Essential Role of Gap Junctions in NO- and Prostanoid-Independent Relaxations Evoked by Acetylcholine in Rabbit Intracerebral Arteries

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Background and Purpose—Direct intercellular communication via gap junctions may play a central role in endothelium-dependent relaxations that are mediated by a conducted hyperpolarization and do not involve the synthesis of NO and prostanooids. In the present study, inhibitory peptides homologous to the Gap27 domain of the second extracellular loop of connexin37/connexin43 and connexin40, designated as 37,43 Gap27 and 40 Gap27, respectively, were used to evaluate the role of this mechanism in intracerebral arteries.

Methods—Isolated rings of rabbit middle cerebral artery were constricted by histamine (10 μmol/L) in the presence of Nω-nitro-L-arginine methyl ester (300 μmol/L) and indomethacin (10 μmol/L). Concentration-relaxation curves for acetylcholine were constructed in the presence and absence of 37,43 Gap27 and 40 Gap27. Specific antibodies were used to delineate the distribution of connexin37, connexin40, connexin43, and connexin45 within the arterial wall.

Results—Individually, 37,43 Gap27 and 40 Gap27 minimally affected endothelium-dependent relaxations to acetylcholine at concentrations of 300 μmol/L, whereas their combination (at 300 μmol/L each) inhibited the maximal response by ≈70% and increased the EC50 value for relaxation by ≈15-fold. In endothelium-denuded rings, this peptide combination did not attenuate responses to sodium nitroprusside, an exogenous source of NO. Gap junction plaques, whose incidence was highest in endothelium, were constructed from connexin40 and connexin43 in the media and connexin37, connexin40, and connexin43 in the endothelium.

Conclusions—The findings confirm that direct communication via gap junctions contributes to agonist-induced relaxations of intracerebral arteries. More than one connexin subtype appears to participate in such responses. (Stroke. 2003;34:544-550.)

Key Words: acetylcholine ■ connexins ■ endothelium-derived hyperpolarizing factor ■ histamine

In many peripheral vessels, an endothelium-derived hyperpolarizing factor (EDHF) has been widely hypothesized to underpin smooth muscle relaxations that are independent of the synthesis of NO and prostanooids by the endothelium. Although less well documented, agonists such as acetylcholine (ACh), ATP, and substance P also stimulate EDHF-type relaxations in isolated cerebral vessels, including human vessels,1–6 and analogous responses have been observed in the pial microcirculation in vivo.6 EDHF-type relaxations appear to play a more prominent role in small branch arteries and arterioles from peripheral beds and the intracerebral vasculature than in major supply vessels, whereas the importance of NO-mediated relaxations may diminish progressively along the vascular tree.4,7 Such observations suggest that the EDHF phenomenon may be a significant determinant of microvascular resistance and cerebral perfusion under physiological conditions.

Although a diverse variety of agents, ranging from ions (specifically, K+) to arachidonic acid metabolites,8,9 have been proposed as EDHFs, there is growing evidence that direct electrotonic endothelium–to–smooth muscle signaling is central to the EDHF phenomenon, inasmuch as agents that interrupt intercellular communication via gap junctions inhibit EDHF-type responses in peripheral arteries, veins, and arterioles.7,10–19 In arterioles, myoendothelial gap junctions behave as simple ohmic resistors that allow endothelial hyperpolarizations induced by ACh to be conducted passively to immediately subjacent smooth muscle cells.20,21 In thick-walled conduit arteries, the dissipation of electrical current within the media may be offset by an associated endothelium-dependent synthesis of cAMP evoked by ACh that increases the permeability and electrical conductance of myoendothelial and homocellular smooth muscle gap junctions, thereby facilitating a passive spread of hyperpolarizing current through the media.22–24 The endothelial hyperpolarization that initiates this current follows the opening of endothelial Ca2+-dependent K+ channels (Kcs, channels) and...
can be inhibited by coadministration of the peptide toxins apamin and charbydotoxin, a property now widely regarded as a hallmark of the EDHF phenomenon.25

Gap junctions are formed by the docking of 2 hemichannels, each constructed from 6 connexin protein subunits, which surround an aqueous central pore that allows passage of polar molecules <1000 Da in size and confers electrical continuity between coupled cells.26 Communication occurs predominantly at focal sites in the plasma membrane where gap junctions cluster in plaques of up to several hundred individual units,27 and the distinctive punctate appearance of such structures, abundant in the endothelial monolayer but relatively sparse in the media, is readily demonstrated by immunostaining of the arterial wall with specific antibodies.7,18,28,29 Myoendothelial plaques are much smaller and less numerous than plaques coupling endothelial cells but can nevertheless be visualized by electron microscopy.30 Three main connexin subtypes, connexin37 (Cx37), connexin40 (Cx40), and connexin43 (Cx43), classified according to molecular mass in kilodaltons, are expressed in cerebral and peripheral arteries,7,18,26,28,29 and histochemical and electrophysiological evidence for the expression of connexin 45 (Cx45) has also been obtained in rat cerebral arterioles.31 Each connexin subunit crosses the cell membrane 4 times to form 2 extracellular loops, and similarities and differences in the amino acid sequences of the Gap26 and Gap27 domains of these loops in Cx37, Cx40, and Cx43 can be exploited to synthesize peptides that interrupt direct intercellular coupling in a connexin-specific fashion.18,32 These peptides can be designated as 40Gap27, 37,40Gap26, 43Gap26, and 37,43Gap27, according to sequence overlap, and are capable of inhibiting communication via both myoendothelial and homocellular smooth muscle gap junctions.18,22,32 Although their molecular mechanism of action remains unknown, these connexin-mimetic peptides do not disrupt the structural integrity of gap junction plaques at points of intercellular contact7 and do not exert nonspecific effects on endothelial hyperpolarization or smooth muscle constriction and relaxation.10,12,13,19

In the present study, the functional role of signaling via gap junctions has been investigated in the rabbit middle cerebral artery, a vessel in which the mechanisms involved in the response to ACh remain to be conclusively established. In early studies with this vessel, Brayden13 reported large perpolarizations were dominated by an EDHF-type response subsequently demonstrated that ACh-evoked relaxations and hyperpolarizations were dominated by an EDHF-type response sensitive to the Kc channel blocker apamin rather than NO or prostanoids. By contrast, Dong et al14 concluded that prostanoids underpin NO-independent relaxations in this vessel and that there is no contribution from an EDHF-type mechanism. In the present study, we have investigated the nature of the response to ACh with Gap27 peptides targeted to the second extracellular loop of Cx40 and Cx37/Cx43, whose effects on relaxation are correlated with connexin expression as visualized by immunostaining and confocal microscopy. The findings provide evidence that direct communication via gap junctions constructed from >1 connexin subtype may underpin NO- and prostanoid-independent responses in the rabbit intracerebral circulation.

Materials and Methods

Isolated Rings

Male New Zealand White rabbits (2 to 2.5 kg) were euthanized with sodium pentobarbitone (120 mg/kg IV) according to institutional guidelines, and the proximal middle cerebral artery was removed and transferred to cold Holmans buffer of the following composition (mmol/L): NaCl 120, KCl 5, CaCl2 2.5, NaH2PO4 1.3, NaHCO3 25, glucose 11, and sucrose 10. The vessels were stripped of adherent tissue, and rings 1.5 to 2 mm wide were cut and suspended in a Mulvany Multi Myograph (Danish Myo Technology) containing gassed (95% O2/5% CO2, pH 7.4) buffer at 37°C. Tension was set at 0.1 to 0.2 mN, and during an equilibrium period of 1 hour, the tissues were repeatedly washed with fresh buffer, and the tension was readjusted after stress relaxation. The rings were incubated for 40 minutes with Nω-nitro-L-arginine methyl ester (300 μmol/L) and indomethacin (10 μmol/L), and after constriction with histamine (10 μmol/L), cumulative concentration-relaxation curves to ACh were constructed. After washout for 1 hour, this protocol was repeated with rings being additionally preincubated for 40 minutes with either 40Gap27 or 37,40Gap27 (at 300 μmol/L or 600 μmol/L) or a combination of these gap junction peptides (300 μmol/L for each peptide). Control concentration-relaxation curves were derived from repeat responses to ACh in time-matched experiments with rings that had not been incubated with the connexin-mimetic peptides. To confirm the EDHF-type nature of the responses to ACh, the effects of apamin (100 nmol/L) and charbydotoxin (100 nmol/L) were evaluated in a separate series of experiments, and the requirement for an intact endothelium was confirmed with rings that had been denuded by gentle abrasion. Denuded rings were also used to test the effects of 40Gap27 plus 37,40Gap27 (at 300 μmol/L each) on relaxations evoked by sodium nitroprusside.

Immunohistology

Freshly isolated rabbit middle cerebral arteries were cryopreserved in OCT compound (Agar Scientific) cooled by liquid N2. Transverse cryosections 10 μm thick were prepared and mounted onto polylysine-coated slides (Surigaph), air-dried, and stored at −20°C. Immediately before immunostaining, the sections were fixed in −20°C methanol for 10 minutes and then rehydrated in PBS (120 mmol/L NaCl and 2.7 mmol/L Na2PO4 · 2H2O, pH 7.4) for 10 minutes. Permeabilization was performed in PBS containing 0.1% (vol/vol) Triton X-100 for 30 minutes, and the sections were blocked with PBS containing 1% (wt/vol) BSA for 30 minutes at room temperature. Sections were labeled with the following primary antibodies: for Cx43, a mouse monoclonal antibody generated against amino acids 252 to 270 (Chemicon, 5 μg/mL) was used; for Cx37 and Cx40, rabbit polyclonal anti-peptide antibodies prepared against amino acids sequences of each connexin, respectively, were used (Alpha Diagnostics, 5 μg/mL); and for Cx45, a mouse monoclonal antibody generated against amino acids 354 to 367 was used (Chemicon, 10 μg/mL). Primary antibodies were incubated for 2 hours at 37°C, followed by washing for 30 minutes at room temperature in PBS. Secondary antibodies of goat anti-mouse–conjugated Alexa 488 or goat anti-rabbit–conjugated Alexa 546 (Molecular Probes, 1:500 dilution) were incubated for 45 minutes at 37°C, followed by an extensive washing to remove unbound antibody. The endothelial cell layer was also stained with an anti–factor VIII FITC-conjugated antibody (Serotec, 1:100 dilution). Sections were mounted in Fluorsave (Calbiochem), and a series of images was collected at 0.5-μm steps through each section by use of a Leica TCS 4D confocal laser scanning microscope equipped with an argon-krypton laser (Leica). A maximum projection image was then obtained with Leica Scanware software.
Drugs
All reagents were obtained from Sigma Chemical Co unless otherwise stated. The purity of 40 Gap27 (sequence SRPTEKNVFIV) and of 37,43 Gap27 (sequence SRPTEKTIFII) was >95%.

Statistical Analysis
All data are given as mean±SEM. Concentration-relaxation curves to ACh were evaluated by 1-way ANOVA, with the Bonferroni multiple comparisons procedure as a further method of analysis. Concentrations of ACh causing half-maximal relaxation (EC50 value) and maximal relaxations (expressed as percent reversal of histamine-induced constriction, Rmax) were compared by a Student t test. A value of P<0.05 was considered significant.

Results
Inhibition of Relaxation by Gap Junction Peptides
EDHF-type relaxations were maximal at a concentration of ≈30 μmol/L ACh, with a maximal response equivalent to ≈90% of histamine-induced tone and an EC50 value of 0.66±0.04 μmol/L (n=26, Figures 1A and 2 and Table). Preincubation with 300 μmol/L 37,43 Gap27 or 40 Gap27 significantly attenuated maximal relaxations evoked by ACh by ≈15%, in association with a ≈2-fold increase in EC50 compared with control (P<0.05 and n=5 for both, Figures 1B, 1C, and 2 and Table). Increasing the concentration of each of these peptides to 600 μmol/L caused maximal EDHF-type relaxations to ACh to be significantly inhibited by ≈70% (P<0.001 for both, n=7 and 9; Figures 1D, 1E, and 2 and Table), although there was no further increase in EC50 (Table). Incubation with a combination of 37,43 Gap27 and 40 Gap27 (300 μmol/L for each component) also inhibited maximal relaxations by ≈70% (P<0.001, n=6; Figures 1F and 2 and Table), but there was then an associated increase in the EC50 for relaxation to 10.5±0.5 μmol/L (P<0.001 compared with control, Table). Incubation with 100 nmol/L apamin and 100 nmol/L charybdotoxin attenuated maximal relaxations to ACh by ≈80% and caused a ≈30-fold increase in the EC50 value for relaxation compared with control (P<0.01, n=3; Figures 1H and 2 and Table). Relaxation was

Figure 1. Representative traces demonstrating control EDHF-type relaxations evoked by ACh (A) and the effects of incubation with 40 Gap27 or 37,43 Gap27 at concentrations of 300 μmol/L (B and C), 600 μmol/L (D and E), and their combination at 300 μmol/L each (F). Relaxation was absent in rings denuded of their endothelium (G) and was inhibited by the combination of apamin and charybdotoxin (H). Endothelium-independent relaxations evoked by sodium nitroprusside (SNP) were unaffected by the combination of 40 Gap27 and 37,43 Gap27 at 300 μmol/L each (I). W indicates washout.
middle cerebral artery rings induced by sodium nitroprusside (Figure 1I). This NO donor caused 81.7±9.8% and 86.7±10.3% reductions in histamine-induced tone with EC_{50} values of 0.28±0.08 µmol/L and 0.30±0.08 µmol/L in the absence and presence of the peptides, respectively. Furthermore, 37,43Gap27 and 40Gap27 did not affect the constrictor response to histamine, either individually or in combination (data not shown).

**Connexin Distribution in Rabbit Middle Cerebral Artery**

In general, immunostaining demonstrated higher levels of connexin expression in the endothelium than in the media of the middle cerebral artery, although there was substantial heterogeneity in the connexin composition of the gap junction plaques found in the 2 cell layers. Expression of Cx37 was restricted to the endothelium (Figure 3A and 3B). Cx40 was also highly expressed in the endothelium but was additionally found in the media (Figure 3C and 3D). By contrast, Cx43 was only weakly expressed in the endothelium but was detectable in the media at levels similar to those found with Cx40, and Cx43 was particularly evident in the adventitia (Figure 3E and 3F). Isolated plaques containing Cx45 could be identified in the endothelium and adventitia, but this subtype was consistently absent from the media (Figure 3G and 3H). Staining for factor VIII was used to delineate the morphology and orientation of the endothelial cell layer (Figure 3I and 3J).

**Discussion**

In the present study, synthetic peptides possessing sequence homology with the Gap27 domain of Cx37/Cx43 and Cx40 have been used to delineate the role of gap junctional communication in NO- and prostanoid-independent relaxations of the rabbit middle cerebral artery. These relaxations exhibited the hallmarks of the EDHF phenomenon, inasmuch as they were dependent on the presence of an intact endothelium and sensitive to the blockade of KCa channels with the peptide toxins apamin and charybdoxin in combination. The findings provide evidence that direct intercellular communication, rather than the release of a mediator that diffuses freely through the extracellular space, normally underpins the EDHF-type relaxations in intracerebral vessels. The potential physiological importance of this novel mechanism of cerebral vasodilatation was emphasized by the observation that maximum relaxations to ACh almost completely reversed the smooth muscle constrictor response of these vessels to histamine.

Maximal relaxations to ACh were depressed by only ≈15% after individual incubation with the connexin-mimetic peptide 37,43Gap27 or 40Gap27 at concentrations of 300 µmol/L but were depressed by ≈70% at concentrations of 600 µmol/L. Synergistic inhibitory effects of the peptides were revealed by coadministration at an equivalent total concentration of 600 µmol/L (ie, 300 µmol/L each), because in addition to a marked reduction in the maximal response to ACh, there was then a ≈15-fold increase in the concentration of ACh causing half-maximal relaxation compared with control. These observations are in contrast to previous find-
ings in isolated rabbit ear arteries, in which \textsuperscript{37,43} Gap27 and the Cx43-specific peptide \textsuperscript{43} Gap26 individually inhibited Ach-evoked EDHF-type relaxations almost completely at the lower concentration of 300 \textmu mol/L, consistent with Cx43 being the dominant subtype involved in relaxation in this extracerebral vessel.\textsuperscript{7} Such differences in the ability of connexin-mimetic peptides to attenuate the EDHF phenomenon are likely to reflect heterogeneity in connexin expression. Indeed, immunostaining revealed dual expression of Cx40 and Cx43 in the media of the middle cerebral artery, whereas we have previously shown that Cx43 is the sole connexin subtype detectable in the media of the central ear artery.\textsuperscript{7} Furthermore, the expression of Cx43 within the endothelium was substantially lower than that of Cx40 in the middle cerebral artery, whereas the 2 subtypes are approximately equally expressed in the endothelium of ear arteries.\textsuperscript{7} Heterogeneous patterns of Cx40 and Cx43 expression similarly account for differences in the ability of \textsuperscript{37,43} Gap27 and \textsuperscript{40} Gap27 to inhibit intercellular communication in cultured cells. In confluent COS-7 fibroblasts, which express Cx43 but not Cx40, \textsuperscript{37,43} Gap27 inhibits dye transfer of Lucifer yellow, whereas \textsuperscript{40} Gap27, which differs by just 3 amino acids, is inactive.\textsuperscript{12,13} By contrast, in confluent rat aortic smooth muscle A7r5 cells, which coexpress Cx40 and Cx43, dye transfer is attenuated far more effectively by combined administration of \textsuperscript{40} Gap27 and \textsuperscript{43} Gap26 at concentrations of 300 \textmu mol/L each than by either peptide individually at 600 \textmu mol/L.\textsuperscript{18} As previously noted in rabbit ear arteries,\textsuperscript{7} at the resolution afforded by antibody immunostaining, Cx37 was detectable only in the endothelium of the middle cerebral artery, in which its expression level was similar to that of Cx40. Therefore, the equivalence of \textsuperscript{37,43} Gap27 and \textsuperscript{40} Gap27 in inhibiting relaxation of this vessel at concentrations of 600 \textmu mol/L might reflect an ability of Cx37 to compensate for a relative paucity of Cx43 in the endothelium. Isolated plaques of Cx45 were identified in the endothelium and adventitia of the middle cerebral artery but were so sparsely distributed that they would be unlikely to contribute functionally to relaxation. Indeed, the ability of the \textsuperscript{37,43} Gap27 and \textsuperscript{40} Gap27 peptide combination to almost abolish the response to Ach suggests a negligible role for Cx45 in these vessels, inasmuch as the Gap27 sequence of this connexin subtype (SRPTEK-TIFLL) is not homologous to either peptide.

Because the EDHF phenomenon may involve the spread of endothelial hyperpolarization via smooth muscle gap junctions, the site of action of gap junction peptides against EDHF-type responses might be expected to vary according to the sequence of the peptide(s) used and the exact composition of the myoendothelial and homocellular smooth muscle plaques present in the vascular wall. In rabbit iliofemoral arteries, for example, \textsuperscript{37,43} Gap27 inhibits EDHF-type relaxations by interrupting communication via myoendothelial gap junctions, inasmuch as it prevents diffusion of the fluorescent dye calcein from the endothelium into the media through such channels and abolishes subintimal smooth muscle hyperpolarizations evoked by stimulation of the endothelium with Ach.\textsuperscript{22} By contrast, in the porcine coronary artery, \textsuperscript{37,43} Gap27 inhibits EDHF-type hyperpolarizations evoked by substance P to a greater extent in adventitial than in subintimal smooth
muscle cells, suggesting that its principal effect is then to attenuate the conduction of an initiating endothelial hyperpolarization through the smooth muscle of the media. Because connexin-mimetic peptides are likely to become important tools for evaluating the role of the EDHF phenomenon in vivo, further research is necessary to define the precise connexin composition of myoendothelial gap junction channels and to determine whether the ability of connexin-mimetic peptides to inhibit relaxation is affected by the existence of heterotypic and/or heteromeric gap junctions (i.e., complex channels constructed from mixtures of different connexin subtypes). Indeed, it might be expected that both Gap27 and Gap27 would modulate communication via heterotypic and heteromeric Cx40/Cx43 channels, whose presence in the arterial media can be demonstrated electrophysiologically in certain vessels. It also remains to be determined whether factors known to regulate gap junction permeability and conductance differ between cerebral and peripheral arteries. For example, estrogens, which are known to modulate gap junctional communication, have been reported to upregulate the EDHF phenomenon in peripheral vessels but to depress EDHF-type relaxations in cerebral arteries. Indeed, in pial arterioles Gap27 attenuates the endothelium-dependent component of ADP-induced dilation in ovariectomized rats but not in control rats. Finally, there may be significant regional variations in connexin expression in intracerebral arteries from the same species. For example, Cx40 and Cx43 are both expressed in the media of the rat middle cerebral artery. The functional consequences of such heterogeneity presently remain unknown.

In summary, we have provided evidence that direct intercellular communication via gap junctions underpins EDHF-type relaxations of the rabbit middle cerebral artery. The pattern of connexin distribution observed in endothelial and smooth muscle cells in this vessel is consistent with a synergistic inhibitory effect of peptides simultaneously targeting Cx40 and Cx37/Cx43 against EDHF-type relaxations. The participation of different connexin subtypes, rather than variability in the mechanisms that ultimately mediate such responses, may thus underlie differences in the ability of connexin-mimetic peptides to inhibit EDHF type relaxations in intracerebral and extracerebral vessels.

Acknowledgment

This study was supported by the Medical Research Council.

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Stroke. 2003;34:544-550; originally published online January 23, 2003;
doi: 10.1161/01.STR.0000054158.72610.EC
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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