Actin filaments play a permissive role in the inhibition of store-operated Ca^{2+} entry by extracellular ATP in rat brown adipocytes

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Stimulation of P2 receptors with micromolar concentration of ATP evokes a transient increase in $[\text{Ca}^{2+}]_i$ (intracellular free Ca^{2+} concentration), primarily due to release of Ca^{2+} from intracellular stores; such stimulation also triggers almost complete suppression of thapsigargin-evoked sustained $[\text{Ca}^{2+}]_i$ increase mediated through a store-operated Ca^{2+} entry pathway in rat brown adipocytes. We investigated the role of cytoskeletal actin in the inhibitory effect of the extracellular ATP on store-operated Ca^{2+} entry, using fura 2 fluorescence for continuous measurement of $[\text{Ca}^{2+}]_i$, and using Alexa fluor 488-phalloidin staining of actin. Disassembly of actin networks by cytochalasin D (1 μM) or latrunculin A (3 μM) prevented the inhibitory effect of ATP (10 μM) on the thapsigargin (100 nM)-evoked store-operated Ca^{2+} entry, without changing the effect of ATP in increasing $[\text{Ca}^{2+}]_i$. In normal cells, bath application of ATP induced a

transient $[\text{Ca}^{2+}]_i$ increase, consisting of a rapid increase (the rising phase) and the subsequent decrease (the declining phase) to a lower steady level despite the continued presence of the agonist. Disruption of actin assemblies did not significantly affect the rising phase, but prevented the declining phase. Cells incubated with 10 μM ATP for 4 min demonstrated marked accumulations of actin filaments at the cell periphery, showing protrusions at the cell surface; this actin-assembly process is mediated through P2 receptors. In cells treated with cytochalasin D or latrunculin A, extracellular ATP did not induce actin redistribution. These results suggest that the actin reorganization plays a role in ATP-induced inhibition of store-operated Ca^{2+} entry in rat brown adipocytes.

Key words: actin cytoskeleton, brown adipocyte, extracellular ATP, P2 receptor, store-operated Ca^{2+} entry.

INTRODUCTION

Brown adipose tissue is known to be specialized for non-shivering thermogenesis in mammalian species, and is highly regulated by the sympathetic nervous system [1]. The release of neurotransmitter noradrenaline causes chronic cell differentiation and proliferation [2,3], in addition to acute heat production [1]. It has been recognized that ATP is co-released with noradrenaline from sympathetic nerve terminals, and that ATP functions as a neurotransmitter by binding to P2 receptors on the plasma membrane [4,5]. ATP can also be released from many other types of cells, including mast cells and fibroblasts, through autocrine/paracrine mechanisms mediated via P2 receptors associated with these and/or neighbouring cells [6]. P2 receptors have been classified into two major families based on their molecular structures, namely ionotropic P2X receptors comprising ligand-gated non-selective cation channels, and metabotropic P2Y receptors coupling with G-proteins [6]. In rat brown adipocytes, P2 receptors have been reported to increase cytosolic free Ca^{2+} [7–10] and to modulate proliferation [11] and membrane trafficking [12].

Stimulation of phospholipase C-coupled receptors leads to InsP_3 [inositol(1,4,5)-trisphosphate]-dependent rapid Ca^{2+} release from the endoplasmic reticulum, followed by a slow activation of Ca^{2+} influx across the plasma membrane, known as a store-operated Ca^{2+} entry [13–17]. Store-operated Ca^{2+} entry provides more sustained cytosolic Ca^{2+} increase, and appears to be the predominant Ca^{2+} entry pathway in non-excitable cells to refill the intracellular Ca^{2+} stores. Noradrenaline has been reported to induce InsP_3 -mediated Ca^{2+} release from the endoplasmic reticulum via α_1 -adrenoceptor stimulation, followed by an influx of extracellular Ca^{2+} through a store-operated pathway in brown adipocytes [7,8,18–20]. Store-operated Ca^{2+} entry is also activated by treating cells with an inhibitor of the endoplasmic reticulum Ca^{2+} pump, such as thapsigargin [21], which gradually depletes Ca^{2+} stores by specifically and irreversibly inhibiting Ca^{2+} uptake through the endoplasmic reticulum Ca^{2+} pump [7,8].

In the previous studies [7,8], we have demonstrated that extracellular ATP not only mobilized Ca^{2+} from the intracellular stores but also exerted a potent inhibitory effect on the store-operated Ca^{2+} entry process mediated through multiple subtypes of purinergic receptors in adult rat brown adipocytes. That is, PPADS (pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid)-resistant receptor(s) having high affinity for both ATP and UTP induce the mobilization of the intracellular Ca^{2+} stores, which is probably followed by the activation of store-operated Ca^{2+} entry, and PPADS-sensitive receptor(s) with high affinity for ATP (but not for UTP) inhibit this Ca^{2+} entry process.

The mechanism by which the state of filling of the stores is communicated to the plasma membrane is still unclear. A soluble messenger mechanism has been proposed, by which diffusible chemical messengers, released from the stores, travel to the plasma membrane [22,23]; however, no messenger molecule has been identified yet. Alternatively, a number of studies have been reported to propose a coupling mechanism involving a slowly activating secretion-like trafficking of the stores to the plasma membrane, followed by a direct or indirect association of these membranes [14,15,24–27]. There is increasing evidence indicating that the coupling process of these membranes is modulated by cytoskeletal actin, as in vascular endothelial CPAE

buffer; PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; InsP_3 , inositol(1,4,5)-trisphosphate; KRBH buffer, Krebs–Ringer bicarbonate/Hepes buffer; PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid.

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cells [28], smooth-muscle DDT₁MF-2 and A7r5 cells [26], human platelets [29], mouse pancreatic acinar cells [30], rat hepatocytes [31] and glioma C6 cells [32]. However, the precise role of cytoskeleton in the store-operated Ca²⁺ entry system has not been fully understood.

The aim of the present study was to examine the role of cytoskeletal actin in the ATP-induced inhibition of store-operated Ca²⁺ entry in brown adipocytes. Our results indicate that stimulation of P2 receptors induces accumulation of actin filaments exclusively at the cell periphery, which may play a role in the inhibition of store-operated Ca²⁺ entry in rat brown adipocytes.

EXPERIMENTAL

Materials

Male 3-week-old Sprague–Dawley rats were purchased from Charles River Japan (Yokohama, Japan) and fed *ad libitum* for at least 1 week before use. Fraction V BSA was purchased from Interger (Purchase, NY, U.S.A.), class 2 crude collagenase from Worthington Biochemical (Freehold, NJ, U.S.A.), DNase I from Roche Diagnostics (Indianapolis, IN, U.S.A.), fura 2 acetoxymethyl ester from Dojin Chemicals (Kumamoto, Japan) and Alexa fluor 488-phalloidin from Molecular Probes (Eugene, OR, U.S.A.). Suramin sodium salt and thapsigargin were obtained from Wako Pure Chemicals (Osaka, Japan). Latrunculin A and Y-27632 were from Calbiochem–Novabiochem (San Diego, CA, U.S.A.). ATP (disodium salt), cytochalasin D, PPADS, poly-(L-lysine) and valinomycin were from Sigma (St. Louis, MO, U.S.A.). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Cell isolation and primary culture

The method used for isolating brown adipocytes was described previously [7]. Briefly, rats aged 4–6 weeks were kept at 5 °C for 5–7 h with free access to food and water to deplete stored lipid in brown adipose tissues. The animals were then deeply anaesthetized by an overdose of sodium pentobarbital (50 mg/kg, intraperitoneal injection) and killed by decapitation. Interscapular brown adipose tissue was dissected out and placed in KRBH buffer (Krebs–Ringer bicarbonate/Hepes buffer), supplemented with 1 % (w/v) BSA under sterile conditions. KRBH buffer contained (mM) 120 NaCl, 4 KH₂PO₄, 2 CaCl₂, 1 MgSO₄, 10 NaHCO₃ and 30 Hepes (pH adjusted to 7.4 with NaOH). The tissue was minced and digested with a combination of collagenase and DNase I (7.5 and 0.5 mg/ml respectively). After digesting, the reaction mixture was filtered through 100 µm nylon mesh and washed by centrifugation in KRBH/BSA buffer and further in Dulbecco's modified Eagle's medium, supplemented with 10 % (w/v) foetal bovine serum, 100 µg/ml penicillin and 0.1 mg/ml streptomycin. After the final washing, the cells were seeded on glass coverslips precoated with 0.1 % poly(L-lysine). All procedures were performed in plastic containers. The cells were then maintained at 37 °C in a humidified atmosphere of 95 % O₂ and 5 % CO₂. The mature brown adipocytes cultured for 1–3 days were used in the experiments. All experiments were performed according to the guidelines laid down by the Shiga University of Medical Science Animal Care Committee.

Measurement of [Ca²⁺]_i (intracellular free Ca²⁺ concentration)

The [Ca²⁺]_i was continuously measured using the fluorescent Ca²⁺ indicator fura 2 from cells superfused with the standard solution consisting of KRBH buffer supplemented with 5.6 mM

glucose and 0.05 % BSA as described previously [7]. The cells were loaded with membrane-permeant fura 2 acetoxymethyl ester (5 µM) in the dark for 30 min at 37 °C and were washed twice in the standard solution. Fura 2-loaded cells were then incubated in a dye-free standard solution for 30 min at 37 °C and were transferred to the recording chamber mounted on the stage of an inverted microscope (Diaphot; Nikon, Tokyo, Japan). The recording chamber was maintained at 36 °C and was perfused continuously at a rate of 1.5 ml/min with the standard solution or test solutions. The continuous fluorescence measurement of [Ca²⁺]_i was performed using a microspectrofluorimeter (CAM-220; Japan Spectroscopic, Tokyo, Japan). The [Ca²⁺]_i was calculated from the fluorescence ratios acquired at 340 and 380 nm excitation wavelengths using the equation as described by Grynkiewicz et al. [33].

The fluorescence intensity increased with an increase in incubation time and became saturated after 30–60 min of incubation as described previously [7]. We checked the proportion of dye in the cytoplasmic space and organelles in fura 2-loaded cells using a calcium imaging system consisting of an inverted microscope (Olympus IX70; Olympus Optical, Tokyo, Japan) and a charge-coupled device camera with fitting equipment (Roper Industries, Duluth, GA, U.S.A.). Ratios of imaging data were acquired and analysed in the computer using MetaFluorTM-RIS software (Universal Imaging Corporation, Downingtown, PA, U.S.A.). Intracellular lipid droplets showed no fluorescent signals; nuclear regions were sometimes brighter and of lower resting [Ca²⁺]_i level than the rest of the cells, and [Ca²⁺]_i level at all regions of the cell increased approximately to the same level in response to the stimuli, thus suggesting that compartmentation of the dye did not interfere in the measurements and the cell had no unresponsive dye pools, as those described in neonatal rat brown adipocytes [18].

All data were stored on a digital audiotape using a PCM data recorder (RD-120TE; TEAC, Tokyo, Japan) and were then transferred to a magnetic optical disk through a DigiData 1200 interface (Axon Instruments, Foster City, CA, U.S.A.) for a later computer analysis using pCLAMP software (Axon Instruments). The resting [Ca²⁺]_i level was calculated from the average of continuous measurements for 1 min before the addition of the drugs. In the experiment using thapsigargin, the maximal level of [Ca²⁺]_i was calculated from the average measurement for a period of 10 s, starting when the [Ca²⁺]_i level stopped increasing. The [Ca²⁺]_i level at 2 min was calculated from the average of 4 s, starting 2 s before the 2 min mark. Results are expressed as means ± S.E.M. and *n* is the number of experiments. Statistical comparisons were made using Student's *t* test for paired or unpaired data where appropriate, and differences were considered to be significant at *P* < 0.05.

Staining of actin filaments and confocal laser-scanning microscopy

Cells cultured on the glass coverslips were transferred into 12-well plastic culture plates. The culture medium was replaced with the standard solution, and then the cells were incubated in each test solution at 37 °C for the desired periods. The cells were then rinsed twice with prewarmed PBS (pH 7.4), and were fixed with 3.7 % (w/v) formaldehyde in PBS for 10 min at room temperature (25 °C). The fixed cells were washed three times with PBS for 10 min and were permeabilized with 0.2 % Triton X-100 in PBS for 5 min. The cells were then washed three times with PBS for 10 min. To reduce non-specific background staining, the cells were incubated with 1 % (w/v) BSA in PBS for 30 min and were then stained with 0.33 µM Alexa fluor 488-phalloidin in PBS, supplemented with 1 % BSA for 20 min at room temperature.

After washing three times with PBS for 10 min, each coverslip was mounted on a glass slip with the cell side down in a 1:1 solution of PBS/glycerol. Fluorescent signals were observed with a confocal laser-scanning microscope (BioRad MRC-600UV) mounted on a Nikon Diaphot inverted microscope with a Nikon Fluor $\times 60$ oil immersion objective, 488 nm excitation filter and 510 nm emission filter. ATP-induced redistribution of actin filaments was defined as the acquisition of two or more discernible protrusions accumulating actin filament patches per cell.

Scanning-electron microscopy

Isolated cells were suspended in the standard solution and were incubated with or without 10 μM ATP at 37 $^{\circ}\text{C}$ for 4 min. The solution was then removed by centrifugation (100 g for 5 min) and the cells were rinsed with prewarmed PBS. The cells were pre-fixed with 2.5 % (w/v) glutaraldehyde in PBS for 30 min at 4 $^{\circ}\text{C}$ and were washed twice with PBS for 3 min. The cells were further fixed with 1 % osmium tetroxide in PBS for 30 min at 4 $^{\circ}\text{C}$ and were washed twice with PBS for 3 min. The cells were then dehydrated by immersion in a series of ethanol dilutions, exchanged by immersion in a series of 3-methylbutyl acetate, critical-point dried in CO_2 , and were covered with 20 nm of gold. The final samples were observed with a scanning electron microscope (Hitachi S-570).

RESULTS

Breakdown of actin cytoskeleton prevents ATP-induced decrease in the thapsigargin-evoked $[\text{Ca}^{2+}]_i$ increase

To investigate whether cytoskeletal actin is important for extracellular ATP-induced inhibition of the thapsigargin-evoked $[\text{Ca}^{2+}]_i$ increase in brown adipocytes, we incubated the cells with cytochalasin D, a widely utilized membrane-permeant inhibitor of actin polymerization [34]. Actin filaments were stained with Alexa fluor 488-labelled phalloidin [34] and then observed with a confocal laser-scanning microscope.

Figure 1(A) demonstrates that actin filaments displayed staining, which outlined the cell periphery in control cells. Phalloidin-stained actin filament patches were also detected in intracellular space. In other types of cells, such as in smooth-muscle cells [26] and in fibroblasts [35], phalloidin staining typically reveals a network of actin stress fibres assembled end-to-end on the plasma membrane. Such actin stress fibre structures were not observed in these brown adipocytes. After incubating the cells with 1 μM cytochalasin D for 60 min, actin cytoskeletal structure was completely disassembled, displaying punctate staining throughout the whole cell images (Figure 1B). The breakdown of cortical actin structures by cytochalasin D caused changes in cell morphology. We further experimented with latrunculin A, known to disrupt actin filament organization by forming a complex with actin monomers [36]. As demonstrated in Figure 1(C), the phalloidin staining was observed to be very faint in the cells incubated with 3 μM latrunculin A for 60 min, suggesting a very low level of organized actin filaments in these cells.

We then examined the $[\text{Ca}^{2+}]_i$ responses to thapsigargin and ATP in cells pretreated with these actin-modifying agents. In control cells, 100 nM thapsigargin induced a gradual increase in $[\text{Ca}^{2+}]_i$ with a mean amplitude of 1390 ± 160 nM ($n=8$) above a resting level of 68 ± 5 nM ($n=8$), and $[\text{Ca}^{2+}]_i$ remained stable at around that peak level (Figure 2A) in the presence of extracellular Ca^{2+} . As described in the previous study [7], the thapsigargin-evoked sustained $[\text{Ca}^{2+}]_i$ increase was irreversible and was entirely dependent on the presence of extracellular

Figure 1 Cytochalasin D and latrunculin A disassemble actin cytoskeletal structure

Confocal laser scanning microscopy of rat brown adipocytes stained with Alexa fluor 488-labelled phalloidin. Cells were incubated with the standard solution as control (A), 1 μM cytochalasin D (B) or 3 μM latrunculin A (C) for 60 min at 37 $^{\circ}\text{C}$ before the staining procedure described in the Experimental section. Lipid droplets accumulating in the cells are seen as dark circles. Results are representative of six experiments. Scale bar, 10 μm .

Ca^{2+} , indicating that store-operated Ca^{2+} entry was activated in response to the depletion of intracellular Ca^{2+} stores. As expected, 10 μM ATP applied subsequently in the continuous presence of thapsigargin attenuated the sustained $[\text{Ca}^{2+}]_i$ increase by $98 \pm 1\%$ ($n=8$), suggesting that extracellular ATP inhibits thapsigargin-evoked Ca^{2+} influx (Figure 2B). Although there were some individual differences, there often appeared to be a lag of 5–15 s after ATP application, before the inhibition of thapsigargin-evoked $[\text{Ca}^{2+}]_i$ increase.

Figure 2(C) illustrates that the resting $[\text{Ca}^{2+}]_i$ in the cells preincubated with 1 μM cytochalasin D for 60 min at 37 $^{\circ}\text{C}$ was 83 ± 8 nM ($n=8$), which was not statistically significantly higher ($P > 0.05$) than that of control cells, and bath application of thapsigargin evoked $[\text{Ca}^{2+}]_i$ to the high level with a mean amplitude of 1320 ± 190 nM ($n=8$), showing responses similar to those observed in control cells. However, subsequent application of ATP in the continued presence of thapsigargin reduced the increased $[\text{Ca}^{2+}]_i$ only by $24 \pm 6\%$ ($n=8$) in these cytochalasin D-pretreated cells (Figure 2C), whereas the same concentration of ATP reduced the increase by $98 \pm 1\%$ in control cells (Figure 2B).

Figure 2(D) illustrates the representative $[\text{Ca}^{2+}]_i$ responses of the cells pretreated with 3 μM latrunculin A for 60 min at 37 $^{\circ}\text{C}$. The resting $[\text{Ca}^{2+}]_i$ was 84 ± 9 nM ($n=8$) in latrunculin A-pretreated cells, which was not statistically significantly higher ($P > 0.05$) than that of control cells. Bath application of thapsigargin to these latrunculin A-pretreated cells induced an increase in $[\text{Ca}^{2+}]_i$ with a mean amplitude of 1340 ± 180 nM ($n=8$), similar to the pattern observed in control cells. However, 10 μM ATP applied subsequently in the continued presence of thapsigargin did not reduce the increased $[\text{Ca}^{2+}]_i$ (by $2 \pm 4\%$, $n=8$) in these latrunculin A-pretreated cells. These results suggest that disruption of the actin cytoskeleton does not

Figure 2 Cytochalasin D and latrunculin A block the inhibitory effect of ATP

Chart record of typical $[Ca^{2+}]_i$ responses to 100 nM thapsigargin (TG) and 10 μ M ATP in a control (A, B), cytochalasin D-treated (C) and latrunculin A-treated (D) cell as indicated by the horizontal bars. The experiments were performed in the presence of extracellular Ca^{2+} . (A, B) Cells were stimulated with (A) 100 nM thapsigargin or (B) 100 nM thapsigargin and subsequently with 10 μ M ATP in the continued presence of thapsigargin. (C, D) Cells were incubated with 1 μ M cytochalasin D (C) or 3 μ M latrunculin A (D) for 60 min at 37 °C and then loaded with fura 2, and $[Ca^{2+}]_i$ was measured in the continuous presence of each reagent. The trace is representative of eight experiments.

appreciably affect the signalling pathway by which thapsigargin-induced depletion of the intracellular Ca^{2+} stores leads to opening of the store-operated Ca^{2+} channels, but it prevents ATP-induced inhibition of store-operated Ca^{2+} entry.

Actin disruption affects the ATP-induced $[Ca^{2+}]_i$ increase

We next examined the effect of actin disruption on ATP-induced transient $[Ca^{2+}]_i$ increase in the presence of extracellular Ca^{2+} . Bath application of 10 μ M ATP evoked a rapid increase in $[Ca^{2+}]_i$ (rising phase) of 982 ± 76 nM ($n = 6$) above a resting level of 70 ± 5 nM ($n = 6$), and $[Ca^{2+}]_i$ decreased to a lower level (declining phase) of 80 ± 6 nM ($n = 6$) after 2 min exposure to the agonist, as typically illustrated in Figure 3(A). In the cells preincubated with 1 μ M cytochalasin D for 60 min at 37 °C, 10 μ M ATP evoked a rapid $[Ca^{2+}]_i$ increase of 875 ± 99 nM ($n = 6$) above a resting level of 83 ± 6 nM ($n = 6$) and then $[Ca^{2+}]_i$ decreased to 600 ± 44 nM ($n = 6$) above a resting level after 2 min exposure to ATP (Figure 3B). Figure 3(C) illustrates that 10 μ M ATP induced $[Ca^{2+}]_i$ increase with a peak amplitude of 822 ± 118 nM ($n = 6$) above a resting level of 84 ± 9 nM ($n = 6$) and then $[Ca^{2+}]_i$ decreased to 680 ± 90 nM ($n = 6$) after 2 min exposure to ATP in latrunculin A-preincubated cells (Figure 3C). The increase in resting $[Ca^{2+}]_i$ after preincubation with cytochalasin D or latrunculin A was not statistically significant ($P > 0.05$). $[Ca^{2+}]_i$ increases in response to ATP in those actin-disrupted cells were found to be mostly irreversible. The percentage inhibition of the declining phase of ATP-induced $[Ca^{2+}]_i$ responses, calculated from the decrease in $[Ca^{2+}]_i$ during 2 min exposure to ATP with

Figure 3 Effects of cytochalasin D and latrunculin A on ATP-induced $[Ca^{2+}]_i$ increase

Chart record of typical $[Ca^{2+}]_i$ responses to 10 μ M ATP in a control (A), cytochalasin D-treated (B) and latrunculin A-treated (C) cell as indicated by the horizontal bars. The experiments were performed in the presence of extracellular Ca^{2+} . (B, C) Cells were incubated with 1 μ M cytochalasin D (B) or 3 μ M latrunculin A (C) for 60 min at 37 °C and then loaded with fura 2, and $[Ca^{2+}]_i$ was measured in the continuous presence of each reagent. The trace is representative of six experiments.

reference to the peak amplitude of $[Ca^{2+}]_i$, was $92 \pm 1\%$ ($n = 6$) in control cells, $34 \pm 6\%$ ($n = 6$) in cytochalasin D-treated cells and $29 \pm 3\%$ ($n = 6$) in latrunculin A-treated cells. The results suggest that the declining phase of the ATP-induced transient $[Ca^{2+}]_i$ increase is sensitive to disassembly of the actin cytoskeletal structures, whereas the release of Ca^{2+} from the intracellular stores is not significantly affected.

Extracellular ATP promotes actin filament reorganization and cell-shape change

One possible mechanism by which ATP decreases the $[Ca^{2+}]_i$ level is through cytoskeletal organization in these brown adipocytes. To address this issue, we examined the effect of ATP on the actin cytoskeletal structures. Confocal laser-scanning microscopic analysis revealed that the actin filaments underwent a clear change in distribution after the cells were incubated with 10 μ M ATP for 4 min at 37 °C, displaying peripheral accumulation and protrusions with actin bundles formed on the surface of the cells (Figures 4A and 4B). The extracellular ATP-induced acquisition of two or more protrusions accumulating actin bundles was observed in almost all cells (188/194 cells). Another P2 receptor agonist, UTP, did not induce such a change in the distribution of actin filaments in brown adipocytes (results not shown). We next performed scanning-electron-microscopic analyses to investigate the morphological changes induced by extracellular ATP in isolated cells. Figure 4(C) demonstrates that the isolated brown adipocytes appeared round. Incubation of the cells with 10 μ M ATP for 4 min at 37 °C markedly altered the cell shape, showing a polygonal morphology with numerous angles (Figure 4D). The results thus suggest that extracellular ATP promotes actin repolymerization exclusively at the cell periphery, resulting

Figure 4 Extracellular ATP redistributes actin filaments and alters the cell shape

Confocal laser scanning microscopy of rat brown adipocytes stained with Alexa fluor 488-phalloidin (**A, B**). Cells were incubated with the standard solution as control (**A**) or $10 \mu\text{M}$ ATP (**B**) for 4 min at 37°C before staining (procedure described in the Experimental section). Results are representative of six experiments. Scale bar, $10 \mu\text{m}$. Scanning electron microscopy of isolated rat brown adipocytes (**C, D**). Cells were incubated with the standard solution as control (**C**) or $10 \mu\text{M}$ ATP (**D**) for 4 min at 37°C before the fixation procedure described in the Experimental section. Results are representative of four experiments. Scale bar, $10 \mu\text{m}$.

in the alteration of cell shape. Prolonged washing of the ATP-stimulated cells with the standard solution at 37°C (20 min) slowly reversed both the distribution of actin filaments and the cell shapes (results not shown).

To determine whether ATP-induced changes in the distribution of actin filaments are mediated through P2 receptors, the effects of P2 receptor antagonists PPADS and suramin [6] were examined. The structure of actin filaments in the cells incubated with $10 \mu\text{M}$ PPADS (Figures 5B–5D) or $100 \mu\text{M}$ suramin (Figures 5E–5G) for 6 min at 37°C demonstrated staining patterns similar to those appearing in control cells (Figure 5A). ATP ($10 \mu\text{M}$) did not induce cortical accumulation of actin filaments or protrusions in the presence of $10 \mu\text{M}$ PPADS (78/78 cells, Figures 5H–5J). As demonstrated in Figures 5(K)–5(M), the effect of ATP was also well prevented in most of the suramin-preincubated cells (122/132 cells), although small patches of actin filaments in the plasma membrane were sometimes observed under this condition (10/132 cells). These results suggest that extracellular ATP triggers the rearrangement of actin filaments by binding to a P2 receptor in rat brown adipocytes.

The question then arises as to whether extracellular ATP rearranges actin filaments during the inhibition of store-operated Ca^{2+} inflow by ATP. To address this issue, we investigated the

Figure 5 Suramin and PPADS block the ATP-induced actin reorganization

Confocal laser scanning microscopy of rat brown adipocytes stained with Alexa fluor 488-phalloidin. Cells were incubated with the standard solution for 6 min as control (**A**), $10 \mu\text{M}$ PPADS for 6 min (**B–D**), $100 \mu\text{M}$ suramin for 6 min (**E–G**), $10 \mu\text{M}$ PPADS for 2 min followed by 4 min with $10 \mu\text{M}$ ATP in the continued presence of PPADS (**H–J**) or $100 \mu\text{M}$ suramin for 2 min followed by 4 min with $10 \mu\text{M}$ ATP in the continued presence of suramin (**K–M**) at 37°C before the staining procedure. Results are representative of six experiments. Scale bar, $10 \mu\text{m}$.

ATP-induced changes in the distribution of actin filaments in the presence of thapsigargin; 100 nM thapsigargin alone did not appreciably affect the actin cytoskeletal structure (Figure 6A) compared with control cells (Figure 6I). As demonstrated in Figures 6(B)–6(D), $10 \mu\text{M}$ ATP markedly redistributed actin filaments at the cell periphery displaying two or more protrusions in thapsigargin-preincubated cells (90/94 cells), suggesting that the extracellular ATP reorganizes actin filaments when the inhibition of store-operated Ca^{2+} entry is activated.

We then examined the effects of actin polymerization inhibitors on the ATP-induced actin cytoskeletal reorganizations; $10 \mu\text{M}$ ATP did not induce the peripheral accumulation of actin filaments in cytochalasin D- (Figures 6E–6G) or latrunculin A-pretreated cells (Figure 6H). This suggests that the external ATP could not further repolymerize actin protein in those actin-disassembled cells.

Lee and Pappone [9] reported that extracellular ATP activates a non-selective cation conductance in rat neonatal brown adipocytes. In a previous study [7], we demonstrated that the inhibitory

Figure 6 Effects of thapsigargin, cytochalasin D and latrunculin A on ATP-induced actin reorganization

Confocal laser scanning microscopy of rat brown adipocytes stained with Alexa fluor 488-phalloidin. Cells were incubated with 100 nM thapsigargin for 9 min (A), 100 nM thapsigargin for 5 min followed by 4 min with 10 μ M ATP in the continued presence of thapsigargin (B–D), 1 μ M cytochalasin D for 60 min followed by 4 min with 10 μ M ATP in the continued presence of cytochalasin D (E–G) or 3 μ M latrunculin A for 60 min followed by 4 min with 10 μ M ATP in the continued presence of latrunculin A (H) at 37 °C before the staining procedure. As control, cells were incubated with the standard solution for 9 min (I) or 64 min (J) at 37 °C before the staining procedure. Results are representative of six experiments. Scale bar, 10 μ m.

Figure 7 Effects of valinomycin on ATP-induced actin reorganization

Confocal laser scanning microscopy of rat brown adipocytes stained with Alexa fluor 488-phalloidin. Cells were incubated with the standard solution for 10 min as control (A), 1 μ M valinomycin for 10 min (B, C) or 1 μ M valinomycin for 10 min followed by 4 min with 10 μ M ATP in the continued presence of valinomycin (D, E) at 37 °C before the staining procedure. Results are representative of four experiments. Scale bar, 10 μ m.

action of ATP in rat adult brown adipocytes is not appreciably affected by the K^+ ionophore valinomycin, which is expected to clamp the cell membrane near the reverse potential of K^+ . We then investigated whether ATP-induced redistribution of actin filaments is due to membrane depolarization. Incubating the cells with 1 μ M valinomycin for 10 min (Figures 7B and 7C) showed phalloidin-staining images similar to those that appeared in control cells (Figure 7A). Figures 7(D) and 7(E) typically demonstrate that 10 μ M ATP induced a redistribution of actin filaments with two or more protrusions in the presence of valinomycin (63/65 cells). The result that the reorganization of actin filaments by extracellular ATP was not inhibited by the stabilization of cell-membrane potential with valinomycin suggests that depolarization of the cell membrane is not involved.

These results suggest a possible correlation between the ATP-induced peripheral accumulation of actin filaments and the inhibition of store-operated Ca^{2+} entry. A crucial issue is whether intracellular Ca^{2+} stores, in smooth endoplasmic reticulum, are located relatively near the plasma membrane. To address this question, we first performed a transmission electron microscopic

Figure 8 Effect of Y-27632 on ATP-induced actin reorganization

Confocal laser scanning microscopy of rat brown adipocytes stained with Alexa fluor 488-phalloidin. Cells were incubated with the standard solution for 30 min as control (A), 10 μ M Y-27632 for 30 min (B, C) or 10 μ M Y-27632 for 30 min followed by 4 min with 10 μ M ATP in the continued presence of Y-27632 (D, E) at 37 °C before the staining procedure. Results are representative of four experiments. Scale bar, 10 μ m.

study (Hitachi H-7500) [39,40]. We found that mature brown adipocytes were characterized by a central nucleus and by the presence of numerous mitochondria occupying almost all the cytoplasmic space between lipid droplets (results not shown), consistent with the previous reports [40,41].

It has generally been recognized that low-molecular-mass G-proteins (small G-proteins) of the Rho families act as a molecular switch to regulate a signal transduction pathway that links membrane receptors coupling with heterotrimeric G-proteins to the actin cytoskeleton [42]. We next investigated whether ATP-induced redistribution of actin filaments is mediated through the activation of Rho, using a widely utilized selective Rho kinase inhibitor, Y-27632 [43]. Incubating the cells with 10 μ M Y-27632 for 30 min (Figures 8B and 8C) showed less peripheral phalloidin staining than appearing in control cells (Figure 8A), indicating

that Rho kinase activity contributes to the maintenance of basic actin cytoskeletal structure in these cells. ATP (10 μM) appeared to redistribute actin filaments at the cell periphery in Y-27632-pretreated cells without showing protrusions in most of the cells (60/69 cells, Figures 8D and 8E), and only one or two small protrusions were observed in a small number of cells (5/69 cells; results not shown). These results suggest that reorganization of actin filaments by extracellular ATP is coupled with the activation of Rho kinase in rat brown adipocytes.

DISCUSSION

In the present study, we provide evidence that extracellular ATP in micromolar concentrations markedly redistributes actin filaments towards the plasma membrane, mediated through P2 receptors in rat brown adipocytes. Both ATP-induced inhibition of the thapsigargin-evoked sustained $[\text{Ca}^{2+}]_i$ increase and the declining phase of the ATP-induced transient increase in $[\text{Ca}^{2+}]_i$ are sensitive to the disassembly of actin filaments, suggesting that actin cytoskeletal reorganization may play a role in the inhibitory effect of ATP on the store-operated Ca^{2+} entry process.

In addition to supplying a structural framework for cell shape and polarity, the dynamic properties of cytoskeletal actin provide important signals for diverse cellular functions in all eukaryotic cells, including cell moving and dividing. The nature of the distribution of actin filaments varies depending on cell types. Spindle- or rod-shaped cultured cells, such as fibroblasts [35] and smooth-muscle cells [26], usually display stress fibres running through the cytoplasm constructing the cell frame. In brown adipocytes, actin filaments were primarily found beneath the plasma membrane, supposed to be supporting the round cell shape (Figure 4C). Cytoskeletal actin underwent significant changes in distribution with extracellular ATP, displaying peripheral actin accumulation and protrusions resulting in morphological alteration (Figures 4B and 4D).

The precise role of cytoskeletal actin in the activation of store-operated Ca^{2+} entry is still unclear. Although a majority of the studies on cytoskeletal regulation have relied on the use of actin-filament-modifying toxins, such as actin polymerization inhibitors cytochalasin D and latrunculin A, their effects on store-operated Ca^{2+} entry have tended to vary between cells [28–32,35,44]. Patterson et al. [26] demonstrated that jasplakinolide- or calyculin A-induced cortical actin layer and not the disruption of actin network prevents store-operated Ca^{2+} entry by displacing the Ca^{2+} stores from the plasma membrane, and concluded that cytoskeletal actin does not play a role in driving or stabilizing the coupling process. Jasplakinolide and calyculin A have been widely utilized as inhibitors of store-operated Ca^{2+} entry [26,30,37,38]. However, these agents neither induce peripheral actin accumulation nor block the thapsigargin-evoked Ca^{2+} influx in brown adipocytes (results not shown). One possible reason is that sensitivity to these agents varies depending on the cell type.

Breakdown of actin cytoskeletal structures did not interfere with the P2-receptor-mediated signalling pathway of InsP_3 , originating from the plasma membrane [6], to the Ca^{2+} -releasing channels in the endoplasmic reticulum (Figure 3). Transmission of the subsequent message of 'store depletion', derived from the endoplasmic reticulum, to the plasma membrane, expected to activate the store-operated Ca^{2+} entry channel, was also intact in these actin-disassembled cells (Figure 2). Most importantly, actin disassembly prevented the inhibitory effect of ATP on the thapsigargin-evoked sustained $[\text{Ca}^{2+}]_i$ increase and the declining phase of the ATP-induced transient $[\text{Ca}^{2+}]_i$ increase (Figures 2 and 3). These observations suggest that the ATP-induced cortical accumulation

of actin filaments may be involved in the inhibition of the store-operated Ca^{2+} entry, possibly by impairing the association of these two membranes, which may resemble the mechanism of jasplakinolide- or calyculin A-induced inhibition described in smooth-muscle cell lines [26]. Further research is needed to observe the Ca^{2+} stores in endoplasmic reticulum, in relation to the plasma membrane and the changes in cytoskeletal actin.

Recently, Sauzeau et al. [45] reported that stimulation of G-protein-coupled P2Y receptor subtypes P2Y_1 , P2Y_2 , P2Y_4 and P2Y_6 activates small GTPase RhoA and, subsequently, Rho-dependent signalling pathways, forming actin stress fibres in serum-starved rat aortic smooth-muscle cells. Serum starvation is one technique to verify the assembly and organization of actin filaments by creating a very low level of organized actin filament structure [42,45]. In the present experiments, the cells did not undergo serum-starvation conditions. The stimulation of P2 receptors induced the formation of protrusions on the surface membrane, resulting in cell shape changes (Figure 4), indicating membrane ruffling in these cells. It has generally been accepted that the activation of Rac, another small G-protein of the Rho subfamilies, regulates a signalling pathway linking membrane receptors to the ruffling [42]. Y-27632 is a Rho kinase inhibitor, which inhibits Rho-induced formation of stress fibres but does not prevent Rac-induced membrane ruffling [43]. ATP-induced formation of protrusions was prevented by Y-27632 (Figure 8), suggesting that the activation of Rho kinase plays a role in the P2-receptor-mediated reorganization of actin filaments. It is still possible that P2 receptors are further interacting with other Rho subfamilies, such as Rac and Cdc42, in these cells. Further experiments are necessary to explore whether the existence of dense actin filament structures near the plasma membrane, or the process of the repolymerization of actin filaments, is important for the inhibition of store-operated Ca^{2+} entry in rat brown adipocytes.

We have previously demonstrated the expression of P2X_1 , P2X_3 , P2X_4 , P2X_5 and P2X_7 receptor subtype genes, in addition to P2Y_1 , P2Y_2 , P2Y_4 and P2Y_6 receptor subtype genes, by reverse transcriptase-PCR analysis in rat brown adipocytes [8]. It has been reported that P2X-receptor-mediated membrane depolarization caused an inhibition of P2Y-receptor-mediated store-operated Ca^{2+} entry in human microglia [46]. In brown adipocytes, the K^+ ionophore valinomycin did not affect the ATP-induced inhibition of store-operated Ca^{2+} entry [7] or cortical accumulation of actin filaments (Figure 7), thus ruling out the possible membrane-potential changes in these brown adipocytes. On the other hand, it has been reported that P2X receptor stimulation increases $[\text{Ca}^{2+}]_i$ and actin cytoskeletal disaggregation in rat mammary tumour WRK-1 cells [47]. It also needs to be clarified whether ATP binding to the ionotropic P2X receptor contributes to the actin remodelling inhibitory action of store-operated Ca^{2+} entry.

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REFERENCES

- Nicholis, D. G. and Locke, R. M. (1984) Thermogenic mechanisms in brown fat. *Physiol. Rev.* **64**, 1–64
- Géloën, A., Collet, A. J. and Bukowiecki, L. J. (1992) Role of sympathetic innervation in brown adipocyte proliferation. *Am. J. Physiol.* **263**, R1176–R1181
- Bukowiecki, L. J., Géloën, A. and Collet, A. J. (1986) Proliferation and differentiation of brown adipocytes from interstitial cells during cold acclimation. *Am. J. Physiol.* **250**, C880–C887

- 4 Burnstock, G. (1990) Noradrenaline and AIP as cotransmitters in sympathetic nerves. *Neurochem. Int.* **17**, 357–368
- 5 Harden, T. K., Boyer, J. L. and Nicholas, R. A. (1995) P₂-purinergic receptors: subtype-associated signaling responses and structure. *Annu. Rev. Pharmacol. Toxicol.* **35**, 541–579
- 6 Ralevic, V. and Burnstock, G. (1998) Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50**, 413–492
- 7 Omatsu-Kanbe, M. and Matsuura, H. (1999) Inhibition of store-operated Ca²⁺ entry by extracellular ATP in rat brown adipocytes. *J. Physiol. (Cambridge, U.K.)* **521**, 601–615
- 8 Omatsu-Kanbe, M., Isono, T. and Matsuura, H. (2002) Multiple P2 receptors contribute to a transient increase in intracellular Ca²⁺ concentration in ATP-stimulated rat brown adipocytes. *Exp. Physiol.* **87**, 643–652
- 9 Lee, S. C. and Pappone, P. A. (1997) Effects of P₂ purinergic receptor stimulation in brown adipocytes. *Am. J. Physiol.* **273**, C679–C686
- 10 Leaver, E. V. and Pappone, P. A. (2002) β -Adrenergic potentiation of endoplasmic reticulum Ca²⁺ release in brown fat cells. *Am. J. Physiol.* **282**, C1016–C1024
- 11 Wilson, S. M., Barsoum, M. J., Wilson, B. W. and Pappone, P. A. (1999) Purine nucleotides modulate proliferation of brown fat preadipocytes. *Cell Prolif.* **32**, 131–140
- 12 Pappone, P. A. and Lee, S. C. (1996) Purinergic receptor stimulation increases membrane trafficking in brown adipocytes. *J. Gen. Physiol.* **108**, 393–404
- 13 Putney, Jr, J. W. (1986) A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1–12
- 14 Berridge, M. J. (1995) Capacitative calcium entry. *Biochem. J.* **312**, 1–11
- 15 Parekh, A. B. and Penner, R. (1997) Store depletion and calcium influx. *Physiol. Rev.* **77**, 901–930
- 16 Putney, Jr, J. W. and McKay, R. R. (1999) Capacitative calcium entry channels. *Bioessays* **21**, 38–46
- 17 Elliott, A. C. (2001) Recent developments in non-excitable cell calcium entry. *Cell Calcium* **30**, 73–93
- 18 Lee, S. C., Nuccitelli, R. and Pappone, P. A. (1993) Adrenergically activated Ca²⁺ increases in brown fat cells: effect of Ca²⁺, K⁺, and K channel block. *Am. J. Physiol.* **264**, C217–C228
- 19 Nanberg, E. and Putney, Jr, J. W. (1986) α_1 -Adrenergic activation of brown adipocytes leads to an increased formation of inositol triphosphates. *FEBS Lett.* **195**, 319–322
- 20 Schimmel, R. J., Dzierzanowski, D., Elliott, M. E. and Honeyman, T. W. (1986) Stimulation of phosphoinositide metabolism in hamster brown adipocytes exposed to α_1 -adrenergic agents and its inhibition with phorbol esters. *Biochem. J.* **236**, 757–764
- 21 Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca²⁺ stores by specific inhibition of the endoplasmic reticulum Ca²⁺-ATPase. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- 22 Randriamanpitia, C. and Tsien, R. Y. (1993) Emptying of intracellular Ca²⁺ stores releases a novel small messenger that stimulates Ca²⁺ influx. *Nature (London)* **364**, 809–814
- 23 Csutora, P., Su, Z., Kim, H. Y., Bugrim, A., Cunningham, K. W., Nuccitelli, R., Keizer, J. E., Hanley, M. R., Blalock, J. E. and Marchase, R. B. (1999) Calcium influx factor is synthesized by yeast and mammalian cells depleted of organellar calcium stores. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 121–126
- 24 Irvine, R. F. (1990) 'Quantal' Ca²⁺ release and control of Ca²⁺ entry by inositol phosphates – a possible mechanism. *FEBS Lett.* **263**, 5–9
- 25 Kiselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L. and Muallem, S. (1998) Functional interaction between InSP3 receptors and store-operated Htrp3 channels. *Nature (London)* **396**, 478–482
- 26 Patterson, R. L., van Rossum, D. B. and Gill, D. L. (1999) Store-operated Ca²⁺ entry: evidence for a secretion-like coupling model. *Cell (Cambridge, Mass.)* **98**, 487–499
- 27 Yao, Y., Ferrer-Montiel, A. V., Montal, M. and Tsien, R. Y. (1999) Activation of store-operated Ca²⁺ current in *Xenopus* oocytes requires SNAP-25 but not a diffusible messenger. *Cell (Cambridge, Mass.)* **98**, 475–485
- 28 Holda, J. R. and Blatter, L. A. (1997) Capacitative calcium entry is inhibited in vascular endothelial cells by disruption of cytoskeletal microfilaments. *FEBS Lett.* **403**, 191–196
- 29 Rosado, J. A. and Sage, S. O. (2000) The actin cytoskeleton in store-mediated calcium entry. *J. Physiol. (Cambridge, U.K.)* **526**, 221–229
- 30 Redondo, P. C., Lajas, A. I., Salido, G. M., Gonzalez, A., Rosado, J. A. and Pariente, J. A. (2003) Evidence for secretion-like coupling involving pp60^{src} in the activation and maintenance of store-mediated Ca²⁺ entry in mouse pancreatic acinar cells. *Biochem. J.* **370**, 255–263
- 31 Wang, Y.-J., Gregory, R. B. and Barritt, G. J. (2002) Maintenance of the filamentous actin cytoskeleton is necessary for the activation of store-operated Ca²⁺ channels, but not other types of plasma-membrane Ca²⁺ channels, in rat hepatocytes. *Biochem. J.* **363**, 117–126
- 32 Sabala, P., Targos, B., Caravelli, A., Czajkowski, R., Lim, D., Gragnaniello, G., Santella, L. and Baranska, J. (2002) Role of the actin cytoskeleton in store-operated calcium entry in glioma C6 cells. *Biochem. Biophys. Res. Commun.* **296**, 484–491
- 33 Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440–3450
- 34 Cooper, J. A. (1987) Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105**, 1473–1478
- 35 Ribeiro, C. M. P., Reece, J. and Putney, Jr, J. W. (1997) Role of the cytoskeleton in calcium signaling in NIH 3T3 cells. *J. Biol. Chem.* **272**, 26555–26561
- 36 Spector, I., Schochet, N. R., Kashman, Y. and Groweiss, A. (1983) Latrunculin: novel marine toxins that disrupt microfilament organization in cultured cells. *Science* **219**, 493–495
- 37 Bubb, M. R., Senderowicz, A. M. J., Sausville, E. A., Duncan, K. L. K. and Korn, E. D. (1994) Jaspilkinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* **269**, 14869–14871
- 38 Ma, H.-T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K. and Gill, D. L. (2000) Requirement of the inositol triphosphate receptor for activation of store-operated Ca²⁺ channels. *Science* **287**, 1647–1651
- 39 Kurokawa, K., Yamada, H. and Ochi, J. (1997) Topographical distribution of neurons containing endothelin type A receptor in the rat brain. *J. Comp. Neurol.* **389**, 348–360
- 40 Gölén, A., Collet, A. J., Guay, G. and Bukowiecki, L. J. (1990) *In vivo* differentiation of brown adipocytes in adult mice: an electron microscopic study. *Am. J. Anat.* **188**, 366–372
- 41 Nèchad, M. (1983) Development of brown fat cells in monolayer culture II. Ultrastructural characterization of precursors, differentiation adipocytes and their mitochondria. *Exp. Cell Res.* **149**, 119–127
- 42 Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279**, 509–514
- 43 Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. et al. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature (London)* **389**, 990–994
- 44 Sergeeva, M., Ubl, J. J. and Reiser, G. (2000) Disruption of actin cytoskeleton in cultured rat astrocytes suppresses ATP- and bradykinin-induced [Ca²⁺]_i oscillations by reducing the coupling efficiency between Ca²⁺ release, capacitative Ca²⁺ entry, and store refilling. *Neuroscience* **97**, 765–769
- 45 Sauzeau, V., Jeune, H. L., Cario-Toumaniantz, C., Vaillant, N., Gadeau, A.-P., Desgranges, C., Scalbert, E., Chardin, P., Pacaud, P. and Loirand, G. (2000) P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptors are coupled to Rho and Rho kinase activation in vascular myocytes. *Am. J. Physiol.* **278**, H1751–H1761
- 46 Wang, X., Kim, S. U., van Breemen, C. and McLarnon, J. G. (2000) Activation of purinergic P2X receptors inhibits P2Y-mediated Ca²⁺ influx in human microglia. *Cell Calcium* **27**, 205–212
- 47 Pubill, D., Dayanithi, G., Siatka, C., Andrés, M., Dufour, M.-N., Guillon, G. and Mendre, C. (2001) ATP induces intracellular calcium increases and actin cytoskeleton disaggregation via P2X receptors. *Cell Calcium* **29**, 299–300

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