Peptides homologous to extracellular loop motifs of connexin 43 reversibly abolish rhythmic contractile activity in rabbit arteries

A. T. Chaytor, W. H. Evans* and T. M. Griffith†

*Department of Diagnostic Radiology, Cardiovascular Sciences Research Group, and *Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK*

1. Phenylephrine (10 μM) evoked rises in tension in isolated rings of endothelium-denuded rabbit superior mesenteric artery. These increases consisted of a tonic component with superimposed rhythmic activity, the frequency of which generally remained constant over time but whose amplitude exhibited cycle-to-cycle variability.

2. The amplitude, but not the frequency, of the rhythmic activity was affected by a series of short peptides possessing sequence homology with extracellular loops 1 and 2 of connexin 43 (Cx43). Oscillatory behaviour was abolished at concentrations of 100–300 μM (IC_{50} of 20–30 μM), without change in average tone. No synergy was evident between peptides corresponding to the extracellular loops, and cytoplasmic loop peptides were biologically inactive.

3. The putative gap junction inhibitor heptanol mimicked the action of the extracellular loop peptides and abolished rhythmic activity at concentrations of 100–300 μM without effects on frequency. However, in marked contrast to the peptides, heptanol completely inhibited the contraction evoked by phenylephrine (IC_{50}, 283 $\pm$ 28 μM).

4. The presence of mRNA encoding Cx32, Cx40 and Cx43 was detected in the rabbit superior mesenteric artery by reverse transcriptase-polymerase chain reaction. Western blot analysis showed that Cx43 was the major connexin in the endothelium-denuded vessel wall.

5. We conclude that intercellular communication between vascular smooth muscle cells via gap junctions is essential for synchronized rhythmic activity in isolated arterial tissue, whereas tonic force development appears to be independent of cell–cell coupling. The molecular specificity of the peptide probes employed in the study suggests that the smooth muscle relaxant effects of heptanol may be non-specific and unrelated to inhibition of gap junctional communication.

In vivo, time-dependent oscillatory control of vascular resistance may offer significant physiological advantages over steady-state control. Vasomotion (i.e. rhythmic fluctuations in vascular calibre) has thus been suggested to promote lymphatic drainage, facilitate microcirculatory mass transport and provide a mechanism that allows temporal separation of mutually incompatible detector and effector mechanisms (Mohler & Heath, 1988; Intaglietta, 1991). Vasomotion may also allow highly flexible control of arteriolar resistance without the necessity for precise regulation of arterial and arteriolar calibre (Parthimos, Edwards & Griffith, 1996).

Under appropriate experimental conditions, intrinsic rhythmic activity is also readily evident in intact segments of isolated artery, suggesting the existence of communication pathways that permit macroscopic synchronization of the behaviour of relatively large numbers of individual vascular smooth muscle cells. Most cells in tissues and organs possess gap junctions that allow the direct intercellular communication that is necessary for cell growth and development (Warner, 1992). In the myocardium, gap junctions also permit dynamic co-ordination of the heart beat by allowing propagation of the action potential from one myocyte to another, and, in vascular smooth muscle, it has been suggested that tetraethylammonium induces rhythmic contractions by promoting gap junction activity (Takens-Kwak, Jongsma, Rook & Van Ginneken, 1992; Watts, Tsai, Loch-Caruso & Webb, 1994; Christ, Spray, el-Sabban, Moore & Brink, 1996). Gap junction proteins

† To whom correspondence should be addressed.

(connexins) are present in vascular smooth muscle and direct evidence for their participation in intercellular coupling has been provided by dye-transfer techniques (Watts *et al.* 1994; Christ *et al.* 1996). These observations provide strong evidence that gap junctions contribute to the regulation of vascular behaviour by permitting direct movement of ions and other small molecules between smooth muscle cells.

A gap junction is formed by two connexon hemichannels in register, each connexon consisting of six protein subunits or connexins (Yeager & Nicholson, 1996). A variety of studies have confirmed that there is conserved topography in the membrane among the thirteen connexins identified, with the protein crossing the cell membrane four times (Rahman & Evans, 1991). The amino terminus, the loop between transmembrane segments 2 and 3, and the carboxy terminus are on the cytoplasmic side with two loops on the extracellular side (Kumar & Gilula, 1996) (Fig. 1). It has been shown that peptides containing amino acid sequences corresponding to specific regions of extracellular loops 1 or 2 of connexin 32 (Cx32) delay the achievement of co-ordinated contraction in chick cardiac myocyte aggregates, strongly arguing that gap junction-mediated intercellular communication is necessary for synchrony (Warner, Clements, Parikh, Evans & DeHaan, 1995). Peptide sequences from loop 1 and loop 2 have been shown to be equally effective in inhibiting gap junctions with no synergism between them (Warner *et al.* 1995). In view of the conserved amino acid sequences that are present in the extracellular loops of different connexin proteins, this work suggests that it may be possible to devise a category of mimetic peptides with broad inhibitory characteristics with regard to intercellular communication underwritten by gap junctions.

In the present study we have investigated the role of gap junctional communication in two distinct aspects of vascular control, namely the co-ordination of dynamic time-dependent fluctuations in vascular tone, and the maintenance of time-averaged force development. Peptides with amino acid sequences corresponding to regions of the two extracellular loops of different connexins were used as specific probes, with peptides possessing sequences homologous to the intracellular loop serving as functionally inactive controls (Evans, Carlile, Rahman & Torok, 1992). Gap junctional communication is also thought to be susceptible to inhibition by the straight-chain alcohols heptanol and octanol, although their exact mechanism of action is unknown (Burt & Spray, 1989; Deutsch, Williams & Yule, 1995). The functional effects of gap junction peptides were therefore compared with those of heptanol. To ensure that the intercellular communication studied was exclusively between smooth muscle cells, the endothelium of the isolated ring preparations employed was removed by gentle abrasion at the beginning of all experiments.

METHODS

Isolated tissue preparations

Male New Zealand White rabbits (2–2·5 kg) were killed by administration of sodium pentobarbitone (120 mg kg^{-1} intravenously). The superior mesenteric artery was removed and stripped of adherent connective and adipose tissue. Rings 2–3 mm wide were cut and denuded of endothelium by gentle rubbing of the intimal surface with a roughened probe. Each ring was suspended in a 6 ml tissue bath containing oxygenated (95% O_2–5% CO_2) 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) at 37 °C and was placed under 0·6 g tension. Each preparation was then allowed to equilibrate for $\geqslant$ 1 h in order to allow stress relaxation to occur following washout at 20 min intervals.

Experimental protocol. Following the initial equilibration period, the rings were contracted by phenylephrine (10 μM). Using FT 102 force transducers (ADInstruments, Hastings, UK), fluctuations in tone were observed in 98 out of 117 rings, and were subsequently allowed to stabilize for 20–30 min before cumulative concentration–response curves to gap junction peptides or heptanol were constructed. Some rings were not challenged with the peptides or heptanol in order to obtain matched time controls. To provide a quantitative index of rhythmic activity, the mean amplitude of the oscillatory excursions at the end of the phenylephrine stabilization period was determined by averaging over 5 min. Subsequent changes in amplitude were expressed as a percentage of this control value. This method of quantifying rhythmic activity was adopted because its frequency remained unaltered during the various experimental protocols (see Results). At the end of each experiment, acetylcholine (1 μM) was added to confirm the absence of endothelium. The amino acid sequences of the synthetic peptides employed are given in Fig. 1.

The effects of a range of antibodies generated against specific amino acid sequences found in Cx32 and Cx43 on rhythmic contractile activity were also investigated. The rabbit immunoglobulins used were generated to Gap 7M (VWGDEKSSF ICNTLQPGY), Gap 11 (YVFYLLYPGYAMVRLVK CEAFPCPNTVDCFVSRPTEK), Des 3 (FMKGEIK NEFKDIEC) and Gap 15 (EIKKFKYGIEEHC), which have been extensively characterized (Rahman & Evans, 1991; Becker, Evans, Green & Warner, 1995). The immunoglobulin fractions prepared from serum by protein A sepharose chromatography were diluted to 10 and 1% in buffered Holman's solution. Following addition of phenylephrine (10 μM) to the rings and stabilization of rhythmic contractions (30 min), 5–100 μl IgG was added from either the 1 or 10% solutions. The IgG was incubated with the rings for 30–45 min before addition of the next cumulative concentration. The effects of the antibodies were investigated individually and in combination.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA isolation. The superior mesenteric artery (average individual weight, 12 mg) was removed, cleaned, and gently denuded of endothelium. RNA was extracted by a single-step method using the commercially available Ultraspec™ method (Biotecx Laboratories, Houston, TX, USA). The artery was homogenized in 1 ml Ultraspec™ RNA reagent with a hand-held glass–Teflon homogenizer. The homogenate was stored for 5 min at 4 °C to allow the complete dissociation of the nucleoprotein complexes. Choroform (200 μl) was added and the sample was left for 5 min at 4 °C before centrifugation at 12 000 *g* (4 °C) for 15 min. The RNA-containing

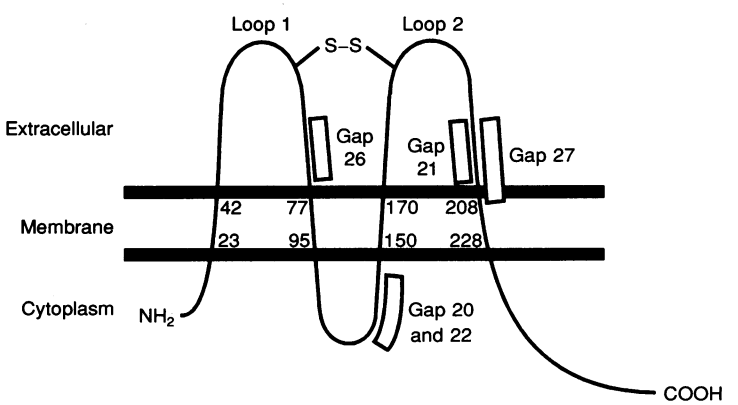
aqueous phase was collected and an equal volume of isopropanol was added; this was left at 4 °C for 10 min and then the RNA pellet was collected by centrifugation (12000 *g*, 4 °C). The supernatant was removed and the pellet was washed by being vortexed in ethanol, followed by centrifugation for 5 min (7500 *g*, 4 °C). The dried pellet was dissolved in 50 μl Ultraspec DEPC-treated water by being vortexed for 1 min. The sample was then incubated for 15 min at 60 °C.

Reverse transcription. Reverse transcription of the mRNA species of the connexins, and subsequent PCR amplification of the cDNA, was carried out using the GeneAmp RNA-PCR protocol (Perkin Elmer, Norwalk, CT, USA). Reverse transcriptase buffer, deoxyribonucleoside triphosphates (dNTPs; 1 μl, 20 mM), 0·1 M dithiothreitol (DTT; 2 μl, 10 mM) and distilled water (to make a total volume of 18 μl) were added to each sample solution. Sample RNA (2 μg, determined by measurement of the absorbance at 260 nm) was used together with positive controls for Cx26, Cx32 and Cx43 from total RNA extracted from rat liver, kidney and heart, respectively; sample cDNA was used as a negative control. Each sample was heated at 80 °C for 4 min to denature the RNA secondary structure and placed on ice. Following centrifugation, RNasin inhibitor (40 u, 1 μl; Promega, Madison, WI, USA) and Moloney Murine Leukaemia virus (MMLV) reverse transcriptase (200 U, 1 μl; Gibco BRL, Life Technologies, Paisley, UK) were added. Each sample was then subjected to 25 °C (10 min), 42 °C (40 min) and 99 °C (2 min) incubations in a programmable thermal cycler (Perkin Elmer) and then placed on ice.

PCR amplification. Taq buffer (Thermobuffer, Promega; 10× concentrated; 5 μl), dNTPs (0·5 μl, 20 mM; Pharmacia Biotech, St Albans, UK), primers 1 (forward primer) and 2 (reverse primer) (1 μl, 20 pmol μl^{-1}), $MgCl_2$ (3 μl, 25 mM), and distilled water (to make the volume up to 50 μl) were added to each sample. The primers were from rat sequences as used by Carter, Chen, Carlile, Kalapothakis, Ogden & Evans (1996). A thin film of mineral oil (50 μl) was introduced to each sample prior to heating. The PCR was hot started at 95 °C for 2 min and Taq polymerase (0·2 μl, 2·5 U; Promega) was added to each sample. The following cycle was repeated 35 times: 1 min at 95 °C, 1 min at 60 °C (annealing step) and 1·5 min at 72 °C (extension step), and finally 10 min at 72 °C (Monaghan, Clarke, Perusinghe, Moss, Chen & Evans, 1996). The PCR samples were visualized using fluorescence photomicrography of an ethidium bromide-stained 5% agarose gel. Full length cDNA to Cx26, Cx32 and Cx43 was used as a positive control; amplified mRNA of Cx37 and Cx40, present in lung, heart and endothelial cells, has been obtained using the same primers (Carter *et al.* 1996).

Western blotting

Western blotting was performed as described by Towbin, Staehelin & Gordon (1979). Superior mesenteric arteries and heart and liver samples were removed and placed individually in 1 mM $NaHCO_3$



Peptide	Connexin	Topographical position	Sequence	Residue no.
Gap 20	43	Intracellular loop	EIKKFKYGC	131–138
Gap 22	40	Intracellular loop	AELSCNKEVNG	130–140
Gap 21	32/43	Extracellular loop 2	SRPTEKT	182–188/204–210
Gap 26	43	Extracellular loop 1	VCYDKSFPISHVR	63–75
Gap 27	43	Extracellular loop 2	SRPTEKIFII	204–214

Figure 1. Amino acid sequences of peptides possessing homology with the extracellular and intracellular loops of connexin proteins that were used to investigate rhythmic vasomotor activity

The positions of the synthetic peptides employed in a topographical map of Cx43 is shown. Gaps 21, 26 and 27 are indicated to be at the interface of the carboxyl end of loops 1 and 2, with Gap 27 penetrating into the lipid bilayer. Gaps 20 and 22 (control peptides) both locate to sequences at the right-hand side of the intracellular loop. Antibodies were generated to the following peptides conjugated to limpet haemocyanin: Gap 7M corresponding to residues 43–59 in loop 1 of Cx32; Gap 11, residues 151–187 in loop 2 of Cx32; Des 3, residues 106–119 in the cytoplasmic loop of Cx26; Gap 15, residues 131–142 in the cytoplasmic loop of Cx43; and Gap 17, residues 331–344 (cytoplasmic tail) of Cx40.

buffer (pH 7·1). Owing to the small size of the arterial segments, tissue was pooled from forty rabbits to give a sample of ~500 mg weight. Tissues were homogenized in buffer at 4 °C containing protease inhibitors (10 μg ml^{-1} each of pepstatin A, leupeptin, chymostatin, antipain and aprotonin) and 1 mM phenylmethyl sulphonyl fluoride. Membrane fractions were prepared from the tissue homogenates by extraction with 20 mM NaOH and sonication and proteins were resolved by SDS–polyacrylamide gel electrophoresis using 12% acrylamide, followed by electrophoretic transfer to nitrocellulose membranes (Hybond, Amersham International). Membranes were blocked overnight at 4 °C with 5% dry skimmed milk in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7·5) containing 0·1% v/v Tween (TBS-T). Membranes were incubated for 1 h with the following primary antibodies (1 : 500 dilution): Gap 15 (Cx43), Gap 11 (Cx32), Gap 17 (Cx40) and Des 3 (Cx26) (see Fig. 1), in TBS-T milk. The membranes were then repeatedly washed in TBS-T for 1 h and then blocked with 5% TBS-T milk for 30 min. They were then incubated with the secondary alkaline phosphatase-linked goat anti-rabbit IgG (1 : 2000 dilution; BioRad Labs, Hemel Hempstead, UK) for 2 h. Chemical detection of the secondary antibody was performed using nitrobluetetrazolium (0·3 mg ml^{-1}) and brom-chloro-indolyl phosphate (0·15 mg ml^{-1}) in bicarbonate buffer (0·1 M $NaHCO_3$, 1·0 mM $MgCl_2 \cdot 6H_2O$, pH 9·8).

Materials

Phenylephrine and acetylcholine were supplied by Sigma Chemical Co. Gap junction peptides were supplied by Severn Biotech Ltd, Kidderminster, UK, and the National Institute for Medical Research, Mill Hill, London, UK.

Statistics

Data were analysed using a one-way analysis of variance (ANOVA) with the Bonferroni multiple comparisons test as a further method of analysis. $P < 0·05$ was considered significant. IC_{50} values are expressed as means ± S.E.M.

RESULTS

Comparison of the effects of intracellular and extracellular gap junction peptides on rhythmic activity and ambient tone

Gap junction peptides 21, 26 and 27, which correspond to amino acid sequences shown by topographical studies to be positioned in the two extracellular loops of connexins (Fig. 1), attenuated the rhythmic responses of rabbit superior mesenteric arteries in a concentration-dependent fashion, generally resulting in complete abolition at 100–300 μM, although in some preparations rhythmic activity was abolished at even lower concentrations (Figs 2*A* and 3*A*). None of these peptides altered the fundamental frequency of the rhythmic activity (see, e.g. gap junction peptide 27; Fig. 2*A*). The mean frequency was thus 0·108 ± 0·002 Hz after administration of phenylephrine and 0·107 ± 0·002 Hz at the highest concentration of peptide at which rhythmic activity could still be detected ($n = 53$ rings; data pooled for peptides 21, 26 and 27). By contrast, the amplitude of the rhythmic activity usually varied between cycles, even in the control situation (Fig. 2*A*). To quantify the effects of the peptides, the average amplitude was determined over a 5 min period immediately prior to the addition of each successive dose and concentrations producing 50% reduction in this statistic relative to control were calculated (Fig. 3*A*). The IC_{50} values thus obtained were 29·9 ± 4·7 μM for gap junction peptide 21 ($n = 16$), 28·4 ± 3·4 μM for gap junction peptide 26 ($n = 25$) and 31·5 ± 4·1 μM for gap junction peptide 27 ($n = 12$); these values were not significantly different from each other.

The inhibitory action of all three peptides was relatively rapid, with tone stabilizing at a non-oscillatory steady-state value 20·5 ± 2·1, 11·9 ± 1·6 and 14·7 ± 2·8 min following administration of gap junction peptides 21, 26 and 27, respectively, at the dose that ultimately abolished rhythmic activity ($n = 16$, 25 and 12, respectively). The reversibility of action of the peptides was tested in a subset of experiments following repeated washout at 10 min intervals for 60 min and reconstriction with 10 μM phenylephrine (e.g. for Gap 27 see Fig. 2*A*, right-hand panel). Overall, rhythmic activity reappeared in 6/6, 18/20 and 8/8 rings in which it was previously abolished by 100–300 μM of gap junction peptides 21, 26 and 27, respectively.

Gap junction peptides 20 and 22, which possess homology with the intracellular loop of connexins, did not have a significant effect on phenylephrine-induced oscillations, the small reduction in rhythmic activity observed over the course of the experiments matching that of appropriate time controls ($n = 12$ for each peptide; Figs 2*C* and 3*B*).

The possibility of synergy between the extracellular gap junction peptides 26 and 27 was also investigated (Fig. 4). When equal concentrations of these peptides were administered simultaneously no enhancement of their individual inhibitory effects was observed, the combined Gap 26–27 IC_{50} value against rhythmic activity being 27·3 ± 4·59 μM. This was not significantly different from that obtained for Gap 26 or 27 peptides alone ($n = 6$). Following administration of the combined dose that ultimately abolished rhythmic activity, a non-oscillatory steady state became established after 11·4 ± 1·8 min ($n = 6$, not shown). Combined application did not therefore significantly enhance the rate of inhibition observed with either agent alone.

Control preparations usually exhibited a small time-dependent decline in ambient force development over the course of the experiments, but this was not enhanced by administration of gap junction peptides 21, 26 and 27 (e.g. Gap 27, Fig. 5*B*). The lack of effect of gap junction peptide 27 on ambient phenylephrine-induced tone at concentrations up to 3 mM was demonstrated quantitatively by pooling data from preparations that exhibited rhythmic activity and preparations in which responses were essentially tonic in nature ($n = 6$; Fig. 6). Analogous experiments confirmed that gap junction peptide 20 did not have a direct effect on ambient tone at concentrations up to 1 mM (Fig. 6). Contraction to a second dose of 10 μM phenylephrine was not significantly impaired by exposure to 3 mM gap junction

peptide 27; after repeated washout of all agents for 1 h a second challenge with phenylephrine induced 75·3 ± 12·9% of the initial force development, compared with 87·8 ± 12·5% in control preparations not previously exposed to the peptide (n.s. (not significant); $n = 6$ in both cases).

In a further series of experiments, the effects of antibodies generated to specific amino acid sequences in connexin proteins on rhythmic activity and ambient tone were also investigated. These included the Gap 15 antibody, which is specific for an intracellular loop of Cx43; the Gap 7M and Gap 11 antibodies, which are specific for regions found in Cx32, with Gap 7 being directed against a region of the extracellular loop 1 and Gap 11 against a major part of extracellular loop 2; the Des 3 antibody, which is directed

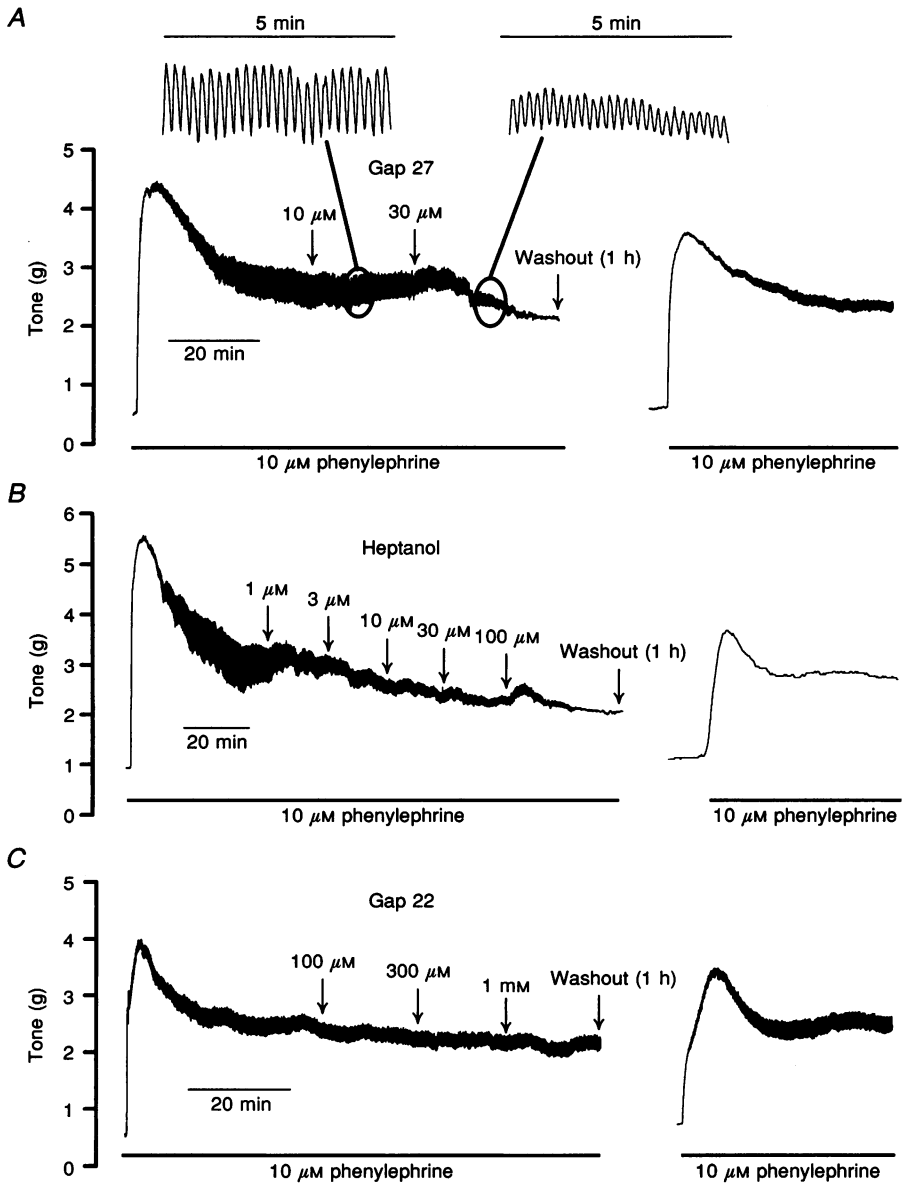


Figure 2. Representative traces illustrating the effects of synthetic gap junction peptides and heptanol on rhythmic activity induced by 10 μM phenylephrine

Peptides homologous to the extracellular loops of Cx43, e.g. Gap 27 (*A*) and the straight-chain alcohol heptanol (*B*) inhibited oscillations in a concentration- and time-dependent fashion, whereas peptides homologous to the intracellular loop, e.g. Gap 22 (*C*), were inactive. Peptides and heptanol were added at the times indicated by the arrows. The insets in *A* show that the responses induced by phenylephrine normally exhibited cycle-to-cycle fluctuations in amplitude, but that their frequency remained constant even following intervention, e.g. administration of gap junction peptide 27. The right-hand panels in *A–C* show that the effects of gap junction peptides were reversible on washout, but that persistent depression of rhythmic activity was observed following washout of heptanol in the example illustrated.

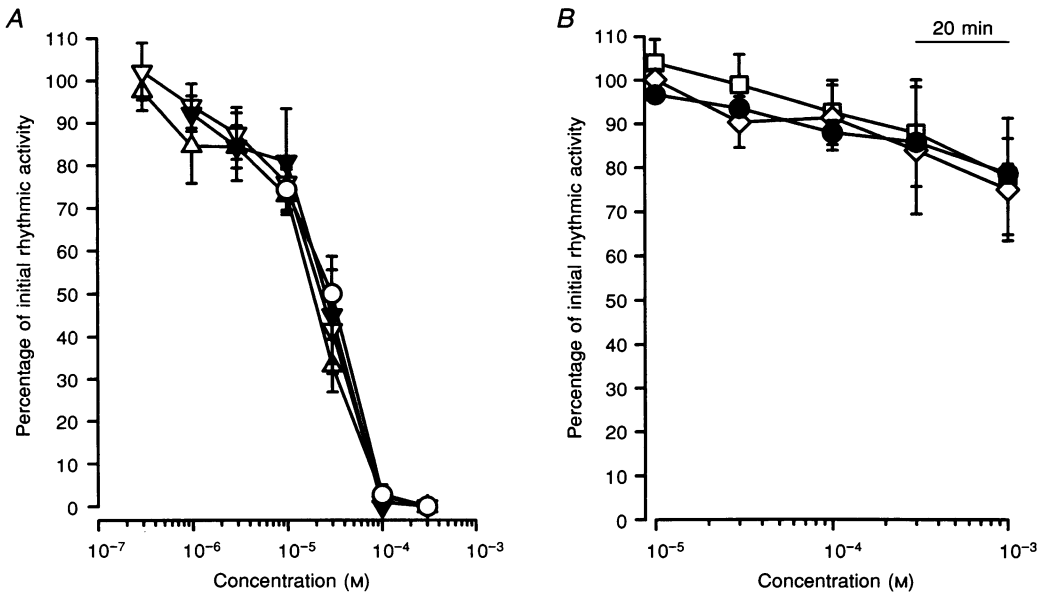


Figure 3. Concentration–inhibition curves comparing the effects of gap junction peptides and heptanol on phenylephrine-induced rhythmic activity

A, heptanol (▼) and gap junction peptides 21 (○), 26 (△) and 27 (▽) caused complete cessation of rhythmic activity at concentrations of 100–300 μM. *B*, gap junction peptides 20 (□) and 22 (◇) were inactive at concentrations up to 1 mM. Note the small reduction in rhythmic activity over time in experiments in which no exogenous agent was administered (●).

against the intracellular loop of Cx26; and the Gap 17 antibody, which is directed against the cytoplasmic tail of Cx40 (Carter *et al.* 1996). Neither of the two antibodies ganerated against extracellular loops, viz. Gap 7M and Gap 11, either individually or together, altered rhythmic activity or influenced ambient tone (not shown; $n = 4$).

Effects of heptanol on rhythmic vasomotor activity and ambient tone

Heptanol closely mimicked the effects of gap junction peptides 21, 26 and 27 by causing a concentration-dependent reduction in the amplitude of the rhythmic activity induced by phenylephrine. Again there was no change in the frequency of the oscillatory behaviour, which was 0·110 ± 0·002 Hz in preparations constricted by phenylephrine and 0·110 ± 0·001 Hz at the highest concentration at which fluctuations could still be detected in the presence of heptanol (n.s., $n = 7$; Fig. 2*B*). Usually, there was complete cessation of rhythmic activity with 100–300 μM heptanol, and this occurred on average some 13 ± 3 min after the administration of the highest inhibitory dose. The IC_{50} was 45·4 ± 8·2 μM ($n = 7$), which was not significantly different

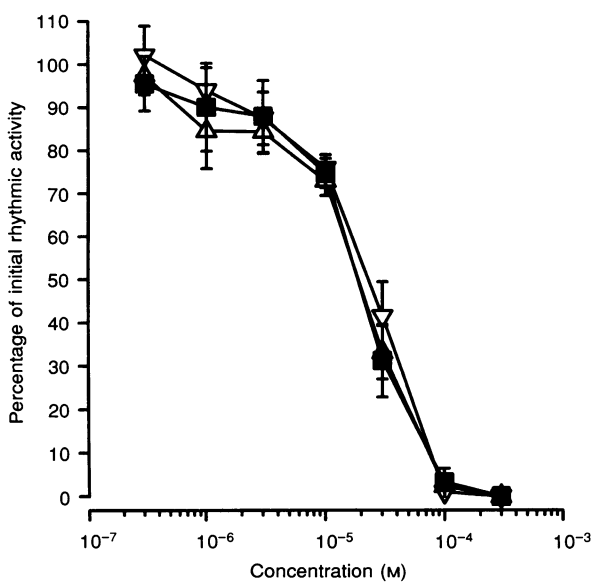


Figure 4. Lack of synergy between gap junction peptides 26 and 27

Concentration–inhibition profiles for these peptides were similar whether administered singly or in combination. △, Gap 26; ▽, Gap 27; ■, Gap 26 + Gap 27.

from the IC_{50} values for the three extracellular loop gap junction peptides, so that the concentration–inhibition curves for the peptides and heptanol could be superimposed (Fig. 3*A*). In marked contrast to the peptides, however, heptanol caused concentration-dependent relaxation such that ambient phenylephrine-induced tone ultimately declined back to baseline at a concentration of 3 mM (Figs 5 and 6). When data were pooled for preparations exhibiting oscillatory and tonic responses, half-maximal relaxation was found at an IC_{50} value of 283 ± 28 μM ($n = 13$).

In preparations in which rhythmic activity was abolished by 100–300 μM heptanol, oscillatory behaviour was restored in only 2/7 rings that were repeatedly washed for 1 h and then reconstricted with phenylephrine (e.g. Fig. 2*B*). Furthermore, the smooth muscle relaxant action of 3 mM heptanol was only partially reversible after ~60 min washout, the repeat contraction to 10 μM phenylephrine on a second challenge being 37 ± 9% of that obtained in appropriate control preparations ($n = 5$, $P < 0{\cdot}01$). In two of these rings, the tone regained after washout was < 10% of control.

RT-PCR and Western blot analysis

RT-PCR was used to characterize connexin mRNA in the rabbit superior mesenteric artery. Figure 7 shows the RT-

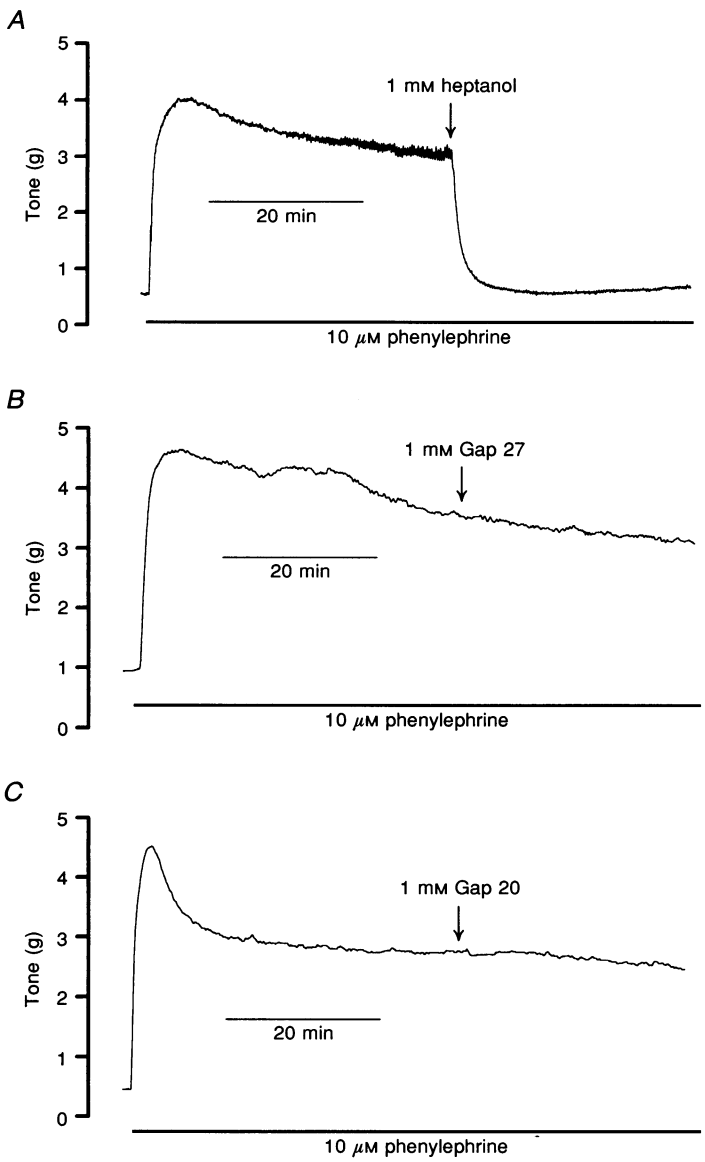


Figure 5. Representative traces contrasting the effects of heptanol and gap junction peptides on ambient phenylephrine-induced tone

Heptanol at a concentration of 1 mM caused marked relaxation (*A*) whereas neither gap junction peptide 27 (1 mM; *B*) nor gap junction peptide 20 (1 mM; *C*) enhanced the time-dependent decline in tone that followed peak contraction. Heptanol and peptides were added at the times indicated by the arrows.

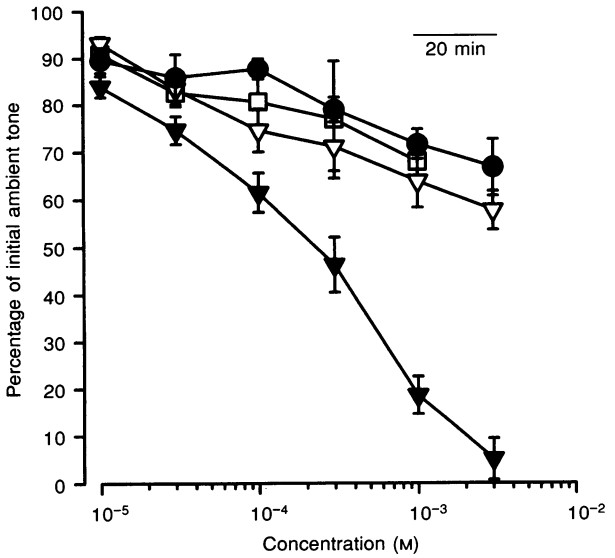


Figure 6. Concentration–relaxation curve for heptanol against ambient phenylephrine-induced tone

Heptanol caused a concentration-dependent decrease in tone, which ultimately fell to preconstriction levels. The small fall in tension observed during similar experiments with gap junction peptides 20 and 27 was non-specific as it matched that found in control preparations. □, Gap 20; ▽, Gap 27; ▼, heptanol; ●, time control.

PCR products obtained using primers directed towards Cx26, Cx32, Cx37, Cx40 and Cx43. Primers to Cx32, Cx40 and Cx43 produced PCR products of the predicted size (386, 399 and 295 bp, respectively), whereas primers to Cx26 and Cx37 revealed no products.

Western blotting using sequence-specific antibodies to Cx26 (Des 3), Cx32 (Gap 10), Cx40 (Gap 17) and Cx43 (Gap 15) were used to investigate the presence of connexin proteins in the rabbit endothelium-denuded superior mesenteric artery, heart and liver. Figure 8 shows that the Gap 15 antibody detected Cx43 as a band at ~43 kDa in the rabbit superior mesenteric artery and heart. Bands of the appropriate molecular mass were not detected for Cx26, Cx32 and Cx40 in the superior mesenteric artery.

DISCUSSION

The present experiments have demonstrated for the first time that peptides homologous to specific regions of the extracellular loop sequences of connexin proteins reversibly abolish rhythmic vasomotor activity in isolated endothelium-denuded arterial rings. The specificity of these molecular probes is emphasized by results showing that peptides containing sequences homologous to the intracellular loops of connexins, by contrast, were completely without effect. Previous investigations by Warner *et al.* (1995) have shown that extracellular loop gap junction peptides corresponding in sequence to Cx32 increased the time taken for aggregates of chick heart myocytes ('myoballs') to synchronize their beating, with peptide chains as short as seven amino acids significantly delaying gap junction formation. Although the

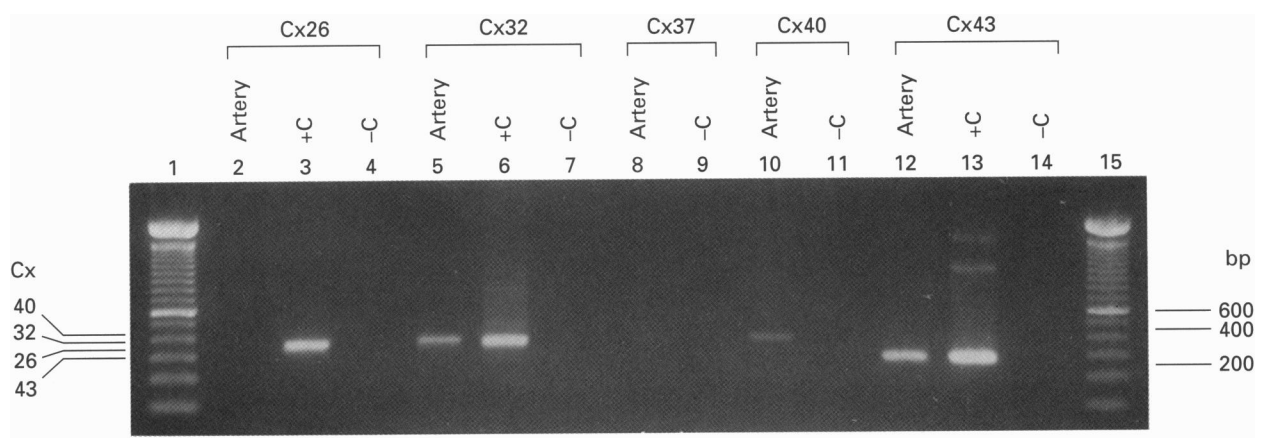


Figure 7. RT-PCR analysis of the rabbit superior mesenteric artery

Fluorescence photomicrograph of ethidium bromide-stained agarose (5%) revealed mRNA-encoded sequences for Cx32, Cx40 and Cx43 in lanes 5, 10 and 12, respectively. Lanes 1 and 15 contain markers. +C, positive control (for Cx26, Cx32 and Cx43; full length cDNA amplified from total RNA extracted from rat liver, kidney and heart, respectively); −C, negative control (sample cDNA).

length of these peptide chains did not correlate exactly with inhibitory action, this approach provided compelling evidence that connexon docking and gap junction formation are essential for synchronizing the contractile behaviour of cardiocytes. One novel implication of the present study is that peptides possessing homology with extracellular connexin loops are also able to interfere with the function of preformed gap junctions, and thereby interupt or abolish intercellular communication. In marked contrast to their effects on rhythmic behaviour, extracellular loop gap junction peptides exerted no effect on the ambient level of tone induced by phenylephrine, even at concentrations considerably higher than those that abolished oscillatory behaviour. Gap junction-mediated intercellular communication between smooth muscle cells thus plays a central role in the dynamic behaviour of the arterial wall, but does not contribute to the maintenance of time-averaged force development.

Warner *et al.* (1995) found that the amino acid motifs QPG and SHVR of extracellular loop 1 and the SRPTEK motif of extracellular loop 2 were important for achieving synchrony in cardiocytes. In the present study, gap junction peptides 21 and 27, which both contain the motif SRPTEK, and gap junction peptide 26, which contains the motif SHVR, were analogously found to inhibit rhythmic activity in vascular smooth muscle. By contrast, gap junction peptides 20 and 22, which correspond to intracellular loop regions of Cx43 and Cx40, respectively, were functionally inactive, and this presumably reflects the fact that intracellular loops cannot directly contribute to connexon docking. Previous studies have detected both Cx43 and Cx40 proteins in the artery wall (for review, see Christ *et al.* 1996). In the rabbit superior mesenteric arteries employed in the present study, Western blot analysis identified Cx43 as the major connexin actually expressed, although RT-PCR revealed the presence of mRNA encoding Cx32, Cx40 and Cx43. These findings are consistent with the ability of gap junction peptides 26 and 27, which correspond to extracellular loops 1 and 2 of Cx43, respectively, to inhibit rhythmic vasomotor activity in this vessel type. As the level of Cx32 protein present was too low for detection by Western blot analysis, it is likely that gap peptide 21, which contains the SRPTEK motif present in extracellular loop 2 of Cx43 (Fig. 1), also inhibited rhythmic activity through effects on gap junctions containing Cx43 rather than Cx32. Interestingly, no synergy of action, either in terms of potency or speed of inhibition, was found between gap junction peptides 26 and 27, even though they target different extracellular loops of Cx43. A similar lack of synergy was also noted by Warner *et al.* (1995) in their experiments with cardiocytes. The finding that antibodies specific to the different connexins did not affect rhythmic activity suggests that they are probably too large to penetrate into the vessel wall and in particular to gain access to the extracellular space inside or in the vicinity of gap junctions.

After administration of each successive dose of the gap junction peptides 21, 26 or 27, the amplitude of the rhythmic activity declined progressively over 10–20 min to a new steady-state level. While the molecular details of complementarity of interaction between the two extracellular loops of each connexin (and 12 in a connexon) are at present unknown (see Yeager & Nicholson (1996) for review), this rapid time course of action suggests that these peptides are somehow able to gain access to the essential functional components of the connexon docking sites. Indeed, these gap junction peptides also rapidly (within 30 min) block the transfer of Lucifer Yellow between stably transfected confluent HeLa cells expressing Cx43 when administered at

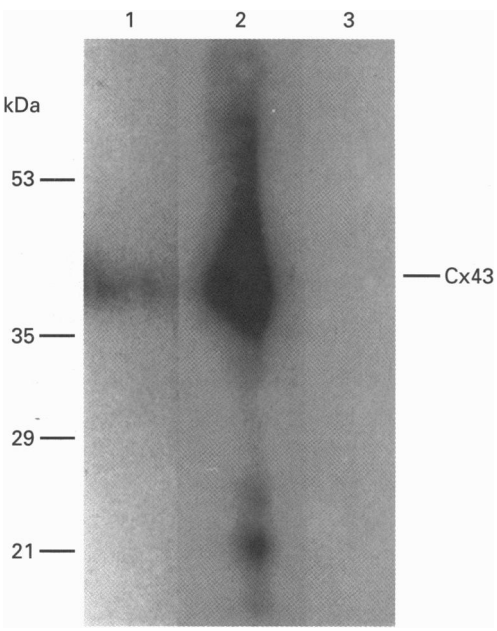


Figure 8. Western blot analysis of the rabbit superior mesenteric artery, heart and lung

Immunoblotting with Gap 15 IgG confirmed the presence of Cx43 protein in the superior mesenteric artery (lane 1) and also demonstrated Cx43 in the heart (lane 2) but not in the liver control (lane 3).

similar concentrations to those employed in the present study (C. George & W. H. Evans, unpublished observations). One possible explanation for the mode of action of the gap junction peptides is that the interactions between the extracellular connexin loops are not fixed and immobile, but involve a dynamic docking–undocking mechanism. Indeed, unpaired non-junctional connexons have been demonstrated in the plasma membrane (Li *et al.* 1996; Perkins, Goodenough & Sosinsky, 1997) and gap junctions in other tissues and in cultured cells are characterized by a high rate of turnover of the constituent connexins (Darrow, Laing, Lampe, Saffitz & Beyer, 1995). In principle, the peptides could thus inhibit the recruitment or accretion of these connexons into functional gap junction plaques, although this process might be expected to occur over longer time periods than the 10–20 min required for the peptides to exert their inhibitory effects on rhythmic arterial activity. Another possibility is that specific synthetic peptides destabilize the molecular interactions between the extracellular loops of connexins and thereby actively reverse the docking of the connexon hemichannels. A less probable scenario is that the peptides modulate the function of intact gap junctions by binding to externally located peptide sequences of the extracellular connexin loops and causing a conformational change which is transmitted to intracellular or transmembrane protein domains. This could then modulate channel function through mechanisms that operate primarily from the cytoplasmic face of the gap junction. Intercellular communication across gap junctions has been shown to be regulated mainly by intracellular calcium and hydrogen ions, transjunctional voltage, and phosphorylation of amino acids at the carboxyl terminal tail of the connexin. The voltage sensor in Cx32 and Cx26 has been mapped to a proline residue located in the second membrane traverse, with an amino terminal aspartic acid at position 2 determining polarity, and gating by pH in Cx43 is mediated by a histidine at position 95 in the intracellular loop (reviewed by Yeager & Nicholson, 1996). The effects of calcium and pH on dye transfer were shown to be synergistic in cardiac muscle cells (Burt, 1987).

A spectrum of drugs and chemicals has been shown to inhibit gap junction communication with varying degrees of efficacy and specificity. These include volatile anaesthetics such as halothane (Nedergaard, 1994), the straight-chain alcohols heptanol and octanol (Nedergaard, 1994; Donahue *et al.* 1995), phorbol esters (Enkvist & McCarthy, 1994), anandamide (Venance, Piomelli, Glowinski & Giaume, 1995), and glycerrhetinic acid (Davidson & Baumgarten, 1988). In the present study, we therefore compared the effects of the apparently specific extracellular loop connexin gap junction peptides with heptanol which is thought to uncouple gap junctions by decreasing the open-state probability of the junction channel (Takens-Kwak *et al.* 1992), and/or decreasing the fluidity of cholesterol-rich domains of the plasma membrane and causing mechanical uncoupling of cells (Bastiaanse, Jongsma, Van der Laarse & Takens-Kwak, 1993). Previous reports have shown that heptanol abolishes rhythmic vasomotor activity in isolated arteries at concentrations in the range 100–300 μM and inhibits the increased dye-coupling between vascular smooth muscle cells induced by tetraethylammonium (Watts *et al.* 1994; Watts & Webb, 1996). The present study confirms these reports as heptanol was found to abolish rhythmic activity at similar concentrations in rabbit superior mesenteric arteries, its overall concentration–inhibition curve closely resembling that of the extracellular loop gap junction peptides.

In marked contrast to the peptide inhibitors, however, heptanol also caused a concentration-dependent decrease in ambient tone, which ultimately fell to baseline pre-constriction levels at a concentration of 3 mM. Analogously, others have shown that pre-incubation of isolated rat thoracic aortic strips with heptanol significantly depresses subsequent phenylephrine-induced contraction (Watts & Webb, 1996). The nature of the relaxant response to heptanol remains controversial, although it has been the subject of considerable experimental investigation. In the case of α-agonist-evoked responses in vascular tissues it does not appear to involve altered Ca^{2+} mobilization from stores, transmembrane Ca^{2+} influx, agonist binding, or changes in the Ca^{2+} sensitivity of contractile proteins (see Christ (1995) for review). Furthermore, in the case of vascular contraction evoked by high extracellular $[K^+]$, heptanol is reportedly without effect on tension development (Christ, 1995). In view of the molecular specificity of the peptide probes used in the present study, it may be speculated that depression of vascular smooth muscle tone by heptanol in the rabbit superior mesenteric artery is 'non-specific' and unrelated to effects on gap junctions. Indeed, after exposure to high concentrations of heptanol (3 mM) repeat contractions to phenylephrine were reduced by ~60% in the present experiments, even after 1 h of repeated washout, in marked contrast to the reversible effects of the antagonistic extracellular loop peptides. It should be noted, however, that other workers have reported that the biological effects of heptanol may be reversible (Takens-Kwak *et al.* 1992; Bastiaanse *et al.* 1993; Watts *et al.* 1994; Watts & Webb, 1996). These differences may result, at least in part, from inter-species variability, the duration of the exposure time and the washout protocol. Thus, a brief 5 min exposure to 2·5 mM heptanol reversibly inhibits gap junctional-dependent synchronization of cultured rat cardiac myocytes, with washout taking 5 min, whereas synchrony reappears only after washouts over 60 min following exposure to the higher concentration of 3 mM heptanol (Kimura, Oyamada, Ohshika, Mori & Oyamada, 1995).

In the present study, the amplitude of the oscillatory behaviour induced by phenylephrine generally varied in an irregular fashion over time (e.g. Fig. 2*A*, insets). Previous studies from this laboratory with isolated rabbit ear arteries have shown that irregular vasomotor activity is a low-dimensional chaotic process whose intrinsic complexity,

assessed by a standard correlation dimension algorithm, is equivalent to that of a non-linear system governed by four interacting dynamic control variables (Griffith & Edwards, 1994*a*, 1994*b*, 1995). Non-linear analysis has similarly permitted classification of the responses of rabbit superior mesenteric arteries as an example of low-dimensional chaos (T. M. Griffith & D. H. Edwards, unpublished observations). Pharmacological probes have shown that the fundamental determinants of rhythmic behaviour in rabbit arteries are oscillatory release of Ca^{2+} from internal stores (through a ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release mechanism) and oscillatory extracellular Ca^{2+} influx; both mechanisms operate at the level of the smooth muscle cell (Griffith & Edwards, 1994*b*). The emergence of 'macroscopic' oscillations in arterial segments and their sensitivity to extracellular loop gap junction peptides thus implies that synchronization of Ca^{2+} oscillations in large numbers of cells occurs as a direct result of intercellular communication. Indeed, the spatially distributed nature of internal Ca^{2+} stores in myocytes and endothelial cells may permit waves of elevated $[Ca^{2+}]_i$ to propagate through the cytosol, and then between cells as a consequence of diffusion of inositol 1,4,5-trisphosphate and/or Ca^{2+} through gap junctions (Iino, Kasai & Yamazawa, 1994; Sanderson, Charles, Boitano & Dirksen, 1994; Sneyd, Keizer & Sanderson, 1995; Ying, Minamiya, Fu & Bhattacharya, 1996; Carter *et al.* 1996). Theoretical studies have explained how large populations of biological oscillators may entrain spontaneously when they are weakly coupled through phase, resulting in recruitment to a 'global' pacemaker that exhibits a narrow range of frequencies because many possible degrees of freedom of the system are suppressed (Mirollo & Strogatz, 1990). Variability in the patterns of $[Ca^{2+}]_i$ oscillations observed in individual vascular smooth muscle cells may thus be reduced in confluent as compared with subconfluent cultures because of increased intercellular coupling (Ambler, Poenie, Tsien & Taylor, 1988). As the behaviour of isolated arteries can, in practice, be characterized as low-dimensional chaos, synchronization mediated by gap junctional communication also appears to limit the intrinsic complexity of the responses induced by constrictor agonists. Loss of this communication pathway would allow cells to oscillate asynchronously and thereby explain why extracellular loop gap junction peptides did not affect ambient force development in the rabbit superior mesenteric arteries employed in the present study. If there is no phase coherence between the behaviour of different cells, small scale fluctuations in tone will tend to 'average out', thereby masking 'global' oscillatory behaviour but leaving net force development unchanged.

In conclusion, we have shown that peptides designed to mimic short amino acid sequences in either of the extracellular loops of connexins, including one peptide that penetrates partially into the fourth transmembrane segment, appear to be relatively rapid and specific in their actions. The fact that they act as inhibitors in two disparate functional systems, viz. synchronization of chick myocyte contraction (Warner *et al.* 1995) and abolition of rhythmic activity in rabbit arteries, suggest that they may emerge as inhibitors of a broad range of integrative biological systems that are underwritten by the necessity of the participating cells to communicate directly across gap junctions. While the precise mechanism of action of these peptides remains to be established, their inhibitory properties are probably explained by a mimetic action with the ability to interact with the extracellular loops of the connexins in the interacting connexons of gap junctions.

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Author's email address

T. M. Griffith: Griffith@Cardiff.ac.uk

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