# Der Einfluss von Dipyridamol auf die Gap Junction-abhängige Kommunikation in Zellen des vaskulären Systems: physiologische und molekulare Analysen

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**Zusammenfassung**

Eine Dysregulation der Gap Junction-abhängigen direkten Zell-Zell-Kommunikation in vaskulären Endothel- und glatten Muskelzellen ist assoziiert mit Erkrankungen wie Hypertonie oder Arteriosklerose. Deshalb werden Gap Junctions vaskulärer Zellen als neues therapeutisches Ziel zur Behandlung dieser Erkrankungen postuliert. Dipyridamol ist ein Wirkstoff, der in der Schlaganfall-Therapie und -Prävention eingesetzt wird. Obwohl für ihn vasoprotektive Eigenschaften nachgewiesen wurden, wird er derzeit als Inhibitor der Thrombozyten-Aggregation eingesetzt. Das Ziel dieser Dissertation war daher die Analyse eines möglichen Einflusses von Dipyridamol auf die Gap Junction-abhängige Kommunikation in vaskulären Endothel- und glatten Muskelzellen.

Durch die *Scrape Loading*/Farbstofftransfer-Methode konnte eine konzentrations- und zeitabhängige Erhöhung der Gap-Junction-abhängigen Kopplung durch therapeutisch relevante Konzentrationen zwischen 1 µM und 100 µM Dipyridamol vermutlich nach Aktivierung einer cAMP-abhängigen Signalkaskade nachgewiesen werden. Proteinkinase A (PKA)-Inhibitoren konnten die Dipyridamol-induzierte Erhöhung nur teilweise reduzieren, sodass die Gap Junction-abhängige Kopplung vermutlich über PKA-abhängige und -unabhängige Signalwege reguliert wurde. Die durch Dipyridamol erhöhte Kopplung korrelierte mit einer über Immunfärbungen nachgewiesenen vermehrten Akkumulation von Connexin 43 (Cx43) in der Zellmembran. Während einer 6-stündigen Inkubationszeit wurde im *Scrape Loading*/Farbstofftransfer die Kopplung durch Dipyridamol signifikant im Vergleich zu Kontrollbedingungen erhöht, ohne eine Veränderung von Cx43 auf mRNA- und -Proteinebene hervorzurufen. Diese erhöhte Kopplung konnte zudem nicht durch Brefeldin A inhibiert werden, was auf eine Regulation bereits existierender Connexone/Gap Junctions nach dem Passieren des Golgi-Apparats durch Dipyridamol hindeutete. Nach 24 h Applikation von Dipyridamol wurde die Cx43-mRNA- und -Proteinmenge erhöht, bei einer gleichzeitigen signifikanten Steigerung der Gap Junction-Kopplung im *Scrape Loading*/Farbstofftransfer. Durch die 24-stündige Applikation wurde zudem die Migrationsform von Cx43 in der SDS-Polyacrylamidgelelektrophorese verändert, was auf eine Erhöhung der Cx43-assoziierten Gap Junction-Plaques hindeutete. Eine Langzeitapplikation von Dipyridamol führte daher vermutlich durch die Neusynthese und die posttranslationale Modifikation von Cx43 zur einer erhöhten Assemblierung der Gap Junctions in der Zellmembran und damit zu einer erhöhten Gap Junction-Kopplung.

Weiterhin konnte in dieser Dissertation gezeigt werden, dass die Gap Junction-abhängige Kopplung vaskulärer Zellen das therapeutische Ziel eines pharmakologisch verwendeten Wirkstoffs ist, der für die Therapie und Prävention von Schlaganfällen verwendet wird. Der therapeutische Einfluss von Dipyridamol bzw. anderen Wirkstoffen auf diese Kopplung, sowie die Rolle der Gap Junctions in vaskulär-assoziierten Erkrankungen sollte zukünftig in Modellsystemen erforscht werden, die beispielsweise hypoxische, inflammatorische oder hyperglykämische Bedingungen nachahmen, wie sie während eines Schlaganfalls oder Diabetes vorherrschen.

**Schlagworte**: Gap Junction, Dipyridamol, vaskuläre Zellen.

**Abstract**

Dysregulations of the gap junction-dependent direct cell to cell communication in vascular endothelial and smooth muscle cells are commonly associated with diseases like hypertension or arteriosclerosis. Therefore gap junctions of the vascular cells have been proposed as target of therapeutic treatment for these pathologies. Dipyridamole is a drug used in therapy and prevention of stroke. Although it has vasoprotective effects, dipyridamole currently develops its therapeutic effect as an inhibitor of thrombocytes aggregation. The aim of the present thesis was to analyse whether dipyridamole could also affect the gap junction-dependent cell to cell communication in vascular cells.

The scrape loading/dye transfer (SL/DT) technique revealed that dipyridamole induced an increase in gap junction coupling in aortic endothelial and smooth muscle cell lines. Pharmacological experiments showed that the dipyridamole-induced increase in gap junction coupling was observed at therapeutic relevant drug concentrations of 1 μM to 100 μM and was related to an activation of cAMP-dependent signalling cascades. The dipyridamole-induced increase in gap junctional coupling was partly antagonized by inhibitors of protein kinase A (PKA), indicating that probably PKA-dependent and PKA-independent pathways are involved. Immunostaining revealed a correlation between the dipyridamole-induced increase in gap junction coupling and an increased accumulation of connexin 43 (Cx43) in the membrane of endothelial cells. A dipyridamole application time of 6 h correlated with a significant increase in gap junction coupling in comparison to control conditions using SL/DT without showing any changes in Cx43 mRNA or protein. Moreover, brefeldin A could not antagonise the dipyridamole-induced increase in gap junction coupling observed after 6 h which indicated a short-term effect of dipyridamole on connexins beyond the Golgi apparatus. An increase in cellular content of Cx43 mRNA or protein was observed after a dipyridamole application time of 24 h which correlated with the significant increase in gap junction coupling. Additionally, the 24 h application time correlated with a change in Cx43 migration behaviour in SDS-polyacrylamide gel electrophoresis. Concluding, dipyridamole induced an increase in gap junction coupling probably by activating cAMP synthesis in vascular cells followed by an enhanced accumulation of Cx43 in the cell membrane. During the first 6 h, this increase was related to already synthesized connexins beyond the Golgi apparatus. After 24 h dipyridamole probably stimulated new synthesis of connexins as well as connexin posttranslational modifications.

Furthermore, the results of the present thesis show that gap junction coupling in the vascular system is a pharmacological target of a drug used in treatment and prevention of stroke and cardiovascular insults. In future studies, the therapeutic applicability of dipyridamole or other drugs on the gap junction-dependent cell to cell communication as well as the role of gap junctions in vascular-associated diseases should be investigated using model systems mimicking for example hypoxic, inflammatory or hyperglycaemic conditions that are associated with stroke or diabetes.

**Keywords:** Gap junction, dipyridamole, vascular cells.

# 1 Einleitung

## 1.1 Gap Junctions und Connexine

Gap Junctions sind Kanäle, die das Zytoplasma benachbarter Zellen verbinden. Sie ermöglichen einen direkten Austausch von Ionen, Metaboliten und sekundären Botenstoffen bis zu einem Molekulargewicht von etwa 1 kDa (Alexander & Goldberg 2003, Kumar & Gilula 1996). Die Expression von Gap Junctions konnte in fast allen Zellarten bis auf wenige Ausnahmen, wie reife Samenzellen, Thrombozyten und differenzierte Skelettmuskeln, gezeigt werden. Die elektrische und metabolische Kopplung von Zellen durch Gap Junctions (Goodenough et al. 1996, Rackauskas et al. 2010, Saez et al. 2003) kann die Entwicklung, Proliferation und Differenzierung der Zellen regulieren, indem räumliche Gradienten von Signalmolekülen oder Nährstoffen verändert werden (Goodenough & Paul 2009).

Funktionsstörungen von Gap Junctions sind häufig assoziiert mit verschiedenen Krankheitsbildern wie zum Beispiel neurodegenerativen Erkrankungen, Hauterkrankungen oder Taubheit (Bergoffen et al. 1993, Duman & Tekin 2012, Macari et al. 2000, Maestrini et al. 1999, Willecke et al. 1999). Eine Dysregulation der Gap Junction-abhängigen Kommunikation wurde ebenfalls während der Tumorgenese beobachtet (Mesnil et al. 2005, Mroue et al. 2011).

### 1.1.1 Struktur und Aufbau der Gap Junctions

Gap Junctions sind aus Proteinuntereinheiten, den sogenannten Connexinen aufgebaut. Im Menschen sind bis dato 21 Connexin-Isoformen bekannt, in der Maus 20 Isoformen (Söhl & Willecke 2003, Söhl & Willecke 2004). Connexine sind Transmembranproteine mit vier Transmembrandomänen, die über zwei extrazelluläre und eine intrazelluläre Schleife miteinander verbunden sind. Der Amino- und der Carboxylterminus (N- und C-Terminus) der Connexine sind im Zytoplasma lokalisiert. Während der N-Terminus, die Transmembrandomänen und die extrazellulären Schleifen hochkonserviert sind, gibt es starke Sequenzvariationen zwischen den einzelnen Isoformen in der intrazellulären Schleife und dem C-Terminus (Laird 2006, Yancey et al. 1989, Zimmer et al. 1987). Zur

Unterscheidung der verschiedenen Connexin-Isoformen haben sich zwei Nomenklaturen durchgesetzt. Bei der ersten werden die Connexine (Cx) nach ihrem Molekulargewicht in kDa eingeteilt. Cx43 ist demnach ein Connexin mit einem Molekulargewicht von 43 kDa. In der zweiten Nomenklatur werden die Connexine nach dem phylogenetischen Ursprung in drei Untergruppen entsprechend ihrer Sequenzhomologie und der Länge ihrer C-Termini eingeteilt. Es werden α-, β-und γ-Connexine unterschieden (Eiberger et al. 2001, Söhl & Willecke 2003, Söhl & Willecke 2004).

Von einer Zelle werden meistens mehrere Connexin-Isoformen exprimiert, wobei die Expression gewebe- und zelltypspezifisch ist. Einige Connexine werden seltener, andere wie zum Beispiel Cx43 dagegen in vielen Zelltypen exprimiert (Goodenough et al. 1996, Laird 2006, Willecke et al. 2002). Jede Connexin-Isoform hat eine Reihe eigener funktioneller Eigenschaften, die nicht oder nur teilweise durch die Expression einer anderen Isoform übernommen werden können (Johnstone et al. 2009). Durch *Knock-In*-Experimente wurde die Rolle spezifischer Connexin-Isoformen nachwiesen. Cx43, dessen Fehlen in *Knock-Out*-Mäusen zu starken kardiovaskulären Defekten führt, wurde durch Cx26, Cx32 oder Cx40 ersetzt. Keine der drei *Knock-In*-Varianten konnte die durch Cx43 erfüllten Funktionen vollständig ersetzen, sondern zeigte neue Abnormalitäten wie z. B. ventrikuläre Arrhytmien oder kardiologische Malformationen (Plum et al. 2000, Reaume et al. 1995, Winterhager et al. 2007).

Einzelne Connexine lagern sich zu einem Hexamer zusammen, einem sogenannten Connexon oder Hemikanal. Jede Zelle steuert für die Ausbildung eines vollständigen Gap Junction-Kanals an der Plasmamembran ein Connexon bei. Bei der Expression mehrerer Connexin-Isoformen in einer Zelle können sich Connexone aus nur einer oder aus verschiedenen Isoformen zusammensetzen und bilden dann Homo- oder Heteromere. Verbinden sich an der Plasmamembran zwei gleich zusammengesetzte Connexone, kommt es zu homotypischen Gap Junctions. Unterschiedlich zusammengesetzte Connexone bilden heterotypische Gap Junctions aus. Nicht alle Connexin-Isoformen sind miteinander kompatibel, vermutlich können nur Isoformen der gleichen Untergruppe (z. B. α:α) miteinander interagieren (Falk et al. 1997, Goodenough & Paul 2009, Segretain & Falk 2004).

Die zahlreichen homo- und heterotypischen Gap Junctions weisen, neben den Connexin-spezifischen Funktionen, physiologische Unterschiede unter anderem in der Selektivität und der Permeabilität von Molekülen auf (Bevans et al. 1998, Elfgang et al. 1995, Mroue et al. 2011, Veenstra 1996). Weiterhin können auch gleich aufgebaute Gap Junction-Kanäle unterschiedliche Funktionen wahrnehmen, die wahrscheinlich durch ihre Interaktionspartner wie zum Beispiel interzelluläre Adhäsionsproteine reguliert werden können (Giepmans et al. 2001, Mroue et al. 2011, Singh & Lampe 2003, Singh et al. 2005).

### 1.1.2 Synthese und Degradierung von Connexinen und Gap Junctions

Connexine als Transmembranproteine werden während der Protein-Biosynthese durch spezifische Signalsequenzen cotranslational in die Membran des endoplasmatischen Retikulums (ER) integriert (Falk et al. 1994, Falk & Gilula 1998). Im weiteren Verlauf der Connexin-Biosynthese wird ein Transport von dem ER über das *endoplasmatic reticulum-Golgi intermediate compartment* (ERGIC) und den Golgi-Apparat vermutet (Pfeffer & Rothman 1987, Rothman & Wieland 1996). Während dieses Transports findet die Oligomerisierung der Connexine zu Connexonen statt, wobei der genaue Zeitpunkt der Oligomerisierung zelltyp- und connexinspezifisch ist. Cx37, Cx40 und Cx45 beispielsweise bilden schon im ER Connexone aus, Cx43 oligomerisiert hingegen erst im *trans*-Golgi-Bereich (Das Sarma et al. 2002, Maza et al. 2005, Musil & Goodenough 1993). Über einen Mikrotubuli- und Actinfilament-abhängigen Vesikeltransport werden die fertigen, aber geschlossenen Hemikanäle an die Membran transportiert (Falk et al. 1997, Falk & Gilula 1998, George et al. 1999). In der Membran liegen Gap Junctions als Plaques zu Dutzenden bis Tausenden nebeneinander vor. Die Connexone werden jedoch vermutlich nicht direkt zu bestehenden Gap Junction-Plaques gebracht, sondern in distalen Regionen in die Plasmamembran integriert. Sie diffundieren dann lateral in der Membran zu bestehenden Gap Junction-Plaques (Gaietta et al. 2002). Die Ausbildung eines neuen Kanals an der Peripherie des Plaques erfolgt durch die Verbindung eines Connexons mit einem Connexon der benachbarten Zelle über eine Interaktion der extrazellulären Schleifen der einzelnen Connexine (Bao et al. 2004, Foote et al. 1998). Die funktionellen Gap Junction-Kanäle können anschließend durch multiple

Regulationsmechanismen in ihrem Öffnungszustand und ihrer Öffnungszeit reguliert werden (Solan & Lampe 2005). Der Abbau von Gap Junctions/Connexinen ist Gegenstand derzeitiger Forschungen und scheint sowohl proteasomale als auch lysosomale Wege zu beinhalten (Laing & Beyer 1995, Laing et al. 1997, Musil et al. 2000, Qin et al. 2003). Dabei konnten *Annular Junctions* beobachtet werden, große vesikelartige Strukturen, die durch Invagination der gesamten Gap Junction Struktur mitsamt der Plasmamembran der Nachbarzelle entstehen (Jordan et al. 2001, Larsen et al. 1979).

### 1.1.3 Regulationsmechanismen der Gap Junction-abhängigen Kommunikation

Zellen und Gewebe besitzen diverse Möglichkeiten sich an verändernde Bedingungen anzupassen und durch die umgebenden Reize die Kommunikation über Gap Junctions zu regulieren. Entgegen früherer Meinungen besitzen Gap Junctions eine Halbwertszeit von wenigen Stunden, je nach Zelltyp und Connexin-Zusammensetzung (Musil et al. 2000, Saffitz et al. 2000). Gap Junction-Plaques sind daher kein starrer Verbund von zusammengelagerten Kanälen, sondern eine dynamische und variable Struktur, die sich innerhalb von Minuten an veränderte Umgebungsparameter anpassen kann. Im Uterus zum Beispiel nimmt kurz vor der Geburt die Menge der Gap Junctions um das Fünffache zu und wird kurz nach der Geburt wieder auf das Ausgangsniveau herunter reguliert (Hendrix et al. 1992, Risek et al. 1990, Winterhager et al. 1991).

Zur Modifikation der Gap Junction-abhängigen Kommunikation können zum einen von bestehenden Gap Junction-Kanälen die Permeabilität für bestimmte Moleküle, die Öffnungswahrscheinlichkeit oder die Öffnungszeit reguliert und zum anderen die Anzahl funktioneller Gap Junction-Kanäle in der Plasmamembran verändert werden (Goodenough & Paul 2009, Martin & Evans 2004). Eine direkte Regulation bereits bestehender Gap Junction-Kanäle kann durch Veränderungen der Spannung, der intrazellulären Calciumkonzentration, des pH-Werts oder der Phosphorylierung erfolgen (Lazrak & Peracchia 1993, Liu et al. 1993, Noma & Tsuboi 1987, Solan & Lampe 2005). Das Schließen der Gap Junctions durch erhöhte Calciumkonzentrationen ist zum Beispiel essentiell für Zellen, die sich von verletzten Zellen abgrenzen und so eine Depolarisierung der intakten Zelle und den Verlust von Metaboliten über die

angrenzende, sterbende Zelle vermeiden können (Deleze 1970). Auch Phosphorylierungen der Connexine durch spezifische Kinasen können einen Einfluss auf existierenden Gap Junction-Kanäle haben. So führen Phosphorylierungen durch die Proteinkinase A (PKA) meistens zu einem Öffnen der Gap Junctions, während Phosphorylierungen durch die Proteinkinase C (PKC) eher zu einem Schließen der Kanäle führen (Berthoud et al. 1997, Berthoud et al. 2000, Lampe & Lau 2004).

Die Anzahl funktioneller Gap Junctions in der Zellmembran kann durch die Beeinflussung des gesamten Biosynthese-Prozesses von Connexinen reguliert werden. So ist die Expression von Connexinen ein dynamischer Prozess und wird sowohl während der Entwicklung, als auch während des Zellzyklus modifiziert (Mroue et al. 2011, Solan & Lampe 2007). Auch über Phosphorylierungen durch verschiedene Kinasen kann die Expression der Connexine gesteuert werden - nachgewiesen sind zum Beispiel PKA-, *mitogen activated protein kinase* (MAPK)-, Phosphoinositid-3-Kinase/Proteinkinase B- oder β-Catenin-vermittelte Veränderungen der Connexin-Genexpression (Salameh et al. 2006, Salameh & Dhein 2011, Xia et al. 2010, Zhang et al. 2005). Auch die Proteinsynthese (Burghardt et al. 1995), der Transport (Hunter et al. 2005, Olk et al. 2009), die Gap Junction-Assemblierung und die Degradierung (Lan et al. 2005, Musil et al. 2000) können reguliert werden (Goodenough & Paul 2009, Segretain & Falk 2004).

Eine Schlüsselrolle in der Regulation der Gap Junction-abhängigen Kommunikation nimmt der Second Messenger zyklisches AMP (cAMP) ein. Abhängig von Zelltyp und Versuchsmethode wird über eine intrazelluläre Erhöhung von cAMP die Gap Junction-abhängige Kopplung auf verschiedenen Ebenen verstärkt. So kann cAMP bei Cx43 die Expression und Menge an mRNA, die Proteinmenge, den Transport an die Plasmamembran, die Gap Junction-Formation und/oder die Größe der Plaques erhöhen (Atkinson et al. 1995, Holm et al. 1999, Mehta et al. 1992, Paulson et al. 2000, TenBroek et al. 2001). Nach einer Aktivierung durch cAMP und der Rekrutierung von weiteren Signalmolekülen ist beispielsweise der Transkriptionsfaktor *cAMP response element binding protein* (CREB) an einer erhöhten Genexpression von *cx43* beteiligt (Salameh et al. 2009).

Eine weitere wichtige Rolle in der gesamten Regulation der Connexine und Gap Junctions nimmt der Phosphorylierungszustand der Connexine ein. Bis auf Cx26 besitzen Connexine multiple Phosphorylierungsstellen in ihrer Aminosäuresequenz (Lampe & Lau 2000, Saez et al. 1998, Solan & Lampe 2005). Gerade Cx43 als eines der am häufigsten exprimierten Connexine ist strukturell intensiv untersucht worden. Es sind Phosphorylierungsstellen für zahlreiche Kinasen nachgewiesen worden, wie beispielsweise für die PKA, PKC, Caseinkinase 1 (CK1) oder MAPK (Cooper & Lampe 2002, Lampe 1994, Warn-Cramer et al. 1998, Yogo et al. 2006). Diese Kinasen phosphorylieren spezifisch unterschiedliche Serin- oder Tyrosinreste des C-Terminus. Die Phosphorylierungen der spezifischen Aminosäurereste erfolgen nicht nur in Gap Junction-integrierten Molekülen, sondern schon kurz nach der Synthese des Proteins. Hierbei ist das Phosphorylierungsmuster mit einer spezifischen Lokalisation von Cx43 in der Zelle assoziiert und wird daher während des Transports bis zur Plasmamembran und bis zum endgültigen Einbau in Gap Junctions verändert (Solan & Lampe 2007, Solan & Lampe 2009). Phosphorylierungen durch die PKA oder die CK1 werden meist mit positiven Modifikationen wie zum Beispiel dem Einbau der Connexone in die Plasmamembran assoziiert (Cooper & Lampe 2002, Paulson et al. 2000), wohingegen Phosphorylierungen durch andere Kinasen eine Negativregulation der Connexine zur Folge haben können (Berthoud et al. 1993). So forcieren Phosphorylierungen durch die PKC oder MAPK die Internalisierung von Cx43-Gap Junctions (Leithe & Rivedal 2004, Solan & Lampe 2008).

## 1.2 Gap Junctions im vaskulären System

Gefäßwände besitzen grundlegend alle den gleichen Aufbau und können in die drei Schichten Intima, Media und Adventitia unterteilt werden. Die Intima als innerste Schicht kleidet mit einer Lage von Endothelzellen die Gefäßwände aus und hat dadurch direkten Kontakt mit dem Blut und den darin enthaltenen vasoaktiven Substanzen. Hinter der Intima liegt die Schicht der Media, dessen zellulärer Hauptbestandteil die glatten Muskelzellen sind, die direkt mit den Endothelzellen der Intima gekoppelt sind. Die Endothelzellen der Intima sind längs der Gefäßachse in Richtung des Blutflusses angeordnet, glatte Muskelzellen der Media hingegen helikal. In der angrenzenden

Adventitia liegt das Bindegewebe, das die Gefäße in ihrer Umgebung verankert. Dieser Grundaufbau der Gefäße kann sich zwischen Arterien und Venen in der Ausprägung unterscheiden - zum Beispiel ist die Muskelzellschicht der Media bei Venen geringer ausgeprägt als bei Arterien (Beny et al. 2006, Brisset et al. 2009, Haefliger et al. 2004, Johnstone et al. 2009).

Die Zellen innerhalb der Gefäßwand sind untereinander über Gap Junctions verbunden. Neben homozellulären Gap Junctions zwischen glatten Muskelzellen oder Endothelzellen bilden sich auch heterozelluläre Gap Junctions aus, die sogenannten myoendothelialen Gap Junctions (Johnstone et al. 2009, Rhodin 1967). In Zellen des vaskulären Systems sind die Kanäle aus einer Vielfalt von Connexinen aufgebaut, wobei die Expression abhängig von der untersuchten Gefäßart ist (Figueroa et al. 2004, Severs et al. 2001). Die Expression der Connexin-Isoformen ist in der Literatur kontrovers diskutiert. Zumeist wird allerdings das Vorkommen von Cx37, Cx40, Cx43 und Cx45 in vaskulären Zellen beschrieben (Bruzzone et al. 1993, Hoffmann et al. 2003). Glatte Muskelzellen exprimieren dabei hauptsächlich Cx43 sowie Cx40 und Cx45 (Gabriels & Paul 1998, Kruger et al. 2000). In Endothelzellen werden neben Cx40 auch Cx37 und Cx43 exprimiert (Gabriels & Paul 1998, Little et al. 1995, van Kempen & Jongsma 1999). Die essentielle Rolle der vaskulär exprimierten Connexine konnte durch spezifische *Knock-Outs* gezeigt werden (de Wit et al. 2000, Figueroa et al. 2004, Willecke et al. 2002). Neben der Regulation des vaskulären Tonus haben die Deletionen Einfluss auf den Blutdruck und auf die kardiovaskuläre Entwicklung in Mäusen. Ein *Knock-Out* von Cx40 ruft eine Hypertonie und eine irreguläre Vasomotion in Mäusen hervor (de Wit et al. 2006, Kurtz et al. 2007, Wagner et al. 2007). Andere *Knock-Outs* sind letal, wie bei Cx45 (Kruger et al. 2000, Willecke et al. 2002) oder Cx43 (Reaume et al. 1995). Ein *Knock-Out* von Cx43 spezifisch in Endothelzellen von Mäusen führt zu Hypotonie und Bradykardie (Liao et al. 2001).

### 1.2.1 Physiologie der vaskulären Gap Junctions

Die Regulation des Blutdrucks und des Blutflusses in einem Blutgefäß wird durch eine Feinabstimmung zwischen den Endothel- und den glatten Muskelzellen innerhalb der

Gefäßwand erreicht. Blutgefäße stellen ein komplexes multizelluläres System dar, das durch eine Gap Junction-vermittelte Kommunikation als Einheit funktionieren kann. Verglichen zur Gesamtgröße eines Gefäßes koordinieren die relativ kleinen Zellen damit eine gleichmäßige Regulation über Strecken des Gefäßes, sowie die gleichmäßige Regulation zwischen einzelnen Gefäßen in einem weit verästelten Gefäßsystem. Diese Regulation kann den Gefäßtonus und den luminalen Gefäßdurchmesser verändern, indem die glatten Muskelzellen kontrahieren oder relaxieren. Hierbei beeinflussen sich glatte Muskelzellen und Endothelzellen gegenseitig, so dass erst durch die direkte Zell-Zell-Kommunikation über Gap Junctions die Funktion der vaskulären Zellen erfüllt werden kann (Figueroa et al. 2004, Figueroa & Duling 2009).

Die Kommunikation über Gap Junctions findet in der Gefäßwand entlang des Gefäßes (longitudinal) oder von Zellschicht zu Zellschicht (transversal) statt (Beny et al. 2006). Eine longitudinale Kommunikation findet über homozelluläre Gap Junctions statt. In den glatten Muskelzellen zum Beispiel kann die nach einem Stimulus hervorgerufene Änderung des Membranpotentials longitudinal über Gap Junctions elektrisch fortgeleitet werden. Die Änderung des Membranpotentials in den benachbarten glatten Muskelzellen kann dann zur Öffnung spannungsabhängiger $Ca^{2+}$-Kanäle führen und eine Kontraktion der glatten Muskelzellen hervorrufen. Glatte Muskelzellen synchronisieren über diese Zell-Zell-Kommunikation den vaskulären Tonus über mehrere Millimeter eines Gefäßes (Christ et al. 1991, Christ et al. 1992, Segal & Duling 1986).

Eine transversale Kommunikation ergänzt die longitudinale Kommunikation und erfolgt über die myoendothelialen Gap Junctions (Beny et al. 2006, Emerson & Segal 2000, Goto et al. 2002, Griffith 2004). In der relaxatorischen Regulation des vaskulären Tonus wird angenommen, dass durch einen lokalen Stimulus der Endothelzellen ein *endothelium-derived hyperpolarizing factor* (EDHF) freigesetzt wird (Feletou & Vanhoutte 1996, Vanhoutte 2004), das Signal über Gap Junctions weitergeleitet wird und zur Hyperpolarisierung der glatten Muskelzellen führt (Busse et al. 2002, Dora et al. 2003, Griffith et al. 2004).

Die Beteiligung der spezifischen Connexin-Isoformen an dieser Signalausbreitung nach einem Stimulus ist nicht vollständig geklärt. Cx40 der Endothelzellen und myoendotheliale Gap Junctions scheinen an der Weiterleitung des EDHFs aus den Endothelzellen beteiligt zu sein (Griffith 2007, Isakson et al. 2006, Wolfle et al. 2007). Die longitudinale Ausbreitung des hyperpolarisierenden Signals in den glatten Muskelzellen wird wahrscheinlich über Cx43-assoziierte Gap Junctions vermittelt (Chaytor et al. 2005). Die EDHF-vermittelte Reaktion erfolgt zusätzlich zu der *endothelium-derived relaxation factor* (EDRF)-vermittelten Reaktion. Sie wirkt Gap Junction-unabhängig von den Endothel- auf die glatten Muskelzellen und wird vermutlich durch Stickstoffmonoxid (NO) als EDRF hervorgerufen (Goodenough & Paul 2009).

Eine Dysregulation oder Dysfunktion der vaskulär exprimierten Connexine in diesem komplexen System führt oftmals zu vaskulären Beeinträchtigungen und ist mit Erkrankungen, wie zum Beispiel Hypertonie oder Arteriosklerose assoziiert (Brisset et al. 2009, Figueroa et al. 2006, Kwak et al. 2002). In Gefäßbereichen mit starkem Scherstress, wie er an Verzweigungsstellen auftreten kann, konnte ebenfalls eine veränderte Connexin-Expression nachgewiesen werden (Gabriels & Paul 1998). Gap Junctions sind für die Wundheilung von Gefäßen essentiell und Connexine werden während der Wundheilung stark reguliert (Figueroa et al. 2004, Kwak et al. 2001). Eine Rolle spielen veränderte Expressionsmuster von Connexinen ebenso in Krankheitsbildern wie Diabetes oder Adipositas (Heilbronn & Campbell 2008, Inoguchi et al. 1995), die als chronisch gelten. Chronische Erkrankungen zeigen Eigenschaften von Entzündungen oder Wunden, die das vaskuläre System angreifen und zu einer veränderten Gap Junction-Regulation führen können. Green und Nicholson postulieren daher eine Verbesserung der chronischen Erkrankungen, indem durch die Behandlung der dysregulierten Gap Junctions der dauerhafte Entzündungszustand aufgehalten werden und eine Regeneration des vaskulären Systems erfolgen könnte (Green & Nicholson 2008). Die Gap Junctions und ihre Regulation werden daher als therapeutisches Ziel vorgeschlagen (Brisset et al. 2009, Green & Nicholson 2008).

## 1.3 Dipyridamol

Dipyridamol wird primär in Kombination mit Acetylsalicylsäure (ASS) zur sekundären Prophylaxe von transienten ischämischen Attacken (TIA, vorübergehende Durchblutungsstörungen) und Schlaganfällen eingesetzt (Aldenhoff et al. 1997). Nach der *European Stroke Prevention Study* 2 und der *European/Australasian Stroke Prevention in Reversible Ischaemia Trial* ist eine kombinierte Therapie doppelt so effektiv im Vergleich zur Monotherapie in der Reduktion des Schlaganfall- und TIA-Risikos (Diener et al. 1996, ESPRIT Study Group et al. 2006). Der von Dipyridamol vermittelte Effekt ist die Inhibierung der Thrombozyten-Aggregation, indem membranständige *equilibrative nucleoside transporter* (ENT) inhibiert werden und folglich extrazellulär eine erhöhte Adenosin-Konzentration entsteht (Molina-Arcas et al. 2009, Podgorska et al. 2005). Adenosin bindet an die Adenosin-Rezeptoren der Thrombozyten und aktiviert intrazellulär über ein trimeres $G_S$-Protein die Adenylyl-Zyklase (Gao & Jacobson 2011, Haslam & Rosson 1975). Durch diese Aktivierung steigt der intrazelluläre cAMP-Spiegel an. cAMP wiederum nimmt in Thrombozyten Einfluss auf die Gerinnungskaskade und inhibiert die Thrombozytenaggregation, indem der Schritt der Konformationsänderung des Glykoproteins IIb/IIIa und die resultierende Bindung an den Von-Willebrand-Faktor oder an Fibrinogen inhibiert wird (Kehrel 2008).

Dipyridamol wird ebenfalls für einen Einsatz nach Angioplastien oder Implantation von Herzklappen oder Gefäßprothesen diskutiert, da in Studien eine proliferationshemmende Wirkung auf glatte Muskelzellen gezeigt werden konnte. In Folge von Angioplastien sind vaskuläre Stenosen dokumentiert worden, die durch eine myointimale Hyperplasie (übermäßige Proliferation der glatten Muskelzellen) hervorgerufen werden. Weiterhin ist eine übermäßige Kolonisation der Implantate durch glatte Muskelzellen beobachtet worden, die oftmals zu einer Stenose führt (Zhuplatov et al. 2006). Eine proliferationshemmende Wirkung von Dipyridamol konnte auch in vaskulären Endothelzellen nachgewiesen werden (Liem et al. 2001). Zudem kann Dipyridamol durch die Erhöhung des extrazellulären Adenosin-Spiegels Apoptose in glatten Muskelzellen induzieren und könnte damit einen Einfluss auf die Ausbildung einer Arteriosklerose nehmen (Peyot et al. 2000).

Neben diesen Anwendungen sind für Dipyridamol weitere Effekte bekannt. So wirkt es antioxidativ, indem es unter anderem die Bildung reaktiver Sauerstoffspezies in Endothelzellen und Thrombozyten verringert (Chakrabarti et al. 2005). Durch die Potenzierung des NO-Systems wirkt es als Vasodilator. Dipyridamol kann in ischämischen Geweben den Blutfluss wieder herstellen und stimuliert die Angiogenese über endokrine Nitrit/NO-Signalwege (Venkatesh et al. 2010). Ebenso konnte die Adhäsion von Neutrophilen, die aus ischämischen Schlaganfallpatienten isoliert wurden, an die vaskulären Endothelzellen durch Behandlung mit Dipyridamol verhindert werden. Diese während Entzündungsreaktionen vorkommende Adhäsion spielt vermutlich eine Rolle in der Ausbildung von arteriosklerotischen Plaques, die zum Schlaganfall führen können. Diese Vermutung wird durch die Beobachtung unterstützt, dass Neutrophile an instabilen und rupturierten Plaques detektiert wurden (Hallevi et al. 2007).

Zusammenfassend hat Dipyridamol also neben seiner Rolle als Thrombozyten-Aggregationshemmer vielfältige Effekte, die zusammengenommen vasoprotektiv wirken können. Dipyridamol könnte demnach eine bedeutende Rolle im vaskulären System zugesprochen werden. Die positiven, entzündungshemmenden Effekte auf das vaskuläre System könnten vor allem im Kontext von Entzündungsreaktionen, zum Beispiel während und nach Schlaganfällen relevant sein (Guo et al. 2010, Schaper 2005).

## 1.4 Zielsetzung der Dissertation

Gap Junctions in vaskulären Endothel- und glatten Muskelzellen sind grundlegend an der Aufrechterhaltung der Gefäß-Funktion beteiligt. Dysregulationen vaskulärer Connexine und der vaskulären Zell-Zell-Kommunikation führen meist zu kardiovaskulären Beeinträchtigungen und sind assoziiert mit Erkrankungen wie Arteriosklerose oder Hypertonie, die beide Risikofaktoren für Schlaganfälle sind (Figueroa et al. 2006, Kwak et al. 2002, Simmons et al. 2012). Daher werden Gap Junctions als neues Therapieziel in vaskulär-assoziierten Erkrankungen postuliert (Brisset et al. 2009, Green & Nicholson 2008). Der Wirkstoff Dipyridamol wird in der Therapie und Prävention von Schlaganfällen als Thrombozyten-Aggregationshemmer

vor allem in Kombination mit ASS eingesetzt (Weber et al. 2012). Für Dipyridamol sind jedoch nachweislich vasoprotektive Eigenschaften bekannt (Guo et al. 2010, Schaper 2005). Dieser Wirkstoff könnte daher neben der thrombotischen Therapie eine Verwendung in der vaskulären Therapie mit besonderem Augenmerk auf die Gap Junctions finden. Ziel dieser Arbeit war daher erstmals einen möglichen Einfluss von Dipyridamol auf die Gap Junction-abhängige Kommunikation in Zellen des vaskulären Systems zu analysieren. Als Modell für die vaskulären Zellen wurden zwei etablierte Zelllinien des Gefäßsystems verwendet. Die Zelllinie GM-7373 stammt aus Endothelzellen einer Rinderaorta, die Zelllinie A-10 aus glatten Muskelzellen der Aorta einer Ratte. Ziel dieser Arbeit war es zunächst mit Hilfe der *Scrape Loading*/Farbstofftransfer-Methode die Kopplung der Zellen über Gap Junctions zu untersuchen und mögliche Dipyridamol-induzierte Veränderungen in der Kopplung nachzuweisen. Zudem sollte der Signalweg von Dipyridamol, der Einfluss auf die Gap Junction-abhängige Kopplung hat, aufgedeckt werden. Durch Variation der Applikationszeiten von Dipyridamol und durch Verwendung von Inhibitoren und Aktivatoren des Signalwegs sollte der Wirk-mechanismus der Dipyridamol-vermittelten Regulation der Gap Junction-abhängigen Kopplung untersucht werden. Schließlich sollte untersucht werden auf welcher Ebene Dipyridamol die Regulation des Connexin-Biosynthese-Prozesses beeinflusst. Mittels einer Analyse der Expression, der Proteinsynthese und der Proteinlokalisation von Cx43 in Endothelzellen wurden diese Fragestellungen beantwortet.

# 2 Ergebnisse

Die vorliegende Arbeit zeigt den Einfluss von Dipyridamol auf die Gap Junction-abhängige Kommunikation in vaskulären Endothel- und glatten Muskelzellen. In drei verschiedenen Veröffentlichungen wurde dieser Einfluss analysiert. Die Publikation „*Dipyridamole increases gap junction coupling in bovine GM-7373 aortic endothelial cells by a cAMP-protein kinase A dependent pathway*" (Begandt et al. 2010, Anhang A) konnte den Effekt von Dipyridamol auf die aortalen Endothelzellen der Rinderzelllinie GM-7373 zeigen. Die zweite Publikation *„Biphasic increase of gap junction coupling induced by dipyridamole in the rat aortic A-10 vascular smooth muscle cell line"* (Begandt et al. 2013a, Anhang B) analysiert das Verhalten der Gap Junction-abhängigen Kopplung nach der Stimulation mit Dipyridamol in glatten Muskelzellen der Rattenzelllinie A-10. In der dritten Publikation *„Dipyridamole-related enhancement of gap junction coupling in the GM-7373 aortic endothelial cells correlates with an increase in the amount of connexin 43 mRNA and protein as well as gap junction plaques"* (Begandt et al. 2013b, Anhang C) wurde in den GM-7373 Endothelzellen analysiert, auf welcher Ebene Dipyridamol die Cx43-abhängige Gap Junction-Kopplung reguliert. Die Ergebnisse der Publikationen werden nachfolgend zusammengefasst wiedergegeben.

## 2.1 Dipyridamol erhöht konzentrationsabhängig die Gap Junction-abhängige Kopplung in vaskulären Endothel- und glatten Muskelzellen

Vaskuläre Endothel- und glatte Muskelzellen wurden mit Dipyridamol in Konzentrationen von 1 bis 100 µM inkubiert. Mithilfe der *Scrape Loading*/Farbstofftransfer-Methode wurden Änderungen der Gap Junction-abhängigen Kopplung *in vitro* untersucht. Hierzu wurden vollständig konfluente Zellen in Gegenwart des membranimpermeablen Farbstoffes Lucifer Yellow (0,25 %, 457 Da) angeschnitten. Während einer definierten Inkubationszeit konnte die Diffusion des Farbstoffes durch die Gap Junction-gekoppelten Zellen erfolgen. Lucifer Yellow zeigte danach in unbehandelten oder mit 0,5 % Ethanol behandelten Endothelzellen eine Diffusionsweite von 93 bzw. 97 Pixeln (Abbildung 2.1), in glatten Muskelzellen eine Diffusionsweite von 81 bzw. 82 Pixeln (Abbildung 2.2). Dipyridamol konnte nach einer Inkubationszeit von 24 h die

Diffusionsweite konzentrationsabhängig erhöhen. Relativ zu den unbehandelten Zellen wiesen Endothelzellen eine erhöhte Diffusionsweite von Lucifer Yellow auf 104, 127, 130, 137, 153, 160, 173 und 146 % nach der Inkubation mit Ethanol, 1, 5, 10, 25, 50, 75 und 100 µM Dipyridamol auf (Abbildung 2.1).

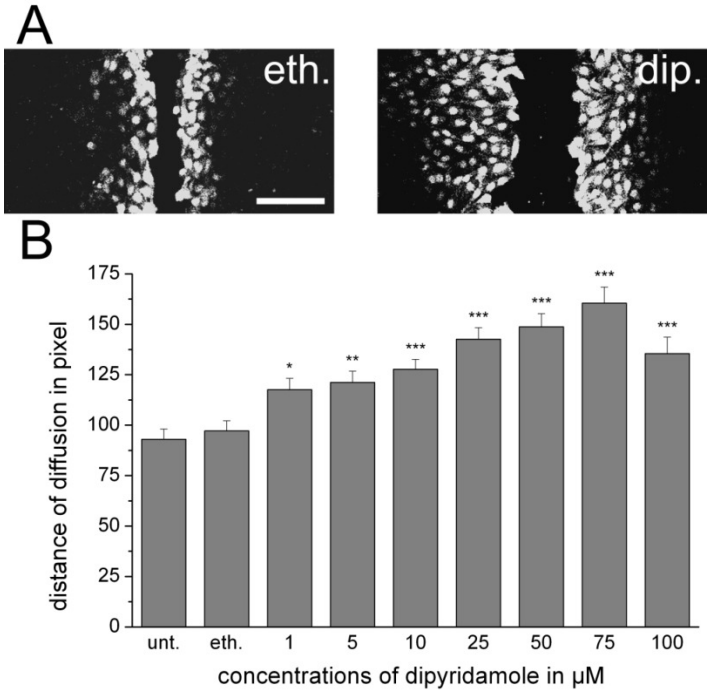


**Abbildung 2.1: Dipyridamol verstärkte die Gap Junction-abhängige Kopplung in bovinen GM-7373 Endothelzellen. Dipyridamol wurde 24 h vor dem *Scrape Loading*/Farbstofftransfer zugegeben. (A) Fluoreszenzaufnahmen der Diffusion von Lucifer Yellow in Ethanol- (eth., 0,5 %) oder Dipyridamol- (dip., 50 µM) behandelten Zellen. Die Skala entspricht 100 µm. (B) Quantitative Auswertung des Effektes von Dipyridamol auf die Gap Junction-abhängige Kopplung. Soweit nicht anders gekennzeichnet wurden immer mindestens sechs unabhängige Versuche durchgeführt. In diesen und allen nachfolgenden Versuchen unterschieden sich die Ethanol-behandelten Zellen nicht signifikant im Vergleich zu unbehandelten Zellen (unt.). Die Ergebnisse sind als mittlere Diffusionsweite ± Standardfehler (SEM) dargestellt. Signifikante Änderungen sind mit * für $p<0,05$, ** für $p<0,01$ und *** für $p<0,001$ gekennzeichnet und gelten nachfolgend für alle Abbildungen (modifiziert nach Begandt et al. 2010).**

Glatte Muskelzellen wiesen im Vergleich zu Ethanol-behandelten Zellen eine Veränderung der relativen Diffusionsweiten auf 103, 113, 128, 137, 158, 165 und 178 %

nach Inkubation mit den entsprechenden Dipyridamol-Konzentrationen auf (Abbildung 2.2).

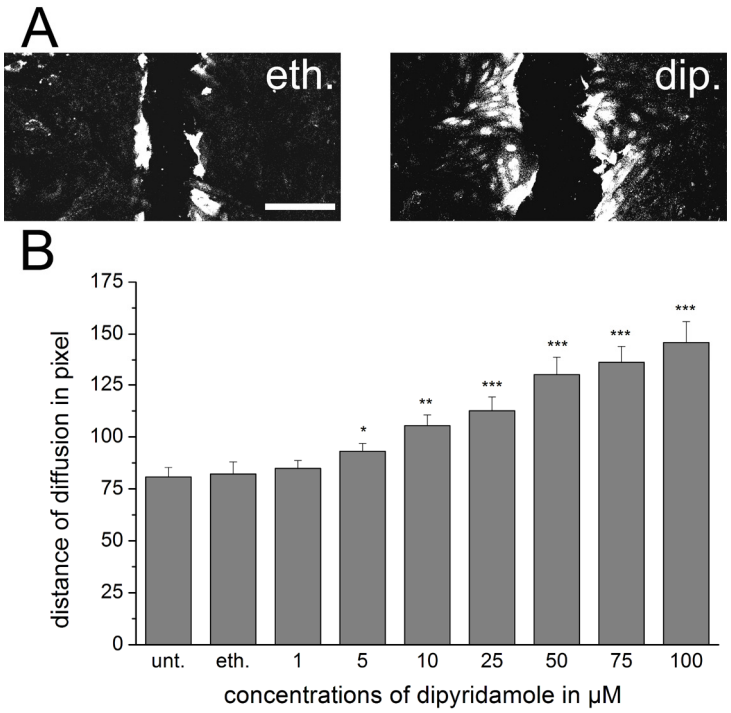


**Abbildung 2.2: Dipyridamol verstärkte die Gap Junction-abhängige Kopplung in A-10 glatten Muskelzellen der Ratte. (A) Die Fluoreszenzaufnahmen zeigen die Diffusion von Lucifer Yellow in Zellen unter Kontrollbedingungen (eth.) und mit 50 µM Dipyridamol (dip.) nach 24 h Inkubation. Die Skala repräsentiert 100 µm. (B) Quantitative Auswertung der Diffusionsweiten bei verschiedenen Dipyridamol-Konzentrationen (modifiziert nach Begandt et al. 2013a).**

## 2.2 Die Gap Junction-abhängige Kopplung in vaskulären Zellen wird durch Dipyridamol über einen cAMP/PKA-abhängigen Weg verstärkt

Dipyridamol führt zu einer extrazellulären Erhöhung der Adenosin-Konzentration durch Inhibierung der ENTs (Molina-Arcas et al. 2009, Podgorska et al. 2005). In Thrombozyten aktiviert Adenosin nach der Bindung an Adenosin-Rezeptoren die Adenylyl-Zyklase und ruft einen intrazellulären Anstieg von cAMP hervor (Anfossi et al. 2002, Eisert 2006, Kim & Liao 2008). cAMP ist ein bekannter Aktivator der PKA (Solan & Lampe 2005), sodass eine Erhöhung der Gap Junction-abhängigen Kopplung von

vaskulären Zellen durch Dipyridamol über einen cAMP/PKA-abhängigen Signalweg hervorgerufen worden sein könnte. Um diese Hypothese zu überprüfen, wurde der Einfluss der spezifischen PKA-Inhibitoren H-89 oder Rp-cAMPS auf die Dipyridamol-induzierte Zunahme der Gap Junction-abhängigen Kopplung untersucht.

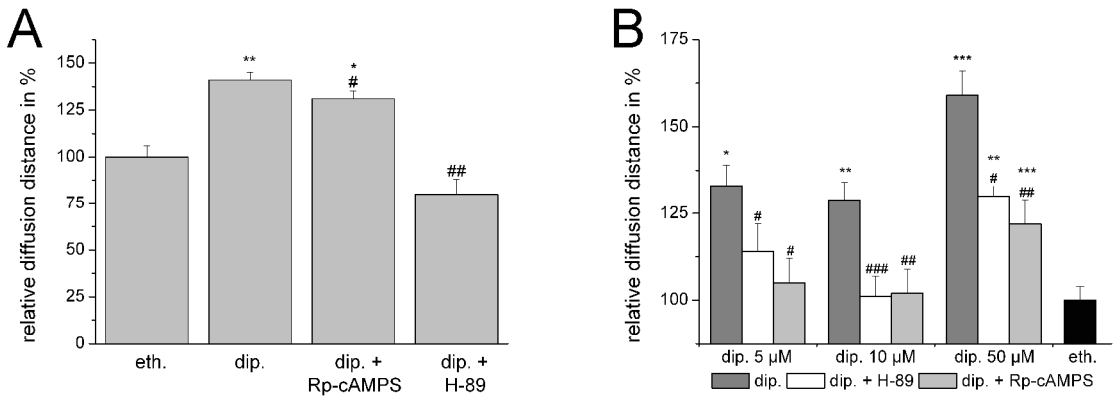


**Abbildung 2.3: Die PKA-Inhibitoren Rp-cAMPS (50 µM in A, 200 µM in B) und H-89 (15 µM) hemmten nach 24 h in Endothelzellen (A) und nach 6 h in glatten Muskelzellen (B) die durch Dipyridamol (dip., 50 µM) hervorgerufene Verstärkung der Gap Junction-abhängigen Kopplung. Signifikante Änderungen der durch die Inhibitoren reduzierten Diffusionsweiten verglichen mit Dipyridamol-behandelten Zellen sind mit dem Symbol # gekennzeichnet (modifiziert nach Begandt et al. 2010 (A) und nach Begandt et al. 2013a (B)).**

Nach einer Inkubationszeit von 24 h konnte in Endothelzellen die Dipyridamol-vermittelte Erhöhung der Kopplung von 141 % (50 µM) durch die gemeinsame Applikation von Dipyridamol mit 15 µM H-89 auf 80 % reduziert werden. In Kombination mit 50 µM Dipyridamol konnte die Applikation von 50 µM Rp-cAMPS die Diffusion von 141 % auf 131 % verringern (Abbildung 2.3A). In ähnlicher Weise konnten beide PKA-Inhibitoren in glatten Muskelzellen eine Reduktion der Dipyridamol-induzierten verstärkten Kopplung nach 6 h hervorrufen. 15 µM H-89 konnte die relativen Diffusionsweiten bei Dipyridamol-Behandlung von 133 % auf 114 % (5 µM), von 129 % auf 101 % (10 µM) und von 159 % auf 130 % (50 µM) reduzieren. Ebenso wurden durch die Applikation von 200 µM Rp-cAMPS die relativen Diffusionsweiten von 133 % auf 105 % (5 µM), von 129 % auf 102 % (10 µM) und von 159 % auf 122 % (50 µM) verringert (Abbildung 2.3B). Die PKA-Inhibitoren konnten die durch hohe

Dipyridamol-Konzentrationen induzierte Reaktion nur teilweise reduzieren (Abbildung 2.3).

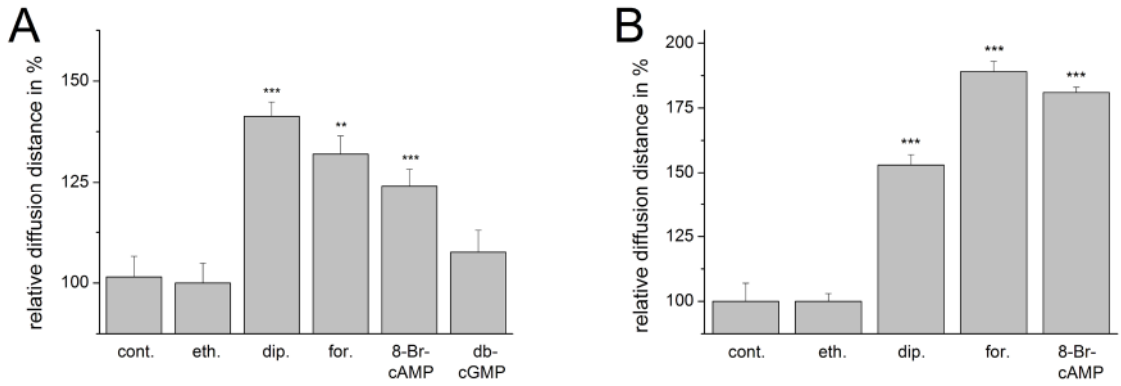


**Abbildung 2.4: Forskolin (for., 100 µM) und 8-Br-cAMP (1 mM) erhöhten die Diffusionsweite von Lucifer Yellow in einer mit Dipyridamol vergleichbaren Weise in Endothelzellen signifikant nach 24 h (A) und in glatten Muskelzellen signifikant nach 6 h (B). Dagegen zeigte Dibutyryl-cGMP (db-cGMP, 400 µM) keinen Einfluss auf die Gap Junction-Kopplung (modifiziert nach Begandt et al. 2010 (A) und Begandt et al. 2013a (B)).**

Weiterhin konnte durch eine Aktivierung der Adenylyl-Zyklase mit 100 µM Forskolin und durch die Applikation von 1 mM 8-Bromo-cAMP (8-Br-cAMP) in beiden Zelltypen eine ähnliche, signifikante Steigerung der Diffusionsweite wie durch Dipyridamol hervor-gerufen werden. Nach einer 24-stündigen Applikation erhöhten Forskolin und 8-Br-cAMP die Kopplung auf 133 % bzw. 115 % in vaskulären Endothelzellen, nach 6 h auf 189 % bzw. 181 % in glatten Muskelzellen (Abbildung 2.4).

Der mögliche Einfluss von Dipyridamol auf die Gap Junction-abhängige Kopplung durch einen cAMP/PKA-abhängigen Signalweg konnte durch die Ergebnisse der Aktivierung des cAMP-abhängigen Signalweges und der Inhibierung der PKA bestätigt werden.

## 2.3 Dipyridamol reguliert zeitabhängig die Gap Junction-abhängige Kommunikation in vaskulären Zellen

Zur Analyse der zeitabhängigen Wirkung der Dipyridamol-induzierten Regulation der Gap Junction-abhängigen Kopplung, wurde die Applikationszeit von Dipyridamol auf die vaskulären Zellen variiert. Hierfür wurden beide Zelltypen zwischen 15 min und 24 h

mit Dipyridamol behandelt. Die vaskulären Endothelzellen wurden nach 1, 3, 6, 9 und 24 h Applikation von 25 und 50 µM Dipyridamol mithilfe des *Scrape Loading*/Farbstofftransfers in ihrem Kopplungsverhalten analysiert. Erst nach 3 h Applikation konnte eine signifikante Erhöhung der Kopplung auf 113 % bzw. 114 % durch 25 und 50 µM Dipyridamol detektiert werden. Zwischen einer 6- und 9-stündigen Applikation von Dipyridamol erhöhte sich die Gap Junction-abhängige Kopplung im Vergleich zu Kontrollbedingungen auf 126 % und 125 % (25 und 50 µM) bzw. 129 % und 123 % (25 und 50 µM). Nach einer Inkubationszeit von 24 h wurde diese Kopplung noch einmal auf 153 % und 160 % gesteigert (Abbildung 2.5A). Die Applikation von Dipyridamol erhöhte somit zeitabhängig die Gap Junction-abhängige Kopplung kontinuierlich, wobei die maximale Diffusionsweite des Farbstoffes bei einer Inkubation von Dipyridamol für 24 h beobachtet werden konnte.

Ebenso zeigten die glatten Muskelzellen eine zeitabhängige Veränderung in der Gap Junction-abhängigen Kopplung nach Dipyridamol-Zugabe. 10 und 50 µM Dipyridamol wurden hierbei für 15 min, 30 min, 1, 2, 3, 4, 5, 6, 9 und 24 h zugegeben. Bei kurzen Applikationszeiten bis zu 5 h zeigten mit 50 µM Dipyridamol behandelte Zellen eine relative Diffusionsweite von 114, 100, 121, 108, 129, 104 und 113 % zu den entsprechenden Zeitpunkten. Dieses oszillatorische Verhalten konnte nicht in Zellen beobachtet werden, die mit 10 µM Dipyridamol behandelt wurden. Nach der Applikation für mindestens 6 h konnte für beide Konzentrationen ein deutlicher Anstieg beobachtet werden, der sich durch eine Langzeitapplikation von Dipyridamol von bis zu 24 h nicht wesentlich veränderte. Dabei erreichte die Gap Junction-Kopplung einen maximalen Anstieg auf ca. 124 % (10 µM) und 144 % (50 µM) (Abbildung 2.5B).

Beide Zelltypen zeigten demnach eine Dipyridamol-induzierte, zeitabhängige Erhöhung der Gap Junction-Kopplung. In den glatten Muskelzellen ließ sich diese zeitabhängige Erhöhung unterscheiden in einen Kurzzeiteffekt, der bis zu einer Inkubationszeit von 6 h auftrat, und in einen Langzeiteffekt, der zwischen einer Inkubationszeit von 6 und 24 h detektiert wurde. In Endothelzellen stieg die Dipyridamol-induzierte Kopplung kontinuierlich in Abhängigkeit der Zeit an und erreichte nach einer Applikationszeit von 24 h das Maximum.

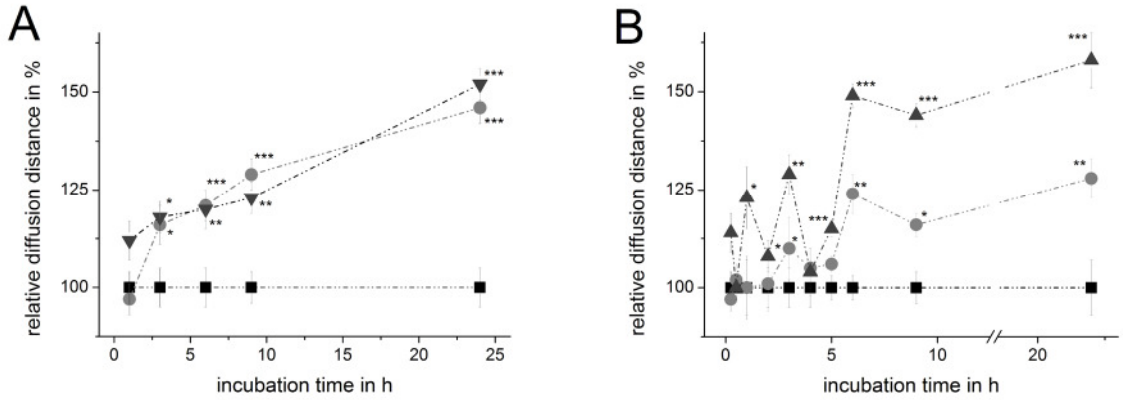


**Abbildung 2.5: Zeitabhängiger Einfluss von Dipyridamol auf die vaskulären Zellen. (A) 25 µM (●) und 50 µM (▼) Dipyridamol verstärkten die Gap Junction-Kopplung in GM-7373 Endothelzellen. (B) Zeitabhängiger Einfluss von 10 µM (●) und 50 µM Dipyridamol (▲)auf die Gap Junction-Kopplung in glatten Muskelzellen verglichen mit Kontrollzellen (0,5 % Ethanol, ■) (modifiziert nach Begandt et al. 2013a (B) und Begandt et al. 2013b (A)).**

## 2.4 Dipyridamol reguliert in Endothelzellen die Cx43-mRNA- und -Proteinmenge, sowie die Lokalisation von Cx43

Dipyridamol konnte eine Erhöhung der Gap Junction-abhängigen Kopplung durch einen cAMP/PKA-vermittelten Signalweg hervorrufen. Zur Analyse auf welcher Ebene Dipyridamol die Cx43-abhängige Gap Junction-Kopplung reguliert, wurden molekularbiologische und proteinbiochemische Untersuchungen durchgeführt. In der Literatur sind Änderungen der Gap Junction-abhängigen Kopplung durch eine Regulation auf verschiedenen Ebenen beschrieben worden: Die mRNA- und Proteinmenge der Connexine kann ebenso verändert werden wie die Assemblierungs-Rate von Gap Junctions in der Membran (Segretain & Falk 2004). Posttranslationale Modifikationen der Connexine können zu Veränderungen in der Assemblierung oder dem Öffnungszustand der Gap Junctions führen (Solan & Lampe 2007). In den bovinen GM-7373 Endothelzellen wurden nach 6 bzw. 24 h Inkubationszeit sqRT-PCR- und Western Blot-Versuche durchgeführt, sodass ein Einfluss von Dipyridamol auf Cx43-mRNA- und -Proteinmenge, sowie auf posttranslationale Modifizierungen von Cx43 analysiert werden konnte. Ebenso wurde ein möglicher Dipyridamol-induzierter Effekt auf die Lokalisierung von Cx43 mithilfe der Immunfluoreszenz untersucht.

Während der sqRT-PCR-Versuche wurde das *Housekeeping*-Gen *glyceraldehyd-3-phosphat dehydrogenase* (*gapdh*) verwendet und das Verhältnis aus *cx43*-Amplifikaten und *gapdh*-Amplifikaten gebildet, sodass Dipyridamol-behandelte Zellen zu Ethanol- oder unbehandelten Zellen verglichen werden konnten. In Western Blot-Versuchen wurde dafür das Kontrollprotein β-Tubulin verwendet. Nach einer 6-stündigen Dipyridamol-Applikation konnten weder in der Cx43-mRNA-Menge (Abbildung 2.6A und C) noch in der Cx43-Protein-Menge (Abbildung 2.8A und C) signifikante Änderungen verglichen zu den Ethanol-behandelten Zellen detektiert werden. Diese Ergebnisse deuten darauf hin, dass Dipyridamol die Gap Junction-abhängige Kopplung bei Dipyridamol-Inkubationen von bis zu 6 h nicht durch eine erhöhte Expression oder Synthese von Cx43 regulierte.

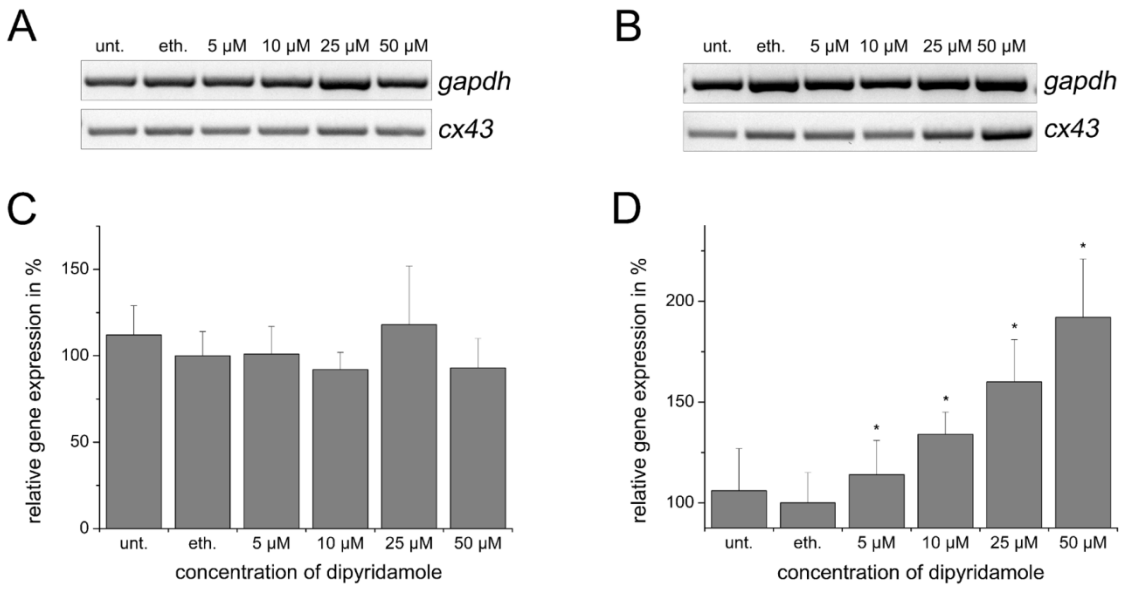


**Abbildung 2.6: Effekt von Dipyridamol auf die mRNA-Menge von Cx43 in Endothelzellen. Agarosegel-Bilder einer semi-quantitativen RT-PCR (sqRT-PCR) mit *cx43* und Housekeeping-Gen *gapdh* nach 6 h (A) und 24 h (B). (C und D) Quantifizierung der Ergebnisse der sqRT-PCR aus (A) und (B). Es wurden mindestens drei unabhängige Versuche durchgeführt (modifiziert nach Begandt et al. 2013b).**

Diese Hypothese wurde durch Versuche mit Brefeldin A (BFA, 5 µg/ml) bestätigt (Abbildung 2.7). BFA inhibiert den Proteintransport anterograd vom ER zum Golgi und verstärkt ihn retrograd vom Golgi zum ER (Klausner et al. 1992). Für 6 h wurden

vaskuläre Endothelzellen mit BFA oder BFA und 50 µM Dipyridamol behandelt und die Gap Junction-abhängige Kopplung mittels *Scrape Loading*/Farbstofftransfer analysiert. Relativ zu BFA-behandelten Zellen konnte durch Dipyridamol in Anwesenheit von BFA die Kopplung auf 116 % gesteigert werden (Abbildung 2.7). Diese Erhöhung war vergleichbar mit der allein durch Dipyridamol induzierten Steigerung der Kopplung auf 125 % nach 6 h (Abbildung 2.5A).

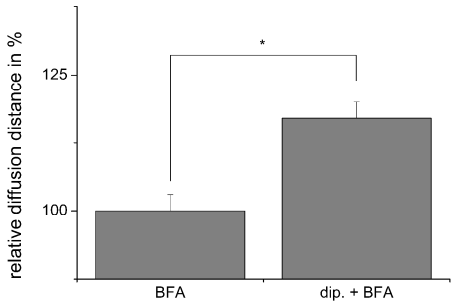


**Abbildung 2.7: Effekt der Inhibierung des vesikulären Transports durch Brefeldin A (BFA) auf die durch 50 µM Dipyridamol (dip.) erhöhte Gap Junction-Kopplung in Endothelzellen nach *Scrape Loading*/Farbstofftransfer (modifiziert nach Begandt et al. 2013b).**

Die Applikation von Dipyridamol für 24 h konnte in vaskulären Endothelzellen eine konzentrationsabhängige Erhöhung der Cx43-mRNA-Menge hervorrufen. Relativ zur Ethanol-Kontrolle wurde mittels der sqRT-PCR ein signifikanter Anstieg der Cx43-mRNA-Menge auf 114, 134, 160 und 192 % durch 5, 10, 25 und 50 µM Dipyridamol detektiert (Abbildung 2.6B und D). Weiterhin konnte im Western Blot die Proteinmenge von Cx43 konzentrationsabhängig durch Dipyridamol auf 103, 135, 196 und 432 % im Vergleich zu Kontrollbedingungen erhöht werden (Abbildung 2.8B und D).

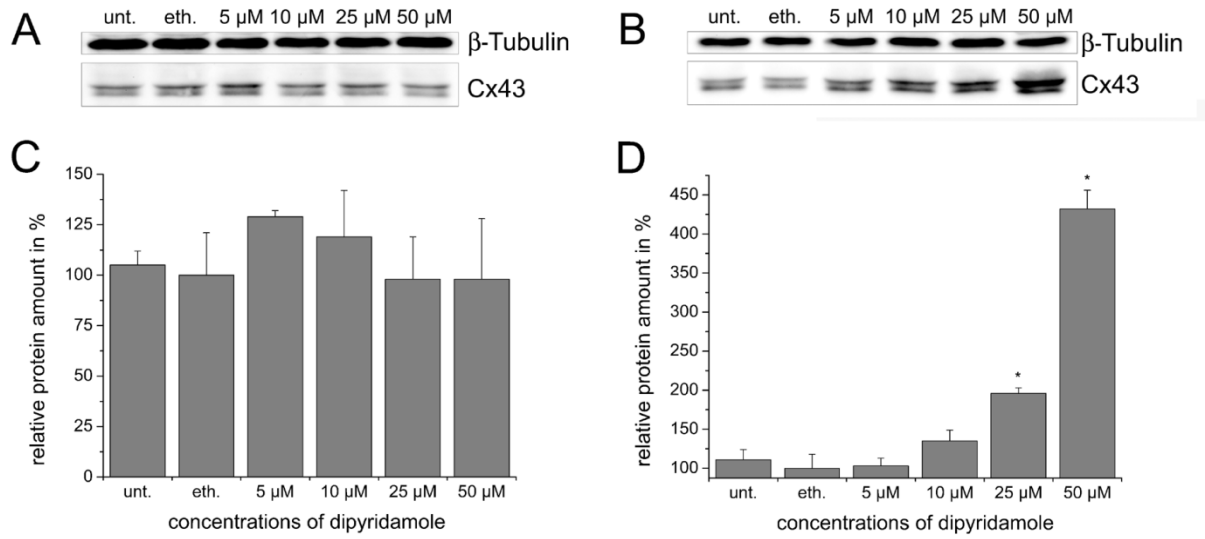


**Abbildung 2.8: Effekt von Dipyridamol auf die Cx43-Proteinmenge. (A und B) Western Blots mit Cx43 und Kontrollprotein β-Tubulin nach 6- (A) und 24-stündiger (B) Inkubation. (C und D) Quantifizierung der Western Blot-Ergebnisse nach 6 h (C) und 24 h (D). Es wurden mindestens drei unabhängige Versuche durchgeführt (modifiziert nach Begandt et al. 2013b).**

Neben der signifikanten Erhöhung der mRNA- und Proteinmenge von Cx43 wurde durch eine Langzeitapplikation von Dipyridamol in Endothelzellen eine veränderte Migration von Cx43 in der SDS-PAGE detektiert (Abbildung 2.9). Unbehandelte oder mit Ethanol behandelte Zellen zeigten nach der Auftrennung in der SDS-PAGE im Blot zwei distinkte Banden mit ca. 42 und 44 kDa für Cx43. Nach der Stimulation mit Dipyridamol konnte eine weitere Bande mit ca. 46 kDa detektiert werden (Abbildung 2.9).

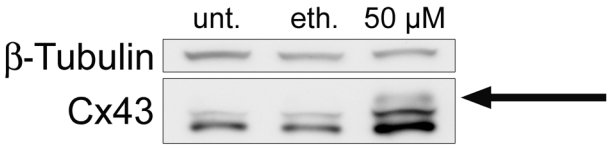


**Abbildung 2.9: Dipyridamol (50 µM) rief eine posttranslationale Modifikation von Cx43 nach einer Langzeitinkubation von 24 h hervor (siehe Pfeil). Es wurden mindestens drei unabhängige Versuche durchgeführt (modifiziert nach Begandt et al. 2013b).**

Die Verteilung von Cx43 innerhalb der Endothelzellen, sowie der mögliche Effekt von Dipyridamol auf die Lokalisierung von Cx43 wurde über eine Immunofärbung validiert (Abbildung 2.10).

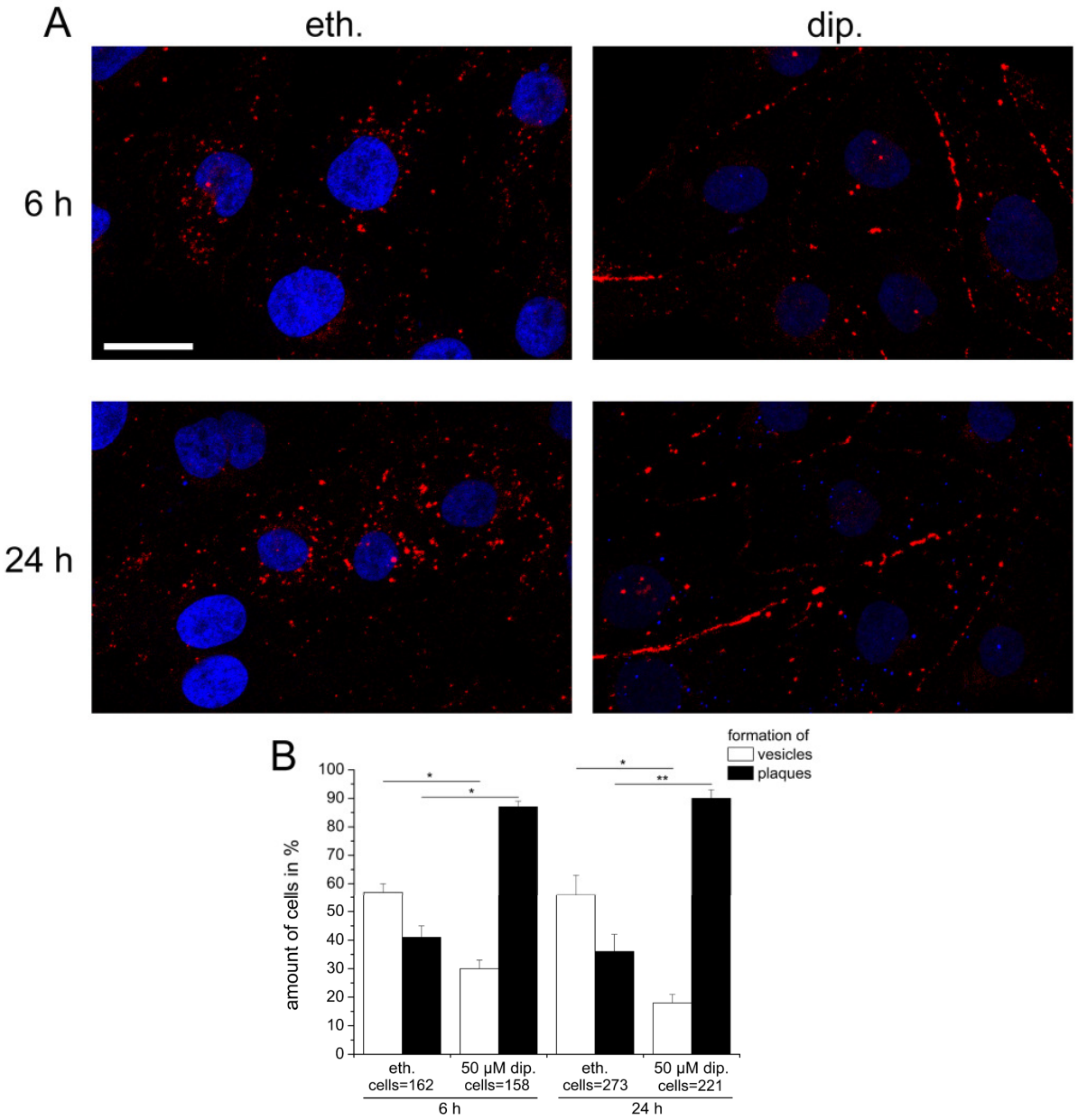


**Abbildung 2.10: Einfluss von 50 µM Dipyridamol (dip.) auf die Lokalisierung von Cx43 in Endothelzellen im Vergleich zu 0,1 % Ethanol-behandelten Zellen (eth.). (A) Fluoreszenzaufnahmen und (B) Quantifizierung der Anzahl an Zellen, die Cx43-assoziierte Gap Junction-Plaques und Vesikel aufwiesen. Es wurden mindestens drei unabhängige Experimente durchgeführt (modifiziert mach Begandt et al. 2013b).**

Kontrollzellen zeigten ein intrazelluläres Cx43-Signal um den Nukleus herum, das dem ER und Golgi-Apparat zugeordnet werden konnte. Eine große Anzahl an Cx43-assoziierten Vesikeln konnte zwischen dem Golgi-Apparat und der Plasmamembran

detektiert werden. Im Gegensatz zu diesem Verteilungsmuster war in mit 50 µM Dipyridamol behandelten Endothelzellen nach 6 bzw. 24 h die Anzahl der Cx43-gefärbten Vesikel zwischen dem Golgi-Apparat und der Plasmamembran deutlich reduziert. Dagegen zeigten Dipyridamol-behandelte Zellen eine vermehrte Färbung im Bereich der Zell-Grenzen zwischen zwei benachbarten Zellen, die Gap Junction-Plaques zugeordnet werden konnten (Abbildung 2.10A). Demnach reguliert Dipyridamol vermutlich die Ausbildung Cx43-assoziierter Vesikel und/oder Gap Junction-Plaques. Diese Vermutung konnte durch die Quantifizierung von Cx43-assoziierten Vesikel- und Plaque-bildenden Zellen bestätigt werden. Die Anzahl Vesikel-bildender Zellen sank nach einer Applikationszeit von 6 h von 57 % bei Ethanol-Behandlung auf 30 % bei Dipyridamol-behandelten Zellen. Dagegen stieg die Anzahl der Zellen, die Plaques ausbildeten, von 41 % auf 87 %. Nach 24 h Ethanol- oder Dipyridamol-Applikation konnten ähnliche Ergebnisse erzielt werden. Die Anzahl Vesikel-bildender Zellen sank von 56 % auf 18 % und die Anzahl Plaque-bildender Zellen stieg von 36 % auf 90 % in Ethanol-bzw. Dipyridamol-behandelten Zellen (Abbildung 2.10B).

Zusammenfassend ist die durch Dipyridamol erhöhte Gap Junction-abhängige Kopplung assoziiert mit einem Einfluss von Dipyridamol auf den Biosynthese-Prozess der Gap Junctions. Nach einer 6-stündigen Applikation von Dipyridamol wurden bereits vorhandene Connexone/Gap Junctions nach der Passage des Golgi-Apparats reguliert. Nach 6- und 24-stündiger Inkubation mit Dipyridamol wurde die Anzahl Cx43-assoziierter Vesikel-bildender Zellen verringert, wohingegen die Anzahl Plaque-bildender Zellen erhöht wurde. Durch eine Applikation von Dipyridamol über 24 h wurde in Endothelzellen zusätzlich eine erhöhte Cx43-mRNA- und -Proteinmenge, sowie eine veränderte Migration von Cx43 in der SDS-PAGE hervorgerufen.

# 3 Diskussion

Störungen im komplexen und feinregulierten Prozess der direkten Zell-Zell-Kommunikation über Gap Junctions sind innerhalb des vaskulären Systems assoziiert mit Erkrankungen wie Hypertonie oder Arteriosklerose, die zu einem Schlaganfall führen können. Daher wurden Gap Junctions als ein neues therapeutisch relevantes Ziel postuliert. In der derzeitigen Therapie von Schlaganfällen werden als Therapieziel nur die Thrombozyten reguliert, wie durch den Wirkstoff Dipyridamol. Obwohl Dipyridamol bereits vielfältige vasoprotektive Eigenschaften nachgewiesen worden sind, wird es derzeit als Thrombozyten-Aggregations-Inhibitor eingesetzt. Das Ziel dieser Arbeit war daher erstmals einen möglichen Effekt von Dipyridamol auf die Gap Junction-abhängige Kommunikation in vaskulären Zellen zu analysieren, was mithilfe physiologischer und molekularbiologischer Methoden erfolgreich gelang. In den ersten beiden Publikationen konnte der Effekt von Dipyridamol auf die Gap Junction-abhängige Kopplung in Endothelzellen der Zelllinie GM-7373 und in glatten Muskelzellen der Zelllinie A-10, sowie der Dipyridamol-induzierte Signalweg nachgewiesen werden. Die dritte Publikation analysierte auf welcher Ebene Dipyridamol durch einen cAMP-abhängigen Signalweg die Cx43-abhängige Gap Junction-Kopplung regulierte.

## 3.1 Effekte von Dipyridamol auf Zellen des vaskulären Systems

Erstmals konnte in dieser Arbeit der Einfluss von Dipyridamol auf die Gap Junction-abhängige Kommunikation in Zellen des vaskulären Systems *in vitro* nachgewiesen werden. In vaskulären Endothelzellen und glatten Muskelzellen wurde die Kopplung über Gap Junctions nach einer Dipyridamol-Applikation von bis zu 24 h erhöht (Abbildung 2.1, Abbildung 2.2). Signifikante Unterschiede im Vergleich zu Kontrollbedingungen wurden ab einer Konzentration von 1 µM (in Endothelzellen) und von 5 µM (in glatten Muskelzellen) festgestellt. Diese Konzentrationen sind therapeutisch relevant, da Patienten bei einer täglichen Dosis von 200 mg Dipyridamol ähnliche Plasmakonzentrationen von mindestens 1,5 µM aufweisen (Eisert 2006). Eine steigende Dipyridamol-Konzentration bis zu 75 µM bzw. 100 µM korrelierte mit steigender Gap

Junction-abhängiger Kopplung in Endothel- bzw. glatten Muskelzellen (Abbildung 2.1, Abbildung 2.2).

In Thrombozyten erhöht Dipyridamol die intrazelluläre cAMP-Konzentration durch die Hemmung der Adenosin-Transporter und einer nachfolgenden Bindung von Adenosin an die Adenosin-Rezeptoren. Über die Rezeptor-vermittelte Aktivierung der Adenylyl-Zyklase wird die intrazelluläre cAMP-Synthese gesteigert (Anfossi et al. 2002, Gao & Jacobson 2011, Kim & Liao 2008). Dieser Signalweg wurde vermutlich auch in den vaskulären Zellen aktiviert. Die Stimulation der Zellen mit dem Adenylyl-Zyklase-Aktivator Forskolin und die Applikation von membranpermeablem 8-Br-cAMP resultierten beide in einer erhöhten Gap Junction-abhängigen Kopplung in Endothelzellen und in glatten Muskelzellen (Abbildung 2.4). Eine Steigerung der Gap Junction-Kopplung durch erhöhte intrazelluläre cAMP-Konzentrationen ist aus früheren Studien bekannt (Hoffmann et al. 2003, Holm et al. 1999, van Rijen et al. 2000), sodass Dipyridamol vermutlich über eine Steigerung der intrazellulären cAMP-Konzentration die Gap Junction-Kopplung erhöhte.

Die in dieser Arbeit gezeigten Dipyridamol-induzierten Erhöhungen der Gap Junction-Kopplung waren PKA-abhängig, da eine zusätzliche Applikation der spezifischen PKA-Inhibitoren H-89 und Rp-cAMPS das Dipyridamol-induzierte Signal abhängig von der Dipyridamol-Konzentration reduzieren konnte. Nach 6 h oder 24 h konnte mithilfe der Inhibitoren in vaskulären Endothelzellen und glatten Muskelzellen die über einen Farbstofftransfer ermittelte Kopplung reduziert werden (Abbildung 2.3). Eine Aktivierung der PKA durch intrazellulär erhöhte cAMP-Konzentrationen mit dem Resultat einer erhöhten Gap Junction-abhängigen Kopplung ist beschrieben worden (Lampe & Lau 2004, Salameh et al. 2009, Zhang et al. 2005), dabei wurde zum Beispiel die *de novo*-Ausbildung von Gap Junction-Kanälen stimuliert (Hoffmann et al. 2003). Neben cAMP-induzierten PKA-abhängigen Steigerungen der Gap Junction-abhängigen Kopplung durch Dipyridamol könnten allerdings auch PKA-unabhängige Wege aktiviert worden sein. Bei hohen Dipyridamol-Konzentrationen konnte beispielsweise keine vollständige Hemmung der Dipyridamol-induzierten Gap Junction-Kopplung mithilfe der PKA-Inhibitoren erreicht werden (Abbildung 2.3).

Die Variation der Applikationszeiten von Dipyridamol zwischen 15 min und 24 h resultierte in einer zeitabhängigen Veränderung der Gap Junction-abhängigen Kopplung in beiden Zelltypen des vaskulären Systems (Abbildung 2.5). Die durch Dipyridamol signifikant gesteigerte Gap Junction-abhängige Kopplung nahm in Endothelzellen zwischen 9 h und 24 h kontinuierlich zu (Abbildung 2.5A). In den glatten Muskelzellen wurde ein biphasisches Verhalten detektiert. Die Gap Junction-Kopplung zeigte durch eine Kurzzeitapplikation von bis zu 5 h von 50 µM Dipyridamol ein oszillatorisches Verhalten. Ab einer Applikationszeit von 6 h bis zu einer Langzeitinkubation von 24 h steigerte sich die Kopplung stabil auf ca. 144 % für 50 µM Dipyridamol (Abbildung 2.5B).

Das oszillatorische Verhalten der Kopplung in glatten Muskelzellen könnte durch einen Agonisten-induzierten Desensitivierung/Resensitivierungs-Kreislauf der Adenosin-Rezeptoren erklärt werden. Stimulierte Adenosin-$A_{2A}$- und -$A_{2B}$-Rezeptoren werden nach der Bindung des Agonisten internalisiert, recycelt und für eine neue Agonisten-abhängige Aktivierung wieder an die Plasmamembran gebracht (Mundell & Kelly 2011). Die oszillatorisch veränderte Kopplung in glatten Muskelzellen könnte ein Resultat dieser Recyclingszyklen sein und durch eine Oszillation der intrazellulären cAMP-Konzentration hervorgerufen werden. Mundell und Kelly (1998) konnten über die intrazelluläre Erhöhung von cAMP eine Recyclingzeit von 60 bis 90 Minuten für den $A_{2A}$-Rezeptor nachweisen, was die hier gezeigten Oszillationen erklären könnte (Mundell & Kelly 1998).

Aufgrund der beobachteten zeitabhängigen Wirkung von Dipyridamol auf die vaskulären Endothel- und glatten Muskelzellen (Abbildung 2.5) wurde eine biphasische Regulation postuliert. Die Steigerung der Gap Junction-abhängigen Kopplung nach der Kurzzeitapplikation bis zu 6 h konnte vermutlich durch die Regulation der bereits existierenden Connexine/Gap Junctions hervorgerufen werden. Die 24-stündige Langzeitapplikation konnte vermutlich durch eine Neusynthese von Connexinen zusätzlich zu der Regulation der existierenden Connexine/Gap Junctions eine weitere Verstärkung der Kopplung erwirken. In beiden Zelltypen wurde die Aktivierung einer

cAMP-abhängigen Kaskade angenommen, die zu einer erhöhten Gap Junction-abhängigen Kopplung führte.

In den GM-7373 Endothelzellen konnte die Dipyridamol-vermittelte Gap Junction-Kopplung auf ca. 130 % nach 6 h und auf ca. 150 % nach 24 h erhöht werden. In beiden Zeiträumen, der Kurz- und Langzeitapplikation, konnte durch die Dipyridamol-Applikation eine Veränderung in der Lokalisierung von Cx43 innerhalb der Zelle beobachtet werden. Die Anzahl der Zellen mit Cx43-assoziierten Vesikeln sank durch die Dipyridamol-Zugabe, wohingegen die Anzahl der Zellen mit Cx43-assoziierten Gap Junction-Plaques anstieg. Dipyridamol induzierte demnach eine erhöhte Akkumulation von Cx43 in den Bereichen der Zellmembran (Abbildung 2.10). Allerdings konnten durch eine Kurzzeitapplikation von 6 h mit 50 µM Dipyridamol weder Änderungen in der Cx43-mRNA-Menge noch Änderungen in der Cx43-Proteinmenge nachgewiesen werden (Abbildung 2.6A und C, Abbildung 2.8A und C). Demnach konnte der Dipyridamol-induzierte Effekt der erhöhten Kopplung in *Scrape Loading*/Farbstofftransfer-Versuchen durch bereits existierende Connexine hervorgerufen werden. Dies geht einher mit der Beobachtung, dass in *Scrape Loading*/Farbstofftransfer-Versuchen während einer Kurzzeitapplikation mit Dipyridamol und BFA der Dipyridamol-induzierte Effekt nicht durch BFA gehemmt werden konnte (Abbildung 2.7). BFA führt zu einer Inhibierung des Proteintransports anterograd vom ER zum Golgi (Klausner et al. 1992). Eine Dipyridamol-induzierte erhöhte Kopplung in Anwesenheit von BFA deutet daher auf den Einfluss von Dipyridamol auf die Connexine hin, die den Golgi-Apparat passiert haben und schon zu Connexonen oligomerisiert sind. Da Dipyridamol vermutlich einen cAMP-abhängigen Signalweg aktiviert (Abbildung 2.4), kann angenommen werden, dass cAMP die existierenden Connexone/Gap Junctions nach dem Passieren des Golgi-Apparats regulierte. In Verbindung mit der Dipyridamol-induzierten vermehrten Akkumulation von Cx43 in der Zellmembran (Abbildung 2.10), erhöht cAMP vermutlich den Transport von vorhandenen Connexonen in die Plasmamembran oder inhibiert die Degradierung der Gap Junction-Kanäle in der Zellmembran.

Diese Ergebnisse stimmen mit Beobachtungen überein, bei denen eine erhöhte Gap Junction-abhängige Kopplung nach einer kurzen Applikationszeit von cAMP durch eine

Änderung der Gap Junction-Assemblierung hervorgerufen wurde (Burghardt et al. 1995, Holm et al. 1999, Paulson et al. 2000). Ähnlich zu den hier erlangten Ergebnissen konnte eine cAMP-induzierte Neuassemblierung der Gap Junctions nicht durch BFA inhibiert werden, im Gegensatz dazu aber durch Monensin. Monensin inhibiert den letzten Schritt des Vesikeltransports an die Plasmamembran (Paulson et al. 2000). Ein verändertes Öffnungsverhalten der vorhandenen Gap Junctions könnte ebenso eine Rolle in der 6-stündigen durch Dipyridamol veränderten Gap Junction-abhängigen Kopplung spielen. Studien belegen den Einfluss von cAMP auf die existierenden Gap Junction-Kanäle, die durch cAMP in ihrem Öffnungszustand oder ihrer Permeabilität positiv verändert werden können (Atkinson et al. 1995, Chanson et al. 1996, Faucheux & Nagel 2002, Loewenstein 1981).

Zusammenfassend konnte die Dipyridamol-vermittelte Aktvierung eines vermutlich cAMP-induzierten Signalwegs nach einer 6-stündigen Inkubation die Gap Junction-Kopplung erhöhen, indem bereits existierende Connexone/Gap Junctions vermehrt in der Zellmembran akkumulierten. Zum einen könnte die Regulation durch eine erhöhte Gap Junction-Kanal-Assemblierung oder zum anderen durch eine verminderte Kanal-Degradierung erfolgen. Ebenso könnte Dipyridamol den Öffnungszustand und die Permeabilität der vorhanden Gap Junction-Kanäle regulieren.

Die nach 24-stündiger Dipyridamol-Applikation induzierte erhöhte Gap Junction-abhängige Kopplung vaskulärer Endothelzellen (Abbildung 2.1), korrelierte mit einer erhöhten Menge von Cx43-mRNA und -Protein (Abbildung 2.6B und D, Abbildung 2.8B und D). Die erhöhte Cx43-mRNA-Menge könnte das Resultat einer durch Dipyridamol aktivierten Transkription sein oder durch einen verminderten Abbau der mRNA hervorgerufen werden. Eine durch cAMP aktivierte erhöhte Transkription des *cx43*-Gens ist bereits von Mehta et al. (1992) beschrieben worden. Welcher Mechanismus den gemessenen Dipyridamol-induzierten Anstieg der Cx43-mRNA erklärt, ist Bestandteil weiterer Forschungen.

Nach einer Langzeitapplikation von Dipyridamol konnte zusätzlich zu der erhöhten Cx43-mRNA-Menge eine erhöhte Cx43-Proteinmenge detektiert werden

(Abbildung 2.8B und D). Diese erhöhte Cx43-Proteinmenge könnte ein direktes Resultat der erhöhten Cx43-mRNA-Menge sein. Ebenso könnte die Syntheserate erhöht oder die Degradierung von Cx43-Proteinen verringert sein. In anderen Studien konnte belegt werden, dass mit einer cAMP-Stimulation eine erhöhte Cx43-Proteinmenge und in Verbindung damit eine gesteigerte Gap Junction-abhängige Kopplung einhergeht (Salameh et al. 2009, Yogo et al. 2002).

Zusätzlich war eine Migrationsveränderung von Cx43 in der SDS-PAGE nach einer Langzeitapplikation mit Dipyridamol zu beobachten (Abbildung 2.9). Cx43 aus Kontrollzellen zeigte nach der Auftrennung in der SDS-PAGE zwei distinkte Banden von ca. 42 kDa und 44 kDa, Dipyridamol induzierte zusätzlich die Ausbildung einer weiteren Bande von ca. 46 kDa (Abbildung 2.9). Diese Banden sind nach Solan und Lampe (2005) als Migrationsformen P0, P1 und P2 bekannt und durch die multiple Phosphorylierung spezifischer Serinreste charakterisiert. Die Regulation von Cx43 durch eine cAMP-vermittelte Phosphorylierung ist zahlreich nachgewiesen worden (Lampe et al. 2006, Musil & Goodenough 1991, Solan & Lampe 2005, Solan & Lampe 2009, Sosinsky et al. 2007). Alle drei Migrationsformen können theoretisch die Plasmamembran erreichen. Dabei ist die P1 Migrationsform primär in der Plasmamembran und zum Teil in Gap Junction-Plaques zu detektieren. Die P2 Migrationsform ist ausschließlich in Gap Junction-Strukturen nachzuweisen (Solan & Lampe 2007, Solan & Lampe 2009). Durch die Dipyridamol-vermittelte Migrationsveränderung konnte folglich in Kombination mit der durch die Immunofärbungen gezeigten vermehrten Akkumulation von Cx43 in der Zellmembran eine Erhöhung der Cx43-assoziierten Gap Junction-Plaques nachgewiesen werden.

Nach einer 24-stündigen Applikationszeit erhöhte Dipyridamol die Gap Junction-abhängige Kopplung über eine vermutlich cAMP-vermittelte Erhöhung von Cx43-mRNA und -Protein, sowie über eine erhöhte Akkumulation von Cx43 in Gap Junction-Plaques.

## 3.2 Gap Junction-Regulation durch cAMP

Eine Erhöhung der intrazellulären cAMP-Konzentration durch Dipyridamol in Thrombozyten ist in der Literatur beschrieben (Anfossi et al. 2002, Gao & Jacobson 2011, Kim &

Liao 2008). Die Signalkaskade der cAMP-vermittelten Verstärkung der Gap Junction-abhängigen Kopplung nach Dipyridamol-Applikation ist allerdings nicht vollständig aufgeklärt. Die PKA wurde für lange Zeit als einziger cAMP-Effektor betrachtet (Sands & Palmer 2008, Duquesnes et al. 2010). Eine Beteiligung der PKA konnte im Dipyridamol-vermittelten cAMP-abhängigen Mechanismus nachgewiesen werden, da die Applikation von PKA-Inhibitoren einer erhöhten Kopplung in *Scrape Loading*/Farbstofftransfer-Versuchen entgegenwirken konnte (Abbildung 2.3). Allerdings konnte der Effekt bei hohen Dipyridamol-Konzentrationen nur teilweise durch die PKA-Inhibitoren reduziert werden. Ebenso konnten PKA-Inhibitoren die Dipyridamol-vermittelte, cAMP-induzierte Erhöhung der Cx43-mRNA- und-Proteinmenge in sqRT-PCR- und Western Blot-Experimenten nicht reduzieren (Ergebnisse nicht gezeigt). Demnach könnten durch einen Dipyridamol-vermittelten cAMP-Anstieg auch PKA-unabhängige Signalwege aktiviert werden und die Gap Junction-abhängige Kopplung erhöhen, zum Beispiel durch eine Regulation auf Ebene von mRNA oder Protein (Abbildung 3.1). Somekawa und Kollegen (2005) konnten nach einer cAMP-Applikation die Aktivierung eines PKA-unabhängigen, *exchange protein directly activated by cAMP* (Epac)-vermittelten Signalwegs in Kardiomyozyten nachweisen: Neben dem cAMP-induzierten PKA-abhängigen erhöhten Farbstofftransfer, wurde die Gap Junction-abhängige Kopplung zusätzlich durch eine veränderte Lokalisierung von Cx43 über den PKA-unabhängigen, Epac-vermittelten Weg verstärkt. Epac ist ein cAMP-stimuliertes Protein, dass auf Ebene der Zellproliferation, der Gentranskription oder des Vesikeltransports die Zellen regulieren kann (Bos 2003, Bos 2006, Roscioni et al. 2008, Somekawa et al. 2005). Eine Beteiligung des Epacs in den GM-7373 Endothelzellen sollte in zukünftigen Studien validiert werden.

Während einer kurzen Applikationszeit von 6 h von Dipyridamol wurde in vaskulären Zellen die Gap Junction-Kopplung durch eine erhöhte Akkumulation bereits existierender Cx43-assoziierter Connexone/Gap Junctions in der Zellmembran verstärkt und/oder die bereits existierenden Gap Junctions in ihrem Öffnungsverhalten reguliert. Eine Modulation von Cx43 auf der mRNA- oder Proteinebene konnte nicht nachgewiesen werden. Inwiefern die anderen exprimierten Connexin-Isoformen auf

diesen Ebenen, während dieser Applikationszeit oder generell durch Dipyridamol reguliert werden, sollte in zukünftigen Studien beurteilt werden. In schon veröffentlichten Studien anderer Arbeitsgruppen konnte neben Cx43 zum Beispiel Cx40 durch intrazellulär erhöhte cAMP-Konzentrationen reguliert werden (Hoffmann et al. 2003, van Rijen et al. 2000). Ebenso konnten gegensätzliche Regulationen unterschiedlicher Isoformen innerhalb eines Zelltyps nachgewiesen werden: Nach Stimulation mit dem Adenovirus-Vektor E4 wurde Cx40 positiv, Cx43 negativ reguliert (Zhang et al. 2005). Nachweislich besteht eine Interaktion zwischen den Connexin-Isoformen, die in einer Zelle exprimiert werden (Isakson et al. 2006): Nach dem *Knock-Out* von Cx40 in vaskulären Endothelzellen ist die Expression von Cx37 verändert. Dahingegen hat ein *Knock-Out* von Cx37 in Mäusen scheinbar geringeren Einfluss auf die Expression von Cx40 (Simon & McWhorter 2003).

Mithilfe der Dipyridamol-induzierten Effekte konnte in dieser Dissertation ein Einblick in die komplexe Regulation der Gap Junctions im vaskulären System gewonnen werden (Abbildung 3.1). Aspekte wie die aktivierten PKA-abhängigen und -unabhängigen Signalwege oder die vermehrte Akkumulation von Cx43 in der Zellmembran, durch erhöhte Translokation von Connexons oder verminderte Degradierung der Gap Junctions, sind wichtige Regulationspunkte, die in zukünftigen Studien weiter analysiert werden sollen. Ebenso ist das Expressions- und Lokalisierungsverhalten der Connexine in glatten Muskelzellen nach einer Dipyridamol-Behandlung zu analysieren, da die Untersuchungen auf mRNA- und Proteinebene von Cx43 und zur Lokalisierung von Cx43 derzeit nur in den Endothelzellen durchgeführt wurden.

### 3.3 Dipyridamol und Gap Junctions im Kontext – ein Ausblick

Nach den Ergebnissen dieser Arbeit wird angenommen, dass Dipyridamol seine Wirkung auf die Gap Junction-abhängige Kommunikation über einen cAMP-gesteuerten Signalweg in Zellen des vaskulären Systems als Folge der Inhibierung der ENTs ausübt. (Anfossi et al. 2002, Gao & Jacobson 2011, Kim & Liao 2008, Molina-Arcas et al. 2009, Podgorska et al. 2005). Durch die Inhibierung der ENTs kommt es allerdings neben einem Anstieg von Adenosin im extrazellulären Raum ebenfalls zur lokalen Erhöhung

weiterer Substrate der ENTs, also neben Adenosin zur Erhöhung der Purin- und Pyrimidin-Nukleoside und ihrer Basen (Baldwin et al. 2004). Ein Einfluss dieser Substrate auf die Gap Junction-abhängige Kopplung könnte daher ebenfalls möglich sein (Abbildung 3.1).

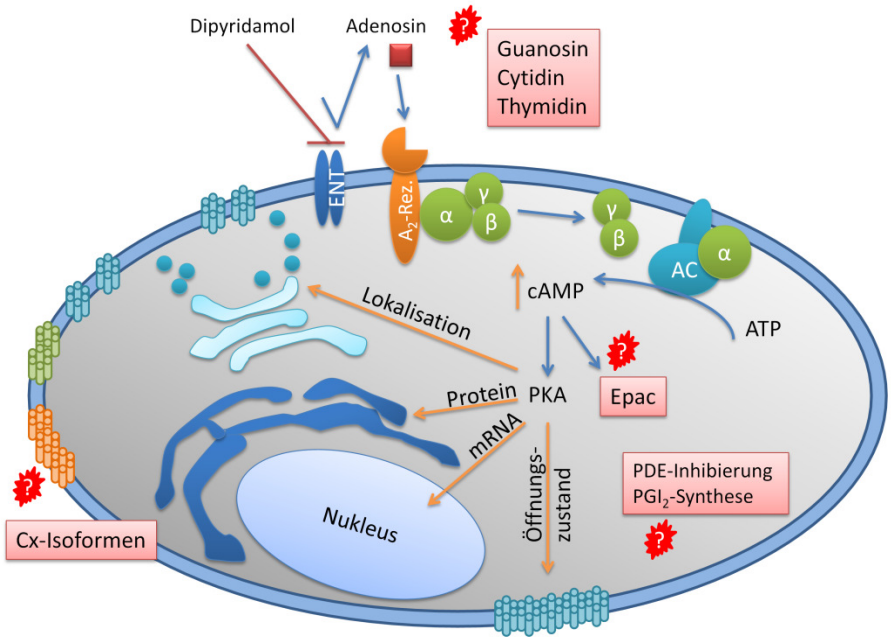


**Abbildung 3.1: Mögliche durch Dipyridamol-induzierte Signalwege auf die Zellen des vaskulären Systems. Durch die Inhibierung der *equilibrative nucleoside transporter* (ENT) wird Adenosin extrazellulär akkumuliert, so dass es vermehrt an den Adenosin-$A_2$-Rezeptor ($A_2$-Rez.) binden kann. Über die Aktivierung eines trimeren $G_s$-Proteins (grün, α-, β-, γ-Untereinheiten) wird die Adenylyl-Zyklase (AC) zur Synthese von cAMP stimuliert. Proteinkinase A (PKA) als Effektor-Enzym von cAMP könnte an multiplen Regulationspunkten, wie der Expression, der Proteinsynthese, der Lokalisierung oder dem Öffnungszustand von Gap Junctions die Gap Junction-abhängige Kopplung modifizieren. Cx: Connexin, Epac: *exchange protein directly activated by cAMP*, PDE: Phosphodiesterase, $PGI_2$: Prostaglandin $I_2$.**

Ebenso können weitere Eigenschaften von Dipyridamol die Gap Junction-abhängige Kopplung und den cAMP-induzierten Signalweg beeinflussen. Neben der Inhibierung der ENTs ist bekannt, dass Dipyridamol konzentrationsabhängig die cGMP- und cAMP-

spezifischen Phosphodiesterasen (PDE) inhibiert und dadurch die intrazellulären cGMP- und cAMP-Konzentrationen erhöht (Ahn et al. 1989, Eisert 2006, Gillespie & Beavo 1989). Zudem aktiviert Dipyridamol cAMP-abhängig die Prostaglandin $I_2$ ($PGI_2$)-Synthese (Blass et al. 1980, Mehta & Mehta 1982). Durch die Bindung von $PGI_2$ an die $PGI_2$-Rezeptoren wird intrazellulär der cAMP-Spiegel ebenfalls erhöht, sodass eine Verstärkung der zuvor erhöhten cAMP-Konzentration erfolgt (Abbildung 3.1). Der Einfluss dieser weiteren Dipyridamol-vermittelten Signale auf die Gap Junction-Kopplung wurde in dieser Arbeit nicht untersucht. Inwiefern die Dipyridamol-induzierte Erhöhung der Gap Junction-Kopplung daher ein Effekt aus allen drei Signalen ist (ENT-Inhibierung/PDE- Inhibierung /$PGI_2$-Synthese), aus zeitlich versetzten Effekten oder nur aus einem einzigen, bedarf weiterer Untersuchungen.

Die in dieser Arbeit erstmals dokumentierte Dipyridamol-induzierte Erhöhung der Gap Junction-abhängigen Kopplung korrelierte in den Endothelzellen mit einer erhöhten Akkumulation von Cx43 in der Zellmembran und nach 24-stündiger Inkubation mit einer erhöhten Cx43-mRNA- und -Proteinmenge. Im Gegensatz zu dieser Dipyridamol-induzierten Erhöhung sinkt die Gap Junction-abhängige Kommunikation vaskulärer Zellen durch hohe Zuckerkonzentrationen wie sie während Diabetes-Erkrankungen vorkommen können (Inoguchi et al. 1995, Inoguchi et al. 2001, Kuroki et al. 1998). Diese verminderte Kopplung wurde durch eine erhöhte PKC-Aktivität hervorgerufen und führte in glatten Muskelzellen zu einer spezifischen Phosphorylierung von Cx43 (Kuroki et al. 1998) und in mikrovaskulären Endothelzellen der Ratte zu einer verminderten Cx43-mRNA- und -Protein-Menge (Sato et al. 2002). Eine erhöhte PKC-abhängige Phosphorylierung ist ebenfalls während ischämischer und hypoxischer Bedingungen spezifisch an dem Serin-Rest 368 (S368) des C-Terminus von Cx43 nachgewiesen worden (Ek-Vitorin et al. 2006, Hund et al. 2007, Richards et al. 2004, Solan et al. 2007). Durch die Dipyridamol-induzierte spezifische posttranslationale Modifikation der Cx43-Moleküle (Abbildung 2.9) könnte eine PKC-spezifische Phosphorylierung, wie sie bei hyperglykämischen oder hypoxischen Bedingungen auftreten kann, vielleicht verhindert werden. Ein solcher Effekt ist bereits von Solan et al. beschrieben worden - die PKC-abhängige Phosphorylierung an S368 und die daraus resultierende verminderte Gap

Junction-Kopplung konnte durch eine Phosphorylierung von Cx43 an S365 unterdrückt werden (Solan et al. 2007). Die Serin-Reste S364/365 werden durch intrazellulär erhöhte cAMP-Konzentrationen über Kinasen phosphoryliert und stehen in Zusammenhang mit einem erhöhten Transport von Cx43 an die Zellmembran und dem Einbau in Gap Junctions (TenBroek et al. 2001, Yogo et al. 2002, Yogo et al. 2006). Da Dipyridamol vermutlich die erhöhte Gap Junction-Kopplung über eine Steigerung der intrazellulären cAMP-Konzentration hervorruft, könnte dieser von Solan et al (2007) beschriebene Einfluss durch Dipyridamol aktiviert werden. Dabei könnte Dipyridamol einen inhibierenden, prophylaktischen Effekt auf die PKC-abhängige Ausbildung der Gap Junction-abhängigen Dysfunktionen oder auch Einfluss auf bereits angegriffene Gefäße während einer Diabetes-Erkrankung haben. Diese mögliche therapeutische Anwendbarkeit von Dipyridamol oder anderen Wirkstoffen auf eine dysregulierte Gap Junction-abhängige Zell-Zell-Kommunikation sowie die Rolle der Gap Junctions in vaskulär-assoziierten Erkrankungen sollte zukünftig in Modellsystemen analysiert werden, die hyperglykämische, hypoxische oder inflammatorische Bedingungen nachstellen wie sie während des Diabetes, eines Schlaganfalls oder chronischen Erkrankungen vorkommen können (Green & Nicholson 2008).

Damit versprechen die in dieser Dissertation vorgestellten Ergebnisse eine mögliche erweiterte Anwendung von Dipyridamol in der Therapie vaskulärer Erkrankungen, die mit einer Dysregulation der Gap Junction-abhängigen Kommunikation assoziiert sind. Zudem wurde in dieser Arbeit erstmals gezeigt, dass die Gap Junction-abhängige Kommunikation vaskulärer Zellen ein neues potentielles therapeutisches Ziel eines pharmakologisch in der Schlaganfall-Therapie verwendeten Wirkstoffs darstellt.

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## Abkürzungsverzeichnis

| | |
|---|---|
| 8-Br-cAMP | 8-Bromoadenosin-3',5'-zyklisches Monophosphat |
| AC | Adenylyl-Zyklase |
| ASS | Acetylsalicylsäure |
| BFA | Brefeldin A |
| CK1 | Caseinkinase 1 |
| CREB | *cAMP response element binding protein* |
| C-Terminus | Carboxylterminus |
| Cx | Connexin |
| db-cGMP | Dibutyryl-zyklisches Guanosinmonophosphat |
| dip. | Dipyridamol |
| EDHF | *endothelium-derived hyperpolarizing factor* |
| EDRF | *endothelium-derived relaxation factor* |
| ENT | *equilibrative nucleoside transporter* |
| Epac | *exchange protein directly activated by cAMP* |
| ER | Endoplasmatisches Retikulum |
| ERGIC | *endoplasmatic reticulum-Golgi intermediate compartment* |
| eth. | Ethanol |
| for. | Forskolin |
| H-89 | *N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide* |
| MAPK | *mitogen activated protein kinase* |

| | |
|---|---|
| NO | Stickstoffmonoxid |
| N-Terminus | Aminoterminus |
| PDE | Phosphodiesterase |
| $PGI_2$ | Prostglandin $I_2$ |
| PKA | Proteinkinase A |
| PKC | Proteinkinase C |
| Rp-cAMPS | Rp-Adenosin-3′,5′-zyklisches monophosphorothioat |
| SDS-PAGE | Natriumdodecylsulfat-Polyacrylamidgelelektrophorese |
| SEM | Standardfehler |
| sqRT-PCR | semiquantitative Reverse Transkription-PCR |
| TIA | transiente ischämische Attacke |
| unt. | unbehandelt |

# Abbildungsverzeichnis

# Anhang A

Dipyridamole increases gap junction coupling in bovine GM-7373 aortic endothelial cells by a cAMP-protein kinase A dependent pathway (Begandt et al. 2010)



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

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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.

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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 $1\times10^6$ 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

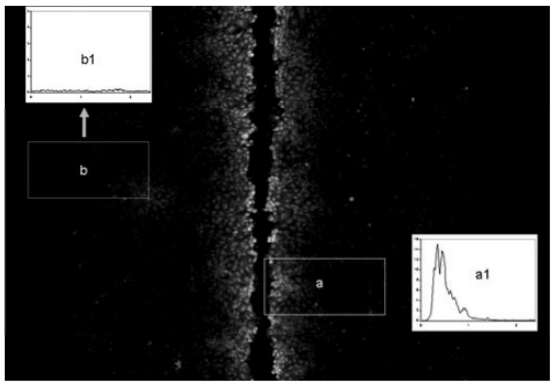


**Fig. 1** A sharp razor was used to scrape a cell monolayer in the presence of 0.25% Lucifer Yellow (LY). The injured cells at the edge of the scrape absorbed (strong fluorescence) and transmitted LY to neighbouring cells via gap junctions. For quantitative evaluation of gap junction coupling in bovine 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

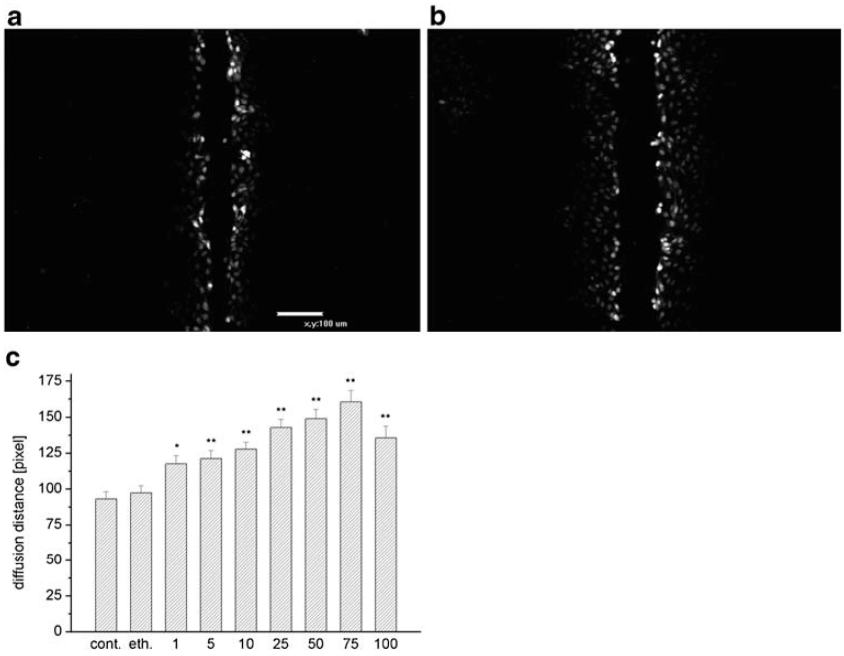


**Fig. 2** Dipyridamole affects gap junction coupling of bovine aortic GM-7373 endothelial cells. Cells were cultivated until they formed a monolayer. Dipyridamole was added 24 h before scrape loading. The fluorescent micrographs show the diffusion of LY in cells cultivated in the presence of 0.5% ethanol (**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

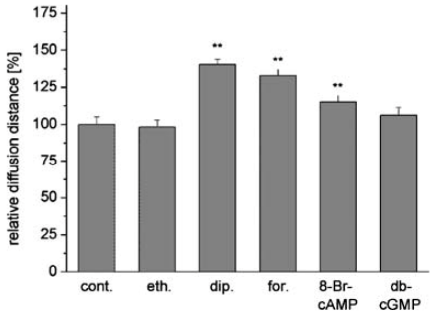


**Fig. 3** Similar to dipyridamole (50 μM), forskolin (for., 100 μM) or 8-Br-cAMP (400 μM) significantly increased the gap junction coupling ($P<0.01$; Student´s t-test). The cGMP analogue db-cGMP (400 μM) did not affect the gap junction coupling. The results are given as average ± SEM for six experiments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

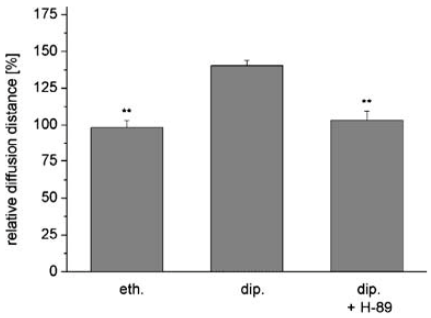


**Fig. 4** The inhibitor of PKA H-89 (15 μM) could suppress the dipyridamole (50 μM) induced increase of gap junction coupling. The results are given as average ± SEM for six experiments for each treatment ($P<0.01$; Student´s t-test)

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.

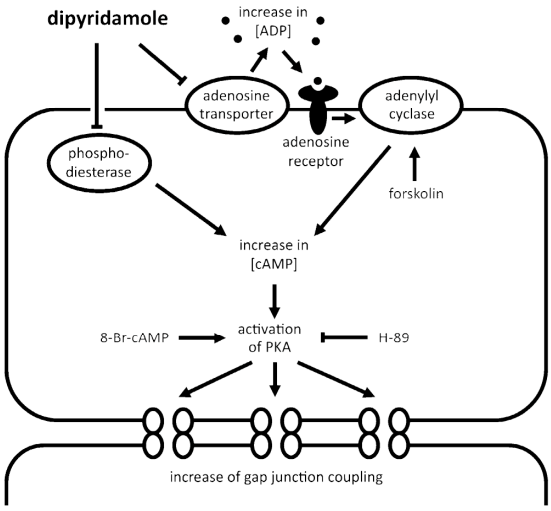


**Fig. 5** A model summarising how dipyridamole increases gap junction coupling in endothelial cells. One effect of dipyridamole is the inhibition of the adenosine transporters. This inhibition results in a local increase of adenosine concentration in the interstitial space. This in turn leads to increased 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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# Anhang B

Biphasic increase of gap junction coupling induced by dipyridamole in the rat aortic A-10 vascular smooth muscle cell line (Begandt et al. 2013a)



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RESEARCH ARTICLE

# 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 μM and 100 μM 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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**Keywords** cAMP · Dipyridamole · Gap junction · PKA · Vascular smooth muscle cells

**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 monophosphorothioate 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% $CO_2$. 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 $2 \times 10^5$ 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 $NaHCO_3$, 5.5 mM glucose, 0.8 mM $MgCl_2$, 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 $MgCl_2$, 5 Glucose, 10 Hepes and 0.25% LY (pH: 7.4, 295 mOmsmol/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 $CaCl_2$ 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 $Na_2HPO_4$, and 1.14 $KH_2PO_4$ (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

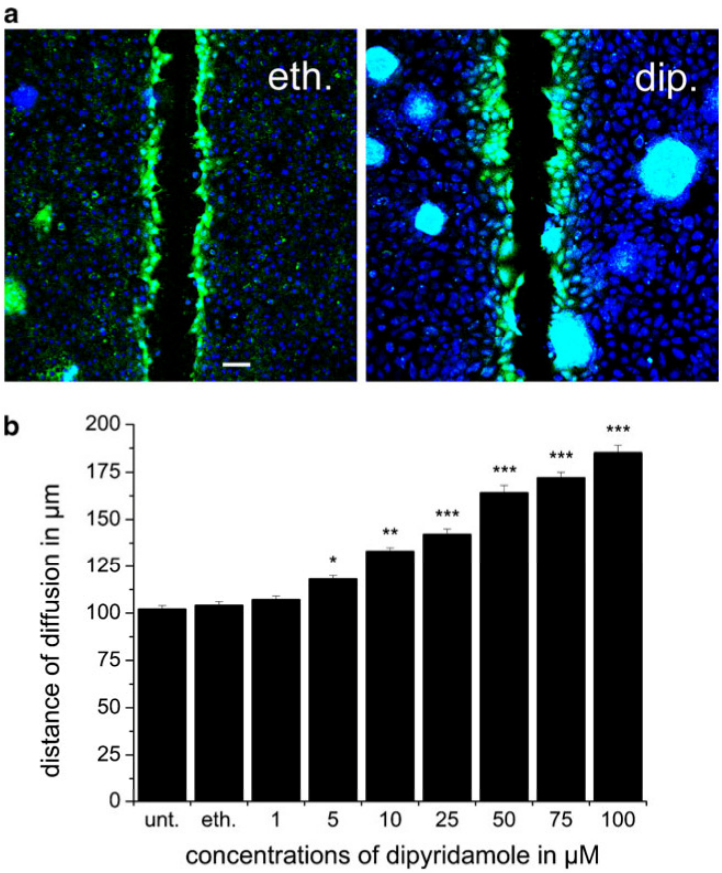


**Fig. 1** Dipyridamole affects gap junction coupling of rat aortic A-10 smooth muscle cells. Cells grown to a monolayer were treated with dipyridamole (dip.) for 24 h before SL/DT experiments. **a** The fluorescence micrographs show the diffusion of LY (*green*) in cells cultured under control conditions (eth.) and incubated with 50 µM dip. The cellular nuclei were stained with DAPI (*blue*). The scale bar corresponds to 100 µm. **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 µM

diffusion distance (Fig. 2a and b). Furthermore, the application of the adenylyl cyclase activator forskolin (100 µM) 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 µM), whereas a low dose of dipyridamole (5 µM and 10 µM) 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 µM 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

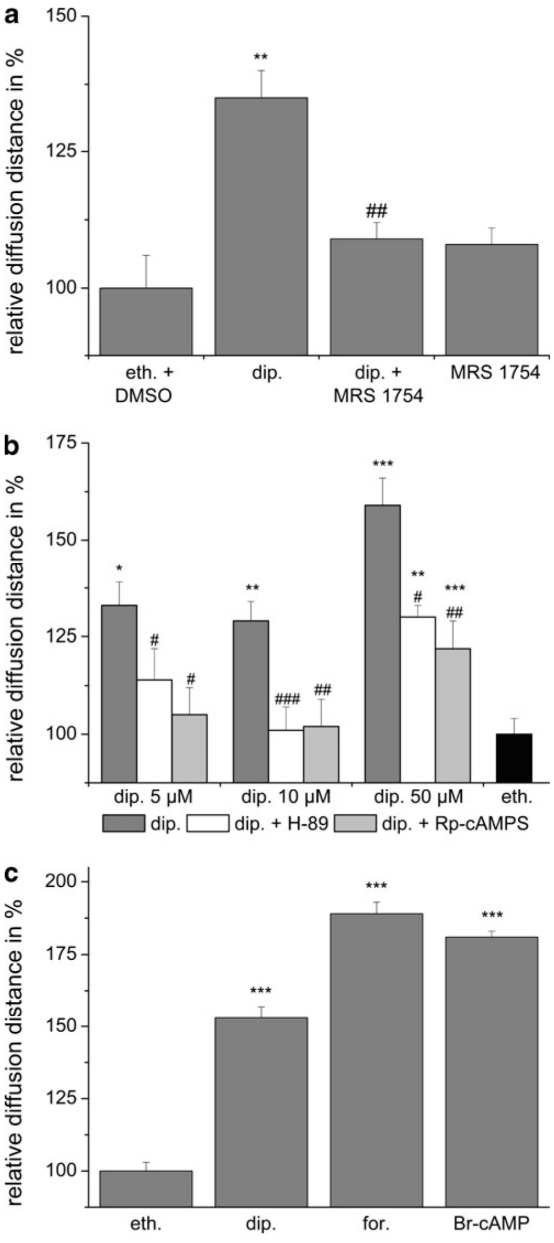


**Fig. 2** **a** The antagonist of the adenosine receptor $A_{2B}$ MRS 1754 as well as (**b**) the PKA inhibitors Rp-cAMPS (200 µM) and H-89 (15 µM) significantly (# for $p<0.05$) inhibited the dipyridamole- (dip., 50 µM) induced enhancement of gap junction coupling. **c** In contrast, forskolin (for.,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

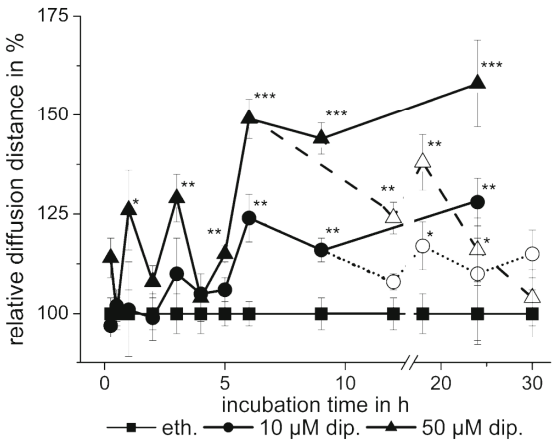


**Fig. 3** Time-dependent effect of dipyridamole on gap junction coupling. The figure shows the change of the diffusion distance induced by 10 µM (●) or 50 µM (▲) dipyridamole (7) after an application time of 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 9 h or 24 h compared with control cells (0.5% ethanol (■)). 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 µM) and high concentrations (50 µM) 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 µM and *dotted line* for 10 µM)

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 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 µM 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 µM) (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 μM 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 ± 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 μM dip. | relative diffusion distance in % ± SEM | |
|---|---|---|
| 0.25 | 113.6 ± 5.1 | |
| 0.5 | 99.7 ± 3.5 | # |
| 1 | 126.4 ± 9.5 | # |
| 2 | 108.2 ± 3.7 | # |
| 3 | 128.9 ± 6.4 | # |
| 4 | 104.2 ± 5.9 | |
| 5 | 114.9 ± 2.3 | # |
| 6 | 148.9 ± 5.2 | |
| 9 | 144.4 ± 4.1 | |
| 24 | 158.1 ± 10.9 | |

24 h, indicating that the effect of dipyridamole on gap junction coupling was reversible. The diffusion distance of cells treated with 50 μM 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 μM 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 μM), 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 μM) 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 μM 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 μM or 50 μM dipyridamole (dip.) for indicated durations relative to ethanol-treated cells. The cells were incubated with dipyridamole for the indicated duration times. Thereafter, dipyridamole 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 dipyridamole application. The results are given as average ± 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 % ± SEM | |
|---|---|---|
| | 10 μM dip. | 50 μM dip. |
| 0.08 | 109.2±2.7 | 111.8±1.3 |
| 0.25 | 113.9±7.9 | 131.1±8.4* |
| 0.5 | 104.0±6.4 | 109.8±4.7 |
| 1 | 108.6±4.8 | 126.6±9.0* |
| 2 | 105.9±3.1 | 116.1±3.2* |
| 3 | 112.1±3.3 | 118.9±3.8* |
| 4 | 111.1±2.9* | 118.9±1.7* |
| 5 | 105.5±3.0 | 121.8±4.5* |
| 6 | 127.2±6.4* | 152.1±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

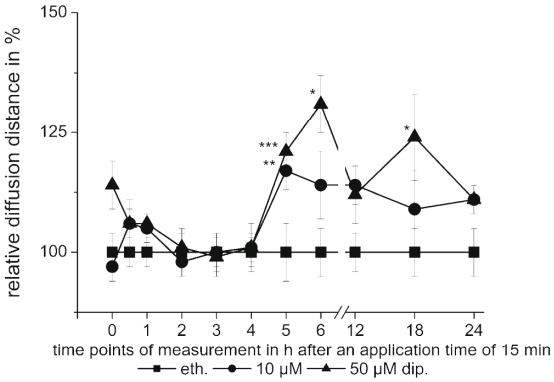


**Fig. 4** A short period of dipyridamole application for 15 min induced a long-term effect on gap junction coupling that lasted for 18 h. Cells were incubated for 15 min with 10 μM (●) or 50 μM (▲) dipyridamole and were compared with control cells (■). After drug washout, the gap junction coupling was 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 dipyridamole 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 dipyridamole-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 dipyridamole acts by many steps before activating adenylyl cyclase to produce cAMP. Taken together, these results support the hypothesis that the observed dipyridamole-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 dipyridamole. 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 dipyridamole 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 dipyridamole 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 dipyridamole, 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 dipyridamole (50 μM), 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). Dipyridamole 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 dipyridamole 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 dipyridamole 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 dipyridamole 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 dipyridamole concentrations (≤ 10 μM) 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 dipyridamole-induced inhibition of equilibrative nucleoside transporters (ENT), which are characterised by $k_i$ values in a range of 0.05–10 μM 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 dipyridamole with a $k_i$ value of 5–10 μM (Visser et al. 2002).

The long-term effect of dipyridamole treatment showed a constant increase of gap junction coupling for high and low dipyridamole 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 dipyridamole. 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 dipyridamole 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 dipyridamole-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 dipyridamole 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 dipyridamole in blood plasma is about 4 μM (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 dipyridamole-induced mechanisms is not clear. Finally, an increase in gap junction coupling in vascular smooth muscle cells together with the previously reported dipyridamole-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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# Anhang C

Dipyridamole-related enhancement of gap junction coupling in the GM-7373 aortic endothelial cells correlates with an increase in the amount of connexin 43 mRNA and protein as well as gap junction plaques (Begandt et al. 2013b)



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# Dipyridamole-related enhancement of gap junction coupling in the GM-7373 aortic endothelial cells correlates with an increase in the amount of connexin 43 mRNA and protein as well as gap junction plaques

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**Abstract** Previous data showed that dipyridamole enhanced gap junction coupling in vascular endothelial and smooth muscle cell lines by a cAMP-dependent mechanism. The present study investigates the level at which dipyridamole affects gap junction coupling. In the GM-7373 endothelial cell line, scrape loading/dye transfer experiments revealed a rapid increase in gap junction coupling induced during the first 6 h of dipyridamole treatment, followed by a slow increase induced by further incubation. Immunostaining analyses showed that the rapid enhancement of gap junction coupling correlated with an increased amount of Cx43 gap junction plaques and a reduced amount of Cx43 containing vesicles, while the amount of Cx43 mRNA or protein was not changed during this period, as found by semiquantitative RT-PCR and Western blot. Additionally, brefeldin A did not block this short-term-induced enhancement of gap junction coupling. Along with the dipyridamole-induced long-term enhancement of gap junction coupling, the amount of Cx43 mRNA and protein additionally to the amount of Cx43 gap junction plaques were increased. Furthermore, the anti-Cx43 antibody detected only two bands at 42 kDa and 44 kDa in control cells and cells treated with dipyridamole for 6 h, while long-term dipyridamole-treated cells showed a third band at 46 kDa. We propose that a dipyridamole-induced cAMP synthesis increased gap junction coupling in the GM-7373 endothelial cell line at different levels: the short-term effect is related to already oligomerised connexins beyond the Golgi apparatus and the long-term effect involves new expression and synthesis as well as posttranslational modification of Cx43.

**Keywords** Dipyridamole · Gap junction · cAMP · Endothelial cells · Connexin 43

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## Introduction

Gap junctions allow the direct exchange of ions or molecules, such as second messengers and metabolites (< 1 kDa). Gap junction channels connect the cytoplasm of adjacent cells, where each cell contributes one hemichannel, called a connexon, to the functional gap junction channel. A connexon is formed by connexin hexamerisation (Bruzzone et al. 1996; Kumar and Gilula 1996), which for most connexins takes place in the endoplasmatic reticulum and the trans-Golgi network (Berthoud et al. 2003; Maza et al. 2005). In the vascular system, connexin 37 (Cx37), Cx40, Cx43 and Cx45 are expressed and form gap junction channels which play a key role in longitudinal/transversal signal transduction and thereby regulate, amongst other things, the vascular tone (Figueroa et al. 2004; Johnstone et al. 2009). Pathophysiological conditions such as hypertension, ischemia or arteriosclerosis are associated with altered connexin expression in either endothelial or smooth muscle cells, as confirmed in connexin knock-out mouse experiments (Chadjichristos and Kwak 2007;

Haefliger et al. 1999; Haefliger and Meda 2000). Therefore, gap junctions were proposed as target for the therapeutic treatment of vascular impairments (Green and Nicholson 2008).

Regulation of gap junction-dependent communication could involve controlling the number of gap junction channels in the cell membrane, the regulation of their open probability or their permeability. The number of gap junction channels in the membrane can be modulated at the level of mRNA expression and protein synthesis, oligomerisation and trafficking of connexons, connexon insertion into the plasma membrane, gap junction formation by docking of connexons in the membranes of adjacent cells, and the degradation rate of channels (Beardslee et al. 1998; Saffitz et al. 2000). Different mechanisms, such as phosphorylation, were shown to modulate not only the open probability and permeability of gap junctions (Cruciani and Mikalsen 2002) but also the insertion of connexons into the membrane and gap junction plaque formation (Solan and Lampe 2007, 2009). The effect of phosphorylation is complex and appears to be related to the activated kinases. While protein kinase C-dependent phosphorylation reduced gap junction coupling, a protein kinase A (PKA)-dependent phosphorylation frequently potentiated gap junction coupling. This was found particularly for gap junctions composed of Cx43 and Cx40 (Cruciani and Mikalsen 2002; Harris 2001; Olk et al. 2009; van Rijen et al. 2000). Many factors, including increasing levels of intracellular cAMP, lead to the regulation of gap junction coupling (Civitelli et al. 1998; Murray and Shah 1998).

The antithrombotic drug dipyridamole increases intracellular cAMP concentrations via the stimulation of adenosine receptors as a result of the inhibition of nucleoside transporters (Eisert 2006; Molina-Arcas et al. 2009; Podgorska et al. 2005). The European Stroke Prevention Study 2 (ESPS2) and the European/Australasian Stroke Prevention in Reversible Ischemia Trial (ESPRIT) have shown that a combined therapy of dipyridamole and aspirin is twice as effective in reducing the risk of a secondary stroke compared to aspirin alone (Diener et al. 1996; ESPRIT Study Group et al. 2006). Because dipyridamole was shown to inhibit the aggregation of thrombocytes, the possible effects of dipyridamole on other cells, such as the vascular cells, were neglected, whereas Guo et al. (Guo et al. 2010) proposed to consider dipyridamole as a drug for treatment of pathophysiological conditions in vascular cells. Recently, we showed that therapeutically relevant concentrations of dipyridamole increased gap junction coupling in vascular endothelial and smooth muscle cell lines most likely due to the stimulation of cAMP synthesis in these cells (Begandt et al. 2010, 2013). The goal of the present study was to investigate which level of gap junction formation is affected by dipyridamole in an endothelial cell line.

## Methods

### Chemicals

Dipyridamole was kindly provided by Boehringer Ingelheim International GmbH (Ingelheim, Germany). The drug as well as brefeldin A (BFA, Sigma-Aldrich, Taufkirchen, Germany) were dissolved in ethanol and diluted to the desired concentration in cell culture medium, with a final ethanol concentration of 0.1 %. Untreated cells and cells treated with 0.1 % ethanol were used as controls.

### Cell culture

Bovine GM-7373 aortic endothelial cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany) were cultivated using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foetal calf serum (FCS), penicillin and streptomycin (100 U/ml and 0.1 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. Dipyridamole or other agents were added at the indicated time points prior to experimental investigation.

### Scrape loading/dye transfer

For scrape loading/dye transfer (SL/DT) experiments, cover slips (Ø 10 mm) were placed into the wells of a 24 multiwell plate containing 1 ml of DMEM culture medium. The cells were seeded at a density of $4\times10^5$ cells/well. Additional cultivation for 24 h allowed the cells to adhere and form a monolayer on the cover slip. Analyses of functional gap junction coupling were performed using the SL/DT technique as previously described (Begandt et al. 2010).

### Quantification of SL/DT experiments

The SL/DT experiments were documented using a confocal laser scanning microscope with a 10× objective (Nikon, Düsseldorf, Germany) using the software program EZ-C1 3.50 (Nikon). A view area of 1,024×1,024 pixels was recorded. Estimation of the dye transfer was performed as described previously (Begandt et al. 2010) with the software ImageJ (http://rsweb.nih.gov/ij/docs/menus/analyze.html#plot). For each treatment, at least four experiments were performed.

### Immunofluorescence

For immunostaining, the cells were seeded at a density of $6\times10^4$ cells/well on Ø 10 mm cover slips and cultivated for 24–48 h reaching a density of 70–80 %. The cells were washed with PBS, fixed with 4 % formaldehyde and blocked

with 0.5 % bovine serum albumin (BSA) dissolved in PBS. The primary antibody rabbit anti-connexin 43 (Sigma-Aldrich) was diluted 1:4,000 in 0.5 % Triton X-100 dissolved in PBS and incubated overnight at 4 °C. After washing, the cells were incubated for 1 h at room temperature in TRITC-labelled secondary antibody goat anti-rabbit IgG (1:600, Sigma-Aldrich). Simultaneous with the secondary antibody incubation, a DAPI staining (2 μM, Calbiochem Millipore, Darmstadt, Germany) was performed. Actin filaments were stained using phalloidin-Alexa Fluor 488® (132 nM; Invitrogen Life Technologies, Darmstadt, Germany). As a negative control, secondary antibody only (with no prior primary antibody) was used. After a final washing step with PBS, images were taken with an inverted Nikon Eclipse TE2000-E confocal laser scanning microscope with a 60× objective (Nikon), with excitation lasers of 408 nm for DAPI, 488 nm for Alexa Fluor and 543 nm for TRITC. The software program EZ-C1 3.50 (Nikon) was used to record the images. For each treatment, at least three experiments were performed.

For quantification of the immunofluorescence results, we analyzed the localization of Cx43 signals using the software ImageJ (http://rsbweb.nih.gov/ij/). 16 bit-images were colour-split, and for downstream analyses, only the red channel was used. The image was changed to an 8 bit-image and a threshold was applied. Cell-free regions were used to define the background, which was set at a grey value of 39. Additionally, we used the staining of the actin filaments with phalloidin to distinguish the cell body and cell borders. We defined structures with a grey value above 70 as highly fluorescent structures, as observed in the cell membrane and in the region between the ER/Golgi apparatus and the cell membrane. This allowed us to quantify the immunostaining experiments by counting the number of cells that contained highly fluorescent vesicles in the cell body and gap junction plaques in membrane regions of adjacent cells both in ethanol- and dipyridamole-treated cells.

## Semiquantitative reverse transcription-PCR (sqRT-PCR)

Cells were cultivated for 24 h in Petri dishes (Ø 6 cm) and allowed to grow to 80–90 % confluence. They were then treated with ethanol or dipyridamole for 6 h or 24 h. The cells were washed twice with PBS containing 1 g/l EDTA before isolation of total RNA using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Potential genomic DNA contamination was removed through RNase-free DNase I digestion (Fermentas, St. Leon-Rot, Germany). Total RNA (500 ng) was reverse transcribed into cDNA in 20 μl of reaction mixture (Fermentas) containing 0.2 μg random hexamer primer, 0.5 mM of each dNTP, 1× reaction buffer, 20 U RNase Inhibitor and 40 U of M-MuLV Reverse Transcriptase. Random hexamer primer and DNA-free RNA were pre-incubated at 65 °C for 5 min before adding the rest of the reaction mix. After an incubation time of 5 min at 25 °C and 60 min at 37 °C, the reaction was stopped at 70 °C for 5 min. After the first-strand cDNA was prepared, 62.5 ng cDNA was used as template for a PCR reaction with a final volume of 25 μl containing 0.2 μM of each primer, 200 μM of each dNTP, 1× GoTaq green reaction buffer and 1.25 U GoTaq DNA polymerase (Promega, Mannheim, Germany). The amplification was carried out using the following temperature profile: 95 °C for 3 min followed by cycles of 95 °C for 15 s, 59 °C for 30 s and 72 °C for 45 s with a final step at 72 °C for 5 min. A qualitative expression analysis of the vascular connexins Cx37, Cx40, Cx43 and Cx45 within the GM-7373 endothelial cells was carried out using the primer pairs for the *connexins* and the g*lyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) given in Table 1. For a semiquantitative estimation of *connexin* expression, *gapdh* was used as reference. The number of cycles was determined using a titration curve corresponding to the range in which an exponential increase in products could be detected according to Trosko et al. (Trosko et al. 2000). PCR products were separated in 2 % agarose gel stained with GelRed (Biotrend, Cologne, Germany).

## Western blot

For Western blotting, the cells were cultivated as described for sqRT-PCR experiments. Total protein was extracted using RIPA buffer composed of 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 % IGEPAL, 1 % SDC, 0.1 % SDS,

**Table 1** List of the primers used for PCR analysis

| Target | Primer sequence 5′-3′ | | Accession no. | Amplicon in bp |
|---|---|---|---|---|
| *gapdh* | for | TGTGCTGTGCCAGCCGCATC | NM_001034034 | 538 |
| | rev | CACGATGCCAAAGTGGTCATGGAT | | |
| cx37 | for | CTCGACCGTGGTGGGCAAGAT | NM_001083738 | 475 |
| | rev | ACGCCACTGGCCGTACAGGAA | | |
| cx40 | for | ACCTGGTTGCGAGAACGTCTGCTA | NM_001078022 | 418 |
| | rev | ATAGCAGTTGACGGGGTGGGG | | |
| cx43 | for | GAGAAAGAGCAGTACCCAGGCAAC | NM_174068 | 488 |
| | rev | GTTCTCAGCAAGCCCCCTCGC | | |
| cx45 | for | AAGAAGGCCGCTCGCAGCAA | NM_001046076 | 545 |
| | rev | TAGCCAGGGGGAGCAGATGGC | | |

supplemented with 1 mM PMSF, 1 mM $Na_3VO_4$, 10 mM NaF and 0.5 % protease inhibitor cocktail (Sigma-Aldrich). Cells were washed twice with ice cold PBS, scraped from the Petri dish in the presence of 1 ml PBS and centrifuged at 750×g for 3 min. The cell pellet was dissolved in 15 μL RIPA buffer, incubated for 15 min on ice and centrifuged for 15 min at 14,000×g. Concentration of the protein supernatant was determined using a Bradford Assay (Thermo Scientific Pierce Protein Biology Products, Rockford, IL, USA). Total protein was mixed with Laemmli buffer composed of 65 mM Tris–HCl, 10 % glycerin, 2 % SDS, 0.01 % bromophenol blue, (pH 6.8), heated at 70 °C for 10 min and 40 μg protein per slot was loaded onto a 5 % stacking and 12 % running SDS-polyacrylamide gel. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto a nitrocellulose membrane (0.2 μm; Roth, Karlsruhe, Germany) by a semi-dry blot technique, blocked with 5 % non-fat dry milk diluted in PBS with 0.1 % Tween-20 (PBST). Washing steps were performed with PBST. Primary antibodies (rabbit anti-Cx43 and mouse anti-β-Tubulin, Sigma Aldrich) were diluted 1:20,000 in PBST and applied to the nitrocellulose membrane overnight. Goat anti-rabbit-peroxidase and rabbit anti-mouse-peroxidase secondary antibodies (1:100,000 in PBST, Sigma Aldrich) were applied for 1 h each. The detection was carried out with super signal west pico chemiluminescent substrate (Thermo Scientific Pierce Protein Biology Products) and recorded with a 16 bit Kodak CCD Sensor camera (KAF 3200ME) system (Intas, Göttingen, Germany) and 1×1 binning.

To analyse whether or not Cx43 was phosporylated, protein isolated as described above was treated with calf intestinal alkaline phosphatase (CIAP; Promega) following the protocol given by the manufacturer. After treatment, the protein solution was mixed with Laemmli buffer. SDS-PAGE and western blotting were performed as described above.

For estimation of the distribution of Cx43 in the cell membrane versus in the membranes of intracellular compartments, proteins were separated into triton-soluble and -insoluble protein fractions according to Musil and Goodenough (Musil and Goodenough 1991) and Hoffmann et al. (Hoffmann et al. 2003). Cells treated with 0.1 % ethanol or 50 μM dipyridamole for 24 h were harvested as described above. After centrifugation the cell pellet was resuspended in 30 μl lysis buffer (20 mM $KH_2PO_4$, pH 7.0 and 1 mM EDTA supplemented with 1 mM PMSF, 1 mM $Na_3VO_4$, 10 mM NaF and 0.5 % protease inhibitor cocktail (Sigma-Aldrich)). Benzonase (Novagen Millipore, Darmstadt, Germany) and $MgCl_2$ were added at a final concentration of 25 U and 2.5 mM, respectively. The lysates were incubated for 1 h at 4 °C. Thereafter, Triton X-100 was added to a final concentration of 1 % and the lysates were incubated for additional 30 min at 4 °C. From each lysate one third was removed and saved (whole cell lysate), the remaining two thirds were centrifuged at 20,000×g for 1 h at 4 °C. The supernatant (triton-soluble fraction) was transferred into a new tube and the pellet (triton-insoluble fraction) was resuspended in lysis buffer. Laemmli buffer was added to each fraction. SDS-PAGE and western blotting were then performed as described above.

### Quantification of sqRT-PCR and western blot

To quantify the results from Western blot and sqRT-PCR techniques, the software ImageJ was used (http://rsbweb.nih.gov/ij/). The rolling ball radius was used for background correction of the images. The intensity of bands was estimated using the gel analysis tool. To compare the amount of connexin mRNA in dipyridamole- and ethanol-treated, as well as in untreated aortic cells, the ratio of *connexin* amplicons to the *gapdh* amplicons was built. For Western blots, the ratio of Cx43 protein to β-Tubulin protein was calculated. For each treatment at least three experiments (sqRT-PCR as well as Western blotting) were performed and results are given as average, the error bars represent the SEM.

### Statistical analysis

For statistical analyses, the two-sample Student's *t*-test was used. The significance is given as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$.

## Results

Previously, SL/DT experiments have shown that a 24 h treatment with dipyridamole increased gap junction coupling in the bovine aortic GM-7373 endothelial cell line. A cAMP-dependent signalling cascade was proposed to explain the effect of dipyridamole on gap junction coupling in this cell line (Begandt et al. 2010). The aim of the present study was to evaluate at which level of gap junction formation dipyridamole might affect gap junction coupling of the endothelial cell line.

First, we analysed the time dependence of the dipyridamole-induced effect on gap junction coupling. SL/DT technique revealed an averaged LY diffusion of 134 μm in cells cultivated under control conditions and a dye diffusion distance of 135 μm in cells cultivated in the presence of 0.1 % ethanol.

Treatment of the cells with 25 μM dipyridamole for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 9 h, enhanced the gap junction coupling up to 101 %, 109 %, 97 %, 110 %, 116 %, 123 %, 116 %, 121 %, and 129 % respectively, compared to 0.1 % ethanol-treated cells. Stimulation with 50 μM dipyridamole correlated with an increase in gap junction coupling up to 103 %, 103 %, 112 %, 117 %, 118 %, 129 %, 118 %, 120 %, and 123 % for the time points indicated above (Fig. 1). By an application time of 24 h, an increase in gap junction coupling up to 147 % and 154 % was found for

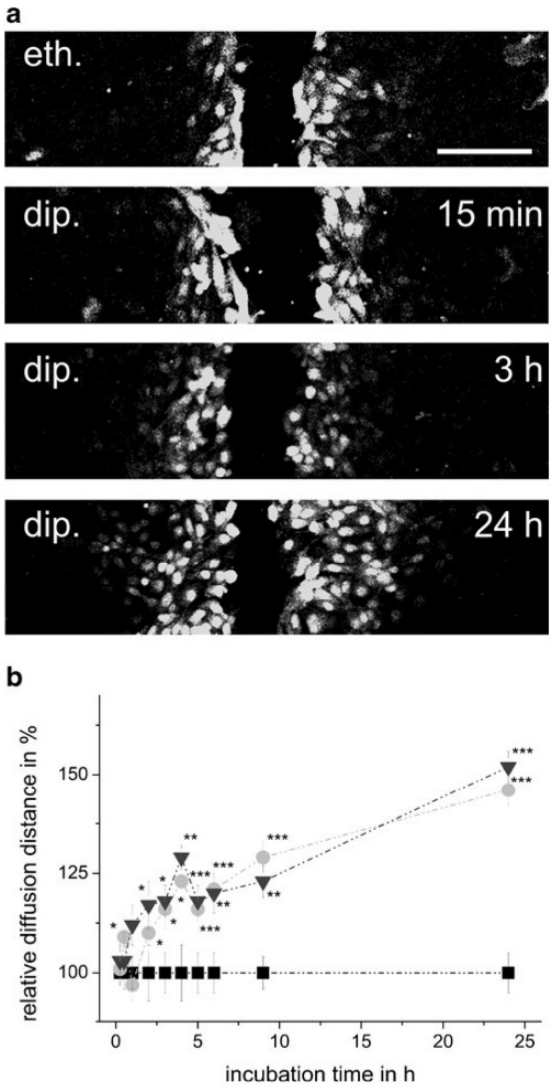


**Fig. 1** Dipyridamole increased gap junction coupling in GM-7373 endothelial cell line in a time-dependent manner. **a** Exemplary micrographs of cells after SL/DT. Dipyridamole (dip., 50 μM) was applied for the indicated time spans, cells treated with ethanol (eth., 0.1 %) served as control. *Scale bar* represents 100 μm. **b** Dipyridamole was applied at concentrations of 25 μM (symbol: grey filled circle) and 50 μM (symbol: black down pointing triangle) for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 9 h and 24 h. SL/DT technique revealed a rapid increase of gap junction coupling within the first 6 h of drug application. This continuous increase was already significantly different from gap junction coupling observed in 0.1 % ethanol-treated cells (symbol: black filled square) after 2 h. A further presence of the drug for the following 18 h induced a moderate increase of the gap junction coupling

25 μM and 50 μM dipyridamole (Fig. 1, (Begandt et al. 2010)). Additionally, we found that dipyridamole enhanced gap junction coupling in other vascular cells such as the human blood–brain barrier cell line hCMEC/D3 (Weksler et al. 2005; Bintig et al. 2011) or the HUVEC cells (results not shown) as well as in the rat aortic smooth muscle cell line A-10 (Begandt et al. 2013).

In agreement with our previous findings for 24 h (Begandt et al. 2010), the adenylyl cyclase activator forskolin, or the membrane permeable cAMP analogue 8-Bromo-cAMP, increased gap junction coupling in the same range as dipyridamole after an application time of 6 h (result not shown), indicating that dipyridamole might increase gap junction coupling in the GM-7373 endothelial cell line by stimulating cAMP-dependent signalling.

Gap junction coupling can be affected at different levels of its regulation. The amount of connexin mRNA, or of connexin protein, can be changed. The rate of connexin oligomerisation, as well as the rate of connexon assembly within the membrane, can also be varied. Posttranslational modification of the connexins could occur which would influence the assembly process or the permeability of the gap junction channels (Solan and Lampe 2007).

PCR experiments revealed that GM-7373 cells expressed Cx37, Cx40, Cx43 and Cx45 (Fig. 2a). However, dipyridamole differentially affected the expression of these connexins as revealed by sqRT-PCR experiments. The expression of Cx37 and Cx45 was not affected by dipyridamole (results not shown). The amount of Cx40 mRNA was maximally increased after incubation with 10 μM dipyridamole compared to ethanol-treated cells and stayed significantly increased at a concentration of 25 μM. A further elevation of the dipyridamole concentration up to 50 μM correlated with a decrease of the Cx40 mRNA amount to the level found in untreated cells (Fig. 2b and c). Only the amount of Cx43 mRNA was elevated with increasing dipyridamole concentrations, in the same manner as the concentration-dependent increase of the gap junction coupling (Fig. 3, (Begandt et al. 2010)). Therefore the regulation of Cx43 by dipyridamole is the main focus of this report.

At protein level Cx43 could be detected by an antibody raised against bovine Cx43. Western blotting experiments were undertaken to analyse whether the dipyridamole-induced increase in gap junction coupling was related to an increased amount of Cx43 protein and/or to a posttranslational modification of Cx43. A possible dipyridamole-induced effect on connexin localisation was investigated using immunofluorescence experiments.

At the mRNA and protein level, sqRT-PCR experiments using *gapdh* as a housekeeping gene and Western blotting experiments using β-tubulin as a reference were performed. We found that dipyridamole did not change the Cx43 mRNA and protein content of the cells when applied for 6 h (Fig. 3a and c; Fig. 4a and c), indicating that the observed dipyridamole-induced increase in gap junction coupling during the first 6 h was not related to new mRNA or protein

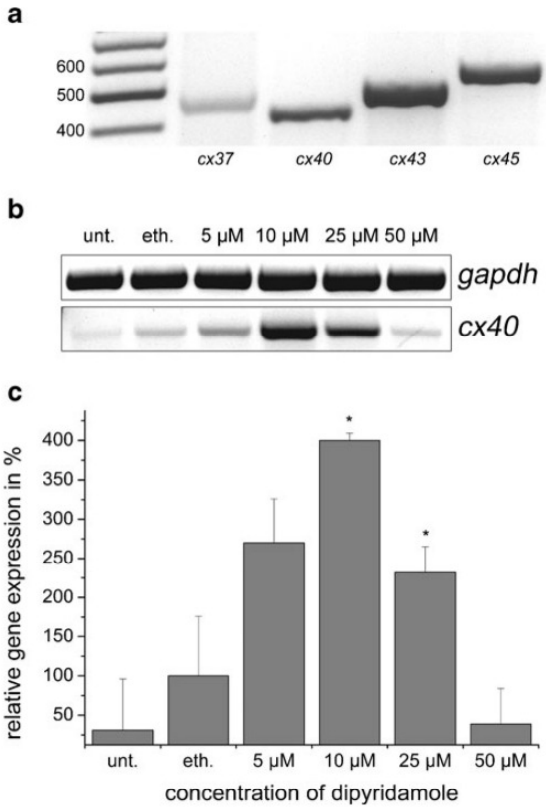


**Fig. 2** Analysis of connexin expression in GM-7373 endothelial cells by PCR. **a** The isoforms Cx37, Cx40, Cx43 and Cx45 were found. **b** Representative semiquantitative RT-PCR agarose gel with *connexin40* and housekeeping gene *gapdh* amplicons. **c** Quantification of (**b**) showing the increase in Cx40 mRNA amount after the application of different dipyridamole concentrations for 24 h. Results are given as average, the *error bars* represent the SEM for three different experiments

synthesis. This is also supported by the observation that BFA could not antagonize the short-term effect of dipyridamole on gap junction coupling: co-treatment of GM-7373 endothelial cells with 5 μg/ml BFA and 50 μM dipyridamole enhanced gap junction coupling of cells up to 117 % compared to BFA alone (Fig. 5a). Notably, the degree of enhancement of gap junction coupling measured after 6 h incubation with dipyridamole in the presence of BFA (Fig. 5a), or by dipyridamole alone (Fig. 1), was comparable.

After an application time of 24 h, sqRT-PCR and Western blotting experiments showed an increased amount of both Cx43 mRNA and Cx43 protein (Fig. 3b and d, Fig. 4b and d). When compared to ethanol-treated cells, dipyridamole induced an increase of Cx43 mRNA by up to 114 %, 134 %, 160 % and 192 % for 5, 10, 25 and 50 μM dipyridamole, respectively (Fig. 3b and d). The Cx43 protein content was increased up to 103 %, 135 %, 196 %, and 432 %, by the respective dipyridamole concentrations (Fig. 4b and d). In addition to the elevation in Cx43 mRNA and protein content, the long-term treatment (24 h) with dipyridamole led to a shift in Cx43 migration in SDS-PAGE. For untreated or ethanol-treated cells, the anti-Cx43 antibody detected two bands at approximately 42 kDa and 44 kDa (Fig. 5b). For cells treated for 24 h with 50 μM dipyridamole, a third band at approximately 46 kDa was detected (Fig. 5b). It is worthy to note that treatment of the proteins with the calf intestinal alkaline phosphatase (CIAP) before separation on SDS-PAGE eliminated the bands detected at approximately 44 kDa and 46 kDa of proteins from cells treated with 50 μM dipyridamole for 24 h and yielded one single band at approximately 42 kDa (Fig. 5b). Musil and Goodenough (Musil and Goodenough 1991) proposed to consider the 42 kDa band as a non-phosphorylated Cx43 (Cx43-$P_0$), and the 44 kDa and the 46 kDa bands as phosphorylated

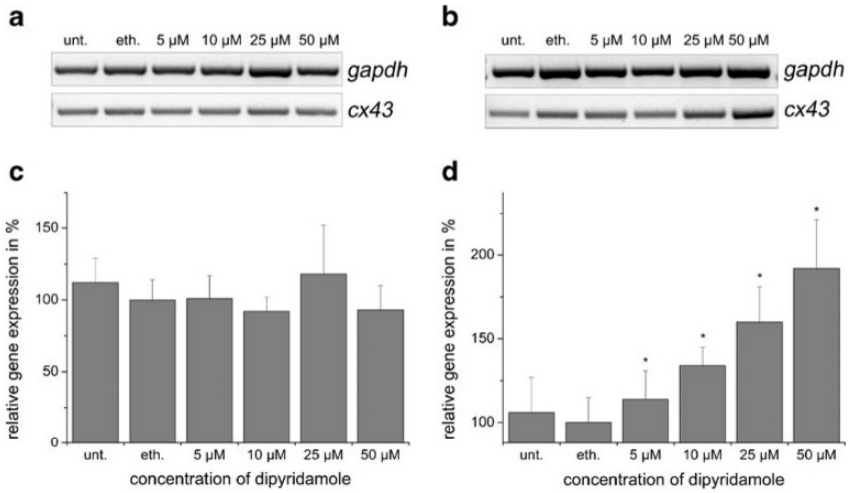


**Fig. 3** Effect of dipyridamole on the amount of Cx43 mRNA. **a** and **b** Original semiquantitative RT-PCR (sqRT-PCR) agarose gel with *connexin43* and housekeeping gene *gapdh* after 6 h (**a**) and 24 h (**b**) dipyridamole application. **c** and **d** Quantification of the sqRT-PCR results. **c** A dipyridamole application time of 6 h did not change the amount of Cx43 mRNA. **d** Applied for 24 h, dipyridamole induced a significant increase in the amount of cellular Cx43 mRNA

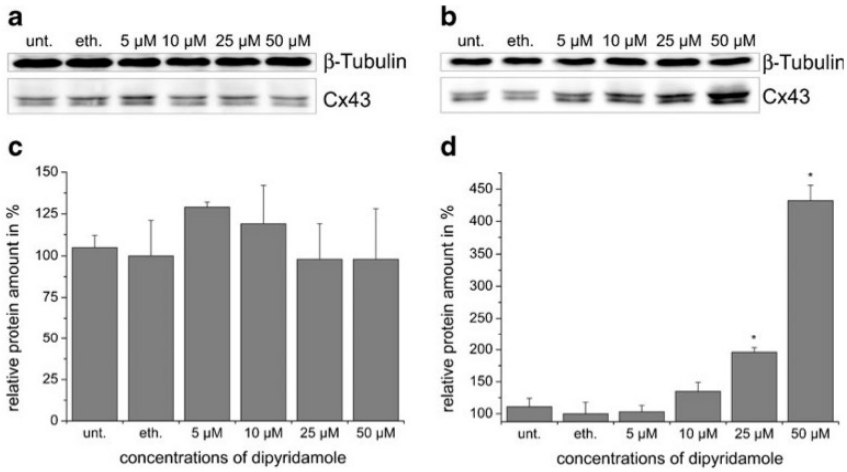


**Fig. 4** Effect of dipyridamole on the amount of Cx43 protein. **a** and **b** Original Western blot with Cx43 and control protein β-Tubulin after a dipyridamole application time of 6 h (**a**) and 24 h (**b**). **c** and **d** Quantification of the Western blot. Applied for 6 h (**c**), dipyridamole did not change the amount of Cx43 protein. Applied for 24 h (**d**), dipyridamole induced an increase in the amount of Cx43 protein

Cx43 (Cx43-$P_1$ and Cx43-$P_2$). Musil and Goodenough also showed that the Cx43-$P_2$ was localized in gap junction plaques and was therefore triton-insoluble, while the triton-soluble Cx43-$P_0$ was predominantly located in intracellular membranes. Using the protocol established by Musil and Goodenough (Musil and Goodenough 1991) and modified by Hoffmann et al. (2003), we separated the triton-soluble and triton-insoluble proteins from cells treated with ethanol or dipyridamole for 24 h. From the whole cell lysate of dipyridamole-treated cells, the 42 kDa, 44 kDa, and the 46 kDa bands were detected by Western blotting experiments. In the triton-soluble fraction only the 42 kDa band was found, while the 46 kDa band was only observed in the triton-insoluble fraction (Fig. 5c). The results suggest that the dipyridamole treatment pushed Cx43 in gap junction plaques. This suggestion is also in agreement with immunostaining experiments (Fig. 6). In cells treated with ethanol Cx43 was present in intracellular regions around the nucleus, corresponding to the ER and the Golgi apparatus. Moreover, a large number of vesicles stained for Cx43 were found in the region between the Golgi apparatus and cell membrane region. In cells treated with dipyridamole for 6 h or 24 h, the number of vesicles stained for Cx43 between the Golgi apparatus and membrane area diminished. Cells treated with dipyridamole showed increased Cx43 staining in the membrane regions where cell pairs were in close proximity (Fig. 6a), suggesting that dipyridamole reduced the number of intracellular vesicles containing Cx43 whilst increasing the size and number of gap junction plaques formed by Cx43. We quantified this behaviour by counting cells with vesicles containing Cx43 and cells with gap junction plaques formed by Cx43. For a 6 h treatment, Cx43-positive vesicles and gap junction plaques were found in 57 % and 41 % of ethanol-treated cells, respectively. In comparison, 30 % and 87 % of the cells treated with 50 µM dipyridamole showed vesicles and gap junction plaques stained for Cx43, respectively (Fig. 6b). A 24 h treatment did not change the situation in ethanol-treated cells. In dipyridamole-treated cells, Cx43-immunostained vesicles were found in 18 % of the cells and gap junction plaques positive for Cx43 immunostaining were found in 90 % of the cells (Fig. 6b).

In summary, the dipyridamole-related enhancement of gap junction coupling takes place in a biphasic manner. A short-term effect observed in the first 6 h was related to a dipyridamole effect only on connexons and gap junction channels beyond the Golgi apparatus. This effect resulted in an increase of Cx43 gap junction plaques. A long-term effect added an increase in the cellular content of Cx43 mRNA and Cx43 protein to the short-term effect, as well as a shift in the migration of Cx43 protein.

## Discussion

In this report, we observed a dipyridamole-related increase in gap junction coupling of the aortic GM-7373 endothelial cell line in a time-dependent manner (Fig. 1). Compared to control cells, the increase in gap junction coupling in cells treated with dipyridamole began within the first hour of drug application and achieved a significant increase of approximately 110 % after 2 h. After an application time of 6 h, gap junction coupling in dipyridamole-treated cells was increased by approximately 120 % relative to control cells (Fig. 1). This accelerated increase in gap junction coupling was followed by a slow increase, which achieved a maximum of approximately 150 % after a drug application time of 24 h (Fig. 1).

With respect to the increase observed after an application time of 24 h, which we designate the long-term effect of dipyridamole on gap junction coupling, the present results reproduce our previous observations using the aortic endothelial cell line (Begandt et al. 2010) and are comparable to

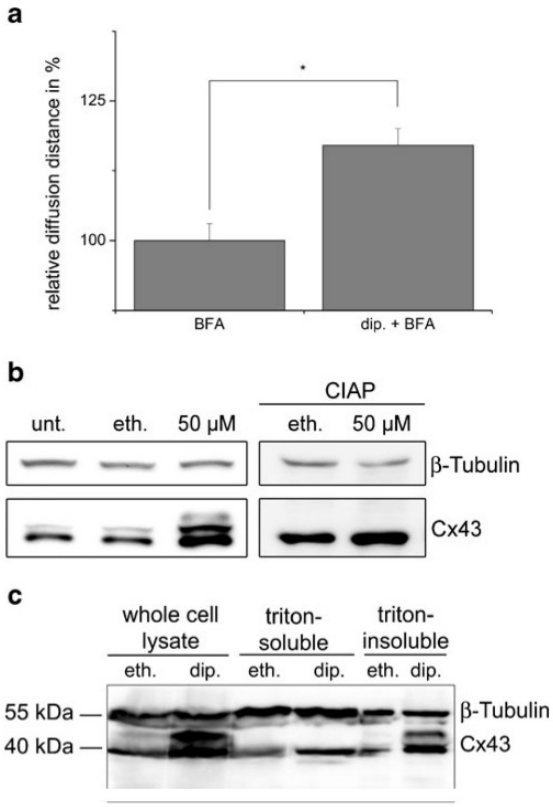


**Fig. 5** (**a**) Brefeldin A (BFA, 5 µg/ml) did not inhibit the dipyridamole-induced (50 µM) short-term increase (6 h) of gap junction coupling in GM-7373 endothelial cell line. However, (**b**) Dipyridamole changed the migration behaviour of Cx43 in SDS-PAGE after an application time of 24 h. Under control conditions, two bands at approximately 42 kDa and 44 kDa were found. In 50 µM dipyridamole-treated cells, a third band at approximately 46 kDa was observed. Treatment of proteins with calf intestinal alkaline phosphatase (CIAP) prior to SDS-PAGE suppressed the 44 kDa and the 46 kDa band. Only one band was detected at approximately 42 kDa. Moreover (**c**) Dipyridamole changed the solubility of Cx43 in Triton X-100. Proteins of 0.1 % ethanol-treated (eth.) and 50 µM dipyridamole-treated (dip.) cells for 24 h were separated into triton-soluble and triton-insoluble fractions. Western blots showed that the 42 kDa, 44 kDa and 46 kDa bands were present in the whole cell lysate. In the triton-soluble fraction only the 42 kDa band was found and in the triton-insoluble fraction only the 46 kDa band could be detected

recently observed results in an aortic smooth muscle cell line (Begandt et al. 2013). However, with respect to the short-term effects observed in the first 6 h of drug application in endothelial cells, the results shown here differ from observations made in aortic smooth muscle cells. In the rat aortic smooth muscle cell line A-10, an oscillatory behaviour was observed in the increase in dipyridamole-induced gap junction coupling for application times of 5–6 h (Begandt et al. 2013). In both aortic endothelial and aortic smooth muscle cell lines a cAMP signalling cascade is assumed to explain the dipyridamole-induced gap junction increase. In smooth muscle cells, an oscillatory cAMP synthesis with a direct effect on already existing connexins was proposed for the short-term effect of dipyridamole. For the long-term effect, a cAMP-induced new synthesis of connexin mRNA and protein was proposed (Begandt et al. 2013).

In this report, we observed that treatment of the GM-7373 endothelial cell line with 25 µM or 50 µM dipyridamole induced a rapid increase in gap junction coupling up to 120 % within the first 6 h, followed by a slow increase up to 150 % within the following 18 h (Fig. 1). In immunofluorescence experiments, the anti-Cx43 antibody detected a reduction in Cx43 vesicle-containing cells and an increase in Cx43 plaque-containing cells after a 6 h and 24 h dipyridamole treatment (Fig. 6). We assume that by inducing cAMP synthesis, dipyridamole induced an accumulation of Cx43 in the gap junction plaques. This assumption is supported by the observations of Atkinson et al. (1995) who showed that cAMP increased the proportion of Cx43 protein used in channel formation. The findings by Wang and Rose (Wang and Rose 1995), who showed a cAMP-induced clustering of Cx43 in gap junctions, also support our assumption.

At the mRNA and protein level, sqRT-PCR and Western blotting experiments did not show an increase in the cellular amounts of Cx43 mRNA (Fig. 3) and protein (Fig. 4) for an application time of 6 h. The dipyridamole-induced increase in gap junction coupling in the short-term is mostly due to an effect on already synthesized connexin molecules. Furthermore, BFA did not inhibit the short-term effect of dipyridamole on gap junction coupling (Fig. 5a) indicating that the dipyridamole-induced increase in gap junction coupling after short-term treatment could affect connexins beyond the Golgi apparatus. In our previous studies, we proposed a dipyridamole-induced increase in gap junction coupling via the activation of cAMP-dependent signalling cascades. Thus, we assume that cAMP might increase gap junction coupling after short-term stimulation by affecting the connexins beyond the Golgi apparatus, which are already oligomerised in connexons. Because we observed an increase in gap junction plaques between adjacent cells (Fig. 6), we assume that cAMP might accelerate insertion of connexons into the membrane or might reduce the removal of gap junction channels from the membrane. Moreover, many experiments have shown that cAMP can increase gap junction coupling by affecting proteins already inserted in the membrane. This effect seems to be related to an increase in the open probability of gap junction channels (Chanson et al. 1996) or to an increase in Cx43 channel permeability (Atkinson et al. 1995; Faucheux and Nagel 2002; Loewenstein 1981).

During short-term stimulation, cAMP seems to affect Cx43 connexons/gap junction channels to induce an increase in gap junction coupling. This effect is due to either an increased formation of Cx43 gap junction channels or to a reduced

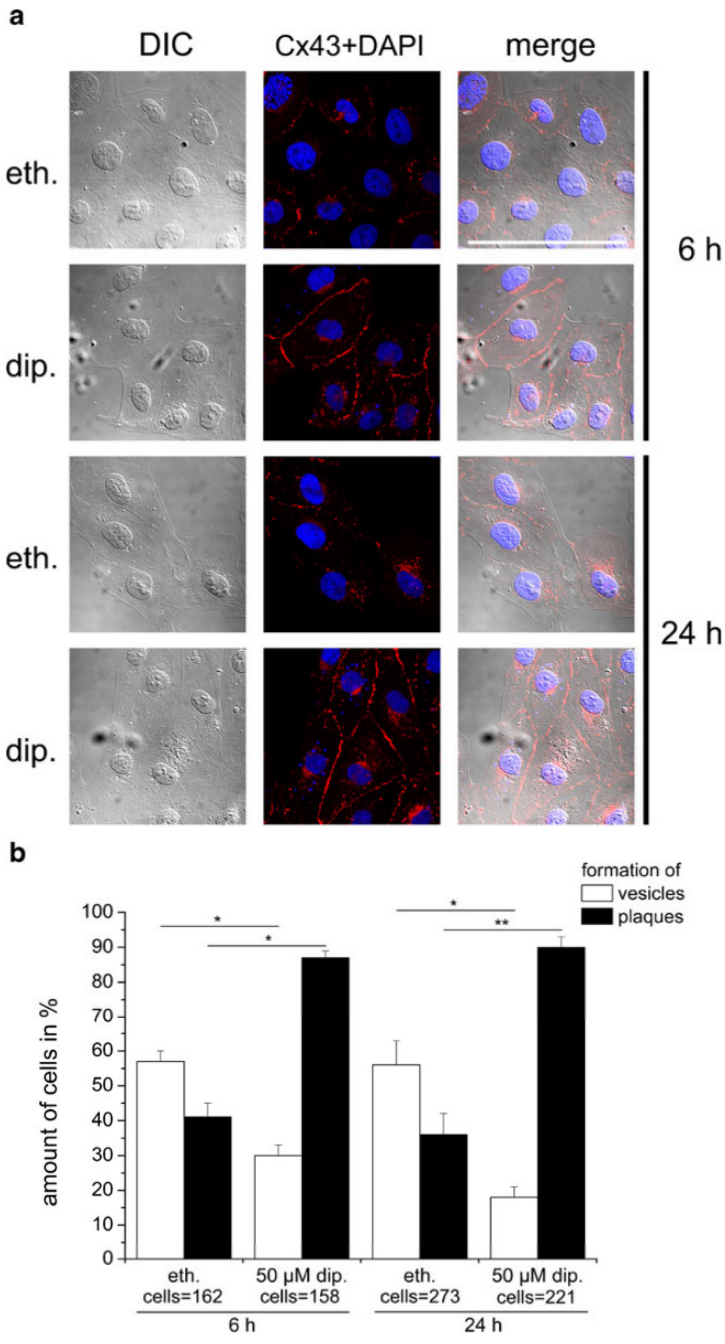


**Fig. 6** Effect of dipyridamole on Cx43 localization. **a** Representative micrographs showing control cells cultivated with 0.1 % ethanol with many vesicles containing Cx43 (*red*) and cells cultivated in the presence of 50 µM dipyridamole with large gap junction plaques containing Cx43. The micrographs shown here are 240×240 pixels sections of 1,024×1,024 pixel original images. *Scale bar* represent 25 µm. Cell nuclei were stained with DAPI (*blue*) and cells were imaged with the differential interference contrast (DIC). **b** Quantification of the Cx43 immunostaining. Dipyridamole treatment for 6 h or 24 h induced an increase in the number of cells with gap junction plaques containing Cx43, while reducing the number of the cells with Cx43-containing vesicles

removal of channels from the membrane. Additionally, cAMP can positively change the gating or open probability of gap junction channels. Regarding the long-term effect of dipyridamole (Fig. 1), we found a correlation between the increase in gap junction coupling and the increase in the amount of Cx43 mRNA (Fig. 3) and protein (Fig. 4). As previously proposed, the dipyridamole-induced long-term effect in gap junction coupling is related to activation of the cAMP-dependent pathway (Begandt et al. 2010). The cAMP signalling cascade could initiate enhanced expression of the

*cx43* gene leading to the observed increase in Cx43 mRNA. The cAMP-induced signalling cascade could also result in reduced mRNA degradation yielding an accumulation of Cx43 mRNA. An enhancement of the transcription rate of *cx43* in response to stimulation of cAMP synthesis has already been shown (Mehta et al. 1992). Therefore, we can assume that the observed dipyridamole-related increase in cellular mRNA is due, at least in part, to cAMP-induced transcription of Cx43 mRNA.

At the protein level, the long-term dipyridamole-induced increase in gap junction coupling correlated with an increase in Cx43 protein content (Fig. 4) and a shift in the migration of Cx43 in SDS-PAGE, hallmarked by the appearance of a third band at 46 kDa that was not observed in control cells or in cells treated with dipyridamole for only 6 h (Fig. 5b). The increase in the amount of Cx43 protein could be directly due to the increase in Cx43 mRNA, or due to a reduction in protein degradation, as well as a product of an increased rate of protein synthesis in response to the dipyridamole-induced increase of cAMP. Salameh et al. and Yogo et al. corroborate our findings by showing that cAMP elevation induced an upregulation of Cx43 protein amount and an increase in gap junction coupling (Salameh et al. 2009; Yogo et al. 2002). With respect to Cx43 migration behaviour (Fig. 5b), in cells cultivated under control conditions or treated with dipyridamole for only 6 h, two bands were observed at approximately 42 kDa and 44 kDa (Fig. 5b). In cells treated with 50 μM dipyridamole for 24 h, a third band at 46 kDa was found (Fig. 5b). These bands could be reduced to a single band at 42 kDa by treatment with CIAP (Fig. 5b). These results suggest that these bands represent different phosphorylation states of Cx43, defined as Cx43-$P_0$ (non Phosphorylated), Cx43-$P_1$ and Cx43-$P_2$ by Musil and Goodenough (1991) and later by Solan and Lampe (2007, 2009). Phosphorylation of Cx43 by a cAMP-induced mechanism is well documented (Cooper and Lampe 2002; Lampe et al. 2006; Musil and Goodenough 1991; Solan and Lampe 2005, 2009; Sosinsky et al. 2007). In a combination of triton solubilisation and in situ extraction, Musil and Goodenough (1991) showed that the triton-insoluble Cx43-$P_2$ was localised in gap junction plaques and the triton-soluble Cx43-$P_0$ was predominantly found in intracellular membranes. The Cx43-$P_1$ was partly found in triton-soluble and in triton-insoluble fractions. The results of Musil and Goodenough were substantially confirmed by other authors (Solan and Lampe 2007, 2009). In our experiments, we found a triton-insoluble Cx43 form of approximately 46 kDa in dipyridamole-treated cells (Fig 5c). In parallel, we observed a dipyridamole-induced increase in the amount of cells with Cx43 gap junction plaques (Fig. 6). We therefore assume that endothelial cells respond to long-term treatment with dipyridamole by activating a cAMP-related mechanism that not only induced an accumulation of Cx43 mRNA and protein but also activated phosphorylation of Cx43 that could enhance the Cx43 gap junction assembly in the membrane. These modifications in turn could lead to the observed increase in gap junction coupling.

For both short- and long-term effects, the nature of the cAMP-related mechanisms is not clear. cAMP is a classical activator of PKA and we showed in a previous report that PKA inhibitors could partly antagonize the dipyridamole-induced increase in gap junction coupling in the GM-7373 endothelial cell line (Begandt et al. 2010), suggesting that PKA-dependent signalling is involved in the response of endothelial cells to dipyridamole. In this report, inhibitors of PKA could not suppress the observed dipyridamole-related increase in the amount of cellular Cx43 mRNA or protein (results not shown). It is tempting to speculate that PKA-independent mechanisms were also activated. PKA-independent signals downstream of cAMP, such as the activation of exchange protein directly activated by cAMP (Epac), that regulate transcription and enhance functional gap junction neoformation were described (Sands and Palmer 2008; Somekawa et al. 2005). Whether such mechanisms were activated in endothelial cells should be clarified in the future.

In summary, the present report shows a dipyridamole-induced, biphasic increase in gap junction coupling in the GM-7373 endothelial cell line by activating cAMP synthesis. The short-term increase in gap junction coupling observed within the first 6 h of drug application might be related to an accumulation of Cx43 in the plasma membrane leading to an elevation in Cx43 gap junction plaques. This effect seems to be related to already synthesised and oligomerised Cx43 proteins beyond the Golgi apparatus. During the long-term effect, dipyridamole evokes an increase in the cellular amount of Cx43 mRNA and protein, in addition to a posttranslational modification and an increase in Cx43 gap junction plaques. By stimulation of cAMP synthesis, dipyridamole most likely induced the new synthesis of connexin mRNA and protein as well as phosphorylation of the connexins, thus inducing the enhancement of gap junction coupling in GM-7373 endothelial cells.

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# Lebenslauf

## Ausbildung

| | |
|---|---|
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## Weiterbildungen

Absolvierter Kurs “Gene Technology, Biosafety and Biosecurity” (2008)

Absolvierter Kurs „Versuchstierkunde nach FELASA B und GV-SOLAS“ (2008)

# Publikationsliste

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## Erklärung

Hierdurch erkläre ich, dass ich die Dissertation mit dem Titel

“Der Einfluss von Dipyridamol auf die Gap Junction-abhängige Kommunikation in Zellen des vaskulären Systems: physiologische und molekulare Analysen”

selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Daniela Begandt

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