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Effect of diadenosine polyphosphates in achondroplasic chondrocytes: Inhibitory effect of Ap_4A on FGF9 induced MAPK cascade

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ABSTRACT

Achondroplasia is characterised by a mutation in the gene that encodes for the FGF receptor type 3 (FGFR3), producing a hyperactivation of this receptor and a subsequent increase in MAPK activity. We have tested the ability of nucleotides to decrease the activation of MAPK in chondrocytes with achondroplasic FGFR3 receptor. Diadenosine tetraphosphate, Ap_4A, reduced the phosphorylation of pERK1/2 triggered by FGF9 (38% reduction). Ap_4A diminished the expression of achondroplasic FGFR3 receptor (65% reduction), stimulating FGFR3 receptor degradation. The action of Ap_4A seems to be mediated by a dinucleotide receptor rather than by any other ATP receptor.

1. Introduction

Achondroplasia, also known as dwarfism, is one of the most representative types of congenital skeletal dysplasias. This pathology is produced by a mutation in the gene that encodes for the FGFR3 receptor, being the change of Gly^{380} to Arg the most common mutation in achondroplasia.

FGFR3 is activated after the binding of FGF. This activation induces receptor dimerization and a subsequent receptor autophosphorylation at the intracellular kinase domain, thus, triggering a downstream activation of intracellular signalling [1]. In achondroplasia the ''pathological receptor'' shows a gain of function, leading to an alteration the normal equilibrium between proliferation and maturation and thereby inhibiting the normal growth of the bone [2].

Several mechanisms have been reported to explain how mutant FGFR3 enhances these signals. One of the mechanisms described suggests that the mutation stabilizes the dimeric

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinases 1 and 2; FGFR3, fibroblast growth factor receptor 3; FGF9, basic fibroblast growth factor 9; Ap_3A, diadenosine triphosphate; Ap_4A, diadenosine tetraphosphate; Ap_5A, diadenosine pentaphosphate; ATPγS, adenosine 5′-3-O-thiotriphosphate; 2MeSADP, 2-(methylthio)-ADP; 2MeSATP, 2-(methylthio)-ATP; AMP-PCP, adenyl 5′-(beta, gamma-methylene)-diphosphonate; IP3, inositol triphosphate; cGMP, cyclic guanosine monophosphate; PLC, phospholipase C; PKC, protein kinase C

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state of the receptor, thus, permitting its prolonged signalling [3]. On the other hand, a slow down-regulation of the mutant receptor [4], and a defective lysosomal targeting of mutant FGFR3, has also been observed [5].

Concerning the signal transduction pathways downstream of FGFR3, two main pathways seem to play an important role in the inhibition of the proliferation of chondrocytes: STAT-1 and ERK1/2 (MAPKs) cascades [6,7]. In the case of ERK1/2, this pathway is mainly involved in chondrocyte differentiation process [8]. In addition, MAPK pathway reduces the synthesis of the components of the extracellular matrix [9].

Nucleotides and dinucleotides are biologically active substances that can modify the physiology of many tissues. For this reason, mononucleotides such as ATP and specially dinucleotides like dinucleoside polyphosphates can be used for the treatment of some pathologies such as chronic bronchitis, cystic fibrosis, dry eye disease and atherosclerotic events [10].

Apart from mononucleotides, dinucleoside polyphosphates exert their actions through P2X, P2Y and dinucleotide receptors [11]. P2Y receptors are classically coupled to phospholipase C activation, IP3, diacylglicerol formation and intracellular calcium mobilization. Some subtypes, such as the $P2Y_{12}$ are negatively coupled to adenylate cyclase, and most of them are also able to stimulate MAPK cascade. It has been also described that in the endothelium $P2Y_1$ and $P2Y_2$ receptors can stimulate guanylate cyclase inducing the formation of cGMP [12]. cGMP, on the other hand, has been described as an inhibitor of the MAPK pathway in achondroplasic chondrocytes [9]. Recently, we have demonstrated the presence of $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ in achondroplasic chondrocytes [13], and how they modify intracellular calcium concentration. Apart from these metabotropic ATP receptors it is necessary to bear in mind the possible existence of specific dinucleotide receptors, which can be coupled to both ionotropic and metabotropic mechanisms [14,15].

The aim of the present experimental work is to see whether or not a group of dinucleotides, the diadenosine polyphosphates can modify the activity of the MAPK cascade which is stimulated in prolonged a way by mutated FGFR3 in achondroplasia, therefore, becoming a possible alternative for the treatment of this pathology.

2. Materials and methods

2.1. Reagents and antibodies

Tetracycline, α-MEM, heat-inactivated foetal bovine serum and antibiotics (penicillin, streptomycin and hygromicin) were purchased from Invitrogen (Carlsbad, CA, USA).

Nucleotides, dinucleotides and FGF9 were purchased from Sigma (St. Louis, MO, USA). Tris, NaCl, Triton X-100, phenylmethylsulphonylfluoride (PMSF), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), SDS, Tween 20, BSA, *p*-formaldehyde, aprotinin, pepstatin leupeptin, MG132 (N-CBZ-Leu-Leu-Leu-AL) and chloroquine were obtained from Sigma.

Antibodies against phospho-ERK1/2, ERK2, FGFR3, β-tubulin and horseradish peroxidase-conjugated goat anti-mouse and goat anti-mouse FITC-conjugated were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell lines and cell culture

Non-transformed rat chondrocytes (RCJ3.1C5.18) were transfected with full-length human wild type (WT) FGFR3 or mutant (ACH) FGFR3, $FGFR3^{G380R}$, as described elsewhere [4]. Expression of FGFR3 was regulated by a tetracycline suppression system, the receptor is expressed in the absence of tetracycline in the culture medium.

Standard culture medium was α-MEM supplemented with 15% heat-inactivated foetal bovine serum and antibiotics. Cells were incubated at 37 °C with 5% CO_2.

In order to analyze phosphorylation status of ERK1/2 or FGFR3 protein expression 2×10^4 cell/cm^2 cells were plated onto tissue culture dishes. Two days later, culture medium was replaced by fresh one without tetracycline and cells were incubated during 16 h. After starvation for 4 h with serum-free α-MEM, the cells were exposed for 30 min and at the indicated concentrations with the different nucleotides and dinucleotides either without or with FGF9 (25 ng/ml). After stimulation cells were lysed in buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, pepstatin and 2 μg/ml leupeptin). Lysates were clarified at $13{,}000 \times g$ for 20 min at 4 °C. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA, USA).

Cell proliferation was assessed by determining the number of cells after detachment with tripsin. For this purpose, chondrocytes were seeded at a density of 0.4×10^5cells/well (24-well plates) and maintained for 1 day. Culture media was then replaced by fresh one containing FGF9 (25 ng/ml) or FGF9 with Ap_4A (100 μM) for 72 h and counted in a Neuebauer chamber.

2.3. Western blot analysis

Protein extracts from each sample (45 μg) were subjected to 10% SDS-polyacrilamide gels and were transferred to nitrocellulose membranes (Amersham-Pharmacia-Biotech, Buckinghamshire, UK). Thereafter, membranes were blocked and incubated overnight in the primary antibody appropriately diluted in PBS containing 2% skimmed milk and 0.05% Tween 20. After washing, blots were incubated with peroxidase-conjugated secondary antibody. Development was performed using ECL system (Amersham-Pharmacia-Biotech, Buckinghamshire). Films were scanned and a densitometric analysis was performed using Kodak GL 200 Imaging System and Kodak Molecular Imaging Software (Kodak, Rochester, NY, USA). All the data shown are representative of three independent experiments.

2.4. Inmunofluorescence studies

Covers slips with cells were treated with 4% *p*-formaldehyde for 15 min. The fixed cells were washed and incubated overnight in PBS containing 1% BSA and diluted FGFR3 antibody. The covers slips were washed and incubated for 1 h with the secondary antibody. Samples were analyzed by confocal microscopy using a Zeiss Axiovert 200M microscope equipped with a LSM 5 Pascal confocal module (Zeiss, Oberkochen, Germany). All images were taken under the

same conditions (448 nm wavelength excitation, 505–530 nm wavelength emission filter). LSM analysis was performed with the LSM Pascal Software, which assigns an intensity value per pixel. In the case of control images, all the pixel information of the labelled area is taken and a mean of fluorescence is obtained. This value is 100. All the treated cells were analyzed in the same fashion and the pixel mean of each image was normalized with the control value to obtain a relative fluorescence which is equivalent to a relative number of receptors. These experiments were repeated four times per treatment (control, FGF9 and FGF9 + Ap_4A) and a field of each of these experiments were taken for analysis.

2.5. *Inhibition of lysosomal or proteosomal activity*

Chondrocytes transfected with human wild type FGFR3 or mutant (ACH) FGFR3 were pre-incubated with MG132 (50 μM) or chloroquine (500 μM) for 30 min. To measure degradation of FGFR3, FGF9 without or with Ap_4A were added and the cells incubated for other 30 min. Cells were lysed and subjected to inmunoblotting.

2.6. *Extracellular matrix studies*

Cartilage matrix deposition in chondrocytes was quantified by Alcian blue staining. Cells were seeded at a density of 2×10^5 cells/well in six-well dishes. After reaching the confluence, the differentiation was induced by adding 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to the medium. Under these conditions, the presence of cartilage matrix (proteoglycan synthesis) can be detected at 7 and at 10 days of culture, as previously described [16]. FGF9 (25 ng/ml) alone, or FGF9 together Ap_4A (100 μM) were also added with the fresh growth medium. Differentiating cultures were fed supplemented media every 2 days. At different time points cells were washed with PBS and stained with Alcian blue (1% in 3% acetic acid) for 30 min, washed three times for 2 min in 3% acetic acid and rinsed with distilled water. After cells were solubilized in 1% SDS, the absorbance at 605 nm was measured for triplicate samples.

2.7. *Statistical analysis*

The differences between the mean values were analyzed with SigmaPlot v8.02 (SPSS, Chicago, IL, USA) and using Student's *t*-test; statistical significance was considered to be achieved at the $P < 0.05$ level.

3. Results

3.1. *Effect of diadenosine polyphosphates and nucleotides on ERK1/2 phosphorylation*

Diadenosine polyphosphates and nucleotides were applied alone in chondrocytes expressing achondroplasic FGFR3 or WT FGFR3, and their ability to modify ERK1/2 phosphorylation was studied. As it is shown in Fig. 1, diadenosine polyphosphates hardly modified the degree of phosphorylation of ERK1/2 when compared to the basal level, both chondrocytes stably transfected with ACH FGFR3 (Fig. 1A, top panel) and chondrocytes expressing WT FGFR3 (Fig. 1B, top panel). In contrast, natural mononucleotides or synthetic mononucleotides increased the levels of phosphorylated ERK1/2, but to a lesser extent than after FGF9, the preferred ligand for FGFR3, exposure (Fig. 1A and B, mid and lower panels).

When diadenosine polyphosphates and FGF9, were applied together (Fig. 2A, top panel) on achondroplasic chondrocytes, Ap_3A and Ap_5A produced no variation on ERK1/2 phosphorylation stimulated by FGF9. On the contrary, Ap_4A significantly reduced ERK1/2 phosphorylation induced by FGF9 (around 40% compared to FGF9 alone). Concerning mononucleotides, after the application of FGF9 together with the naturally occurring or with the synthetic mononucleotides, an increase in ERK1/2 phosphorylation was observed in chondocytes expressing achondroplasic FGFR3 compared to the control (Fig. 2A, mid and lower panels).

We also examined whether the dinucleotide Ap_4A was able to decrease ERK1/2 phosphorylation in chondrocytes expressing WT (Fig. 2B, top panel). Chondrocytes transfected with WT FGFR3 showed a similar behaviour to that obtained in achondroplasic chondrocytes after dinucleotide treatment. No significant differences were found on ERK1/2 phosphorylation induced by FGF9 after addition of Ap_3A or Ap_5A together with FGF9, whereas Ap_4A together with FGF9 induced a noticeable decrease on ERK1/2 phosphorylation (34% compared to FGF9 alone). On the other hand, an increase in ERK1/2 phosphorylation was similarly detected after the application of FGF9 together with the naturally occurring or with the synthetic mononucleotides compared to the untreated chondrocytes (Fig. 2B mid and lower panels), following the pattern shown by chondrocytes transfected with the mutant.

3.2. *Effect of Ap_4A on FGFR3 protein levels*

Ap_4A reduces ERK1/2 phosphorylation only in presence on FGF9. Taking into account that the binding of FGF9 is necessary to receptor internalization and subsequent degradation, a possible mechanism, which could accomplish for this decrease, is an action of the dinucleotide on FGFR3 protein levels, for example, stimulating the degradation of the receptor.

To investigate this possibility, we have analyzed FGFR3 protein levels in the presence of FGF9, Ap_4A or FGF9 with Ap_4A in achondroplasic chondrocytes. As it is shown in Fig. 3A, only a slight decrease in the amount of the achondroplasic mutant FGFR3 receptor was detected after FGF9 addition (14% of reduction). In contrast, a marked decrease was observed when achondroplasic chondrocytes were exposed to FGF9 together with Ap_4A, the reduction being 65%, when compared to control. Treatment with Ap_4A alone did not induce any significant change in achondroplasic FGFR3 levels. Regarding cells expressing the wild type of the FGFR3, a decrease in levels of wild type receptor was also obtained after FGF9 addition or FGF9 together with Ap_4A (Fig. 3B). However, in both cases, the reduction observed was higher than the decrease detected in achondroplasic chondrocytes. In particular, 31% of reduction was determined after FGF9 addition

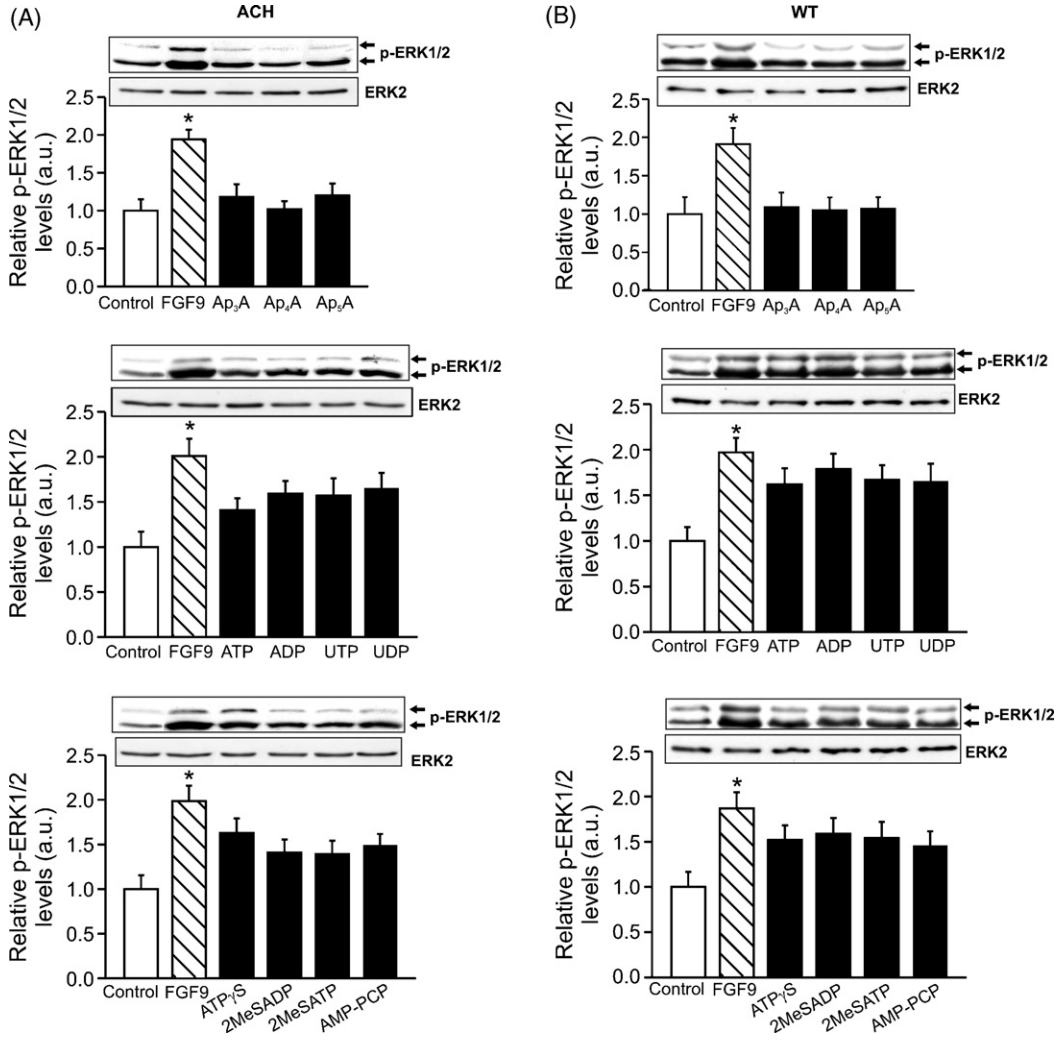

Fig. 1 – Effect of dinucleotides and nucleotides on ERK1/2 phosphorylation. Chondrocytes expressing ACH FGFR3 (A) or WT FGFR3 (B) were stimulated with FGF9 alone (25 ng/ml) or with nucleotides alone (100 μM) for 30 min. Cell lysates were successively inmuno blotted with antiphospho-ERK1/2 and anti-ERK2 antibody to verify equal loading. The histograms represent the levels of phosphorylated ERK1/2. Data (mean ± S.D.) are represented in arbitrary units (a.u.) and normalized to the intensity of the band corresponding control; $^{*}P < 0.05$.

and a marked decrease of 74% was found when chondrocytes expressing wild type FGFR3 were exposed to FGF9 together with Ap_4A.

On the other hand, no variations were found in FGFR3 protein levels after simultaneous treatment with FGF9 and different mononucleotides and other dinucleotides compared to levels obtained after application of FGF9 alone, both in achondroplasic chondrocytes (Fig. 3C) and in chondrocytes expressing the wild type (Fig. 3D).

Similar to the results obtained by Western blot, using inmunofluorescence microscopy, a marked down-regulation of achondroplasic mutant FGFR3 was observed when cells were incubated with FGF9 and Ap_4A (Fig. 4A). Under these conditions, a weak labelling of the FGFR3 intensity was obtained in achondroplasic cells compared with the same cells in the absence of any substance. Ap_4A in the presence of FGF9 produced a reduction of the ACH FGFR3 labelling of 63%. In the case of chondrocytes expressing WT (Fig. 4B), the decrease of WT FGFR3 staining was around 73%.

3.3. *Effect of proteasome and lysosome inhibition on FGFR3 degradation stimulated by Ap_4A*

In order to identify if the effect of Ap_4A is linked to a more active rate of FGFR3 degradation, MG132 and chloroquine, inhibitors of proteosomal and lysosomal degradation, respectively, were assayed.

Treatment with FGF9 and Ap_4A produced a dramatic decreased in the levels of mutant receptor with an observed reduction of 58% (Fig. 5A). As expected, a more noticeable decrease of WT FGFR3 levels (75%) was detected after identical treatment (Fig. 5B). In both cases, the effect was blocked with either MG132 or chloroquine, suggesting that Ap_4A is involved in FGFR3 down-regulation by both the proteasome and lysosomal pathways.

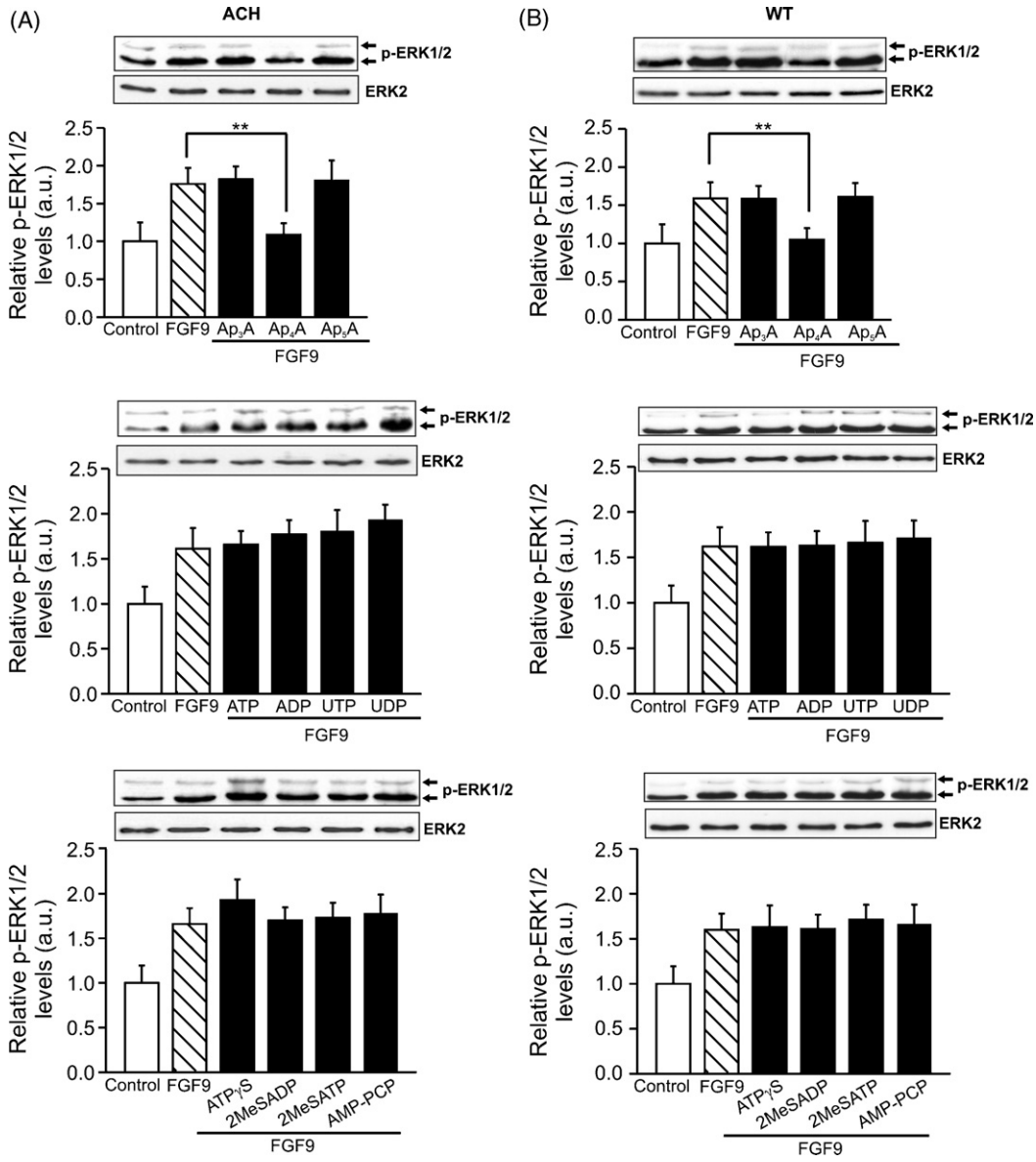

Fig. 2 – Effect of dinucleotides and nucleotides in the presence of FGF9 on ERK1/2 phosphorylation. Cells transfected with ACH FGFR3 (A) or with WT FGFR3 (B) were stimulated with FGF9 (25 ng/ml) alone or together with nucleotides (100 μM) for 30 min. Cell lysates were successively inmunoblotted with antiphospho-ERK1/2 and anti-ERK2 antibody to verify equal loading. The histograms represent the levels of phosphorylated ERK1/2. Data (mean ± S.D.) are represented in arbitrary units (a.u.) and normalized to the intensity of the band corresponding control; $^{**}P < 0.01$.

3.4. *Effect of Ap_4A on FGF9-induced cellular responses*

We have also studied the influence of Ap_4A on biological process induced by FGF9 in chondrocytes transfected with mutant FGFR3. Previous studies have revealed that sustained ERK kinase activity induced by FGFR3 signalling is involved in growth arrest [7]. In good agreement with this result, as shown in Fig. 6A, exposure of chondrocytes transfected with mutant FGFR3 to FGF9 resulted in a dramatic decrease of cell number (70% compared with untreated cells). In contrast, treatment with FGF9 together Ap_4A led to reversal of FGF9 effect on proliferation.

Apart from inhibition of proliferation, prolonged activation of ERK1/2 cascade triggered by activated FGFR3 produces the loss of extracellular matrix in chondrocytes [9,17]. For this reason, we assessed the amount of extracellular matrix by Alcian blue staining in chondrocytes treated with FGF9 alone or FGF9 together with Ap_4A (Fig. 6B). As expected, FGF9 produced a significant reduction of Alcian blue stainable extracellular matrix. The loss of extracellular matrix was, at least partially, prevented by Ap_4A.

4. Discussion

The present work describes the effect of the diadenosine polyphosphates and mononucleotides on the ERK1/2 cascade triggered by FGFR3 receptor. Either alone or in the presence of

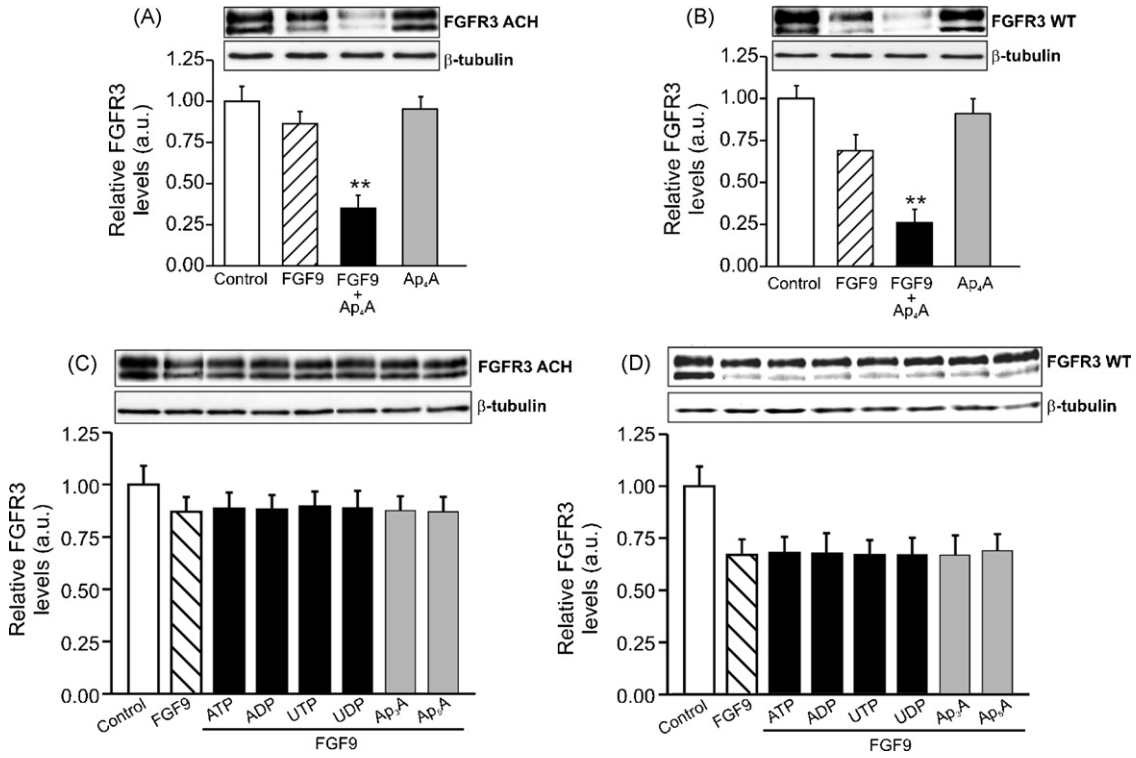

Fig. 3 – Effect of Ap_4A and other nucleotides on FGFR3 expression. Chondrocytes expressing ACH FGFR3 (A) or expressing WT (B) were treated with FGF9 (25 ng/ml), FGF9 together with Ap_4A (100 μM) or Ap_4A alone. In addition, achondroplasic chondrocytes (C) and chondrocytes expressing WT (D) were exposed to FGF9 alone or together with natural nucleotides or other dinucleotides for 30 min. FGFR3 levels were analyzed by Western blot. To ensure equal loading, the membranes were reprobed with an antibody detecting β-tubulin. The histograms represent the levels of FGFR3. Data (mean ± S.D.) are represented in arbitrary units (a.u.) and normalized to the intensity of the band corresponding control; $^{**}P < 0.01$.

FGF9 most of the substances tested did not reduce the phosphorylation of ERK1/2, and even in some cases phosphorylation was increased. Despite these results, the dinucleotide diadenosine tetraphosphate, Ap_4A, offered remarkable results. Ap_4A significantly counteracts FGF9-mediated phosphorylation of ERK1/2 in achondroplasic chondrocytes and as well in chondrocytes transfected with wild type FGFR3. This effect seems to be a consequence of the reduction of FGFR3 protein levels after the treatment by Ap_4A. This dinucleotide facilitates the rate of FGFR3 degradation, therefore, reducing the presence of this protein in the membrane and attenuating the phosphorylation of ERK1/2.

In our experiments, the presence of FGF9 is necessary to observe the decrease of ERK1/2 phosphorylation elicited by the dinucleotide. Taking into account that Ap_4A seems to exert its action by stimulating FGFR3 degradation, it is easy to explain this requirement because it has been reported that ligand binding is essential for phosphorylation and resulting internalization of the receptor [4]. Several authors have indicated that the achondroplasic mutant of the FGFR3 receptor shows a defective internalization and degradation compared to the wild type receptor [4,5,18]. Consistent with those results, we detected a higher reduction of wild type FGFR3 levels after FGF9 addition compared to achondroplasic FGFR3. As a result of this defective degradation, the mutant receptor accumulates at the cell surface and prolongs the signalling. Therefore, our finding about the ability of Ap_4A promoting FGFR3 degradation is especially relevant in the case of achondroplasic chondrocytes. Thus, the enhanced signalling initiated by the achondroplasic FGFR3 was clearly moderated. As a consequence of this less pronounced signalling those biochemical processes that are linked to the MAPK overstimulation induced by activated FGFR3, such as growth inhibition and loss of the extracellular matrix were reversed by Ap_4A.

In addition, we have analyzed the degradation pathway by which Ap_4A contributes to FGFR3 down-regulation. It has previously been detected in RCJ cells that activated FGFR3 receptor is usually targeted for lysosomal degradation [5]. In spite of this possibility, a possible involvement of the proteosome has also been demonstrated [18]. Our results are in accordance with both observations since the proteosomal and the lysosomal inhibitors, blocked FGF9-dependent degradation of the receptor. Moreover, FGFR3 down-regulation stimulated by Ap_4A was also prevented by both inhibitors.

As commented at the beginning of this discussion, other dinucleotides and mononucleotides assayed did not modify or increased the levels of phosphorylated ERK1/2 in chondrocytes. This fact is interesting because it should be reasonable to think that some of the mononucleotides should mimic the inhibitory effect elicited by Ap_4A. The lack of such a result demands some explanation. A possible reason to understand

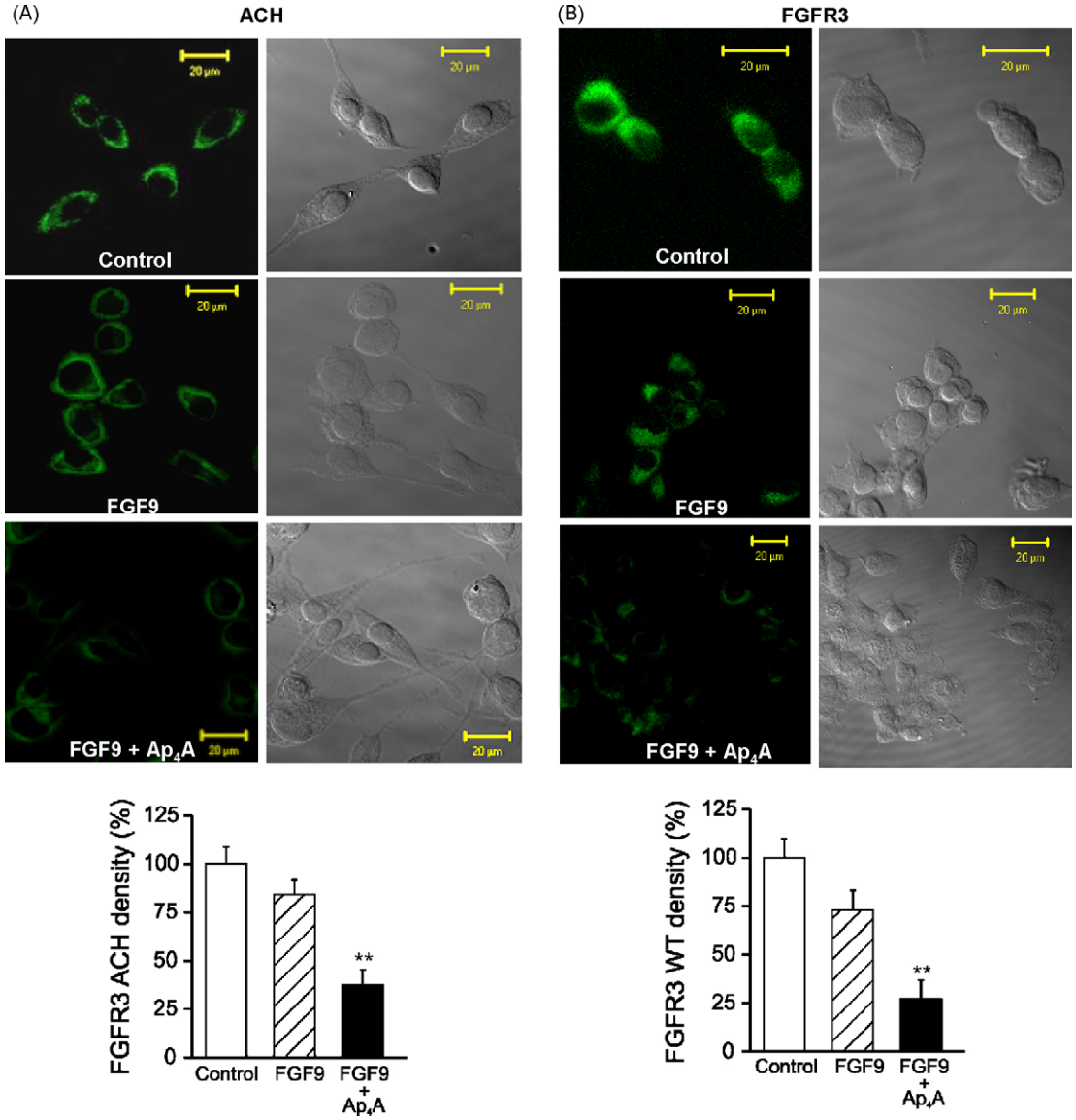

Fig. 4 – Confocal laser microscopy analysis of FGFR3 expression. Chondrocytes transfected with achondroplasic FGFR3 (A) and chondrocytes expressing WT (B) were incubated in the absence (control) or in the presence of FGF9 alone, or together with Ap_4A for 30 min, then cells were fixed and stained. The histograms represent the density of receptors. The experiment was repeated four times and a field of each of these experiments were taken for analysis; $^{**}P < 0.01$.

the different behaviour shown by nucleotides compared to Ap_4A is to take into account the hydrolysis of these compounds after incubation for 30 min together with the chondrocytes. However, the use of slowly degraded nucleotide analogues and the development of stability studies (data not shown), have allowed us to rule out this possibility. In fact, the half-life of ATP on chondrocytes had previously been found to be 3 h [19]. So, presumably other alternatives need to be found, these may be dealing with the activation of ATP receptors.

In a previous work we have described the existence of $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ in achondroplasic chondrocytes [13]. The presence of these P2Y receptors would suggest the involvement of any of them in the action of the diadenosine tetraphosphate. In this sense, Ap_4A has been described as an agonist in different P2Y receptors such as the $P2Y_1$, $P2Y_2$ and $P2Y_4$ [20]. Nevertheless, none of the tested mononucleotides, which are known to be selective P2Y agonists, were able to reproduce the behaviour of Ap_4A.

Under these circumstances, the participation of a different receptor, sensitive to diadenosine tetraphosphate and insensitive to the other di and mononucleotides, should be taken into consideration. Thus, the existence of a specific Ap_4A receptor coupled to G proteins and distinct from P2Y receptors has been described in lung and human tracheal gland cells [21,22]. In a similar way, a specific Ap_5A receptor, which presents metabotropic features, has been identified in cerebellar astrocytes [15,23]. In particular, it has been published that this receptor is coupled to PLC by means of a G_q protein [24], being able to activate Src kinase protein [25]. Therefore, in chondrocytes, the existence of a specific receptor for Ap_4A cannot be ruled out

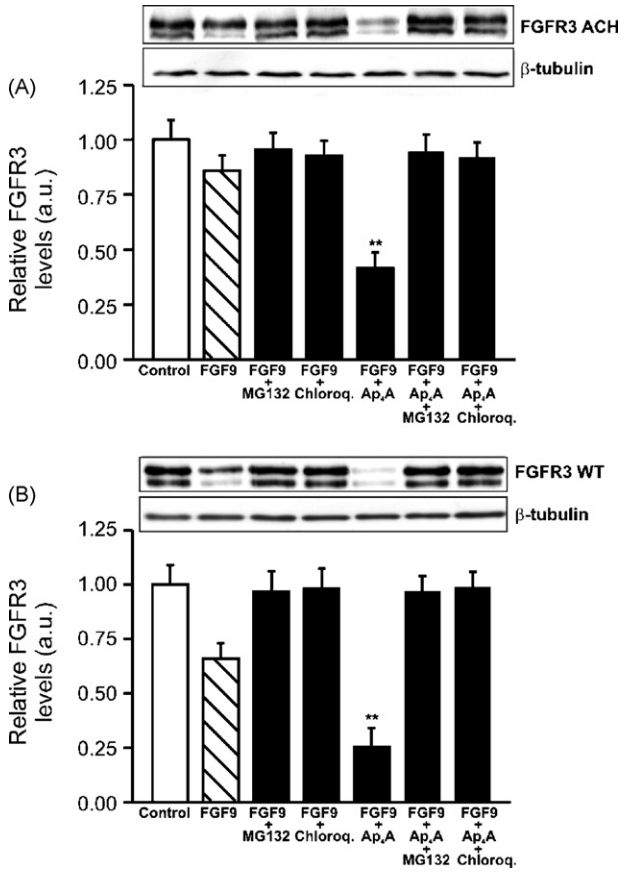

Fig. 5 – Effect of proteosome and lysosome inhibitors on FGFR3 degradation stimulated by Ap_4A. Chondrocytes expressing ACH FGFR3 (A) or expressing WT (B) were pre-treated with inhibitors, MG132 (50 μM) or chloroquine (500 μM) for 30 min before treatment with FGF9 alone or with Ap_4A (30 min). FGFR3 levels were analyzed by Western blot. To ensure equal loading, the membrane was reprobed with an antibody detecting β-tubulin. The histograms represent the levels of FGFR3. Data (mean ± S.D.) are represented in arbitrary units (a.u.) and normalized to the intensity of the band corresponding control; $^{**}P < 0.01$.

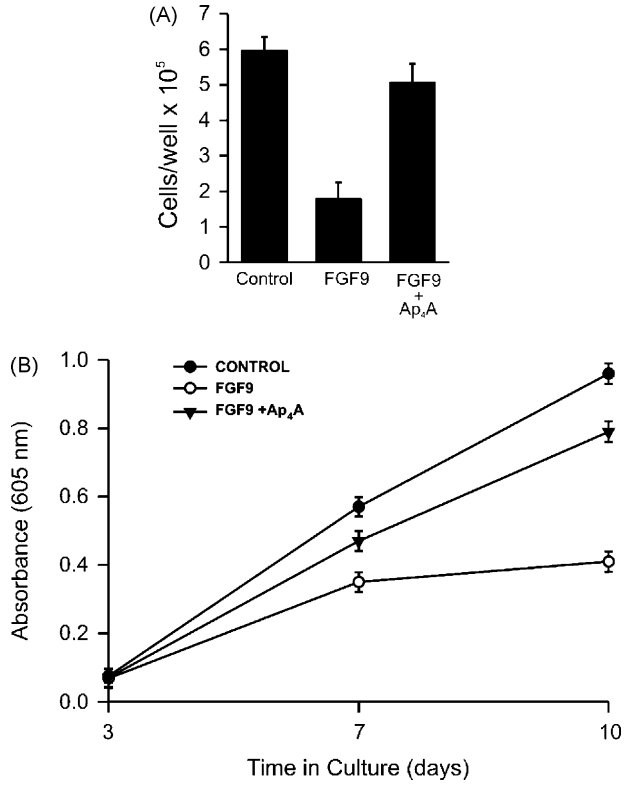

Fig. 6 – Effect of Ap_4A on FGF9-induced cellular responses. (A) Chondrocytes expressing ACH FGFR3 were seeded at a density of 0.4×10^5cells/well (24-well plates). After 24 h, the medium was replaced, then cells were treated with FGF9 (25 ng/ml) or FGF9 with Ap_4A (100 μM) for 72 h and counted. Data are the mean (±S.D.) of three independent experiments with duplicates samples. (B) Chondrocytes expressing ACH FGFR3 were treated as described in Section 2. At the indicated times, extracellular matrix was quantified by Alcian blue staining of cell layers, following solubilization, dye uptake was quantified at 605 nm for triplicate samples.

according to the evidences here reported, nevertheless, more research is necessary to fully characterise this receptor.

Regarding the underlying mechanism used by the dinucleotide receptor to increase FGFR3 degradation several possibilities could be proposed. Lysosomal targeting of FGFR3 and its consequent degradation is initiated by recruitment of the receptor into clathrin-coated pits at the plasma membrane. In this process, ubiqutin system has been shown as an important regulatory system. Upon internalization, the endocytic pathway to the lysosome, involves both regulatory proteins and phosphoinositides [26]. The role of phosphoinositide metabolites in this process would allow to suggest the involvement of PLC coupled to dinucleotide receptor.

Apart from ubiquitylation, other important post-translational modification involved in receptor endocytosis is phosphorylation. The activation of PLC coupled to dinucleotide receptor leads to Ca^{2+} release and to activation of PKC. Thus, activated PKC may phosphorylate different components of the cellular internalization machinery, resulting in receptor internalization and concomitant degradation. Other modifications of the endocytic machinery may also accelerate receptor degradation. For instance, clathrin tyrosine phosphorylation plays an outstanding role in the regulation of endocytosis [27], being Src the kinase responsible for clathrin phosphorylation [28]. Taking into account that the dinucleotide receptor can mediate Src activation [25], this finding could provide other possible molecular link between dinucleotide receptor signalling and the increase of FGFR3 down-regulation observed after Ap_4A treatment.

Achondroplasia is an orphan pathology with no pharmacological treatment so far. Approaches for the treatment of this disease are focused to limit the activity of the FGFR3 receptor among other strategies. The use of selective antibodies for the FGFR3 receptor, natriuretic peptides or the parathyroid hormone and analogues are the technologies currently used as an approach to the pharmacological treatment of achondroplasia [29]. In our case, we present

evidences supporting that Ap_4A, acting through a mechanism which needs to be fully investigated, significantly increases mutant FGFR3 down-regulation. This fact consequently attenuates sustained ERK signalling, as well as the inhibitory actions on proliferation and extracellular matrix production induced by FGF9. These findings open a new perspective inviting to consider Ap_4A as a new therapeutic drug for the treatment of achondroplasia.

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