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ORIGINAL ARTICLE

$P2X_1$ receptor-mediated inhibition of the proliferation of human coronary smooth muscle cells involving the transcription factor NR4A1

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Abstract Adenine nucleotides acting at $P2X_1$ receptors are potent vasoconstrictors. Recently, we demonstrated that activation of adenosine A_{2B} receptors on human coronary smooth muscle cells inhibits cell proliferation by the induction of the nuclear receptor subfamily 4, group A, member 1 (NR4A1; alternative notation Nur77). In the present study, we searched for long-term effects mediated by $P2X_1$ receptors by analyzing receptor-mediated changes in cell proliferation and in the expression of NR4A1. Cultured human coronary smooth muscle cells were treated with selective receptor ligands. Effects on proliferation were determined by counting cells and measuring changes in impedance. The induction of transcription factors was assessed by qPCR. The P2X receptor agonist α,β-methylene-ATP and its analog β,γ-methylene-ATP inhibited cell proliferation by about 50 % after 5 days in culture with half-maximal concentrations of 0.3 and 0.08 μM, respectively. The effects were abolished or markedly attenuated by the $P2X_1$ receptor antagonist NF449 (carbonylbis-imino-benzene-triylbis-(carbonylimino)tetrakis-benzene-1,3-disulfonic acid; 100 nM and 1 μM). α,β-methylene-ATP and β,γ-methylene-ATP applied for 30 min to 4 h increased the expression of NR4A1; NF449 blocked or attenuated this effect. Small interfering RNA directed against NR4A1 diminished the antiproliferative effects of α,β-methylene-ATP and β,γ-methylene-ATP. α,β-methylene-ATP (0.1 to 30 μM) decreased migration of cultured human coronary smooth muscle cells in a chamber measuring changes in impedance; NF449 blocked the effect. In conclusion, our results demonstrate for the first time that adenine nucleotides acting at $P2X_1$ receptors inhibit the proliferation of human coronary smooth muscle cells via the induction of the early gene NR4A1.

Keywords $P2X_1$ receptor · Coronary artery · Nuclear factor NR4A1 · Early growth response factor · Proliferation · Migration

Introduction

Vascular smooth muscle cells possess ligand-gated P2X receptors mediating vasoconstrictor responses to ATP released as sympathetic co-transmitter [1–5]. The cells also express a variety of G-protein coupled receptors for extracellular nucleotides and its breakdown product adenosine. These G-protein coupled receptors contribute to both the control of the vascular tonus by adenosine and nucleotides [5–9] and the control of the proliferation of the vascular smooth muscle [5, 9–17].

Adenosine is known to inhibit the proliferation of rat and human aortic smooth muscle cells via adenosine A_{2B} receptors [12, 13, 18, 19]. In a recent study on cultured human coronary smooth muscle cells, we demonstrated that the activation of adenosine A_{2B} receptors induces the expression of the transcription factor nuclear receptor subfamily 4, group A, member 1 (NR4A1) via an activation of the exchange protein activated by cAMP pathway (Epac [20]). NR4A1 (alternative notation Nur77) is subsequently involved in the antiproliferative effects mediated by adenosine A_{2B} receptors as shown by a loss of these antiproliferative effects in cells treated with small interfering RNA (siRNA) against NR4A1 [20]. Previous studies indicate a cellular

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expression of transcription factors of the NR4A subfamily in human atherosclerotic lesions and an involvement of these transcription factors in regulatory mechanisms during atherosclerosis [21]. Genetically induced overexpression of NR4A1 and drug-induced induction of NR4A1 are known to inhibit proliferation of both arterial and venous smooth muscle cells [21–24]. In contrast to the effects mediated by adenosine A_{2B} receptors, activation of adenosine A_3 receptors by the selective agonist 2-chloro-IB-MECA increased the proliferation of cultured human coronary smooth muscle cells [25]. 2-chloro-IB-MECA did not change the expression of NR4A1, but increased the expression of the transcription factors early growth response protein (EGR)-2 and EGR-3 [25].

The P2X receptor agonist α,β-methylene-ATP [26–28] failed to affect the proliferation of cultured smooth muscle cells derived from rat aorta [11, 29]. However, culturing of vascular smooth muscle cells has been reported to possibly cause a loss in the expression of $P2X_1$ receptors [30, 31]. Moreover, coronary smooth muscle cells and myocardial cells are likely to be derived from a common precursor cell [32], suggesting potential differences of coronary smooth muscle on the one hand and noncoronary vascular smooth muscle on the other hand. Therefore, we now examined the effects of α,β-methylene-ATP and its analog β,γ-methylene-ATP on the proliferation of human coronary smooth muscle cells cultured under conditions suitable to maintain the contractile smooth muscle phenotype [20, 25]. In contrast to α,β-methylene-ATP, β,γ-methylene-ATP has been shown to exert mitogenic effects in rat vascular smooth muscle cells [11, 29]. β,γ-methylene-ATP is degraded to adenosine within a relatively short period of time [7, 33, 34], suggesting that a part of the effects of β,γ-methylene-ATP is mediated by adenosine receptors [30]. Some of the results have been represented in abstract form [35] and in a dissertation [36].

Materials and methods

Cell culture Primary human coronary artery smooth muscle cells (HCASMCs) were commercially obtained (PromoCell, Heidelberg, Germany). HCASMCs from two donors (female Caucasians, 36 and 64 years of age) were used for the experiments. Cells were cultured at 37 °C and 5 % CO_2 in smooth muscle cell medium (PromoCell) containing 5 % fetal calf serum as well as the growth factors epidermal growth factor, basic fibroblast growth factor, and insulin as supplements.

Real-time RT-PCR For the experiments (passage number three to six), cells were seeded into 24-well plates. Forty hours prior to the experiment, the medium was replaced by serum-free medium without any supplements. In most experiments, cells were incubated for 1 h with compounds. Cells were then lysed, and total RNA was extracted using a magnetic bead-based RNA extraction kit (Roche, Mannheim, Germany). After reverse transcription of total RNA by random primers (Transcriptor First Strand DNA synthesis kit, Roche), PCR reactions were set up for the Light Cycler 480 instrument (Roche) in 96-well format. Amplification was performed over 45 cycles with denaturation at 95 °C for 10 s and annealing/elongation at 72 °C for 105 s. The primers used to quantify the early genes have been described previously [20]. For $P2X_1$ receptor detection, the following pair was used: forward 5′-GTGGGCGTTATCTTCCGACTGATCC-3′ and reverse 5′-TGACTCTTGCACCACGTAGCCAAGC-3′). Crossing point (CP) values were used for measuring cDNA by quantitative PCR using the second derivative maximum method as described previously [20]. The CP value is inversely correlated to the concentration of target cDNA in the sample on a logarithmic scale; drug-induced changes were calculated as delta crossing point (ΔCP) values. The CP values were created by the software of the PCR machine using a standard algorithm provided by the manufacturer. Neither the researcher nor the operator could influence or bias this process. Hence, the CP values are the raw data of this evaluation. The ΔCP values, which are shown in the Results section, were calculated by subtracting the CP value of the housekeeping gene (GAPDH). In pilot experiments, other housekeeping genes were also tested, but there were no relevant differences to the widely used housekeeping gene GAPDH, so that the latter was chosen for the pivotal experiments.

Analysis of cell migration Cells were cultured in smooth muscle cell medium (see above). The 16-well plate for analysis of cell migration (CIM-Plate; Roche) comprises an upper chamber and a lower chamber. The bottom of the upper chamber consists of a microporous polyethylene terephthalate membrane containing gold electrode arrays on the bottom side of the membrane. Cells were seeded into the upper chamber (8,000 per well), and their migration into the lower chamber was assessed by changes in impedance. Cells were treated for 20 h with the test substances which were added to the upper chamber. Platelet derived growth factor (PDGF) (0.1 μg/ml) was present in the lower chamber to direct the cells.

Analysis of cell growth Cells were cultured in smooth muscle cell medium (see above). Twenty thousand cells were seeded into one well of a 24-well plate and 5,000 cells into one well of a 96-well plate designed for measuring changes in impedance. Twenty-four hours prior to the experiment, the medium was replaced by serum-free medium without any supplements. Then cells were treated for 5 days with adenine nucleotides or its solvent (water) in the absence and presence of the test substances. The medium was replaced by fresh

medium containing the drugs after 48 h. Thus, by refreshing the test substances, the experiments were not compromised by a potentially limited stability of the agonists and antagonists in cell culture medium. Five days after the start of the experiment, cells were treated with trypsin (PromoCell) and removed from the plate. Then the cells were counted using a Cedex XS counter (Roche) or stained using trypane blue (0.5 %) (Sigma, Steinheim, Germany). In some experiments, changes in impedance in a 96-well plate culture chamber with specialized gold electrodes were determined every 10 min over 5 days as a measure of cell numbers using an xCELLigence system (Roche). The change in impedance reflects the change in cell number; changes over time are given relative to the time point of addition of ligands.

siRNA knockdown In some experiments, cells in 24-well plates were treated with negative control siRNA or siRNA directed against NR4A1 (control siRNA-A, catalog# sc-37007, and Nur77 siRNA h2, catalog# sc-156146, respectively, from Santa Cruz Biotechnology, Heidelberg, Germany). The siRNA directed against NR4A1 was a mixture of three target-specific oligonucleotides, each 19–25 nucleotides in length. The negative control siRNA was a scrambled sequence of the same length that was designed not to lead to a specific degradation of any known human cellular mRNA. The siRNA was used according to the provider's recommendation. In brief, it was dissolved in RNase-free water to yield a concentration of 10 μM. Of this solution, 5 μl per well were diluted with 25 μl medium and mixed with 5 μl transfection reagent (also provided by Santa Cruz Biotechnology) suspended in 25-μl medium. After 45 min at room temperature, this mixture was further diluted in 200 μl cell culture medium and added to the cells 24 h before the addition of agonists.

Chemicals The following drugs were used: adenosine 5′-triphosphate disodium salt hydrate (ATP, Sigma); 8-[4-(4-benzylpiperazide-1-sulfonyl)phenyl]-1-propylxanthine (PSB-601; Biotrend; Cologne, Germany); 4,4',4",4"'-(carbonylbis (imino-5,1,3-benzene-triylbis-(carbonylimino))) tetrakis-benzene-1,3-disulfonic acid sodium salt (NF449, Tocris, Bristol, UK); α,β-methylene-adenosine 5′-triphosphate lithium salt (α,β-methylene-ATP, Sigma); β,γ-methylene-adenosine 5′-triphosphate disodium salt (β,γ-methylene-ATP, Sigma); 2-(methylthio)adenosine 5′-diphosphate trisodium salt hydrate (2-methylthio-ADP, Sigma); platelet-derived growth factor-BB (PDGF-BB; Promokine; Heidelberg, Germany); control siRNA (Santa Cruz Biotechnology); NR4A1 siRNA (Santa Cruz Biotechnology). Stock solutions of drugs were prepared either with distilled water or dimethyl sulfoxide (used for PSB-601). The solvents were added at the desired concentrations to the buffer used for control incubations.

Statistics Results are presented as means ± SEM from *n* experiments. Differences between means were tested for significance by the Student's *t* test or (for multiple comparisons with the same control) by an analysis of variance followed by the Bonferroni posttest. $P<0.05$ or lower was the significance criterion.

Results

Effects of adenine nucleotides on cell proliferation RT-PCR revealed the expression of mRNA encoding $P2X_1$ receptors in the cultured human coronary smooth muscle cells (Fig. 1a). Effects of adenine nucleotides on the proliferation of human coronary smooth cells were examined by incubating cells for 5 days with solvent (water) or adenine nucleotides in the absence of serum, insulin, and other growth factors. ATP (3 and 10 μM) inhibited cell proliferation (measured as cell counts) by about 15 % after 5 days of culturing ($P<0.001$; not shown). The metabolically more stable analogs α,β-methylene-ATP and β,γ-methylene-ATP were used for further experiments. α,β-methylene-ATP (Fig. 1b) and β,γ-methylene-ATP (Fig. 1c) reduced proliferation by up to 50 % in a concentration-dependent manner in the absence of PDGF (open symbols and dashed lines in Fig. 1b, c). The half-maximal effect was reached at about 0.3 μM α,β-methylene-ATP and 0.08 μM β,γ-methylene-ATP, respectively. Neither treatment with α,β-methylene-ATP nor treatment with β,γ-methylene-ATP increased the number of trypan blue positive cells (not shown). PDGF (0.1 μg/ml) increased the proliferation of the cells by about 120 % (legend to Fig. 1). In the presence of PDGF (0.1 μg/ml), the inhibitory effect on proliferation of α,β-methylene-ATP or β,γ-methylene-ATP was markedly attenuated or even completely abolished (Fig. 1b, c). Cell proliferation was also studied in a chamber designed to detect increases in impedance as a measure of increases in proliferation. α,β-methylene-ATP decreased the proliferation observed in the absence of that drug (control) after 36 h of treatment (Fig. 2a). In contrast, PDGF increased proliferation in respect to control experiments (Fig. 2a). As a further positive control, the P2Y receptor agonist 2-methylthio-ADP (100 nM and 1 μM [37]) was used. This compound also increased the impedance as a measure of proliferation of human coronary smooth muscle cells (Fig. 2b). Next, the interaction of α,β-methylene-ATP and β,γ-methylene-ATP with the $P2X_1$ receptor-selective antagonist NF449 (100 nM and 1 μM [38]) was studied. NF449 itself did not affect cell proliferation (legend to Fig. 3) but markedly attenuated or completely abolished the inhibitory effects of α,β-methylene-ATP and β,γ-methylene-ATP on cell proliferation (Fig. 3). This is in line with the assumption that vascular

$P2X_1$ receptors are involved in this response. Previously, we have demonstrated that adenosine analogs inhibit the proliferation of human coronary smooth muscle via A_{2B} receptors [20]. The A_{2B} receptor antagonist PSB-601 (1 μM) [39] did not affected responses to α,β-methylene-ATP (Fig. 4a), but attenuated the effects of β,γ-methylene-ATP (Fig. 4b). This suggests that a portion of the effect of β,γ-methylene-ATP is mediated by the breakdown product adenosine. PSB-601 (1 μM) itself did not change cell counts (legend to Fig. 4).

Involvement of the transcription factor NR4A1 The induction of NR4A1 has been shown to play a crucial role in the inhibition of proliferation of vascular smooth muscle cells [20–24]. Therefore, we analyzed effects of nucleotides on the expression of the early gene NR4A1. In the absence of PDGF, both α,β-methylene-ATP and β,γ-methylene-ATP (Fig. 5) induced the expression of mRNA encoding NR4A1 when the nucleotides were applied for 30 min to 4 h. In contrast, transcription factors of the EGR family were not induced by α,β-methylene-ATP and β,γ-methylene-ATP (not shown). All subsequent experiments on gene expression were performed with an incubation period of 1 h. PDGF (0.1 μg/ml) also induced the expression of NR4A1 (not shown). In the presence of PDGF, α,β-methylene-ATP and β,γ-methylene-ATP did not increase the expression of NR4A1 beyond the effect of PDGF alone (not shown). Concentration-responses curves for α,β-methylene-ATP and β,γ-methylene-ATP inducing the expression of mRNA for NR4A1 in the absence of PDGF are summarized in Fig. 6. The $P2X_1$ receptor antagonist NF449 itself did not change the expression of NR4A1 when used at the concentrations of 100 nM and 1 μM (legend to Fig. 6). NF449 (100 nM and 1 μM) abolished the increases in expression of mRNA for NR4A1 in response to α,β-methylene-ATP (Fig. 6a) and attenuated the responses to β,γ-methylene-ATP (Fig. 6b).

Knockdown of NR4A1 Next, we studied the involvement of NR4A1 in the effects of adenine nucleotides on cell proliferation by using siRNA directed against NR4A1. Cells were pre-incubated with siRNA (10 μM) directed against NR4A1 24 h before the addition of the agonist; nonsense siRNA served as negative control. NR4A1 is a transcription factor and an early gene which triggers further events (proliferation inhibition here). This does not require permanent expression of NR4A1. In fact, the expression level (mRNA) decreases again at 2 h after onset of stimulation (Fig. 5), even if the agonist is further present. Due to this transient presence of NR4A1 mRNA, expression of NR4A1 protein was measured early (1 h) after the onset of agonist stimulation. It was observed that in the presence of control siRNA, NR4A1 protein was strongly induced by α,β-methylene-ATP, whereas no NR4A1 protein expression due to α,β-

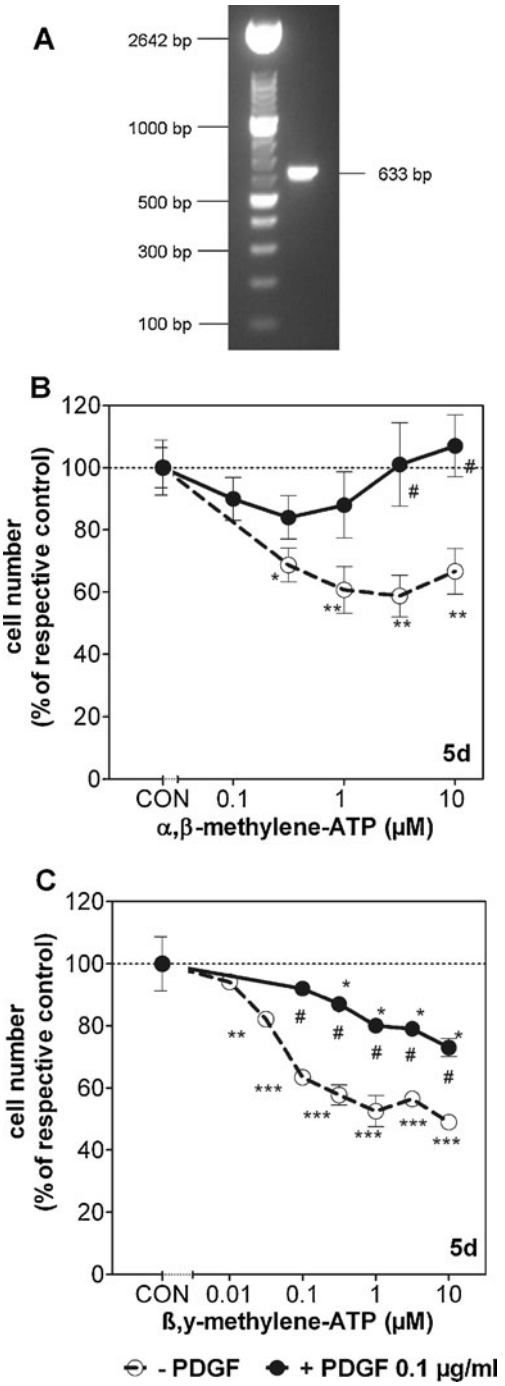

Fig. 1 RT-PCR indicating the expression of mRNA encoding $P2X_1$-receptors (**a**) and effects of α,β-methylene-ATP (**b**) and β,γ-methylene-ATP (**c**) on cell counts of cultured human coronary smooth muscle cells in the absence (*open symbols*) and presence of PDGF (0.1 μg/ml). Cells were cultured for 5 days in serum-free medium with the agonists indicated and, when used, PDGF. Then the cell count was determined using the Cedex XS counter and expressed as percentage of respective values without an agonist (control, *CON*; **b** without PDGF, 124,579±3,707 cells/ml, $n=6$; with PDGF, 200,462±7,818 cells/ml, $n=6$; **c** without PDGF, 129,644±8,081 cells/ml, $n=6$; with PDGF, 216,468±9,237 cells/ml, $n=6$). Data points are means ± SEM from four to eight determinations. *, **, *** $P<0.05$, 0.01 and 0.001 versus vehicle control; # $P<0.05$ versus response in the absence of PDGF

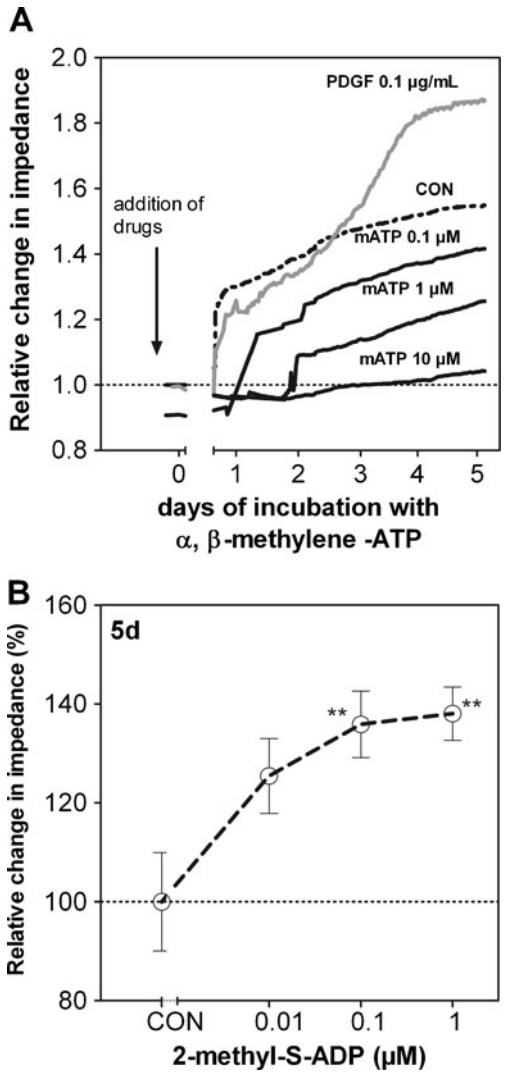

Fig. 2 Effects of α,β-methylene-ATP (**a**, mATP; 0.1, 1, and 10 μM) and 2-methylthio-ADP (**b**, 2-methyl-S-ADP; 0.01 to 1 μM) on changes in impedance as a measure of cell proliferation of human coronary smooth muscle cells. Panel **a** shows representative traces of changes in relative impedance induced by α,β-methylene-ATP (*mATP*) or platelet-derived growth factor (*PDGF*) analyzed with the xCELLigence system over 5 days. Representative traces from eight determinations. **b** Data points are means ± SEM from four determinations after 5 days (5 d). ** $P<0.01$ versus control (*CON*, no 2-methylthio-ADP)

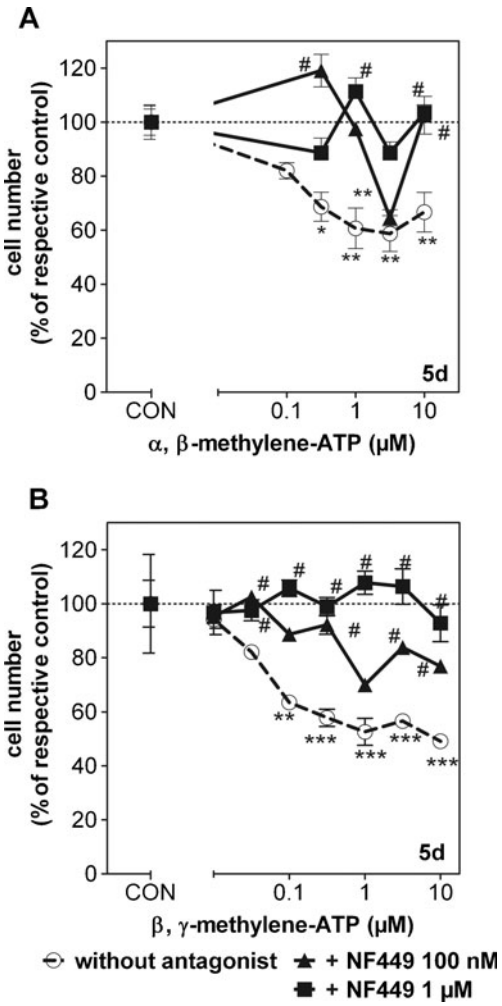

Fig. 3 $P2X_1$ receptor mediated inhibition of proliferation of human coronary smooth muscle cells. Cells were cultured for five days (5 d) in serum-free medium with the agonists indicated and, when used, the $P2X_1$ receptor selective antagonist NF449. Then the cell count was determined using the Cedex XS counter and expressed as percentage of respective values without an agonist (control, *CON*; w/o—without antagonist: **a** 169,333±10,885 cells/ml, $n=6$, **b** 161,500±33,333 cells/ml, $n=6$; with NF449 100 nM: **a** 161,500±11,126 cells/ml, $n=6$, **b** 166,833±23,559 cells/ml, $n=6$; with NF449 1 μM: **a** 161,275±12,023 cells/ml, $n=6$, **b**, **a** 158,500±15,711 cells/ml, $n=6$). Data points are means ± SEM from six to nine determinations. *, **, *** $P<0.05$, 0.01 and 0.001 versus vehicle control; # $P<0.05$ versus response in the absence of the antagonist

methylene-ATP was observed in the presence of siRNA against NR4A1 (Fig. 7a). Knockdown of NR4A1 alone did not affect cell proliferation; it remained at the same level as with control siRNA (10 μM; legend to Fig. 7). However, siRNA knockdown of NR4A1 attenuated the effect of α,β-methylene-ATP on cell proliferation (Fig. 7b) and blocked the effect of β,γ-methylene-ATP (Fig. 7c).

Effects on migration of coronary smooth muscle cells Finally, we studied the effects of α,β-methylene-ATP on cell migration by measuring changes in impedance in a two-chamber wells over 20 h (see "Materials and methods" section). Migration was directed towards the lower chamber by the addition of PDGF to the latter. α,β-methylene-ATP inhibited cell migration in a concentration-dependent manner by up to about 30 % ($P<0.001$, Fig. 8). NF449 (1 μM) abolished the inhibitory effects of α,β-methylene-ATP on cell migration (Fig. 8).

Discussion

The present study shows for the first time that adenine nucleotides inhibit the proliferation of human coronary

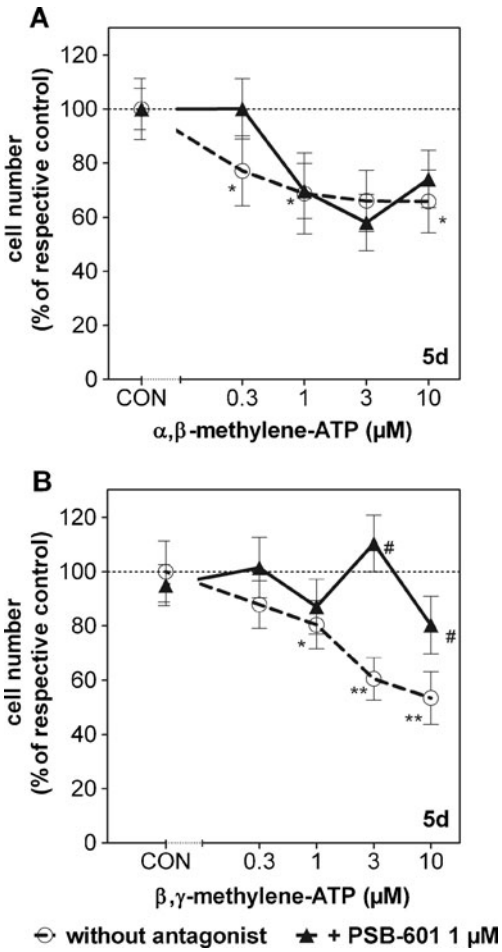

Fig. 4 Attenuation of the effects of β,γ-methylene-ATP (**b**), but not α,β-methylene-ATP (**a**) by the adenosine A_{2B} receptor antagonist PSB-601 (1 μM). Cells were cultured for 5 days in serum-free medium with the agonists indicated and, when used, PSB-601. Then the cell count was determined using the Cedex XS counter and expressed as percentage of respective values without an agonist (control, *CON*; w/o—without antagonist: **a** 149,696±5,634 cells/ml, n=12, **b** 153,743±5,412 cells/ml, n=12; with PSB-601 1 μM: **a** 130,235±4,946 cells/ml, n=11, **b** 133,756±6,626 cells/ml, n=11). Data points are means ± SEM from 6 to 12 determinations. *, ** P<0.05 and 0.01 versus vehicle control; # P<0.05 versus response in the absence of the antagonist

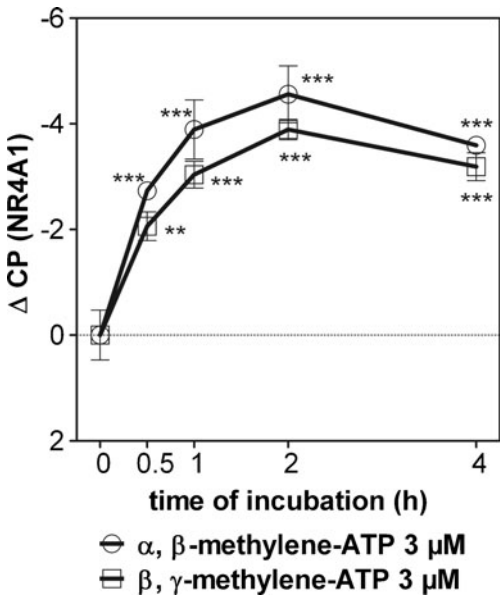

Fig. 5 Time course of the induction of mRNA encoding the transcription factor NR4A1 by α,β-methylene-ATP and β,γ-methylene-ATP (3 μM each). Human coronary smooth muscle cells were incubated for the periods indicated. mRNA encoding the transcription factor NR4A1 was determined by quantitative real-time PCR (control gene: GAPDH). Results are expressed as CP (crossing point) values (see "Materials and methods"). Data points are means ± SEM from six determinations. **, *** P<0.01 and 0.001 versus control (*CON*), respectively

smooth muscle cells via an activation of $P2X_1$ receptors and the subsequent induction of the transcription factor NR4A1.

RT-PCR revealed the expression of mRNA encoding $P2X_1$ receptors in cultured human coronary smooth muscle cells in agreement with the presence of mRNA encoding $P2X_1$ receptors in rat coronary arteries [40]. The presence of functional receptor protein could be demonstrated by using the highly selective ligand NF449. NF449 is the most selective $P2X_1$ ligand available [41]. It was developed and thoroughly investigated in recombinant rat P2X receptors by Rettinger et al. [42]. The following IC_{50} values were obtained: 0.3 nM, 0.7 nM, 0.3 μM, 1.8 μM, and >300 μM for $rP2X_1$, $rP2X_{1+5}$, $rP2X_{2+3}$, $rP2X_3$, and $rP2X_4$ receptors, respectively. These data indicate that there is factor 1,000 between IC50 for $P2X_1$ (0.3 nM) and IC_{50} towards the P2X type with the highest affinity not carrying a $P2X_1$ subunit, namely $P2X_{2+3}$ (0.3 μM). NF449 can probably not distinguish between P2X1 and the heteromeric receptor $P2X_{1+5}$. But even the presence of $P2X_{1+5}$ heteromeric receptor would demonstrate the functional expression of $P2X_1$ protein in the HCASMCs used. No such extensive affinity data are available for the human P2X receptors, but the same group [38] reported an IC_{50} of 0.05 nM for $hP2X_1$ as compared to 40 μM for $hP2X_7$. This is in high agreement with the situation in rats. In our experiment, the effects of α,β-methylene-ATP on proliferation and gene expression were virtually completely blocked by 100 nM NF44. This clearly argues against a major involvement of P2X receptors other than $P2X_1$ or $P2X_{1+5}$. In case of β,γ-methylene-ATP, the inhibition by NF449 was incomplete, but the situation here is different. It is known that β,γ-methylene-ATP is less stable than α,β-methylene-ATP, and therefore part of the former was degraded to adenosine as revealed by our experiments, using the adenosine A_{2B} receptor antagonist PSB601. The prototypic P2X receptor agonist α,β-methylene-ATP and its analog β,γ-methylene-ATP caused both an inhibition in proliferation and an increase in the expression of the transcription factor NR4A1. The nucleotides acted with half-maximal concentrations in the submicromolar range compatible with an action on $P2X_1$ receptors. The interaction with

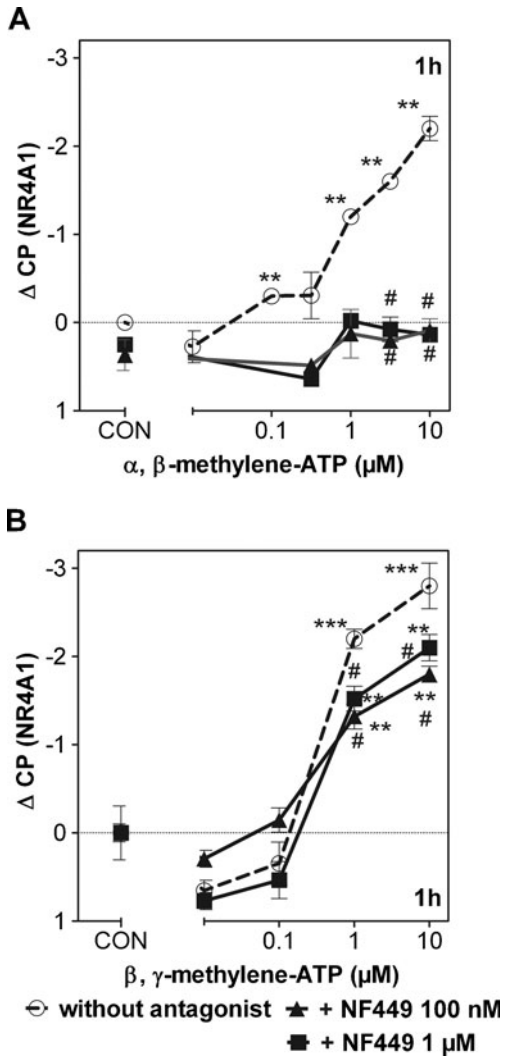

Fig. 6 P2X1 receptor mediated induction of the transcription factor NR4A1 in human coronary smooth muscle cells. Cells were treated for one hour (1 h) in serum-free medium with the agonists indicated and, when used, the antagonist NF449. The expression of mRNA for NR4A1 was determined by quantitative real-time PCR (control gene: GAPDH). Results are expressed as ΔCP (delta crossing point). Data points are means ± SEM from three to six determinations. **, *** $P<0.01$ and 0.001 versus respective control (*CON*, no agonist); # $P<0.05$ versus response in the absence of the antagonist

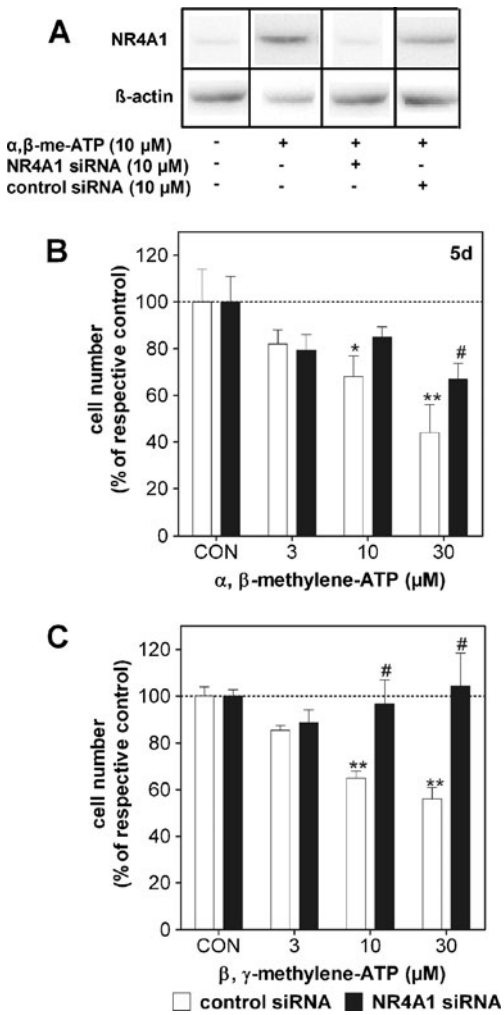

Fig. 7 Involvement of NR4A1 in the $P2X_1$ receptor-mediated proliferation of human coronary smooth muscle cells. Cells were cultured for five days (5 d) in serum-free medium with the agonists indicated and either control siRNA (10 μM; *open columns* in **b**, **c**) or siRNA directed against NR4A1 (10 μM; *filled columns* in **b**, **c**, see "Materials and methods" section for details) before the determination of the cell number. Part **a** demonstrates knockdown of NR4A1 protein in response to the specific, but not to the control siRNA by Western blotting (polyclonal rabbit antibody Nur77 P15 from Cell Signaling, Technology, Inc.; the provider's recommendations were followed). For Western blotting, cells were harvested after 1 h stimulation with α,β-methylene-ATP; siRNA had been added 24 h before adding the agonists. Since the expression of NR4A1 is transient, the timing for Western blotting had to be different from the timing for cell counting. In parts **b** and **c**, the cell count is indicated, expressed as percentage of respective values without an agonist (control, *CON*; control siRNA: **b** 254,735±35,735 cells/ml, $n=3$, **c** 230,200±9,129 cells/ml, $n=5$; with NR4A1 siRNA: **a** 311,439±33,709 cells/ml, $n=6$, **c** 210,023±5,728 cells/ml, $n=6$). Data points are means ± SEM from three to six determinations. *, ** $P<0.05$ and 0.01 versus respective control; # $P<0.05$ versus response in the presence of control siRNA

the selective $P2X_1$ receptor antagonist NF449 used at the low concentrations of 100 nM and 1 μM [38, 41–43] clearly characterizes the involved subtype as $P2X_1$ receptor as discussed above. Rat coronary arteries express $P2X_4$ receptors [43, 44] in addition to $P2X_1$ receptors. $P2X_4$ receptors are, however, less sensitive to stimulation by α,β-methylene-ATP [26, 27, 44] and are not blocked by NF449 in concentrations up to 30 μM [43]. This argues against an involvement of $P2X_4$ receptors in the observed responses of human coronary smooth muscle cells to α,β-methylene-ATP. Previously, a loss in expression of $P2X_1$ receptors has been shown in cultured vascular smooth muscle cells [30, 31].

The maintenance of expression and function of $P2X_1$ receptors in our experimental system may be due to the culture conditions used and correlates with the continuous expression of smooth muscle α-actin in these cultured cells [25].

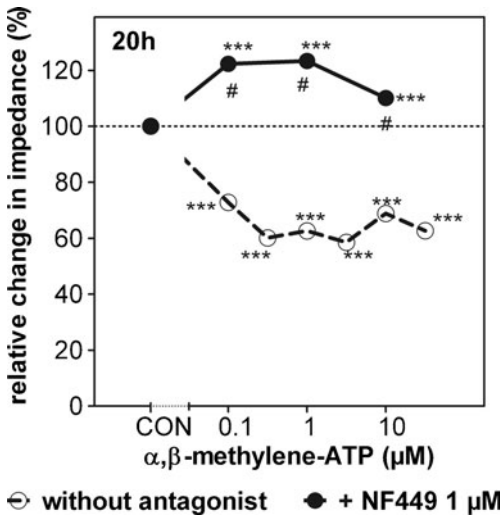

Fig. 8 P2X receptor-mediated inhibition of migration of human coronary smooth muscle cells. Cells were cultured for 20 hours (20 h) in serum-free medium with the agonist α,β-methylene-ATP at the indicated concentrations and, when used, the $P2X_1$ receptor selective antagonist NF449 (1 μM). Migration was determined using the xCELLigence system and expressed as percentage of relative changes in impedance (control, *CON*). Data points are means ± SEM from six determinations. *** $P<0.001$ versus vehicle control # $P<0.0$ versus response in the absence of the antagonist

There is the possibility that P2X analogs, which are ATP derivates, become degraded to adenosine via 5′-nucleotidases located on the surface of the cells. However, α,β-methylene-ATP will be converted to α,β-methylene-ADP in a first step, and the latter compound is a highly potent inhibitor of 5′-nucleotidases [45], so that degradation of α,β-methylene-ATP to adenosine is prevented. In contrast, β,γ-methylene-ATP could be at least in part converted to adenosine, and this would explain our finding depicted in Fig. 4 that the adenosine A_{2B} receptor antagonist PSB601 can inhibit the effect of β,γ-methylene-ATP on proliferation but not the effect of α,β-methylene-ATP.

In contrast to the inhibition of proliferation due to the activation of $P2X_1$ receptors as observed in the present study, P2Y receptor subtypes including $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors mediate increases in proliferation of vascular smooth muscle cells [5, 11, 29, 30, 46–51]. In cultured human vascular smooth muscle cells pretreated with thrombin, the agonist 2-methylthio-ADP induced mitogenic effects by an action on $P2Y_{12}$ receptors [50]. In agreement with these findings, 2-methylthio-ADP also increased the proliferation of cultured human coronary smooth muscle cells in our present experiments.

Our present study provides direct evidence for a signaling of $P2X_1$ receptors in human coronary smooth muscle cells via the transcription factor NR4A1 (alternative notation Nur77) to inhibition of proliferation. First, α,β-methylene-ATP and β,γ-methylene-ATP induced the expression of the early gene NR4A1 in a manner sensitive to blockade of $P2X_1$ receptors by the selective antagonist NF449 [38, 42, 43]. And secondly, knockdown of the NR4A1 by treatment of cells with siRNA directed against the NR4A1 sequence attenuated the inhibitory effects of α,β-methylene-ATP and β,γ-methylene-ATP on cell proliferation. Interestingly, stimulation of cells by PDGF abolished or attenuated the effects of the nucleotides on both increases in NR4A1 expression and inhibition of cell proliferation. This contrasts with the adenosine A_{2B} receptor-mediated inhibition of proliferation of human coronary smooth muscle cells. The adenosine A_{2B} receptor-mediated inhibition of proliferation was observed in the presence and not in the absence of PDGF [20]. The responses to stimulation of adenosine A_{2B} receptors differ in intracellular signaling; they are mediated by increases in intracellular cAMP and a subsequent activation of EPAC, followed by the induction of NR4A1 [20]. Increases in intracellular Ca^{2+} concentration followed by an activation of Ca^{2+}/calmodulin-dependent protein kinase $II\delta_2$ and an expression of myocyte-enhancer factor 2 have recently been shown to serve as an alternative signaling pathway for the induction of NR4A1 in vascular smooth muscle cells [52] (see also ref [53]). Activation of the $P2X_1$ receptors is known to increase intracellular Ca^{2+} concentration in vascular smooth muscle cells [3, 5] and is, therefore, likely to signal via the Ca^{2+}-sensitive pathway. The nucleotides α,β-methylene-ATP and β,γ-methylene-ATP did not affect the viability of the cells as indicated by trypane blue staining, arguing against direct toxic effects of the adenine nucleotides used.

As with proliferation, migration was also inhibited by α,β-methylene-ATP, and this effect was again reversed by NF449, so that P2X1 receptors are obviously involved. PDGF was present in the migration experiments in order to direct the cells towards the lower chamber of the migration apparatus (Roche xCELLigence system). Thus, PDGF directs but does not induce migration because in the latter case, the presence of PDGF in the upper chamber (where the cells are located at the beginning of the experiment) would be necessary. Therefore, our findings in regard to migration do not contradict the observations made with proliferation, where α,β-methylene-ATP did affect the PDGF-mediated effect.

We observed a further effect of α,β-methylene-ATP on cultured human coronary smooth muscle cells mediated by $P2X_1$ receptors. α,β-methylene-ATP inhibited with a half-maximal concentration of about 0.1 μM the migration of the cells. P2Y receptors including the $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ subtypes mediate the opposite effect—an increase in migration [54–59]. Migration facilitated by the activation of $P2Y_2$ receptors involved the ERK1/2 as well as the arrestin2 pathway [54, 58]. Interestingly, migration induced by $P2Y_{12}$ receptor stimulation has been proposed to play a major role in transplant arteriosclerosis [57]. In agreement with that

notion, treatment of animals with clopidogrel reduced the progression of arteriosclerosis in a murine aortic allograft model [60].

Proliferation and migration of vascular smooth muscle are involved in the progression of arteriosclerosis. Our present results demonstrate for the first time that the $P2X_1$ receptor agonist α,β-methylene-ATP inhibits both proliferation and migration of human coronary smooth muscle cells. Blockade of $P2Y_{12}$ receptors by antagonists such as ticagrelor [61] or by active metabolites of clopidogrel or prasugrel [62, 63] may direct effects of endogenous adenine nucleotides to inhibition of the progression of arteriosclerosis by an action on smooth muscle $P2X_1$ receptors.

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References

1. von Kügelgen I, Starke K (1985) Noradrenaline and adenosine triphosphate as co-transmitters of neurogenic vasoconstriction in rabbit mesenteric artery. J Physiol 367:435–455
2. Burnstock G, Kennedy C (1986) A dual function for adenosine 5'-triphosphate in the regulation of vascular tone. Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. Circ Res 58:319–330
3. Burnstock G (1990) Noradrenaline and ATP as co-transmitters in sympathetic nerves. Neurochem Int 17:357–368
4. von Kügelgen I, Starke K (1991) Noradrenaline-ATP co-transmission in the sympathetic nervous system. Trends Pharmacol Sci 12:319–324
5. Erlinge D, Burnstock G (2008) P2 receptors in cardiovascular regulation and disease. Purinergic Signal 4:1–20. doi:10.1007/s11302-007-9078-7
6. Olsson RA, Pearson JD (1990) Cardiovascular purinoceptors. Physiol Rev 70:761–845
7. Ralevic V, Burnstock G (1991) Roles of P2-purinoceptors in the cardiovascular system. Circulation 84:1–14
8. Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492
9. Schuchardt M, Tölle M, van der Giet M (2012) P2Y purinoceptors as potential emerging therapeutical target in vascular disease. Curr Pharm Des 18:6169–6180
10. Teuscher E, Weidlich V (1985) Adenosine nucleotides, adenosine, and adenine as angiogenesis factors. Biomed Biochim Acta 44:493–495
11. Erlinge D, Yoo H, Edvinsson L, Reis DJ, Wahlestedt C (1993) Mitogenic effects of ATP on vascular smooth muscle cells versus other growth factors and sympathetic co-transmitters. Am J Physiol 265:H1089–H1097
12. Dubey RK, Gillespie DG, Osaka K, Suzuki F, Jackson EK (1996) Adenosine inhibits growth of rat aortic smooth muscle cells. Possible role of A2B receptor. Hypertension 27:786–793
13. Dubey RK, Gillespie DG, Mi Z, Jackson EK (1998) Adenosine inhibits growth of human aortic smooth muscle cells via A2B receptors. Hypertension 31:516–521
14. Clark AN, Youkey R, Liu X, Jia L, Blatt R, Day YJ, Sullivan GW, Linden J, Tucker AL (2007) A_1 adenosine receptor activation promotes angiogenesis and release of VEGF from monocytes. Circ Res 101:1130–1138
15. Ryzhov S, McCaleb JL, Goldstein AE, Biaggioni I, Feoktistov I (2007) Role of adenosine receptors in the regulation of angiogenic factors and neovascularization in hypoxia. J Pharmacol Exp Ther 320:565–572
16. Burnstock G, Verkhratsky A (2010) Long-term (trophic) purinergic signaling: purinoceptors control cell proliferation, differentiation, and death. Cell Death Dis 1:e9. doi:10.1038/cddis.2009
17. Jackson EK, Ren J, Gillespie DG (2011) 2',3'-cAMP, 3'-AMP, and 2'-AMP inhibit human aortic and coronary vascular smooth muscle cell proliferation via A_{2B} receptors. Am J Physiol Heart Circ Physiol 301:H391–H401. doi:10.1152/ajpheart.00336.2011
18. Dubey RK, Rosselli M, Gillespie DG, Mi Z, Jackson EK (2010) Extracellular 3',5'-cAMP-adenosine pathway inhibits glomerular mesangial cell growth. J Pharmacol Exp Ther 333:808–815. doi:10.1124/jpet.110.166371
19. Jackson EK, Ren J, Gillespie DG, Dubey RK (2010) Extracellular 2',3'-cyclic adenosine 5'-monophosphate is a potent inhibitor of preglomerular vascular smooth muscle cell and mesangial cell growth. Hypertension 56:151–158. doi:10.1161/HYPERTENSIONAHA.110.152454
20. Mayer P, Hinze AV, Harst A, von Kügelgen I (2011) A_{2B} receptors mediate the induction of early genes and inhibition of arterial smooth muscle cell proliferation via Epac. Cardiovasc Res 90:148–156. doi:10.1093/cvr/cvq371
21. Bonta PI, Pols TW, de Vries CJ (2007) NR4A nuclear receptors in atherosclerosis and vein-graft disease. Trends Cardiovasc Med 17:105–111
22. Arkenbout EK, de Waard V, van Bragt M, van Achterberg TA, Grimbergen JM, Pichon B, Pannekoek H, de Vries CJ (2002) Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. Circulation 106:1530–1535
23. de Waard V, Arkenbout EK, Vos M, Mocking A, Niessen HWM, Stooker W, de Mol B, Quax P, Bakker E, van Bavel E, Pannekoek H, de Vries CJM (2006) TR3 nuclear orphan receptor prevents cyclic stretch-induced proliferation of venous smooth muscle cells. Am J Pathol 168:2027–2035
24. Pires NM, Pols TW, de Vries MR, van Tiel CM, Bonta PI, Vos M, Arkenbout EK, Pannekoek H, Jukema JW, Quax PH, de Vries CJ (2007) Activation of nuclear receptor Nur77 by 6-mercaptopurine protects against neointima formation. Circulation 115:493–500
25. Hinze AV, Mayer P, Harst A, von Kügelgen I (2012) Adenosine A3 receptor-induced proliferation of primary human coronary smooth muscle cells involving the induction of early growth response genes. J Mol Cell Cardiol 53:639–645. doi:10.1016/j.yjmcc.2012.08.003
26. Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Séguéla P, Voigt M, Humphrey PP (2001) International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev 53:107–118
27. Vial C, Roberts JA, Evans RJ (2004) Molecular properties of ATP-gated P2X receptor ion channels. Trends Pharmacol Sci 25:487–493
28. Gever JR, Cockayne DA, Dillon MP, Burnstock G, Ford AP (2006) Pharmacology of P2X channels. Pflugers Arch 452:513–537
29. Erlinge D, You J, Wahlestedt C, Edvinsson L (1995) Characterization of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. Eur J Pharmacol 289:135–149
30. Erlinge D (1998) Extracellular ATP: a growth factor for vascular smooth muscle cells. Gen Pharmacol 31:1–8
31. Erlinge D, Hou M, Webb TE, Barnard EA, Möller S (1998) Phenotype changes of the vascular smooth muscle cell regulate P2 receptor expression as measured by quantitative RT-PCR. Biochem Biophys Res Commun 248:864–870
32. Wu SM, Fujiwara Y, Cibulsky SM, Clapham DE, Lien CL, Schultheiss TM, Orkin SH (2006) Developmental origin of a

bipotential myocardial and smooth muscle cell precursor in the mammalian heart. Cell 127:1137–1150
33. Ohkubo S, Kumazawa K, Sagawa K, Kimura J, Matsuoka I (2001) Beta, gamma-methylene ATP-induced cAMP formation in C6Bu-1 cells: involvement of local metabolism and subsequent stimulation of adenosine A2B receptor. J Neurochem 76:872–880
34. Joseph SM, Pifer MA, Przybylski RJ, Dubyak GR (2004) Methylene ATP analogs as modulators of extracellular ATP metabolism and accumulation. Br J Pharmacol 142:1002–1014
35. Hinze AV, Harst A, Mayer P, von Kügelgen I (2011) P2X1 receptor-mediated inhibition of proliferation of primary human coronary smooth muscle cells via induction of NR4A1. Naunyn-Schmiedebergs Arch Pharmacol 383(Supplement 1):1–11
36. Hinze AV (2012) Purinorezeptor-vermittelte Änderung der Expression Proliferations-regelnder Gene in humanen Koronarglattmuskelzellen. Dissertation, University of Bonn
37. von Kügelgen I, Harden TK (2011) Molecular pharmacology, physiology, and structure of the P2Y receptors. Adv Pharmacol 61:373–415. doi:10.1016/B978-0-12-385526-8.00012-6
38. Hülsmann M, Nickel P, Kassack M, Schmalzing G, Lambrecht G, Markwardt F (2003) NF449, a novel picomolar potency antagonist at human P2X1 receptors. Eur J Pharmacol 470:1–7
39. Yan L, Bertarelli DC, Hayallah AM, Meyer H, Klotz KN, Müller CE (2006) A new synthesis of sulfonamides by aminolysis of p-nitrophenylsulfonates yielding potent and selective adenosine A2B receptor antagonists. J Med Chem 49:4384–4391
40. Nori S, Fumagalli L, Bo X, Bogdanov Y, Burnstock G (1998) Coexpression of mRNAs for P2X1, P2X2, and P2X4 receptors in rat vascular smooth muscle: an in situ hybridization and RT-PCR study. J Vasc Res 35:179–185
41. Kassack MU, Braun K, Ganso M, Ullmann H, Nickel P, Böing B, Müller G, Lambrecht G (2004) Structure-activity relationships of analogs of NF449 confirm NF449 as the most potent and selective known P2X1 receptor antagonist. Eur J Med Chem 39:345–357
42. Rettinger J, Braun K, Hochmann H, Kassack MU, Ullmann H, Nickel P, Schmalzing G, Lambrecht G (2005) Profiling at recombinant homomeric and heteromeric rat P2X receptors identifies the suramin analog NF449 as a highly potent P2X1 receptor antagonist. Neuropharmacology 48:461–468
43. Hechler B, Magnenat S, Zighetti ML, Kassack MU, Ullmann H, Cazenave JP, Evans R, Cattaneo M, Gachet C (2005) Inhibition of platelet functions and thrombosis through selective or nonselective inhibition of the platelet P2 receptors with increasing doses of NF449 [4,4',4",4'"-(carbonylbis(imino-5,1,3-benzenetriylbis-(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid octasodium salt]. J Pharmacol Exp Ther 314:232–243
44. Lewis CJ, Evans RJ (2000) Comparison of P2X receptors in rat mesenteric, basilar, and septal (coronary) arteries. J Auton Nerv Syst 81:69–74
45. Naito Y, Lowenstein JM (1985) 5'-nucleotidase from rat heart membranes. Inhibition by adenine nucleotides and related compounds. Biochem J 226:645–651
46. Schulze-Lohoff E, Zanner S, Ogilvie A, Sterzel RB (1995) Vasoactive diadenosine polyphosphates promote growth of cultured renal mesangial cells. Hypertension 26:899–904
47. Malam-Souley R, Seye C, Gadeau AP, Loirand G, Pillois X, Campan M, Pacaud P, Desgranges C (1996) Nucleotide receptor P2u partially mediates ATP-induced cell cycle progression of aortic smooth muscle cells. J Cell Physiol 166:57–65
48. Vonend O, Grote T, Oberhauser V, von Kügelgen I, Rump LC (2003) P2Y-receptors stimulating the proliferation of human mesangial cells through the MAPK42/44 pathway. Br J Pharmacol 139:1119–1126
49. Shen J, Seye CI, Wang M, Weisman GA, Wilden PA, Sturek M (2004) Cloning, upregulation, and mitogenic role of porcine P2Y2 receptor in coronary artery smooth muscle cells. Mol Pharmacol 66:1265–1274
50. Rauch BH, Rosenkranz AC, Ermler S, Böhm A, Driessen J, Fischer JW, Sugidachi A, Jakubowski JA, Schrör K (2010) Regulation of functionally active P2Y12 ADP receptors by thrombin in human smooth muscle cells and the presence of P2Y12 in carotid artery lesions. Arterioscler Thromb Vasc Biol 30:2434–2442. doi:10.1161/ATVBAHA.110.213702
51. Gui Y, He G, Walsh MP, Zheng XL (2011) Signaling mechanisms mediating uridine adenosine tetraphosphate-induced proliferation of human vascular smooth muscle cells. J Cardiovasc Pharmacol 58:654–662. doi:10.1097/FJC.0b013e318231e929
52. Ginnan R, Sun LY, Schwarz JJ, Singer HA (2012) MEF2 is regulated by CaMKIIδ2 and a HDAC4-HDAC5 heterodimer in vascular smooth muscle cells. Biochem J 444:105–114. doi:10.1042/BJ20120152
53. Ruiz-Hurtado G, Morel E, Domínguez-Rodríguez A, Llach A, Lezoualc'h F, Benitah JP, Gomez AM (2012) Epac in cardiac calcium signaling. J Mol Cell Cardiol. doi:10.1016/j.yjmcc.2012.11.021, in press
54. Chaulet H, Desgranges C, Renault MA, Dupuch F, Ezan G, Peiretti F, Loirand G, Pacaud P, Gadeau AP (2001) Extracellular nucleotides induce arterial smooth muscle cell migration via osteopontin. Circ Res 89:772–778
55. Pillois X, Chaulet H, Belloc I, Dupuch F, Desgranges C, Gadeau AP (2002) Nucleotide receptors involved in UTP-induced rat arterial smooth muscle cell migration. Circ Res 90:678–681
56. Yu N, Erb L, Shivaji R, Weisman GA, Seye CI (2008) Binding of the P2Y2 nucleotide receptor to filamin A regulates migration of vascular smooth muscle cells. Circ Res 102:581–588. doi:10.1161/CIRCRESAHA.107.162271
57. Harada K, Matsumoto Y, Umemura K (2011) Adenosine diphosphate receptor P2Y12-mediated migration of host smooth muscle-like cells and leukocytes in the development of transplant arteriosclerosis. Transplantation 92:148–154. doi:10.1097/TP.0b013e318221d407
58. Morris GE, Nelson CP, Brighton PJ, Standen NB, Challiss RA, Willets JM (2012) Arrestins 2 and 3 differentially regulate ETA and P2Y2 receptor-mediated cell signaling and migration in arterial smooth muscle. Am J Physiol Cell Physiol 302:C723–C734. doi:10.1152/ajpcell.00202.2011
59. Wiedon A, Tölle M, Bastine J, Schuchardt M, Huang T, Jankowski V, Jankowski J, Zidek W, van der Giet M (2012) Uridine adenosine tetraphosphate (Up4A) is a strong inductor of smooth muscle cell migration via activation of the P2Y2 receptor and cross-communication to the PDGF receptor. Biochem Biophys Res Commun 417:1035–1040. doi:10.1016/j.bbrc.2011.12.088
60. Abele S, Weyand M, Wollin M, Hiemann NE, Harig F, Fischlein T, Ensminger SM (2006) Clopidogrel reduces the development of transplant arteriosclerosis. J Thorac Cardiovasc Surg 131:1161–1166
61. van Giezen JJ, Nilsson L, Berntsson P, Wissing BM, Giordanetto F, Tomlinson W, Greasley PJ (2009) Ticagrelor binds to human $P2Y_{12}$ independently from ADP but antagonizes ADP-induced receptor signaling and platelet aggregation. Thromb Haemost 7:1556–1565. doi:10.1111/j.1538-7836.2009.03527
62. Savi P, Zachayus JL, Delesque-Touchard N, Labouret C, Hervé C, Uzabiaga MF, Pereillo JM, Culouscou JM, Bono F, Ferrara P, Herbert JM (2006) The active metabolite of clopidogrel disrupts $P2Y_{12}$ receptor oligomers and partitions them out of lipid rafts. Proc Natl Acad Sci U S A 103:11069–11074
63. Algaier I, Jakubowski JA, Asai F, von Kügelgen I (2008) Interaction of the active metabolite of prasugrel, R-138727, with cysteine 97 and cysteine 175 of the human $P2Y_{12}$ receptor. J Thromb Haemost 6:1908–1914. doi:10.1111/j.1538-7836.2008.03136