Effects of diadenosine tetraphosphate on FGF9-induced chloride flux changes in achondroplastic chondrocytes

Fernando Huete · Ana Guzman-Aranguez ·
Javier Ortín · Charles H. V. Hoyle · Jesús Pintor

Received: 14 January 2011 / Accepted: 28 April 2011 / Published online: 11 May 2011
© Springer Science+Business Media B.V. 2011

Abstract Achondroplasia, the most common type of dwarfism, is characterized by a mutation in the fibroblast growth factor receptor 3 (FGFR3). Achondroplasia is an orphan pathology with no pharmacological treatment so far. However, the possibility of using the dinucleotide diadenosine tetraphosphate (Ap₄A) with therapeutic purposes in achondroplasia has been previously suggested. The pathogenesis involves the constitutive activation of FGFR3, resulting in altered biochemical and physiological processes in chondrocytes. Some of these altered processes can be influenced by changes in cell volume and ionic currents. In this study, the action of mutant FGFR3 on chondrocyte size and chloride flux in achondroplastic chondrocytes was investigated as well as the effect of the Ap₄A on these processes triggered by mutant FGFR3. Stimulation with the fibroblast growth factor 9 (FGF9), the preferred ligand for FGFR3, induced an enlarged achondroplastic chondrocyte size and an increase in the intracellular chloride concentration, suggesting the blockade of chloride efflux. Treatment with the Ap₄A reversed the morphological changes triggered by FGF9 and restored the chloride efflux. These data provide further evidence for the therapeutic potential of this dinucleotide in achondroplasia treatment.

Keywords Diadenosine tetraphosphate · Dinucleotides · Achondroplasia · Chondrocytes · Chloride flux · Fibroblast growth factor receptor 3

Fernando Huete and Ana Guzman-Aranguez contributed equally to this work.

F. Huete · A. Guzman-Aranguez · J. Ortín · C. H. V. Hoyle · J. Pintor (✉)
Departamento de Bioquímica, E.U. Óptica,
Universidad Complutense de Madrid,
c/Arcos de Jalón 118,
28037 Madrid, Spain
e-mail: jpintor@vet.ucm.es

Introduction

Achondroplasia is a disorder in which the gene that encodes for the fibroblast growth factor receptor 3 (FGFR3) is mutated, and in 97% of the patients the mutation a single glycine to arginine substitution at position 380 (G380R). FGFR3 is a tyrosine kinase receptor which is activated by the binding of fibroblast growth factors. This activation induces receptor dimerization and a subsequent receptor autophosphorylation in the intracellular domain, triggering a downstream activation of intracellular signalling. In achondroplasia, the activated mutant FGFR3 is resistant to down-regulation and internalization [1, 2]. Consequently, it remains constitutively active and over-expressed in the cell membrane, causing an excessive intracellular signalling, that produces critical changes in the biochemistry of chondrocytes. In particular, chondrocyte proliferation and differentiation as well as cartilage matrix production are disturbed, leading to a remarkably foreshortened epiphyseal growth plate cartilage [3, 4]. Processes altered in achondroplasia such as chondrocyte proliferation or extracellular matrix homeostasis can be influenced by factors such as changes in cell volume and ionic currents. In this sense, it has been shown that the modification of normal chondrocyte volume or ionic composition can affect the turnover (synthesis and degradation) of the extracellular matrix [5]. Moreover, a relationship between ion channel activity and proliferative behaviour has been suggested in normal chondrocytes [6, 7]. Despite these findings, little is known about changes in cell volume or intracellular ion concentrations in achondroplastic chondrocytes.

There is currently no accepted pharmacological treatment for achondroplasia, but it has been suggested that diadenosine tetraphosphate (Ap₄A) could have a therapeutic potential. Ap₄A belongs to group of dinucleotides

termed diadenosine polyphosphates, which are formed by two adenosine moieties connected by a chain of phosphates of variable length (Ap_nA , where n ranges from 2 to 7). Their activity has been reported in the eye, central nervous system, cardiovascular system and many visceral organs (for reviews, see [8–11]), among other locations, suggesting not only a role in the normal physiology of these tissues and organs but also some interesting properties as therapeutic agents.

Achondroplastic chondrocytes present important amounts of metabotropic P2Y receptors, some of which can be stimulated by the dinucleotide Ap_4A [12]. This naturally occurring dinucleotide is more stable than others such as ATP or UTP [9], thus indicating it might be a good candidate to stimulate some of those P2Y receptors, in particular P2Y_1 and P2Y_2 [10]. We have previously shown that Ap_4A can ameliorate the FGFR3-induced over-activation in achondroplastic cells, reducing the expression of the achondroplastic FGFR3 receptor and reducing its presence in the cell membrane. FGFR3 receptors are not down-regulated in the achondroplastic state when compared with the wild type. Under these conditions, Ap_4A stimulates the lysosomal and proteasomal degradation of FGFR3 permitting a reduction in p-ERK cascade and returning the achondroplastic cells to an apparent normal phenotype [13]. Indeed, Ap_4A , was able to rescue achondroplastic chondrocytes by both, restoring extracellular matrix and avoiding rapid cell death [13]. Furthermore, in achondroplastic chondrocytes obtained from transgenic mice [14] Ap_4A can restore the normal calcium signalling by fibroblast growth factor 9 (FGF9) [12]. All these actions are due to the existence of a variety of P2Y receptors in these cells that after stimulation can reduce the presence of the achondroplastic FGFR3 receptor in the cell membrane as previously indicated [12].

In the present experimental work, we studied whether FGF9, the preferred ligand for FGFR3, produces morphological changes in RCJ-FGFR3-G380R-tet cells and, since these changes might be due to a water load, we also evaluated the effect of FGF9 on the chloride flux, as an important anion facilitating water movement. Likewise, considering that Ap_4A could be an attractive candidate for the treatment of achondroplasia, we analysed the action that Ap_4A exerts on FGF9-mediated effects on morphology and chloride flux in these cells.

Materials and methods

Reagents

Tetracycline, minimum essential medium (MEM) alpha medium, heat-inactivated foetal bovine serum, antibiotics (penicillin, streptomycin and hygromycin) and *N*-(6-methoxyquinolyl) acetoethyl ester (MQAE) were pur-

chased from Invitrogen (Carlsbad, CA, USA). Ap_4A , FGF9, inhibitors of FGFR3 activity (PD173074) and mitogen-activated protein kinase pathway (U0126) were purchased from Sigma (St. Louis, MO, USA).

Cells

RCJ3.1C5.18-cells are a sub-clonal mesenchymal cell line that differentiate into chondrocytes and become chondrogenic [15]. They can be stably transfected with full-length human wild-type or the G380R mutant FGFR3 [1, 2] that is the characteristic of the most common achondroplasia. RCJ3.1C5.18 transfected with full-length human wild-type (RCJ-FGFR3-WT-tet cells) or with the G380R mutant FGFR3 (RCJ-FGFR3-G380R-tet cells) were kindly provided by Prochon Biotech (Israel). 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 MEM alpha medium was supplemented with 15% heat-inactivated foetal bovine serum, 1% penicillin–streptomycin and 50 $\mu\text{g}/\text{ml}$ hygromycin. Cells were incubated at 37°C with 5% CO_2 .

Microscopy and cell size relative quantification

Cells were observed and monitored by confocal microscopy using a Zeiss Axiovert 200 M microscope equipped with a LSM 5 Pascal confocal module (Zeiss, Oberkochen, Germany). All images were taken with the LSM Pascal Software, using $\times 20$ plastic DIC objective also supplied by Zeiss, which permits interferential Nomarski contrast images of living cells plated in a plastic well.

Cell size was calculated and normalized by the LSM Pascal Software (Zeiss, Oberkochen, Germany). Briefly, cells perimeters were calculated at time 0 (100%). This value was originally expressed in microns by means of the “Physiology” software package. The cells were individually selected with the region of interest tool (ROI) and any single cell was analysed by means of the “distance” tool. The ROI tool allows you to select the threshold to differentiate the cells from the background. This permits an accurate profile (perimeter) of the cell under study (and its prolongations) that can be calculated into microns by means of the “distance” tool. The obtained value was 100%. At the indicated times (105 and 210 min), cell perimeters were calculated again (under the same threshold conditions) and the values (expressed in microns) normalized with the value obtained at time 0.

Pharmacology

For the concentration-response studies, FGF9 was assayed at concentrations ranging from 10^{-12} to 5×10^{-8} g/mL in

order to display the corresponding dose–response curve. The pD_2 value was taken as the $-\log(EC_{50})$, i.e. the (FGF9) that produced 50% of the maximal effect.

The effect of Ap_4A was studied at a fixed dose of 25 ng/mL of FGF9 and graded concentrations of the dinucleotide from 10^{-11} to 10^{-4} M were used. The $-\log$ dose of Ap_4A that inhibited the response to FGF9 by 50%, i.e. the pD_2 , was calculated by means of the computer programme MICROCAL ORIGIN 8.0 (Microcal Software Inc., Northampton, MA, USA).

MQAE measurements

Intracellular chloride concentration was measured using MQAE as a fluorescent indicator [16]. MQAE has a high sensitivity to chloride, which interacts with and quenches the dye in its excited state. Changes in MQAE fluorescence should, therefore, inversely reflect changes in intracellular chloride concentration. Thus, a decrease in intracellular MQAE fluorescence indicates an increase in intracellular chloride concentration [17].

RCJ-FGFR3-G380R-tet cells were grown to confluence in 96-well plates and loaded overnight with 0.8 mM MQAE at 37°C. Before the experiment started the medium was removed, and each well was washed three times with chloride-containing buffer. The composition of the chloride-containing buffer was: 2.4 mM K_2HPO_4 , 0.6 mM KH_2PO_4 , 1 mM $CuSO_4$, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM D-glucose and 130 mM NaCl. The wells were incubated with chloride-containing buffer (to induce chloride channel activation) for 10 min at 37°C. The buffer was then removed and replaced by 100 μ l of chloride-free buffer containing the different agonists (FGF9, Ap_4A and FGF9+ Ap_4A) at the desired concentration. The composition of this buffer was identical to that with chloride except that the NaCl was replaced with equimolar $NaNO_3$.

In experiments where inhibitors were used, these were added to both chloride-containing buffer for 10 min and chloride-free buffer with agonists.

The plates were then read on a Fluoroskan Ascent FI (Thermo Systems) at 360 nm excitation wavelength and emission at wavelength 460 nm. Data are expressed as fluorescence variation ($F_t - F_0$), where F_t is the fluorescence at final time recorded in the experiment and F_0 is the initial fluorescence.

Statistical analysis

All data are presented as the mean $\pm$ SE mean. Significant differences were determined by two-tailed Student's *t* test. The plotting and fitting of dose–response curves was carried out with the computer programme MICROCAL ORIGIN 8.0 (Microcal Software Inc., Northampton, MA, USA).

Results

Changes in RCJ-FGFR3-G380R-tet cell morphology

RCJ-FGFR3-G380R-tet cells challenged with FGF9 (25 ng/ml), changed their morphology as it can be observed in Fig. 1a (upper panels). Cells, after being challenged with FGF9 started to increase their size suggesting an inflow of water from the extracellular medium.

When the dinucleotide diadenosine tetraphosphate, Ap_4A (10^{-4} M), was tested alone it did not modify cell morphology (Fig. 1a (middle panels)). When this dinucleotide was present in the medium together with FGF9, RCJ-FGFR3-G380R-tet cells did not change their morphology as when they were incubated with FGF9 alone (Fig. 1a (lower panels)).

Normalization of the size of cells can be seen in Fig. 1b. RCJ-FGFR3-G380R-tet cells were measured as described in methods to obtain the relative size changes in the presence of FGF9 ($n=156$) as well as in the presence of, Ap_4A ($n=118$) and FGF9+ Ap_4A ($n=125$). There was a maximal change in size of 126% in the cells treated with FGF9 and a maximal variation of 105% in the case of FGF9+ Ap_4A , showing that Ap_4A was able to prevent the change in size triggered by FGF9.

Changes in chloride flux

When tetracycline was present in the medium and consequently FGFR3 expression is inhibited, no variations in chloride flux were observed in neither of the cell lines RCJ-FGFR3-G380R-tet or RCJ-FGFR3-WT-tet (Fig. 2a).

Removal of tetracycline induced FGFR3 expression. Under these experimental conditions and in the absence of FGF9, an increase in MQAE fluorescence occurred in RCJ-FGFR3-G380R-tet, indicating an initial chloride efflux that produced a decrease in intracellular chloride concentration (Fig. 2a). In contrast, addition of FGF9 induced a decrease in intracellular MQAE fluorescence in RCJ-FGFR3-G380R-tet that reflected an increase in intracellular chloride concentration. Concerning RCJ-FGFR3-WT-tet no changes in chloride flux were detected with or without FGF9 exposure.

Exposure of RCJ-FGFR3-G380R-tet cells to varying concentrations of FGF9 permitted to create a concentration–response relationship (Fig. 2b). The resulting curve yielded a pD_2 value of 9.3 ± 0.2 ($n=6$). As expected, when G380R FGFR3 mutant was not expressed (medium containing tetracycline was used) any modification in chloride flux was found.

Effect of Ap_4A on FGF9-induced chloride flux changes

The possible effect of Ap_4A on the chloride flux was tested (Fig. 3). Ap_4A alone did not modify the cytosolic chloride

Fig. 1 Morphology of RCJ-FGFR3-G380R-tet cells. **a** Cells were grown in wells and exposed to FGF9 alone (25 ng/ml), Ap_4A (10^{-4} M) or FGF9 with Ap_4A (10^{-4} M). Phase contrast images were taken at the indicated times with an objective magnification of $\times 20$. Scale bars = 20 μm . **b** Relative quantification of the size of RCJ-FGFR3-G380R-tet cells treated FGF9 alone (25 ng/ml), Ap_4A (10^{-4} M) or FGF9 with Ap_4A (10^{-4} M). *** $P < 0.0001$; ** $p < 0.01$

levels in RCJ-FGFR3-G380R-tet cells. At a fixed dose of FGF9 (25 ng/ml), diadenosine tetraphosphate reversed the FGF9-induced increase in intracellular chloride. As shown in Fig. 3, the behaviour of Ap_4A was concentration dependent, the pA_2 value being 7.9 ± 0.3 ($n=6$).

Effect of inhibitors on FGF9-induced chloride flux changes

To examine the role of FGFR3 and ERK1/2 phosphorylation in FGF9-induced chloride flux changes, selective inhibitors were used (Fig. 4). Treatment with Ap_4A

prevented the FGF9-induced reduction in MQAE fluorescence (indicative of cytosolic chloride concentration increase).

Pre-treatment of RCJ-FGFR3-G380R-tet cells with PD173074, which inhibits the FGFR3 autophosphorylation, abolished the FGF9-induced changes in chloride flux, confirming that the increase in the cytosolic chloride concentration is mediated by FGFR3 activation. On the other hand, treatment with U0126, a selective inhibitor of activation of mitogen-activated protein kinases (ERK1/2), prevented the FGF9-induced increase in intracellular

Fig. 2 Effect of FGF9 on chloride flux in RCJ-FGFR3-WT-tet and RCJ-FGFR3-G380R-tet cells. **a** Cells were examined pre- and post-tetracycline withdrawal as well as in the absence or in the presence of FGF9. Summary bar graphs show the changes in MQAE fluorescence. **b** Concentration-response curves obtained after treatment of RCJ-FGFR3-G380R-tet cells with graded doses of FGF9 (from 10^{-12} to 5×10^{-8} g/mL). Fitting was performed by means of the programme Microcal Origin as indicated in “Materials and methods”. Each data point is the mean $\pm$ s.e.m. of six independent experiments. *** $P < 0.0001$

chloride concentration pointing to the involvement of ERK1/2 signalling in FGF9-induced chloride flux variations. In the absence of FGF9, Ap₄A and the inhibitors PD173074 and U0126 did not modify chloride flux.

Discussion

RCJ-FGFR3-G380R-tet cells have been widely used as a cellular model for achondroplasia and therefore they are an interesting tool to understand and find possible treatments for this pathology [2, 18]. It is necessary, nevertheless to be aware that these cells overexpress the FGFR3 receptor. The present work describes the effect of FGF9, ligand for FGFR3 on cell size and chloride flux in RCJ-FGFR3-G380R-tet cells, as well as the action of Ap₄A on FGF9-induced processes.

Fig. 3 Concentration-response analysis of Ap₄A effect on changes in intracellular chloride concentration induced by FGF9 in RCJ-FGFR3-G380R-tet cells. The action of Ap₄A alone or Ap₄A together with FGF9 (25 ng/ml) were tested at doses from 10^{-11} to 10^{-4} M, creating a concentration-response curve which was fitted by means of the programme Microcal Origin as indicated in “Materials and methods”. Each data point is the mean $\pm$ SEM of six independent experiments

Our data show that FGFR3 activation induced by FGF9 leads to an enlarged RCJ-FGFR3-G380R-tet cell size and an increase in the intracellular chloride concentration, presumably as a consequence of blocking chloride efflux.

Several reports have shown the existence of different chloride channels in chondrocytes [19, 20] as well as a Na⁺/K⁺/2Cl⁻ cotransporter [21]. Chloride channels contribute to

Fig. 4 Effect of FGFR3 and pERK1/2 inhibitors on changes in chloride flux elicited by FGF9. RCJ-FGFR3-G380R-tet cells were treated with 25 ng/ml FGF9 alone or together with Ap₄A (10^{-4} M). The FGFR3 antagonist PD173074 (100 nM) reversed the effect of FGF9, and the inhibitor of ERK1/2 phosphorylation U0126 (200 μ M) also blocked the effect of FGF9. Data corresponding to treatment with Ap₄A or inhibitors alone are also shown. Values represent the mean $\pm$ SE of six independent experiments. *** $P < 0.0001$

various cellular functions in non-excitable cells including regulation of cell volume, regulation of the intracellular pH in cooperation with other transporters, and setting the membrane potential [22]. Multiple signalling pathways have been proposed to control chloride channel activity. These include tyrosine kinase signalling pathways. In articular chondrocytes, using the protein tyrosine phosphatase inhibitor sodium orthovanadate, it has been shown that activity of endogenous protein tyrosine kinases is required for the regulation of chloride currents [23]. The precise role of protein tyrosine kinase seems to vary according to the cell type, and diverse protein tyrosine kinases can exhibit antagonistic effects [24]. In this sense, the receptor protein tyrosine kinase epidermal growth factor promotes a swelling-activated chloride current in cardiac myocytes whereas the non-receptor protein tyrosine kinase Src inhibits it [25–27]. In the present study, the activation of the tyrosine kinase receptor G380R mutant FGFR3 by FGF9 results in chloride flux blockade. This effect was inhibited by a selective inhibitor of FGFR3 autophosphorylation, confirming the link between mutant FGFR3 tyrosine phosphorylation and the decrease in chloride flux. Thus, our results provide additional data about the role of this tyrosine kinase receptor, mutant FGFR3, in chloride channel regulation.

Moreover, considering that the activation of mitogen-activated protein kinase pathway (ERK1/2 pathway) is one of the immediate downstream signal transduction events in response to FGFR3 autophosphorylation, the involvement of this intracellular pathway in FGF9-induced changes in chloride flux was also evaluated. The use of a selective inhibitor of ERK1/2 activity showed that ERK1/2 phosphorylation takes part in the decrease in chloride flux observed after FGF9 exposure. The relationship between mitogen-activated protein kinase pathway and chloride current modulation has already demonstrated in astrocytes and cardiac myocytes [28, 29].

The antagonistic effect produced by the ERK1/2 phosphorylation inhibitor, U0126, was not as great as that induced by the inhibitor of FGFR3 autophosphorylation, PD173074. The application of both compounds alone, in the absence of FGF9, did not significantly contribute to any change in the chloride efflux. Altogether these data suggest that the contribution of other downstream signal transduction pathways activated by mutant FGFR3 cannot be ruled out, but it remains elusive at present.

When the dinucleotide Ap₄A was added to the medium together with FGF9 morphological changes were not observed in RCJ-FGFR3-G380R-tet cells and at concentrations from 1 nM to 100 μ M Ap₄A counteracted the FGF9-mediated intracellular chloride concentration increase. In particular, Ap₄A reversed the effect of FGF9 on chloride flux in a similar manner to that provoked by the

inhibitor of ERK1/2 phosphorylation. These results are consistent with our previous work [13], in which we described the ability of Ap₄A to attenuate the sustained ERK1/2 phosphorylation elicited by FGF9. Thus, again, it would seem that ERK1/2 phosphorylation is associated with closure of the chloride ion channel.

Concerning the mechanism by which Ap₄A produces the reversion of the FGF9 effect on chloride and cell size in these cells, it is very likely that it is related to the lack of internalization of the mutated receptor [1]. As it has been previously demonstrated, Ap₄A is capable to modify the degradation rate of the receptor by activating both proteosomal and lysosomal pathways [13]. This means that the number of mutant receptors in the membrane after the application of the dinucleotide is reduced and therefore the effect on chloride flux is affected by that drop of receptors.

Our results have been obtained with a cell line and not with achondroplastic chondrocytes. This suggests that some differences in the results may occur between our cell line and the native achondroplastic cells. Nevertheless, this RCJ 3.1C5.18 cell line expresses differentiated and terminally differentiated chondrocyte phenotypes in a sequence that mimics chondrocytes in the endochondral growth plate [30, 31]. For this reason, RCJ 3.1C5.18 cell line has been widely used until now to analyse different aspects related to biochemical chondrocyte behaviour [32–34]. In addition, RCJ 3.1C5.18 cell line produces both collagen types II and X at different stages of their development. For all these reasons we think that our results may mimic what happened in the achondroplastic cartilage growth plate.

In summary, the changes in cell size and chloride flux observed after G380R mutant FGFR3 activation by FGF9 could contribute to the progressive dysfunction and hypertrophy of achondroplastic chondrocytes observed in this disease. These changes are mediated, at least in part, by ERK1/2 phosphorylation. Ap₄A counteracted the FGF9-induced responses in RCJ-FGFR3-G380R-tet cells including changes in cell volume and chloride flux. Our findings indicate that Ap₄A is a potential pharmacological agent to alleviate the biochemical and pathophysiological signs that occur in achondroplasia.

Acknowledgements This work has been supported by research grants from Fundacion Ramon Areces (Acondroplasia 2010) and Fundacion Magar. We express our gratitude to PROCHON BIOTECH laboratories for the provision of RCJ-FGFR3-G380R-tet cells.

References

1. Monsonego-Ornan E, Adar R, Feferman T, Segev O, Yaron A (2000) The transmembrane mutation G380R in fibroblast growth factor receptor 3 uncouples ligand-mediated receptor activation from down-regulation. *Mol Cell Biol* 20:516–522

2. Monsonogo-Ornan E, Adar R, Rom E, Yayon A (2002) FGF receptors ubiquitylation: dependence on tyrosine kinase activity and role in downregulation. *FEBS Lett* 528:83–89
3. L'Hote CG, Knowles MA (2005) Cell responses to FGFR3 signalling: growth, differentiation and apoptosis. *Exp Cell Res* 304:417–431
4. Yasoda A, Komatsu Y, Chusho H, Miyazawa T, Ozasa A, Miura M, Kurihara T, Rogi T, Tanaka S, Suda M, Tamura N, Ogawa Y, Nakao K (2004) Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat Med* 10:80–86
5. Urban JP, Hall AC, Gehl KA (1993) Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J Cell Physiol* 154:262–270
6. Wohlrab D, Lebek S, Kruger T, Reichel H (2002) Influence of ion channels on the proliferation of human chondrocytes. *Biorheology* 39:55–61
7. Wohlrab D, Vocke M, Klapperstuck T, Hein W (2004) Effects of potassium and anion channel blockers on the cellular response of human osteoarthritic chondrocytes. *J Orthop Sci* 9:364–371
8. Guzman-Aranguel A, Crooke A, Peral A, Hoyle CH, Pintor J (2007) Dinucleoside polyphosphates in the eye: from physiology to therapeutics. *Prog Retin Eye Res* 26:674–687
9. Miras-Portugal MT, Gualix J, Mateo J, Diaz-Hernandez M, Gomez-Villafuertes R, Castro E, Pintor J (1999) Diadenosine polyphosphates, extracellular function and catabolism. *Prog Brain Res* 120:397–409
10. Pintor J, Diaz-Hernandez M, Gualix J, Gomez-Villafuertes R, Hernando F, Miras-Portugal MT (2000) Diadenosine polyphosphate receptors. from rat and guinea-pig brain to human nervous system. *Pharmacol Ther* 87:103–115
11. Stavrou BM (2003) Diadenosine polyphosphates: postulated mechanisms mediating the cardiac effects. *Curr Med Chem Cardiovasc Hematol Agents* 1:151–169
12. Guzman-Aranguel A, Irazu M, Yayon A, Pintor J (2008) P2Y receptors activated by diadenosine polyphosphates reestablish Ca (2+) transients in achondroplastic chondrocytes. *Bone* 42:516–523
13. Guzman-Aranguel A, Irazu M, Yayon A, Pintor J (2007) Effect of diadenosine polyphosphates in achondroplastic chondrocytes: inhibitory effect of Ap4A on FGF9 induced MAPK cascade. *Biochem Pharmacol* 74:448–456
14. Segev O, Chumakov I, Nevo Z, Givol D, Madar-Shapiro L, Sheinin Y, Weinreb M, Yayon A (2000) Restrained chondrocyte proliferation and maturation with abnormal growth plate vascularization and ossification in human FGFR-3(G380R) transgenic mice. *Hum Mol Genet* 9:249–258
15. Grigoriadis AE, Heersche JN, Aubin JE (1996) Analysis of chondroprogenitor frequency and cartilage differentiation in a novel family of clonal chondrogenic rat cell lines. *Differentiation* 60:299–307
16. Koncz C, Dargatzis JT (1994) Use of MQAE for measurement of intracellular [Cl⁻] in cultured aortic smooth muscle cells. *Am J Physiol* 267:H2114–H2123
17. Marandi N, Konnerth A, Garaschuk O (2002) Two-photon chloride imaging in neurons of brain slices. *Pflügers Arch* 445:357–365
18. Guzman-Aranguel A, Crooke A, Yayon A, Pintor J (2008) Effect of PPADS on achondroplastic chondrocytes: inhibition of FGF receptor type 3 over-activity. *Eur J Pharmacol* 584:72–77
19. Sugimoto T, Yoshino M, Nagao M, Ishii S, Yabu H, Tsuga K, Tohse N, Yoshino M, Sugimoto T, Yamashita T, Ishii S, Yabu H (1996) Voltage-gated ionic channels in cultured rabbit articular chondrocytes. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 115:223–232
20. Tsuga K, Tohse N, Yoshino M, Sugimoto T, Yamashita T, Ishii S, Yabu H (2002) Chloride conductance determining membrane potential of rabbit articular chondrocytes. *J Membr Biol* 185: 75–81
21. Trujillo E, Alvarez de la Rosa D, Mobasher A, Gonzalez T, Canessa CM, Martin-Vasallo P (1999) Sodium transport systems in human chondrocytes. II. Expression of ENaC, Na⁺/K⁺/2Cl⁻ cotransporter and Na⁺/H⁺ exchangers in healthy and arthritic chondrocytes. *Histol Histopathol* 14:1023–1031
22. Nilius B, Droogmans G (2003) Amazing chloride channels: an overview. *Acta Physiol Scand* 177:119–147
23. Okumura N, Imai S, Toyoda F, Isoya E, Kumagai K, Matsuura H, Matsusue Y (2009) Regulatory role of tyrosine phosphorylation in the swelling-activated chloride current in isolated rabbit articular chondrocytes. *J Physiol* 587:3761–3776
24. Franco R, Panayiotidis MI, de la Paz LD (2008) Autocrine signaling involved in cell volume regulation: the role of released transmitters and plasma membrane receptors. *J Cell Physiol* 216:14–28
25. Du XL, Gao Z, Lau CP, Chiu SW, Tse HF, Baumgarten CM, Li GR (2004) Differential effects of tyrosine kinase inhibitors on volume-sensitive chloride current in human atrial myocytes: evidence for dual regulation by Src and EGFR kinases. *J Gen Physiol* 123:427–439
26. Ren Z, Baumgarten CM (2005) Antagonistic regulation of swelling-activated Cl⁻ current in rabbit ventricle by Src and EGFR protein tyrosine kinases. *Am J Physiol Heart Circ Physiol* 288:H2628–H2636
27. Walsh KB, Zhang J (2005) Regulation of cardiac volume-sensitive chloride channel by focal adhesion kinase and Src kinase. *Am J Physiol Heart Circ Physiol* 289:H2566–H2574
28. Crepel V, Panenka W, Kelly ME, MacVicar BA (1998) Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. *J Neurosci* 18:1196–1206
29. d'Anglemont de Tassigny A, Berdeaux A, Souktani R, Henry P, Ghaleh B (2008) The volume-sensitive chloride channel inhibitors prevent both contractile dysfunction and apoptosis induced by doxorubicin through PI3kinase, Akt and Erk 1/2. *Eur J Heart Fail* 10:39–46
30. Lunstrum GP, Keene DR, Weksler NB, Cho YJ, Cornwall M, Horton WA (1999) Chondrocyte differentiation in a rat mesenchymal cell line. *J Histochem Cytochem* 47:1–6
31. Spagnoli A, Hwa V, Horton WA, Lunstrum GP, Roberts CT Jr, Chiarelli F, Torello M, Rosenfeld RG (2001) Antiproliferative effects of insulin-like growth factor-binding protein-3 in mesenchymal chondrogenic cell line RCJ3.1C5.18. relationship to differentiation stage. *J Biol Chem* 276:5533–5540
32. Lau WF, Tertinegg I, Heersche JN (1993) Effects of retinoic acid on cartilage differentiation in a chondrogenic cell line. *Teratology* 47:555–563
33. Weksler NB, Lunstrum GP, Reid ES, Horton WA (1999) Differential effects of fibroblast growth factor (FGF) 9 and FGF2 on proliferation, differentiation and terminal differentiation of chondrocytic cells in vitro. *Biochem J* 342:677–682
34. Fernandez C, Jami S, Loredi G, Ko F, Hahn T, McDougall S, Peters JH (2010) Recognition of the alternatively spliced segments of fibronectin by the RCJ 3.1C5.18 chondrocytic rat cell line. *Osteoarthritis Cartilage* 18:228–239