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Tyrphostin inhibition of ATP-stimulated DNA synthesis, cell proliferation and Fos-protein expression in vascular smooth muscle cells

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1 We and others have shown that extracellular ATP (adenosine triphosphate), released from sympathetic nerves and platelets, stimulates growth of vascular smooth muscle cells (SMC). To study the importance of tyrosine kinases for ATP-mediated proliferation in vascular smooth muscle cells we used tyrphostins, a recently developed group of highly specific inhibitors of tyrosine kinases.

2 ATP induced a powerful concentration-dependent increase in DNA synthesis measured by [^{3}H]-thymidine incorporation in rat aorta SMC (RASMC) and an increase in total cell number after 72 h of incubation as measured by an enzymatic cell proliferation assay. Tyrphostin 25 (10^{-5} M) had no effect *per se* on basal DNA synthesis but reduced ATP-stimulated DNA synthesis and increase in cell number in a dose-dependent manner. Higher concentrations of ATP could not reverse the inhibitory effect of tyrphostin 25. The potency of several (six) other tyrphostins was also examined and found to be slightly greater than tyrphostin 25 with equal efficacy.

3 When RASMC were incubated with 10^{-5} M ATP for 2 h, nearly all of the cells ($87 \pm 5\%$) were intensely stained with an antibody to the Fos protein while in the controls only $1 \pm 2\%$ of the cells were weakly stained. Tyrphostin 25 greatly reduced the Fos-protein staining ($14 \pm 2\%$).

4 ATP induced a concentration-dependent increase in $^{45}Ca^{2+}$-influx and formation of inositol phosphates (IP_{total}) in RASMC. These effects were not inhibited by tyrphostin 25.

5 Tyrphostin 25 did not alter ATP-induced contraction in ring segments of rat aorta.

6 In conclusion, tyrphostin 25 inhibited ATP-induced DNA synthesis, cell proliferation and Fos-protein expression, but not ATP-induced $^{45}Ca^{2+}$-influx, inositolphosphate-production or vasoconstriction. This indicates that the mitogenic effect of ATP on vascular smooth muscle cells is dependent on tyrosine kinases in contrast to the contractile effect of ATP in blood vessels.

Keywords: ATP; Fos-protein; mitogen; smooth muscle cell; tyrosine kinase; tyrphostin; vasoconstriction

Introduction

Intracellular adenosine triphosphate (ATP) plays a fundamental role in nucleic acid synthesis, energy metabolism and enzyme regulation. Over the last two decades evidence has demonstrated that *extracellular* ATP can act as a transmitter via membrane bound receptors with biological actions in many tissues and cells. In the vascular system, ATP mediates constriction through direct stimulation of vascular smooth muscle cells or vasodilatation via endothelium-dependent relaxation (Burnstock, 1990). In addition, we and others have recently shown that extracellular ATP is a potent mitogen for vascular smooth muscle cells from rat (Erlinge *et al.*, 1993), pig (Wang *et al.*, 1992) and man (Erlinge *et al.*, 1994). ATP is also mitogenic for other cell types, e.g. astrocytes and PC12 cells (Rathbone *et al.*, 1992). Furthermore, extracellular ATP induces immediate early genes associated with the initiation of cell division, in vascular smooth muscle cells (Malam-Souley *et al.*, 1993).

Growth factors can be divided into at least two classes that act through different signal transduction pathways. One class constitutes the family of polypeptide growth factors that bind to receptors with intrinsic tyrosine kinase activity, the prototype for this is epidermal growth factor (EGF) (Carpenter, 1987). Another class of mitogen is the 'Ca^{2+}-mobilizing' mitogens such as bombesin, substance P, angiotensin II, endothelin, neuropeptide Y and 5-hydroxytryptamine. Receptor characterization indicates that the mitogenic effect of ATP is mediated by a G-protein coupled nucleotide-receptor similar to the receptor linked to inositol phosphate formation and $^{45}Ca^{2+}$-influx in rat aorta smooth muscle cells (Erlinge *et al.*, 1993; 1995).

Protein tyrosine kinases have a key role in cell division and an enhanced protein tyrosine kinase activity has been associated with proliferative disorders such as atherosclerosis (Ross, 1989). Recently, a new group of inhibitors called tyrphostins have been found to block phosphorylation of tyrosine residues (Yaish *et al.*, 1988; Gazit *et al.*, 1989; 1991). Tyrphostins have little or no effect on other protein kinases or other signal transduction systems (Levitzki & Gilon, 1991), which explains the low cytotoxicity of tyrphostins in cell cultures (Levitzki & Gilon, 1991). Tyrphostins have been shown to inhibit both membrane-bound (e.g. intrinsic receptor protein tyrosine kinases), and cytosolic protein tyrosine kinases (O'Dell *et al.*, 1991; Anafi *et al.*, 1992). Bombesin, a transmitter substance that induces growth through activation of receptors coupled to other second messengers than intrinsic receptor protein tyrosine kinase, has been shown to phosphorylate cytosolic protein tyrosine kinases. This effect could be blocked by adding tyrphostins (Seckl & Rosengurtz, 1993).

Since ATP is a co-transmitter in sympathetic nerves and is released from platelets upon aggregation, (Stjärne & Lishajko 1966; Gordon, 1986) it might contribute to the development of vascular hypertrophy seen in hypertension and in atherosclerosis. It may therefore be of value to develop inhibitors of ATP-induced mitogenesis. In our attempts to define the mechanisms underlying the mitogenic effects of ATP we studied

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the effects of several different tyrphostins on ATP-induced DNA synthesis and cell proliferation. The results demonstrate that inhibitors of tyrosine kinase activity prevent ATP-mediated mitogenesis.

Methods

Cell culture

A primary culture of vascular smooth muscle cells (VSMC) was prepared essentially as described previously (Erlinge *et al.*, 1993). Briefly, aortae from female Sprague-Dawley rats were removed under sterile conditions and placed in Earle's balanced salt solution (EBSS) containing streptomycin (100 $\mu g\ ml^{-1}$), penicillin (100 u ml^{-1}) and 2 mg ml^{-1} collagenase type I-S for 30 min at 37°C. Under a binocular microscope, the adventitia and outer media were stripped off. The vessels were opened by a longitudinal cut and the intima removed by scraping. The vessels were placed into fresh EBSS, minced and incubated with collagenase for 2 h at 37°C. After the incubation, the tissues were flushed through 16 and 18 gauge needles, centrifuged gently and the cells were then resuspended in collagenase-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with streptomycin (100 $\mu g\ ml^{-1}$), penicillin (100 u ml^{-1}) and 10% foetal calf serum (FCS). The resulting suspension was then plated into 25 cm^2 flasks and cultured at 37°C in humidified 5% CO_2, 95% air atmosphere until cells reached confluence (7–10 days). VSMC were passaged 4 times before use. Cell viability was tested by exclusion of trypan blue (>95%). VSMC were identified by immunofluorescence staining of α-actin filaments using a monoclonal antibody labeled with FITC (Boehringer Mannheim, Germany). There were more than 95% SMC in the cell cultures.

Determination of DNA synthesis

DNA synthesis was measured by use of [^{3}H]-thymidine-incorporation. VSMC cultures were suspended by trypsinization, counted and replated into 24 well plates at a density of 30 000 cells/well in media (see above) containing 10% FCS. The cells were of passage 5–11. After 48 h the cells were starved in 0.5% FCS for another 48 h to decrease proliferation and induce quiescence. All the studied substances were then added at the same time and were present for 19 h, except for antagonists which were added 20 min earlier. The cells were incubated with [^{3}H]-thymidine (1 $\mu Ci\ ml^{-1}$) during the last 4 h. The medium was then aspirated, the cells washed three times with PBS and twice with ice-cold 10% trichloroacetic acid. The fixed cellular material was solubilized in 1 ml 0.2 M NaOH for 2 h or overnight. All the NaOH from each well (1 ml) was mixed with 4 ml of OptiPhase 'HiSafe' 3 liquid scintillation cocktail. The amount of [^{3}H]-thymidine taken up by the cells was estimated by counting in a Wallac 1410 liquid scintillation counter.

Determination of cell number by a colorimetric assay

The tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) is bioreduced by viable cells into a formazan that is soluble in tissue culture medium (Carmichael *et al.*, 1987). The conversion of MTS is accomplished by dehydrogenase enzymes found in living cells. The amount of formazan can then be determined by measuring absorbance at 490 nm in an ELISA plate reader. The quantity of formazan product, and thus the amount of 490 nm absorbance, is directly proportional to the number of living cells in culture. We used the CellTiter 96 AQ Non-Radioactive Cell Proliferation Assay from Promega, U.S.A.

VSMC cultures were suspended by trypsinization, counted and replated into 96 well plates at a density of 5000 cells/well in media (see above) containing 0.5% FCS and starved for 48 h to decrease proliferation and induce quiescence. The cells were of passage 5–11. All the studied substances were then added at the same time and present for 72 h. Tyrphostins were added 30 min earlier. The plates were kept at 37°C in a humidified 5% CO_2 atmosphere. Each well was then supplemented with 20 μl of MTS reagent and left for another 60 min before recording the absorbance at 490 nm using an ELISA plate reader.

Immunocytochemistry

Rat aortic smooth muscle cells were seeded into 96 well plates with 5000 cells/well in DMEM supplemented with streptomycin (100 $\mu g\ ml^{-1}$) and penicillin (100 u ml^{-1}) but without foetal calf serum. After 3 days the cells were stimulated with ATP in the presence or absence of tyrphostin for 2 h. The cells were fixed with ice-cold methanol for 5–10 min and incubated with blocking solution (5% milk powder and 0.2% Triton-X 100 in PBS) for 45 min at room temperature. The blocking solution was removed and a polyclonal rabbit antiserum specific for amino acid 1-16 of the Fos-protein (SC-52, Santa Cruz Biotechnology, U.S.A.; diluted 1:1000 in blocking solution) was added. Incubation was 48 h at 8°C. The wells were washed three times with PBS and incubated with 100 μl/well PBS with a secondary biotinylated goat anti-rabbit antibody in a dilution of 1:400 for 60 min at room temperature. After washing with PBS three times the cells were incubated with an avidine and biotinylated horseradish peroxidase reagent (Vectastain *Elite* ABC kit, Vector Laboratories, U.S.A.) for 60 min at room temperature. The cells were washed with PBS three times and incubated with diaminobenzidin (DAB) and H_2O_2 for 5–8 min. After additional washing the cells were examined in an inverted microscope (Nikon).

Vasomotor responses

Rats were killed with an overdose of mebumal (NordVacc, Sweden). The abdominal aorta was dissected out under a microscope and immersed in a cold oxygenated buffer solution. The vessels with the endothelium still present, were cut into cylindrical segments (2 mm long) which were immediately used in the experiments. Each cylindrical segment was mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer (FT03C) attached to a Grass Polygraph for continuous recording of the isometric tension, and the other to a displacement device (Högestedt *et al.*, 1983). The position of the holder could be changed by means of a movable unit allowing fine adjustments of the vascular tension by varying the distance between the metal prongs. The mounted specimens were immersed in temperature controlled (37°C) tissue baths containing a buffer solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 11. The solution was continuously gassed with 5% CO_2 in O_2 giving a pH of 7.4.

A tension of 4 mN was applied to the aortic segments and they were allowed to stabilize at this level of tension for 1.5 h. The contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 mM) buffer solution which had the same composition as the standard solution except that some of the NaCl was exchanged for an equimolar concentration of KCl. When two reproducible contractions had been achieved the vessels were used for further studies (variation less than 10%).

$^{45}Ca^{2+}$ uptake by VSMC

Cells were grown to confluence in 35 mm, 6 well plates for 7 days. Prior to uptake assays, cells were washed twice in a HEPES buffer of the following composition (mM): NaCl 137, KCl 5.4, KH_2PO_4 0.44, $CaCl_2$ 1.26, $MgSO_4$ 0.81, HEPES 20 mM and 0.3% bovine serum albumin with a pH of 7.4, and incubated in the same buffer for 1 h. The uptake assay mix-

tures consisting of 3 μCi $^{45}Ca^{2+}$ per well, with appropriate concentration of agonists in HEPES buffer were added to the cells in final volume of 1 ml and incubated for 5 min at room temperature. The uptake was terminated by rapid aspiration of the incubation mixture. Cells were then washed four times with ice-cold HEPES buffer in order to remove the residual $^{45}Ca^{2+}$ and lysed with 1 ml of 0.2 M NaOH. All the NaOH from each well (1 ml) was mixed with 4 ml of OptiPhase 'HiSafe' 3 liquid scintillation cocktail. The amount of $^{45}Ca^{2+}$ taken up by the cells was estimated by counting in a Wallac 1410 liquid scintillation counter.

Inositol phosphate production by VSMC

Cells were grown to confluence in 35 mm, 6 well plates for 3–5 days. Prior to uptake assays, cells were loaded with 1 μCi/well [^{3}H]-inositol for 16 h. After this they were washed twice and 1 ml of DMEM with 0.5% FCS with LiCl 10^{-2} M and imipramin 10^{-6} M were added. After 15 min at 37°C the cells were stimulated by the nucleotide for 15 min. The reaction was terminated by rapid aspiration of the medium and addition of 800 μl ice-cold methyl alcohol (MeOH) and 0.12 M HCl (1:1) to stop the reaction. Plates were put on ice for 1 h. The wells were scraped to loosen the cells and the mixture was transferred to vials containing 400 μl chloroform and homogenized for 20 s. To extract the aqueous phase containing the water soluble inositol phosphates we added another 600 μl chloroform, vortexed for 30 s and 1.2 ml distilled H_2O vortexed 30 s, and centrifuged the tubes at 2500 *g* for 15 min (Pavenstädt *et al.*, 1992). The aqueous phase was poured onto a Dowex 1 × 8, (1 ml, 100–200 mesh) anion exchange column (Bio-Rad Poly-Prep column). Inositol phosphates were eluted sequentially as described by Berridge (1983). Columns were washed with 12 ml 10 mM inositol to rinse the columns of free inositol. The different inositol phosphates were eluted with 12 ml 5 mM disodium tetraborate, 60 mM ammonium formate for glycerophosphoinositol, 200 mM ammonium formate, 100 mM formate for inositol monophosphate, 400 mM ammonium formate, 100 mM formate for inositol diphosphate, 1 M ammonium formate, 100 mM formate for inositol triphosphate. The total 12 ml of each fraction was placed in a scintillation vial and mixed with 9 ml OptiPhase 'HiSafe' 3 liquid scintillation cocktail. The amount of [^{3}H]-inositol in the different elutions was estimated by counting in a Wallac 1410 liquid scintillation counter.

Materials

The following were used: ATP and DMEM (Sigma, U.S.A.), Dowex (Fluka, Switzerland), foetal calf serum (FCS), EBSS and penicillin/streptomycin (Gibco, U.S.A.), $^{45}Ca^{2+}$, [^{3}H]-inositol, [^{3}H]-leucine, [^{3}H]-thymidine (Amersham, U.K.), tyrphostins (Calbiochem, U.S.A.).

Statistical analyses

Values represent mean ± s.e.mean except where otherwise stated. Statistically significant differences between groups were determined with Students' *t* test using StatView II on a Macintosh PowerBook 180. $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$.

Results

ATP-induced DNA synthesis: inhibitory effects of tyrphostins

ATP (10^{-5} M) induced a powerful concentration-dependent increase in DNA synthesis measured by [^{3}H]-thymidine incorporation (225 ± 30% increase relative to control (0 ± 12%, 635 ± 76 c.p.m.)). Tyrphostin 25 (10^{-5} M) had no effect *per se* on basal DNA synthesis. The ATP-stimulated DNA synthesis was reduced by 89% (down to 26 ± 8%) in the presence of tyrphostin 25 10^{-5} M (Figure 1a). Higher doses of ATP ($10^{-4.5}$ M: 29 ± 12% or 10^{-4} M: 13 ± 7%) could not reverse the inhibitory effect of tyrphostin 25 (10^{-5} M). Tyrphostin 1, a negative control for other tyrphostins has no inhibitory effects on protein tyrosine kinases and was without effect on ATP induced mitogenesis (Figure 1a). The potency of several other tryphostins was also examined and are presented in Table 1. They were all slightly more potent than tyrphostin 25. However, all tyrphostins except tyrphostin 1 were able to block completely ATP-induced DNA synthesis.

ATP-induced cell proliferation

ATP (10^{-5} M) increased total cell number after 72 h of incubation by 28 ± 4% as measured by an enzymatic cell proliferation assay by optical density determination using an ELISA counter (control = 0 ± 3%, 0.244 ± 8 OD, 490 nm). Tyrphostin 25 (10^{-5} M) inhibited the increase in cell number significantly ($P<0.01$, $n=9$). Tyrphostin 25 had no inhibitory effect *per se*. Tyrphostin 1 at the same concentration had no inhibitory effect on ATP stimulated proliferation (Figure 1b).

Fos-protein expression

When VSMC were incubated with 10^{-5} M ATP for 2 h nearly all of the cells (87 ± 5%) were intensely stained with an antibody to the Fos-protein (Figures 2 and 3). The Fos-protein

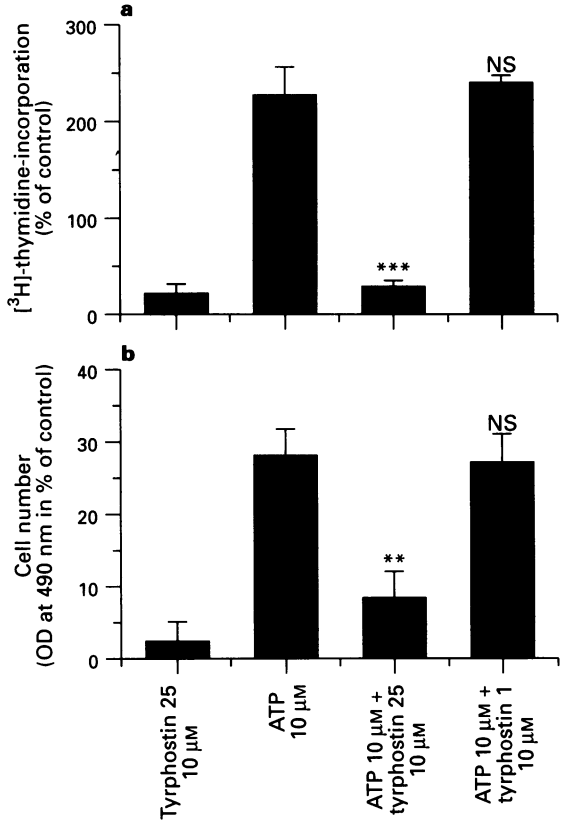

Figure 1 (a) Effect of extracellular ATP on DNA synthesis in rat aortic smooth muscle cells measured by incorporation of [^{3}H]-thymidine expressed as % difference from control. Inhibitory effect of tyrphostin 25 (10^{-5} M). Lack of inhibitory effect of tyrphostin 1. (b) Effect of extracellular ATP on cell proliferation in rat aortic smooth muscle cells measured by a colorimetric cell proliferation assay expressed as % difference from control. Inhibitory effect of tyrphostin 25 (10^{-5} M). Lack of inhibitory effect of tyrphostin 1. Values represent the mean ± s.e.mean, $n=9$. Statistical differences compared to ATP (10^{-5}M) alone are expressed as $^{***}P<0.001$, $^{**}P<0.01$ or NS, not significant.

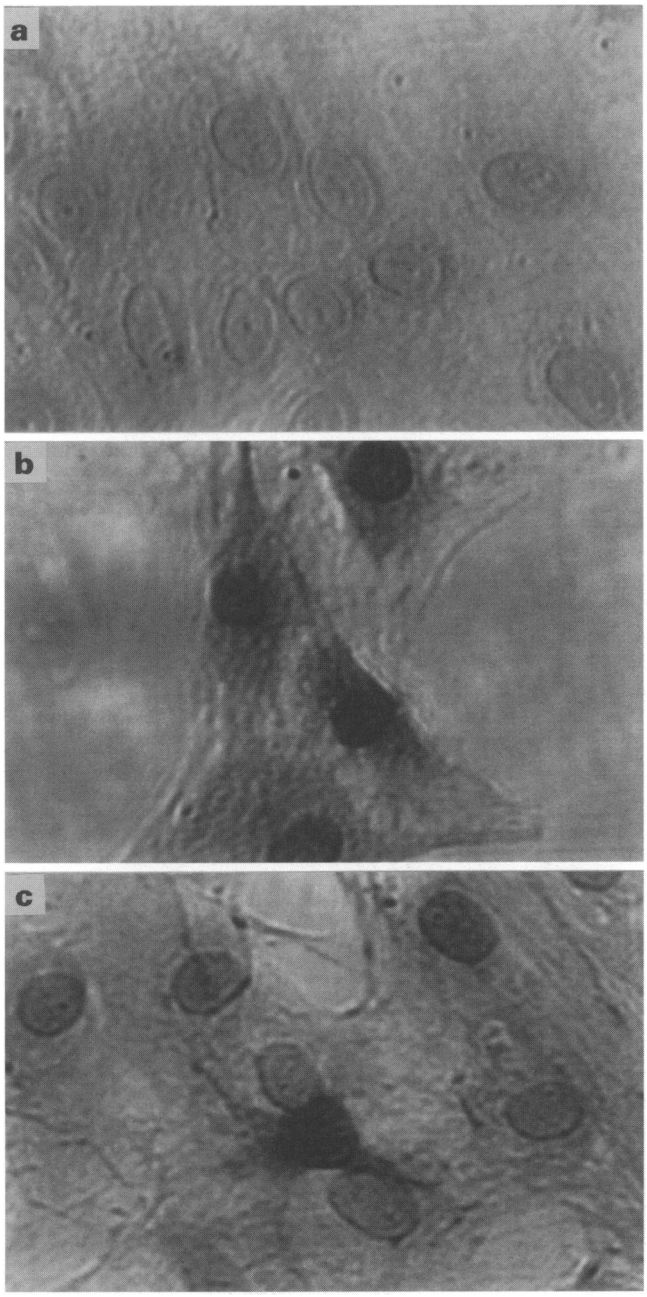

Figure 2 Effect of extracellular ATP on Fos-protein expression in rat aortic smooth muscle cells shown by antibody to the Fos-protein. (a) Control; (b) stimulation with ATP 10 μM; (c) inhibitory effect of tyrphostin 25 (10^{-5} M) on ATP (10^{-5} M)-stimulated Fos-protein expression.

was mainly localized to the cell nucleus (Figure 2). In the controls only $1 \pm 2\%$ of the VSMC were weakly stained. When the cells were preincubated with 10^{-5} M tyrphostin 25 the effect of 10^{-5} M ATP was radically diminished to $14 \pm 2\%$ staining (Figure 3). Tyrphostin 25 (10^{-5} M) alone had no effect on Fos-protein expression.

$^{45}Ca^{2+}$ uptake by VSMC

ATP induced a concentration-dependent increase in $^{45}Ca^{2+}$-influx with a pD_2 value of $10^{-5.67}$ M. Tyrphostins by themselves did not alter $^{45}Ca^{2+}$-influx. When 10^{-5} M tyrphostin 25 was added 30 min before 10^{-5} M ATP the $^{45}Ca^{2+}$-influx was slightly but not significantly reduced (Figure 4a).

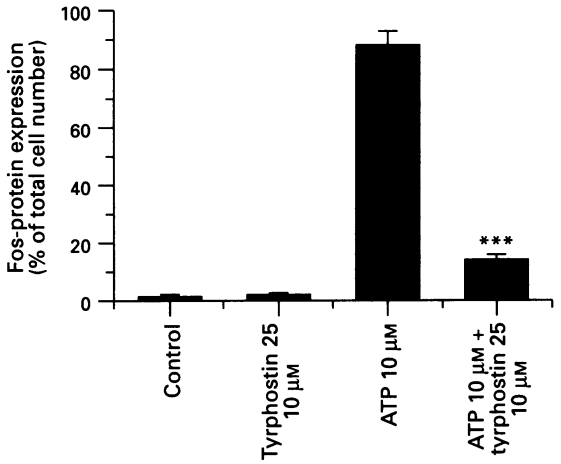

Figure 3 Effect of extracellular ATP on Fos-protein expression in rat aortic smooth muscle cells. Number of positively stained cells, expressed as % difference from control. Inhibitory effect of tyrphostin 25 (10^{-5} M). Inhibitory effect of tyrphostin 25 (10^{-5} M). Values represent the mean $\pm$ s.e.mean, $n=9$. Statistical differences compared to ATP (10^{-5} M) alone are expressed as ***$P<0.001$.

Inositol phosphate production by VSMC

We did a time study on the ATP-induced formation of inositol monophosphate (IP_1), inositol diphosphate (IP_2) and inositol trisphosphate (IP_3), where IP_3 reached its maximum after 5 min, IP_2 after 10 min while IP_1 did not reach maximum even after 30 min. The results were most stable at 15 min and this time point was selected for the rest of the experiments. Most of the inositol phosphates at 15 min were IP_1 and IP_2 with less IP_3. The results are presented as IP_{total} ($IP_1 + IP_2 + IP_3$).

ATP induced formation of inositol phosphates (IP_{total}) in VSMC in a concentration-dependent manner with a pD_2 value of $10^{-4.19}$ M. Tyrphostin 25 did not modify either basal or ATP-stimulated IP_{total}-formation (Figure 4b).

Vasoconstriction

ATP induced a concentration-dependent contraction of circular segments of rat aorta examined *in vitro* (Figure 5). The contractile response to ATP was not altered by 30 min preincubation with 10^{-5} M tyrphostin 25 (Figure 5).

Discussion

Pharmacological studies and receptor cloning have shown that extracellular ATP acts through membrane bound receptors that are coupled to G-proteins or ion-channels and lack intrinsic tyrosine kinase activity (Barnard *et al.*, 1994). Acting on a nucleotide receptor ATP has potent effects on vascular smooth muscle cell proliferation through an increase in intracellular Ca^{2+} via extracellular influx and inositol phosphate formation (Erlinge *et al.*, 1993; 1995). This is in contrast to an important group of polypeptide growth factors that act through receptors with intrinsic tyrosine kinase activity, e.g. platelet derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin. Thus, it places ATP together with bombesin, substance P, angiotensin II, endothelin, neuropeptide Y and 5-hydroxytryptamin in the group of growth factors that act through receptors with no intrinsic tyrosine kinase activity. Intracellular mechanisms involved in their mitogenic effects are elevation of intracellular Ca^{2+}, inositol phosphate formation, activation of protein kinase C, arachidonic acid metabolites and phospholipase D. Furthermore, it was recently shown that bombesin, bradykinin, vasopressin and endothelin could increase tyrosine phosphorylation of protein substrates in intact

Swiss 3T3 cells (Leeb-Lundberg & Song, 1991 Zachary *et al.*, 1991a, b).

Recently, Neary & Zhou (1994) were able to demonstrate that extracellular ATP stimulates mitogen-activated protein kinase (MAPK) activity in astrocytes by a signal pathway independent of the Raf kinase pathway used by for example bFGF. Similar to our findings in vascular smooth muscle cells (Erlinge *et al.*, 1993; 1995), Huwiler & Pfeilschifter (1994) showed that activation of a nucleotide receptor by ATP and UTP stimulates mesangial cell proliferation. Furthermore, they showed that ATP and UTP stimulated phosphorylation of MAPK and mitogen-activated protein kinase (MAPKK), suggesting that tyrosine kinases are responsible for the mitogenic effect of the nucleotides (Huwiler & Pfeilschifter, 1994).

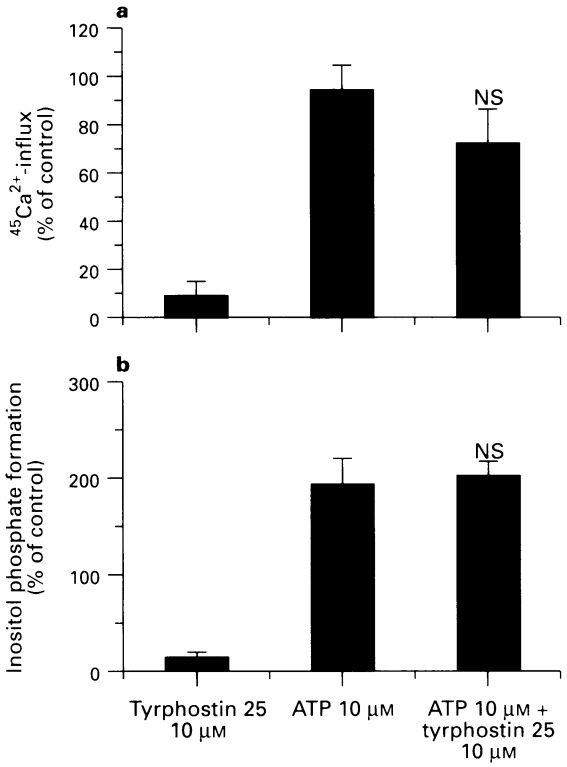

Figure 4 (a) Effect of extracellular ATP on $^{45}Ca^{2+}$-influx in rat aortic smooth muscle cells expressed as % difference from control. No significant inhibitory effect of tyrphostin 25 (10^{-5} M). (b) Effect of extracellular ATP on inositol phosphate formation in rat aortic VSMC ($IP_{total} = IP_1 + IP_2 + IP_3$) expressed as % difference from control. No inhibitory effect of tyrphostin 25 (10^{-5} M). Values represent the mean ± s.e.mean, $n = 9$. Statistical differences compared to ATP (10^{-5} M) alone are expressed as NS, not significant.

Thus, we wanted to examine whether protein tyrosine kinases are involved in mediating the mitogenic effect of ATP in vascular smooth muscle cells.

The ability to examine the role of tyrosine kinases in intracellular events has improved considerably since the development of tyrphostins, a group of compounds that selectively inhibit tyrosine kinases (Levitzki & Gilon, 1991). Using tyrphostins, Seckl & Rosengurtz (1993), were able to discover a role for tyrosine kinases in the mitogenic signalling of the bombesin receptor, a receptor lacking intrinsic tyrosine kinase activity, through inhibition of cytosolic tyrosine kinases. The tyrphostins permeate the cells quickly since maximal effects have been observed within 30 min (Bilder *et al.*, 1991). Based on this, we added the tyrphostins 30 min before addition of ATP in our experiments. In most of the experiments tyrphostin 25 was used since it is the most extensively documented and characterized tyrphostin and since it has been found to inhibit selectively bombesin induced tyrosine phosphorylation.

Tyrphostin 25 inhibited in a concentration-dependent manner ATP-induced DNA synthesis as measured by [^{3}H]-thymidine incorporation with an IC_{50} of $10^{-5.25}$ M, with no effect on basal DNA synthesis. The six other tyrphostins examined were noted to be even more potent. Tyrphostin 25 prevented the increase in cell number induced by ATP after a 3 day incubation. In contrast, the negative control, tyrphostin 1, was without effect. Thus, all the tyrphostins known to inhibit tyrosine kinases inhibited ATP-induced mitogenesis in a potent and complete way.

The expression of the immediate-early gene c-*fos* is an early nuclear signal induced by a variety of growth factors (Green-

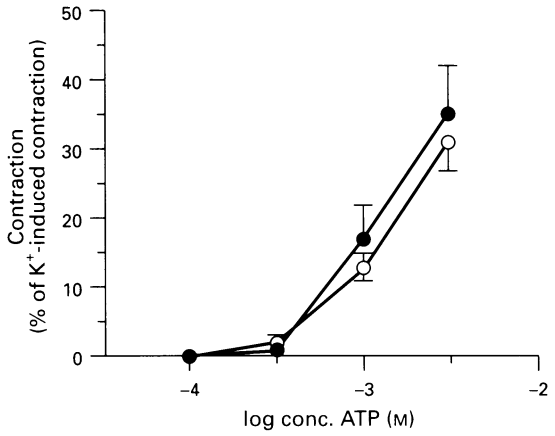

Figure 5 Contractile effect of extracellular ATP on rat aorta as % of 60 mM KCl. No inhibitory effect by tyrphostin 25 (10^{-5} M) added 30 min prior to ATP: (○) ATP alone; (●) ATP + tyrphostin 25. Values represent the mean ± s.e.mean, $n = 6$. There were no statistical differences at any ATP concentration.

Table 1 Potency and maximum inhibition of different tyrphostins as inhibitors of ATP induced DNA synthesis in rat aorta smooth muscle cells

	IC_{50} (log conc)	*IC_{50} (μM)*	*Maximum inhibition (% of [^{3}H]-thymidine-incorporation induced by ATP 10 μM)*
Tyrphostin 1	ND	ND	0
Tyrphostin 25	−5.25 ± 0.08	5.62	100
Tyrphostin B42	−5.73 ± 0.10*	1.86	100
Tyrphostin B44(−)	−5.86 ± 0.06*	1.38	100
Tyrphostin B46	−5.58 ± 0.09*	2.63	100
Tyrphostin B48	−5.90 ± 0.11*	1.26	100
Tyrphostin B50 (+)	−5.67 ± 0.08*	2.14	100
Tyrphostin 56	−5.77 ± 0.06*	1.70	100

pD_2 and E_{max} values, ND = no detectable effect. All the other active tyrphostins were significantly more potent than tyrphostin 25. *$P < 0.05$ compared to tyrphostin 25. Values represent the mean ± s.e.mean, $n = 6-9$.

berg, 1984). It is thought to be important for an optimal mitogenic response to PDGF (Rollins & Stiles, 1988), while it is considered neither to be necessary nor sufficient for the mitogenic response. In a study on PDGF-dependent proliferation in vascular smooth muscle cells, tyrphostins inhibited both the mitogenic response and the c-*fos* mRNA expression (Bilder *et al.*, 1991). We examined the effect of ATP on the expression of the Fos-protein with a monoclonal antibody. Two hours after stimulation with 10^{-5} M ATP, the cells exhibited high immunoreactivity in the nucleus. This is consistent with the role of the Fos-protein as a regulator of gene expression by DNA binding in the nucleus. In vascular smooth muscle cells that had been pretreated with tyrphostin 25 (10^{-5} M) the immunoreactivity in the nucleus was absent. It has previously been demonstrated that stimulus-induced phosphorylation of the cyclic AMP response element-binding protein (CREB) at the Ser-133 residue, or of the serum response factor (SRF) at the Ser-103 position induces an expression of *c-fos* (Ginty *et al.*, 1993; 1994; Rivera *et al.*, 1993). To our knowledge, tyrosine phosphorylation is a novel signal for *c-fos* expression, thus stressing the need for characterizing the proteins involved (see below).

The inhibitory effects of tyrphostin 25 on Fos-protein expression, DNA synthesis and cell proliferation show that the tyrphostins blocked the mitogenic response of the cells triggered by ATP. The stimulation of tyrosine kinases must then be an earlier event than the activation of immediate-early genes. However, it did not seem to interfere with ATP stimulation of the membrane receptor since the ATP-stimulated $^{45}Ca^{2+}$-influx and inositol phosphate-formation were unaltered by tyrphostin 25. The lack of effect by tyrphostins on ATP-induced $^{45}Ca^{2+}$-influx and IP-formation shows that the tyrphostins do not block ATP-induced mitogenesis through competitive inhibition at the receptor site. This is also less probable since higher doses of ATP could not reverse the inhibitory effect of the tyrphostins, which would be the case if competitive displacement were possible.

The mitogenic effects of ATP are mediated through activation of tyrosine kinases since tyrphostins are selective with no effect on protein kinase C or protein kinase A (Seckl & Rosengurtz, 1993). The inhibitory effect is probably mediated via cytosolic tyrosine kinases although an effect on membrane bound receptor-coupled tyrosine kinases cannot be excluded. To study this we have initiated experiments where total protein extracts from vascular smooth muscle cells were separated by anti-phosphorylated tyrosine-sepharose, SDS-page and incubated with radiolabelled antibodies to phosphorylated tyrosine. Preliminary data shows that bands at 20 kDa and 40 kDa appear after ATP-stimulation but these are absent in controls and tyrphostin-pretreated cells. Since MAP kinase is a 42–44 kDa protein it could represent one of the bands. At present we are trying to establish the identity of the proteins.

What roles do the tyrosine kinases play in the mediation of other physiological effects of ATP on vascular smooth muscle cells, e.g. contraction? Receptors with intrinsic tyrosine kinase activity are potent mitogens but are usually poor smooth muscle cell constrictors. When EGF and PDGF do induce contraction this contraction is blocked with low concentrations (micromolar) of tyrphostins (Sauro & Thomas, 1993; Hollenberg, 1994). Some contractions mediated by G-protein coupled receptors have also been reported to be blocked by high concentrations of tyrphostins where the selectivity might be questioned, while others were unaffected (Di Salvo *et al.*, 1993; Hollenberg, 1994). ATP induced a concentration-dependent constriction in circular segments of rat aorta that was unaffected by tyrphostin 25 (10^{-5} M) pretreatment. This indicates that the contractile effect of ATP is not dependent on tyrosine kinases in contrast to the mitogenic effect of ATP.

In conclusion, tyrphostin 25 inhibits ATP-induced DNA synthesis, cell proliferation and Fos-protein expression, but not ATP-induced $^{45}Ca^{2+}$-influx, inositol phosphate-production or vasoconstriction. Our findings suggest that extracellular ATP stimulates a membrane receptor coupled to G-proteins and activates second messenger mechanisms such as inositol phosphates and Ca^{2+}-influx through ion channels. This may lead to activation of cytosolic tyrosine kinases with a subsequent activation of immediate-early genes like c-*fos*, which initiates the mitogenic process leading to DNA synthesis, cell division and proliferation of vascular smooth muscle cells. The new class of agents, the tyrphostins, inhibit the proliferation. The contractile effect of ATP on the other hand is mediated independently of tyrosine kinases.

Vascular smooth muscle cell proliferation is involved in the pathophysiological development of atherosclerosis and possibly hypertension (Schwartz *et al.*, 1986; Mulvany, 1993). ATP might be one of the mitogenic stimuli that triggers the cell proliferation, since ATP can be released from platelets, from damaged cells during vascular injury and from perivascular sympathetic nerves. Tyrphostins may be useful inhibitors of these mitogenic processes since they inhibit both PDGF and ATP-induced mitogenesis.

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References

ANAFI, M., GAZIT, A., GILON, C., BEN-NERIAH, Y. & LEVITZKI, A. (1992). Selective interactions of transforming and normal abl proteins with ATP, tyrosine-copolymer substrates, and tyrphostins. *J. Biol. Chem.*, **267**, 4518–4523.

BARNARD, E.A., BURNSTOCK, G. & WEBB, T.E. (1994). G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol. Sci.*, **15**, 67–70.

BERRIDGE, M.J. (1983). Rapid accumulation of inositol triphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.*, **212**, 849–858.

BILDER, G.E., KRAWIEC, J.A., MCVETY, K., GAZIT, A., GILON, C., LYALL, R., ZILBERSTEIN, A., LEVITZKI, A., PERRONE, M.H. & SCHREIBER, A.B. (1991). Tyrphostins inhibit PDGF-induced DNA synthesis and associated early events in smooth muscle cells. *Am. J. Physiol.*, **260**, C721–C730.

BURNSTOCK, G. (1990). Overview: purinergic mechanisms. *Ann. NY Acad. Sci.*, **603**, 1–17.

CARMICHAEL, J., DEGRAFF, W.G., GAZDAR, A.F., MINNA, J.D. & MITCHELL, J.B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.*, **47**, 936–942.

CARPENTER, G. (1987). Receptors for EGF and other polypeptide mitogens. *Annu. Rev. Biochem.*, **56**, 881–914.

DI SALVO, J., STEUSLOFF, A., SEMENCHUK, L., SATOH, S., KOLQUIST, K. & PFITZER, G. (1993). Tyrosine kinase inhibitors suppress agonist-induced contraction in smooth muscle. *Biochem. Biophys. Res. Commun.*, **190**, 968–974.

ERLINGE, D., BRUNKWALL, J. & EDVINSSON, L. (1994). Neuropeptide Y stimulates proliferation of human vascular smooth muscle cells: cooperation with noradrenaline and ATP. *Regul. Pept.*, **50**, 259–265.

ERLINGE, D., YOU, J., WAHLESTEDT, C. & EDVINSSON, L. (1995). Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur. J. Pharmacol., Mol. Pharmacol. Sect.*, **289,** 135–149.

ERLINGE, D., YOO, H., EDVINSSON, L., REIS, D.J. & WAHLESTEDT, C. (1993). Mitogenic effects of extracellular ATP on vascular smooth muscle cells vs. other growth factors and sympathetic cotransmitters. *Am. J. Physiol.*, **265,** (34), H1089–H1097.

GAZIT, A., OSHEROV, N., POSNER, I., YAISH, P., PORADOSU, E., GILON, C. & LEVITZKI, A. (1991). Tyrphostins. 2: Heterocyclic and α-substituted benzylidenemalononitrile tyrphostins as potent inhibitors of EGF receptor and ErbB2/neu tyrosine kinases. *J. Med. Chem.*, **34,** 1896–1907.

GAZIT, A., YAISH, P., GILON, C. & LEVITZKI, A. (1989). Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors. *J. Med. Chem.*, **32,** 2344–2352.

GINTY, D.D., BONNI, A. & GREENBERG, M.E. (1994). Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell*, **77,** 713–725.

GINTY, D.D., KORNHAUSER, J.M., THOMPSON, M.A., BADING, H., MAYO, K.E., TAKAHASHI, J.S. & GREENBERG, M.E. (1993). Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science*, **260,** 238–241.

GORDON, J.L. (1986). Extracellular ATP: Effects, sources and fate. *Biochem. J.*, **233,** 309–319.

GREENBERG, M.E. & ZIFF, E.B. (1984). Stimulation of 3T3 cells induces transcription of the c-*fos* protooncgene. *Nature*, **311,** 433–438.

HÖGESTEDT, E.D., ANDERSSON, K-E. & EDVINSSON, L. (1983). Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. *Acta Physiol. Scand.*, **117,** 449–461.

HOLLENBERG, M.D. (1994). Tyrosine kinase pathways and the regulation of smooth muscle contractility. *Trends Pharmacol. Sci.*, **15,** 108–114.

HUWILER, A. & PFEILSCHIFTER, J. (1994). Stimulation of extracellular ATP and UTP of the mitogen-activated protein kinase cascade and proliferation of rat renal mesangial cells. *Br. J. Pharmacol.*, **113,** 1455–1463.

LEEB-LUNDBERG, L.M.F. & SONG, X.H. (1991). Bradykinin and bombesin rapidly stimulate tyrosine phosphorylation of a 120-kDa group of proteins in Swiss 3T3 cells. *J. Biol. Chem.*, **266,** 7746–7749.

LEVITZKI, A. & GILON, C. (1991). Tyrphostins as molecular tools and potential antiproliferative drugs. *Trends Pharmacol. Sci.*, **12,** 171–174.

MALAM-SOULEY, R., CAMPAN, M., GADEAU A-P. & DESGRANGES, C. (1993). Exogenous ATP induces limited cell cycle progression of arterial smooth muscle cells. *Am. J. Physiol.*, **264,** C783–C788.

MULVANY, M.J. (1993). Resistance vessels structure and the pathogenesis of hypertension. *J. Hypertension*, **11** (suppl. 5), S7–S12.

NEARY, J.T. & ZHU, Q. (1994). Signaling by ATP receptors in astrocytes. *Neuroreport*, **5,** 1617–1620.

O'DELL, T.J., KANDELL, E.R. & GRANT, S.G.N. (1991). Long term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature*, **353,** 558–560.

PAVENSTÄDT, H., SPÄTH, G., SCHLUNCK, M., NAUCK, R., FISCHER, R., WANNER, C. & SCHOLLMEYER, P. (1992). Effect of nucleotides on the cytosolic free calcium activity and inositol phosphate formation in human glomerular epithelial cells. *Br. J. Pharmacol.*, **107,** 189–195.

RATHBONE, M.P., DEFORGE, S., DELUCA, B., GABEL, B., LAURENSSEN, C., MIDDLEMISS, P. & PARKINSON, S. (1992). Purinergic stimulation of cell division and differentiation: Mechanisms and pharmacological implications. *Medical Hypotheses*, **37,** 213–219.

RIVERA, V.M., MIRANTI, C.K., MISRA, R.P., GINTY, D.D., CHEN, R.H., BLENIS, J. & GREENBERG, M.E. (1993). A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. *Mol. Cell Biol.*, **13,** 6260–73.

ROLLINS, B.J. & STILES, C.D. (1988). Regulation of c-*myc* and c-*fos* protooncgene expression by animal cell growth factors *in vitro*. *Cell. Dev. Biol.*, **24,** 81–84.

ROSS, R. (1989). Platelet-derived growth factor. *Lancet*, **1,** (8648) 1179–1182.

SAURO, M.D. & THOMAS, B. (1993). Tyrphostin attenuates platelet-derived growth factor-induced contraction in aortic smooth muscle through inhibition of protein tyrosine kinase(s). *J. Pharmacol. Exp. Ther.*, **267,** 1119–1125.

SCHWARTZ, S.M., CAMPBELL, G.R. & CAMPBELL, J.H. (1986). Replication of smooth muscle cells in vascular disease. *Circ. Res.*, **58,** 427.

SECKL, M. & ROSENGURTZ, E. (1993). Tyrphostin inhibits bombesin stimulation of tyorsine phosphorylation, c-fos expression, and DNA synthesis in Swiss 3T3 cells. *J. Biol. Chem.*, **268,** 9548–9554.

STJÄRNE, L. & LISHAJKO, F. (1966). Comparison of spontaneous loss of catecholamines and ATP in vitro from isolated bovine adrenomedullary, vesical gland, vas deferens and splenic nerve granules. *J. Neurochem.*, **13,** 1213–1216.

WANG, D-J., HUANG, N-N. & HEPPEL, L.A. (1992). Extracellular ATP and ADP stimulate proliferation of porcine aortic smooth muscle cells. *J. Cell. Physiol.*, **153,** 221.

YAISH, P., GAZIT, A., GILON, C. & LEVITZKI, A. (1988). Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. *Science*, **242,** 933–935.

ZACHARY, I., GIL, J., LEHMANN, W., SINNETT-SMITH, J. & ROSENGURTZ, E. (1991a). Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation in intact Swiss 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 4577–4581.

ZACHARY, I., SINNETT-SMITH, J. & ROSENGURTZ, E. (1991b). Stimulation of tyrosine kinase activity in anti-phosphotyrosine immune complexes of Swiss 3T3 cell lysates occurs rapidly after addition of bombesin, vasopressin, and endothelin to intact cells. *J. Biol. Chem.*, **266,** 24126–24133.

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