

SPECIAL REPORT

Identification of interstitial cells of Cajal in corporal tissues of the guinea-pig penis

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This study shows for the first time the presence of interstitial cells of Cajal (ICC) and their possible role in the initiation of spontaneous excitation in the corporal tissue of the guinea-pig penis. ICC, which were identified by their c-kit immunoreactivity, were abundantly distributed in the corporal smooth muscle meshwork. Spontaneous increases in the intracellular calcium concentration ($[Ca^{2+}]_i$; calcium transients) were visualized in preparations loaded with the fluorescent dye fura-2. Ca transients originated from the boundary of muscle bundles and then spread throughout the meshwork (Ca waves). Ca waves were strongly suppressed by either CPA (10 μ M), ryanodine (50 μ M) or 2-APB (10 μ M), and their synchronicity was disrupted by 18 β -GA (30 μ M). These results suggest that ICC in the corporal tissue may have a role as pacemakers to drive the bulk of smooth muscles, and that intracellular Ca^{2+} stores and gap junctions are critical for the generation of spontaneous excitation. *British Journal of Pharmacology* (2004) 141, 199–204. doi:10.1038/sj.bjp.0705622

Keywords: Interstitial cells of Cajal (ICC); corporal tissue; calcium wave; gap junction

Abbreviations: 2-APB, 2-aminoethoxydiphenylborate; 18 β -GA, 18 β -glycyrrhetic acid; $[Ca^{2+}]_i$, intracellular concentration of free calcium ions; CICR, calcium-induced calcium release; CPA, cyclopiazonic acid; DMSO, dimethyl sulphoxide; ICC, interstitial cells of Cajal; InsP₃, inositol 1,4,5-trisphosphate

Introduction Penile erection and detumescence result from the increased activity of parasympathetic and sympathetic nerves, respectively (Andersson & Wagner, 1995). There is general agreement that nitric oxide (NO) released from both autonomic nerves and endothelium is the principal mediator for the relaxation of corporal smooth muscles and erection. Detumescence predominantly results from the activation of α -adrenoreceptors by neurally released noradrenaline, which contracts the corporal smooth muscles. Continuous input from sympathetic nerves and spontaneous 'myogenic activity' may both contribute to the sustained smooth muscle tone in the flaccid penis (Andersson & Wagner, 1995).

Corporal smooth muscle strips taken from several species, including man, exhibit spontaneous contractions. These contractions rely largely on Ca^{2+} influx through L-type Ca^{2+} channels, and are inhibited either by L-type Ca^{2+} channel blockers or by K^+ channel openers, which hyperpolarize the membrane (Holmquist *et al.*, 1990; Christ *et al.*, 1990; Hashitani, 2000; Hashitani *et al.*, 2002). Underlying the contractions are spontaneous action potentials and associated Ca^{2+} transients (Hashitani *et al.*, 2002). However, the origin and basis of spontaneous excitation in corporal smooth muscles is still to be elucidated.

Interstitial cells of Cajal (ICC) are now considered to play a key role in pacemaking and signal transmission in gastrointestinal tissues (Sanders, 1996; Hirst & Ward, 2003). ICC-like cells have also been identified in the urinary tract of the guinea-pig and mouse, in which spontaneous activity is generated, either by their immunoreactivity for c-kit, a cell surface marker specific for ICC, or through morphological criteria (Klemm *et al.*, 1999; Sergeant *et al.*, 2000; McCloskey

& Gurney, 2002; Pezzone *et al.*, 2003). Since corporal smooth muscles are also spontaneously active, it would be reasonable to speculate that ICC are involved in the generation of spontaneous excitation in corporal tissue.

ICC generate electrical slow waves which coordinate activity in the bulk of smooth muscle cells. Regardless of the varied sensitivity of slow waves for L-type Ca^{2+} channel blockers (Hashitani *et al.*, 1996; Suzuki & Hirst, 1999; Yoneda *et al.*, 2002), they are readily blocked by chemicals which disrupt the function of either intracellular Ca^{2+} stores or mitochondria (Hashitani *et al.*, 1996; Fukuta *et al.*, 2002; Yoneda *et al.*, 2002). Consistently, the underlying currents recorded from isolated ICC are also highly sensitive to these chemicals, indicating that Ca^{2+} handling by intracellular Ca stores and mitochondria is crucial in generating slow waves (Ward *et al.*, 2000; Sergeant *et al.*, 2001). Although spontaneous excitation in corporal smooth muscles is known to be blocked by Ca^{2+} channel blockers, the contribution of intracellular Ca handling to their generation remains unknown.

Generally, gap junctions play a key role in signal transmission, and thus signals generated from either ICC or smooth muscles require intact gap junctions in coordinating multicellular units. Cultured corporal smooth muscles are shown to be electrically coupled to each other through gap junction channels (Christ *et al.*, 1992). Consistently, in intact corporal smooth muscle preparations, electrical coupling between two separate cells measured using independent electrodes was blocked by 18 β -glycyrrhetic acid (18 β -GA), a gap junction blocker (Hashitani, 2000). However, we have not yet elucidated how the blockade of gap junctions alters the propagation of spontaneous excitation in the corporal tissue.

In the present study, first, ICC were identified in corporal tissues of the guinea-pig, using an antibody for c-kit, a selective marker for ICC. Second, Ca waves in intact corporal tissues

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were visualized, and the effects of a gap junction blocker and inhibitors for the function of intracellular Ca^{2+} stores were examined. The possible roles of ICC in generating spontaneous excitation in the corporal tissue will be discussed.

Methods *General* The procedures described have been approved by the animal experimentation ethics committee at Nagoya City University Medical School. Male guinea-pigs weighing 400–600 g were killed by a blow to the head, followed by cervical dislocation. After removal of the bulbospongiosum muscle, which covered the bottom of the penile shaft, the tunica albuginea was slit open with a lateral incision and the corpus spongiosum was dissected free. Corpus spongiosum preparations were pinned out in a dissecting chamber and several smooth muscle layers were carefully removed, leaving a single layer of the meshwork of corporal smooth muscle. These procedures did not cause disruption of either corporeal smooth muscles or nerves (Hashitani, 2000; Hashitani *et al.*, 2002).

Immunohistochemistry To visualize cells expressing c-kit immunoreactivity, preparations were incubated for 1 h in physiological saline containing rat monoclonal antibodies raised against the c-kit protein (ACK-2, diluted 1:100, Cymbus Biotechnology Ltd, Hants, U.K.). The tissue was washed and then incubated for another 1 h in anti-rat IgG antibody labelled with a fluorescent marker (IgG-Alexa Fluor 488, diluted 1:100, Molecular Probes, OR, U.S.A.). After washing with physiological saline, the preparations were observed using confocal laser microscopy (LSM5 PASCAL, Zeiss, Germany).

Intracellular calcium measurements For measurements of changes in the concentration of intracellular calcium ($[\text{Ca}^{2+}]_i$), preparations were pinned out on a block of Sylgard (silicone elastomer, Dow Corning Corporation, Midland, MI, U.S.A.) which had a window of some 3 mm \times 3 mm in the centre. The Sylgard block was turned over and then placed at the bottom of the recording chamber, so that the preparation faced a glass coverslip. After 30 min incubation with warmed (35°C) physiological saline, spontaneous movements of the tissues were detected visually, and then the preparations were loaded with fluorescent dye, fura-2, by incubation in nominally Ca^{2+} -free solution containing 10 μM fura-2 AM for 1 h at room temperature. After loading, the preparations were superfused with dye-free, warmed (35°C) physiological saline, at a constant flow (about 2 ml min⁻¹) for 30 min. Preparations, loaded with fura-2, were viewed under either an oil-immersion objective (UPlanApo 40, Olympus) or a normal objective (UPlanApo 10 and 20, Olympus), and were illuminated with two periods of ultraviolet light, wave lengths 340 and 380 nm, alternating at a frequency of higher than 40 Hz. The ratio of the emission fluorescence ($R_{340/380}$) in a desired size of rectangular window was measured through a barrier filter of 510 nm (sampling interval 25–100 ms), using a micro-photoluminescence measurement system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan).

Solutions The composition of physiological saline was (in mM): NaCl, 120; KCl, 5.9; MgCl₂, 1.2; CaCl₂, 2.5; NaHCO₃,

15.5; KH₂PO₄, 1.2 and glucose, 11.5. The solution was bubbled with 95% O₂ and 5% CO₂, and pH was 7.2–7.3.

The drugs used were 2-aminoethoxydiphenylborate (2-APB), fura-2 AM and ryanodine (from Calbiochem-Novabiochem Ltd, San Diego, CA, U.S.A.), cyclopiazonic acid (CPA), 18 β -GA, nifedipine (from Sigma, St Louis, MO, U.S.A.). Nifedipine was dissolved in 100% ethanol; 2-APB, CPA, 18 β -GA and ryanodine were dissolved in dimethyl sulphoxide (DMSO). The final concentration of these solvents in the physiological saline did not exceed 1:1000.

Results *Identification of c-kit-positive cells in the corporal tissue* Staining using antibody for c-kit identified two different cell types in the corporal tissue (Figure 1a). Based on their morphological characteristics, these were determined to be ICC and mast cells. ICC had a spindle-shaped cell body, some 50 μm in length and less than 10 μm width (Figure 1b), and sometimes had a few processes (Figure 1c). Mast cells, which are known to be another c-kit-positive cell type (Hagger *et al.*, 1998), had a round-shaped cell body, some 10 μm in diameter (Figure 1b). ICC were preferentially located near the boundaries of the smooth muscle meshwork rather than in the middle (Figure 1d).

Propagation of Ca waves in corporal smooth muscles When changes in $[\text{Ca}^{2+}]_i$ were visualized in the corporal smooth muscle meshwork, increases in $[\text{Ca}^{2+}]_i$ generally arose from either single or multiple sites near the boundary of muscle bundles and then spread throughout the meshwork (Ca waves). Figure 2Aa shows the propagation of Ca waves originating from two different sites. When changes in $[\text{Ca}^{2+}]_i$ were recorded from separate areas with a distance between areas of some 100 μm , synchronous Ca signals were recorded in all areas (Figure 2Ab). Figure 2Ba shows a Ca wave that originated from one boundary of the muscle bundle and spread to the other boundary (Figure 2Ba). When changes in $[\text{Ca}^{2+}]_i$ were recorded from separate areas with a distance between areas of some 40 μm , Ca signals concurrently occurred in all areas (Figure 2Bb). However, on a fast time scale, there was a delay of several hundred ms between the peaks of the Ca signal recorded from one boundary and that from the other boundary (Figure 2Bc). The estimated conduction velocities of Ca waves ranged between 0.44 and 1.2 mm s⁻¹ (mean 0.73 \pm 0.23 mm s⁻¹, $n=8$), and were not very different from the values reported in gastrointestinal and urinary tissues (Stevens *et al.*, 1999; Hashitani *et al.*, 2001).

Role of gap junctions and intracellular Ca stores in Ca waves To investigate the role of gap junctions in the propagation of Ca waves, the effects of 18 β -GA, a gap junction blocker (Hashitani, 2000), were examined. In control conditions, synchronous Ca transients were recorded from two separate areas located some 100 μm apart (Figure 3Aa). 18 β -GA (30 μM) reduced the amplitude of Ca transients recorded from the area in which the leading Ca transients were generated, while almost completely suppressing the follower Ca signals (Figure 3Ab), indicating that cell-to-cell communication was effectively disrupted by 18 β -GA.

As shown previously, Ca transients recorded from corporal smooth muscles were readily abolished by nifedipine (1 μM , $n=4$), indicating that they resulted from Ca influx through

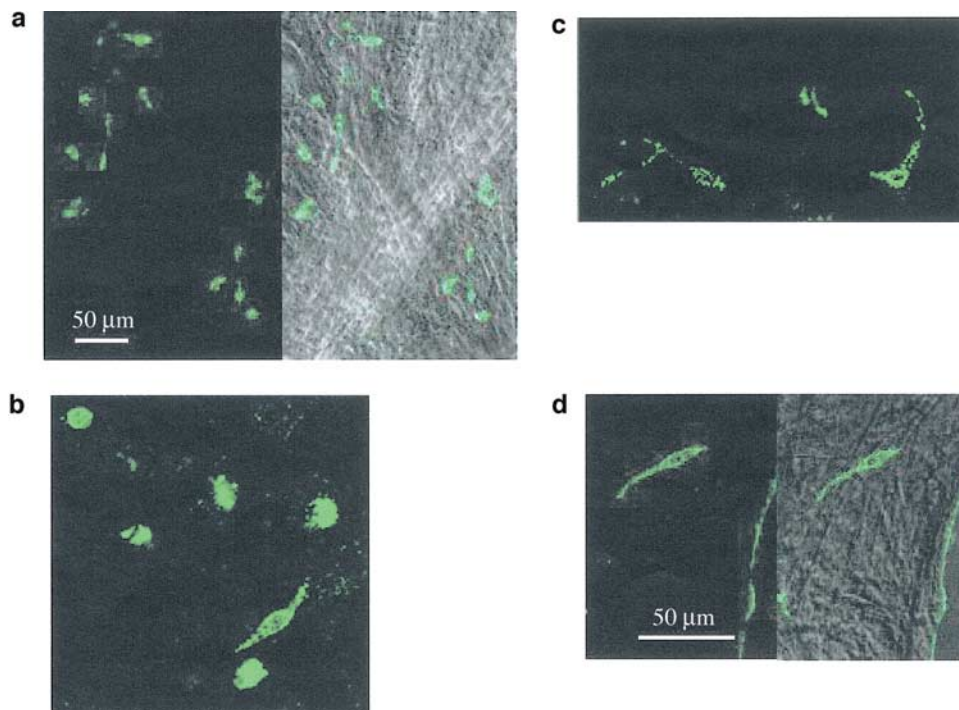


Figure 1 Identification of ICC in corporal tissue of the guinea-pig penis. ICC identified using ACK2 antibody against c-kit labelled with Alexa 488 are seen in the corporal tissue of the guinea-pig (a). The left panel shows fluorescent images of ICC and the right panel shows the image superimposed on the phase image of smooth muscle bundles. Two distinct populations of cells expressed c-kit immunoreactivity (b). Spindle-shaped cells were identified as ICC, while round-shaped cells were considered to be mast cells. ICC had a spindle-shaped cell body, some 50 μm in length and less than 10 μm in width, and sometimes had a few processes (c). ICC are preferentially located near the boundary of the smooth muscle meshwork (d). The scale bar in (a) refers to a. The scale bar in (d) refers to b–d.

L-type Ca channels (Hashitani *et al.*, 2002). To investigate the cellular basis of Ca transients in corporal tissues further, the role of intracellular Ca stores in the generation Ca waves was examined.

In control conditions, synchronous Ca transients were recorded from separate areas at a distance of some 100 μm apart (Figure 3Ba). CPA (10 μM), an inhibitor of the sarcoplasmic reticulum Ca^{2+} ATPase, reduced the amplitude of Ca transients and almost completely suppressed them in about 10 min (Figure 3Bb). Ryanodine (50 μM), a blocker of Ca-induced Ca release from the store, also reduced the amplitude of Ca transients and strongly suppressed them in some 20 min (Figure 3Cb). 2-APB (10 μM), a known blocker for InsP_3 -induced Ca release (Maruyama *et al.*, 1997), reduced the frequency of Ca transients and prevented their generation in about 10 min (Figure 3Db).

Discussion In the present study, ICC have for the first time been identified in corporal tissue of the guinea-pig penis using an antibody for c-kit, a specific marker of ICC. Spontaneous Ca signals originated from the boundary of muscle bundles and spread throughout the muscle meshwork to generate synchronous Ca waves. The Ca waves were blocked by CPA, ryanodine and 2-APB, suggesting that interplay between the receptors for InsP_3 and ryanodine on intracellular Ca store membrane is crucial in generating Ca waves. The synchronicity of Ca waves was disrupted by 18β -GA, indicating a key role of gap junctions in signal transmission in the corporal tissue.

ICC can be identified from their ultrastructural characteristics with electron microscopy, but since these cells in both the

gastrointestinal and urinary tracts also express immunoreactivity for c-kit, a surface tyrosin kinase receptor (Dickens *et al.*, 1999; McCloskey & Gurney, 2002; Yoneda *et al.*, 2002), staining using anti-c-kit antibody is the most convenient way to identify ICC. In the corporal tissue, two populations of c-kit positive cells were identified. The other cell type known to express c-kit immunoreactivity is the mast cell (Hagger *et al.*, 1998). Based on morphological criteria, spindle-shaped cells with some branches were considered to be ICC, while round-shaped cells were considered to be mast cells. In the canine corpus cavernosum, mast cells have been identified by toluidine blue staining (Iwamoto *et al.*, 2001). ICC are now believed to play a key role in pacemaking and signal transmission in the gastrointestinal and urinary tracts (Sanders, 1996; Hirst & Ward, 2003), and this may also be the case in the corporal tissue. Mast cells are also known to release a number of biologically active substances such as prostaglandins and histamine (Ennis *et al.*, 1984). Furthermore, they are often closely associated with nerves and are possibly themselves innervated (Newson *et al.*, 1983; Stead *et al.*, 1989). It has indeed been reported that spontaneous contractions in human corporal tissues are markedly reduced by indomethacin, suggesting an involvement of cyclo-oxygenase products in the generation of the contractions (Christ *et al.*, 1990). Therefore, not only ICC but also mast cells may play an important role in pacemaking or the transmission of spontaneous excitation in the corporal tissues.

Spontaneous contractions in corporal smooth muscles result from action potentials and associated Ca transients, indicating the critical role of Ca influx through L-type Ca channels

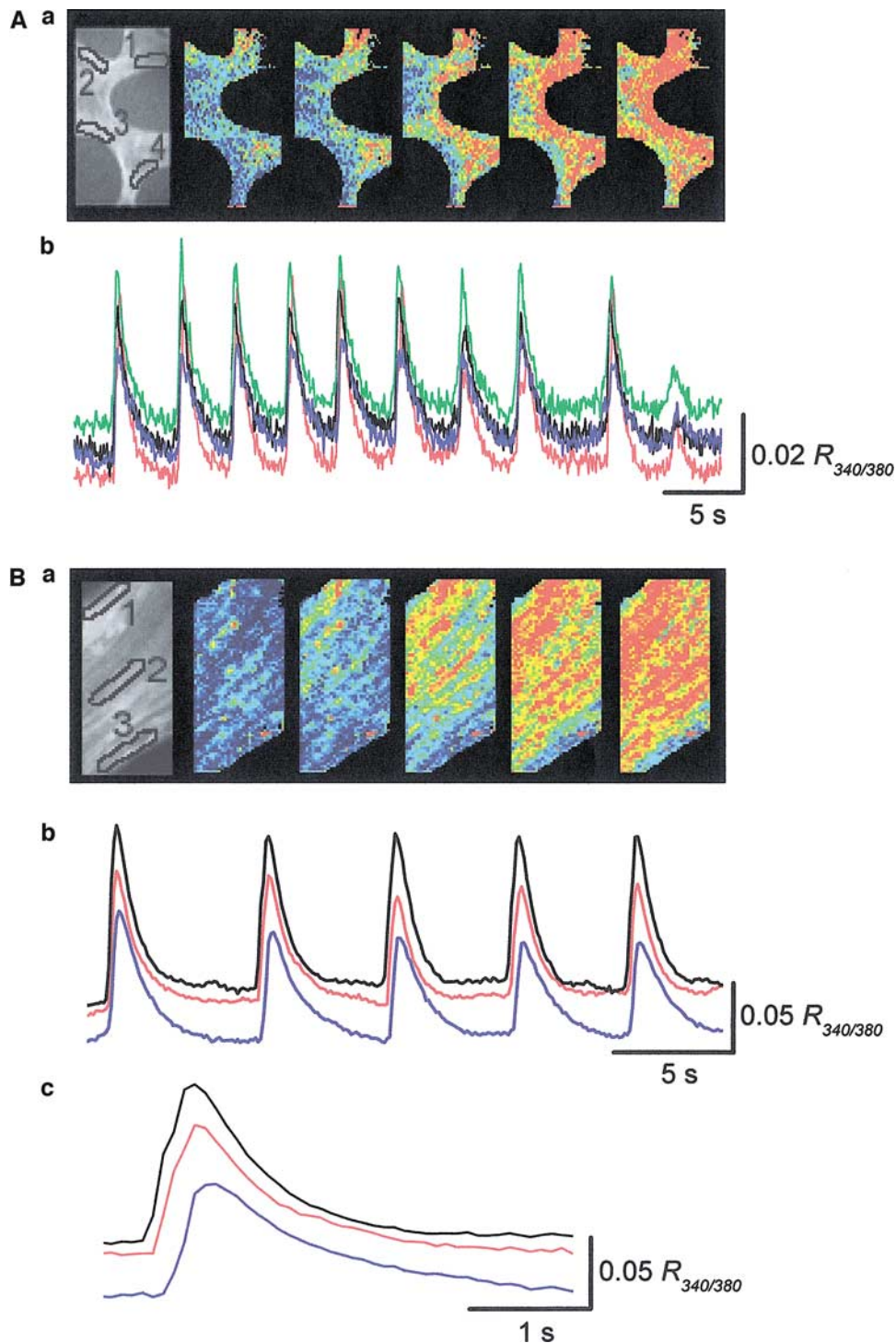


Figure 2 Calcium waves in the corporal smooth muscle meshwork. (A) Changes in $[Ca^{2+}]_i$ were simultaneously recorded from separate areas located with a separation between each area of some $100\ \mu\text{m}$. The series of frames show Ca waves originating from two separate sites and spreading throughout the tissue within the imaged area (Aa). When changes of $[Ca^{2+}]_i$ were simultaneously recorded from four different areas, transient increase in $[Ca^{2+}]_i$ occurred synchronously in all areas (Ab). (B) Changes in $[Ca^{2+}]_i$ were simultaneously recorded from three separate areas located on the muscle bundle, with a separation between each area of some $40\ \mu\text{m}$. The series of frames demonstrate a Ca wave originating from the boundary of the muscle bundle and spreading to the other boundary (Ba). When changes of $[Ca^{2+}]_i$ were simultaneously recorded from each area, synchronous increase in $[Ca^{2+}]_i$ occurred in all areas (Bb). Ca^{2+} transients recorded from the areas had almost identical time courses, but a delay of some 200 ms was observed between the rising phases of Ca transients in both boundaries (Bc). Black, red and blue traces were recorded from areas 1, 2 and 3, respectively.

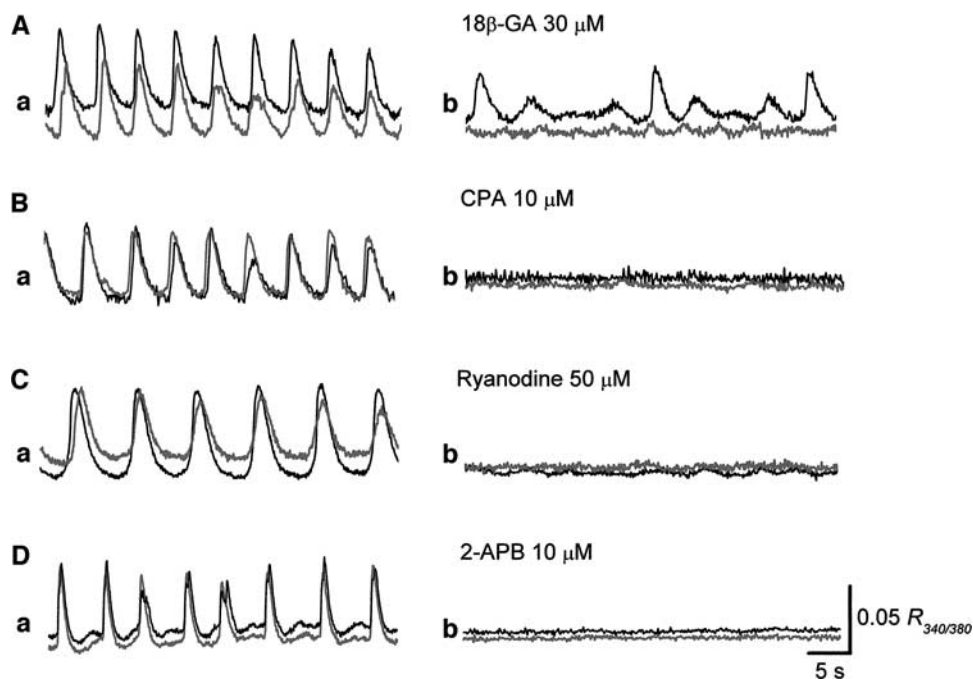


Figure 3 Role of gap junctions and intracellular Ca stores in the generation of Ca waves. (A) In control solution, synchronous Ca waves were recorded from two areas separated by a distance of some 100 μm (Aa). $18\beta\text{-GA}$ (30 μM) reduced the amplitude of Ca transients in the leading area, while almost completely suppressing Ca transients in the follower area (Ab). (B) In control solution, Ca waves occurred synchronously in two separate areas (Ba). After some 10 min application of CPA (10 μM), Ca waves in both areas were almost completely suppressed (Bb). (C) In a different preparation, 20 min of application of ryanodine (50 μM) strongly inhibited Ca waves, which had synchronously arisen from two separate areas (Cb). (D) 2-APB (10 μM) abolished Ca waves recorded from two areas in some 10 min (Db). The scale bar on the right of Db refers to all traces.

(Hashitani, 2000; Hashitani *et al.*, 2002). In the present study, Ca waves in corporal smooth muscles of the guinea-pig penis were inhibited by chemicals, which disrupt the function of intracellular Ca stores. Unlike action potentials, slow waves originating from ICC in gastrointestinal tissues are generally insensitive to L-type Ca channel blockers (Dickens *et al.*, 1999). In the mouse colon, however, slow waves were blocked by both Ca antagonists and blockers for the function of Ca stores, indicating an interplay between L-type Ca channels and Ca release from the stores (Yoneda *et al.*, 2002). In the urethra, the plateau phase, but not the initial component of slow waves recorded in multicellular preparations, was blocked by nifedipine (Hashitani *et al.*, 1996). Therefore, although ICC are widely distributed in visceral smooth muscle tissues, the basic mechanisms involved in generating spontaneous excitation appear to vary between tissues. In corporal tissue, as in the case of the mouse colon, both the activation of L-type Ca channels and the Ca release from intracellular stores may be required in generating spontaneous excitation.

Interestingly, both 2-APB and ryanodine strongly suppressed the Ca waves. It is generally agreed that Ca^{2+} handling by intracellular stores and mitochondria play a critical role in the generation of slow waves (Ward *et al.*, 2000; Sergeant *et al.*, 2001; Fukuta *et al.*, 2002). Accumulated evidence suggests a central role of InsP_3 receptors (Suzuki *et al.*, 2000; Van Helden *et al.*, 2000), while the role of ryanodine receptors appears to be controversial. In gastrointestinal tissues, ryanodine had almost no effects on spontaneous activity (Liu *et al.*, 1995; Van Helden *et al.*, 2000; Fukuta *et al.*, 2002). Conversely, spontaneous currents recorded from isolated ICC in the urethra were readily blocked by ryanodine (Sergeant *et al.*,

2001). In corporal tissues, there seems to be a complex interplay between L-type Ca channels, InsP_3 and ryanodine receptors in the generations of spontaneous excitation.

$18\beta\text{-GA}$, a gap junction blocker, almost completely suppressed Ca signals in the follower area, while only reducing the amplitude of Ca signals in the leading area, suggesting that $18\beta\text{-GA}$ inhibited signal transmission through gap junctions rather than the generation of Ca signals. Both cultured and intact corporal smooth muscles have been shown to be coupled to each other *via* gap junctions (Christ *et al.*, 1992; Hashitani, 2000). Although we do not have any information about the coupling between ICC and corporal smooth muscles, we have confirmed that the transmission of both electrical and calcium signals requires intact gap junctions.

In conclusion, ICC identified using their immunoreactivity for c-kit may be crucial in pacemaking and signal transmission in corporal tissue, and thus contribute to the corporal muscle tone to maintain detumescence in the penis. ICC are also thought to be involved in neuromuscular transmission, including inhibitory transmission mediated by NO (Ward *et al.*, 1998; Hirst *et al.*, 2002); therefore, they may also play an important role in the NO-mediated relaxation of corporal smooth muscles, which appears to be fundamental for the penile erection. Further investigation of the physiological role of corporal ICC might enable advances in the pharmacological treatment of erectile dysfunction.

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