Role of gap junctions in endothelium-derived hyperpolarizing factor-mediated vasodilatation in rat renal artery[1]

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KEY WORDS carbachol; endothelium-derived hyperpolarizing factor; gap junction; gap 27 peptide; renal artery

ABSTRACT

AIM: To investigate the effects of gap junction inhibitors on endothelium-derived but nitric oxide (NO)- and prostacyclin (PGI_2)-independent vasodilatations induced by carbachol in the rat isolated renal artery. **METHODS:** Isolated renal arteries were mounted on a wire myograph apparatus were tissues treated with the nitric oxide synthase inhibitor N^{ω}-nitro-*L*-arginine methyl ester hydrochloride (NAME; 100 μmol/L) and indomethacin (10 μmol/L) and precontracted with phenylephrine (0.1 μmol/L). NAME and indomethacin treated Carbachol (0.01-10 μmol/L)- or sodium nitroprusside (SNP; 1-300 nmol/L)-induced mediated relaxations were observed in the presence of gap junction inhibitors. **RESULTS:** Carbachol produced a concentration-dependent relaxation in tissues treated with NAME (100 μmol/L) and indomethacin (10 μmol/L). This relaxation was not affected by hemoglobin (3 μmol/L), but was inhibited by charybdotoxin (200 nmol/L) and ouabain (30 μmol/L). The putative gap junction inhibitors, GAP 27 peptides with sequence homology to connexins 40 and 43 respectively reduced carbachol- but not SNP-induced relaxations mediated by endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxations. The inhibition by the connexin 43 inhibitor was greater than that of the connexin 40 inhibitor. **CONCLUSION:** The results indicate the presence of gap junctions sensitive to ^{43}GAP 27 and ^{40}GAP 27 in the rat renal artery and each of these different types of gap junctions plays a role in the NO- and PGI_2-independent relaxations induced by carbachol in this blood vessel. However, connexin 43 appears to play a more predominant role in mediating gap junction communications in the rat renal artery.

INTRODUCTION

It is well recognized that the vascular tone is regulated by the release of various endothelial factors such as nitric oxide (NO), prostacyclin (PGI_2), and an endothelium-derived hyperpolarizing factor (EDHF)[1]. As far as EDHF is concerned, its nature and role in the regulation of vascular tone has been subjected to considerable debate recently. Nevertheless it is generally believed that EDHF acts by hyperpolarizing vascular smooth muscle cells to produce relaxation, and that the identity and mechanism of action of EDHF may vary between tissues and species[2,3].

A number of studies have suggested that gap junctions are involved in EDHF-induced hyperpolarizations, since putative gap junction inhibitors, such as GAP27 peptide block EDHF-mediated relaxations of some vascular beds in different species[2,4]. Gap junctions permit direct movement of electrical current and small molecules (<1 kDa in size) between smooth muscle cells

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and are formed by the docking of connexins that contain extracellular loops[5]. Connexins show overlapping tissue-specific patterns of expression, although expression of connexins differs between vessels, species and under different physiological conditions[6]. To date, at least thirteen connexins have been identified[7], among which connexins 40 and 43 were found in rodent vasculature[8,9].

Pharmacological tools used to disrupt gap junction formation include GAP 27 peptides, which possess sequence homology to specific regions of connexin proteins. The interactions between the extracellular loops of connexins involve a dynamic docking-undocking mechanism. The connexin mimetic peptide binds to the essential components of the connexin docking sites and thus interferes with the docking of two connexons[4]. Previous studies have shown that EDHF-mediated relaxations are attenuated by the GAP27 peptide with sequence homology to connexins 43 and 40 (denoted ^{43}GAP27 and ^{40}GAP27 respectively) in mesenteric and ear arteries from the rabbit[10,11], the internal carotid artery from the guinea pig[12], the coronary artery from the porcine[13] and the retina from the rat and bovine[14].

Previously, we have identified a carbachol induced and EDHF-mediated relaxation in the rat renal artery. However, the role of gap junctions in this response has not been elucidated. Immunohistochemical and biochemical studies demonstrated expression of multiple connexins in the rat kidney, including 40 and 43[7,15]. Therefore in this study we investigated the effect of specific gap junction inhibitors GAP 27 peptides (connexins 40 and 43 peptides) on EDHF-mediated relaxations in the rat renal artery.

MATERIALS AND METHODS

Tissue preparation Sprague-Dawley rats (250-400 g) of either sex were asphyxiated with carbon dioxide inhalation followed by decapitation. All procedures involving animal studies conformed to the Australian National Health and Medical Research Council guidelines with protocols that had prior approval from the RMIT University Animal Experimentation Ethics Committee.

Kidneys with the renal pedicle attached were removed and placed in a Petri dish containing physiological salt solution (PSS) at room temperature. The branches of the renal artery were isolated under a dissecting microscope (Olympus SZ-30). Care was taken not to damage the endothelium. The branches had an average outer diameter of 300-400 μm and were cut into 1 mm segments and set up in a wire myograph (Model 410A, JP Trading I/S, Denmark) to measure the mechanical activity. Two stainless steel wires of 50 μmol/L diameter were placed in the lumen of the segment. Each wire was mounted on a holder. One holder was fixed to a tension transducer. The other holder was attached to a micrometer that adjusted the distance between the two wires in order to control the passive tension loaded on the tissue.

The preparation was immersed in a chamber containing 6 mL of PSS and maintained at 37 ºC and gassed with 5 % CO_2 and 95 % O_2. The isometric tension of the vessel was recorded with a MacLab data recording system (MacLab/4, model MKIII, AD Instruments Pty Ltd Australia).

The composition of the PSS was as follows (mmol/L): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1, disodium ethylenediaminetetraacetic acid (EDTA) 0.067 and ascorbic acid 0.14.

Experimental protocol After an initial equilibration period of 10 min, the tissue was normalized to 90 % of the inner circumference which corresponds to 100 mmHg blood pressure[16], using a software program developed by GA McPherson[17]. This setting represents a resting tension of 2-3 milli Newtons (mN) for the tissue. The preparation was then equilibrated for a further 45 min.

Tissues were treated with a nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-*L*-arginine methyl ester hydrochloride (NAME; 100 μmol/L) and a cyclo-oxygenase (COX) inhibitor indomethacin (10 μmol/L) for 20 min and the effects of NO-PGI_2-independent relaxations were studied. The relaxation to carbachol (0.01-10 μmol/L) and sodium nitroprusside (SNP; 1-300 nmol/L) in tissue precontracted with phenylephrine (0.1 μmol/L) were observed in the presence of the following agents or solution control, that had been incubated for 20 min: the NO scave nger hemoglobin (3 μmol/L), the Ca^{2+} activated and/or voltage-dependent K^+ channel inhibitor charybdotoxin (200 nmol/L), the Na^+/K^+ATPase inhibitor ouabain (30 μmol/L) and the gap junction inhibitors, GAP 27 peptides which posses sequence homology to connexins 40 and 43 (300 μmol/L).

Drugs The following drugs were purchased from Sigma Chemical Co, St Louis, MO, USA: N^{ω}-nitro-*L*-arginine methyl ester hydrochloride (NAME),

L-phenylephrine hydrochloride, carbamylcholine chloride (carbachol), sodium nitroprusside (SNP), G-strophanthin (ouabain) and bovine haemoglobin. Other drugs used were indomethacin, purchased from Merck Sharp & Dohme, Australia, charybdotoxin, ^{43}GAP 27 peptide (sequence H-SRPTEKTIFII-NH_2) and ^{40}GAP 27 peptide (sequence H-SRPTEKNVFIV-NH_2) purchased from Auspep, Melbourne, Australia.

Stock concentrations of various drugs were dissolved in distilled water, except for the GAP 27 peptides and charybdotoxin, which were dissolved in 100 % dimethylsulphoxide (Me_2SO); indomethacin was dissolved in 0.1 μmol/L Na_2CO_3. In experiments with hemoglobin, a small amount of Antifoam A (30 % aqueous emulsion of Antifoam A concentrate (Sigma Chemical Co, St Louis, MO, USA) was placed on the rim of the myograph chamber to reduce the foam formed by hemoglobin. All effects of hemoglobin on agonist-induced responses were compared to the antifoam vehicle control.

Statistical analysis The tension of the tissue was measured in mN. Relaxations were expressed as a percentage of phenylephrine-precontraction. Data were presented as mean±SD and statistically analysed with two-way repeated measures analysis of variance (two-way ANOVA) followed by Student-Newman-Keuls test (SigmaStat, Jandel Scientific Software, Chicago, USA). A value of $P<0.05$ was regarded as statistically significant.

RESULTS

Effects of hemoglobin, ouabain, and charybdotoxin on carbachol- and SNP-induced relaxations The mean tension of the renal artery constricted with phenylephrine (0.1 μmol/L) in the presence of NAME (100 μmol/L) and indomethacin (10 μmol/L) was 6.8± 0.5 mN, n=18.

The NO trapping agent, hemoglobin (3 μmol/L) had no significant effect on the NO- and PGI_2-independent relaxations to carbachol (0.01-10 μmol/L) compared with the solvent control (Fig 1A). However, SNP (1-300 nmol/L)-induced relaxations were significantly reduced in the presence of hemoglobin 3 μmol/L compared with the solvent control (Fig 1B) ($P<0.05$, n=4).

The large conductance calcium activated, voltage-dependent K^+ channel inhibitor charybdotoxin 200 nmol/L reduced NO- and PGI_2-independent carbachol (0.01-10 μmol/L)-induced relaxations (Fig 1C) ($P<0.05$, n=5). The Na^+/K^+ATPase inhibitor ouabain (30 μmol/L) also significantly reduced carbachol (0.01-10 μmol/L)-induced relaxations (Fig 1D) ($P<0.05$, n=5).

Effects of GAP27 peptides on carbachol- and SNP-induced relaxation The ^{43}GAP27 peptide produced a transient increase in tone, resembling a peak that would revert back to the base line upon completion of incubation (Fig 2A) in approximately half of the total preparations. Whereas, the ^{40}GAP27 peptide had a variable effect on tone, with either a sustained small increase in tone or no obvious change in tone in two out of four preparations.

Both ^{43}GAP 27 300 μmol/L and ^{40}GAP 27 300 μmol/L significantly inhibited carbachol (0.01-10 μmol/L)-induced NO/PGI_2-independent relaxations ($P<0.05$, n=4-5), as illustrated in a typical trace in Fig 2A, 2B and shown in Fig 3A, 3B. Furthermore, the inhibition of carbachol-induced relaxations by ^{43}GAP 27 300 μmol/L was significantly greater than that by ^{40}GAP 27 300 μmol/L (Fig 3C) ($P<0.05$, n=4-5). SNP (1-300 nmol/L)-induced NO/PGI_2-independent relaxations in the presence of ^{43}GAP 27 300 μmol/L were not significantly altered compared with initial controls ($P>0.05$, n=5, Fig 3D).

DISCUSSION

The primary aim of this study was to investigate the effect of the gap junction inhibitor, GAP 27 peptide on carbachol-induced NO- and PGI_2-independent relaxations in the rat renal artery. The major finding was that intercellular gap junctions might be involved in EDHF-mediated vasodilatations in the isolated renal artery from the rat.

Firstly, we confirmed that the carbachol- induced relaxations in the rat renal artery were indeed endothelium-dependent and involved two different components, NO-dependent and NO- and PGI_2-independent component[18]. The NO- and PGI_2-independent relaxation elicited by carbachol in this study was sensitive to the K^+ channel inhibitor charybdotoxin which was in accord with other studies of the renal vasculature[18,19]. It is now generally accepted that EDHF opens K^+ channels in the vascular smooth muscle, thereby causing membrane hyperpolarization and relaxation[3]. In addition, the Na^+/K^+ATPase inhibitor ouabain partially inhibits the NO- and PGI_2-independent relaxation elicited by carbachol in this study, which suggests EDHF responses may by mediated through the activation of Na^+/K^+ATPase in the smooth muscle membrane and is also in accord with previous findings in the rat and rabbit renal artery[18,19].

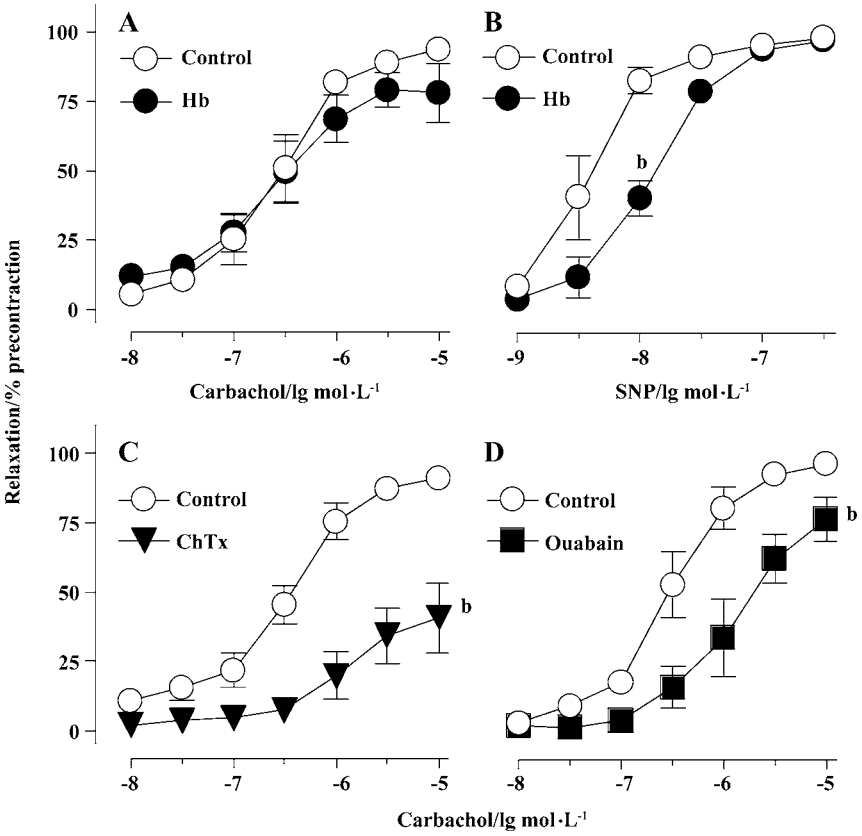

Fig 1. Effect of haemoglobin (Hb; 3 μmol/L, A & B), charybdotoxin (ChTx; 0.2 μmol/L, C), and ouabain (30 μmol/L, D) on carbachol (CCh; 0.01-10 μmol/L, A, C & D) and SNP (1-300 nmol/L, B)-induced relaxations in the rat renal arteries precontracted with phenylephrine (0.1 μmol/L). Tissues treated with NAME (100 μmol/L) and indomethacin (10 μmol/L). *n*=4-5. Mean±SD. $^{b}P<0.05$ *vs* control.

Furthermore, in the present study, the NO scavenger hemoglobin reduced the NO- and PGI_2-independent SNP-induced relaxations without affecting carbachol-induced relaxation. Therefore the NO- and PGI_2-independent responses induced by carbachol observed in the study were attributed to EDHF and not endothelium-derived NO.

Recent studies suggested that gap junction communication may play an important role in EDHF-mediated responses, especially as vessel size decreases[20,21]. In particular, a number of studies using gap junction inhibitors, ie GAP 27 peptide, found that vasodilator responses of the renal vasculature depended on the activity of gap junctions[22]. The exact mode of action of GAP 27 peptide has not been determined but it is likely to disrupt the formation and stability of the intercellular pathways involving gap junctions[10]. The homologous proteins, connexins, which form a gap junction have been shown to be present with tissue specificity and developmental stage specific expression[23]. To date, at least 5 different connexins 32[24], 26[25], 37, 40[15], and 43[26] have been identified in the rat kidney. Connexin 40 and connexin 43 are each present in both smooth muscle and vascular endothelial cells[8,27]. The two forms of the connexin mimetic peptide used in this study have sequence homologies to the second extracellular loop of either connexin 40 or 43 (denoted ^{40}GAP 27 and ^{43}GAP 27, respectively)[28].

Both peptides partially inhibited the NO- and PGI_2-independent EDHF-mediated responses induced by carbachol, indicating a role for gap junctions in this relaxation. The inhibitory action of ^{43}GAP 27 was greater than that of ^{40}GAP 27, which is in agreement with previous findings where connexin 43 has been described as the dominant gap junction protein present in endothelial cells[29]. However an *in vivo* study had demonstrated that connexin 40 had a more important role than connexin 43 in the rat renal vascular bed, since ^{40}GAP 27 peptide but not ^{43}GAP 27 abolished the NO/PGI_2-independent renal blood flow response to acetylcho-line[4]. It is important to note that in the De Vriese *et al* study, GAP 27 peptide effects were based

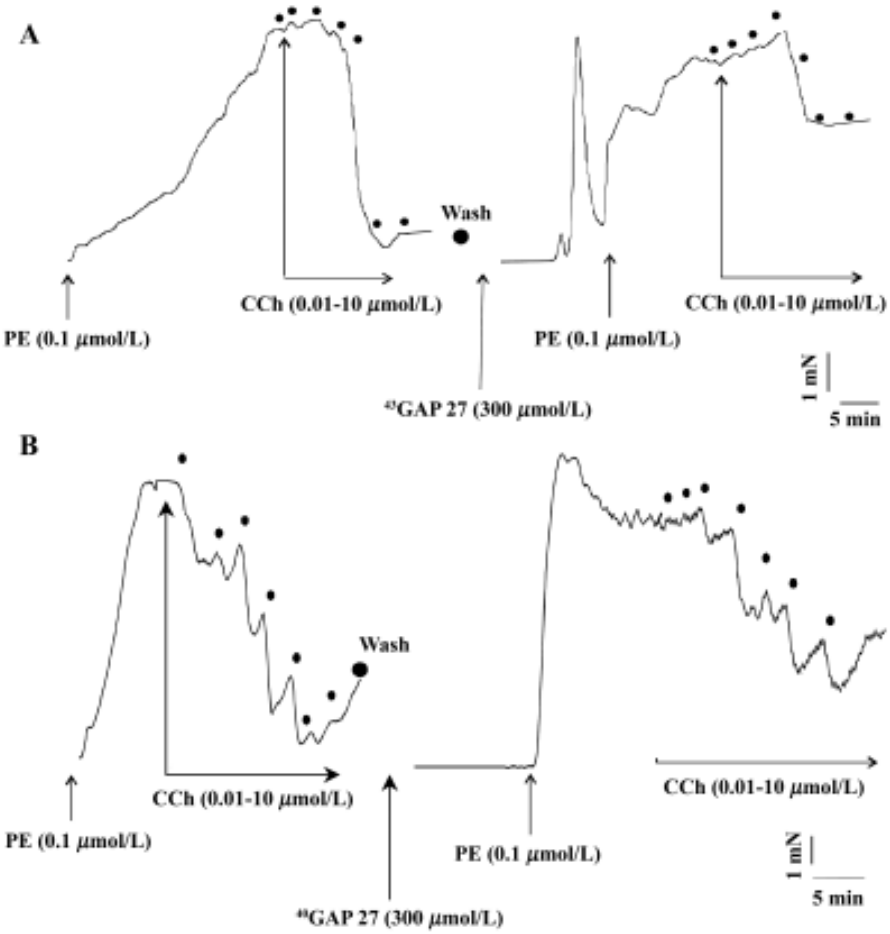

Fig 2. A typical trace illustrating the effect of ^{43}GAP 27 peptide 300 μmol/L (A) and ^{40}GAP 27 peptide 300 μmol/L (B) on carbachol (CCh; 0.01-10 μmol/L)-induced relaxations in the rat renal artery precontracted with phenylephrine (PE; 0.1 μmol/L). Tissues were treated with NAME 100 μmol/L and indomethacin 10 μmol/L.

on the renal blood flow response, which involved the overall renal vascular bed. However the present study specifically examined vessels from the second branch of the renal artery. Hence vessel specificity may account for the discrepancies in responses to the two different GAP 27 peptides between the two studies.

Since GAP 27 did not completely abolish the EDHF-mediated response it is possible that this peptide may not have an affinity for other connexins present in this vasculature. The specificity of GAP 27 peptide has been previously reported where a control peptide, GAP 20, which possesses homology with a sequence of the intracellular loop of connexin 43 was inactive[10]. The relaxations induced by SNP were not affected by ^{43}GAP 27 suggesting that the effect of ^{43}GAP 27 in this preparation may be specific for the endothelium and not at the smooth muscle level. This observation is also consistent with previous findings where ^{43}GAP 27 produced an inhibitory effect on the NO- and PGI_2-independent relaxations induced by acetylcholine, ATP, and cyclopiazonic acid (receptor independent) in rabbit conduit arteries without affecting relaxations to endogenous or exogenous sources of NO[10]. However, intercellular communication via gap junctions may occur between endothelial cells or between endothelium and smooth muscle cells or even between adjacent smooth muscle cells. Since no gap junction-blocking agent can discriminate between inhibition of myoendothelial gap junctions and inhibition of gap junctional coupling within the endothelium or smooth muscle cell[4], in this study we cannot be certain of the direction of gap junction communication and therefore inhibition of gap junctions at any of these locations might suppress relaxations, depending on how the hyperpolarisation is spread. Although, immunohistochemical evidence suggests that connexins 43 and 40 are predominantly expressed in the endothelium of muscular arteries[30]. However this does not exclude the possibility that connexins other

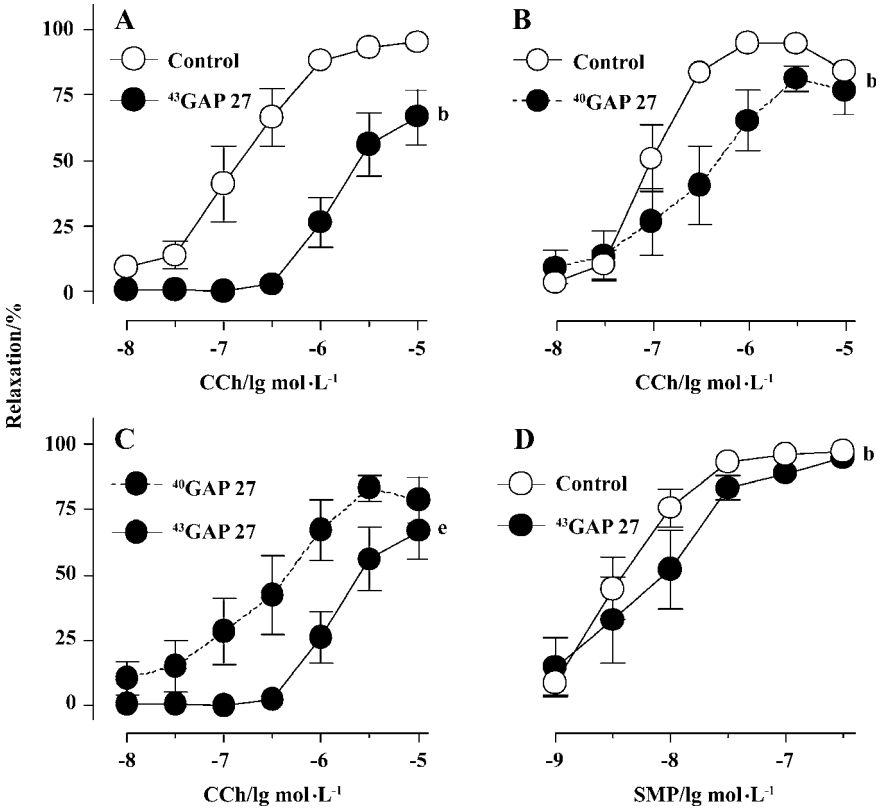

Fig 3. Effect of ^{43}GAP 27 peptide 300 μmol/L (A), ^{40}GAP 27 peptide 300 μmol/L (B), and a comparison between both peptides (C) on carbachol (CCh; 0.01-10 μmol/L)-induced relaxations and the effect of ^{43}GAP 27 peptide 300 μmol/L on SNP (0.01-10 μmol/L)-induced relaxations in rat renal arteries precontracted with phenylephrine 0.1 μmol/L (D). Tissues treated with NAME (100 μmol/L) and indomethacin (10 μmol/L). *n*=4-7. Mean±SD. $^{b}P<0.05$ *vs* control. $^{e}P<0.05$ *vs* ^{40}GAP 27.

than 40 and 43 may be present on smooth muscle cells in the rat renal artery. *In vivo* analyses of microvessels from the brain and cremaster of the rat and arterioles from the hamster cheek pouch indicate connexins 43 and 40 are more abundant on the endothelium than the smooth muscle, suggesting greater coupling within the endothelium[27]. However connexin 43 but not connexin 37 or 40 has been demonstrated in smooth muscle cells of coronary arteries[6]. Hence, there is tissue specificity of gap junction and connexin coupling.

In conclusion the present study demonstrates that in the rat renal artery EDHF-induced vasodilatations do involve gap junctions, which are sensitive to GAP 27 peptides predominantly affecting connexins 43.

REFERENCES

1 Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109-42.

2 Ujiie H, Chaytor AT, Bakker LM, Griffith TM. Essential role of Gap junctions in NO- and prostanoid-independent relaxations evoked by acetylcholine in rabbit intracerebral arteries. Stroke 2003; 34: 544-50.

3 Triggle CR, Dong H, Waldron GJ, Cole WC. Endothelium-derived hyperpolarizing factor(s): species and tissue heterogeneity. Clin Exp Pharmacol Physiol 1999; 26: 176-9.

4. De Vriese AS, Van de Voorde J, Lameire NH. Effects of connexin-mimetic peptides on nitric oxide synthase- and cyclooxygenase-independent renal vasodilation. Kidney Int 2002; 61: 177-85.

5 Yeager M, Nicholson BJ. Structure of gap junction intercellular channels. Curr Opin Struct Biol 1996; 6: 183-92.

6 Severs NJ. Cardiovascular disease. Novartis Found Symp 1999; 219: 188-206; *discussion 206-11*.

7 Silverstein DM, Thornhill BA, Leung JC, Vehaskari VM, Craver RD, Trachtman HA, *et al*. Expression of connexins in the normal and obstructed developing kidney. Pediatr Nephrol 2003; 18: 216-24.

8 Christ GJ, Spray DC, El-Sabban M, Moore LK, Brink PR. Gap junctions in vascular tissues: evaluating the role of intercellular communication in the modulation of vasomotor tone. Circ Res 1996; 79: 631-46.

9 Kumar NM, Gilula NB. The gap junction communication channel. Cell 1996; 84: 381-8.

10 Chaytor AT, Evans WH, Griffith TM. Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. J Physiol 1998; 508:

561-73.

11 Berman RS, Martin PE, Evans WH, Griffith TM. Relative contributions of NO and gap junctional communication to endothelium-dependent relaxations of rabbit resistance arteries vary with vessel size. Microvasc Res 2002; 63: 115-28.

12 Edwards G, Feletou M, Gardener MJ, Thollon C, Vanhoutte PM, Weston AH. Role of gap junctions in the responses to EDHF in rat and guinea-pig small arteries. Br J Pharmacol 1999; 128: 1788-94.

13 Edwards G, Thollon C, Gardener MJ, Feletou M, Vilaine J, Vanhoutte PM, *et al*. Role of gap junctions and EETs in endothelium-dependent hyperpolarization of porcine coronary artery. Br J Pharmacol 2000; 129: 1145-54.

14 Matesic D, Tillen T, Sitaramayya A. Connexin 40 expression in bovine and rat retinas. Cell Biol Int 2003; 27: 89-99.

15 Arensbak B, Mikkelsen HB, Gustafsson F, Christensen T, Holstein-Rathlou NH. Expression of connexin 37, 40, and 43 mRNA and protein in renal preglomerular arterioles. Histochem Cell Biol 2001; 115: 479-87.

16 Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 1977; 41: 19-26.

17 McPherson GA. Assessing vascular reactivity of arteries in the small vessel myograph. Clin Exp Pharmacol Physiol 1992; 19: 815-25.

18 Jiang F, Li CG, Rand MJ. Mechanisms of nitric oxide-independent relaxations induced by carbachol and acetylcholine in rat isolated renal arteries. Br J Pharmacol 2000; 130: 1191-200.

19 Kitagawa S, Yamaguchi Y, Kunitomo M, Sameshima E, Fujiwara M. N^G-nitro-*L*-arginine-resistant endothelium-dependent relaxation induced by acetylcholine in the rabbit renal artery. Life Sci 1994; 55: 491-8.

20 Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, *et al*. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. J Cardiovasc Pharmacol 1996; 28: 703-11.

21 Tomioka H, Hattori Y, Fukao M, Sato A, Liu M, Sakuma I, *et al*. Relaxation in different-sized rat blood vessels mediated by endothelium-derived hyperpolarizing factor: importance of processes mediating precontractions. J Vasc Res 1999;36: 311-20.

22 De Vriese AS, Van de Voorde J, Blom HJ, Vanhoutte PM, Verbeke M, Lameire NH. The impaired renal vasodilator response attributed to endothelium-derived hyperpolarizing factor in streptozotocin-induced diabetic rats is restored by 5-methyltetrahydrofolate. Diabetologia 2000; 43: 1116-25.

23 Kumar NM, Gilula NB. Molecular biology and genetics of gap junction channels. Semin Cell Biol 1992; 3: 3-16.

24 Paul DL. Molecular cloning of cDNA for rat liver gap junction protein. J Cell Biol 1986; 103: 123-34.

25 Zhang JT, Nicholson BJ. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. J Cell Biol 1989; 109: 3391-401.

26 Beyer EC, Paul DL, Goodenough DA. Connexin 43: a protein from rat heart homologous to a gap junction protein from liver. J Cell Biol 1987; 105 (Pt 1): 2621-9.

27 Little TL, Beyer EC, Duling BR. Connexin 43 and connexin 40 gap junctional proteins are present in arteriolar smooth muscle and endothelium *in vivo*. Am J Physiol 1995; 268 (2 Pt 2): H729-39.

28 Chaytor AT, Evans WH, Griffith TM. Peptides homologous to extracellular loop motifs of connexin 43 reversibly abolish rhythmic contractile activity in rabbit arteries. J Physiol 1997;503 (Pt 1): 99-110.

29 Christ GJ, Spektor M, Brink PR, Barr L. Further evidence for the selective disruption of intercellular communication by heptanol. Am J Physiol 1999; 276: H1911-7.

30 Hill CE, Rummery N, Hickey H, Sandow SL. Heterogeneity in the distribution of vascular gap junctions and connexins: implications for function. Clin Exp Pharmacol Physiol 2002; 29: 620-5.