INTRODUCTION
Blood flow through vascular beds is regulated through neural action on vascular smooth muscle, and the release of local mediators from cells of the vessel wall, in particular from endothelial cells. Direct cell-cell coupling between endothelial cells, smooth muscle cells, and between endothelial and smooth muscle cells or pericytes in the vessel wall (Larson, 1988; Moore et al., 1991; Spagnoli et al., 1982; Cuevas et al., 1984; von der Weid and Beny, 1993), are thought to co-ordinate vasomotor responses to neural action or local mediators by propagating changes of membrane potential through and along the vessel wall (see Segal and Duling, 1989). Junctional communication has also been implicated in the co-ordinated migration of endothelial cells during repair of the endothelial lining or in angiogenesis (Larson and Haudenschild, 1988; Pepper et al., 1992; Reed et al., 1993). are thought to coordinate vasomotor responses to neural action or local mediators by propagating changes of membrane potential through and along the vessel wall (see Segal and Duling, 1989). Junctional communication has also been implicated in the co-ordinated migration of endothelial cells during repair of the endothelial lining or in angiogenesis (Larson and Haudenschild, 1988; Pepper et al., 1992; Reed et al., 1993).

SUMMARY
Gap junction channels permit the direct intercellular transfer of ions and small molecules and allow electrotonic coupling within tissues. Porcine aortic endothelial cells were extensively coupled, as assessed by gap junctional transfer of Lucifer yellow and the fluorescent calcium indicators fluo-3 and furaptra, but were not permeable to rhodamine B isothiocyanate-dextran 10k. The subunit composition of gap junction channels of porcine aortic endothelial cells was characterised using both northern blot analysis and RT-PCR techniques. Messenger RNA encoding connexins 37 and 43, but not 26, 32 or 40, were found in freshly isolated and cultured porcine aortic endothelial cells. Western blots using antipeptide antibodies raised to unique sequences of connexins 37, 40 and 43 showed the presence of connexins 37 and 43, but no connexin 40 was detected. Immunostaining with anticonnexin 43 antibodies showed extensive punctate fluorescent decoration of contacting membranes, whilst antibodies to connexin 37 showed predominantly intracellular staining. Caged InsP3 was found to readily permeate endothelial gap junctions. These results show that primary cultures of porcine aortic endothelial cells express connexins 37 and 43, and provide strong evidence that the second messenger molecule InsP3 can permeate porcine endothelial gap junctions.

Key words: Gap junction, Connexin 37 and 43, InsP3, Patch-clamp, Endothelial cell

INTRODUCTION
Blood flow through vascular beds is regulated through neural action on vascular smooth muscle, and the release of local mediators from cells of the vessel wall, in particular from endothelial cells. Direct cell-cell coupling between endothelial cells, smooth muscle cells, and between endothelial and smooth muscle cells or pericytes in the vessel wall (Larson, 1988; Moore et al., 1991; Spagnoli et al., 1982; Cuevas et al., 1984; von der Weid and Beny, 1993), are thought to coordinate vasomotor responses to neural action or local mediators by propagating changes of membrane potential through and along the vessel wall (see Segal and Duling, 1989). Junctional communication has also been implicated in the co-ordinated migration of endothelial cells during repair of the endothelial lining or in angiogenesis (Larson and Haudenschild, 1988; Pepper et al., 1989, 1992). Direct cell-cell communication is mediated by a family of proteins, the connexins (Beyer et al., 1990), which assemble to form a hexameric connexon hemichannel; the docking of two connexons provided by co-operating cells leads to the formation of a gap junction channel. The nature and regulation of the gap junction proteins in the vascular wall, and the signals that pass between cells that may propagate or co-ordinate vasomotor responses and regulate cell migration, are of great interest, since these represent potential targets for modifying blood flow, repair and growth of blood vessels.

Most of the large vessel and microvascular endothelial cells, smooth muscle cells and pericytes that have been studied, contain connexin 43 (Cx43) (Larson et al., 1990; Pepper et al., 1992; Reed et al., 1993). In addition, connexin 40 (Cx40) has been described in bovine, rat and human vascular smooth muscle (Beyer et al., 1992; Reed et al., 1993; Moore and Burt, 1995; Little et al., 1995), and in rat, hamster and mouse vascular endothelial cells (Bruzzone et al., 1993; Bastide et al., 1993; Dahl et al., 1995; Little et al., 1995). In contrast, it appears to be absent from porcine coronary artery smooth muscle (Moore and Burt, 1995), and from human umbilical vein and bovine aortic endothelial cells (Reed et al., 1993). Connexin 37 (Cx37) has been identified, along with Cx43, in human umbilical vein, bovine aortic and retinal microvascular endothelial cells and bovine retinal pericytes, but not in vascular smooth muscle (Reed et al., 1993). The release of vasoactive local mediators from endothelium, such as prostacyclin and nitric oxide, is mediated by an elevation of the intracellular free calcium concentration (see Carter et al., 1991). Many agonists which release these vasoactive mediators, including acetylcholine, bradykinin, histamine and adenine nucleotides, are generated in blood or adjacent tissues at sites of vessel damage and inflammation, and these
stimulate repetitive calcium oscillations that can be synchronised across many endothelial cells, both in culture (Sage et al., 1989; Carter et al., 1991) and in situ (Laskey et al., 1993). In addition, mechanical stimulation or direct injury of a single endothelial cell within a confluent monolayer can lead to a propagated calcium wave that extends across many cells (Drumheller and Hubbell, 1991; Demer et al., 1993). Both types of behaviour occur in many different cell types (see Berridge, 1993), and are believed to depend on functional gap junction communication, although the exact mechanisms responsible are not known (Sage et al., 1989; Sanderson et al., 1990; Charles et al., 1992). Close temporal synchronisation of rapid calcium signals over many cells argues against a diffusible factor, but rather suggests some rapid form of intercellular signal, which may be mediated by direct electrical coupling via gap junctions. In contrast, calcium signals that initiate in one cell and then spread to adjacent cells, often with delays at cell-cell junctions, are thought to be mediated by diffusible second messengers such as calcium and/or InP3 (Dunlap et al., 1987; Brehm et al., 1989; Demer et al., 1993; see also Berridge, 1993; Sanderson, 1995). There is evidence that calcium can cross gap junctions as an intercellular messenger (Dunlap et al., 1987; Brehm et al., 1989). The evidence that InP3 acts as an intercellular messenger is less direct, and is based on several observations. Firstly, calcium waves can be propagated in the absence of external calcium (Sanderson et al., 1990; Charles et al., 1991; Demer et al., 1993), and are blocked under conditions where InsP3-sensitive calcium stores have been depleted by thapsigargin (Charles et al., 1993). Secondly, calcium waves can be initiated by micro-injection of InP3 and are blocked in cells loaded with heparin (Saez et al., 1989; Sanderson et al., 1990; Boitano et al., 1992). These experiments do not provide direct evidence that InP3 itself passes through gap junctions, though they are suggestive of this. It is equally possible, however, that some other signal is generated that permeates gap junctions to produce the responses seen, perhaps by triggering InsP3 production in adjacent cells.

The aim of the present study was to identify the gap junction proteins present in cultured porcine aortic endothelial cells, and to investigate whether caged InP3 can permeate between cells in confluent monolayers of these endothelial cells. A demonstration that caged InP3 could permeate these gap junctions would provide more direct evidence that InP3 can also permeate these junctions and spread to adjacent cells. The approach of using caged InP3 has an important advantage over microinjection experiments in that caged InP3 is biologically inactive, so that cells are not stimulated during the period that the caged InP3 is allowed to diffuse and spread across the monolayer. Photolysis then releases InP3 in the cells into which it has spread, and the resulting intracellular calcium mobilisation can be monitored selectively in different cells across the monolayer.

MATERIALS AND METHODS

Tissue culture

Porcine aortic endothelial cells were isolated and cultured as previously described (Hallam and Pearson, 1986). Experiments were carried out using freshly isolated cells, primary (within 3-5 days of isolation) or passage 1 cultures (~2 weeks in culture). These cells were identified as endothelial cells by uptake of acetylated low-density lipoprotein (Di-Ac-LDL; Biogenesis), whilst the lack of positive staining with monoclonal anti-(a smooth muscle actin) antibody (Sigma) suggests that these cultures were essentially pure.

Determination of junctional communication

Fluorescent dye transfer

Primary isolates were seeded onto 35 mm square or 13 mm diameter glass coverslips (No.1) and grown to confluence (2-3 days). The cells were then transferred to a Hepes-buffered physiological saline (145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM Hepes, 1 mM CaCl2, 10 mM glucose, pH 7.3) and placed onto the stage of an inverted microscope (Nikon Diaphot). Lucifer yellow CH (Sigma) or rhodamine B isothiocyanate-dextran 10S were dissolved in an internal solution (153 mM K gluconate, 5.6 mM Na gluconate, 1 mM MgSO4, 3 mM disodium-ATP, 10 mM Hepes, pH 7.2) at concentrations of 1-10 μM and introduced into endothelial cells by diffusion from a patch pipette using the standard tight seal whole cell patch-clamp configuration (Hamill et al., 1981). Membrane potential was recorded as previously described (Carter and Ogden, 1994). Patch pipettes (2-5 MQ) were made of Pyrex with a microfilament insert (Clark Electromedical). Lucifer yellow or rhodamine fluorescence was detected by epifluorescence microscopy (lucifer yellow; 400-440 nm excitation, emitted light >470 nm, rhodamine; 510-560 nm excitation, emitted light >590 nm). Evidence of cell-cell coupling was taken if three or more cells showed lucifer yellow fluorescence.

Identification of porcine aortic connexins

Northern blot analysis

Isolation and electrophoresis of Poly(A)+ RNA was carried out as described by Sambrook et al. (1989). The gels were capillary blotted using sterile 1.5 M NaCl, 0.15 M sodium citrate (treated with 0.05% diethyl pyrocarbonate (DEPC)) onto nitrocellulose. Blots were pre-hybridised in a solution containing 0.1% (w/v) bovine serum albumin (BSA) fraction 5, 0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinyl-pyrolidone, 0.9 M NaCl, 0.09 M sodium citrate (treated with 0.05% DEPC). 0.5% SDS and 100 μg of sheared denatured salmon sperm DNA, at 68°C for 12 hours. Following prehybridisation, denatured 32P-labelled probe (rat connexin 43, 32 or 26 cDNA; specific activity 106 cpm/μg) was added. Filters were washed under high stringency conditions (0.015 M NaCl, 0.9 mM sodium citrate (treated with 0.5% DEPC), at 68°C before exposure at −70°C to Kodak film fitted in a cassette with an intensifying screen.

RNA purification for reverse transcriptase-PCR

RNA was purified from porcine tissues and from isolated aortic endothelial cells according to the method of Auffray and Rougeon (1980). Briefly, tissues or cultured cells were solubilised in 5-10 ml of lysis buffer (3 M LiCl, 6 M urea) per gram of cells, and sonicated on ice for 1-2 minutes. After 24 hours at 0-4°C, the sample was centrifuged for 10 minutes at 12,000 rpm, the supernatant discarded and 5 ml of lysis buffer added prior to recentrifugation (12,000 rpm, 10 minutes). The supernatant was discarded and the pellet resuspended in 2 ml TES buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% SDS, pH 7.5) and an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) added. The RNA was precipitated, washed with ethanol and finally dissolved in distilled water and stored at −20°C.

Reverse transcriptase-PCR

The RT-PCR method was modified to facilitate treatment of the sample with DNaseI to remove any contaminating DNA (Dilworth and McCarrey, 1992), and ‘hot PCR’ (Chou et al., 1992). Briefly, before the reverse transcription reaction, 0.5-1.0 μg total RNA in 10 μl DEPC-treated water was mixed with 1 μl RNase-free DNaseI (10 U/ml) and incubated at 37°C for 30 minutes. The reaction was stopped by...
heating to 95°C for 5 minutes and then cooling to room temperature. The DNaseI-treated total RNA (or DEPEC-treated water alone as control) was added to 1 μl of each of dNTP (25 mM), 0.5 μl of 40 μg/ml RNasine (Promega), 100 pmol of both the upstream and downstream primers (for sequences, see Table 1) and 5-10 units of AMV transcriptase (Promega), to give a total volume of 20 μl in reverse transcription buffer. The sample was incubated at 42°C for 60 minutes, then at 95°C for 3 minutes before being quick-chilled on ice. The sample was made up to 99.5 μl with 8 μl of 10X PCR buffer (KCl 0.5 M Tris-HCl, pH 8.4, 25 mM dNTP, BSA 20 mg/ml), MgCl₂ (to final concentration 2.5 mM) and dH₂O. A thin film of mineral oil was placed over the solution prior to heating to 85°C for 5 minutes before addition of 0.5 μl (5 uM) Taq polymerase (Perkin Elmer Cetus). Thermal cycling was performed using a programmable thermal cycler (Perkin Elmer 480). The samples were subjected to 30-32 cycles of amplification as follows: the samples were initially heated to 95°C for 1 minute to ensure complete denaturation of DNA (30 seconds for subsequent cycles) followed by 1 minute at 60°C to anneal the primers (30 seconds in subsequent cycles) and 1 minute at 72°C for extension of the annealed primer (30 seconds in subsequent cycles). After the final cycle the samples were incubated at 72°C for 5 minutes to ensure that the final extension step was complete. The PCR product(s) (10 μl) were visualised on 3% agarose gel. Both strands of the RT-PCR products were sequenced using a deoxyoligonucleotide kit (Perkin Elmer) and an automatic sequence machine (AB1373 Perkin Elmer). PCR primers were designed from mouse or rat sequences (Table 1). To control for contamination by genomic DNA, DNase I-treated samples of total RNA were subjected to PCR amplification without reverse transcription, and 10 μl of the sample run on 3% agarose gel, as described above.

Table 1. Primers used for connexin mRNA PCR amplification

<table>
<thead>
<tr>
<th>Peptide Connexin no.</th>
<th>Sequence</th>
<th>Topological position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP 23</td>
<td>37</td>
<td>ALLAEBHQMAKIC</td>
</tr>
<tr>
<td>GAP 17</td>
<td>40</td>
<td>ATEEVNPQIEQIPFEQ</td>
</tr>
<tr>
<td>GAP 13</td>
<td>43</td>
<td>CVEMHKLQIEIKFFK</td>
</tr>
<tr>
<td>GAP 14</td>
<td>43</td>
<td>SAEQNNMGGQAGS</td>
</tr>
<tr>
<td>GAP 18</td>
<td>43</td>
<td>MGDSALGKLDKQVAC</td>
</tr>
</tbody>
</table>

P1, forward primer; P2, reverse primer.

Western blots and antibodies

Western blots were carried out as described by Towbin et al. (1979). Cells (freshly dissociated or cultured) or tissues were disrupted using an ultratrarrax and a tight Dounce homogeniser, and dissolved in Laemmli buffer (Laemmli, 1970), and the protein resolved on 10% polyacrylamide gels (Rahman et al., 1993). Following transfer of protein to nitrocellulose membranes (constant current 80 mA, 24 hours), the membranes were washed in phosphate-buffered saline (PBS) (NaCl, 8 g l⁻¹; KCl, 200 mg l⁻¹; Na₂HPO₄, 1.15 g l⁻¹; KH₂PO₄, 200 mg l⁻¹; CaCl₂, 1 mM; MgCl₂, 0.5 mM, pH 7.4), followed by 5% skimmed milk with 0.1% Triton X-100 (1 hour). The membranes were then incubated with the first antibody (1/1,000 dilution) in PBS containing 1% skimmed milk and 0.1% Triton X-100 for 2 hours at 37°C. After extensive washing, the second antibody (goat anti-rabbit HRP (Amersham)) was added, and the membrane incubated for 45 minutes followed by extensive washes (1-2 hours). Detection of protein was carried out using the Amersham ECL western blotting analysis system. All primary antibodies were antipeptide antibodies raised to unique regions of known connexins (see Table 2, and Monaghan et al., 1994; Becker et al., 1995). Peptides were synthesised by FMOC-polymamide synthesis. The specificity of connexin antibody staining was confirmed in the following ways: (1) by omission of primary antibody; (2) using preimmune sera; (3) by comparison with staining in other tissues (Becker et al., 1995); and (4) by elimination of staining in the presence of excess peptide.

Immunocytochemistry

Endothelial cells grown on 13 mm diameter glass coverslips (No.1) were rinsed three times in PBS and fixed in 70% ethanol at −20°C for 20 minutes. The cells were then rinsed three more times in PBS and incubated for 1-2 hours at 37°C with a 1:30 dilution of the rabbit polyclonal anti-connexin 43 antibody GAP 13 or GAP 14 (see Becker et al., 1995 and Table 2) the anti-connexin 37 antibody GAP 23 (see Table 2), the anti-connexin 40 antibody GAP 17, or with appropriate dilutions of preimmune serum. FITC-labelled anti-rabbit IgG or PBS, in PBS containing 1% BSA. Following a further three 10 minute washes, the second antibody (anti-rabbit IgG, FITC-conjugated; Sigma), at a 1:80 dilution in PBS with 1% BSA, was added and the cells incubated for 2 hours at room temperature. After rinsing three times in PBS (no BSA) coverslips were mounted on slides with a drop of 0.1% p-phenylenediamine/glycerol/PBS 10:1 (v/v), and examined on a Zeiss Axiovert microscope fitted with filters for fluorescein detection.

Table 2. Sequence and topographical details of synthetic peptides to connexins 37, 40 and 43

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Connexin no.</th>
<th>Sequence</th>
<th>Topological position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP 23</td>
<td>37</td>
<td>ALLAEBHQMAKIC</td>
<td>Int. loop</td>
</tr>
<tr>
<td>GAP 17</td>
<td>40</td>
<td>ATEEVNPQIEQIPFEQ</td>
<td>Int. carboxy-terminal tail</td>
</tr>
<tr>
<td>GAP 13</td>
<td>43</td>
<td>CVEMHKLQIEIKFFK</td>
<td>Int. loop</td>
</tr>
<tr>
<td>GAP 14</td>
<td>43</td>
<td>SAEQNNMGGQAGS</td>
<td>Int. carboxy-terminal tail</td>
</tr>
<tr>
<td>GAP 18</td>
<td>43</td>
<td>MGDSALGKLDKQVAC</td>
<td>Int. amnio-terminal tail</td>
</tr>
</tbody>
</table>

Int. = Intracellular.

RESULTS

Junctional coupling in porcine aortic endothelial cells

Primary or passage 1 cultures of endothelial cells showed
extensive coupling, as determined by the junctional transfer of Lucifer yellow. Fig. 1 shows the transfer of Lucifer yellow between endothelial cells in a confluent primary culture, recorded approximately 4 minutes after obtaining a whole cell recording. Junctional transfer was seen in all primary and passage 1 cultures tested (n=58), with dye transfer extending to >20 cells within 5 minutes. Endothelial gap junctions were also found to be readily permeable to a range of other fluorescent molecules, including the fluorescent calcium indicators fluo-3 (see Fig. 6) and furaptra, but were not permeable to rhodamine B isothiocyanate-dextran 10S (9.4 kDa) (data not shown).

mRNA detection in porcine aortic endothelial cells

Northern blot analysis revealed Cx43 mRNA (Fig. 2), but mRNA encoding connexins 26 or 32 was not detected (data not shown). RT-PCR was used to detect the presence of connexin mRNA in extracts derived from porcine liver, lung, heart and either cultured or freshly isolated aortic endothelial cells. Fig. 3 shows the RT-PCR products obtained using primers directed towards Cx26, Cx32, Cx37, Cx40 and Cx43 (see Table 1) in tissues from mouse (lung, liver and kidney) and pig (liver, lung, heart and cultured aortic endothelial cells). The primers to Cx26 and Cx32 produced PCR products of the predicted size (365 bp and 386 bp, respectively) in both mouse and pig tissues, but not in the cultured endothelial cells. The identity of these PCR products was confirmed by sequencing. Primers for Cx37, Cx40 and Cx43 yielded PCR products of the predicted size (413 bp, 399 bp and 295 bp, respectively) in both the pig tissues and in the cultured aortic endothelial cells. The identity of the DNA products of Cx37 and 43 primers was confirmed by sequencing (sequences available from EMBL Nucleotide Sequence Database, accession numbers X86024 for Cx37 and X86023 for Cx43). The predicted amino acid sequences for the Cx43 and Cx37 PCR products correspond to amino acid positions 247-332 (Cx43) and 3-140 (Cx37), by alignment comparison with corresponding sequences from other species (Beyer et al., 1987; Fishman et al., 1990; Beyer and Steinberg, 1991; Musil et al., 1990; Haeflinger et al., 1992; Reed et al., 1993; Willecke et al., 1991). The sequence of the PCR product in endothelial cells obtained using the Cx40 primers did not correspond to any connexin.

Western blots and immunocytochemistry

Western blots using antipeptide antibodies to unique sequences of Cx37 (GAP 23), Cx40 (GAP 17) and Cx 43 (GAP 14, GAP 18), were used to determine the presence of these connexins in cultured porcine endothelial cells. Fig. 4 shows that GAP 18 and GAP 14 detect Cx43 as a band between 43-48 kDa in guinea-pig, rat and pig heart, pig lung and cultured pig aortic endothelial cells. Experiments with connexin 43-specific antibodies can reveal several bands on western blots, and these are thought to be associated with differential post-translational processing of this gap junction protein (Kadle et al., 1991). GAP 23 detects Cx37 as a band at ~37 kDa in rat heart and cultured pig aortic endothelial cells, whilst GAP 17 detects Cx40 as a weak band at ~41 kDa in rat heart, and a weak band in pig lung, but failed to detect a corresponding band in
cultured pig aortic endothelial cells. Cx43 was immuno-
localised within monolayers of pig aortic endothelial cells
using two antipeptide antibodies to Cx43, GAP 13 or GAP 14.
Immunolabeling was observed as punctate fluorescence
between contacting membranes of endothelial cells (Fig. 5A).
Endothelial cells immunostained with antibodies to connexin
37 showed a predominant intracellular staining (Fig. 5B), cor-
responding to the region where the Golgi apparatus is located.
No immunostaining was seen with the anti-connexin 40
antibody GAP 17 (not shown). No immunolabeling of porcine
aortic endothelial cell was observed with preimmune serum, or
following incubation with the second antibody or PBS alone
(not shown).

**Junctional transfer of caged InsP3**
The spread of caged InsP3 to cells adjacent to the patch-clamped
cell was studied by recording the time course of calcium release,
recorded by fluo-3 fluorescence, from intracellular stores in
either the whole cell patch-clamped cell or in cells distant to the
patch-clamped cell in confluent cultures following photolytic
release of InsP3 (see Fig. 6i). In cells further removed from the
patch-clamped cell, the fluo-3 fluorescence was generally lower,
indicating that the concentration of fluo-3 in these cells was
lower than that of the patch-clamped cell. Fig. 6ii shows the flu-
orescence record from a whole cell patch-clamped endothelial
cell (Cell A) following photolysis of 10 μM caged InsP3 to
release 1.4 μM InsP3. The time course of the calcium-fluo-
rescence response shows a delay of 90 milliseconds (range 60-
100 milliseconds, n=7), peaks by 1 second before declining with
a t1/2 of 10 seconds. The fluorescence record from a cell separated
by two cells (~30 μm) from the patch-clamped cell (Cell B; Fig.
6iii) following an identical pulse of near-UV light shows a time
course comprising a delay of 180 milliseconds (range 120-230
milliseconds, n=6), a time to peak of 1 second before declining
with a t1/2 of 10 seconds. In both cases there is a transient
membrane hyperpolarisation (Vm, 10-15 mV), delayed with
respect to the fluorescence response in the patch-clamped cell,
which recovers to close to pre-photolysis values within 5-10
seconds (delays in Vm changes, 120-310 milliseconds). Exposure to a pulse of UV light in the absence of caged InsP3
in the patch-pipette produced no change in fluo-3 fluorescence,
either in the patch-clamped cell or in adjacent cells (data not
shown). To control for the effects of membrane potential, hyper-
polarisation of single voltage-clamped endothelial cells did not
elicit intracellular calcium mobilisation (data not shown).

**DISCUSSION**
Endothelial cells in situ and in culture can express (and co-
express) several different connexins, which include Cx37,
Cx40 and Cx43. The combination and relative abundance of
different connexins expressed varies from species to species, and between endothelial cells located at different anatomical sites (Larson et al., 1990; Pepper et al., 1992; Beyer et al., 1992; Reed et al., 1993; Bruzzone et al., 1993; Bastide et al., 1993). The ratio of expression of connexin isoforms may be affected by tissue culture conditions or damage to the endothelial cells (Pepper et al., 1992), which may occur during isolation or subculture of the cells.

In confluent monolayer cultures of porcine aortic endothelial cells, intercellular communication, as assessed by the junctional transfer of Lucifer yellow, was rapid and extensive. Northern blot analysis shows the presence of mRNA encoding Cx43 but not Cx26 or 32, whilst RT-PCR shows that mRNA encoding Cx37 and 43, but not Cx26 or 32, was present in cultures of these endothelial cells. No Cx40 mRNA could be identified in these cells. Western blot analysis revealed the presence of Cx37 and 43, but Cx40 could not be detected. These results show that the cultured porcine aortic endothelial cells used in this study appear to have a connexin expression profile similar to that of cultured bovine aortic endothelial cells and human umbilical vein endothelial cells (Reed et al., 1993). The failure to detect Cx40, as indicated by several approaches used in the present work, may reflect an absence of this connexin from pig aortic endothelia or a change in phenotype due to isolation and tissue culture procedures. In this respect, other studies using pig tissue found Cx40 to be absent from arterial smooth muscle (Moore and Burt, 1995), a tissue which in other species is reported to contain this connexin (see above), whilst in pig heart, the distribution of Cx43 and 40 shows considerable variation when compared to other species, being in large absent except in the conduction fibres (Van Kempen et al., 1995).

The immunostaining seen with the antibodies to Cx43 and 37 was different. The Cx43 immunostain was located primarily as plaques at the cell periphery, presumably coinciding with large aggregates of gap junctions. In contrast, the Cx37 was located mainly intracellularly, at perinuclear areas where the Golgi apparatus and the endoplasmic reticulum-Golgi interface is located. Differential intracellular/cell surface location of connexins has been reported in breast luminal cells (Monaghan et al., 1996) and perfused rat liver (Kojima et al., 1995), where multiple connexins are expressed, and may contribute a mechanism for rapidly modulating the connexin composition of gap junctions (Kojima et al., 1995).

The central role of gap junctions in the co-ordinated contraction of uterine smooth muscle (Cole and Garfield, 1985), and cardiac muscle (Spray and Burt, 1990) is well documented. In blood vessels, and in endothelium in particular, the physio-

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**Fig. 6.** Intracellular transfer of caged InsP3 in confluent primary cultures of porcine aortic endothelial cells. (i) Shows the experimental procedure whereby a cell (cell A) is patch-clamped, and fluo-3 and caged InsP3 (10 μM patch-pipette concentration) are allowed to diffuse to adjacent cells before photolysis of the caged InsP3. (ii and iii; left) Show on a slow time base, the time course of the intracellular calcium release (upper of pair of traces) and membrane potential changes (lower of pair of traces) recorded from either the patch-clamped cell (cell A, ii), or a distant cell (cell B, iii) following photolytic release of InsP3 in two separate experiments. The initial time course of these responses are shown on the expanded records (ii and iii; right). The concentration of InsP3 released in cell A was approximately 1.4 μM, whilst the concentration in cell B can be estimated from the time course of the fluorescence response (see Discussion).
logical role of gap junction communication is less clear. Junctional communication has been implicated in migration of endothelial cells during wound repair or angiogenesis (Larson and Haudenschild, 1988; Pepper et al., 1989), and in the coordination of vasomotor responses (see Segal and Duling, 1989). Gap junction communication is also implicated in the spatial synchronisation of calcium oscillations over many cells (Sage et al., 1989; Carter et al., 1991), and the propagation of calcium waves following mechanical stimulation or cell damage (Drumheller and Hubbell, 1991; Sanderson et al., 1990; Charles et al., 1992; Demer et al., 1993). The spatial synchronisation of calcium changes may have important implications for calcium-dependent secretion of vasoactive mediators, such as nitric oxide, and hence the local control of blood flow, whilst propagated responses that arise as a result of mechanical injury may mediate rapid localised secretion of procoagulant molecules such as von Willebrand factor. The mechanisms that regulate these two types of co-ordinated response, synchronised and propagative calcium signalling, are not known. Changes of membrane potential can be propagated rapidly between cells via the low resistance pathways afforded by gap junctions, providing a potential mechanism for co-ordinating cell behaviour. A membrane hyperpolarisation, as seen in these experiments following photolytic release of InsP₃, or in agonist-stimulated endothelial cells in situ (Carter and Ogden, 1994), may provide a means of synchronising calcium responses by promoting calcium entry, necessary for sustained signalling (see Carter et al., 1991). In this respect calcium oscillations have been shown to be closely synchronised with membrane potential changes recorded in functionally coupled endothelial cells in situ (Carter and Ogden, 1994). Propagative calcium signals involve a calcium wave initiated in one cell, which spreads to adjacent cells, often with delays of up to a second at cell-cell junctions (see Charles et al., 1992). It has been suggested that soluble second messengers such as InsP₃ and calcium may be involved in initiating or mediating the spread of such calcium waves across cells (see Berridge, 1993; Sanderson, 1995). There is evidence that calcium can cross gap junctions and act as a physiological intercellular messenger (Brehm et al., 1989), but calcium diffusion is slow (Allbritton et al., 1992). The evidence that InsP₃, which diffuses faster (Brehm et al., 1989), but calcium diffusion is slow (Allbritton et al., 1992), can pass gap junctions is less direct, and has in the main relied on the microinjection of InsP₃ into coupled cells. In previous experiments, transfer of InsP₃ was assessed by calcium mobilisation in adjacent cells (Saez et al., 1989). However, these types of experiments do not show directly that the InsP₃ injected is responsible for the calcium mobilisation seen in adjacent cells, because it is possible that some other signal mediates this behaviour by stimulating InsP₃ production in the adjacent cell.

In this study, porcine aortic endothelial cells were found to be permeable to polyvalent anions ranging in relative molecular mass (Mr) from 457.2 Da to 854.7 Da (Lucifer yellow, 457.2; furaptra, 586.7; fluo-3, 854.7). On this basis alone, it seemed likely that the anion caged InsP₃ (valency 5, Mr 635 Da) could also permeate gap junctions. However, it has recently become clear that gap junctions provide a much greater degree of selectivity in what molecules they pass (Little et al., 1995; Elfgang et al., 1995). Molecules are selected not only on the basis of size, but on other factors such as net charge, chemical structure and the presence of reactive groups (Little et al., 1995; Elfgang et al., 1995). The caged InsP₃ used in this study differs from free InsP₃ (valency 6, Mr 437 Da) by the addition of a dinitrophenyl group at the 4-phosphate position (Walker et al., 1989). This alters the net charge by one and increases the overall molecular mass. However, the published NMR spectral data (Walker et al., 1989) for free and caged InsP₃ show unequivocally that the conformation of the inositol is unperturbed in the caged compound, and the lack of any significant differences in chemical shift for the inositol protons strongly suggest that there is no interaction between the cage moiety and the inositol ring. Hence, the demonstration that caged InsP₃ is permeable would provide good evidence that InsP₃ itself can permeate junctions and spread to adjacent cells. Fig. 6 shows that caged InsP₃ diffuses across porcine aortic endothelial gap junctions. There was a difference in the delay in onset of the Ca²⁺ responses between the patch-clamped cell and distant coupled cells. This most likely reflects a lower concentration of caged InsP₃, and hence of InsP₃ released by photolysis, in these cells (Carter and Ogden, 1992), and is supported by lower fluo-3 fluorescence intensities in distant cells, suggesting a gradient of concentration of fluo-3 away from the patch-clamped cell. The delays seen in distant cells (e.g. Cell B, Fig. 6) are too short to be accounted for by diffusion of calcium, or of InsP₃ photo-released in the patch-clamped cell (Allbritton et al., 1992). The velocity of calcium waves measured in other mammalian cells, 5-40 µM s⁻¹ (Jaffe, 1991), are too slow to account for the delay. Indeed, propagated calcium waves show substantial delays during the transfer from one cell to the next (see Charles et al., 1992 and refs therein). Similarly, the InsP₃ concentration arriving by diffusion in a distant cell 30 µm away with the observed delay (180 milliseconds) would be approximately 50 nM, too small to evoke calcium release (Carter and Ogden, 1992). Hyperpolarisation of single porcine aortic endothelial cells does not itself elicit intracellular calcium mobilisation, so changes of membrane potential cannot account for the Ca²⁺ responses seen in coupled distant cells. However, the results can be readily explained by diffusion of caged InsP₃ during the period of about 10 minutes between establishing whole cell patch-clamp and eliciting calcium release in the distant cell. To account quantitatively for the slower response in distant coupled cells the concentration of caged InsP₃ would have to be approximately one third that in the patch-clamped cell (see Carter and Ogden, 1992).

In summary, we have shown that cultured porcine aortic endothelial cells express predominantly connexins 37 and 43, and that the functional gap junctions linking these cells are permeable to a variety of anions, including caged InsP₃. These experiments provide strong evidence that InsP₃ is able to permeate gap junctions in endothelial cells, and could function as an intercellular messenger.

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REFERENCES


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