An Electron-Microscopic Study of Smooth Muscle Cell Dye Coupling in the Pig Coronary Arteries
Role of Gap Junctions

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Arterial smooth muscles behave like a syncytium, since they are electrically coupled. It is generally assumed that electrical coupling and dye coupling are mediated by gap junctions. No gap junctions could be detected by transmission electron microscopy in media of coronary arteries. We looked for the presence of gap junction protein in vascular smooth muscle by immunohistochemistry with light microscopy. Immunohistologically detectable connexin is expressed by smooth muscle cells of the media of pig coronary arteries, where staining occurs as a discrete punctuation. We investigated the dye coupling in strips of pig coronary artery. The fluorescent dye lucifer yellow was microiontophoretically injected into a smooth muscle cell through an intracellular microelectrode. The dye was visualized on the entire strip, then on semithin sections with a fluorescence microscope, and at the ultrastructural level by using an anti-lucifer yellow antibody revealed by the protein A-gold technique. In all the tissues examined, the cells were dye-coupled. We conclude that in arterial media the smooth muscle cells are dye-coupled, despite the absence of detectable gap junctions by transmission electron microscopy, and suggest that dye coupling could occur via isolated gap junction channels. (Circulation Research 1992;70:49-55)

The visceral smooth muscles behave like a syncytium, since they are electrically coupled.1-3 This electrical coupling could be due to gap junctions that constitute cytoplasmic continuities between neighboring cells. They are formed by hexameric cylinders, called connexons, that are embedded in the apposed membranes of adjacent cells in such a way that the connexons of the two cells in contact are aligned (for review see Reference 4). By electron microscopy, gap junctions are recognized because there is apparent fusion of plasma membranes, which results in a pentamaminal membrane complex in a low-power electron micrograph and in a seven-layered complex in a high-power electron micrograph, since the outer leaflets of plasma membrane are separated by a gap of 2-3 nm.5 These junctions mediate the passage of ions and small hydrophilic molecules between the cells.6,7 Such pathways allow electrical coupling8 as well as chemical signaling in intercellular communication.9-11 However, not all the visceral smooth muscles have morphologically detectable gap junctions.12 In particular, the smooth muscle cells of arteries are electrically coupled,13 but gap junctions are rare or absent, as detected by transmission electron microscopy in media of some arteries (e.g., dog coronary arteries).14,15

To solve this paradox, first, we searched for the presence of gap junctions in the media of the anterior descending branch of pig coronary artery by transmission electron microscopy and immunohistochemical detection using an antibody against a synthetic peptide from connexin43.16 Second, we injected the fluorescent dye lucifer yellow into one smooth muscle cell of this tissue to test whether these cells possess functional intercellular communications and are thus dye-coupled. The fluorescent dye lucifer yellow is a low-toxicity molecule that spreads quickly from the injected cell to nearby cells through gap junctions without crossing the cell membrane.17 This phenomenon is known as “dye coupling.” The limits of the individual cells are not perceptible by fluorescence light microscopy on semithin sections. Thus, we also examined thin sections with a transmission electron microscope. Lucifer yellow was visualized at the cytological level using an anti-lucifer yellow antibody revealed by the protein A-gold technique.
Materials and Methods

Preparation of Tissues

The method was presented elsewhere. Briefly, anterior descending branches of pig coronary arteries obtained at a slaughterhouse were dissected in an oxygenated Krebs’ solution containing (mM) NaCl 118.7, KCl 4.7, CaCl2 2.5, KH2PO4 1.2, NaHCO3 24.8, MgSO4 1.2, and glucose 10.1, at 4°C and pH 7.3. Strips of ~5 mm in length were dissected and pinned on a silicon rubber surface in a 100-µl Plexiglas bath with the intimal surface upward. Then, it was continuously perfused with oxygenated Krebs’ solution (1,250 µl/min) at 37°C.

Membrane Potential Recording and Lucifer Yellow Injection

The cell membrane potential was measured with a glass microelectrode. The tip of the electrode was filled with a lucifer yellow solution (5% in water), back-filled with 150 mM LiCl. The resistance of the resulting electrodes was >400 MΩ. This corresponds to a resistance of 80–150 MΩ when the same electrode was filled with 3 M KCl. The LiCl electrode was connected to an amplifier (model 707, World Precision Instruments, Sarasota, Fla.) with an Ag–AgCl half cell in contact with the tissue bath with an LiCl-agar-agar bridge. The membrane potential was monitored on an oscilloscope (model 1242, Gould, Cleveland, Ohio) and recorded on a potentiometric recorder (W&W Electronics, Switzerland). The microiontophoretic injection of the fluorescent dye was done by passing a direct current of 0.35 nA through the electrode for 2–5 minutes. The criteria for accepting a record were a stable membrane potential below ~40 mV before and after the lucifer yellow injection and a sharp rise to 0 mV when the electrode was withdrawn from the injected cell. This ensured that the injection was within the impaled cell.

Cytological Detection of the Dye

To evaluate the extent of dye coupling, after the dye injection, the tissue was fixed with 4% paraformaldehyde. The luminal face of the strip was examined with a fluorescence microscope (Nikon diaphot) (excitation wavelength, 450–490 nm) and photographed. The tissue was then embedded in polyethylene glycol (MW, 1,000) following the methods described by Smithson et al. The embedded tissue was cut with a tissue chopper in 50-µm slices. The polyethylene glycol was removed from the slices by immersion in phosphate-buffered saline (PBS), and the slices were examined with the fluorescence microscope. The positive slice(s) was then embedded in Epon without osmification for 1-µm serial semithin sectioning. After having reached the first semithin sections containing fluorescent material, thin sections were cut (~60 nm). Lucifer yellow–immunoreactive sites were localized on these thin sections mounted on parlodion carbon–coated nickel grids by the protein A–gold–citrate technique. Colloidal gold particles (particle size, 15 nm) were prepared according to Frens. Thin sections were placed first for 5 minutes on a droplet of 0.5% ovalbumin in PBS; then they were transferred to an anti–lucifer yellow antiserum (diluted 1:400) (gift of H. Reichert) for 2 hours at room temperature. The sections were washed twice with doubly distilled water for 5 minutes and incubated for 1 hour at room temperature with the protein A–gold solution (diluted 1:100). After rinses with doubly distilled water, the sections were counterstained with uranyl acetate and lead citrate. The thin sections were examined with a Zeiss EM 10 electron microscope.

Electron Microscopy

To search for gap junctions by ultrastructural examination, freshly dissected tissue was fixed with a 2.5% glutaraldehyde solution in PBS. Subsequently, the tissue was postfixed in 2% osmium tetroxide, embedded in Epon, and processed for conventional electron microscopy according to standard procedures. The thin sections were stained with uranyl acetate and lead citrate and were examined with a Zeiss EM 10 electron microscope.

Immunohistological Detection of Gap Junctions

To search for connexin43, indirect immunofluorescence was performed on 10-µm frozen sections of coronary arteries according to the protocol described by Harfst et al and Gourdie et al. Antibodies were centrifuged (7,000 rpm for 5 minutes) before use to prevent the presence of precipitate on the preparation. The primary antibodies used are described in References 16 and 22. They were raised in the rabbit against the HJ and HH synthetic peptides and were kindly supplied by Dr. Green (Department of Anatomy and Developmental Biology, University College, London) and diluted 1:10 in PBS. Secondary antibody (GAR-FITC, Nordic, The Netherlands) was used (diluted 1:50). Preimmune serum (diluted 1:10) was used as primary antibody for the control experiment.

Results

Ultrastructural Research of Gap Junctions

The ultrastructural examination of the arterial media by transmission electron microscopy to search for cell to cell junctions showed close appositions of smooth muscle cell membranes without specialized intercellular junctions (Figure 1) and less tight appositions with surface vesicles, or caveolae, in the abutting plasma membranes (Figure 2). These caveolae are invaginations of the cell membrane of smooth muscle cells, endothelial cells, or fibroblasts. Gap junctions, as defined in the introductory section, were not observed between any of the smooth muscle cells. By contrast, typical gap junctions between endothelial cells were frequently encountered (Figure 3). The failure to see gap junctions by transmission electron microscopy led us to search for gap junctions using immunocytochemical techniques.
Immunohistochemical Detection of Connexin by Light Microscopy

Immunocytochemistry using an antibody raised against a synthetic peptide located in the intracellular domain of connexin43 was performed on more than a hundred frozen sections of coronary arteries from four different animals. Myocardial tissue was examined as a positive control, since in this tissue, detectable gap junctions occur and are ordered along intercalated disks. A typical positive result was obtained on the myocardium adjacent to the coronary artery (Figure 4A). Such evident labeling was never observed on the
coronary arterial media. However, on 25% of the preparations, a staining was noticed as a discrete punctation on some regions of the media (Figure 4B). This labeling was not present when we used the preimmune serum, and we can thus assume that this weak staining was due to very small gap junctions.

**Figure 3.** Photomicrograph of thin section of left anterior descending branch of coronary artery from pig. RER, rough endoplasmic reticulum; GJ, gap junction; V, vesicle. Site of contact between two contiguous endothelial cells with gap junctions (arrows) is shown. The arrow on the right indicates apparently internalized gap junctions as described in smooth muscle cells isolated from dog carotid artery.24 Magnification, ×68,800; bar, 0.2 μm.

**Figure 4.** Photomicrographs showing immunocytochemistry on frozen sections of pig coronary and heart using an anti-connexin43 antibody. Panel A: Immunolocalization of gap junctions on the heart. The antibody has bound to the intercalated disk regions (arrow) where the gap junctions occur. At this magnification, individual gap junctions are not resolved. Bar, 50 μm. Panel B: Small gap junctions (arrows) visualized in the coronary arterial media (SM). L, lumen of the vessel; E, endothelial side; A, adventitia. Bar, 50 μm.
Dye Coupling

We succeeded four times in performing the entire procedure from the impalement of a smooth muscle cell to the cytological observation. The examination of the whole strip with the fluorescent microscope allows rapid checking (after 20 minutes in paraformaldehyde) of the success of an injection (Figure 5). When the injected cell was deep in the media, the picture was not clearly focussed, because the thickness of the inhomogeneous tissue obscured the view of the fluorescent cells. If the injection was near the surface, the image was in sharper focus (Figure 5B). The longitudinal axis of the fluorescent tissue was orientated between $+45^\circ$ and $-45^\circ$ of the axis perpendicular to the longitudinal axis of the vessel. The cells appeared fusiform. By comparison, the axis of the fluorescent endothelial cells injected with the same dye was parallel to the longitudinal axis of the vessel. The endothelial cells looked like ellipsoidal cells (Figure 5A). In each case, the orientation and shape of the fluorescent cells allow precise distinction between endothelial and smooth muscle cells at this stage. This constitutes a rapid method for identifying the recorded tissue.

The examination of the semithin sections did not give much information, because no counterstaining was satisfactory for recognizing the limits of the cells: it was impossible at this stage to know whether the dye was confined to the injected cell or had diffused outside. The only information available was the localization of the injected cells in the depth of the arterial wall (Figure 6).

Consequently we performed cytological observations. In all four cases examined, the dye was localized to the interior of the cells and not outside, and the cells were dye-coupled. The fact that no dye was found in the extracellular space implies that the cells were coupled, since the electrophysiological method used guarantees that the dye was injected in one cell. We could observe dilution of the gold particles when passing from one cell to the other, but in each cell the particles were homogeneously distributed (Figure 7).

Discussion

Using electron microscopy, our morphological observations of regions of close membrane apposition without identifiable gap junctions between pig coronary arterial smooth muscle cells were similar to previous observations in canine coronary arteries.\textsuperscript{14} The scarcity of morphologically detectable gap junctions between the smooth muscle cells of some arteries has led to the controversial issue of whether gap junctions are required for the existence of electrical coupling.\textsuperscript{12} If gap junctions are present in this tissue, they must be quite small, perhaps consisting of dispersed gap junction channels. Nevertheless, these few channels could provide the pathway for electrical and chemical signaling. The alternative possibility that gap junctions are not necessary for coupling in arterial smooth muscle would imply the existence of other mechanisms of electrical transmission.\textsuperscript{25} The physiological consequences of this latter alternative would be that only electrical coupling would take place between cells linked in this way, without chemical signaling. In this context, it is noteworthy that dye-coupled cells are necessarily electrically coupled, whereas electrically coupled cells are not necessarily dye-coupled.\textsuperscript{26} The present study shows

![Figure 5](image-url) Intimal face of a pig coronary arterial strip seen with a fluorescence microscope. The longitudinal axes of the vessels run vertically. An endothelial cell (panel A) and a smooth muscle cell (panel B) were injected with the fluorescent dye lucifer yellow. Magnification, $\times96$; bar, 100 $\mu$m.
conclusively that there are hydrophilic channels between smooth muscle cells in the interior of the pig coronary artery that allow intercellular diffusion of lucifer yellow from cell to cell. Consequently, the presence of morphologically observable gap junctions by electron microscopy of thin sections of a tissue is not necessary for the existence of dye coupling between the cells. Our observation of discrete punctate labeling using peptide-specific antibodies on coronary arterial media and the observation that different vessels ex-

FIGURE 6. Photomicrographs of semithin (1-μm) section of a pig coronary arterial strip. IEL, internal elastic lamina; SM, smooth muscles (media); L, position of the lumen; E, endothelium. The section was illuminated with ultraviolet light to show the fluorescent cells (panel A) and with both white and ultraviolet light to show the location of the injected cells in the tissue (panel B). The arrowheads show the fluorescing injected cells. Magnification, ×480; bar, 20 μm.

FIGURE 7. Photomicrograph of thin section of a pig coronary arterial strip. SM, smooth muscle cells; BM, basal membrane and collagenous fibrils. The gold particles (black spots) indicate the presence of lucifer yellow made apparent by an anti-lucifer yellow antibody coupled to protein A–gold. Magnification, ×9,200; bar, 2 μm.
pressed connexin43 mRNA\textsuperscript{27} are compatible with the idea that the inability to see gap junctions by transmission electron microscopy is caused by the fact that the channels are not grouped in large clusters in this tissue, which could be similar to the tissue described in \textit{Xenopus} (amphibian) myocardium.\textsuperscript{28} In this tissue, no gap junctions were found by thin-section electron microscopy, although cells were electrically coupled. Application of the freeze-fracture technique showed linear or circular arrays of particles, which would be difficult to detect using conventional thin-section electron microscopy. What are the signals that could be transmitted through these cell to cell junctions? Although pig coronary arteries were considered to be electrically silent, the endothelium-dependent relaxations caused by the peptides substance \textit{P} and bradykinin are associated with hyperpolarizations.\textsuperscript{18,29} Such hyperpolarizations are related to arterial relaxation\textsuperscript{20} and could be spread as electrical signals through these gap junctions. The propagation of such signals could serve to coordinate relaxation of the arterial wall. In addition to such electrical coupling, water-soluble second intracellular messengers could diffuse through these junctions, thus providing a route for direct intercellular chemical signaling. This type of communication could participate in the spread of hyperpolarization and could also coordinate tissue development or repair.\textsuperscript{8–11}

We conclude that in arterial media the cytosolic compartments of the smooth muscle cells are coupled by hydrophilic channels that are not likely aggregated into large plaques, as judged by the absence of morphologically detectable gap junctions in this tissue by transmission electron microscopy.

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\section*{References}

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