# Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries

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1. The contribution of gap junctions to endothelium-dependent relaxation was investigated in isolated rabbit conduit artery preparations pre-constricted by 10 $\mu$M phenylephrine (PhE).

2. Acetylcholine (ACh) relaxed the thoracic aorta by ~60% and the superior mesenteric artery (SMA) by ~90%. A peptide possessing sequence homology with extracellular loop 2 of connexin 43 (Gap 27, 300 $\mu$M) inhibited relaxation by ~40% in both artery types. Gap 27 also attenuated the endothelium-dependent component of the relaxation induced by ATP in thoracic aorta but did not modify force development in response to PhE.

3. $N^G$-nitro-L-arginine methyl ester (L-NAME, 300 $\mu$M), an inhibitor of NO synthase, attenuated ACh-induced relaxation by ~90% in the aorta but only by ~40% in SMA ($P < 0{\cdot}05$). Residual L-NAME-insensitive relaxations were almost abolished by 300 $\mu$M Gap 27 in aorta and inhibited in a concentration-dependent fashion in SMA (~50% at 100 $\mu$M and ~80% at 10 mM). Gap 27 similarly attenuated the endothelium-dependent component of L-NAME-insensitive relaxations to ATP in aorta.

4. Responses to cyclopiazonic acid, which stimulates endothelium-dependent relaxation through a receptor-independent mechanism, were also attenuated by Gap 27, whereas this peptide exerted no effect on the NO-mediated relaxation induced by sodium nitroprusside in preparations denuded of endothelium.

5. ACh-induced relaxation of 'sandwich' mounts of aorta or SMA were unaffected by Gap 27 but completely abolished by L-NAME.

6. We conclude that direct heterocellular communication between the endothelium and smooth muscle contributes to endothelium-dependent relaxations evoked by both receptor-dependent and -independent mechanisms. The inhibitory effects of Gap 27 peptide do not involve homocellular communication within the vessel wall or modulation of NO release or action.

Following the discovery of endothelium-dependent relaxation (Furchgott & Zawadzki, 1980), synthesis of nitric oxide (NO) by the constitutive endothelial NO synthase (NOS) has emerged as one of the major mechanisms modulating vascular tone in response to agonist stimulation and shear stress (for review see Griffith, 1994). Agonists such as acetylcholine (ACh) and adenosine triphosphate (ATP) may also contribute to relaxation by causing endothelium-dependent hyperpolarization of subjacent smooth muscle (Chen & Suzuki, 1991; for review see Garland, Plane, Kemp & Cocks, 1995). This phenomenon can involve NO and prostanoid synthesis (Parkington, Tare, Tonta & Coleman, 1993), but evidence for the release of a distinct endothelium-derived hyperpolarizing factor (EDHF) has been obtained in bioassay studies in which the effluent from an upstream donor hyperpolarizes downstream vascular myocytes under conditions of combined NOS and cyclo-oxygenase blockade (Popp, Bauersachs, Hecker, Fleming & Busse, 1996). Candidate mediators include cytochrome P450-derived arachidonic acid metabolites (Popp *et al.* 1996) and anandamide, an endogenous cannabinoid (Randall *et al.* 1996). Many studies suggest that endothelium-dependent hyperpolarization involves $K^+$ channel opening, but also demonstrate considerable regional and species heterogeneity. Subtypes implicated include large conductance $K_{Ca}$ channels (Hwa, Ghibaudi, Williams & Chatterjee, 1994; Hansen & Olesen, 1997), small conductance $K_{Ca}$ channels (Adeagbo & Triggle, 1993), $K_{ATP}$ channels (Brayden, 1990; Chen, Yamamoto, Miwa & Suzuki, 1991), and the dual involvement of $K_V$ and $K_{Ca}$ channels has also been suggested (Petersson, Zygmunt & Högestätt, 1997). This high degree of experimental variability may reflect the existence of more

than one EDHF and different mechanisms of $K^+$ channel activation. Indeed, NO can itself open $K_{Ca}$ channels via both cGMP-dependent and direct cGMP-independent mechanisms (Robertson, Schubert, Hescheler & Nelson, 1993; Bolotina, Najibi, Palacino, Pagano & Cohen, 1994).

Hyperpolarization of arterial smooth muscle is associated with hyperpolarization of the endothelium itself, and dye transfer techniques for detecting intercellular continuity confirm direct heterocellular coupling (Bény & Pacicca, 1994; Little, Xia & Duling, 1995). Although preferential passage of certain dye tracers from endothelium to smooth muscle has suggested polarity in the direction of information transfer (Little *et al.* 1995), signals can be transmitted in the reverse direction thereby leading to elevated endothelial $[Ca^{2+}]_i$ and enhanced NO synthesis (Dora, Doyle & Duling, 1997). Furthermore, synchronous fluctuations in smooth muscle and endothelial membrane potential during spontaneous rhythmic activity are driven from the media (Von der Weid & Bény, 1993; Xia, Little & Duling, 1995). Bidirectional communication through gap junctions may permit heterocellular movements of ions and other small molecules, provide electrical continuity, and thus facilitate the co-ordinated behaviour of the arterial wall.

Gap junctions are formed by the docking of two connexon hemichannels contributed by interacting cells. Each connexon is constructed of six connexin protein subunits that cross the cell membrane four times, and to date thirteen rodent connexin subtypes have been identified (Yeager & Nicholson, 1996). The amino terminus, the loop connecting transmembrane segments 2 and 3, and the carboxy-terminus of connexins are located on the cytoplasmic side of the plasma membrane, with two further loops projecting into the extracellular 'gap' (Kumar & Gilula, 1992). Previous studies employing the putative gap junction uncoupler heptanol have yielded conflicting results concerning the possible contribution of gap junctions to endothelium-dependent relaxation (Kühberger, Groschner, Kukovetz & Brunner, 1994; Javid, Watts & Webb, 1996; Zygmunt & Högestätt, 1996). However, heptanol possesses 'non-specific' pharmacological actions that are unrelated to gap junction function (Chaytor, Evans & Griffith, 1997). In the present study we therefore investigated the effects of a specific inhibitory gap junction peptide (Gap 27; amino acid sequence: SRPTEKTIFII), which possesses conserved sequence homology to a portion of the 2nd extracellular loop leading into the 4th transmembrane connexin segment. Short synthetic peptides possessing the amino acid motifs QPG and SHVR of extracellular loop 1 and the SRPTEK motif of extracellular loop 2 have previously been shown to delay the achievement of co-ordinated contractions in chick cardiac myocyte aggregates and to inhibit rhythmic contractile activity in rabbit conduit arteries without affecting ambient tone (Warner, Clements, Parikh, Evans & Dehaan, 1995; Chaytor *et al.* 1997).

Endothelial cells express mRNA encoding connexins Cx37, Cx40 and Cx43 (Larson, Haudenschild & Beyer, 1990; Bruzzone, Haefliger, Gimlich & Paul, 1993; Carter, Chen, Carlile, Kalapothakis, Ogden & Evans, 1996). In rabbit superior mesenteric artery Western blot analysis has identified Cx43 as the major connexin expressed in endothelium-denuded vessels, although reverse transcriptase-polymerase chain reaction also revealed the presence of mRNA encoding Cx32, Cx40 and Cx43 (Chaytor *et al.* 1997). Gap 27 peptide would be expected to inhibit junctions constructed of those connexins known to be present in the arterial wall, since the 2nd extracellular loop of each connexin isoform contains the crucial conserved sequence SRPTEK (Kumar & Gilula, 1992). In view of reports suggesting that the NO–prostanoid-independent component of agonist-induced relaxation may vary with vessel diameter, the effects of Gap 27 peptide were compared in two arteries of different size (Hwa *et al.* 1994; Shimokawa *et al.* 1996). Gap junction peptide 20, which possesses sequence homology with the intracellular loop of Cx43, was used as a biologically inactive control (Chaytor *et al.* 1997). The findings provide new insights into the role of intercellular communication in the regulation of vascular tone.

## METHODS

### Isolated ring preparations

Male New Zealand White rabbits (2–2·5 kg) were killed by administration of sodium pentobarbitone (120 mg $kg^{-1}$ intravenously). The thoracic aorta and superior mesenteric artery were removed, stripped of adherent connective and adipose tissue and placed in Holman's buffer (composition (mM): 120 NaCl, 5 KCl, 2·5 $CaCl_2$, 1·3 $NaH_2PO_4$, 25 $NaHCO_3$, 11 glucose and 10 sucrose). Rings of aorta and superior mesenteric artery 2–3 mm wide were dissected and suspended in oxygenated (95% $O_2$–5% $CO_2$) 3 ml tissue baths at 37 °C. In some experiments endothelium was removed by gentle abrasion of the intimal surface of the rings with a roughened probe. Isometric force was measured using FT102 force transducers with a MacLab/4e (ADInstruments, Hastings, UK) and placed under 0·6 g (superior mesenteric artery) or 1·5 g (thoracic aorta) tension. Each preparation was allowed to equilibriate for ~1 h to allow stress relaxation to occur following washout at 20 min intervals.

### Experimental protocol

Following the initial equilibration period, the rings were contracted with phenylephrine (10 $\mu$M) and subsequently allowed to stabilize for 20–30 min before cumulative concentration–response curves were constructed. In thoracic aorta cumulative concentration–response curves were obtained for endothelium-dependent relaxation to ACh, ATP and cyclopiazonic acid (CPA) in the presence and in the absence of L-NAME (300 $\mu$M). Responses to sodium nitroprusside (SNP), CPA and ATP were also investigated in endothelium-denuded preparations. In superior mesenteric artery cumulative concentration–response curves were constructed for ACh in the presence and absence of L-NAME (300 $\mu$M). To evaluate the role of prostanoid synthesis, in some experiments indomethacin (10 $\mu$M) was included in the buffer. Following the initial responses and washout period, a second cumulative concentration–response

curve was constructed in the presence of gap junction peptides Gap 20 (3 mM) and Gap 27 (100 $\mu$M–10 mM) or heptanol (300 $\mu$M–3 mM) after a 20 min preincubation period. To assess the reversibility of Gap 27 peptide and heptanol all agents were washed out over a 1 h period, the tissue reconstricted, and a further cumulative concentration–response curve constructed. In order to obtain time-matched controls some rings were not exposed to Gap 27 peptide, Gap 20 peptide or heptanol. At the end of each experiment involving denuded rings ACh (1 $\mu$M) was added to confirm the absence of endothelium.

### 'Sandwich' experiments

In this series of experiments rings of aorta or superior mesenteric artery were cut open to provide longitudinal strips. Some of these were denuded of endothelium by gentle abrasion and attached to endothelium-intact strips by means of two stitches with the intimal surfaces of the tissues apposed. The composite preparation was placed under 1·5 g (thoracic aorta) or 0·6 g (superior mesenteric artery) tension and subjected to a 1 h equilibration period, tension being monitored only in the endothelium-denuded component. To examine the 'transferable' nature of endothelium-dependent relaxation in such composite preparations, responses to ACh and substance P were determined in the presence of Gap 27 peptide (3 mM) or L-NAME (300 $\mu$M).

### Materials

ACh, ATP, substance P, cyclopiazonic acid, indomethacin, L-NAME, phenylephrine and sodium nitroprusside were supplied by Sigma. Gap junction peptides 20 (E I KK F KYGC) and 27 (SRP TEK T I F I I) were synthesized by Severn Biotech Ltd, Kidderminster, UK. All compounds were dissolved in buffered Holman's solution except CPA (DMSO) and indomethacin (5% bicarbonate solution).

### Statistics

Concentration–response curves were compared by one-way analysis of variance (ANOVA) employing the Bonferroni multiple comparisons test. The magnitude of contractile responses to phenylephrine and maximal relaxations in the two artery types were compared by Student's $t$ test. $P < 0{\cdot}05$ was considered significant.

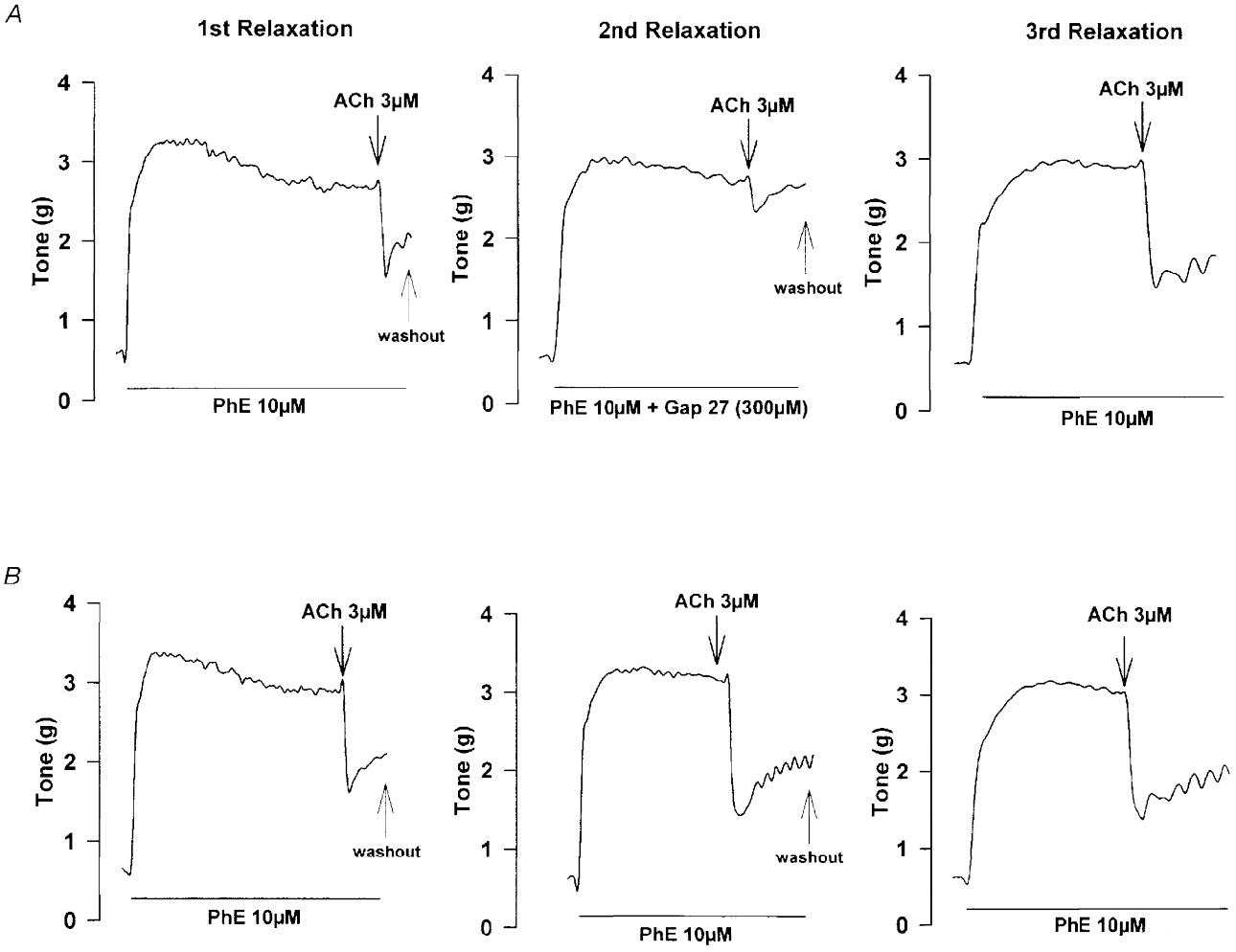


**Figure 1. Original traces demonstrating reversible inhibition of ACh-induced relaxation by gap junction peptide 27**

*A*, Gap 27 (300 $\mu$M) markedly inhibited the relaxant response to ACh, an effect that was fully reversible on washout for ~60 min. *B*, in matched experiments relaxations to ACh showed no significant attenuation over time.

## RESULTS

### Effects of gap junction peptide 27 on acetylcholine- and ATP-induced relaxation in the thoracic aorta

ACh induced concentration-dependent relaxations of phenylephrine-constricted rings that were maximal at ~3 $\mu$M under all experimental protocols (Figs 1 and 2). No significant variation was observed over time when control ACh responses were repeated after washout and further constriction, maximal relaxation being of the order of ~60%. Responses to ACh in the presence of Gap 27 peptide (300 $\mu$M) were significantly attenuated with a rightward shift in the concentration–relaxation curve (Fig. 2*A*), such that there was a 40% reduction in maximal relaxation and an ~3-fold increase in the $EC_{50}$ value from 280 ± 20 to 870 ± 30 nM ($P < 0{\cdot}05$, $n = 8$). The effects of Gap 27 peptide were fully reversible on washout (Fig. 1*A*). The concentration–relaxation response to ACh was markedly depressed (by ~90%) in the presence of L-NAME (300 $\mu$M) with an increase in the $EC_{50}$ value to 1·05 ± 0·08 $\mu$M ($n = 5$, $P < 0{\cdot}001$). The residual relaxation observed in the presence of L-NAME was reduced further by Gap 27 peptide (300 $\mu$M), resulting in a further increase in $EC_{50}$ to 2·8 ± 0·14 $\mu$M ($P < 0{\cdot}05$, $n = 5$). These findings indicate that the inhibitory effects of L-NAME and Gap 27 peptide against ACh-induced relaxation were synergistic.

Endothelium-denuded rings of thoracic aorta relaxed significantly with ATP only at concentrations ⩾ 300 $\mu$M, whereas endothelium-intact rings exhibited relaxation that plateaued over the range 10–100 $\mu$M at a value representing ~20% of initial phenylephrine-induced tone (Fig. 2*B*). As in the case of ACh, concentration–relaxation curves to ATP did not vary significantly over time. Following exposure to Gap 27 peptide (300 $\mu$M), the plateau in relaxation to ATP at concentrations ⩽ 100 $\mu$M was reduced by ~60% with an ~2-fold increase in the associated $EC_{50}$ value from 1·8 ± 0·6 to 3·4 ± 0·8 $\mu$M ($P < 0{\cdot}05$, $n = 5$). Incubation with L-NAME (300 $\mu$M) produced a similar ~60% reduction in response and an ~2-fold rightward shift in $EC_{50}$ from 1·8 ± 0·6 to 3·6 ± 0·6 $\mu$M ($P < 0{\cdot}05$, $n = 5$). The residual ATP-induced relaxation observed in the presence of L-NAME was attenuated further by Gap 27 peptide (300 $\mu$M) with an ~1·5-fold shift in $EC_{50}$ from 3·6 ± 0·6 to 5·1 ± 0·8 $\mu$M ($P < 0{\cdot}05$, $n = 5$).

### Effects of gap junction peptide 27 on cyclopiazonic acid (CPA)- and sodium nitroprusside (SNP)-induced relaxation in thoracic aorta

CPA evoked a concentration-dependent relaxation in endothelium-intact rings of thoracic aorta that was maximal at concentrations of 30–100 $\mu$M when the maximal response represented ~50% of phenylephrine-induced contraction (Fig. 3*A*). Relaxation did not vary over time when a second concentration–response curve was constructed following washout of all drugs. Preincubation with Gap 27 peptide (300 $\mu$M) significantly attenuated this relaxation with a rightward shift in the concentration–response curve, such that there was an ~30% reduction in maximal relaxation and an ~2-fold shift in the $EC_{50}$ value from 3·8 ± 0·2 to 6·9 ± 0·4 $\mu$M ($P < 0{\cdot}05$, $n = 10$). CPA-induced relaxation was markedly attenuated (by ~80%) in the presence of L-NAME (300 $\mu$M) with an increase in the $EC_{50}$ value to 14 ± 3 $\mu$M ($P < 0{\cdot}05$, $n = 5$). In the additional presence of Gap 27 peptide (300 $\mu$M), there was further inhibition of the response to CPA with an increase in $EC_{50}$ to 22 ± 3 $\mu$M

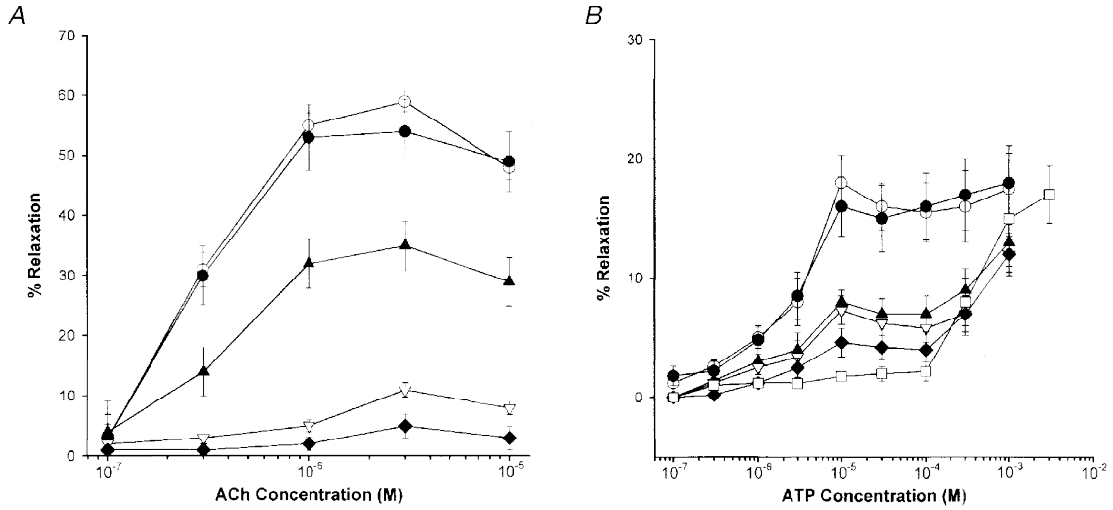


**Figure 2. Concentration–relaxation curves showing the effects of gap junction peptide 27 against ACh and ATP in thoracic aorta in the presence and absence of L-NAME**

*A*, Gap 27 (300 $\mu$M, ▲) inhibited the relaxation induced by ACh by ~40% whereas L-NAME (300 $\mu$M, ▽) inhibited relaxation by ~90%. ○, control responses; ●, time-matched controls. Gap 27 further attenuated the residual relaxation observed in the presence of L-NAME (◆). *B*, analogous effects were found with ATP, symbols denoting the same experimental protocols as in *A*. In endothelium-denuded rings ATP evoked relaxation only at concentrations ⩾ 300 $\mu$M (□).

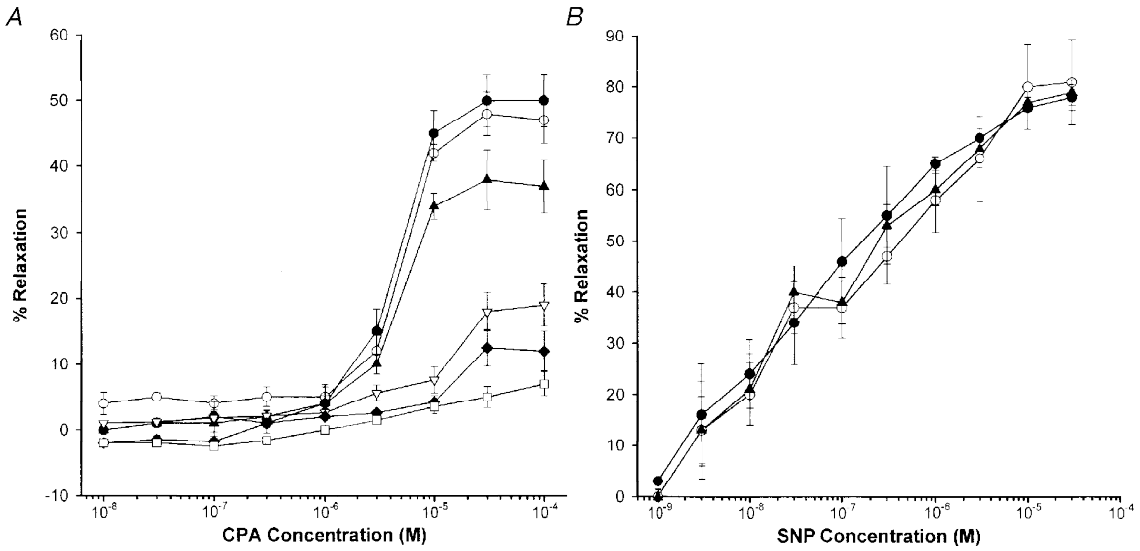


**Figure 3. Concentration–relaxation curves showing the effects of gap junction peptide 27 against CPA and SNP in the thoracic aorta**

*A*, Gap 27 (300 $\mu$M, ▲) inhibited the relaxation to CPA by ~30%, whereas relaxation was attenuated by ~80% in the presence of L-NAME (300 $\mu$M, ▽). The residual relaxation observed in the presence of L-NAME was further reduced by Gap 27 (◆). CPA itself caused < 5% relaxation in endothelium-denuded preparations (□). *B*, SNP concentration–relaxation curves obtained in endothelium-denuded rings were not affected by Gap 27 (300 $\mu$M, ▲). In both panels: ○, control responses; ●, time-matched controls.

($P < 0{\cdot}05$, $n = 5$). In endothelium-denuded rings relaxation to CPA amounted to less than 5% of phenylephrine-induced contraction at 100 $\mu$M CPA.

SNP caused ~80% relaxation of endothelium-denuded rings at a concentration of 30 $\mu$M, with a repeat concentration–response not varying significantly over time. Preincubation with Gap 27 peptide (300 $\mu$M) did not significantly affect the maximal response to SNP or the $EC_{50}$ value for relaxation ($55 \pm 7$ *vs.* $62 \pm 9$ nM; Fig. 3*B*).

### Effects of gap junction peptide 27 on phenylephrine-induced contraction in thoracic aorta

Gap 27 peptide did not significantly affect the contractile response to 10 $\mu$M phenylephrine in either endothelium-intact preparations ($2{\cdot}8 \pm 0{\cdot}3$ *vs.* $2{\cdot}9 \pm 0{\cdot}4$ g, data pooled from experiments with ACh, ATP and CPA, $n = 15$) or in endothelium-denuded preparations ($3{\cdot}2 \pm 0{\cdot}5$ *vs.* $3{\cdot}1 \pm 0{\cdot}8$ g, data pooled from SNP and CPA experiments, $n = 10$).

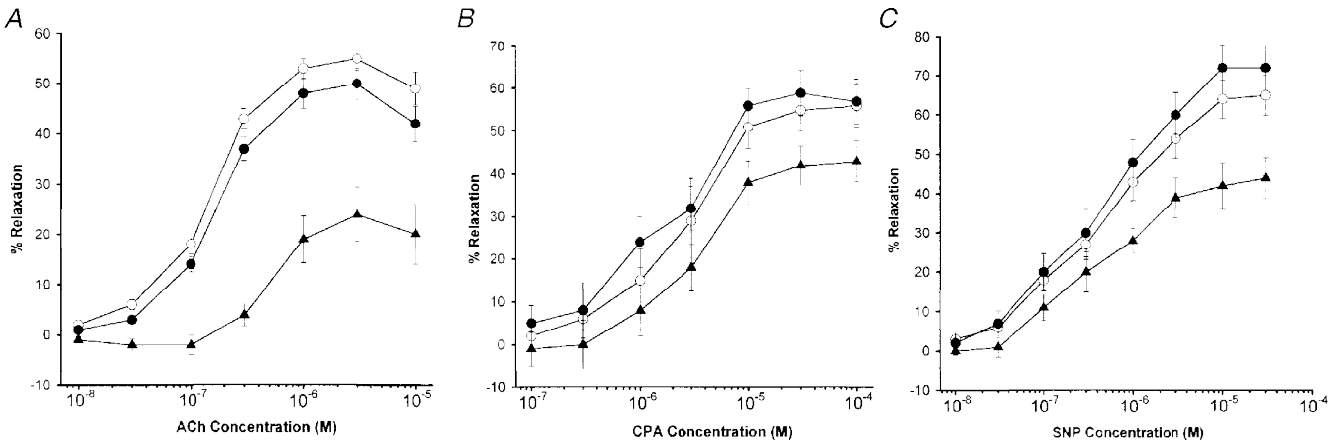


**Figure 4. Concentration–relaxation curves showing the effects of heptanol against ACh- (*A*), CPA- (*B*) and SNP- (*C*) induced relaxation in the thoracic aorta**

Heptanol (3 mM, ▲) significantly reduced relaxation to all three agents. ○ and ●, control and time-matched relaxations to each agent, respectively. SNP concentration–relaxation curves were obtained in endothelium-denuded rings.

### Effects of heptanol on ACh-, CPA- and SNP-induced relaxation in thoracic aorta

Heptanol (3 mM) mimicked the effect of Gap 27 peptide on the responses to ACh and CPA (Fig. 4*A* and *B*, $n = 4$ in each case) but unlike Gap 27 peptide also caused a significant concentration-dependent inhibition of SNP-induced relaxation (Fig. 4*C*, $P < 0{\cdot}05$, $n = 4$). There was a rightward shift in the concentration–relaxation curves to all three agents, with depression of maximal responses. The $EC_{50}$ value for ACh-induced relaxation increased 5-fold from $110 \pm 15$ to $610 \pm 28$ nM, and there were 2-fold increases in the $EC_{50}$ values for SNP ($320 \pm 25$ *vs.* $615 \pm 42$ nM) and CPA ($2{\cdot}8 \pm 0{\cdot}18$ *vs.* $6{\cdot}4 \pm 0{\cdot}32$ $\mu$M).

Heptanol (3 mM) also depressed the contraction evoked by 10 $\mu$M phenylephrine, data pooled from the ACh and CPA experiments showing that this agent caused an overall reduction in developed tone of $24 \pm 4\%$ ($n = 8$). In endothelium-denuded preparations a similar reduction of $21 \pm 4\%$ ($n = 4$, n.s.) was recorded.

### Effects of gap junction peptides 20 and 27, L-NAME and indomethacin on ACh-induced relaxation in the superior mesenteric artery

In the superior mesenteric artery maximal relaxation to ACh was ~90% of developed tension and significantly larger than that observed in the thoracic aorta on a relative basis ($P < 0{\cdot}01$). In contrast to the aorta also, L-NAME (300 $\mu$M) and Gap 27 peptide (300 $\mu$M) both attenuated maximal relaxation to ACh to a similar extent (i.e. ~40%; Fig. 5*A*). L-NAME caused a rightward shift in the concentration–relaxation curve such that there was an ~2·5-fold increase in the $EC_{50}$ value from $225 \pm 110$ to $520 \pm 60$ nM ($P < 0{\cdot}5$, $n = 10$). In the presence of L-NAME (300 $\mu$M) exposure to Gap 27 peptide at concentrations of 100 $\mu$M, 300 $\mu$M, 3 mM and 10 mM caused decreases in the maximal ACh-induced relaxation to ~47, ~32, ~28 and ~23%, respectively (Fig. 5*B*). There were corresponding rightward shifts in the concentration–response curves with $EC_{50}$ values for ACh-induced relaxation in the presence of L-NAME increasing ~2·5-fold for Gap 27 peptide at 100 $\mu$M ($215 \pm 26$ *vs.*

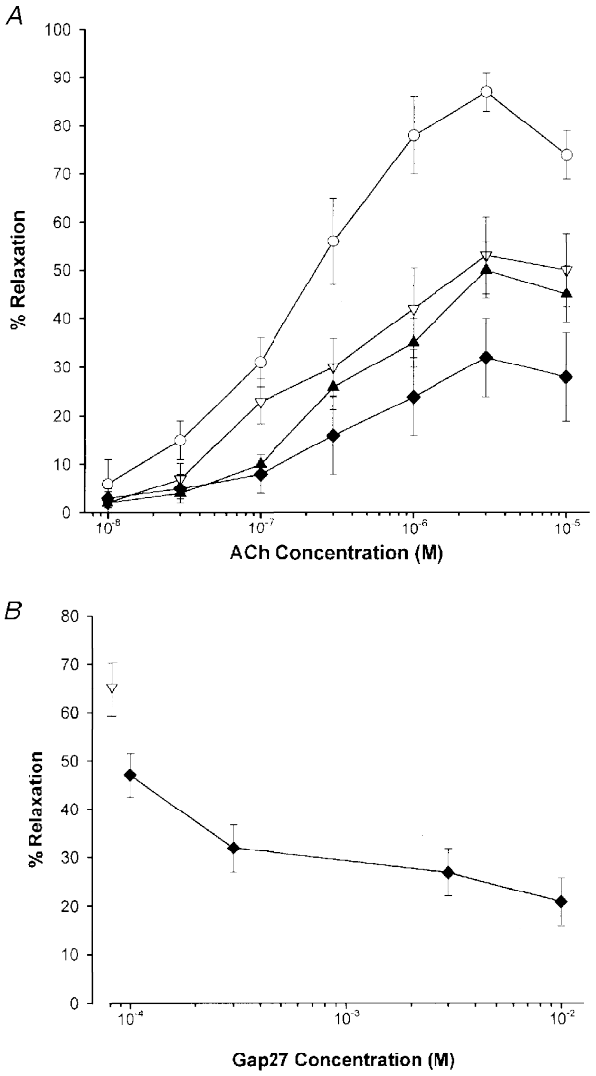


**Figure 5. Concentration–relaxation curves showing the effects of gap junction peptide 27 against ACh in superior mesenteric artery in the presence and absence of L-NAME**

*A*, maximal control ACh-induced relaxation (○) was greater in the superior mesenteric artery than in the aorta, although Gap 27 (300 $\mu$M) again resulted in ~40% inhibition (▲). In contrast to the aorta, however, L-NAME (300 $\mu$M) inhibited the maximum response to ACh by only ~40% (▽). These inhibitory effects of Gap 27 and L-NAME were synergistic (◆). *B*, concentration-dependent augmentation of the inhibitory effects of L-NAME (300 $\mu$M) by Gap 27 over the range 100 $\mu$M to 10 mM. ▽, maximum relaxation induced by ACh in the presence of L-NAME; ◆, maximum relaxations to ACh in the additional presence of Gap 27.

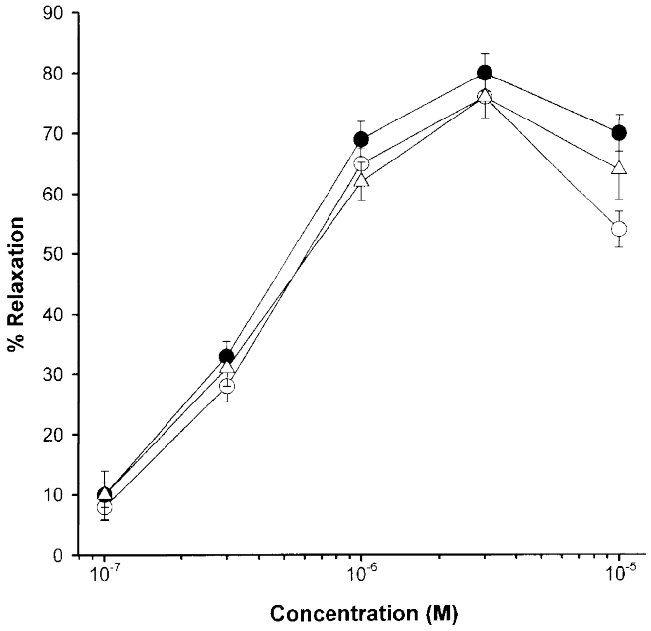


**Figure 6. Concentration–relaxation curves showing no effect of gap junction peptide 20 (3 mM, △) against responses to ACh in superior mesenteric artery**

○ and ●, control and time-matched responses to ACh, respectively.

510 ± 18 nM) and up to ~7-fold for 10 mM of the peptide (220 ± 30 *vs.* 1506 ± 21 nM).

Gap junction peptide 20 exerted no significant effect on ACh-induced relaxation in the superior mesenteric artery ($n = 4$, $P > 0{\cdot}05$, Fig. 6). As the L-NAME-insensitive component of the net relaxation was significantly larger in the superior mesenteric artery than in the aorta, Gap 20 peptide was employed as a control only in this artery type.

Preincubation with indomethacin (10 μM) did not significantly affect concentration–relaxation curves to ACh either in the presence or absence of L-NAME (300 μM, $n = 5$), Gap 27 peptide (300 μM, $n = 5$), or the combination of both inhibitors ($n = 5$; Fig. 7).

### Effects of gap junction peptide 27 and L-NAME on phenylephrine-induced contraction in endothelium-intact preparations in superior mesenteric artery

Administration of Gap 27 peptide (300 μM) did not significantly amplify the contractile response to 10 μM phenylephrine (2·3 ± 0·2 *vs.* 2·2 ± 0·15 g, $n = 10$, $P > 0{\cdot}05$) whereas L-NAME (300 μM) caused a significant increase in tone from 2·4 ± 0·2 to 3·1 ± 0·3 g ($n = 6$, $P < 0{\cdot}05$), attributable to inhibition of basal NO activity (Fig. 8). This

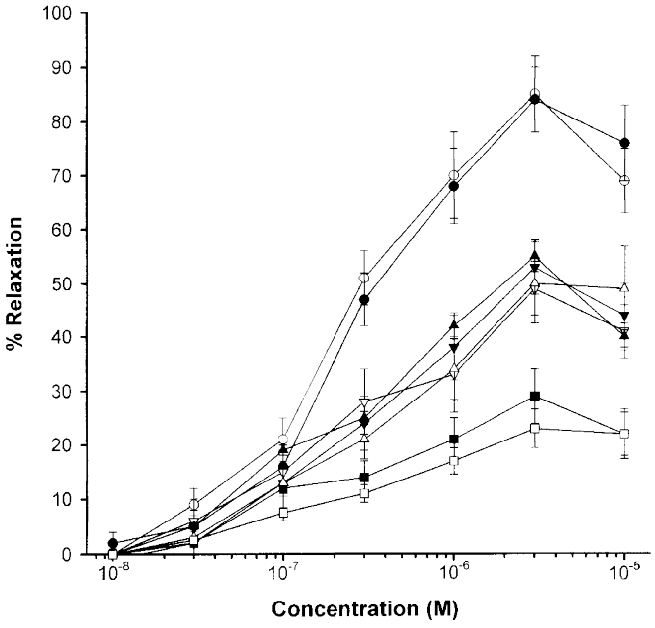


**Figure 7. Concentration–relaxation curves for ACh in superior mesenteric artery in the presence and absence of indomethacin**

Indomethacin (10 μM, ●) did not modulate control ACh-induced relaxations (○). The inhibition observed with Gap 27 (300 μM, △) or L-NAME (300 μM, ▽) was not amplified by indomethacin (▲ and ▼, respectively). Inhibition by the combination of Gap 27 and L-NAME (□) was similarly unaffected by indomethacin (■).

contractile effect of L-NAME was not modified by preincubation with Gap 27 peptide, tension increasing from $2{\cdot}5 \pm 0{\cdot}3$ to $3{\cdot}2 \pm 0{\cdot}35$ g ($n = 4$, $P < 0{\cdot}05$).

### 'Sandwich' experiments with thoracic aorta and superior mesenteric artery

ACh caused concentration-dependent relaxation of sandwich preparations, and matched control experiments revealed no significant decline in relaxation over time (Figs 9 and 10). As in intact ring segments, maximal relaxation to ACh was found at concentrations of ~3 $\mu$M, and constituted ~34% of developed tension with an $EC_{50}$ value of $1{\cdot}2 \pm 0{\cdot}14$ $\mu$M in aortic sandwich preparations ($n = 5$), and ~21% of developed tension with an $EC_{50}$ value of $1{\cdot}4 \pm 0{\cdot}16$ $\mu$M in the case of superior mesenteric artery ($n = 5$). In marked contrast to the situation in intact rings, in neither artery type was relaxation to ACh affected by Gap 27 peptide (3 mM) whereas L-NAME (300 $\mu$M) abolished relaxation completely (Fig. 10).

To confirm that diffusional factors did not account for the failure of Gap 27 peptide to inhibit relaxation in these composite preparations, substance P (10 nM), a peptidergic endothelium-dependent dilator, was used to confirm that peptides of similar length to Gap 27 were able to gain unimpeded access to the endothelium of the 'donor' arterial strip (Fig. 11). As in the case of ACh, relaxation with 10 nM substance P in sandwich preparations was unaffected by Gap 27 peptide (3 mM) whereas L-NAME (300 $\mu$M) abolished relaxation completely ($n = 5$; Fig. 11).

## DISCUSSION

The present experiments demonstrate that a major component of endothelium-dependent relaxation in rabbit conduit arteries can be attributed to direct endothelial–smooth muscle communication via gap junctions. The peptide Gap 27, which contains the motif SRPTEK present in the 2nd extracellular loop of connexins present in the vascular wall, attenuated ACh- and ATP-induced relaxations of endothelium-intact thoracic aorta and mesenteric arteries, whereas a control peptide Gap 20, which possesses homology with a sequence in the intracellular loop of Cx43, was inactive. This extends previous studies in which Gap 27 peptide inhibited rhythmic contractile activity in

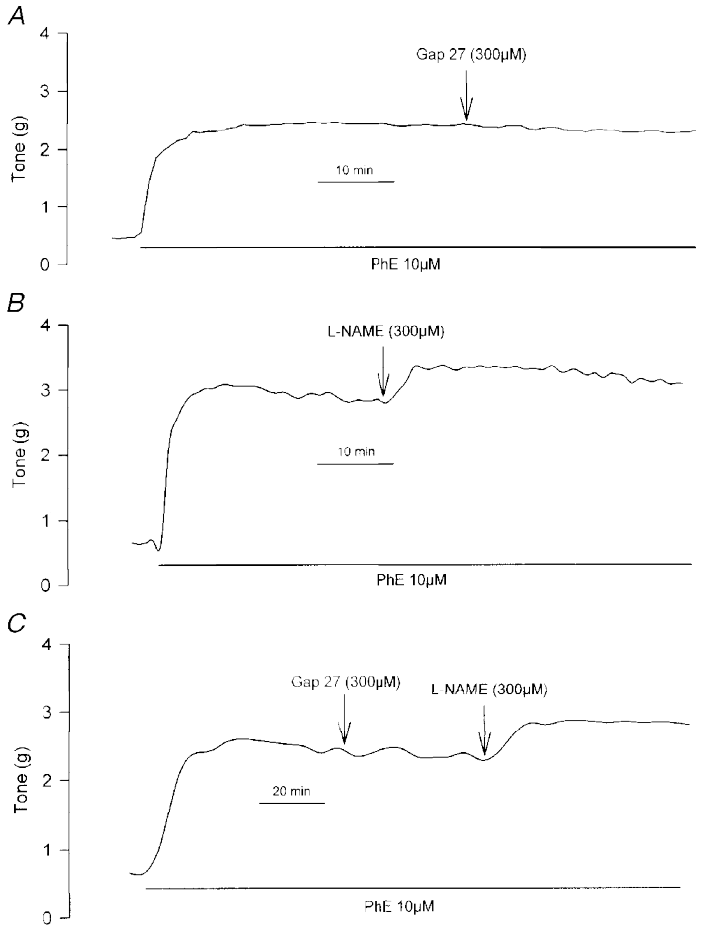


**Figure 8. Original traces showing effects of Gap 27 peptide and L-NAME on phenylephrine-induced contraction**

Force development was not enhanced by Gap 27 (300 $\mu$M, *A*) and rises in tension induced by L-NAME (300 $\mu$M) were similar in the presence (*C*) or absence (*B*) of this peptide.

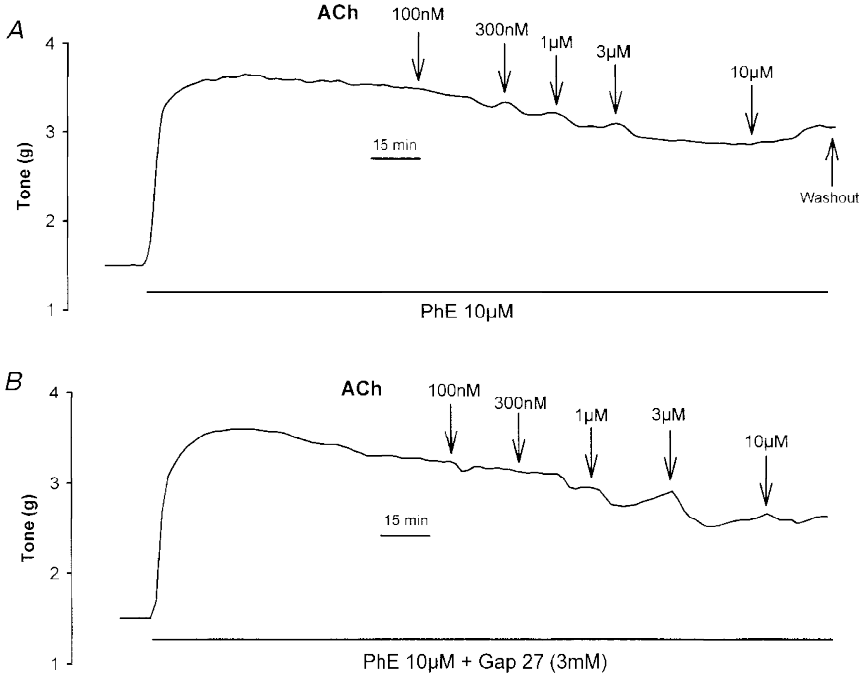


**Figure 9. Original traces showing ACh-induced relaxation in sandwich mounts of endothelium-intact and -denuded strips of thoracic aorta in the presence and absence of gap junction peptide 27**

*A*, control relaxation following constriction by phenylephrine (PhE). *B*, repeat protocol in the presence of Gap 27 (3 mM) showing no loss of relaxation. Gap 27 was similarly without effect on responses to ACh in sandwich mounts of mesenteric artery (not shown).

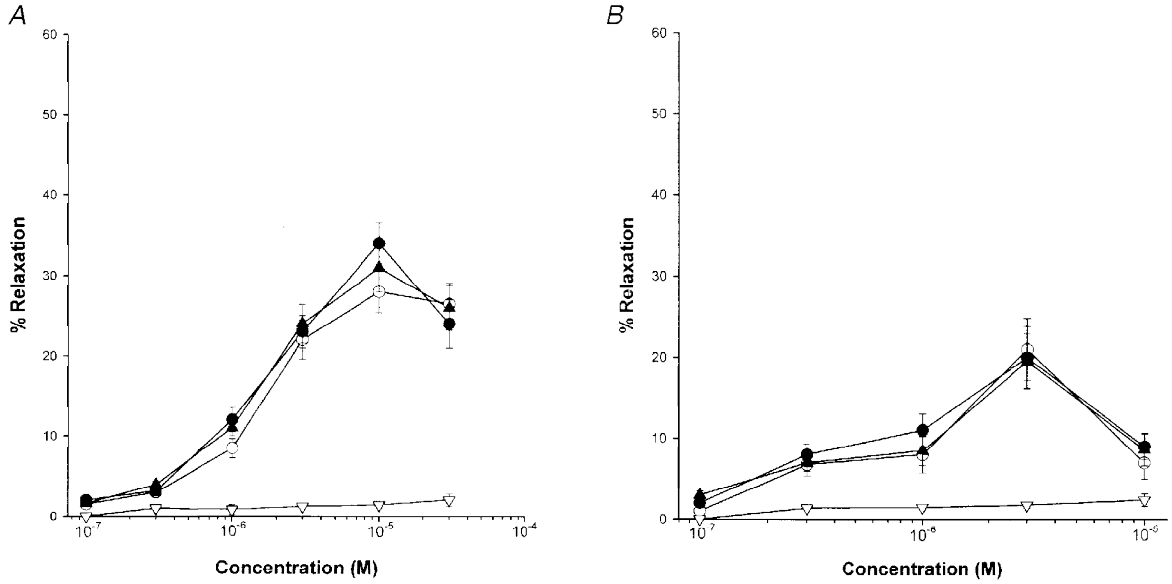


**Figure 10. Concentration–relaxation curves for ACh in sandwich preparations from thoracic aorta (*A*) and superior mesenteric artery (*B*)**

In contrast to the findings with intact rings Gap 27 (3 mM, ▲) was completely without effect, whereas L-NAME (300 μM, ▽) abolished responses to ACh in both artery types. ○ and ●, control and time-matched controls, respectively, in both panels.

endothelium-denuded rabbit arteries (Chaytor *et al.* 1997). The results show that this connexin mimetic undecapeptide may be regarded as a specific inhibitor of both hetero- and homocellular gap junctional communication in the vascular wall. Ultrastructural studies have confirmed the characteristic pentalaminar appearance of gap junctions in the myo-endothelial bridges present in rabbit conduit arteries (Spagnoli, Villaschi, Neri & Palmieri, 1982).

Maximal ACh-induced relaxations were smaller in the aorta (~60%) than in the superior mesenteric artery (~90%) and these responses exhibited differential susceptibility to inhibition of gap junctional communication and NO synthase. Gap 27 peptide (300 $\mu$M) thus caused a similar overall degree of inhibition (~40%) in both artery types, whereas 300 $\mu$M L-NAME caused ~90 and ~40% reductions in the response to ACh, respectively. In contrast, the endothelium-dependent component of ATP-induced relaxation in the aorta was attenuated by ~80% in the presence of either Gap 27 or L-NAME. These findings confirm the important contribution of NO to agonist-induced relaxation in both vessel types. However, the differing potencies of L-NAME and Gap 27 against ACh in the two artery types, and their equivalence against ACh and ATP in the aorta, suggest that the relative contribution of NO-dependent and -independent mechanisms of vasorelaxation varies between vessels and for different agonists.

Gap junctional communication was also shown to contribute to endothelium-dependent relaxation evoked by cyclopiazonic acid (CPA), an agent which stimulates capacitative $Ca^{2+}$ influx by inhibiting the endoplasmic reticulum $Ca^{2+}$ ATPase and depleting internal $Ca^{2+}$ stores (Graier, Simecek & Sturek, 1995). The resulting increase in cytosolic [$Ca^{2+}$] promotes NO synthesis (Pasyk, Inazu & Daniel, 1995) and, in some arteries, an NO-independent hyperpolarizing response that may contribute to relaxation (Fukao, Hattori, Kanno, Sakuma & Kitabatake, 1995). The characteristics of CPA-induced relaxation in thoracic aorta were comparable to those obtained with ACh, being of similar magnitude and equally susceptible to inhibition by L-NAME (300 $\mu$M). Furthermore, Gap 27 peptide (300 $\mu$M) attenuated relaxations to CPA by ~30%. The contribution of gap junctional communication to relaxation is therefore not restricted to activation of the endothelial cell via specific receptor-linked pathways.

ACh-induced relaxations of sandwich preparations of aorta and superior mesenteric artery were completely abolished by

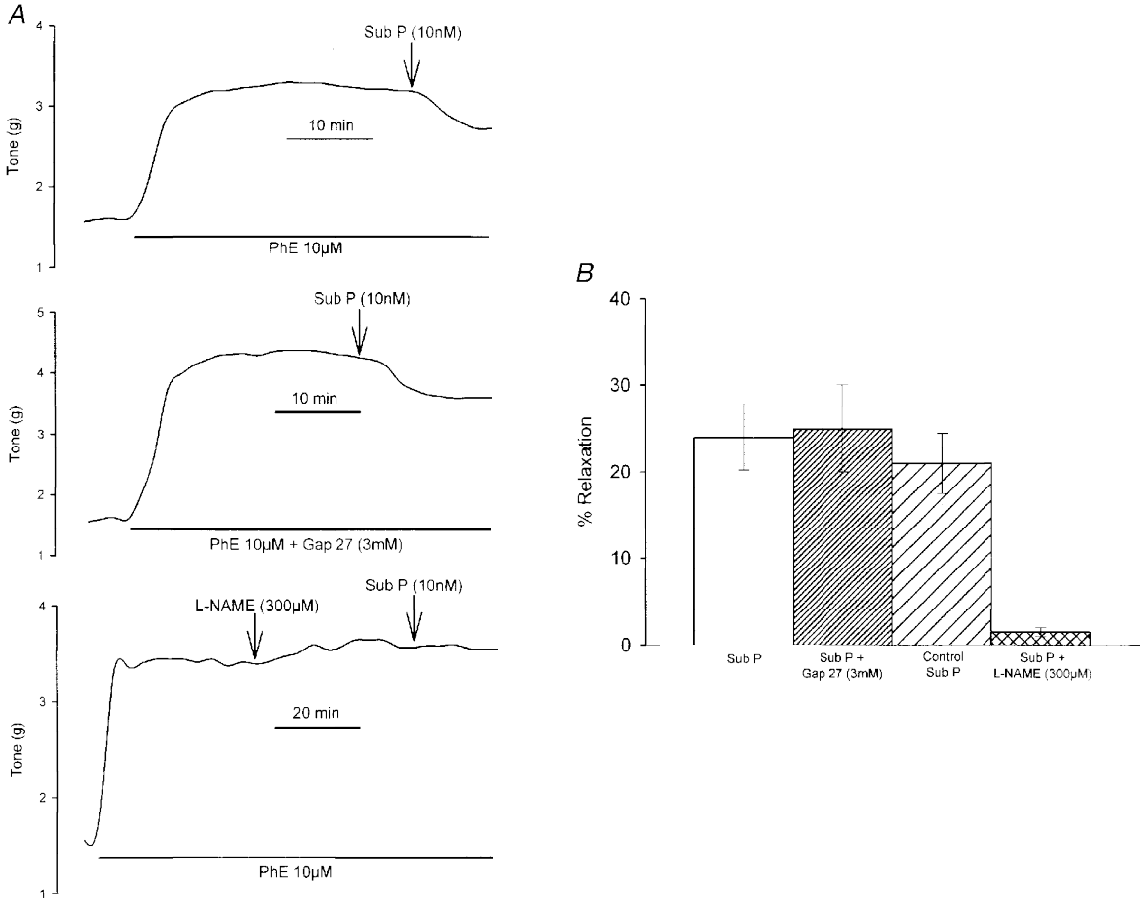


**Figure 11. Responses of sandwich preparations to substance P**

Original traces (*A*) and histograms (*B*) showing that Gap 27 did not affect responses to substance P (10 nM) in sandwich mounts from thoracic aorta, whereas L-NAME (300 $\mu$M) abolished relaxation completely.

L-NAME, and therefore mediated exclusively by NO. In such composite preparations the transit time between the endothelium and the media is very short so that a freely diffusible EDHF, even if unstable, should exert similar effects as in intact rings. The complete lack of effect of Gap 27 against relaxations to ACh and substance P in sandwich preparations confirms that this peptide does not inhibit the synthesis or release of NO by the endothelium in a non-specific fashion. It also confirms that the measured contractile responses of sandwich preparations derive solely from the endothelium-denuded strip (otherwise Gap 27 effects would have been detectable). Simple calculations thus suggest that the smaller maximal percentage relaxation to ACh in sandwich experiments compared with intact rings can be attributed to loss of gap junctional communication. In intact rings, the NO-dependent component of the ACh response can be estimated as the difference between the maximal relaxation obtained in the presence of Gap 27 and that obtained in the presence of Gap 27 and L-NAME. This gives ~30% (35–5%) for the aorta and ~18% (50–32%) for the SMA, which closely match the maximal relaxation of the sandwich preparations (34 and 21%, respectively).

As Gap 27 peptide was ineffective in sandwich experiments, the findings are also consistent with the view that direct endothelial–endothelial communication does not contribute to the release of NO (or other relaxant factors) in response to ACh and substance P. Indeed, elevations in endothelial $[Ca^{2+}]_i$ stimulated by local application of agonists do not appear to propagate within the endothelial monolayer (Honda, Goldhaber, Demer & Weiss, 1996). The lack of effect of Gap 27 peptide also excludes the possibility that NO preferentially relaxes/hyperpolarizes smooth muscle cells immediately subjacent to the endothelium and that these responses are then conducted regeneratively via gap junctions from superficial to deep layers of the media, thereby enhancing overall relaxation. Confirmation that the smooth muscle response to NO was independent of gap junctional communication was obtained in endothelium-denuded aortic rings in which Gap 27 peptide did not affect relaxation to SNP, an exogenous NO donor that would be expected to release NO uniformly throughout the media. Other studies have shown that the relative contributions of pathways mediating endothelium-dependent relaxation may be stimulus specific. In sandwich preparations of rabbit femoral artery Plane, Pearson & Garland (1995) found complete inhibition of ACh-induced relaxation by L-NAME, in agreement with the findings of the present study, whereas relaxation to the $Ca^{2+}$ ionophore A23187 was mediated by an NO-independent pathway involving hyperpolarization and therefore insensitive to L-NAME. The demonstration of such EDHF-type relaxations to A23187 in sandwich preparations confirms that the endothelium of the donor strip is not hypoxic and unable to synthesize EDHF. Indeed, rapid relaxation to ACh and substance P suggests that access of the Gap 27 peptide to the endothelium was not impeded by diffusional limitations resulting from apposition of the two component strips. Substance P and Gap 27 possess a similar number of amino acid residues.

At the present time the exact mode of action of connexin mimetic peptides in disrupting gap junction-mediated communication is unknown. In chick myoballs they delay the assembly of functional gap junctions (Warner *et al.* 1995). However, this is unlikely to be the sole mechanism of action in freshly isolated arterial tissue. These inhibitory peptides could act directly by destabilizing the interaction between the extracellular loops of connexons in preformed gap junctions, possibly by perturbing conformational changes that underlie gating. Residual L-NAME-resistant relaxations to ACh, ATP and CPA were almost abolished by Gap 27 peptide in the aorta. However, in the superior mesenteric artery attenuation of the maximal L-NAME-resistant response to ACh by Gap 27 peptide was concentration dependent, increasing from ~50% at 300 $\mu$M to ~80% at 10 mM, the highest concentration employed. Although relaxation was not completely abolished, we were unable to detect EDHF-like activity in sandwich preparations, and it is conceivable that gap junctional communication may not have been completely inhibited even at high peptide concentrations. Prior to the internalization of gap junctions, a process that allows their rapid turnover and renewal, connexons accrete into tightly packed gap junctional plaques in which there may be intercellular and intermolecular crosslinking due to disulphide rearrangements in the connexon extracellular loops. Such gap junctional heterogeneity could result in the inhibitory effects of Gap 27 peptide being confined mainly to smaller, more accessible gap junction domains which are thought to be more important for functional intercellular communication (Chen & Meng, 1995).

In rabbit mesenteric artery electrophysiological studies have shown that ACh-induced hyperpolarization is not mediated by NO, but becomes more transient in the presence of indomethacin (Murphy & Brayden, 1995). Prostanoids may therefore contribute to agonist-evoked hyperpolarization in this artery type. In the present study, however, concentration–relaxation curves for ACh alone or curves constructed in the presence of L-NAME, Gap 27 peptide, or their combination, were unaffected by inhibition of cyclo-oxygenase with indomethacin in the superior mesenteric artery. This indicates that any possible prostanoid-mediated effects on membrane potential were not coupled to force generation under the experimental conditions employed. Prostaglandin synthesis similarly does not contribute to ACh-induced relaxation in rabbit aorta (Forstermann & Neufang, 1984).

Dora *et al.* (1997) have shown that activation of small resistance arteries by phenylephrine or high $[K^+]$ medium is accompanied by the transmission of a signal from smooth muscle cells to the endothelium, possibly via gap junctions, which elevates endothelial $[Ca^{2+}]_i$ and promotes NO synthesis and release. In the present study contraction of endothelium-

intact aorta by phenylephrine was not enhanced by Gap 27 peptide and L-NAME-induced contractions were not amplified by Gap 27. These observations indicate that the peptide was without effect on the basal level of NO synthesis, and suggest that Gap 27 is unlikely to modulate relaxation to ACh, ATP and substance P in rabbit conduit arteries by interfering with the reverse smooth muscle–endothelial communication pathway proposed by Dora *et al.* (1997). As there may be polarity in the transfer of signals between endothelium and smooth muscle (Bény & Pacicca, 1994; Little *et al.* 1995), it is possible that rectifying properties of heterotypic gap junctions have functional consequences which remain to be evaluated.

Heptanol is often cited as an inhibitor of gap junctional communication but it possesses additional non-specific pharmacological properties, including a potent depressor effect on contraction in rat and rabbit arteries that was confirmed in the present study (Javid *et al.* 1996; Chaytor *et al.* 1997). Furthermore, heptanol has been reported to depress endothelium-independent relaxations to nitrovasodilators (Javid *et al.* 1996), and straight-chain alcohols possessing five to eight carbon atoms directly inhibit the NOS enzyme (Chen & LaBella, 1997). Although several reports have suggested that heptanol depresses relaxations to agonists such as ACh, including those obtained in the presence of indomethacin and inhibitors of NO synthesis or action (Kühberger *et al.* 1994; Javid *et al.* 1996), it is reportedly without effect on endothelium-dependent relaxation in rat hepatic artery (Zygmunt & Högestätt, 1996). In the present experiments, we demonstrated inhibitory effects of heptanol against not only endothelium-dependent ACh and CPA-induced relaxations, but also sodium nitroprusside. These observations reinforce the conclusion that heptanol may exert 'non-specific' effects on nitrovasodilator-induced relaxation as well as phenylephrine-induced contraction.

Our findings suggest that the NO-independent component of relaxation observed in the present study is likely to involve electrical coupling or diffusion of a low molecular weight mediator through gap junctions, rather than the extracellular diffusion of an EDHF. It has been proposed that the $Ca^{2+}$ influx evoked by agonists such as bradykinin and ATPase inhibitors such as CPA is mediated by cytochrome P450 mono-oxygenase-derived metabolites of arachidonic acid (such as 5,6-epoxyeicosatrienoic acid (5,6-EET)) whose formation is promoted by endothelial $Ca^{2+}$ store depletion (Graier *et al.* 1995). Others have shown that such metabolites hyperpolarize vascular smooth muscle by activating $K_{Ca}$ channels and have suggested that they may be identical to a physiological EDHF (Popp *et al.* 1996). It is presently unknown, however, if such arachidonic acid products are able to pass directly from endothlelial to smooth muscle cells via gap junctions, thereby bypassing the extracellular space. It also remains to be determined whether the apparently more pronounced NO-independent component of the response to endothelium-dependent agents observed in resistance as compared with conduit arteries (Hwa *et al.* 1994; Shimokawa *et al.* 1996) can be explained on the basis of anatomical factors. Endothelial–smooth muscle communication via gap junctions might be expected to have greatest functional importance in vessels where the endothelial : smooth muscle cell volume ratio is high. The greater gap junction-dependent component of relaxation seen in the SMA relative to the aorta in the present study is consistent with this hypothesis, but a wider range of vessels need to be studied to test the generality of the findings.

In conclusion, the present experiments have shown that relaxation to NO does not require the involvement of signals conducted via gap junctions, either from endothelium to smooth muscle, or between smooth muscle cells distal to the site of NO synthesis. Observations that a short peptide homologous to a sequence in the extracellular loop 2 of Cx43 is a potent and reversible inhibitor of endothelium-dependent relaxation in rabbit conduit arteries provide a new perspective into the mechanisms underlying the NO-independent component of the phenomenon, and in particular the postulated role of a freely diffusible EDHF.

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