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Dipyridamole increases gap junction coupling in bovine GM-7373 aortic endothelial cells by a cAMP-protein kinase A dependent pathway

D. Begandt · W. Bintig · K. Oberheide · S. Schlie · A. Ngezahayo

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Abstract The scrape-loading/dye transfer technique was applied on the bovine aortic endothelial cell line GM-7373 to analyze the effects of the antithrombolytic drug dipyridamole on gap junction coupling in endothelial cells. We found that a cell treatment for 24 h with dipyridamole in therapeutically relevant concentrations (1–100 μM) increased gap junction coupling in a dose dependent manner. Similar to dipyridamole, forskolin as well as 8-Br-cAMP increased the gap junction coupling, while dibutyryl-cGMP (db-cGMP) did not affect the gap junction coupling of the GM-7373 endothelial cells. In parallel, a pharmacological inhibition of protein kinase A (PKA) with N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), antagonised the action of dipyridamole on gap junction coupling. We propose that the observed dipyridamole induced increase in gap junction coupling in endothelial cells is related to a cAMP-PKA dependent phosphorylation pathway. The report shows that gap junction coupling in endothelial cells is a suitable therapeutic target for treatment of cardiovascular diseases.

Keywords Dipyridamole · Endothelial cells · Gap junction · Connexins · Scrape loading · cAMP · PKA · H-89

Introduction

Dipyridamole has been intensely investigated with respect to stroke prevention. The administration of aspirin together with dipyridamole in a combined therapy reduced stroke risk more than twice compared to aspirin alone (Weber et al. 2009). It is known that dipyridamole inhibited the uptake of adenosine, which resulted in an increase in adenosine concentration in the interstitial space. In platelets, it was shown that the increased extracellular adenosine concentration activated adenylyl cyclase via adenosine receptors and thereby increased cytosolic cAMP (Alheid et al. 1989; Eisert 2007). A second effect of dipyridamole is the blockage of phophodiesterases, which results in further increase in cytosolic cAMP and probably cGMP concentrations (Anfossi et al. 2002; Kim and Liao. 2008). So far, the effects of dipyridamole on endothelial cells were not analysed.

One of the interesting characters of endothelial cells is their expression of gap junction channels composed of connexin37 (Cx37), Cx40 and Cx43 (Figueroa et al. 2004, 2006, 2009; de Wit et al. 2006). Since gap junction coupling in endothelial cells is a target of pathophysiological conditions such as arteriosclerosis (Brisset et al. 2009), it is of great importance to characterise the role of gap junction coupling in these cells. This was partly accomplished by generation of Cx37, Cx40 and Cx43 gene-targeted mice (Chadjichristos and Kwak 2007). Despite contradictory results, analysis of the phenotypes showed that endothelial connexins play an essential role in the regulation of blood pressure (de Wit et al. 2000, 2003). For example, an endothelial cell specific deletion of Cx43 correlated with hypotension (Liao et al. 2001). Alternatively, Cx40 deficient mice were hypertensive and showed an impaired transmission of endothelium-dependent vasodilator response (de Wit et al. 2000). Double Cx37/Cx40 knock-out mice displayed severe vascular abnormalities and the mice died after birth (Simon and McWhorter 2002). The results indicate that gap junction coupling in endothelial cells could be a therapeutic target for vascular impairment.

D. Begandt · W. Bintig · K. Oberheide · S. Schlie · A. Ngezahayo (✉)
Institute of Biophysics, Leibniz University Hannover,
Herrenhäuserstr. 2,
30419 Hannover, Germany
e-mail: ngezahayo@biophysik.uni-hannover.de

Gap junction channels are modulated by $[Ca^{2+}]_i$, pH and phosphorylation (Cruciani and Mikalsen 2002; Harris 2001; Lampe and Lau 2004). An increase in the $[Ca^{2+}]_i$ or a decrease in the internal pH (pH_i) reduces gap junction coupling (Harris 2001). As for phosphorylation, protein kinase C (PKC) dependent phosphorylation correlates with a reduction in gap junction coupling, while PKA dependent phosphorylation frequently correlates with an increase in gap junction coupling. This was found particularly for gap junctions composed of Cx43 and Cx40 (Cruciani and Mikalsen 2002; Harris 2001; Lampe and Lau 2004; van Rijen et al. 2000). In parallel, activation of the cGMP dependent protein kinase G (PKG) reduced gap junction coupling of cardiomyoctes (de Mello 1998). Furthermore, it was found that pathophysiological conditions disrupted gap junction coupling by dephosphorylating PKA specific serine residues of Cx40 (Bolon et al. 2008). Therefore, since dipyridamole was shown to increase the cytosolic cAMP concentration (Anfossi et al. 2002; Kim and Liao 2008), we hypothesised that dipyridamole could increase cellular PKA activity and thereby potentiate gap junction coupling in endothelial cells. Using the bovine aortic endothelial cells GM-7373, we show that dipyridamole treatment for 24 h increased gap junction coupling. An increase in gap junction coupling was also induced by 8-Br-cAMP and forskolin treatment but not by db-cGMP application. H-89, an inhibitor of the protein kinase A antagonised the dipyridamole induced increase in gap junction coupling. These findings highlight the central role of the cAMP-PKA dependent phosphorylation cascade in the dipyridamole induced increase in gap junction coupling. The advantages of the dipyridamole induced gap junction coupling are also discussed.

Materials and methods

Lucifer Yellow, forskolin, 8-Br-cAMP, H-89, Rp-cAMPS and db-cGMP were obtained from Sigma-Aldrich (Taufkirchen, Germany). Dipyridamole was kindly provided by Boehringer Ingelheim International GmbH (Ingelheim, Germany).

Cell culture

GM-7373 endothelial cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany) were cultivated using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal calf serum (FCS), penicillin and streptomycin (100 U/ml and 10 mg/ml, respectively). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was renewed every 2–3 days.

For scrape loading experiments, cover slips (Ø 10 mm) were placed into the wells of a 24 multiwell plate containing 1 ml of the DMEM culture medium. The cells were seeded at a density of 1×10^6 cells/well. A further cultivation for 24 h allowed the cells to adhere and to form a monolayer onto the cover slip. Dipyridamole or other agents were added for an additional 24 h.

Assessment of the effect of dipyridamole

Dipyridamole or forskolin were prepared as a stock solution in ethanol and added to the cell culture at the desired concentration. In all experiments with dipyridamole or forskolin 0.5% ethanol was present. Untreated cells and cells treated with 0.5% ethanol were used as reference.

Scrape loading

The scrape loading technique was modified from El-Fouly et al. (1987). A cover slip with adherent cells grown to monolayer was introduced in a chamber containing Lucifer Yellow (LY) dissolved at 0.25% in a bath solution composed of 121 mM NaCl, 5 mM KCl, 6 mM $NaHCO_3$, 5.5 mM glucose, 0.8 mM $MgCl_2$, and 25 mM HEPES (pH 7.4, 295 mOsmol/l). The monolayer was scraped using a sharp razor (Science Services, Munich, Germany). After a 5 min period for LY uptake and transfer, the cells were washed twice in a fresh bath solution without LY and containing 1.8 mM $CaCl_2$. The cells were fixed with 4% formaldehyde dissolved in phosphate buffer saline (PBS) composed of 136.8 mM NaCl, 2.68 mM KCl, 9.86 mM Na_2HPO_4 and 1.14 mM KH_2PO_4 (pH 7.4) for 10 min and were stored in PBS for further microscopic analysis.

Evaluation of scrape loading experiments

To evaluate the dye transfer, the fluorescence of LY was observed using an inverted Nikon Eclipse TE2000-E confocal laser scanning microscope with a 10× objective (Nikon, Düsseldorf, Germany) after excitation with a 488 nm laser. A view area of 1024×1024 pixels was recorded (Fig. 1). The software program EZ-C1 3.50 (Nikon, Düsseldorf, Germany) was used for recording the images.

Estimation of the dye transfer was performed using the software ImageJ (http://rsbweb.nih.gov/ij/docs/menus/analyze.html#plot). Briefly, frames of 250×100 pixel (length x width) were set perpendicular to the border of the scrape line and into the area of dye diffusion in the monolayer (Fig. 1, Frame a). The fluorescence intensity was plotted using rectangular selections that display a column average plot (Fig. 1, Profile a_1), where the x-axis represents the horizontal distance through the selection and the y-axis the vertically

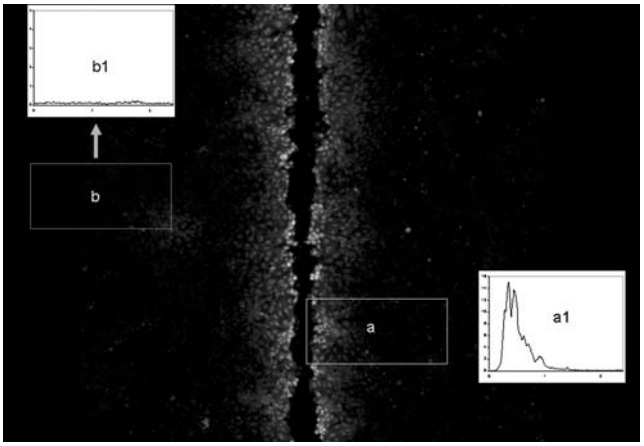

Fig. 1 A sharp razor was used to scrape a cell monolayer in the presence of 0.25% Lucifer Yellow (LY). The injured cells at the edge of the scrape absorbed (strong fluorescence) and transmitted LY to neighbouring cells via gap junctions. For quantitative evaluation of gap junction coupling in bovine aortic GM-7373 endothelial cells, the distance of diffusion was measured by setting a 250×100 pixel frame perpendicular to the scrape line. A fluorescence intensity profile a_1 acquired from frame a was plotted using the software Image J. As background we used a fluorescence intensity profile b_1 acquired in frame b far away from the scrape (for details, s. main text)

averaged pixel intensity of the LY fluorescence (http://rsbweb.nih.gov/ij/docs/menus/analyze.html#plot). A plot profile (Fig. 1, Profile b_1) of a similar frame taken in the cell layer, far away from the scrape line, was used to estimate the background (Fig. 1, Frame b). To quantify distance of diffusion, plot profiles were analysed using a homemade MATLAB-based software. First, the software calculated the average intensity and standard deviation of the background frame (Fig. 1, Profile b_1). To the average the standard deviation was added and this value was used as the background fluorescence. Beginning in the scrape line in frame "a", the software defined the distance of diffusion in pixel units by starting to count pixels at the point in the plot profile (Fig. 1, Profile a_1) where the intensity exceeded the background value and stopped counting when the values of intensity fell to the background value. Six frames were evaluated for each view area of 1024×1024 pixels. For each cover slip four of such areas were recorded. For each treatment, at least six independent experiments were performed.

Results

Dipyridamole increases gap junction coupling

Cells were cultivated with different dipyridamole concentrations. The scrape loading technique revealed that presence of dipyridamole concentrations of 1–100 μM for 24 h affected gap junction coupling (Fig. 2). Cells cultivated under control conditions (untreated) and in the presence of 0.5% ethanol showed a LY diffusion distance of about 93 pixels and 97 pixels respectively. In presence of dipyridamole diffusion distances of 118, 121, 128, 143, 149, 160 and 136 pixels were observed at dipyridamole concentrations of 1, 5, 10, 25, 50, 75, and 100 μM, respectively (Fig. 2c). Relative to control condition, ethanol and the respective dipyridamole concentrations caused an increase in the gap junction coupling to 104, 127, 130, 137, 153, 160, 173 and 146%. Further, experiments performed at different time points of dipyridamole treatment revealed a significant dipyridamole (50 μM) related induction of increase of gap junction coupling after a treatment time of at least 6 h (result not shown). Dipyridamole has been associated with activation of adenylyl cyclase and inhibition of phosphodiesterase. Both, activation of adenylyl cyclase to synthesise cAMP as well as inhibition of phosphodiesterase yield an increase in cytosolic cAMP concentration (Alheid et al. 1989; Anfossi et al. 2002; Eisert 2007; Kim and Liao 2008). Additionally inhibition of phosphodiesterase could also increase the cytosolic cGMP concentration. We therefore analysed whether pharmacological stimulation of adenylyl cyclase by forskolin or treatment of the cells with the membrane-permeable cAMP analogue 8-Br-cAMP could affect gap junction coupling. Both, forskolin and 8-Br-cAMP were able to increase gap junction coupling similar to dipyridamole. Compared to control conditions, forskolin and 8-Br-cAMP increased the diffusion distance to 133% and 115%, respectively (Fig. 3). For 8-Br-cAMP, we could observe that it induced an increase of gap junction coupling after an incubation time of at least 6 h (result not shown). In parallel we found that the cell-permeable cGMP analogue db-cGMP did not affect gap junction coupling (Fig. 3). Since cAMP acts via activation of PKA, we used H-89, an inhibitor of PKA, to study whether the dipyridamole induced gap junction coupling was related to the cAMP-PKA cascade. We found that application of H-89 antagonised the effect of dipyridamole on gap junction coupling (Fig. 4). Furthermore, Rp-adenosine 3′, 5′- cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMPS), another PKA inhibitor could block the dipyridamole induced increase of gap junction coupling similar to H-89 (result not shown). Relative to control conditions or ethanol, 50 μM dipyridamole increased the dye tranfer to 160%. H-89 reduced the dipyridamole (50 μM) induced dye transfer to 103% indicating that activation of PKA was required for the dipyridamole induced increase of gap junction coupling in GM-7373 endothelial cells.

Discussion

The results presented in this report show for the first time that dipyridamole at therapeutically relevant concentrations

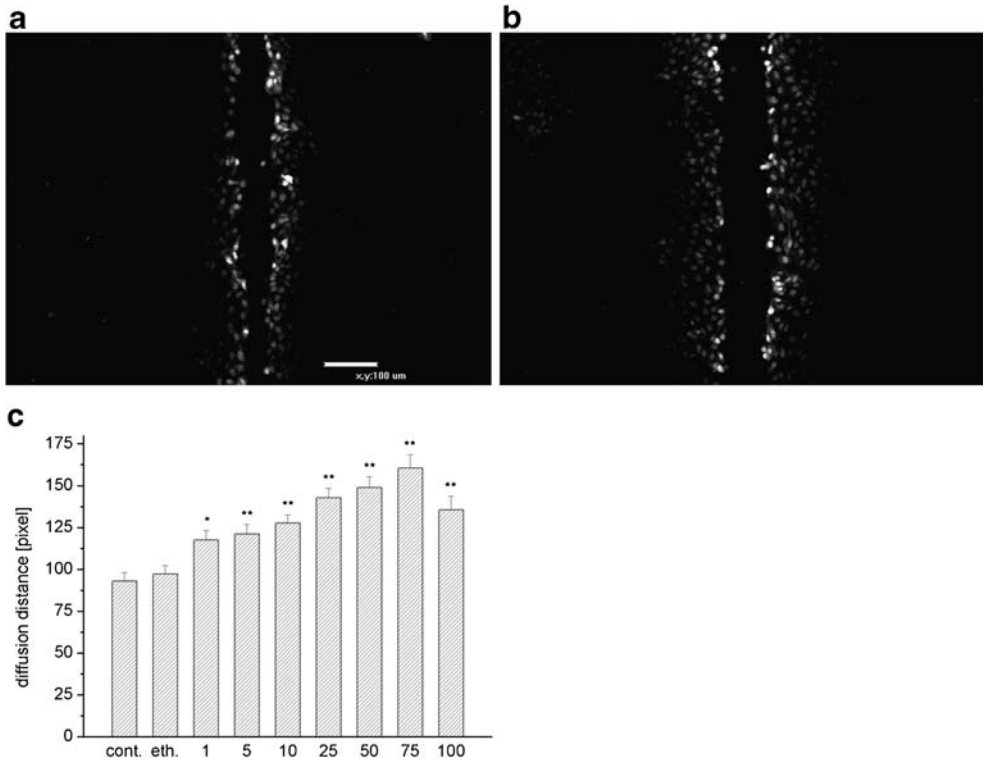

Fig. 2 Dipyridamole affects gap junction coupling of bovine aortic GM-7373 endothelial cells. Cells were cultivated until they formed a monolayer. Dipyridamole was added 24 h before scrape loading. The fluorescent micrographs show the diffusion of LY in cells cultivated in the presence of 0.5% ethanol (**a**) and 50 μM dipyridamole (**b**). (**c**) Quantitative evaluation of the effect of dipyridamole on gap junction coupling. The distance of diffusion was measured for each dipyridamole concentration. We observed that dipyridamole (dip.) induced a significant increase in gap junction coupling compared to 0.5% ethanol (eth.) which alone did not affect the gap junction coupling compared to control (cont.). Dipyridamole significantly increased the gap junction coupling at all tested dipyridamole concentrations ($P<0.01$; Student´s t-test for dipyridamole concentrations above 5 μM and $P<0.05$ for 1 μM dipyridamole). The results are given as average ± SEM for six experiments for each treatment

positively affects gap junction coupling of an arterial endothelial cell line (Fig. 2). Other results from related studies (not shown here) on the aortic smooth muscle cell line A-10 and non vascular cells such as CHO cells revealed that the dipyridamole effects shown here were only reproducible in the aortic smooth muscle cells and not in CHO cells, indicating a possible vascular specific effect.

Gap junctions of endothelial cells are composed of Cx37, Cx40 and Cx43 (Figueroa et al. 2004, 2006, 2009; de Wit et al. 2006). These connexins can associate in

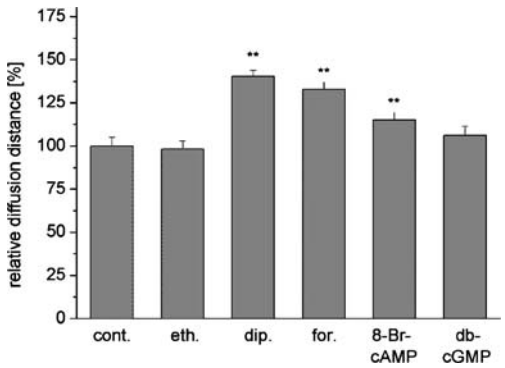

Fig. 3 Similar to dipyridamole (50 μM), forskolin (for., 100 μM) or 8-Br-cAMP (400 μM) significantly increased the gap junction coupling ($P<0.01$; Student´s t-test). The cGMP analogue db-cGMP (400 μM) did not affect the gap junction coupling. The results are given as average ± SEM for six experiments for each treatment

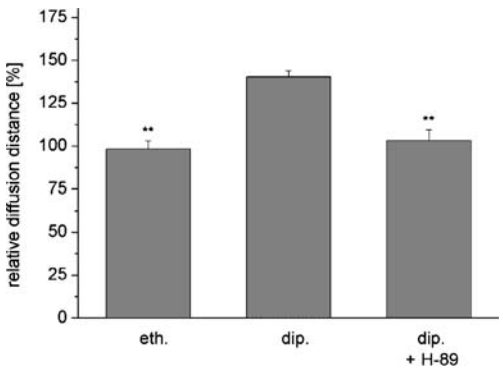

Fig. 4 The inhibitor of PKA H-89 (15 μM) could suppress the dipyridamole (50 μM) induced increase of gap junction coupling. The results are given as average ± SEM for six experiments for each treatment ($P<0.01$; Student´s t-test)

various stoichiometries to form gap junction channels. Gap junction coupling depends on the expression of connexins, their association in connexons as well as in gap junction channels, the turnover of connexins, intracellular modulators, and posttranslational modifications such as phosphorylation (Harris 2001). We observed a dipyridamole dependent increase in gap junction coupling (Fig. 2). Dipyridamole is known to induce an increase in the intracellular cAMP concentration in different cells such as platelets and endothelial cells by stimulation of adenylyl cyclase and inhibition of phosphodiesterases (Anfossi et al. 2002; Eisert 2007; Kim and Liao 2008). We therefore assume that the observed dipyridamole induced increase in gap junction coupling (Fig. 2) could be related to a dipyridamole induced increase in cytosolic cAMP, which would then stimulate the PKA, a kinase known to increase gap junction coupling (Lampe and Lau 2004; van Rijen et al. 2000). This assumption could be supported by following findings: (i) we applied the pharmacological activator of adenylyl cyclase forskolin and the membrane permeable cAMP analogue 8-Br-cAMP and we found that similar to dipyridamole, forskolin as well as 8-Br-cAMP could increase the gap junction coupling of the GM-7373 endothelial cells (Fig. 3). (ii) Moreover we show that H-89 an inhibitor of PKA applied together with dipyridamole could antagonise the dipyridamole induced increase in gap junction coupling (Fig. 4). A similar result could also be produced by application of Rp-cAMPS, another inhibitor of PKA (result not shown). These results indicate an involvement of the cAMP-PKA dependent phosphorylation cascade in the observed dipyridamole induced increase in gap junction coupling (Fig. 5). Interestingly, when we analysed the time course over which dipyridamole (50 μM) or 8-Br-cAMP increased the gap junction coupling, we found that both agents already induced a significant increase of gap junction coupling after an incubation time of 6 h (results not shown). The mechanism by which the cAMP-PKA pathway increases the gap junction coupling, or whether all endothelial connexins (Cx37, Cx40 and Cx43) are equally affected is not clear. Transcription, translation as well as the hexamerisation of connexins into connexons, the insertion of the connexons into the membrane or the connexin turnover could be affected. The turnover of most connexins is in the range of 3–6 h (Hervé et al. 2007). It can therefore be speculated that dipyridamole affects the new synthesized connexins. Further biochemical experiments far beyond the present report are needed to clarify the issue.

By inhibiting phosphodiesterases, dipyridamole could also used cGMP as a second intracellular mediator. However we found that the membrane permeable cGMP analogue db-cGMP did not affect gap junction coupling (Fig. 3) indicating that a cGMP-dependent pathway is not involved in the induction of the observed dipyridamole related increase in gap junction coupling.

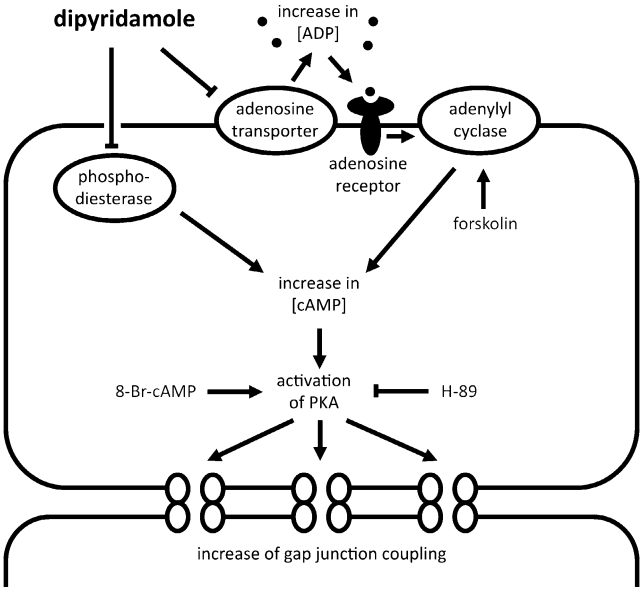

Fig. 5 A model summarising how dipyridamole increases gap junction coupling in endothelial cells. One effect of dipyridamole is the inhibition of the adenosine transporters. This inhibition results in a local increase of adenosine concentration in the interstitial space. This in turn leads to increased stimulation of adenosine receptors and activation of adenylyl cyclase with a subsequent increase in cytosolic cAMP concentration. The second effect of dipyridamole is a blockade of phosphodiesterases, which would reinforce the increase of the cytosolic cAMP and probably cGMP concentration. The increased cAMP stimulates PKA to phosphorylate different proteins leading to the observed increase of gap junction coupling. The increase of gap junction coupling could be achieved by a direct PKA dependent phosphorylation of the connexins of the endothelial cells such as Cx40 and Cx43 but other mechanisms such as expression or turn over of the connexins could also be affected by PKA

We assume that by increasing the cytosolic cAMP concentration, dipyridamole yielded an activation of PKA which in turn phosphorylated different proteins resulting in an increase in gap junction coupling (Fig. 5). As target of the PKA dependent phosphorylation, proteins involved in the transcription, translation or hexamerization of the connexins as well as those participating in targeting and insertion of the connexons in the cellular membrane could be candidates. Moreover, PKA can directly phosphorylate the connexins of GM-7373 endothelial cells leading thereby to an increase of gap junction coupling. This assumption is well established by the literature demonstrating that cAMP activates PKA, which phosphorylates various connexins such as Cx40 and Cx43 and thereby increases gap junction coupling (Lampe and Lau 2004; van Rijen et al. 2000). As for Cx40, it has been shown that pathophysiological conditions disrupted gap junction coupling by dephosphorylating PKA-specific serine residues of Cx40 (Bolon et al. 2008). Further biochemical and

physiological experiments should clarify the connexin specific contribution of each connexin in the observed dipyridamole induced increase in gap junction coupling of the endothelial cells.

The advantage of increasing gap junction coupling in endothelial cells is a matter of speculation, but involvement of gap junction coupling in regulation of vasomotility and vascular tone (Schmidt et al. 2008) as well for transmission of endothelium-dependent vasodilator responses (de Wit et al. 2000) has been documented.

Conclusion

This report shows that therapeutically relevant concentrations of dipyridamole increase gap junction coupling of endothelial cells. This effect is probably due to activation of adenylyl cyclase and inhibition of phosphodiesterase which then increase intracellular cAMP concentration. The increased cAMP level activates PKA which can increase the gap junction coupling by a direct phosphorylation of the new synthesized endothelial cells connexins (Fig. 5) but also other mechanisms such as expression of the connexins could be affected. *In vivo*, an increase in gap junction coupling would lead to an upregulation of vasomotility and allows better blood flow.

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