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ATP modulation of mitogen activated protein kinases and intracellular Ca^{2+} in breast cancer (MCF-7) cells

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Abstract

In the breast tumor cell line MCF-7, extracellular nucleotides induce transient elevations in intracellular calcium concentration ($[Ca^{2+}]_i$). In this study we show that stimulation with ATP or UTP sensitizes MCF-7 cells to mechanical stress leading to an additional transient Ca^{2+} influx. ATP ⩾ ATPγ-S ⩾ UTP >>> ADP = ADPβ-S elevate $[Ca^{2+}]_i$, proving the presence of $P2Y_2/P2Y_4$ purinergic receptor subtypes. In addition, cell stimulation with ATP, ATPγ-S or UTP but not ADPβ-S induced the phosphorylation of ERK1/2, p38 and JNK1/2 mitogen activated protein kinases (MAPKs). The use of Gd^{3+}, La^{3+} or a Ca^{2+}-free medium, inhibited ATP-dependent stress activated Ca^{2+} (SAC) influx, but had no effect on MAPK phosphorylation. ATP-induced activation of MAPKs was diminished by two PI-PLC inhibitors and an IP_3 receptor antagonist. These results evidence an ATP-sensitive SAC influx in MCF-7 cells and indicate that phosphorylation of MAPKs by ATP is dependent on PI-PLC/IP_3/$Ca^{2+}{}_i$ release but independent of SAC influx in these cells, differently to other cell types.

Keywords: ATP; MAPKs; Ca^{2+}; Breast tumor cells; P2Y receptors

In the last few years, biological actions of extracellular nucleotides have been studied in many cell and tissue types. Recently, they have emerged as a novel class of proliferative agents and possible effectors of neoplastic transformation. Extracellular nucleotides act through purinergic receptors from P2 class. The P2 receptors are divided into ligand-gated non-selective ion channels (P2X) and G-protein-coupled receptors (P2Y). At least, seven members of the P2X ($P2X_{1-7}$) and seven subtypes of P2Y ($P2Y_{1,2,4,6,11,12,13}$) have been cloned and characterized in mammals [1,2]. The main signal transduction pathway of P2Y receptor activation involves phospholipase C, which leads to the generation of IP_3 and a transient rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [3]. It has been reported that ATP and UTP both increase $[Ca^{2+}]_i$ and cell proliferation through specific P2Y purinoceptors in MCF-7 breast tumor cells, suggesting that $P2Y_2/_4$ receptor subtypes are mainly expressed in these cells [4].

In various cell types, including breast tumor cells, mitogen-activated protein kinases (MAPKs)[1] signaling pathways have been implicated in the regulation of cell proliferation and differentiation and other cellular responses triggered by extracellular stimuli [5]. Four different subgroups within the MAPK family have been described: extracellular signal-regulated kinases (ERKs1/2), c-jun N-terminal or stress-activated protein kinases (JNK1/2/SAPK), ERK/big MAP kinase 1 (BMK1 or ERK5), and the p38 group of protein kinases [5]. Particularly, ERKs1/2 are involved in cell proliferation/transformation and survival whereas p38 MAPKs are involved in inflammatory responses, cell cycle control, apoptosis, pro-

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[1] *Abbreviations used:* MAPKs, mitogen activated protein kinases; ERKs, extracellular signal-regulated kinases; JNKs, c-jun N-terminal or stress-activated protein kinases; BMK1, big MAP kinase 1; FBS, Fetal bovine serum.

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liferation and differentiation of several cell types including breast cells [6].

Although some studies on the regulation of MAPK signal transduction pathways by G-protein-coupled receptors have been reported in breast tumor cells, the action of ATP on MAPKs in these cells remains poorly understood [7].

Mechanical stimulation has been shown to play a role in the regulation of cell proliferation and differentiation [8,9]. However, the mechanism by which cells sense and respond to mechanical stimuli is not completely known. There is increasing evidence that extracellular nucleotides released from cells by mechanical stimulation may convert mechanical forces into biochemical signals. Thus, in osteoclasts mechanical stimulation induces endogenous ATP release leading to activation of calcium influx through P2X receptors [10]. Also, in astrocytes it has been reported that stretch causes ATP release leading to activation of P2X and P2Y receptors and rapid activation of the ERK pathway [11]. In mammary glands, cell-stretch induced ATP-release and Ca^{2+}-increase through activation of $P2Y_1$ and $P2Y_2$ receptors, and this was associated with an enhancement of milk ejection [12]. In addition, we have recently reported that $P2Y_2$ receptor activation sensitizes osteoblastic cells to mechanical stress leading to Gd^{3+}-sensitive mechanical stress activated Ca^{2+} influx (SAC influx) [13].

In this work we studied ATP regulation of $[Ca^{2+}]_i$ and activation of the MAP kinases ERK1/2, p38 and JNK1/2 in the human breast tumor cell line MCF-7. Our data suggests that ATP through $P2Y_2/_4$ receptors sensitizes breast cells to mechanical stress leading to SAC influx, and stimulates the ERK1/2, p38 and JNK1/2 signaling pathways. However, activation of MAPKs proved to be dependent on PI-PLC/$Ca^{2+}{}_i$ release but independent of extracellular calcium influx induced by ATP in MCF-7 cells.

Materials and methods

Materials

Fura-2/pentaacetoxymethyl ester (Fura-2/AM), neomycin sulfate, ATP, ATPγ-S, UTP, ADPβ-S, RPMI-1640 medium, $GdCl_3$, compound U73122 and diphenylboric acid 2-aminoethyl ester (2-APB) were from Sigma–Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Monoclonal antibodies recognizing dually phosphorylated ERK1/2 (Thr202, Tyr204) and JNK1/2 (Thr183/Tyr185) were from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies recognizing dually phosphorylated p38 (Thr180/Tyr182), polyclonal anti p38 α, 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 of 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 water as vehicle were used. For intracellular calcium measurements, cells were plated onto glass coverslips and allowed to grow until confluence (2–3 days after plating) before use.

Western blot analysis

MCF-7 cells were treated with ATP, ATP-γ-S, UTP or ADPβS in the presence or absence of the calcium channel blocker Gd^{3+} or inhibitors (neomycin, U73122). When the treatment was performed in a Ca^{2+}-free buffer, the medium was replaced by buffer C (see below: Intracellular calcium measurements) 15 min before cell treatment. Then, cells were 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 14,000*g* and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [14]. Lysate proteins dissolved in 6× Laemmli sample buffer 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, 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 BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA).

Stripping and reprobing 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. Then, membranes were washed for 10 min in TBST (1% Tween 20) and blocked, as indicated above, for 1 h at room temperature. After that, membranes were ready to reprobe with the corresponding antibodies.

Intracellular calcium measurements

Intracellular Ca^{2+} changes in MCF-7 cells were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2 as previously described [15,16]. Cells grown onto glass coverslips were loaded with 1 μM of Fura-2/AM in buffer A containing (in mM): 138 NaCl, 5 KCl, 1 $MgCl_2$, 5 glucose, 10 Hepes (pH 7.4), 1.5 $CaCl_2$, and 0.1% bovine serum albumin (BSA), in the dark during 30 min at 37 °C. Unloaded dye was washed out and cells were stored in buffer B (buffer A without BSA and Fura-2/AM) in the dark at room temperature for 30 min prior to use, to allow complete intracellular dye deesterification. For fluorescence measurements, the coverslips containing dye-loaded cells were then mounted on a chamber and placed on inverted microscope (Nikon Diaphot 200) and maintained at 25–30 °C. The excitation wavelength was switched between 340 and 380 nm employing a dual excitation monochromator from an SLM-Aminco 8100 spectrofluorimeter connected to the epifluorescence port of the microscope through an optic fiber. Emitted cellular fluorescence was collected at 510 nm and ratios from 340/380 nm signals were obtained, thus making the measurement independent of variations in cellular dye content, dye leakage or photobleaching. For measurements in Ca^{2+}-free extracellular medium a buffer C (buffer B without Ca^{2+} plus 0.5 mM EGTA) was used. When channels or PI-PLC inhibitors were used, they were added to the medium 3–7 min before the measurements.

Mechanical stimulation

Cells grown onto glass coverslips were subjected to mechanical stimulation by perturbation of the medium during intracellular calcium measurements, by adding an amount of vehicle equivalent to the volume of agonist. Mechanical stimulation was performed before and after treatment of the cells with the agonist.

Cell viability analysis

After experiments, control and pretreated cells with the different inhibitors or channel blockers were washed with PBS buffer and then were treated with 0.1% of Trypan Blue stain. Cells were incubated for 5 min at room temperature with slow agitation in an orbital shaker and then observed in an inverted microscope. Cells were counted per field and the percent of cells that excluded the stain (viable cells) respect to the total cells (viable + non-viable cells) was obtained.

Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test [17] and probability values below 0.01 ($P < 0.01$) were considered highly significant. Quantitative data are expressed as means ± standard deviation (SD) from the indicated set of experiments.

Results

To determine whether ERK 1/2, p38 and JNK1/2 MAPKs are modulated by ATP in MCF-7 cells, we first performed dose (1–50 μM ATP)- and time (0.5–15 min)-response studies. As shown in Fig 1a, the response was dose-dependent, ERK1/2, p38 and JNK1 reached their maximum phosphorylation at 5 μM ATP and remained fully activated even at the highest dose used; however, JNK2 activation was only evident at 50 μM ATP. ATP increased phosphorylation of MAPKs in a time-dependent manner, maximum phosphorylation levels of ERK 1/2, p38 and JNK1 MAPKs were reached within 3–5 min after treatment with 5 μM ATP (Fig. 1b), and remained activated for at least 30 min (not shown). JNK2 activation was observed only after 10–15 min of cell treatment with 5 μM ATP, thus exhibiting a different pattern of phosphorylation/activation in comparison with the other members of the MAPK family.

To evaluate the participation of phospholipase C on ATP-induced activation of MAPKs in MCF-7 cells, we used neomycin (1 and 5 mM), a PI-PLC inhibitor. As shown in Fig. 2a, neomycin decreased by ~70% the phosphorylation of JNK1 and p38 by ATP, while the effect of the PLC inhibitor on ATP-dependent ERK1/2 activation was less pronounced (50%). Partial blockade of PLC-mediated signaling pathways by neomycin has also been observed in other cell types when the inhibitor is applied externally [18]. As observed in Fig. 2b, a more robust inhibition of ATP-induced p38, ERK1/2 and JNK1 phosphorylation was observed by the use of U73122 (10 μM), another PLC inhibitor. As the main signal transduction pathway of P2Y receptors is PLC activation, our results suggest that ATP may be acting at least in part through this purinergic receptor family.

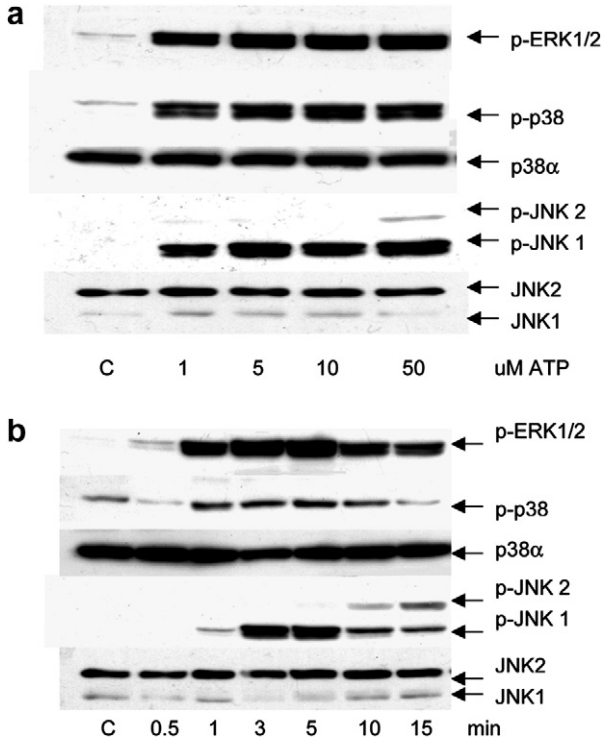

Fig. 1. Dose- and time-response profiles of ATP-dependent ERK 1/2, p38 and JNK1/2 phosphorylation in MCF-7 cells. MCF-7 cells were treated with 1–50 μM ATP during 5 min (a) or with 5 μM ATP during 0.5–15 min (b). Subsequently, Western blot analysis was performed as described in Materials and methods employing antibodies that recognize the active (phosphorylated) forms of ERK 1/2 (pERK 1/2), JNK1/2 (pJNK1/2) and p38 (pp38). Antibodies directed against the total form (active plus inactive) of p38α isoform and JNK1/2 were used as loading controls. Each membrane was stripped and reprobed not more than three or four times. These results are representative of at least three independent experiments.

Since ATP and UTP have been shown to increase $[Ca^{2+}]_i$ and cell proliferation in MCF-7 cells [4], we conducted experiments to identify the possible purinoceptor P2Y subtype involved in ATP-dependent activation of MAPKs. We first performed a dose-response study using different purinergic agonists. As seen in Fig. 3, a strong stimulation of MAPK phosphorylation was observed after cell treatment with ATP (5 μM), ATP-γ-S (1–5 μM) and UTP (5–10 μM), while ADPβS (5–10 μM) was unable to increase phosphorylation of MAPKs. These results suggest the involvement of $P2Y_2$ and/or $P2Y_4$ purinoceptor subtype/s in MAPK regulation by ATP in MCF-7 cells.

To further determine whether activation of MAPKs correlates with changes in $[Ca^{2+}]_i$ levels induced by ATP in MCF-7 cells, we studied the effects of ATP and other purinergic agonists on $[Ca^{2+}]_i$ by spectrofluorimetric measurements using Fura-2-loaded cells. As shown in Fig. 4a, addition of 5 μM ATP in a calcium-free medium (plus 0.5 mM EGTA), induced a rapid rise in $[Ca^{2+}]_i$ that returned to the baseline after 4–5 min (317 ± 79 s; $n = 8$). When $[Ca^{2+}]_i$ measurements were carried out in a medium containing 1.5 mM Ca^{2+}, a similar $[Ca^{2+}]_i$ peak was observed. The slight elevation over the baseline observed

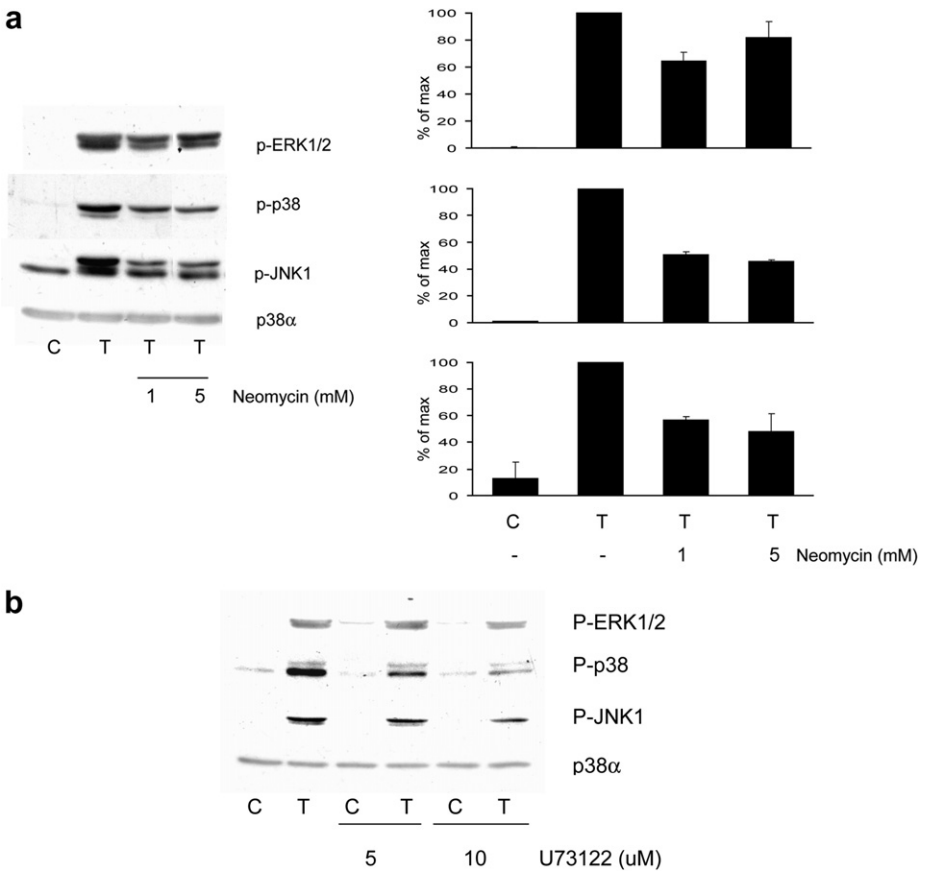

Fig. 2. Effect of the PI-PLC inhibitors neomycin and U73122 on ERK 1/2, p38 and JNK1 activation by ATP in MCF-7cells. (a) MCF-7 cells were preincubated for 15 min in RPMI medium in the presence and absence of 1 or 5 mM neomycin, followed by the addition of either vehicle (C) or 5 μM ATP (T) for 5 min. Cell lysate proteins were immunoblotted with pERK1/2, then the membranes were stripped and reprobed with pp38 and pJNK1/2 phosphospecific antibodies as in Fig. 1, with anti-p38α as loading control, respectively. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown. (b) MCF-7 cells were preincubated for 7 min in RPMI medium containing U73122 or its vehicle (DMSO). Then, cells were stimulated by the addition of either vehicle (C) or 5 μM ATP (T) for 5 min. Cell lysate proteins were immunoblotted with pp38, pERK1/2 and pJNK1/2 phosphospecific antibodies and anti-p38α as loading control. These results are representative of at least three independent experiments.

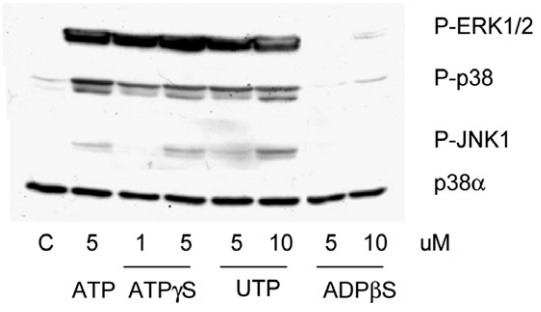

Fig. 3. Modulation of MAPKs by extracellular nucleotides in MCF-7 cells. Breast tumor cells were stimulated by the addition of vehicle (C), 5 μM ATP and different doses of ATPγS, UTP and ADPβS for 5 min. Cell lysate proteins were immunoblotted with pERK1/2 and then the membranes stripped and reprobed with pp38 and pJNK1/2 phosphospecific antibodies and with anti-p38α as loading control, respectively. The immunoblot shown is representative of at least three independent experiments.

in presence of extracellular Ca^{2+}, suggests that calcium release from intracellular stores is the main component of the Ca^{2+} response to ATP. It was also observed that after ATP stimulation, and once $[Ca^{2+}]_i$ returned near basal levels, mechanical perturbation of the medium by the addition of vehicle to the recording chamber, induced a second $[Ca^{2+}]_i$ transient increase, which was not seen in a free-calcium/0.5 mM EGTA buffer (Fig. 4a). This extracellular calcium entry will be referred to as stress activated Ca^{2+} (SAC) influx. Moreover, the application of such mechanical stress prior to purinergic activation did not affect $[Ca^{2+}]_i$ (Fig. 4a). A similar initial $[Ca^{2+}]_i$ transient was observed upon cell stimulation with 5 μM ATPγ-S or UTP, whereas the response to ADP (not shown) and ADPβ-S was very weak (Fig. 4b), further supporting the notion that the $P2Y_2/P2Y_4$ receptor subtypes (responsive to ATP and UTP but not to ADP) are the main P2Y purinoceptor/s expressed in MCF-7 cells. As shown in Fig. 5, SAC influx was only observed when cells were pre-stimulated with ATP or UTP but not ADP.

In osteoblasts, we have previously shown that ATP-dependent SAC influx was inhibited by gadolinium [13], a widely known stretch channel inhibitor [19–21]. Accordingly Fig. 6 shows that the addition of Gd^{3+}, or La^{3+},

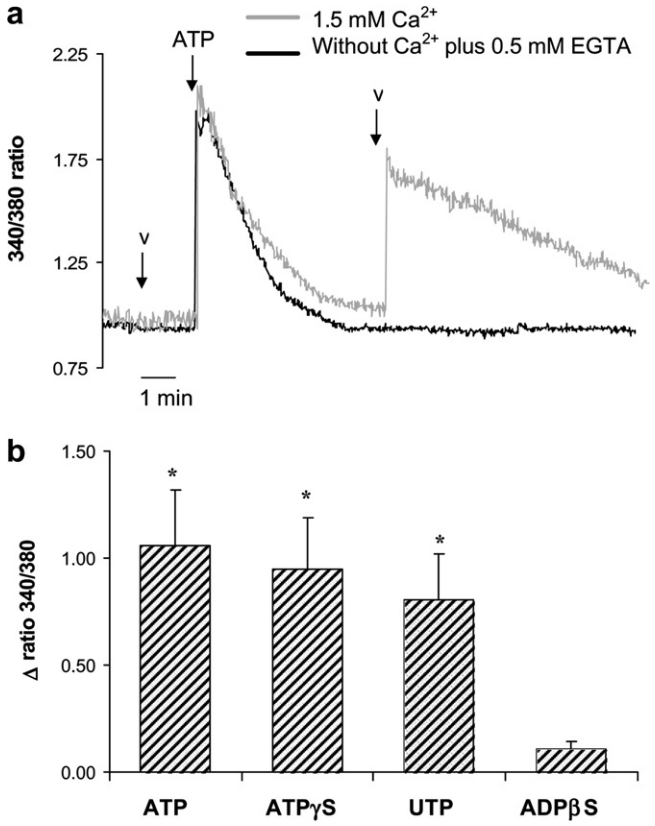

Fig. 4. (a) Effects of ATP and mechanical stress on intracellular calcium levels ($[Ca^{2+}]_i$) in MCF-7 cells. Changes in $[Ca^{2+}]_i$ were measured in Fura-2-loaded MCF-7 cells incubated in 1.5 mM Ca^{2+} or in a Ca^{2+}-free medium as described in Materials and methods. Cells were stimulated with 5 μM ATP as indicate the arrows. Mechanical stress was induced by addition of vehicle before (first V) and after purinergic stimulation (second V). Time traces of ATP treated cells representative from at least five independent experiments each performed onto no less than 40–50 cells are shown. (b) Effects of extracellular nucleotides on $[Ca^{2+}]_i$ in MCF-7 cells. Fura-2-loaded MCF-7 cells incubated in 1.5 mM Ca^{2+} were stimulated with 5 μM of either ATP, ATPγS, UTP and ADPβS; followed by measurement of $[Ca^{2+}]_i$ as described in Materials and methods. The graphic shows the $[Ca^{2+}]_i$ peak expressed as Δ 340:380 nm fluorescence ratio respect to the basal. Means (±SEM) are given; $n = 5$–10/group). $^*p < 0.05$.

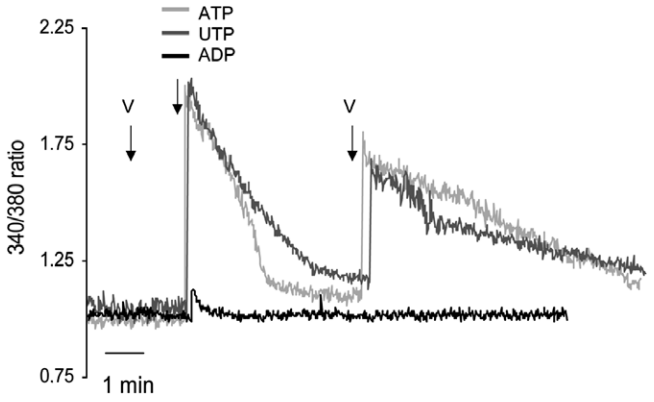

Fig. 5. Purinoceptor stimulation-dependent SAC influx in MCF-7 cells. $[Ca^{2+}]_i$ changes were measured in Fura-2-loaded MCF-7 cells incubated in 1.5 mM Ca^{2+} as described in Materials and methods. Stimulation with 5 μM of either ATP, UTP and ADPβ-S or mechanical stress induced by addition of vehicle (V), are indicated by the arrows. Time traces shown are representative of at least five independent experiments onto no less than 40–50 cells.

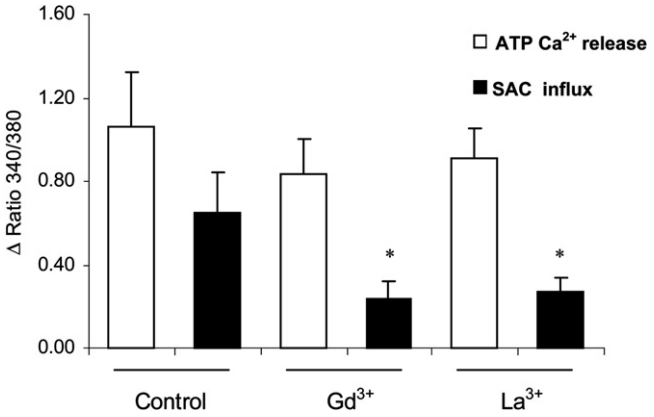

Fig. 6. Effect of Gd^{3+} and La^{3+} on ATP-dependent SAC influx. $[Ca^{2+}]_i$ was measured in Fura-2-loaded MCF-7 cells incubated in 1.5 mM Ca^{2+} in the presence or absence of 10 μM Gd^{3+} or 10 μM La^{3+} as described in Materials and methods. Cells were stimulated with 5 μM ATP and then mechanical stress was induced by addition of vehicle as in Fig. 5. Cell viability was not affected by the pretreatment with Gd^{3+} or La^{3+} since more than 90 % of the cells excluded the Trypan Blue stain after the experiments. Mean (±SEM) data of Δ 340:380 nm fluorescence ratios at the ATP-induced $[Ca^{2+}]_i$ peak or ATP-dependent SAC influx are shown ($n = 5$–10/group). $^*p < 0.05$.

two non-selective Ca^{2+} channel blockers, significantly diminished SAC influx induced by ATP in MCF-7 cells.

Altogether, these results suggest that activation of $P2Y_2$/$P2Y_4$ receptor subtypes sensitizes mechanical stress activated calcium channels leading to calcium influx in MCF-7 cells.

We then evaluated whether stimulation of MAPKs by ATP is mediated by SAC influx. As shown in Fig. 7a, no changes in ATP-dependent MAPK phosphorylation was observed in the presence of Gd^{3+} or La^{3+} suggesting that SAC influx does not participate in ERK1/2, p38 and JNK1 activation by ATP in MCF-7 cells. To investigate if extracellular Ca^{2+} influx through an entry pathway, other than SAC, participates in ATP modulation of MAPKs, cells were pre-incubated for 15 min in a calcium-free buffer containing 0.5 mM EGTA. As shown in Fig. 7b, the absence of extracellular calcium did not affect the phosphorylation state of p38, ERK1/2 and JNK1 in response to ATP, suggesting that activation of MAPKs by the extracellular nucleotide is independent of extracellular Ca^{2+} influx in MCF-7 cells. Cell viability was not affected by incubation in a Ca^{2+}-free medium during 15 min or in the presence of Gd^{3+} or La^{3+}, since more than 90% of the cells excluded the Trypan Blue dye.

In view of the above, to confirm the participation of intracellular Ca^{2+} release in ERK 1/2, p38 and JNK1 regulation by ATP, we employed 2-APB, an IP_3 receptor antagonist [22]. This compound has been shown to potentiate or inhibit some calcium channels at the plasma membrane [23]. In order to avoid these effects, we performed the

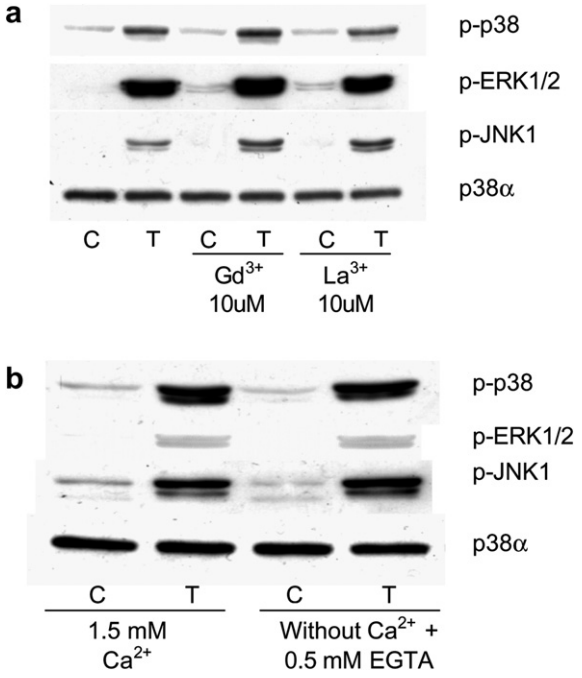

Fig. 7. (a) SAC influx does not participate in the activation of ERK1/2, p38 and JNK1 MAPKs by ATP in MCF-7 cells. MCF-7 cells were pre-incubated for 10 min with La^{3+} or Gd^{3+} (10 μM each). Cell treatment was then performed by the addition of either vehicle (C) or 5 μM ATP (T) for 5 min. Cell lysate proteins were immunoblotted with pERK1/2 and the membranes were stripped and reprobed with p-p38 and p-JNK1/2 phosphospecific antibodies as in Fig. 1, with anti-p38α as a loading control, respectively. (b) ERK1/2, p38 and JNK1 activation in MCF-7 cells is independent of extracellular Ca^{2+} influx. MCF-7 cells were pre-incubated for 15 min in 1.5 mM Ca^{2+} or in a free-calcium medium with 0.5 mM EGTA. Then, cells were stimulated by the addition of vehicle (C) or 5 μM ATP (T) for 5 min. Cell lysate proteins were immunoblotted with pERK1/2, pp38 and pJNK1/2 phosphospecific antibodies as in Fig. 1, with anti-p38α as a loading control. The immunoblots of (a) and (b) are representative of at least three independent experiments.

experiments with 2-APB in a Ca^{2+}-free medium. As shown in Fig. 8a, pretreatment in a Ca^{2+}-free medium with 2-APB (150 μM) completely suppressed ATP-dependent ERK1/2 phosphorylation and also ATP-induced Ca^{2+} release (Fig. 8b). Although 2-APB did not modify ERK1/2 basal phosphorylation, it greatly increased that of p38 and JNK1. These data, together with the previous results obtained using PI-PLC inhibitors, suggest that the PI-PLC/IP_3/Ca^{2+} release is the main signaling pathway involved in ATP activation of MAPKs in MCF-7 cells.

Discussion

The data in the present study demonstrates, in agreement with previous findings in other cell types [4,24], that MCF-7 cells respond to extracellular nucleotides with an elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels due to stimulation of specific purinergic membrane receptors. The P2Y purinoceptor specificity was supported by the observation that $[Ca^{2+}]_i$ response to nucleotides followed the potency order ATPγS ⩾ ATP ⩾ UTP >>> ADP ⩾ ADPβS. In addition, as PLC/IP3/Ca^{2+} is the main signal transduction pathway coupled to P2Y receptor activation, the inhibitory effect of neomycin and U73122 and of the IP_3 receptor antagonist, 2-APB, on ATP-dependent MAPK phosphorylation supports the participation of these receptors. As previously mentioned in the Results section, within the purinergic receptor family, it is known that the P2Y subtype responds to ATP. Moreover, $P2Y_1$ receptors respond to ADP but not UTP, while $P2Y_2$ and $P2Y_4$ receptors are activated by UTP but not ADP. Therefore, the observed response profiles in MCF-7 cells are consistent with activation of the $P2Y_2$ and/or $P2Y_4$ receptor subfamilies [1,2]. However, purinergic receptor isoforms expressed in MCF-7 cells should be cloned and structurally characterized to confirm the identity of the receptor subtypes stimulated by ATP.

Our results indicate that Ca^{2+} release from inner stores, as a consequence of PI-PLC activation, is the main component in the Ca^{2+} response of MCF-7 cells to ATP. Similar findings have been reported for the osteoblastic cell lines ROS-A 17/2.8 [13] and UMR-106 [25].

Evidence was obtained showing that extracellular nucleotide activation of $P2Y_2$/$_4$ purinergic receptors sensitizes stress activated Ca^{2+} (SAC) channels to mechanical perturbation of the medium leading to extracellular calcium influx blocked by Gd^{3+} and La^{3+}. This finding is in keeping with the existence of an ATP-dependent SAC influx in osteoblastic ROS-A 17/2.8 cells [13], chick skeletal muscle and C2C12 cells (Graciela Santillán and Ricardo Boland, unpublished).

Additional evidence suggests that mechanical stresses such as oscillatory fluid flow, hypo/hyperosmolarity, stretch or medium perturbation, can lead to ATP release from cells and trigger cellular responses through activation of P2 receptors [12,26,27]. However, in our case, when mechanical stress was applied without ATP pre-stimulation, SAC influx was not observed, suggesting that under our experimental conditions nucleotide release is insufficient to stimulate calcium influx through activation of ionotropic P2 receptors.

MAPKs, which play a pivotal role in a variety of cell functions, may be modulated by diverse external stimuli such as growth factors, cytokines, and physical stresses (ultraviolet radiation, hyperosmolarity, hypoxia and mechanical stress) [28]. We found, in agreement with a previous report [29], that ATP stimulates ERK1/2 in MCF-7 cells. Of interest, our results also provide, for the first time, information on JNK1/2 and p38 MAPK activation by ATP in these cells. ATP increases JNK1 and p38 MAPK phosphorylation with a similar dose- and temporal-profile patterns. However, the nucleotide regulates JNK2 at higher concentrations and longer treatment intervals than JNK1, implying that the later may be the major contributor to JNK activity in MCF-7 cells exposed to ATP, as has been reported in $JNK1^{-/-}$ and $JNK2^{-/-}$ fibroblasts [30].

There is evidence suggesting that epithelial cells recognize mechanical or physical stress stimulation and trans-

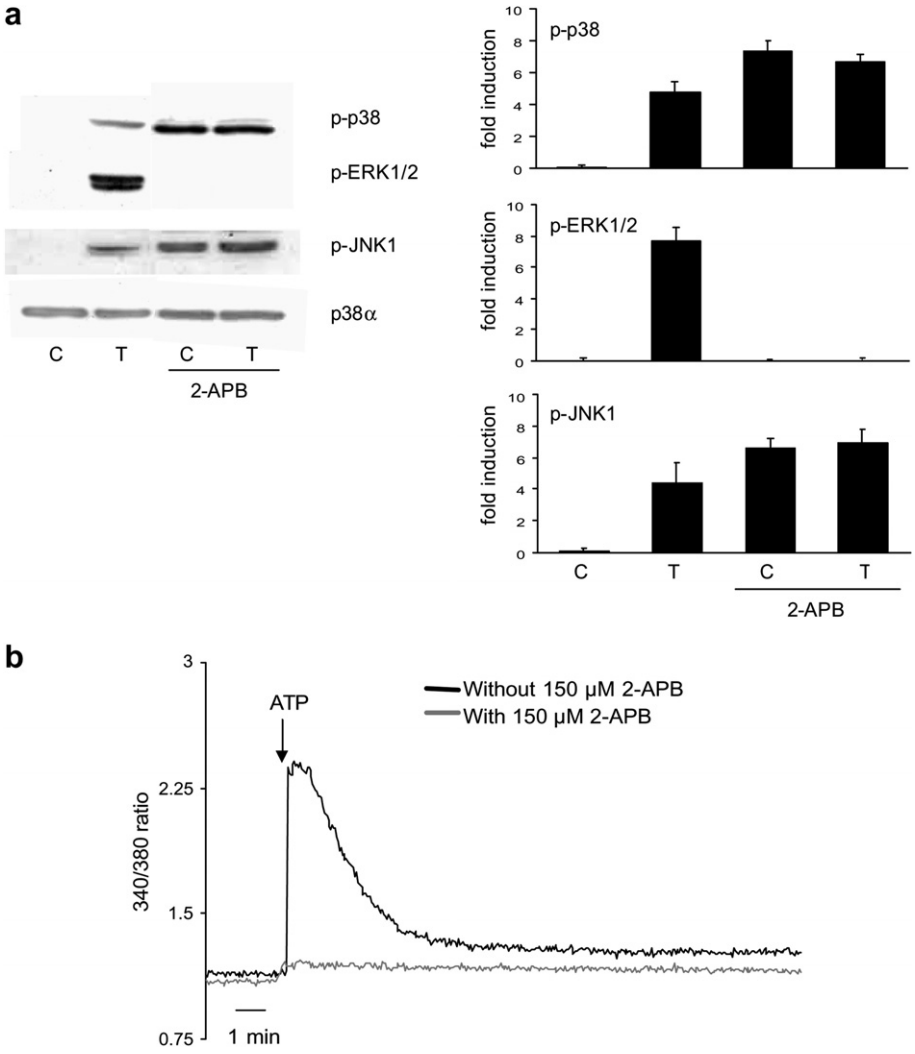

Fig. 8. Involvement of intracellular calcium release in the activation of MAPKs by ATP in MCF-7 cells. (a) Effect of 2-APB on ATP-dependent phosphorylation of ERK1/2, p38 and JNK1. MCF-7 cells were pre-incubated for 7 min in a calcium-free medium containing 0.5 mM EGTA, in the absence and presence of 150 μM 2-APB. Cells were then stimulated by the addition of either vehicle (C) or 5 μM ATP (T) for 5 min. Cell lysate proteins were immunoblotted with pERK1/2 and then the membranes stripped and reprobed with p-p38 and p-JNK1/2 phosphospecific antibodies as in Fig. 1, with anti-p38α as a loading control, respectively. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown. (b) Suppression of ATP-induced Ca^{2+} release by 2-APB. $[Ca^{2+}]_i$ was measured in Fura-2-loaded MCF-7 cells incubated in a Ca^{2+} free medium in the presence or absence of 150 μM 2-APB as described in Materials and methods. Cells were stimulated with 5 μM ATP as indicated by the arrows. The time trace shown is representative of at least five independent experiments onto no less than 40–50 cells.

duce it into signals, which lead to responses in cell proliferation, differentiation, survival and gene expression [31,32]. It has been demonstrated that mechanical stimuli activate a variety of molecules that participate in signal transduction, including ion channels, G-proteins, inositol trisphosphate, and many protein kinases [33,34], thus implying that multiple pathways may be involved in mechanical stress signal transduction. However, the initial sensor that recognizes the mechanical stress has not yet been identified. In vitro experiments have shown that mechanical stimulation increases $[Ca^{2+}]_i$, modulates MAPK cascades and gene expression in epithelial cells [35,36]. MAPK family members were identified as important signaling components linking mechanical stimuli with regulation of cell growth, differentiation, and metabolism in various cell types. In osteoblasts, we previously showed that ATP-induced ERK1/2 and p38 MAPK activation was coupled to ATP-dependent SAC influx. However, in MCF-7 cells, we found that gadolinium and lanthanum, which inhibited the ATP induced SAC influx; and EGTA, an extracellular Ca^{2+} chelator, did not alter ATP-dependent phosphorylation of ERK 1/2, JNK1 and p38 MAPK. Moreover, 2-APB, an IP_3 receptor antagonist, completely eliminated ATP-induced $[Ca^{2+}]_I$ release and phosphorylation of MAPKs, indicating that their activation by ATP in MCF-7 cells is mediated by a mechanism dependent on intracellular calcium release, but independent of calcium influx. The increase on p38 and JNK1 basal phosphorylation induced by 2-APB might be a consequence of some kind of cellular stress caused by the agent, as the MAPKs involved are

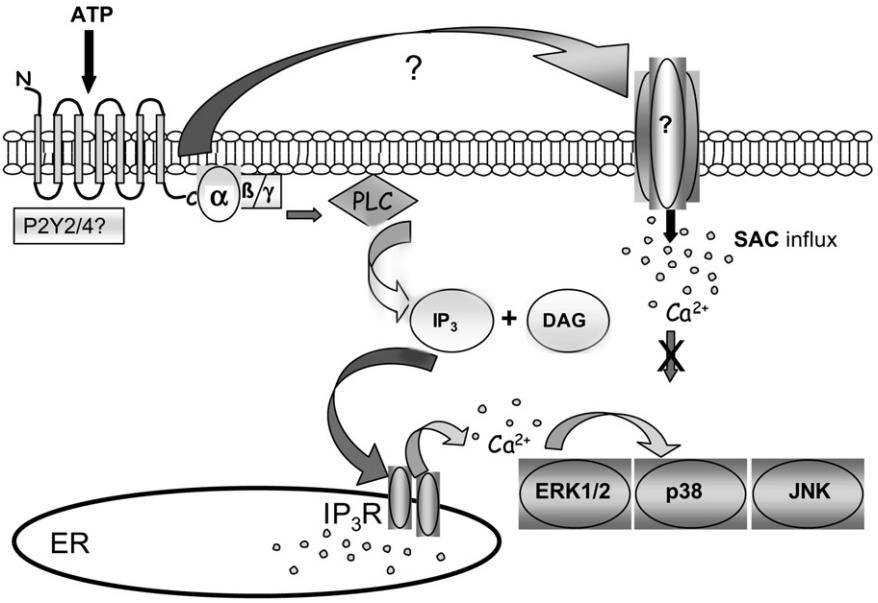

Fig. 9. Schematic model depicting ATP stimulation of MAPKs and stress activated calcium (SAC) influx in MCF-7 breast tumor cells. The $P2Y_2/_4$ purinergic receptor-mediated pathway by which ATP is assumed to modulate ERK 1/2, p38 and JNK 1/2 is shown. The "?" symbol denotes the as yet unknown mechanism of SAC influx activation by ATP.

stress sensitive. Additionally, we can assume that the effect observed is Ca^{2+} independent. On the other hand, given the complex effects of 2-APB, Ca^{2+} independent p38 and JNK1 phosphorylation by ATP can not be ruled out.

These observations raise questions on why SAC influx does not contribute to ATP-dependent MAPK activation in MCF-7 cells, and how do cells discriminate between the calcium message from inner stores and the extracellular space. Clearly, considering the potential non-selective effects of some agents employed, these questions should be addressed in future investigations by silencing specific molecular targets such us IP_3 receptors or SAC channels.

In conclusion, the results of the present study, summarized in Fig. 9, demonstrate that activation of $P2Y_2/P2Y_4$ subtype receptors by ATP sensitizes MCF-7 cells to mechanical stress leading to SAC influx. The data also indicate that MAPK ERK1/2, p38 and JNK1/2 phosphorylation induced by ATP is dependent on PI-PLC/IP_3/$[Ca^{2+}]_i$ release but independent of SAC influx in these breast tumor cells.

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References

[1] V. Ralevic, G. Burnstock, Pharmacol. Rev. 50 (1998) 413–492.
[2] D. Communi, R. Janssens, N. Suarez-Huerta, B. Robaye, J.M. Boeynaems, Cell. Signal. 12 (2000) 351–360.
[3] W.B. Bowler, C.J. Dixon, C. Halleux, R. Maier, G. Bilbe, W. Fraser, J. Gallagher, R. Hipskind, J. Biol. Chem. 274 (1999) 14315–14324.
[4] C. Dixon, W. Bowler, P. Fleetwood, A. Ginty, J. Gallagher, J. Carron, Br. J. Cancer 75 (1997) 34–39.
[5] T. Zarubin, J. Han, Cell Res. 15 (2005) 11–18.
[6] R.J. Santen, R.X. Song, R. McPherson, R. Kumar, L. Adam, M.H. Jeng, W. Yue, J. Steroid Biochem. Mol. Biol. 80 (2) (2002) 239–256.
[7] S. Hart, O.M. Fischer, N. Prenzel, E. Zwick-Wallasch, M. Schneider, L. Hennighausen, A. Ullrich, Biol. Chem. 386 (2005) 845–855.
[8] K. Liang, T. Qin, Z. Yang, Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 21 (2004) 1047–1050.
[9] M. Benjamin, B. Hillen, Eur. J. Morphol. 41 (2003) 3–7.
[10] L.N. Naemsch, S.J. Dixon, S.M. Sims, J. Biol. Chem. 276 (2001) 39107–39114.
[11] J.T. Neary, Y. Kang, K.A. Willoughby, E.F. Ellis, J. Neurosci. 23 (2003) 2348–2356.
[12] K. Furuya, K. Akita, M. Sokabe, Nippon Yakurigaku Zasshi 123 (2004) 397–402.
[13] S. Katz, R. Boland, G. Santillan, Int. J. Biochem. Cell Biol. 38 (2006) 2082–2091.
[14] M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[15] G. Santillán, C. Baldi, S. Katz, G. Vazquez, R. Boland, J. Steroid Biochem. Mol. Biol. 89/90 (2004) 291–295.
[16] G. Santillán, S. Katz, G. Vazquez, R. Boland, Int. J. Biochem. Cell Biol. 36 (2004) 1910–1918.
[17] G.W. Snedecor, W.G. Cochran, Statistical Methods, sixth ed., Ames: Iowa State University Press, Iowa, 1967.
[18] J.P. Hildebrandt, T.D. Plant, H. Meves, Br. J. Pharmacol. 120 (1997) 841–850.
[19] R.V. Sharma, M.W. Chapleau, G. Hajduczok, R.E. Wachtel, L.J. Waite, R.C. Bhalla, F.M. Abboud, Neuroscience 66 (2) (1995) 433–441.
[20] V.V. Chaban, J.A. McRoberts, H.S. Ennes, E.A. Mayer, Brain Res. 903 (2001) 74–85.
[21] H. Tinel, H.W. Denker, M. Thie, Mol. Hum. Reprod. 6 (12) (2000) 1119–1130.
[22] T. Maruyama, T. Kanaji, S. Nakade, T. Kanno, K. Mikoshiba, J. Biochem. 122 (3) (1997) 498–505.
[23] M. Prakriya, R. Lewis, J. Physiol. 536 (1) (2001) 3–19.
[24] M. Flezar, S. Heisler, J. Pharmacol. Exp. Ther. 265 (1993) 1499–1510.

[25] H. Kumagai, H. Sakamoto, S. Guggino, C.R. Filburn, B. Sacktor, Calcif. Tissue Int. 45 (1989) 251–254.
[26] K. Migita, L. Lu, Y. Zhao, K. Honda, T. Iwamoto, S. Kita, T. Katsuragi, Biochem. Biophys. Res. Commun. 328 (2005) 1211–1215.
[27] M. Oike, G. Droogmans, Y. Ito, Nippon Yakurigaku Zasshi 123 (6) (2004) 403–411.
[28] M.A. Arslan, O. Kutuk, H. Basaga, Curr. Cancer Drug Targets 6 (2006) 623–634.
[29] S.C. Wagstaff, W.B. Bowler, J.A. Gallagher, R.A. Hipskind, Carcinogenesis 21 (2000) 2175–2181.
[30] K. Sabapathy, K. Hochedlinger, S.Y. Nam, A. Bauer, M. Karin, E.F. Wagner, Mol. Cell 15 (2004) 713–725.
[31] J. Sanchez-Esteban, L.A. Cicchiello, Y. Wang, S.W. Tsai, L.K. Williams, J.S. Torday, L.P. Rubin, J. Appl. Physiol. 91 (2001) 589–595.
[32] S. Lehoux, Y. Castier, A. Tedgui, J. Int. Med. 259 (2006) 381–392.
[33] Y.S. Li, J.H. Haga, S. Chien, J. Biomech. 38 (10) (2005) 1949–1971.
[34] J. Rubin, C. Rubin, C.R. Jacobs, Gene 15 (367) (2006) 1–16.
[35] S. Yates, T.E. Rayner, Wound Repair Regen. 10 (2002) 5–15.
[36] S. Yano, M. Komine, M. Fujimoto, H. Okochi, K. Tamaki, J. Invest. Dermatol. 122 (2004) 783–790.