Biphasic increase of gap junction coupling induced by dipyridamole in the rat aortic A-10 vascular smooth muscle cell line

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Abstract The rat aortic smooth muscle cell line A-10 was used to investigate the effect of dipyridamole on the gap junction coupling of smooth muscle cells. The scrape loading/dye transfer (SL/DT) technique revealed that dipyridamole concentrations between 5 μ M and 100 μ M significantly increased gap junction coupling. The adenosine receptor antagonist MRS 1754, as well as the PKA inhibitors Rp-cAMPS and H-89 were able to inhibit the dipyridamole-related increase in coupling, while forskolin and Br-cAMP also induced an enhancement of the gap junction coupling. Regarding the time-dependent behaviour of dipyridamole, a short-term effect characterised by an oscillatory reaction was observed for application times of

less than 5 h, while applications times of at least 6 h resulted in a long-term effect, characterised by a constant increase of gap junction coupling to its maximum levels. This increase was not altered by prolonged presence of dipyridamole. In parallel, a short application of dipyridamole for at least 15 min was found to be sufficient to evoke the long-term effect measured 6 h after drug washout. We propose that in both the short-term and long-term effect, cAMP-related pathways are activated. The short-term phase could be related to an oscillatory cAMP effect, which might directly affect connexin trafficking, assembly and/or gap junction gating. The long-term effect is most likely related to the new expression and synthesis of connexins. With previous data from a bovine aortic endothelial cell line, the present results show that gap junction coupling of vascular cells is a target for dipyridamole.

Short summary Dipyridamole is known as an antithrombotic drug, which acts by inhibiting adenosine transport leading to synthesis of cAMP in platelets. The present study shows that dipyridamole also affects vascular smooth muscle cells by enhancing their gap junction coupling through stimulation of the cAMP/PKA pathway. The increase of gap junction coupling was found to be biphasic with a short-term oscillatory behaviour, probably related to oscillatory synthesis of cAMP, which then affected already synthesized connexins and gap junction channels, and a long-term effect, which seemed to depend on a cAMP/PKA-induced expression and synthesis of connexins.

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Abbreviations

Br-cAMP	8-Bromoadenosine-3' 5'-cyclic monophosphate
cAMP	Cyclic adenosine-3' 5'-cyclic monophosphate
Cx	Connexin
Dip.	Dipyridamole
ENT	Equilibrative nucleoside transporter
Eth.	0.5% Ethanol
For.	Forskolin
H-89	N-[2-(p-Bromocinnamylamino)ethyl]- 5-isoquinolinesulfonamide
LY	Lucifer Yellow
PKA	Protein kinase A
Rp-cAMPS	Rp-adenosine-3',5'-cyclic mono- phosphorothioate triethylamine salt
SL/DT	Scrape loading/dye transfer

Introduction

The European Stroke Prevention Study 2 (ESPS2) and the European/Australasian Stroke Prevention in Reversible Ischaemia Trial (ESPRIT) have shown that a combined therapy of dipyridamole and aspirin is twice as effective in the reduction of the risk of a secondary stroke compared to aspirin alone (Diener et al. 1996; ESPRIT Study Group et al. 2006). This beneficial effect of dipyridamole was mainly discussed with respect to the antithrombotic effect of dipyridamole, while its possible effects on other cell types such as vascular cells were neglected. A recent publication proposed that dipyridamole should be considered as a drug targeting vascular cells. Previously, we showed that dipyridamole increased the gap junction coupling of endothelial cells by activating a cAMP-dependent pathway (Begandt et al. 2010). Therefore, in this study, we asked whether dipyridamole could also affect the gap junction coupling of vascular smooth muscle cells.

Dipyridamole inhibits the uptake of adenosine, increasing the concentration of adenosine in the extracellular space. In platelets, adenosine induces cytosolic cAMP synthesis through the activation of adenylyl cyclase via the G_s-protein-coupled adenosine receptors (Alheid et al. 1989; Eisert 2006). As a secondary effect, dipyridamole inhibits phosphodiesterases, inducing a stabilisation of cytosolic cAMP (Anfossi et al. 2002; Kim and Liao 2008).

Vascular smooth muscle cells express gap junctions composed of connexin37 (Cx37), Cx40, Cx43, and Cx45 (de Wit et al. 2006; Figueroa et al. 2004, 2006; Figueroa and Duling 2009; Haefliger et al. 2004). The generation of Cx37, Cx40, Cx43, and Cx45 gene-targeted mice has provided information about pathologies related to the dysfunction of gap junction channels in the vascular system (Chadjichristos and Kwak 2007; Schmidt et al. 2012). Analysis of the phenotypes of these mice revealed the crucial role of vascular connexins in the regulation of blood pressure (Haefliger and Meda 2000; Haefliger et al. 1999; Jobs et al. 2012; Wagner et al. 2007, 2010). The vascular-specific deletion of Cx43 correlated with hypotension (Liao et al. 2001). Alternatively, Cx40-deficient mice were hypertensive and showed an impaired transmission of the endothelium-dependent vasodilator response (de Wit et al. 2000, 2003; Wagner et al. 2007, 2010). In the double Cx37/Cx40 knockout mice, vascular abnormalities were found (Simon and McWhorter 2002). Thus, gap junction coupling in vascular cells is a potential target of pathophysiological conditions, such as arteriosclerosis, hypertension or heterotaxia (Brisset et al. 2009; Levin and Mercola 1998; Nicholson and Bruzzone 1997), and could also be a target for therapeutic strategies in the treatment of vascular impairments (Figueroa and Duling 2009).

Different mechanisms, such as $[Ca^{2+}]_i$ levels, pH and phosphorylation, were shown to modulate gap junction

coupling (Cruciani and Mikalsen 2002; Harris 2001; Lampe and Lau 2004). The effect of phosphorylation is complex and appears to be related to activated kinases. While protein kinase C-dependent phosphorylation reduced gap junction coupling, protein kinase A (PKA)-dependent phosphorylation frequently potentiated gap junction coupling. This was particularly true for gap junctions composed of Cx43 and Cx40 (Cruciani and Mikalsen 2002; Harris 2001; Lampe and Lau 2004; van Rijen et al. 2000). In addition to the regulation of the open probability of gap junction channels, coupling is changed by regulating the trafficking and assembly of existing connexins or by inducing mRNA expression and protein synthesis (Paulson et al. 2000). Intracellular cAMP is credited with playing a key role in this regulation. Intracellular cAMP is credited with playing a key role in this regulation. Depending on the cell type and experimental design, cAMP increased gap junction coupling by elevating Cx43 trafficking (Solan and Lampe 2009), by changing the levels of connexin mRNA or protein (Somekawa et al. 2005; Yogo et al. 2002) or through connexin phosphorylation (Atkinson et al. 1995; TenBroek et al. 2001). Because dipyridamole increases the concentration of cytosolic cAMP (Anfossi et al. 2002; Kim and Liao 2008), we propose that dipyridamole could activate the cAMP signalling cascade, which involves both PKA-dependent and PKA-independent pathways, and thereby potentiate gap junction coupling in vascular smooth muscle cells.

Materials and methods

Materials

H-89, 8-Br-cAMP, forskolin, MRS 1754 and Lucifer yellow (LY) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Rp-cAMPS was purchased from Enzo Life Sciences GmbH (Lörrach, Germany). Dipyridamole was kindly provided by Boehringer Ingelheim International GmbH (Biberach, Germany).

Cell culture

The rat aortic smooth muscle cell line A-10 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany) was cultivated using Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 µg/mL). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was renewed every 2–3 days.

For SL/DT experiments, glass cover slips (Ø 10 mm) were placed into the wells of a 24-multiwell plate containing 1 ml of the culture medium. Cells were seeded at a density of 2×10^5 cells/well and cultivated for 24 h to allow the cells

to adhere and form a monolayer on the cover slip. Dipyridamole or other agents were added for the indicated incubation times.

Treatment with dipyridamole

Dipyridamole and forskolin were prepared as stock solutions in ethanol and added to the cell culture at the desired concentration. In all experiments with dipyridamole or forskolin, a final concentration of 0.5% ethanol was present. All additional chemicals were dissolved in a salt solution containing 121 mM NaCl, 5 mM KCl, 6 mM NaHCO₃, 5.5 mM glucose, 0.8 mM MgCl₂, and 25 mM HEPES (pH 7.4, 295 mOsmol/l). Untreated cells and cells treated with 0.5% ethanol were used as controls.

Scrape loading/dye transfer

Dipyridamole dissolved at appropriated concentration in cell culture medium was applied for a certain time period (0.08 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, etc.) on cell monolayer grown on cover slips. In parallel, a second group of cover slips with cell monolayer was maintained under control conditions with normal cell culture medium and a third group was treated with 0.5% ethanol dissolved in cell culture medium (ethanol was used as vehicle for dipyridamole and was present at 0.5% in all dipyridamole experiments). To each time point which was investigated cells treated with dipyridamole, cells maintained under control conditions and cells treated with ethanol were simultaneously transferred into the scrape loading device (Begandt et al. 2010), which contained a scrape loading solution composed of (in mM): 145 NaCl, 5 KCl, 1 MgCl₂, 5 Glucose, 10 Hepes and 0.25% LY (pH: 7.4, 295 mOsmol/l). The SL/DT experiment as described by el-Fouly et al. (el-Fouly et al. 1987) and modified by Begandt et al. (Begandt et al. 2010) was performed using a sharp razor (Science Services, Munich, Germany). The cells were then maintained in the device for 5 min to allow the absorption of LY by the damaged cells and its further diffusion into the monolayer through their gap junctions. Thereafter the cells were washed with fresh salt solution containing 1.8 mM CaCl₂ but not LY. The cells were then fixed by a treatment of 10 min with 4% formaldehyde dissolved in phosphate buffered saline (PBS) composed of (in mM) 136.8 NaCl, 2.68 KCl, 9.68 Na₂HPO₄, and 1.14 KH₂PO₄ (pH: 7.4). The fixed cells were conserved in PBS for further analysis.

To evaluate the dye transfer, the fluorescence of LY was observed using an inverted Nikon Eclipse TE2000-E confocal laser scanning microscope with a 10x objective (Nikon, Düsseldorf, Germany) after excitation with a 488 nm laser. A viewing area of 1,024×1,024 pixels was recorded. The software program EZ-C1 3.50 (Nikon, Düsseldorf, Germany)

was used to record the images. The estimation of the dye diffusion distance was performed using Image J software, as described in Begandt et al. (Begandt et al. 2010). For a better comparison of the experiments, the diffusion distance of dipyridamole-treated cells was normalised to control experiments.

Statistical analysis

The results are given as the average ± SEM for at least $n=4$ experiments for each treatment. For statistical comparison, the results obtained from ethanol treated cells or untreated cells were used as reference for dipyridamole and pharmacologically treated cells. In all experiments, untreated and ethanol-treated cells showed no significant differences. For significance, Student's *t*-test was applied. The results were indicated as significant with $p<0.05$: *, $p<0.01$: **, and $p<0.001$: ***.

Results

The SL/DT technique (Fig. 1a) was used to study the impact of dipyridamole on gap junction coupling in aortic smooth muscle cells. We found that dipyridamole significantly increased gap junction coupling in A-10 cells, a rat aortic smooth muscle cell line. Untreated cells or cells cultured in the presence of 0.5% ethanol, which was used as a vehicle for dipyridamole, showed a LY diffusion distance of 102 µm and 104 µm, respectively. After treatment with dipyridamole for 24 h at concentrations of 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 75 µM, or 100 µM, a dye diffusion distance of 107 µm, 118 µm, 133 µm, 142 µm, 164 µm, 172 µm, and 185 µm, respectively, was found (Fig. 1b). This increase in gap junction coupling could already be achieved by a drug application time of at least 6 h (Fig. 3).

In platelets, dipyridamole affects the cells by activating adenylyl cyclase and inhibiting phosphodiesterases, which leads to an increase of the concentration of cytosolic cAMP (Alheid et al. 1989; Anfossi et al. 2002; Eisert 2006; Kim and Liao 2008). Recently, we showed that cAMP/PKA-dependent mechanisms were also activated by dipyridamole in aortic endothelial cells (Begandt et al. 2010). To transfer this model to vascular smooth muscle cells, we first studied whether a blockade of the adenosine receptor or an inhibition of PKA could reduce the dipyridamole-related enhancement of gap junction coupling. Secondly, we analysed whether activation of cAMP production by forskolin or the application of 8-Br-cAMP could enhance the gap junction coupling. We found that the antagonist of the adenosine receptor A_{2B} MRS 1754 (1 µM) and the PKA inhibitors H-89 (15 µM) and Rp-cAMPS (200 µM) significantly reduced the dipyridamole-related enhancement of the dye

Fig. 1 Dipyridamole affects gap junction coupling of rat aortic A-10 smooth muscle cells. Cells grown to a monolayer were treated with dipyridamole (dip.) for 24 h before SL/DT experiments. **a** The fluorescence micrographs show the diffusion of LY (green) in cells cultured under control conditions (eth.) and incubated with 50 μ M dip. The cellular nuclei were stained with DAPI (blue). The scale bar corresponds to 100 μ m. **b** Quantitative evaluation of the dye diffusion distance in relationship to dipyridamole concentration. Dipyridamole induced a significant increase of the dye diffusion distance compared with 0.5% ethanol, which alone did not affect the dye diffusion distance compared to untreated cells (unt.). A significant increase in gap junction coupling was observed at dipyridamole concentrations of at least 5 μ M

diffusion distance (Fig. 2a and b). Furthermore, the application of the adenylyl cyclase activator forskolin (100 μ M) or the membrane permeable cAMP analogue 8-Br-cAMP (1 mM) for 6 h increased the dye diffusion distance up to 189% and 181%, respectively (Fig. 2c). The results from the blockade of the adenosine receptors and the inhibition of PKA as well as the activation of the cAMP signalling pathway suggest that dipyridamole could affect gap junction coupling by stimulating the cAMP/PKA-dependent pathway. 8-Br-cAMP or forskolin induced a more pronounced increase of gap junction coupling than dipyridamole (Fig. 2c). Moreover 8-Br-cAMP has a more rapid effect as dipyridamole acting within 1 h of application (result not shown). These differences in kinetic and intensity between dipyridamole and 8-Br-cAMP (or forskolin) might reflect the fact that in case of dipyridamole the different steps from inhibition of adenosine transporter to synthesis of cAMP must take place and are probably limiting, while in case of 8-Br-cAMP the molecule only needs to diffuse into the cells. The inhibition of PKA did not completely block the increase in gap junction coupling induced by a high dose of dipyridamole (50 μ M), whereas a low dose of dipyridamole

(5 μ M and 10 μ M) evoked an increase in gap junction coupling, which could be returned to control levels after inhibition with H-89 or Rp-cAMPS.

To analyse the period of time required for dipyridamole to effect gap junction coupling in smooth muscle cells, cells were treated with dipyridamole for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 9 h, or 24 h. Dye transfer experiments revealed two different behaviours with respect to the application time (Fig. 3). Within the first 6 h, we observed for high dipyridamole concentrations alternating an increase followed by a decrease of gap junction coupling. As shown in Fig. 3, compared to cells cultivated with ethanol, 50 μ M dipyridamole changed the gap junction coupling up to 114%, 100%, 127%, 108%, 129%, 104%, 115% and 149% after application times of 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h, respectively (Fig. 3). Compared to ethanol-treated cells changes observed at 1 h, 3 h, 5 h and 6 h were statistically significant (Table 1). Moreover, the changes induced by dipyridamole after application times of 0.25 h, 1 h, 3 h, 5 h and 6 h were strikingly larger than the changes observed after application times of 0.5 h, 2 h and 4 h (Table 1 and Fig. 3). To analyse whether the differences in

Fig. 2 **a** The antagonist of the adenosine receptor A_{2B} MRS 1754 as well as **(b)** the PKA inhibitors Rp-cAMPS (200 μ M) and H-89 (15 μ M) significantly (# for $p < 0.05$) inhibited the dipyridamole- (dip., 50 μ M) induced enhancement of gap junction coupling. **c** In contrast, forskolin (for., 100 μ M) and 8-Br-cAMP (1 mM) significantly increased gap junction coupling after 6 h of incubation time similar to dipyridamole

the diffusion distance measured at different measuring time points were not just random variations, the diffusion distances found at two consecutive measuring time points were

Fig. 3 Time-dependent effect of dipyridamole on gap junction coupling. The figure shows the change of the diffusion distance induced by 10 μ M (●) or 50 μ M (▲) dipyridamole (7) after an application time of 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 9 h or 24 h compared with control cells (0.5% ethanol (■)). After the respective time period, SL/DT experiments were performed (see material and methods). The analysis of dye diffusion revealed that dipyridamole induced a change in the gap junction coupling of A-10 smooth muscle cell line in a biphasic manner, which could be separated into short-term (0.25 h to 5 h) and long-term effects (over 6 h). Short-term behaviour was characterised by an oscillatory change of coupling by high dose dipyridamole (for more detail see table 1). The long-term effect was observed after at least 6 h of incubation and reached a constant level for both low (10 μ M) and high concentrations (50 μ M) of dipyridamole. The further presence of dipyridamole sustained the enhanced gap junction coupling, while a washout of the drug correlated with a decline of gap junction coupling to a basal level within the next 24 h (dashed line for 50 μ M and dotted line for 10 μ M)

compared. The Student's t test showed that the diffusion distances measured at 0.5 h and 1 h, 1 h and 2 h, 2 h and 3 h, 3 h and 4 h and 5 h and 6 h were significantly different, while the diffusion distances measured after an application time of over 6 h stayed constant at a relative increase of about 150% (Table 1). These results indicate an oscillatory behaviour induced by the high dipyridamole concentration during the first 5–6 h of application time. This oscillatory effect of dipyridamole was not observed for low concentrations, as shown for 10 μ M dipyridamole (Fig. 3). A long-term effect was observed for incubation times exceeding 5 h: the increase of the dye diffusion distance induced by a high dose of dipyridamole was maximal (up to at least 144%) and did not substantially change for a further incubation time, lasting up to 24 h (Fig. 3.). Similarly, a constant enhancement (up to at least 124%) after an incubation time of over 5 h was also observed for low dipyridamole concentrations (10 μ M) (Fig. 3). For both high and low dipyridamole concentrations, a continuous presence of dipyridamole was required to maintain this elevated gap junction coupling. As shown in Fig. 3, removal of dipyridamole after an application time of 6 h led to a continuous decrease of gap junction coupling within the next

Table 1 Results of scrape-loading experiments in cells treated with 50 μ M dipyridamole (dip.) for the indicated durations. The LY diffusion distances of dipyridamole-treated cells were normalised to the diffusion distances of cells treated with ethanol for the corresponding period. The results are given as averages $\pm$ SEM for at least eight experiments for each time period. The oscillatory behaviour was observed as significant differences between the diffusion distances in dipyridamole-treated cells found at two successive measuring time points (# for $p < 0.05$)

incubation time in h with 50 μ M dip.	relative diffusion distance in % $\pm$ SEM
0.25	113.6 $\pm$ 5.1
0.5	99.7 $\pm$ 3.5
1	126.4 $\pm$ 9.5
2	108.2 $\pm$ 3.7
3	128.9 $\pm$ 6.4
4	104.2 $\pm$ 5.9
5	114.9 $\pm$ 2.3
6	148.9 $\pm$ 5.2
9	144.4 $\pm$ 4.1
24	158.1 $\pm$ 10.9

24 h, indicating that the effect of dipyridamole on gap junction coupling was reversible. The diffusion distance of cells treated with 50 μ M dipyridamole for 6 h decreased from 149% to 124%, 138%, 116% and 104% after 6 h, 12 h, 18 h, and 24 h without the presence of the drug. Similarly, in cells treated with 10 μ M dipyridamole for 6 h, a gap junction coupling decrease from 124% to 108%, 117%, 110%, and 115%, was found at respectively 6 h, 12 h, 18 h, and 24 h after removal of the drug (Fig. 3).

The results presented above showed that the effect of dipyridamole on gap junction coupling is variable by an application time of less than 6 h. We therefore analysed how dipyridamole affected the cells when applied only for a short time, and the diffusion distance was measured 6 h after the beginning of the drug application time. Application times of 0.08 h (5 min), 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h and 5 h were tested and compared to a 6 h continuous presence of dipyridamole. SL/DT experiments were performed 6 h after the beginning of the drug application. Normalised to the control conditions the results showed that for high concentrations of dipyridamole, gap junction coupling was already significantly increased for a drug incubation time of the first 15 min. For the period between 1 h and 5 h, the effect of dipyridamole stayed significantly high in comparison to control conditions but remained constant at approximately 120%

of the control level. This 120% increase was further reinforced when the cells were further treated with dipyridamole for at least 6 h (continuous presence) (Table 2). A low concentration of dipyridamole showed no significant effect for short incubation times, i.e., less than 4–5 h. A clear increase was only found after the continuous presence of dipyridamole for 6 h (Table 2). For these short application time experiments, it was striking that, for high dipyridamole concentrations, an application time of 15 min was enough to induce a significant increase of gap junction coupling measured after 6 h, with a mean value of approximately 120% (Table 2). Accordingly, we analysed how the short application time of only 15 min changed the behaviour of gap junction coupling for a long period. Cells were treated for 15 min with dipyridamole, and SL/DT experiments were performed at the indicated time points after drug removal. For the high dipyridamole concentration (50 μ M), an increase of the dye diffusion was observed directly after the removal of the drug. This increase disappeared within the next 2 hours after drug removal, reappeared 5–6 h later to a more pronounced level and declined continuously to the control level by further cultivation of the cells without the drug (Fig. 4). Low concentrations (10 μ M) did not significantly affect the gap junction coupling directly after a 15 min drug application. However, we observed a significant enhancement of gap junction coupling 5–6 h after the cells were treated with the drug for 15 min. This increase was followed by a decline to basal levels with further cultivation time (Fig. 4).

Discussion

The results presented in this report show that dipyridamole affects the gap junction coupling of a vascular smooth muscle cell line in a time- and concentration-dependent manner (Fig. 1). A drug application time of more than 6 h stably increased the gap junction coupling of the A-10 smooth muscle cell line. Compared with control conditions, this increase was already significant at a concentration of 5 μ M dipyridamole. A further increase of the drug concentration correlated with a reinforcement of the enhancement of gap junction coupling (Fig. 1). As an antithrombotic drug, it has been shown that dipyridamole increased the intracellular cAMP concentration in platelets through the inhibition of nucleoside transporters and activation of adenosine receptors, which in turn induced the stimulation of adenylyl cyclase. Additionally, the inhibition of phosphodiesterases by dipyridamole prolonged the increase of cAMP levels (Anfossi et al. 2002; Eisert 2006; Kim and Liao 2008). Therefore, we hypothesised that through the stimulation of cAMP synthesis, dipyridamole could induce the enhancement of gap junction coupling in vascular smooth muscle cells (Figs. 1 and 2). cAMP is known to increase gap junction

Table 2 Dye diffusion distances measured in cells treated with 10 μ M or 50 μ M dipyrindamole (dip.) for indicated durations relative to ethanol-treated cells. The cells were incubated with dipyrindamole for the indicated duration times. Thereafter, dipyrindamole was removed and the cells were incubated in untreated growth medium. The SL/DT experiments (materials and methods) were performed 6 h after the beginning of dipyrindamole application. The results are given as average $\pm$ SEM for at least six experiments. A minimal incubation time of 15 min was enough to significantly affect gap junction coupling measured 6 h after beginning of the drug application (indicated with *)

Incubation time in h with dip. & measurement after 6 h	Relative diffusion distance in % $\pm$ SEM	
	10 μ M dip.	50 μ M dip.
0.08	109.2 $\pm$ 2.7	111.8 $\pm$ 1.3
0.25	113.9 $\pm$ 7.9	131.1 $\pm$ 8.4*
0.5	104.0 $\pm$ 6.4	109.8 $\pm$ 4.7
1	108.6 $\pm$ 4.8	126.6 $\pm$ 9.0*
2	105.9 $\pm$ 3.1	116.1 $\pm$ 3.2*
3	112.1 $\pm$ 3.3	118.9 $\pm$ 3.8*
4	111.1 $\pm$ 2.9*	118.9 $\pm$ 1.7*
5	105.5 $\pm$ 3.0	121.8 $\pm$ 4.5*
6	127.2 $\pm$ 6.4*	152.1 $\pm$ 8.7*

permeability in different types of cells (Holm et al. 1999; Paulson et al. 2000). The effect of cAMP was correlated with changes in mRNA expression, protein synthesis, trafficking and/or gap junction formation (Atkinson et al. 1995; Somekawa et al. 2005; TenBroek et al. 2001; Yogo et al. 2002). One of the known signal transduction pathways of cAMP is the direct stimulation of PKA, a kinase whose

Fig. 4 A short period of dipyrindamole application for 15 min induced a long-term effect on gap junction coupling that lasted for 18 h. Cells were incubated for 15 min with 10 μ M (●) or 50 μ M (▲) dipyrindamole and were compared with control cells (■). After drug washout, the gap junction coupling was analysed using SL/DT experiments as described above (material and methods) at the following time points: 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, 18 h and 24 h. The figure shows that both doses of dipyrindamole evoked a significant long-term increase of gap junction coupling that was first detected 5–6 h after drug washout

activity generally correlates with an increase in gap junction coupling (Lampe and Lau 2004; van Rijen et al. 2000). Additionally, Sands and Palmer and Somekawa and colleagues showed that treating gap junction-coupled cells with cAMP affected gap junction permeability by the stimulation of PKA-dependent and PKA-independent signalling cascades (Sands and Palmer 2008; Somekawa et al. 2005). In our experiments, the antagonist of the adenosine receptor A_{2B} MRS 1754 as well as the PKA inhibitors Rp-cAMPS and H-89 inhibited a large part of the dipyrindamole-related enhancement of gap junction coupling (Fig. 2a and b). Forskolin and 8-Bromo-cAMP could enhance gap junction coupling (Fig. 2b), despite some differences in the kinetic and the amplitude, probably mostly due to high concentrations of the activators as well as the fact that dipyrindamole acts by many steps before activating adenylyl cyclase to produce cAMP. Taken together, these results support the hypothesis that the observed dipyrindamole-dependent enhancement of gap junction coupling in A-10 smooth muscle cells may be largely related to the activation of cAMP/PKA-dependent signalling pathways activated as result of the inhibition of adenosine transporter by dipyrindamole. This hypothesis is in agreement with the observation that a cAMP/PKA pathway activated by 11,12-epoxyeicosatrienoic acid could dynamically modulate gap junction coupling of endothelial cells (Popp et al. 2002). The finding that the stimulation of cAMP/PKA pathway in the vascular system by nitric oxide (NO) enhanced the *de novo* formation of gap junctions (Hoffmann et al. 2003) also supports our hypothesis. Furthermore our hypothesis agrees with the observations by other authors who showed that cAMP treatment affected gap junction permeability simultaneously by PKA-dependent and PKA-independent signalling pathways (Sands and Palmer 2008; Somekawa et al. 2005). Moreover, the results presented in this report indicate that dipyrindamole could affect gap junction coupling in vascular smooth muscle cells in the same manner as it affects gap junction coupling in vascular endothelial cells, probably by inducing the synthesis of cAMP, which in turn could activate PKA (Begandt et al. 2010). The activated PKA could target different proteins, resulting in an increase in gap junction coupling. Proteins involved in the transcription, protein synthesis or hexamerisation of connexins, and those that participate in trafficking, membrane insertion, open probability or turnover of connexons, could be substrates of PKA-dependent phosphorylation (Lampe and Lau 2004; van Rijen et al. 2000).

Parallel to the stationary analysis, in this study, we further investigated the period of time required for dipyrindamole to affect gap junction coupling in aortic smooth muscle cell line. The coupling showed two different behaviours that developed after short-term (from 0.25 h to 5 h) and long-term incubation (over 6 h). During the continuous presence of dipyrindamole, the enhancement of gap junction coupling from the short-term effect was characterised by an oscillatory increase of gap

junction coupling, which lasted for the first 4–5 h during which the drug was present (Table 1, Fig. 3). This oscillatory effect, observed as a short-term response to a high dose of dipyrindamole (50 μ M), appeared to be the result of the continuous presence of the drug. In contrast, if the drug was washed out after a 15 min application time, only the first peak was observed, and the oscillatory response failed to occur for a further 2–5 h (Fig. 4). Instead, the level of gap junction coupling declined after the first peak, and it continued to decline to the level observed for control conditions within the next 2–5 h (Fig. 4). Dipyrindamole is known to inhibit adenosine transporters, leading to elevated adenosine concentrations in the extracellular space. This in turn stimulates adenosine receptors, which activate downstream adenylyl cyclase and PKA (Eisert 2006). Our findings that the stimulation of adenosine receptors evoked an oscillatory behaviour after short-term treatment with dipyrindamole could be explained by the agonist-induced desensitisation-resensitisation cycle of the adenosine receptors. This is in agreement with the known internalisation behaviour of stimulated adenosine A_{2A} and A_{2B} receptors. After internalisation, most of the G-protein-coupled receptors (GPCR) are recycled and transported into the membrane for a new cycle of agonist activation (Mundell and Kelly 2011). This could in turn lead to the oscillatory increase of intracellular cAMP concentrations, thereby influencing the gap junction coupling. Our results are corroborated by Mundell and Kelly (1998) (Mundell and Kelly 1998), who found a turnover time of 60 to 90 min for A_{2A} receptor desensitisation and resensitisation by measuring changes in intracellular cAMP concentration. As a short-term effect, the high dipyrindamole concentration-related rapid cAMP increase could lead to an increase of gap junction coupling within minutes due to changes in assembly and/or open probability of existing connexins and gap junctions (Abudara et al. 2000; Atkinson et al. 1995; Burghardt et al. 1995). Low dipyrindamole concentrations increased gap junction coupling only after at least 6 h application time as a long-term effect independently of a permanent or impermanent drug presence (Figs. 3 and 4). It is possible that low dipyrindamole concentrations ($\leq 10 \mu$ M) did not rapidly elevate intracellular cAMP to the levels that were needed to induce short-term regulation of gap junction coupling. This is most likely related to the dipyrindamole-induced inhibition of equilibrative nucleoside transporters (ENT), which are characterised by k_i values in a range of 0.05–10 μ M for ENT1–4 (Baldwin et al. 2005; Molina-Arcas et al. 2009; Podgorska et al. 2005; Visser et al. 2002). PCR experiments showed that *ent2* is predominantly expressed within the plasma membrane in the vascular smooth muscle cell line A-10 (data not shown), which is only half-inhibited by dipyrindamole with a k_i value of 5–10 μ M (Visser et al. 2002).

The long-term effect of dipyrindamole treatment showed a constant increase of gap junction coupling for high and low

dipyrindamole concentrations after at least 6 h of drug application (Fig. 3). The long-term effect did not need a continuous presence of the drug (Fig. 4), and there is an obvious difference of maximal increase between continuous and short-time drug presence in long-term behaviour (Figs. 3 and 4). An application time as short as 15 min was enough to induce an increase of gap junction coupling in long-term behaviour (Fig. 4). It is assumed that cAMP is the intracellular mediator for the increased coupling induced by dipyrindamole. It is therefore tempting to suppose that the very first increase of cAMP at the beginning of the drug application activated, amongst others, a cAMP signalling cascade that led to the new expression and synthesis of connexins, which are measurable after 6 h in terms of enhanced gap junction coupling (Table 2, Fig. 4). The robust increase of gap junction coupling induced by a continuous presence of dipyrindamole for at least 6 h (Fig. 3) was most likely due to a combination of the short-term (e.g., trafficking, hexamerisation, membrane insertion and/or gap junction formation) and long-term effects (e.g., transcription and/or protein synthesis). We therefore propose that the dipyrindamole-related increase of gap junction coupling is due to the activation of mechanisms that affect existing gap junction channels and induce the synthesis of connexins. At the mRNA level, we found that the A-10 cell line expressed Cx37, Cx40, Cx43 and Cx45 (results not shown). Whether all these isoforms are involved in gap junction formation and whether they are equally affected by dipyrindamole is not clear. Combination of molecular biological, biochemical, immunocytochemical and physiological experiments far beyond the present report will clarify this topic.

The therapeutic concentration of dipyrindamole in blood plasma is about 4 μ M (Eisert 2006). The present report shows that similar concentrations of the drug enhances the gap junction coupling of vascular smooth muscle cells. This effect is most likely due to an increased concentration of intracellular cAMP. The increased cAMP level activates various regulatory mechanisms, which in turn increase gap junction coupling in a biphasic manner that is dependent on short- and long-term observation. We hypothesise that the short-term effects depend on the regulation of existing connexins and gap junctions and that the long-term regulation takes place through the modulation of gene expression and protein synthesis of new connexins. The vascular smooth muscle cells express Cx37, Cx40, Cx43 and Cx45 (de Wit et al. 2006; Figueroa et al. 2004, 2006; Figueroa and Duling 2009; Haefliger et al. 2004; He et al. 1999; Hoffmann et al. 2003; Schmidt et al. 2008). Whether these connexins are equally affected by dipyrindamole-induced mechanisms is not clear. Finally, an increase in gap junction coupling in vascular smooth muscle cells together with the previously reported dipyrindamole-related increase of gap

junction coupling in endothelial cells (Begandt et al. 2010) would lead in vivo to an upregulation of vasomotility and would allow for better blood flow.

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