Function of Interstitial Cells of Cajal in the Rabbit Portal Vein

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Abstract—Interstitial cells of Cajal (ICCs) were identified in the intact fixed media of the rabbit portal vein (RPV) using c-kit staining. The following experiments were performed using single cell preparations of the enzyme-dispersed vessel. Surviving contacts between the processes of single ICCs and the bodies of smooth muscle cells (SMCs) were observed in electron micrographs and by confocal microscopy. Spontaneous rhythmical [Ca$^{2+}$] oscillations were observed in ICCs after loading with the calcium indicator fluo-3 and were associated with depolarizations of the ICCs recorded by tight-seal patch pipette. To investigate signal transmission from ICCs to SMCs in dispersed cell pairs, or within small surviving fragments of the ICC network, an ICC was stimulated under voltage-clamp, while changes in [Ca$^{2+}$] in the stimulated cell as well as in a closely adjacent SMC or ICCs were monitored using fast x-y confocal imaging of fluo-3 fluorescence. After stimulation of single voltage-clamped ICC by a depolarizing step similar in duration to depolarizations associated with spontaneous [Ca$^{2+}$], oscillations, a depolarization and transient elevation of [Ca$^{2+}$] was observed in a closely adjacent SMCs after a delay of up to 4 seconds. In contrast, signal transmission from ICC to ICC was much faster, the delay being less than 200 ms. These results suggest that the ICC may, in addition to generating an electrical signal (such as a slow wave) and thereby acting as a pacemaker for vascular SMCs of RPV, also release some unknown diffusible substance, which depolarizes the SMCs. (Circ Res. 2004;95:619-626.)

Key Words: interstitial cells of Cajal ■ rabbit portal vein ■ calcium waves ■ pacemaker activity ■ vascular smooth muscle

Recently we have demonstrated c-kit-positive cells in the wall of the rabbit portal vein (RPV). After enzymatic dispersion of the RPV as well as guinea pig mesenteric arteries, cells closely resembling morphologically the interstitial cells of Cajal (ICCs) described in other tissues were observed; others have identified such cells in lymphatic vessels and bladder. ICCs have been widely described in the gastrointestinal tract and in urethral smooth muscle layers and are considered to play a pacemaker role in these tissues by generating rhythmical changes in membrane potential, slow waves, which are suggested to be transmitted to SMCs by generating rhythmical changes in membrane potential, slow waves, which are suggested to be transmitted to SMCs, ICCs observed in the gastrointestinal tract express the antigen c-kit, have long processes, a high ability to accumulate methylene blue, and an inability to contract. They form gap junctions with SMCs and may serve to mediate signal transmission from nerves to SMCs.

In enzyme-dissociated cells of RPV contacts between the processes of ICCs and the bodies of SMCs, as well as small fragments of the ICC network, were observed. This observation prompted us to test whether any signal could be transmitted from one cell to another within these multicellular structures. As calcium is involved as a second messenger in the variety of intracellular signaling pathways and is the major controller of SMCs tension, we used imaging of the fluorescence signal from a pair or small group of interconnected cells preloaded with the calcium-sensitive dye, fluo-3 AM, combined with simultaneous electrophysiological recording from two cells to detect if there was any signal transmission between them. We found that signal transmission from ICC to ICC appeared to be much faster than that from ICC to SMC, whereas spontaneous rhythmical [Ca$^{2+}$] waves in ICCs were associated with depolarization of the cell membrane. These observations suggest that the ICC network may act as a pacemaker for SMCs. A preliminary account of some of this work has previously been reported in abstract form.

Materials and Methods

Cell Isolation

A section of RPV ~20 mm long, upstream from the anastomosis of its right and left branches, was removed from male New Zealand rabbits (2 to 3.5 kg, 42 animals; obtained from our animal facilities) immediately after they had been killed by an overdose of pentobarbitone injected into the ear vein as approved under Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. Enzyme dispersion of the cells in the vessel wall was performed as previously described.

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All experiments were performed at room temperature (22 to 24°C) within 6 hours of enzyme dispersion.

**Immunohistochemistry**

To visualize the distribution of ICCs in the wall of RPV, we used the acetone-fixation protocol previously described by Örög et al. The binding of rat monoclonal antibodies, raised against c-kit protein in mouse (RDI) was visualized with Alexa Fluor 488–conjugated chicken anti-rat IgG (Molecular Probes Inc).

**Electron Microscopy**

The cells were fixed in glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, and mounted on aluminum stubs. For viewing under an electron microscope, Zeiss EM 940 SEM, specimens were shadowed with a thin layer of evaporated gold that gives the secondary electron image seen on the monitor.

**Patch-Clamp Recording**

Experiments were performed using amphotericin B (Sigma) perforated-patch tight-seal recording under voltage- or current-clamp conditions. The electrical signals were recorded using an Axopatch 200A patch-clamp amplifier or Multiclamp 700A for double-patch experiments (both Axon Instruments). Voltage protocols were generated and electrical signals were digitized at 1-kHz using a Digidata 1200 or 1322A hosted by a PC running pClamp 6.0 or 8.2 software (Axon Instruments). The cells were bathed in solution of the following composition (in mmol/L): KCl 6, NaCl 120, MgCl₂ 1.2, CaCl₂ 2.5, D-glucose 12, and HEPES 10; pH was adjusted to 7.3 with NaOH. The pipette solution contained the following (in mmol/L): KCl 85, KH₂PO₄ 30, MgSO₄ 5, EGTA 1, HEPES 10 (pH was adjusted to 7.3 with KOH), and 200 μg/mL amphotericin B.

**Confocal Microscopy**

Cells were loaded with the Ca²⁺ sensitive indicator fluo-3AM (Molecular Probes Inc.) as previously described. An Axiovert 100M inverted microscope attached to an LSM 510 laser-scanning unit (Zeiss) was used for x-y time-series or line-scan fluorescence confocal imaging. The acquisition and analysis of the data were performed as described elsewhere.

**Statistics**

Where appropriate, data are presented as mean±SEM for number of the cells (n) analyzed.

**Results**

To identify the location of ICCs in the wall of the RPV, fixed segments of the RPV were incubated with anti-c-kit antibodies followed by staining with Alexa Fluor 488–conjugated IgG. Confocal x-y imaging of Alexa Fluor 488 fluorescence revealed c-kit–positive cells at two locations: subendothelial (Figure 1A) and in a deeper intramuscular location (up to 70 μm from the endothelium; Figure 1B), which was consistent with our previous finding on living preparations of the RPV. We found that c-kit–positive cells were distributed in the wall of RPV at different densities in both subendothelial and intramuscular layers. In some regions, ICCs appeared at high density (up to 150 cell per mm²) (Figure 1C), whereas in the others only a few ICCs were observed (Figure 1D). Such variability in the distribution of ICC along the vein is consistent with heterogeneity of spontaneous contractile activity of different areas of the vein wall, which was frequently observed on dissection of vein fragments (see also Hermsmeyer).

At least two types of ICCs were distinguished in electron micrographs after enzymatic dispersion of the RPV: (1) multipolar cells with stellate-shaped bodies and (2) long cells with spindle-shaped bodies. There was diversity in the morphology of ICCs within each type (Figure 2A), not unlike that previously observed in fluorescence confocal images of fluo-4–loaded ICCs. Scanning electron microscopy, however, allowed a more detailed study of the morphology of ICC processes, which on a number of occasions had a diameter below the optical resolution of the confocal microscope and therefore could not be properly seen in fluorescence images. It was also possible to visualize the microstructure of the contacts between these processes and the body of a SMC (Figure 2B). The ICC processes had a length of between 2 and 260 μm, mean 26±4 μm, n=114 (such variability being probably partially attributable to damage of some processes on cell isolation) and a diameter gradually decreasing from the root of the process (0.4 to 3 μm; mean 1.0±0.1 μm,
Ca$^{2+}$ exhibited spontaneous transient increases in [Ca$^{2+}$]i of ICC (Figure 3A, a) loaded with fluo-3 (Figure 3A, b). It is evident that the initiation point of the [Ca$^{2+}$]i transient varied from one oscillation to another and that there were at least three sites giving rise to [Ca$^{2+}$]i transients in some ICCs: (1) images a17 through a20 and d18 through d22, (2) images b27 through b30, and (3) images c27 through c30 (Figure 3B). It is also notable that when a localized increase in [Ca$^{2+}$]i occurred shortly after a [Ca$^{2+}$]i oscillation, it did not trigger a [Ca$^{2+}$]i wave (images a22, c11, and d13, Figure 3B), possibly because of partial depletion of the calcium stores and/or refractoriness of the release mechanism. The rhythmicity of these oscillations is further emphasized by the plot (Figure 3C) showing the time course of the normalized fluo-3 fluorescence intensity averaged within the boxed region outlined in red (Figure 3A, b). In general, local [Ca$^{2+}$]i oscillations occurred with an average frequency of 3.3±0.3 minute$^{-1}$ (n=21) and were usually observed near the nucleus, which was seen as a darker structure under phase contrast. L-type Ca$^{2+}$ channels previously reported in the ICCs from the RPV are unlikely to contribute to generation of local [Ca$^{2+}$]i oscillations in ICCs as 10 μmol/L nicardipine added to bath solution was without effect (n=4) (Figure 3D).

Subplasmalemmal [Ca$^{2+}$]i changes (Figure 4A) occurred in the central region of spindle-shaped ICCs (position of the scan line is depicted by yellow line on the transmitted light image, Figure 4B) and were associated with spontaneous depolarizations of the cell (plot in Figure 4B). The line-scan image at 667 Hz revealed that the rise in [Ca$^{2+}$]i was initiated at a single site (red arrow, Figure 4A) and spread as a [Ca$^{2+}$]i wave within a restricted region (see also Figure 3B) extending along about half of the scan line. This is further emphasized by the time-course plots (Figure 4C) of the normalized fluorescence signal (F/Fo) at two positions along the scan line, depicted by red and green bars in the line-scan image (Figure 4A), by red and green arrows on the transmitted light image (Figure 4B), and shown in corresponding colors. It is notable that only a large amplitude [Ca$^{2+}$]i transient (initiated at a site depicted by red bars and red arrow, Figure 4A, and red arrow, Figure 4B) was associated with membrane depolarization (Figure 4B), whereas a long-lasting small-amplitude increase in [Ca$^{2+}$]i, in an adjacent region (top part of the line-scan image, Figure 4A, and green plot, Figure 4C) was without effect on membrane potential.

However, can these excitatory signals be transmitted to an adjacent SMC? To test this, ICC-SMC pairs that survived enzymatic dispersion were preloaded with the Ca$^{2+}$-sensitive indicator fluo-3 (Figure 5A). The ICC of a surviving ICC-SMC pair was stimulated by voltage steps (from −60 mV to +30 mV by 30 mV increments) similar in duration to spontaneous depolarizations associated with [Ca$^{2+}$]i oscillations (Figure 4B), whereas fast x-y confocal imaging of fluo-3 fluorescence in both cells was performed (Figure 5B through 5E). Images 15 to 38 of these 48 images are shown in the galleries (panels a in Figure 5B through 5E). The corresponding trace of whole-cell current through the ICC membrane is shown in panels b in Figure 5B through 5E. The gallery
shown in panels c in Figure 5B through 5E is formed by the fluo-3 fluorescence images taken from two boxed areas (1 and 2, Figure 5A, b) located in (1) ICC and (2) SMC. The fluorescence intensity in the images was normalized to the average fluorescence intensity in control (before voltage step was applied) and color coded as indicated by the bar (F/F₀). The time course plots of normalized fluorescence intensity averaged within each box (outlined in green and black, Figure 5A, b) are shown in the corresponding color in panel d (Figure 5B through 5E). When no depolarizing pulse was applied (Figure 5B), no change in the fluorescence signal was observed in both cells. Depolarization of the ICC to −30 mV evoked a [Ca²⁺] transient in the ICC with no [Ca²⁺] change in the adjacent SMC (Figure 5C). When the ICC was depolarized to 0 mV (Figure 5D), the rise in [Ca²⁺], in the ICC was followed by a delayed (up to 4 seconds) rise of [Ca²⁺] in the SMC. The rise of [Ca²⁺] in the ICC caused by the voltage step to +30 mV was followed by a rise in [Ca²⁺] in the adjacent SMC that occurred with a shorter delay, had a larger magnitude, and revealed more than one peak (Figure 5E). (The data shown in Figure 5B through 5E are also available as video clips in online data supplement at http://circres.ahajournals.org). Altogether, 20 ICC-SMC pairs were examined, and in 6 cases, stimulation of the ICC resulted in a [Ca²⁺] elevation in an adjacent SMC that occurred with an average delay of 1.8±0.6 seconds (n=5) when the membrane potential was stepped from −60 mV to 0 mV.

To investigate changes in the membrane potential in the adjacent SMC during stimulation of the ICC in the ICC-SMC pair, we performed double patch experiments where ICC was stimulated under voltage clamp by 5-second step from −60 to 0 mV, and SMC membrane potential was recorded under current clamp (Figure 6). In two of five successful recordings, small (up to 20 mV) delayed depolarizations of the membrane-adjacent SMCs were observed while ICCs were stimulated.

In some cases, small “patches” of the intact ICC network (comprising 2 to 4 ICCs) also survived enzymatic dispersion of the RPV. This allowed the dynamics of signal transmission within the ICC network to be monitored; the same approach as described in Figure 5 was used (ie, one ICC was stimulated while the change in the fluo-3 fluorescence in all intercon-
network appeared to be much faster than from ICC to SMC.

Figure 7A shows a confocal line-scan image of fluo-3 AM-loaded ICCs. The line-scan was performed at 667 Hz and fluorescence intensity was normalized to the average fluorescence intensity (F/F₀) in the insert (B). The line-scan was performed from one ICC to another. Fluorescence intensity was normalized to the average fluorescence intensity (F/F₀) in the insert (B). The line-scan was performed from one ICC to another. Fluorescence intensity was normalized to the average fluorescence intensity (F/F₀) in the insert (B). The line-scan was performed from one ICC to another. Fluorescence intensity was normalized to the average fluorescence intensity (F/F₀) in the insert (B).

Discussion

Because the ICCs in rabbit portal vein were c-kit positive, it seems possible that they have similar functions to those of ICCs in gut, namely, as pacemakers of electrical, and so of contractile, activity and as intermediaries between nerve and muscle. The present experiments do not address the latter possibility but do provide some information about communication between ICCs and SMCs.

Using confocal imaging of fluo-3 fluorescence, we observed for the first time in single ICCs freshly isolated from RPV rhythmic local [Ca²⁺] oscillations (Figure 3). The average frequency of these oscillations (3.3 ± 0.3 minute⁻¹) and allowing for a maximum of 20% change in frequency for 1°C rise in temperature[18] was similar to the frequency of spontaneous depolarizations recorded with microelectrode from the pacemaker regions of multicellular preparations of RPV (8 minute⁻¹ at 38°C). Combining confocal fluorescence imaging with monitoring of the cell membrane potential revealed that [Ca²⁺] oscillations in ICC were coupled to membrane depolarization (Figure 4) and may underlie slow waves previously demonstrated with microelectrode technique.[18] This coupling may occur through activation of Ca²⁺-dependent membrane ion channels, either chloride[19] or cationic[20] channels similar to those previously described in SMCs from RPV.[21,22] In cultured ICCs from the murine small intestine, it was proposed that a Ca²⁺-inhibited cationic conductance may contribute to the pacemaker current and generation of electrical slow waves.[20] In urethral ICCs, unitary currents that could contribute to pacemaker activity had properties similar to spontaneous transient Ca²⁺-dependent inward chloride currents[23] and were shown to be activated by IP₃R-mediated Ca²⁺ release from intracellular stores.[19]

The presence of spontaneous depolarizations associated with increases in [Ca²⁺], is consistent with the ICCs in rabbit portal vein having a pacemaker function. This is in keeping with the spontaneous mechanical and electrical activity that this vessel normally shows. It is well known that spontaneous activity of the RPV is myogenic[18,22] and the presence of the multiple pacemakers in its wall is quite possible.[24] Microelectrode recordings at different positions along the RPV[18] and asynchronous contractile activity of the different fragments of the vein wall[16] suggest the existence of specialized pacemaker regions. In the present study, by screening regions of RPV immunostained with anti-c-kit antibodies, we demonstrated that the density of the ICC network in both subendothelial and intramuscular layers varied widely along the wall of the vessel (Figure 1), which provides a histological basis for the existence of pacemaker regions. This may suggest that in the vasculature, especially in blood vessels with spontaneous contractile and electrical activity, an ICC network may serve as a pacemaker.

Further evidence was provided by our experiments on small clusters of linked ICCs. Depolarization of one ICC spread rapidly in less than 200 ms to other ICCs in the cluster. Thus, an ICC network in the wall of the RPV could be synchronized by such electrical connections and the ICC
network initiate depolarization of adjacent SMCs linked to them by low resistance pathways.

The difficult experiments involving simultaneous recording of electrical activity from adjacent ICCs and SMCs revealed that, on occasions, depolarization of the ICCs could result in a delayed depolarization of the associated SMC (Figure 6). This did not seem to involve an electrotonic spread of depolarization from the ICC to the SMC via the long processes and their “feet” in contact with the SMC (Figure 2) because no obvious depolarization was initiated in the SMC at the beginning of the step depolarization of the ICC. In any case, at present, there is no evidence available as to whether the feet form low resistance gap junctions with the SMCs. Also, it should be borne in mind that in the intact tissue the cell bodies of ICCs are generally closely applied to those of SMCs and likely form gap junctions with them; therefore, additional gap junctions formed at the end of processes may not be necessary, although they may still occur. Rather that the delay before SMC depolarization occurred suggests that some paracrine or vasoactive diffusible substance may start to be released from the ICC on depolarization, and this acts, after a short delay caused by diffusion, or to allow a sufficient concentration to build up, to produce depolarization of the SMC in turn. Presumably the substance can persist after the end of ICC depolarization and give rise to further changes in 

![Figure 5. Signal transmission from ICC to SMC. A, (a) Superimposition of a transmitted light image on a fluo-3 fluorescent confocal image of a ICC-SMC pair and (b) normalized fluorescence confocal image (F/F0) of this pair with two boxed (1, green box in ICC; 2, black box in SMC) regions of interest. ICC was voltage-clamped at −60 mV. Four x-y time series imaging protocols each comprising 48 frames acquired at 5 Hz were performed sequentially, whereas the ICC membrane potential was stepped to different levels (between −60 and +30 mV by 30 mV increments, B through E, respectively). In B through E, panel (a) shows 24 sequential confocal images taken as a part of the x-y time series protocol (see earlier), panel (b) shows the voltage protocol (bottom) and corresponding current record (top), panel (c) is the corresponding gallery formed by sequentially aligning from left to right 24 fluorescence confocal images of the two boxed regions of interest (Ab; labeled 1 in ICC and 2 in SMC) numbered, respectively, on the right hand side of the gallery, panel (d) is the corresponding plot of the time course of the normalized fluorescence (F/F0) averaged within each of two regions of interest (Ab, boxes 1 and 2). Note, that depolarization of the ICC membrane to 0 mV or to +30 mV caused a delayed rise in [Ca2+]i in the adjacent SMC. Also note, that the latency of the SMC response decreased with an increase of the amplitude of the voltage step applied to the ICC.

![Figure 5](image-url)
known. Thus ICCs, in addition to an electrical link to SMCs, also release a diffusible substance that depolarizes them.

We considered some possible sources of artifact. Stimulation of the SMC directly by a current (similar to that generated by the ICC in response to the voltage steps from −60 mV to 0 or 30 mV) applied through the free-tip of a patch pipette within less than 1 μm of the SMC surface (compare to the distance between SMC and ICCs, Figure 5) or even touching but not sealed to the cell, had no effect on 

\[\text{Ca}^{2+}\text{i}\] in the stimulated cell (n=10, unpublished data, 2004). However, stimulation of the ICC under voltage-clamp led to the transmission of the signal to the SMC and activated voltage-gated K⁺ current through the ICC membrane (panels b, Figure 5D and 5E), i.e., an extrusion of K⁺ from the ICC into the extracellular media. This will cause a transient increase in the extracellular K⁺ concentration ([K⁺]o) close to the ICC, which could potentially be sensed by the SMC. We therefore estimated to what extent [K⁺]o could increase in the “inner” space between ICCs and SMCs during this process. The upper limit could be calculated from the assumption that K⁺ released into the “inner” volume between the ICC and neighboring SMC does not immediately diffuse into the “outer” volume and is accumulated in the “inner” volume during the voltage step. The “inner” volume between ICC and SMC (Figure 5) was calculated as a product of the area between these two cells measured from transmitted light image (≈1764 μm²; Figure 5A, a) and the cell thickness (≈4 μm) and was found to be ≈7 pL. The amount of K⁺ extruded from the ICC into the inner volume through K⁺ channels before [Ca²⁺] in adjacent SMCs started to rise was calculated from the integral of the whole-cell K⁺ current. The total charge carried by K⁺ through the IC membrane over this period was 0.8 ± 10⁻⁹ C, what corresponds to 0.008 pmol K⁺ extruded from the cell. Even if a quarter of this K⁺ was released into the inner volume and did not diffuse from it into the extracellular media, it would still be a significant amount.

Figure 6. Simultaneous tight-seal recording from both an ICC and SMC of an ICC-SMC pair. Voltage protocol (A) and corresponding current (B) recorded from stimulated ICC under voltage clamp. C, Membrane potential of the SMC recorded synchronously under current clamp. Note the delayed depolarization of the SMC during stimulation of ICC.

Figure 7. Signal transmission within ICC network. A, (a) Superimposition of a transmitted light image on a fluo-3 fluorescent confocal image of a small “patch” of the ICC network and (b) corresponding normalized confocal fluorescence image (F/F₀) with three boxed (green in stimulated cell, magenta and blue in adjacent cells) regions of interest. One of the ICCs (see position of the patch pipette in Aa) was voltage-clamped at −60 mV using perforated-patch tight-seal technique. Voltage protocol (step to 0 mV) was synchronized with the x-y time series imaging protocol comprising of 48 frames acquired at 5 Hz. B, 40 sequential confocal images taken as part of an x-y time series protocol (see earlier). C, Panel a shows voltage protocol (bottom) and corresponding current record (a, top), panel b is the corresponding gallery formed by the sequential aligning from left to right of 30 fluorescent confocal images of the three boxed regions of interest (green, magenta and blue outlines in Ab: 1 in stimulated ICC, 2 and 3 in two adjacent ICCs) numbered respectively on the left hand side of the gallery. Panel c is the corresponding plot of the time course of the normalized fluorescence (F/F₀) averaged within each of the three regions of interest (green, magenta, and blue outlines in Ab) and shown in the corresponding color. Note that on stimulation of one ICC, [Ca²⁺] increased in all three interconnected ICCs in less than 200 ms.
the outer volume, it would cause only 0.3 mmol/L increase in [K⁺], in the inner volume by the time the SMC started to respond. In the real situation, K⁺ is diffusing freely into the outer volume and an increase in [K⁺] in the inner volume would be therefore substantially smaller. Thus, it seems highly unlikely that K⁺ current generated by an ICC in response to voltage step can stimulate a neighboring SMC either by extracellular current spread (see earlier) or through an increase of [K⁺] in the vicinity of the SMC.

In summary, this study reports spontaneous [Ca²⁺], oscillations associated with depolarizations of the ICC membrane in a vascular tissue and provides for the first time a direct demonstration of signal transmission from ICC to SMC. These findings suggest that the ICC network may serve as a pacemaker in this rhythmically contracting vessel, generating and transmitting low frequency electrical signals to the adjacent SMCs. In addition there was evidence for some unknown paracrine or vasoactive substance which was released from the ICC on depolarization causing depolarization of SMCs.

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