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Diadenosine polyphosphates Ap3A and Ap4A, but not Ap5A or Ap6A, induce proliferation of vascular smooth muscle cells

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ABSTRACT

Depending on the number of phosphate groups, diadenosine polyphosphates (ApnA, Ap3A, Ap4A, Ap5A and Ap6A) differ in properties such as proliferation, apoptosis, vasoconstriction and vasodilatation of vascular smooth muscle cells (VSMCs). Possible signaling pathways leading to effects such as proliferation are still unknown. This study examined the proliferative effects of diadenosine polyphosphates on VSMCs and their intracellular pathways.

Proliferation of VSMCs was measured by the cell count and [^{3}H] thymidine incorporation. Phosphorylation of the MAP kinases ERK1/2 was determined by Western blotting. Single-cell $[Ca^{2+}]_i$ measurements were done to determine the influence of $[Ca^{2+}]_i$ on intracellular signaling. Stress fiber formation was assessed by fluorescence microscopy to detect an influence of $G\alpha_{12}$.

Ap3A and Ap4A, but not Ap5A or Ap6A, were shown to increase proliferation of VSMCs by activating P2Y receptors, which leads to stimulation of the Ras-Raf-MEK-ERK1/2 cascade. Ap3A- and Ap4A-induced activation of the MAP kinases ERK1/2 was dependent on a signaling pathway that included the EGF receptor, PKC, PLCβ and the increase of $[Ca^{2+}]_i$.

In conclusion, Ap3A and Ap4A, but not Ap5A or Ap6A, induce proliferation of VSMCs by a signaling pathway that begins with activation of P2Y receptors and leads to stimulation of the MAP kinases ERK1/2.

1. Introduction

Diadenosine polyphosphates (Ap3A, Ap4A, Ap5A and Ap6A; ApnA) belong to a group of compounds formed by two adenosine molecules linked to a specific number of phosphates. Depending on the number of phosphate groups, diadenosine polyphosphates differ in properties such as vasoconstriction and vasodilatation of vascular smooth muscle cells (VSMCs), as already described [1,2]. Because of their known proliferative properties, diadenosine polyphosphates are thought to be an important additional atherogenic factor [3]. Diadenosine polyphosphates were found to be increased in platelets of patients with high-risk factors for atherosclerosis. Possible signaling transduction pathways that may explain the different effects of diadenosine polyphosphates still remain unclear.

Purinergic receptors that mediate ApnA-induced effects in VSMCs are divided into P2X ligand-gated ion channel and P2Y G-protein-coupled receptor families [4]. Seven P2X subunits that may assemble in different combinations and eight metabotropic P2Y receptors have been characterized thus

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far. Heterotrimeric guanine nucleotide-binding proteins (G-proteins) coupled to P2Y receptors are composed of an α, β and γ subunit and are characterized by the identity of the α subunit that can be classified as $G\alpha_s$, $G\alpha_{i/0}$, $G\alpha_q$ or $G\alpha_{12}$. Except for $G\alpha_{12}$, all G-protein subfamilies have been shown to bind with P2Y receptors. Activation of G-protein receptors can induce different intracellular signaling pathways that evoke a response such as activation of mitogen-activated protein kinases (MAPKs). The MAPK family consists of three subfamilies: extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAP kinase. They are important components of signaling pathways contributing to effects such as proliferation, differentiation or apoptosis [5,6].

The aim of this study was to investigate the proliferative effects of the diadenosine polyphosphates Ap3A, Ap4A, Ap5A and Ap6A on VSMCs. To elucidate the different properties of these diadenosine polyphosphates, we examined potential intracellular signaling pathways by which they contribute to increased proliferation of VSMCs.

2. Material and methods

2.1. Materials

Culture media, fetal calf serum and trypsin were supplied by Invitrogen (Karlsruhe, Germany). PBS was purchased from Seromed (Vienna, Austria). Diadenosine polyphosphates (Ap3A–Ap6A) were obtained from Schlüter et al. (Charité – Universitätsmedizin, Campus Benjamin Franklin, Medical Clinic IV) [7]. Antagonists such as PD98059, bisindolylmaleimid (Bim), U73122, AG1478, AG1295 and substances such as thapsigargin and thrombin were purchased from Calbiochem (San Diego, USA).

2.2. Cell culture

Primary cultures of vascular smooth muscle cells from newborn rats were established as previously described [8]. Cells were grown in minimal essential medium supplemented with 10% fetal calf serum (complete medium, CM), 2% tryptose phosphate

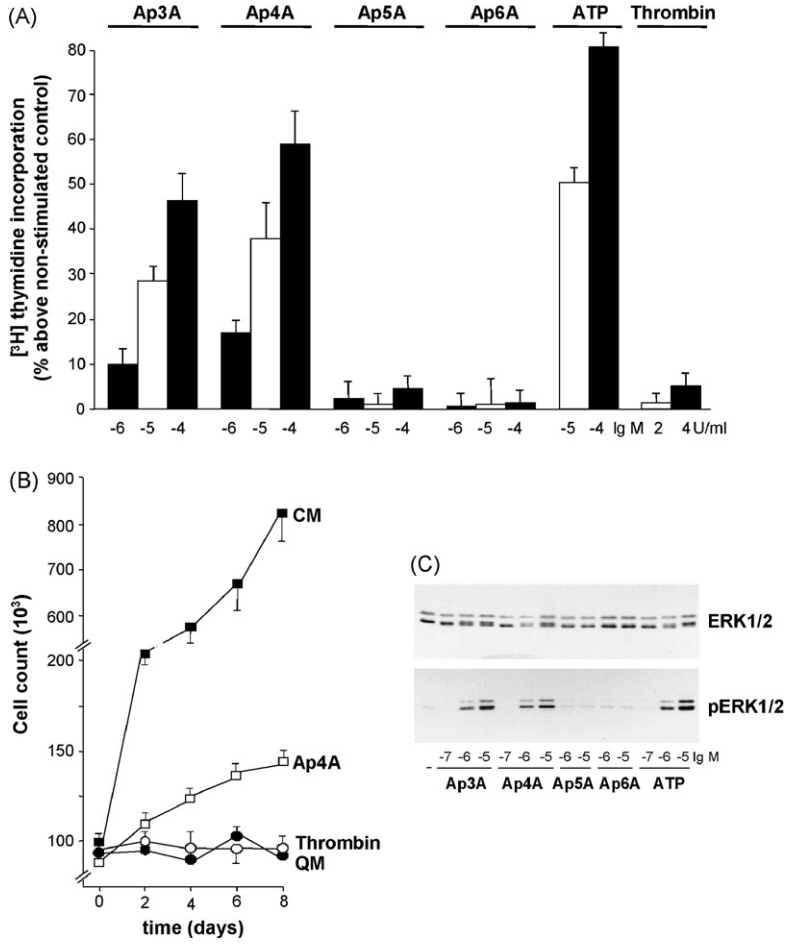

Fig. 1 – Ap3A and Ap4A induce proliferation of VSMCs. Panel A: VSMCs were exposed to the different compounds for 20 h. [Methyl-^{3}H] thymidine was added for 4 h, and radioactivity incorporation was determined. Panel B: VSMCs were maintained in serum-free quiescent medium (QM), complete medium (CM), QM with thrombin (2 U/mL), and QM with Ap4A (0.1 mM). Cells were counted every second day in a Neubauer counting chamber. The depicted means ± S.E. were calculated from at least eight separate counts. Panel C: serum-starved VSMCs were stimulated with Ap3A, Ap4A, Ap5A, Ap6A and ATP (0.1–1.0–10 μM). Whole cell lysates were subjected to Western blot and incubated with ERK1/2 and pERK1/2 antibodies as described above.

broth, penicillin (50 units/mL) and streptomycin (50 units/mL). Cells from passages 8–15 were used in all experiments. Growth arrest was induced in a serum-free quiescent medium (QM) containing 1% bovine serum albumin and 4 mg/mL of transferrin instead of serum. Prior to agonist application, cells were maintained in QM for 72 h. For inhibiting experiments VSMCs were incubated with specific antagonists for 1 h. Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. They were serum-starved by incubation with DMEM supplemented with 25% Ham's F-12, 0.2% $NaHCO_3$, 10 nM Na-Hepes for at least 24 h before exposure to different agonists.

2.3. Western blot

VSMCs were directly lysed in Laemmli buffer containing 10 mM dithiothreitol. ERK1/2 and phosphorylated ERK1/2 were separated on 10% polyacrylamide gels and electroblotted to nitrocellulose membranes. ERK1/2 and phosphorylated ERK1/2 were detected using anti-ERK1/2 and anti-pERK1/2 antibodies (1:1000, New England Biolabs Inc., US). Primary antibodies were detected with horseradish peroxidase-coupled secondary antibody (1:2000, New England Biolabs, Inc., US) and a chemiluminescent substrate (New England Biolabs, Inc., US).

2.4. Single-cell $[Ca^{2+}]_i$ measurements

Cells were seeded on 24 mm glass coverslips and grown for 24 h prior to loading with 2 μM fura-2 in a buffer (Hepes-buffered saline) containing 135 mM CaCl, 6 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 10 mM Hepes pH 7.4 and 0.2% bovine serum albumin. Coverslips were mounted in a monochromator-equipped (TILL-Photonics) inverted microscope (Carl Zeiss). Fura-2 was alternately excited at 340 and 380 nm. Emitted light was filtered

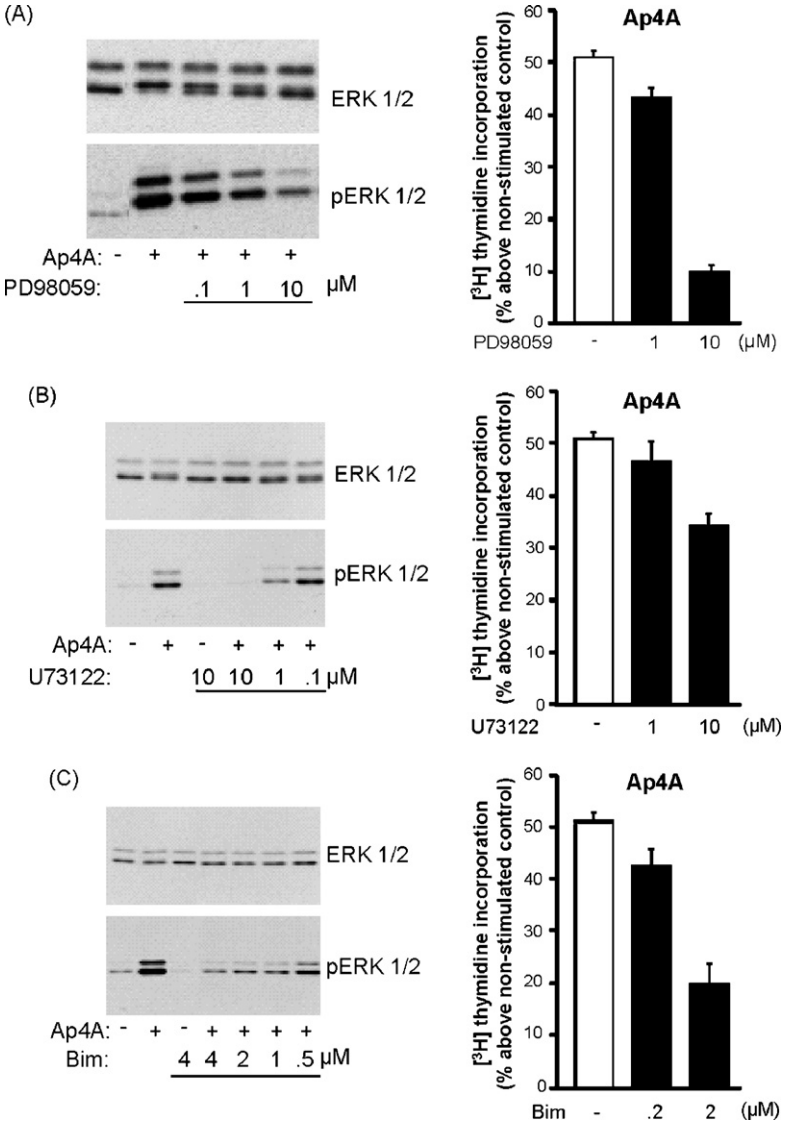

Fig. 2 – Ap4A-induced phosphorylation of ERK1/2 and increase of [methyl-^{3}H] thymidine incorporation are dependent on activation of MEK1/2, PLCβ and PKC. The specific inhibitors of MEK1/2 (PD98059, Panel A), PLCβ (U73122, Panel B), and PKC (Bim, Panel C) were used to determine their impact on Ap4A-induced (10 μM) phosphorylation of ERK1/2 and increase of [methyl-^{3}H] thymidine incorporation. Western blot analysis and measurement of [methyl-^{3}H] thymidine incorporation were done as described above.

(505 nm long pass) and recorded with a 12-bit CCD camera. After correction for background signals, intracellular $[Ca^{2+}]_i$ was calculated as described [9]. R_{max}, R_{min} and F_{max} 380/F_{min} 380 were determined in fura-2-loaded cells equilibrated for 3 h in Hepes-buffered saline supplemented with 1 μM ionomycin and either 10 mM Ca^{2+} or 10 mM EGTA, pH 7.8. The demonstrated figures of single-cell $[Ca^{2+}]_i$ measurements are representative results of at least 4 experiments.

2.5. [^{3}H] thymidine incorporation

VSMCs were kept in 6-well plates for 3 days in CM before being serum-starved for 72 h. Agonists were added for 20 h, and [methyl-^{3}H] thymidine was added for another 4 h. Radioactivity was determined after washing cells twice with 1.5 mL of phosphate saline buffer and three times with 1 mL of ice cold 0.3 NTCA. Cells were lysed by 250 μL of 0.3 NAOH for 30 min at 37 °C. The lysate radioactivity was counted in a β-scintillation counter.

2.6. Fluorescence microscopy

Before being stimulated by different agonists for 30 min at 37 °C, Swiss 3T3 cells were seeded onto 8-well plates and serum-starved for 24 h. Stimulated cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 5 min. To visualize the cytoskeleton, cells were stained for polymerized actin by incubation with 0.5 μg/mL of fluorescein isothiocyanate-phalloidin (Sigma, Munich, Germany) for 40 min. Fluorescence was examined with an inverted microscope (Axiovert 100, Carl Zeiss, Oberkochen)

2.7. Statistical analysis

SPSS statistical software Version 11.0.1 was used for statistical analysis. All data were expressed as mean ± S.E.M. Normally distributed data were analyzed by one-way ANOVA and were Bonferroni-corrected for repeated measures over time. All experiments were performed at least 3 times. Representative results of Western blot analysis are shown. A probability value <0.05 was regarded as significant.

3. Results

3.1. Ap3A and Ap4A, but not Ap5A or Ap6A, induce cell proliferation by ERK1/2-related transduction

The different diadenosine polyphosphates Ap3A, Ap4A, Ap5A and Ap6A were assessed for their stimulatory effect on the proliferation of VSMCs by measuring [methyl-^{3}H] thymidine incorporation after 24-h activation using different concentrations (1–100 μM). Ap3A and Ap4A induced a significant dose-dependent increase of [methyl-^{3}H] thymidine incorporation (Fig. 1A), whereas Ap5A and Ap6A had no significant effect in this connection. Thrombin was the negative and ATP the positive control. To verify the real proliferation increase, VSMCs were incubated for up to 8 days with Ap4A (10 μM), thrombin (2 U/mL), complete medium with 10% fetal calf serum, and quiescent medium (Fig. 1B, complete medium, quiescent medium and medium with thrombin or Ap4A were changed every 24 h). Ap4A induced an up to 50% higher cell proliferation than quiescent medium or thrombin. CM as the positive control induced a proliferation of VSMCs ranging up to 8 times higher than the basal level. Ap3A, but not Ap5A or Ap6A, showed the same property as Ap4A according to the results obtained by measuring [methyl-^{3}H] thymidine incorporation (data not shown). To examine a possible intracellular signaling transduction pathway underlying the Ap3A- and Ap4A-induced increase of cell proliferation, Western blot analysis was done to determine the ratio of the phosphorylated MAP kinases ERK1/2 (Fig. 1C). VSMCs were stimulated for 5 min with different concentrations of Ap3A, Ap4A, Ap5A, Ap6A and ATP (1–100 μM). According to the above results, stimulation by Ap3A and Ap4A, but not by Ap5A or Ap6A, leads to an increased ratio of phosphorylated ERK1/2. To demonstrate equal loading of the lanes, blots were reprobed with anti-total ERK1/2.

3.2. Activation of MEK, PLCβ and PKC alters ERK1/2 phosphorylation

The specific MEK1/2 inhibitor PD98059 (0.1–10 μM), the PLCβ inhibitor U73122 (0.1–10 μM), and the PKC inhibitor bisindolylmaleimide (Bim) (0.5–4 μM) were used to define parts of an

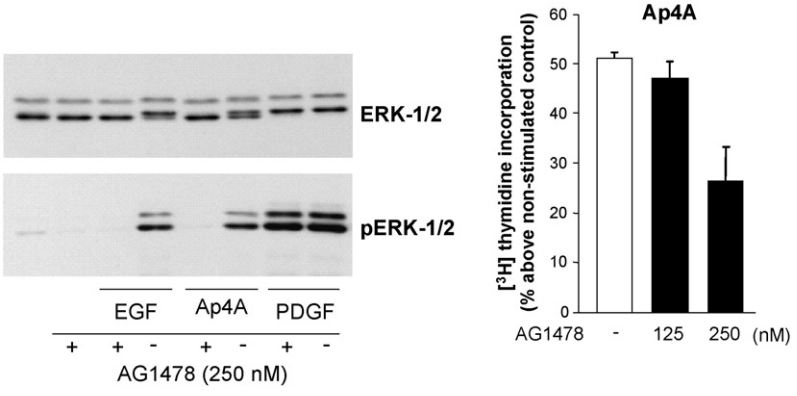

Fig. 3 – Transactivation of the EGF receptor mediates Ap4A-induced phosphorylation of ERK1/2 and increase of [methyl-^{3}H] thymidine incorporation. VSMCs were preincubated with the EGF receptor inhibitor AG1478 at the indicated concentrations before measuring Ap4A-induced (10 μM) phosphorylation of ERK1/2 by Western blot and Ap4A (10 μM)-induced [methyl-^{3}H] thymidine incorporation.

intracellular signaling pathway leading to increased ERK1/2 phosphorylation after stimulation by Ap4A (Ap3A always acted in the same way as Ap4A, data not shown) (Fig. 2). Preincubation of VSMCs with all inhibitors before activation by Ap4A (10 μM) resulted in a significant dose-dependent reduction of [methyl-^{3}H] thymidine incorporation and ERK1/2 phosphorylation, indicating that these signaling proteins play a role in the Ap4A-induced pathway leading to ERK1/2 activation and ultimately to increased proliferation of VSMCs.

3.3. *Ap4A induces transactivation of the EGF receptor*

To investigate a possible transactivation of the EGF and PDGF receptor in VSMCs after stimulation by Ap4A (10 μM), [methyl-^{3}H] thymidine incorporation and ERK1/2 phosphorylation were assessed after preincubation with the specific EGF receptor inhibitor AG1478 (125–250 nM) (Fig. 3) and the PDGF receptor inhibitor AG1295 (data not shown). AG1478, but not AG1295, led to a significant reduction of the Ap4A-induced effect, indicating involvement of the EGF receptor in the investigated pathway.

3.4. *Ap3A and Ap4A increase the intracellular Ca^{2+} concentration*

Single-cell $[Ca^{2+}]_i$ analysis was done to evaluate the change of intracellular calcium concentration in VSMCs after exposure to diadenosine polyphosphates (Fig. 4). Compared to a non-stimulated control, Ap3A and Ap4A, but not Ap5A or Ap6A, induced a significant increase of $[Ca^{2+}]_i$ (Fig. 4A). ATP and thrombin were used as positive controls. To examine whether the detected cytosolic $[Ca^{2+}]_i$ increase is due to extracellular calcium influx or intracellular calcium release, measurements were done with VSMCs cultured in calcium-free and calcium-containing medium. Activation by Ap4A was shown to induce a $[Ca^{2+}]_i$ increase that is independent of extracellular calcium (Fig. 4B).

Preincubation with thapsigargin, a specific inhibitor of intracellular Ca^{2+} transport ATPases [10], inhibited the described Ap4A-induced rise of $[Ca^{2+}]_i$ (Fig. 4C). These results suggest

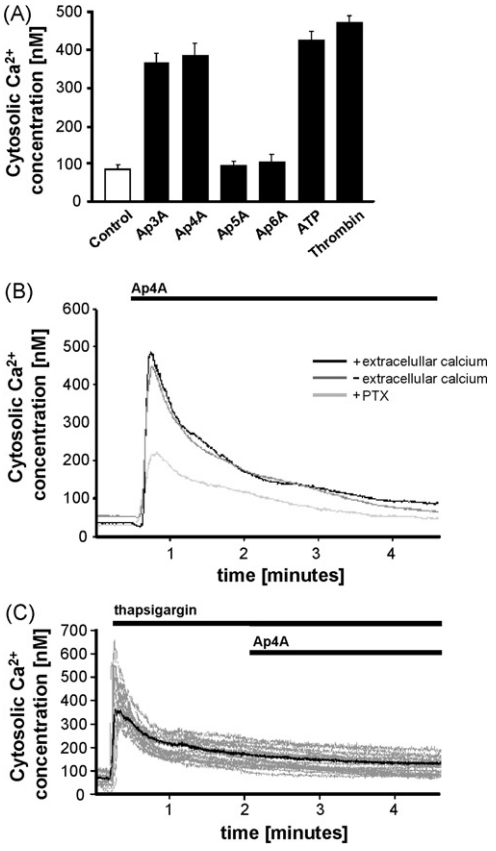

Fig. 4 – Ap3A and Ap4A, but not Ap5A or Ap6A, induce transient elevation of $[Ca^{2+}]_i$ in single-cell VSMCs. VSMCs were subcultured on glass coverslips and loaded with fura-2 (2 μM) before activation by different agonists. $[Ca^{2+}]_i$ was measured as mentioned above. Panel A: VSMCs were exposed to Ap3A, Ap4A, Ap5A, Ap6A and ATP (10 μM) and thrombin (2 U/mL). Panel B: to test the impact of PTX, VSMCs were preincubated overnight in the absence and presence of PTX (200 ng/mL) before measuring the Ap4A-induced elevation of $[Ca^{2+}]_i$. Conversely, Ap4A-induced elevation of $[Ca^{2+}]_i$ was assessed in cells maintained in medium with and without calcium. Mean $[Ca^{2+}]_i$ was calculated from all cells selected in at least 5 experiments. Panel C: VSMCs were preincubated with thapsigargin (2 μM) before exposition with Ap4A (10 μM).

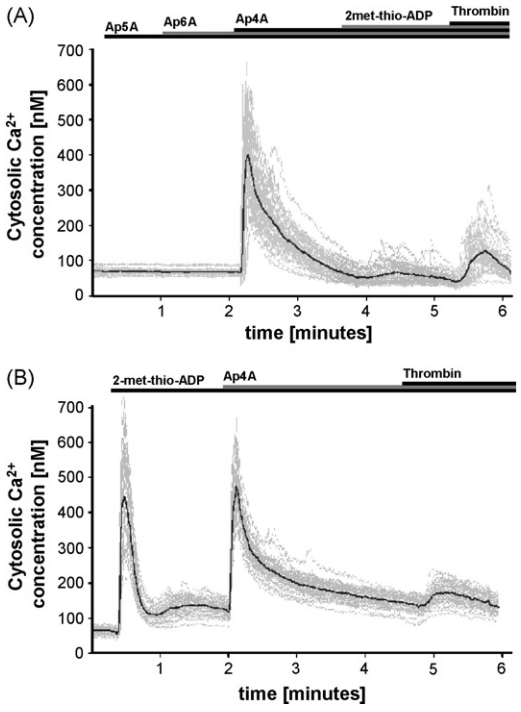

Fig. 5 – P2Y1 receptor mediates Ap4A-induced $[Ca^{2+}]_i$ elevation in VSMCs. Different agonists (diadenosine polyphosphates and 2met-thioADP: 10 mM, thrombin 2 U/mL) were used to assess the role of P2Y1 receptor in the signaling pathway that transduces the Ap4A-induced $[Ca^{2+}]_i$ elevation. Panel A: VSMCs were stimulated by Ap4A before exposure to 2met-thio-ADP. Panel B: VSMCs were activated by 2met-thio-APD before exposure to Ap4A. Mean $[Ca^{2+}]_i$ was calculated from all cells selected in at least 5 experiments.

that the $[Ca^{2+}]_i$ increase induced by Ap3A and Ap4A was due exclusively to release from cellular stores and not to transmembrane calcium influx. Furthermore, this excludes the P2X ligand-gated ion channels as the initial receptors of the Ap3A- and Apn4-induced intracellular pathway.

Possible involvement of $G\alpha_{i/0}$ was assessed by pretreating VSMCs with pertussis toxin (PTX) for 18 h before activation by ApnA. Inhibition of $G\alpha_{i/0}$ by PTX significantly limited the Ap4A-induced $[Ca^{2+}]_i$ increase. The partial inhibition suggests that both $G\alpha_{i/0}$ and $G\alpha_q$ bind with the corresponding Ap4A-activated P2Y receptors.

3.5. *Activation of P2Y1 leads to an Ap4A-induced intracellular Ca^{2+} increase*

In a further step, we investigated possible P2Y receptors that transduced the Ap4A-induced intracellular calcium signaling (Fig. 5). In this context, single-cell $[Ca^{2+}]_i$ was measured after exposure to 2met-thio-ADP, a specific P2Y1 receptor agonist [11], and Ap4A. After preincubation with 2met-thio-ADP, which itself significantly increases $[Ca^{2+}]_i$, Ap4A was found to alter single-cell $[Ca^{2+}]_i$. Conversely, 2met-thio-ADP did not alter $[Ca^{2+}]_i$ after preincubation with Ap4A, that may be explained because of already activated P2Y1 receptors after preincubation with Ap4A. The different effects of Ap4A and 2met-thio-ADP, which depended only in the order of addition of theses substances, indicates that P2Y1 is one of several P2Y receptors that mediate the Ap4A-induced effect on VSMCs.

3.6. *Diadenosine polyphosphates do not induce stress fiber formation*

Stress fiber formation was examined in Swiss 3T3 cells to determine whether ApnA-activated P2Y receptors are coupled with $G\alpha_{12}$ or $G\alpha_{13}$ (Fig. 6). Swiss 3T3 cells were used because non-stimulated VSMCs showed no basal level of actin stress fiber formation. Measurement of single-cell $[Ca^{2+}]_i$ revealed P2Y receptors in Swiss 3T3 cells (data not shown). Stress fiber assembly was induced by the positive controls lysophosphatidic acid (LPA) and thrombin. Neither the applied diadenosine polyphosphates nor ATP changed visualized stress fiber formation, which indicates that ApnA-activated P2Y receptors are not coupled with $G\alpha_{12}$ or $G\alpha_{13}$.

4. Discussion

This study showed that Ap3A and Ap4A, but not Ap5A or Ap6A, increase the proliferation of VSMCs. The Ap4A- and Ap3A-induced effect can be explained by activation of G-protein coupled P2Y receptors leading to increased $[Ca^{2+}]_i$. Other components of the possible intracellular signaling pathway that caused the Ap4A- and Ap3A-induced rise of proliferation were transactivated EGF-receptor, PKC, PLCβ, MEK1/2 and ERK1/2 (Fig. 7).

Diadenosine polyphosphates have already been shown to regulate smooth muscle cell proliferation [12]. Thus, they are thought to be one of several regulatory factors in the development of atherosclerosis. Interestingly, properties of several diadenosine polyphosphates (ApnA) have been reported to differ depending on the variable number of phosphates. For example, different impacts on vasoconstriction and vasodilatation are described for VSMCs [1,13]. To explain these different effects, it is necessary to fully elucidate the intracellular signaling pathways mediating the different impacts of ApnA. To investigate one possible pathway, we assessed the effect of Ap3A, Ap4A, Ap5A and Ap6A on proliferation of VSMCs. Ap3A and Ap4A, but not Ap5A or Ap6A, were found to alter cell growth. The Ap4A-induced effect was mediated by metabotropic P2Y receptors that are coupled with G-proteins. P2Y receptors are known to bind to G_s, $G_{i/0}$ and G_q proteins. We attempted to demonstrate another association with $G\alpha_{12}$ involving stimulation of actin stress fiber formation, which could be dependent on a $G\alpha_{12}$-mediated pathway [14]. In this study we were able to describe a G_q- and G_i- mediated pathway and found no influence of $G\alpha_{12}$. In a second step we assessed Ap4A-induced activation of PLCβ, which is typically transmitted by the βγ subunit of G_i-protein or by the α subunit of G_q protein. After excluding an influence of P2X receptors on the investigated pathway by measuring an ApnA-induced $[Ca^{2+}]_i$ increase, we could attribute the $[Ca^{2+}]_i$ alteration to an inositol-1,4,5-trisphosphate (IP3)-induced effect. IP3 and diacylglycerol (DAG) are generated by hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate. Both DAG and $[Ca^{2+}]_i$

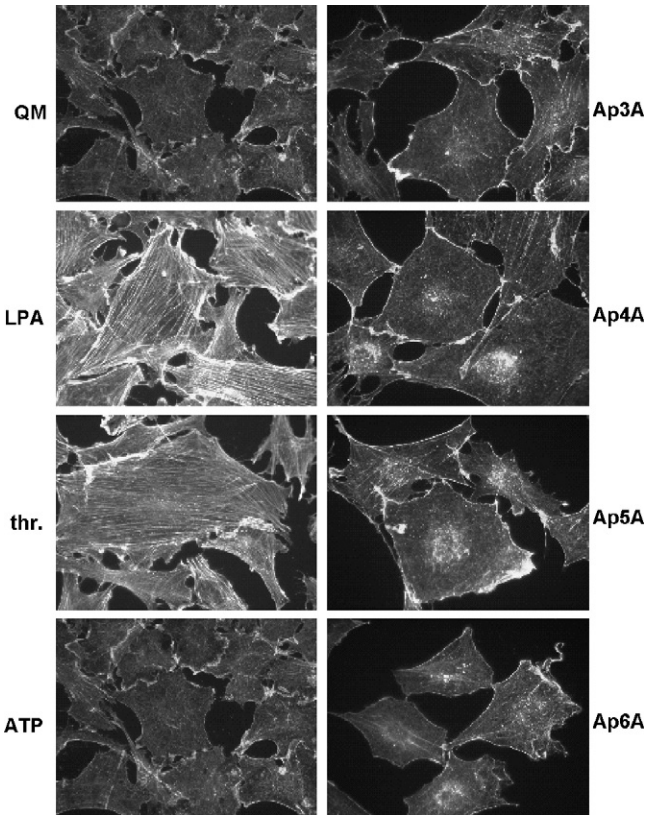

Fig. 6 – Diadenosine polyphosphates have no impact on stress fiber formation in 3T3-fibroblasts. 3T3-fibroblasts were exposed to serum-free quiescent medium (QM), LPA (1 μM), thrombin (2 U/mL), ATP (10 μM) and different diadenosine polyphosphates (10 μM). Actin stress fibers were visualized as described above. Typical examples illustrate stress fibers in individual cells.

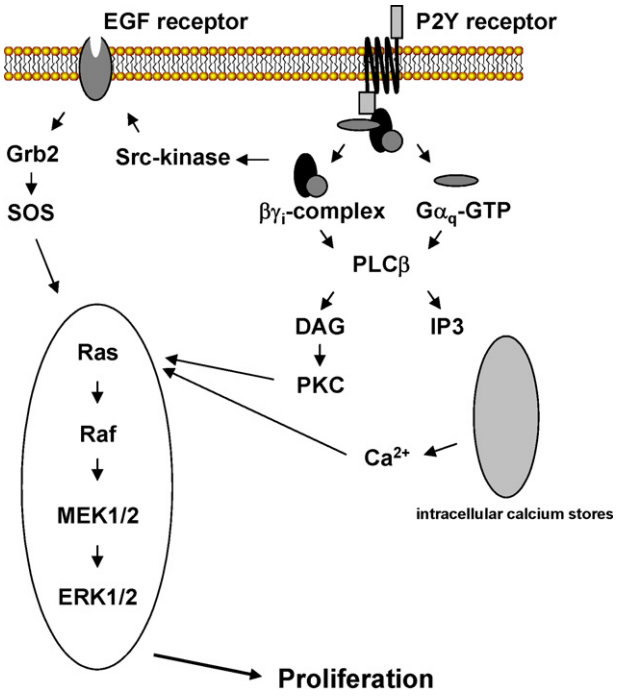

Fig. 7 – Schematic diagram of one possible Ap4A-induced proliferative signaling pathway. The pathway begins with an activation of a G-protein coupled P2Y receptor that leads to increased $[Ca^{2+}]_i$ by activation of PLCβ. Moreover, increased $[Ca^{2+}]_i$, activated PKC and transactivated EGF receptor induce the activation of Ras, Raf, MEK1/2 and ERK1/2 that may cause the described Ap3A- and Ap4A-induced rise of proliferation.

mediate activation of the Ras-Raf-MEK-ERK cascade, which we identified as a regulator of the Ap3A- and Ap4A-induced proliferative impact. On the other hand, evidence disclosed a second intracellular pathway with transactivation of the EGF receptor leading to stimulation of the Ras-Raf-MEK-ERK cascade.

To explain the different proliferative impacts of the applied diadenosine polyphosphates, it will be necessary to obtain more information on the expressed purinergic receptors. The main problem thus far is the lack of specific agonists and antagonists of P2X and P2Y receptors, which are needed to determine which subtype mediates the ApnA-induced effect. Therefore, only 2-met-thio-ADP was used to examine the essential influence of P2Y1 on the Ap4A-activated signaling pathway. It may be hypothesized that the effect of Ap3A and Ap4A differs from that of Ap5A and Ap6A due to diverse expression of purinergic receptors that have further specific affinities to several ApnA. Different expression may also explain the fact that other studies have demonstrated P2Y1-independent proliferative effects of ApnA [12]. A second problem in this study was the possible degradation of ApnA to products such as AMP, ADP or ATP, which themselves can act as proliferative compounds [15]. It can be conjectured that hydrolysis of Ap3A, Ap4A, Ap5A and Ap6A leads to the same compounds [16]. Since no proliferative effects were found for Ap5A and Ap6A, we attributed a proliferative potential to Ap3A and Ap4A but not to their degradation products. Demonstrating no effect of AMP and ADP on the phosphorylation of ERK1/2, we could support our hypothesis that Ap3A and Ap4A, but not their degradation products, induced the demonstrated proliferative effect in VSMCs (data not shown).

In conclusion, this is the first study to demonstrate different proliferative effects of ApnA on VSMCs. Ap3A and Ap4A, but not Ap5A or Ap6A, induced cell growth by activating G-protein-coupled P2Y receptors, which leads to stimulation of the Ras-Raf-MEK-ERK cascade. The activation of this cascade was transduced by stimulation of the EGF receptor, PKC, PLCβ, and the increase of $[Ca^{2+}]_i$.

Disclosure

None.

REFERENCES

[1] Tepel M, Jankowski J, Schluter H, Bachmann J, van der GM, Ruess C, et al. Diadenosine polyphosphates' action on calcium and vessel contraction. Am J Hypertens 1997;10:1404–10.
[2] van der GM, Jankowski J, Schluter H, Zidek W, Tepel M. Mediation of the vasoactive properties of diadenosine tetraphosphate via various purinoceptors. J Hypertens 1998;16:1939–43.
[3] Jankowski J, Hagemann J, Yoon MS, van der GM, Stephan N, Zidek W, et al. Increased vascular growth in hemodialysis patients induced by platelet-derived diadenosine polyphosphates. Kidney Int 2001;59:1134–41.
[4] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998;50:413–92.
[5] Seger R, Krebs EG. The MAPK signaling cascade. FASEB J 1995;9:726–35.
[6] Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.
[7] Jankowski J, Potthoff W, Zidek W, Schluter H. Purification of chemically synthesised dinucleoside(5′,5′) polyphosphates by displacement chromatography. J Chromatogr B Biomed Sci Appl 1998;719:63–70.
[8] Ives HE, Schultz GS, Galardy RE, Jamieson JD. Preparation of functional smooth muscle cells from the rabbit aorta. J Exp Med 1978;148:1400–13.
[9] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[10] Inesi G, Sagara Y. Thapsigargin, a high affinity and global inhibitor of intracellular Ca2+ transport ATPases. Arch Biochem Biophys 1992;298:313–7.
[11] Jiang Q, Guo D, Lee BX, Van Rhee AM, Kim YC, Nicholas RA, et al. A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. Mol Pharmacol 1997;52:499–507.
[12] Verspohl EJ, Hagemann J, Lempka M. Vascular smooth muscle cells (VSMC) proliferation of streptozotocin-diabetic animals induced by diadenosine polyphosphates. Exp Clin Endocrinol Diabetes 2004;112:142–7.
[13] Steinmetz M, Gabriels G, Le TV, Piechota HJ, Rahn KH, Schlatter E. Vasoactivity of diadenosine polyphosphates in

human small renal resistance arteries. Nephrol Dial Transplant 2003;18:2496–504.
[14] Gohla A, Harhammer R, Schultz G. The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor receptor to Rho. J Biol Chem 1998;273:4653–9.
[15] Erlinge D. Extracellular ATP: a growth factor for vascular smooth muscle cells. Gen Pharmacol 1998;31:1–8.
[16] Guranowski A. Specific and nonspecific enzymes involved in the catabolism of mononucleoside and dinucleoside polyphosphates. Pharmacol Ther 2000;87: 117–39.