B). These results suggest that PKC may be involved in the phosphorylation of Akt by ATP.

We previously showed that Src tyrosine kinase is phosphorylated (Tyr 416) by 5 μM ATP in MCF-7 cells, through a mechanism involving PKC [35]. To determine the participation of Src in the activation of Akt by ATP, we used the Src inhibitor PP2. Fig. 9A shows that the phosphorylation of Akt at Ser 473 was completely blocked by PP2, suggesting that Akt activation depends on Src. To

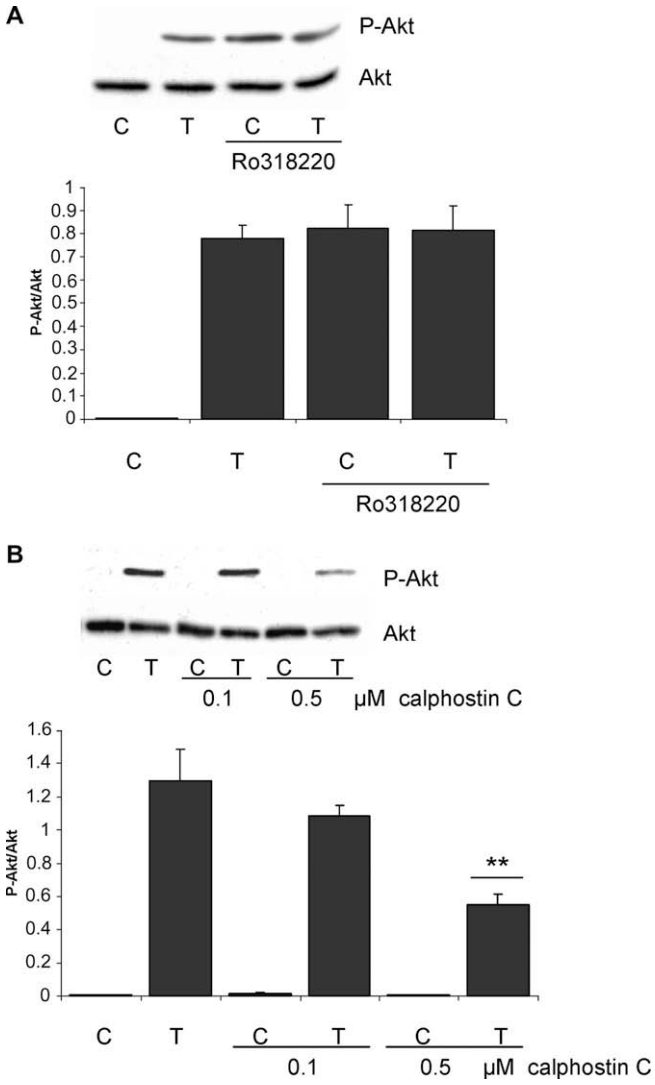

Fig. 8. Effect of PKC inhibitors Ro318220 and calphostin C on the phosphorylation of Akt at Ser 473 induced by ATP. MCF-7 cells were pre-incubated for 20 min with 5 μM Ro318220 (A) or 0.1 and 0.5 μM calphostin C (B). Then, cells were treated with 5 μM ATP (T) or vehicle (C) for 5 min and phosphorylation of Akt at Ser 473 was evaluated by Western blot analysis as described in Materials and Methods, SDS–PAGE and immunoblotting, employing an antibody that recognizes the phosphorylated form of Akt (at Ser 473). Then, the membrane was stripped and re-probed with an antibody directed against the total form (active plus inactive) of Akt. Representative immunoblots and quantifications by scanning volumetric densitometry of blots from three independent experiments are shown. Bars show the quantification of the blots expressed as P-Akt/Akt ± SD. $^{**}p < 0.01$; where p values refer to differences in phosphorylation of Akt by ATP between cells in the presence or absence of calphostin C.

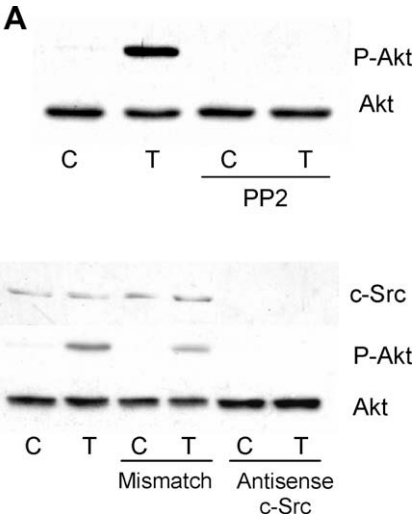

Fig. 9. Src is involved in the phosphorylation of Akt induced by ATP in MCF-7 cells. (A) Cells were pre-incubated for 30 min with 75 μM PP2, and then, were treated with 5 μM ATP (T) or vehicle (C) for 5 min. The phosphorylation of Akt (Ser 473) was evaluated by Western blot analysis, employing specific antibodies, as previously described in Materials and Methods, SDS–PAGE and immunoblotting. (B) Cells were treated with 5 μM ATP (T) or vehicle (C) for 5 min after transfection for 24 h with 20 μM mismatch or antisense oligonucleotides against human c-Src, as described in Materials and Methods, Cell treatment with c-Src antisense and mismatch oligonucleotides. Western blot analysis was then performed, using antibodies that recognize phosphorylated Akt (Ser 473) and total levels of c-Src and Akt (expression and loading control, respectively). Representative immunoblots of at least three independent experiments are shown.

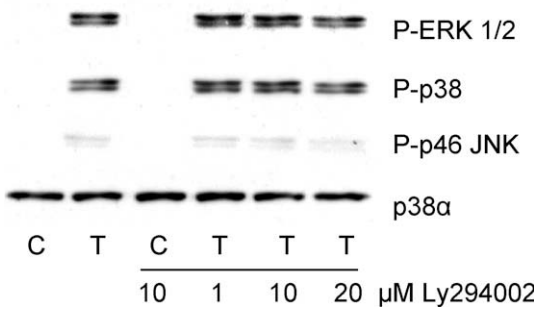

Fig. 10. PI3K is not involved in the activation of MAPKs induced by ATP. MCF-7 cells were pre-incubated for 30 min with the PI3K inhibitor Ly294002 (1–20 μM) and then treated with 5 μM ATP (T) or vehicle (C) for 5 min. The phosphorylation of the MAPKs ERK1/2, p38 and JNK was analyzed by Western Blot as described in Materials and Methods, SDS–PAGE and immunoblotting, employing antibodies that recognize the active (phosphorylated) forms of ERK1/2 (pERK1/2), JNK1/2 (pJNK1/2) and p38 (pp38). An antibody directed against the total form (active plus inactive) of p38α isoform was used as loading control. The figure shows a representative immunoblot of three independent experiments.

further confirm this, MCF-7 cells were transfected with specific antisense oligonucleotides against human c-Src (see Materials and Methods, cell transfection with c-Src antisense and mismatch oligonucleotides). As shown in Fig. 9B, although Src expression was inhibited, the total amount of Akt protein was not affected by cell transfection. Moreover, the phosphorylation of Akt (Ser 473) by ATP was suppressed thus denoting the involvement of Src in its activation.

Recent evidence suggests that a significant amount of cross-talk occurs between the PI3K and MAPK pathways [36,37]. As we previously determined the phosphorylation of ERK1/2, p38 and JNK MAPKs by ATP in MCF-7 cells [4], we now examined the participation of PI3K in ATP-induced MAPKs phosphorylation. Fig. 10 shows that although Ly294002 reduced the phosphorylation of Akt (see Fig. 5A); it had no effect on the phosphorylation of MAPKs by ATP suggesting that their activation is PI3K independent.

It is well established that ATP increases breast cancer cell numbers at low concentrations (<100 μM) [3]. Here, we studied the involvement of the PI3K/Akt signaling pathway in extracellular ATP-mediated cell proliferation. By using MTS cell viability assay, we observed that 5 μM ATP and UTP stimulated cell proliferation (average O.D. values ± S.D. obtained for ATP: 0.563 ± 0.024 and UTP: 0.611 ± 0.073 vs. control: 0.386 ± 0.026). Both PI3K inhibitors, Ly294002 and wortmannin, suppressed the proliferative action of ATP and UTP (ATP + Ly294002: 0.474 ± 0.051; UTP + Ly294002: 0.472 ± 0.034 vs. control Ly294002: 0.412 ± 0.035; ATP + wortmannin: 0.451 ± 0.062; UTP + wortmannin: 0.379 ± 0.020 vs. control

wortmannin: 0.375 ± 0.034). In addition, this result was confirmed by a cell count assay (see Materials and Methods, proliferation assays). Fig. 11 shows that 5 μM ATP and UTP strongly increased the cell number when compared to control condition (C). Inhibitors of PKC, Src, PLC, PI3K and MAPKs significantly suppressed the proliferative action of ATP, suggesting the participation of the PI3K/Akt and the MAPKs signaling pathways in $P2Y_{2/4}$ receptor stimulation-mediated cell growth.

Discussion

Although some reports suggest the activation of the PI3K/Akt pathway by ATP in astrocytes, glial cells and other cell types [20,28,38,39], the present study provides evidence, for the first time, on the activation of the PI3K/Akt signaling pathway by ATP and other extracellular nucleotides in MCF-7 breast cancer cells. This is a relevant observation since it has been determined that the PI3K/Akt signaling pathway has an important role in the development of breast cancer [15,16] and that may contribute to the resistance of human breast cancer cells to ionizing radiation [40]. The results presented here showed that ATP induces phosphorylation of Akt at one of the phosphorylation sites necessary for its full activation, in a dose- and time-dependent manner. Moreover, the EC50 values for the phosphorylation of Akt obtained by the use of different purinergic and pyrimidinergic agonists suggested the involvement of $P2Y_2$ and/or $P2Y_4$ receptor subtypes in agreement with previous molecular evidence demonstrating the expression of both receptor subclasses in MCF-7 cells [35]; as the rank order potency was ATP = UTP = ATPγS ≫ ADP = UDP > ADPβS = adenosine.

The phosphorylation of the serine/threonine kinase Akt can occur through a PI3K-dependent mechanism due to phosphatidyl-inositol triphosphate (PIP_3) formation; or through a PI3K independent mechanism [26–30]. In this work we determined, by the use of two PI3K specific inhibitors, that ATP-induced phosphorylation of Akt is PI3K-dependent in MCF-7 cells.

$P2Y_{2/4}$ receptors are linked to PLC activation that catalyses the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate into the intracellular messenger inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) [30]. Here, the inhibitory effect of neomycin and U73122 (two well known PLC inhibitors) and of the IP_3 receptor antagonist, 2-APB, supports the participation of phospholipase C and IP_3 sensitive-intracellular calcium release on ATP-induced Akt phosphorylation at Ser 473 in MCF-7 cells. Although calcium influx is usually associated to P2X receptor activation, it can also be a secondary signal triggered by the opening of different membrane channels such as stress activated (SAC), store operated (SOC) and voltage-dependent (VDCC) Ca^{2+} channels following P2Y receptor activation [1,5,41,42]. In this work, the use of a nominally Ca^{2+}-free buffer plus EGTA showed that activation of Akt by ATP is mediated by a mechanism independent of calcium influx, thus excluding the participation of membrane calcium channels. Intracellular Ca^{2+} release promoted by activation of $P2Y_{2/4}$ receptor subtypes is probably an important feature in the activation of the Akt pathway. The calcium messenger system is an upstream activator of c-Src and PKCs in several cell types. These kinases can initiate a signaling complex formation or may associate directly with PI3K or Akt leading to their activation [28].

A well-known response to PLC-generated DAG and IP_3/Ca^{2+} is the activation of classical PKCs [30]; and in some cells, PKC occurs upstream Akt and can either activate or inactivate this kinase depending on the cell type and the PKC isoform involved [19]. We found that pharmacological inhibition of PKC by 0.5 μM calphostin C diminished ATP-dependent phosphorylation of Akt, supporting the involvement of PKC as an upstream mediator in the activation of the serine/threonine kinase in MCF-7 cells. We previously showed that 0.1 μM calphostin C significantly reduced the phosphorylation of the MAPKs ERK1/2, p38 and JNK1 [35]. As this compound can cause specific inactivation of both conventional and novel PKC isoforms, the different concentrations needed to inhibit PI3K/Akt and MAPK signaling pathways could be due to the participation of different PKC isoforms [43]. Results obtained using Ro318220, another PKC inhibitor, further support this conclusion.

There is evidence which suggests that the activation of PI3K/Akt by androgen is inhibited by dominant-negative Src [44]. In addition, in rat hepatocytes activation of PI3Kbeta depends on Src [45]. Also, the Src inhibitor PP2 has been shown to inhibit PI3K activity in colon cancer cells [46] suggesting that Src acts upstream PI3K. Accordingly, our studies indicate that Src is crucial to link P2Y receptor activation by ATP with the phosphorylation of Akt as its inhibition or the suppression of its expression abolished the ability of extracellular ATP to activate Akt. In agreement with the role of PKC in Akt activation described above, we have recently shown that ATP leads to Src phosphorylation through a mechanism involving PKC [35].

The MAPKs are a family of serine/threonine kinases that control key cellular functions including proliferation, differentiation, migration and apoptosis, and participate in a number of disease states including chronic inflammation and cancer. They include the extracellular signal-regulated protein kinases (ERK1 and ERK2); c-Jun N-terminal kinases (JNK1, JNK2, JNK3); p38s (p38α,

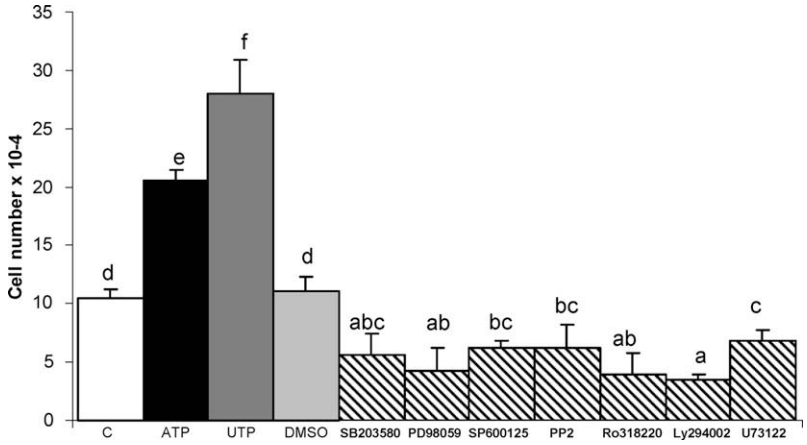

Fig. 11. Participation of the PI3K/AKt and MAPK signaling pathways in MCF-7 cell proliferation induced by ATP and UTP. Number of cells was determined as described in Materials and Methods, Statistical analysis. The data shown are the cell number average ± SD of three independent experiments. An ANOVA test was performed to establish differences between conditions [p(ANOVA) <0.01]. The letters indicate the results of Bonferroni test for mean comparison. Same letters for experimental groups indicate $P > 0.05$, whereas different letters indicate $P < 0.01$.

p38β, p38γ, p38δ) and ERK5/big MAP kinase 1 (BMK1). They play an essential role in signal transduction by modulating gene transcription in the nucleus in response to changes in the cellular environment [47]. In MCF-7 breast cancer cells, we previously showed that ATP through $P2Y_{2/4}$ receptor stimulation increases via PI-PLC/$[Ca^{2+}]_i$/PKC the activation of MAPKs ERK1/2, p38 and JNK1/2 which mediate c-Fos expression and c-Jun and JunD phosphorylation [6,35]. There is evidence which suggests that PI3K may regulate MAPKs in many cell systems [19]. Moreover, in human monocytic and HeLa cells ATP-induced phosphorylation of ERK1/2 MAPKs is mediated by PI3K linked to the $P2Y_2$ receptor [48,49]. Here, we determined that ATP-induced phosphorylation of ERK1/2, p38 and JNK1/2 MAPKs is PI3K independent in MCF-7 cells.

Recent evidence suggests that ATP can induce cell proliferation, differentiation and apoptosis, mediating different pathophysiological functions depending on the target cell and the type of P2 receptors stimulated. Thus, in MCF-7 breast cancer cells it is well established that ATP causes a decrease in cell growth at high doses (100 μM–1 mM), and an increase in cell numbers at lower concentrations [3,50]. This agrees with the fact that P2Y receptors are activated by low ATP concentrations (<100 μM) whereas higher doses (>100 μM) stimulate P2X receptors [51]. Therefore, stimulation of P2Y receptors is generally related with an increase in cell number, whereas P2X receptor activation can induce cell growth inhibition and apoptosis [19,40,52–54]. Most effects of P2YR-mediated activation of PI3K signaling are known to be related to cell proliferation, differentiation and survival [30]. However, the contribution of Akt to cell proliferation was suggested to be cell type specific and stimulus dependent [19]. The mitogenic action of ATP linked to P2Y receptor activation has been shown in some cell types [41,55–57]. Of relevance, the results presented here show, for the first time, the involvement of the PI3K/Akt signaling pathway in extracellular ATP-mediated proliferation of a cell line derived from breast tumor. UTP also stimulated MCF-7 cell proliferation, although the physiological significance of this observation remains to be determined since extracellular concentrations of UTP are much lower than that of ATP [58]. The fact that inhibitors of the MAPK signaling pathway reduced the effect exerted by ATP on MCF-7 cell growth is in keeping with reports which suggest that the Raf/MEK/ERK and PI3K/Akt signaling pathways can act in synergy to promote the G1–S phase cell cycle progression in both normal and cancer cells [59]. In addition, ERK1/2 and PI3K have shown to act independently but in parallel to promote cell growth [60,61]. Also, ATP has been shown to induce proliferation through parallel but independent ERK and PI3K signaling cascades [18].

In addition to its well-known role in various cell survival and metabolic responses, Akt has been reported to play an important function in regulating the cell cycle. Various studies have shown that blockage of Akt signaling results in apoptosis and growth inhibition of tumor cells [62]. Therefore, selective antagonists of the $P2Y_{2/4}$ receptors and of their downstream mediators, such as PI3K/Akt signaling, should be considered in the development of effective breast cancer therapeutic strategies.

Altogether, these data support that stimulation of $P2Y_2$ and $P2Y_4$ receptors subtypes by ATP increases MCF-7 cell growth by a mechanism involving activation of the PI3K/Akt pathway through phospholipase C, intracellular calcium release, protein kinase C and Src. Knowledge on the intracellular signaling pathway regulated by extracellular nucleotides in MCF-7 breast cancer cells is of potential relevance to understand its role in breast tumor growth and progression.

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